

## Theory of Analysis

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## Theory of Real-Time Quantitative PCR Analysis

This is the fifth in a 6 part training series for real-time quantitative PCR.

In the first four sessions, topics covered included:

- 5'-Nuclease Assay and SYBR® Green
- Real-Time PCR instrumentation
- TaqMan® Assays and Custom assays
- Normalization of quantitative experiments
- Real-Time PCR Reagents



## Theory of Real-Time Quantitative PCR Analysis

Topics in this presentation include:

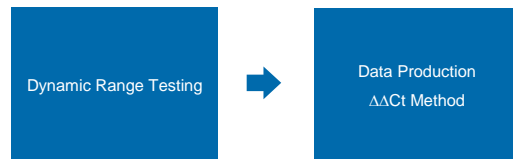
- Dynamic Range
- Standard Curves
- $\Delta\Delta Ct$  Method
- Calibration
- Controls



## Overview

The process of producing real-time PCR data can be divided into two key steps.

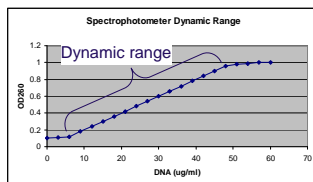
Dynamic range testing occurs first, followed by data production using the  $\Delta\Delta Ct$  Method.



## Dynamic Range

Dynamic range is the minimum and maximum sample amount that may be run in the assay system and produce accurate and precise data.

For example, a spectrophotometer will always produce an OD value, but the OD will only be accurate within a certain sample range.



## Dynamic Range Testing

Dynamic range testing consists of serially diluting a sample and running it in real-time PCR using the Target and Normalizer assays.

Dynamic range testing should be conducted at the beginning of a new assay system.

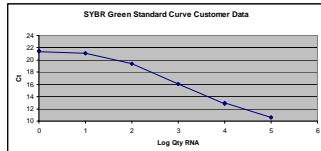
Problems uncovered by this testing should be resolved before data production commences.



## Dynamic Range Testing for Many Targets

For experiments involving many TaqMan® Assays, testing just one Target and the Normalizer should be informative, because problems such as inhibitors should affect all the assays.

A standard curve should be run for every SYBR® Green assay, because each assay may have unique tendencies to produce non-specific products, which may limit dynamic range.



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## Sample Type for Dynamic Range Testing

For valid dynamic range testing, the standard curve must be of the appropriate type.

Sample Type	Standard Curve Type
Extracted DNA	Serially diluted DNA
Extracted RNA	Serially diluted RNA*
Cell lysate**	Serially diluted cells

\* Each RNA concentration is reverse transcribed using the same reaction volume.

\*\* Cell lysates are added directly to the reaction. Cells-to-C<sub>t</sub>™ kits are an example.



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## Selecting a Sample for Dynamic Range Testing

Select a sample to create the standard curve with the following characteristics:

- Derived from the same type as other samples, e.g., use a sample from blood when all other samples are from blood.
- Extracted using the same method as other samples.
- Non-precious.
- Target and normalizer are present in adequate abundance.



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## How Many Standard Curves

Running standard curves on all samples is not necessary.

Running a standard curve using even one representative sample should provide useful information.

Running standard curves on multiple samples is recommended when:

- Highly variable sample quality is suspected, e.g., FFPE.
- Using different extraction methods or sample types, e.g., blood vs. bone.



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## General Standard Curve Recommendations for Dynamic Range Testing

1. Use a minimum of 5 different concentrations or "points" within approximately a 3-log range.
2. Highest concentration should be about 10-fold higher than the expected average sample amount.
3. Use a serial dilution process.

Dilution Factor	Spanned Range with Number of Points			
	5 points	6 points	7 points	8 points
2	16	32	64	128
3	81	243	729	2187
4	256	1024	4096	16384
5	625	3125	15625	78125
6	1296	7776	46656	279936

  Fewest points to span 3-logs.

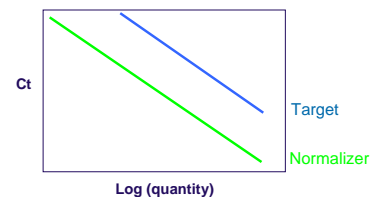


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## Standard Curve Plot

Standard curves are plotted log quantity units on the x-axis and Ct values on the y-axis.

Ideally, the data points should form parallel lines (+/- 0.1) with negative slopes near -3.3 (-3.0 to -3.7).

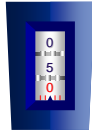


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## Pipette Calibration Error

Pipette calibration error is a volume accuracy error: the volume dispensed is consistently too small or too large.

Calibration errors in the pipettes used to make standard curve will change the slope of the standard curve.

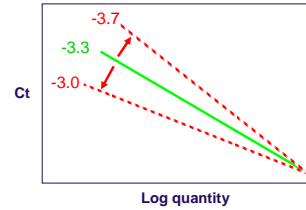


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## Standard Curve Slopes

Standard curve slopes of -3.0 to -3.7 may be considered reasonable pipette calibration error ( $\leq 10\%$ ).



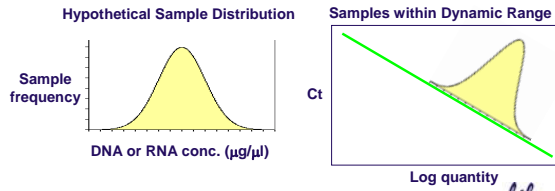
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## Sample Concentration Range

Sample concentrations are expected to differ, perhaps forming a bell-shaped distribution.

As long as the sample distribution falls within dynamic range, samples need not be adjusted so as to run equal amounts for each.



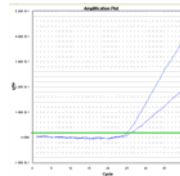
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## Limitations in Real-Time PCR Dynamic Range

Dynamic range in real-time quantitative PCR assays may be limited by:

- Inhibitors.
- Reverse transcription chemistry performance problems.
- Primer-dimer production in SYBR® Green assays.



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## Inhibitor Categories

Inhibitors can be divided into 3 categories:

**Solubility inhibitor** reduces the amount of template participating in the PCR reaction and is usually associated only with genomic DNA.

**Taq inhibitor** reduces the effective concentration of Taq polymerase.

**Reverse transcription (RT) inhibitor** reduces RT efficiency.

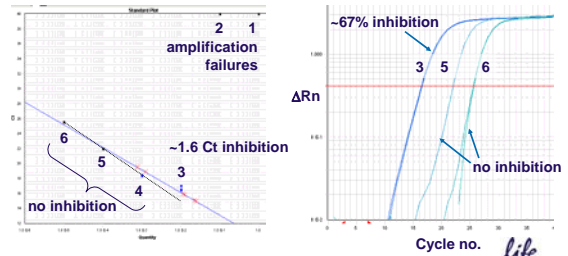
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## Example of DNA Solubility Inhibition

Fungal genomic DNA was serially diluted 10-fold with 6 points.

Solubility inhibition increases the Ct value, but does not affect the  $\Delta Rn$ .

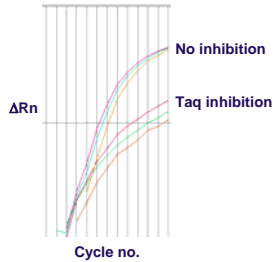


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## Example of Taq Inhibition

Taq inhibitors reduce the  $\Delta Rn$ , but the Ct value is usually unaffected.



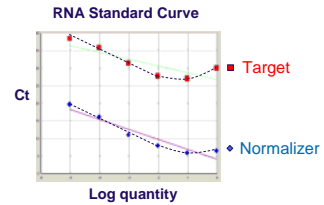
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## Example of Reverse Transcription Inhibition

When RT inhibitor is present, inhibition is strongest at the highest RNA concentration and serial dilution eventually removes the inhibition.

A characteristic "tailing effect" in the RNA standard curve reveals inhibition.

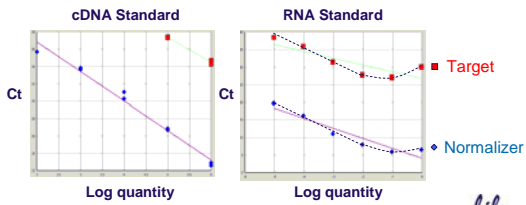


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## cDNA Standard Curves are Not Informative

A serial dilution of cDNA does not reveal reverse transcription inhibition. A direct comparison from a customer case is shown below.



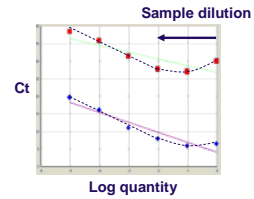
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## Actions Against Inhibition

If inhibition is observed, the researcher may:

- Dilute all the samples to remove the inhibition.
- Reevaluate the extraction method to improve sample quality.



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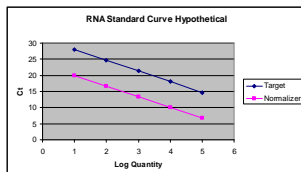


## Reverse Transcription Chemistry Performance

RT chemistries must maintain constant RT efficiency over a range of RNA amounts to produce accurate Ct values.

Poor formulation or improper storage, e.g., frost-free freezer, may result in poor performance.

cDNA from a poorly performing RT chemistry will still produce Ct values, but they will be inaccurate.

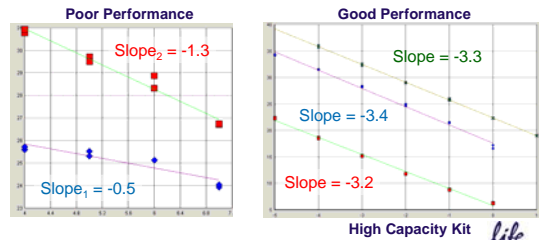


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## RT Chemistry Performance

Abnormally shallow and non-parallel RNA standard curve slopes indicate a poorly performing RT chemistry.



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## Actions Against RT Performance Problems

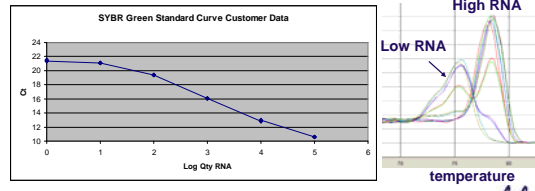
If poor performance is observed:

- Run an RNA standard curve with a fresh RT kit to evaluate whether the old kit has deteriorated.
- Try a different RT kit if the old kit is suspected of having inherently poor performance.



## Primer-Dimer Formation in SYBR® Green Assays

SYBR® Green assays may have a tendency to produce non-specific products, such as primer-dimer, especially at low target concentrations. Primer-dimer tendencies may limit the sensitivity of SYBR® Green assays.



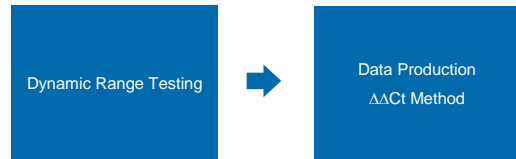
## Actions Against SYBR® Green Primer-Dimer

If the tendency of a SYBR® Green assay to produce non-specific products is a significant problem, actions include:

- Reduce the primer concentration to 50nM final.
- Change the SYBR® Green chemistry, e.g., Power SYBR® Green RNA-to-C<sub>T</sub><sup>™</sup> 1-Step Kit minimizes primer-dimer.
- Redesign the primers.
- Use a TaqMan® probe, e.g., TaqMan® Assay/Custom TaqMan® Assay.

## Data Production

Once dynamic range has been tested and problems resolved, data production may commence.



## ΔΔCt Method

The ΔΔCt Method (a.k.a. Comparative Ct) is a method to produce quantitative data using the equation:

$$\text{Quantity} = 2^{-\Delta\Delta C_t}$$

The ΔΔCt Method is based on the fundamental relationship between initial quantity and Ct:

$$\text{Quantity} \sim e^{-Ct}$$

Where e = geometric efficiency.

## Derivation of ΔΔCt Method

The derivation of the ΔΔCt Method is presented in the Applied Biosystems document User Bulletin #2.

### User Bulletin #2

ABI PRISM 7700 Sequence Detection System

December 11, 1997 (updated 10/2001)

The final step is to divide the  $X_{n,t}$  for any sample  $q$  by the  $X_{n,t}$  for the calibrator ( $c$ ):

$$\frac{X_{n,t,q}}{X_{n,t,c}} = \frac{K \cdot (1 + E)^{-MC_{t,q}}}{K \cdot (1 + E)^{-MC_{t,c}}} = (1 + E)^{-\Delta\Delta C_t}$$

where

$$\Delta\Delta C_t = MC_{t,q} - MC_{t,c}$$

For amplicons designed and optimized according to Applied Biosystems guidelines (amplicon size < 150 bp), the efficiency is close to one. Therefore, the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by:

$$2^{-\Delta\Delta C_t}$$

## Example of $\Delta\Delta Ct$

In the example, RNA is being extracted from 3 different tissues.  
The goal is relative quantification: sample-to-sample comparisons.

Sample	Target Ct	Normalizer Ct
Lung	24.1	14.4
Liver	20.6	11.7
Brain	24.3	13.2

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## Example of $\Delta\Delta Ct$

The first step in  $\Delta\Delta Ct$  is to normalize the Target to the Normalizer.

$$\Delta Ct = Ct_{\text{Target}} - Ct_{\text{Normalizer}}$$

Sample	Target Ct	Normalizer Ct	$\Delta Ct$
Lung	24.1	14.4	9.7
Liver	20.6	11.7	8.9
Brain	24.3	13.2	11.1

Ct values should never be divided, but subtracted instead.

$\Delta$  symbolizes subtraction.

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## Example of $\Delta\Delta Ct$

Next, all the data is normalized to one sample called the calibrator.

$$\Delta\Delta Ct = \Delta Ct_{\text{Sample}} - \Delta Ct_{\text{Calibrator}}$$

Sample	Target Ct	Normalizer Ct	$\Delta Ct$	$\Delta\Delta Ct$
Lung	24.1	14.4	9.7	-1.4
Liver	20.6	11.7	8.9	-2.2
Brain	24.3	13.2	11.1	0

For relative experiments, almost any sample that amplifies may be selected as the calibrator.

Brain was chosen as the calibrator in this case.

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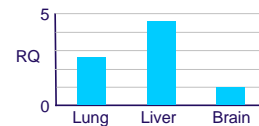
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## Example of $\Delta\Delta Ct$

The last step in this case is to transform  $\Delta\Delta Ct$  to Relative Quantity.

$$RQ = 2^{-\Delta\Delta Ct}$$

Sample	Target Ct	Normalizer Ct	$\Delta Ct$	$\Delta\Delta Ct$	RQ
Lung	24.1	14.4	9.7	-1.4	2.6
Liver	20.6	11.7	8.9	-2.2	4.6
Brain	24.3	13.2	11.1	0	1.0



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## 100% Geometric Efficiency

The number 2 in the  $\Delta\Delta Ct$  equation indicates DNA doubling each cycle, which means the Target and Normalizer assays are 100% efficient.

$$\text{Quantity} = 2^{-\Delta\Delta Ct}$$

Assays designed and run according to Applied Biosystems recommendations should have 100% geometric efficiency.

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## Efficiency Study

An efficiency study using 750 randomly selected TaqMan® Assays, demonstrating conclusively:

- All the assays had 100% geometric efficiency.
- $\Delta\Delta Ct$  produced more accurate results compared to using standard curves.

Application Note: TaqMan® Gene Expression Assays

Amplification Efficiency of  
TaqMan® Gene Expression Assays

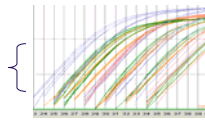
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## 100% Geometric Efficiency

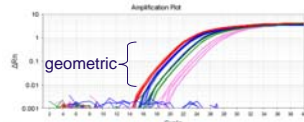
100% efficiency for all assays equates to equal efficiencies.  
Equal efficiencies can be quickly evaluated visually on the amplification plot using a log scale vertical axis.

4 TaqMan® Assays



RpL13 IFN $\gamma$   
CxCl2 IL10

4 TaqMan® Assays

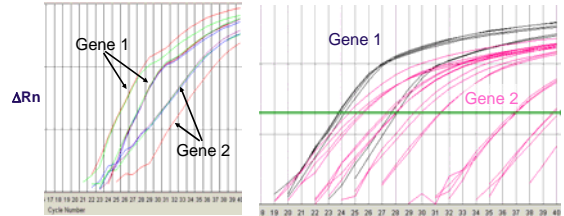


CDKN1B BCL2 PDK1 HPR1

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## Low Efficiency Assays

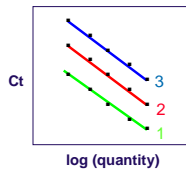
Avoid using assays that do not have parallel geometric phases.  
These SYBR® Green assays were not developed in accordance with Applied Biosystems recommendations.



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## Validation for 100% Geometric Efficiency

User Bulletin #2 describes a method to assess 100% efficiency by comparing the slopes of standard curves.  
Equal slopes are defined as +/- 0.1 in User Bulletin #2.  
However, this method is sensitive to pipette errors, so that +/- 0.1 may be difficult to achieve, even though the assays are 100% efficient.



Assay	Slope
1	-3.5
2	-3.4
3	-3.3

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## Standard Curves for Data Production

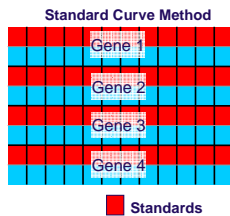
Standard curves are not optimal for data production for a list of reasons:

- Standard curves are more difficult to make for low abundant targets
- Require extra time and labor to prepare
- Increased cost
- Decreased throughput
- Reduced accuracy of results
- Require a greater degree of quality control

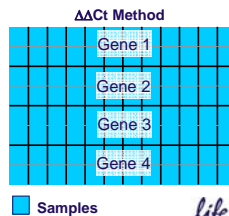
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## $\Delta\Delta Ct$ vs. Standard Curves for Data Production

In this hypothetical example, 4 genes are being run on a 96-well plate. Twice as many samples can be run and the cost per data point is reduced by half using the  $\Delta\Delta Ct$  Method.



Standards



Samples

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## Calibration

Calibration is the quantity unit being used for the data set.

Calibration comes in two categories:

- Absolute
- Relative



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## Absolute Quantification

Absolute quantification (AQ) is determining the molecular concentration of the gene target in the sample.

A general scientific definition of absolute is, "Relating to a fundamental unit of measure."

In real-time PCR, the fundamental unit of measure is the molecule.

AQ allows both sample-to-sample and gene-to-gene quantitative comparisons.

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## Relative Quantification

Relative quantification (RQ) is determining the concentration of the gene target in the sample using a non-molecular, arbitrary unit.

A general definition of relative is, "Meaningful only in relationship."

A single RQ value has no meaning by itself.

RQ allows sample-to-sample, but not gene-to-gene quantitative comparisons.

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## AQ vs. RQ Example

In the example below, sample-to-sample comparisons are valid, e.g., gene 1 is twice as abundant in sample 1 compared to sample 2.

However, gene-to-gene comparisons are not valid, because there is no assurance the RQ's are based on the same number of molecules for gene 1 and 2.

Sample	Relative Quantities	
	Gene 1	Gene 2
1	4	4
2	2	1

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## AQ vs. RQ Example

Hypothetically:

1 RQ for gene 1 = 2000 molecules/sample mass unit

1 RQ for gene 2 = 1000 molecules/sample mass unit

Conversion of RQ to AQ has not affected the sample-to-sample relationships, but has enabled gene-to-gene AQ comparisons.

Sample	Relative Quantities		Absolute Quantities	
	Gene 1	Gene 2	Gene 1	Gene 2
1	4	4	8000	4000
2	2	1	4000	1000

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## AQ and RQ Relationship

The relationship between RQ and AQ is one of proportionality:

$$AQ = RQ \times M$$

Where M = Multiplier for absolute calibration

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## Calibrator

The calibrator is a sample used to set the quantity unit of the data set.

Calibrators may either be Relative or Absolute.

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## Relative Calibrator

A relative calibrator is assigned an RQ value of 1 and all other sample RQ values are relative to that of the calibrator.

The only purpose of a relative calibrator is to make data interpretation easier.

Virtually any sample may be selected as a relative calibrator, provided both the target and normalizer amplified.

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## Relative Calibrator Example

In the example below, calibrating to sample 1 resulted in most RQ values being less than 1, making sample-to-sample comparisons difficult.

Sample 4 had the lowest RQ, so recalibrating to sample 4 makes sample comparisons easier.

Sample	RQ rel to sample 1	RQ rel to sample 4
1	1.000	5.681
2	0.381	2.163
3	0.653	3.712
4	0.176	1.000

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## Absolute Calibrator

An absolute calibrator is assigned an AQ value based on its molecular concentration of the target.

In this hypothetical example:

1 RQ = 1000 molecules target/sample mass unit.

$$RQ \times M = AQ$$

Sample	RQ	AQ
Abs Calibrator	1.000	1000
2	0.381	381
3	0.653	653
4	0.176	176

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## Assigning Absolute Calibration

Creating an absolute calibrator may involve measuring the number of target molecules in cloned or artificial templates.

Measuring the target molecules is a 3-step process:

1. Measure OD<sub>260</sub> for pure template.
2. Convert OD to mass concentration.
3. Convert mass to molecules.

$$1OD_{260} = \begin{matrix} 50\mu\text{g/ml dsDNA} \\ 33\mu\text{g/ml ssDNA} \\ 40\mu\text{g/ml RNA} \end{matrix}$$

$$\frac{\text{Mass (g)}}{\text{Volume}} \times \frac{6.02 \times 10^{23}}{\text{Length} \times 330 \text{ or } 660^*} = \frac{\text{Molecules}}{\text{Volume}}$$

\*330 if single stranded or 660 if double-stranded.

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## Example of Absolute Calibrator for Gene Expression

Absolute quantification for RNA targets requires correction for reverse transcription efficiency, which usually necessitates the use of *in vitro* transcribed RNA for the target.

An absolute calibrator for gene expression may consist of a mixture of *in vitro* transcribed RNA and total RNA in a known ratio:

$$\frac{\text{molecules Target } in \text{ vitro RNA}}{\text{mass total RNA}}$$



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## Example of Absolute Calibrator for Genomic DNA

In this case, the absolute calibrator is DNA from a transgenic mouse known to have two copies of the transgene/diploid genome: AQ = 2.

Mice with only 1 transgene copy/diploid genome should have AQ values near 1.



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## ΔΔCt Software

Most Applied Biosystems real-time PCR instruments come with software that performs ΔΔCt analysis.

ΔΔCt plates must be set up with:

- At least two samples.
- At least one Target.
- At least one Normalizer on the same plate as the Target.

	1	2	3	4	5	6	7	8	9
A	Sample 1 Target	Sample 1 Target	Sample 1 Target	Sample 2 Target	Sample 2 Target	Sample 2 Target	Sample 3 Target	Sample 3 Target	Sample 3 Target
B	Sample 1 Normalizer	Sample 1 Normalizer	Sample 1 Normalizer	Sample 2 Normalizer	Sample 2 Normalizer	Sample 2 Normalizer	Sample 3 Normalizer	Sample 3 Normalizer	Sample 3 Normalizer

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## ΔΔCt Software Analysis

When setting up the ΔΔCt software for analysis:

1. Each assay must be identified as either a Target or Endogenous Control (Normalizer).
2. All Target and Endo wells belonging to the same sample must be identified with the same sample name.

Task

Target

Target

Endogenous Control

	1	2	3
A	Target Sample 1	Target Sample 1	Target Sample 1
B	Normalizer Sample 1	Normalizer Sample 1	Normalizer Sample 1

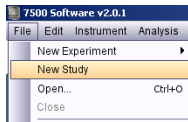
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## ΔΔCt Study

A Study is a collection of one or more plates of data analyzed together using the ΔΔCt Method.

Studies are created from the real-time PCR software main menu, with one exception: the 7900HT software versions 2.3 and 2.4 use a separate application called the "RQ Manager" to do this.



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## DataAssist™

DataAssist™ is free ΔΔCt analysis software for gene expression that may be downloaded from the Applied Biosystems website:

Support > Software Downloads > DataAssist™.

Exported Ct values are imported into DataAssist™.

Assay	Type	Brain (Ct)	Brain (P-value)	Liver (Ct)	Liver (P-value)
β2M	Switched Control	3.0	3.0	3.0	0.1601
β2M3	Target	3.0	3.0	0.4614	0.2861
PKNO2	Target	3.0	3.0	0.4998	0.172
OSCR	Target	3.0	3.0	0.4025	0.4056
OSSEP	Target	3.0	3.0	3.1661	0.7079
SEPPW61	Target	3.0	3.0	11.7324	0.2055



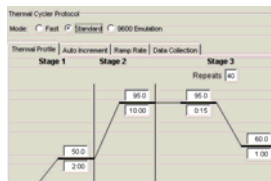
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## ΔΔCt Study Rules

Multiple ΔΔCt plates may be added to the same Study, provided:

- The files are from the same instrument model.
- The plates are the same type, e.g., 96 vs. 384 wells.
- The cycling conditions are the same.
- The same ramp mode is used.



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## Calibrator on One Plate

In the ΔΔCt software, the calibrator is only on one plate of the Study. This method offers 3 key advantages:

- a) A relative calibrator does not need to be chosen ahead of time.
- b) The choice of a relative calibrator may be changed at any time.
- c) Fluctuations due to plate specific calibration are eliminated.

Without a calibrator on each plate, the Study sets the same threshold across all plates for the same target.

Gene Expression

Calibrator Sample: Sample 1

Endogenous Control Detector: D

Control Type:  Multiplexed  Nonmultiplexed

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## Replicates

Replicates fall into two main categories:  
Technical replicates are repetitions of the same sample.  
Biological replicates are different samples from the same treatment group.

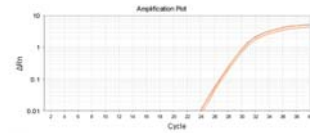


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## Technical Replicates

Technical replicates offer a number of benefits:  
-Rescue sample data when one replicate fails to amplify.  
-Allow assessment of system precision, e.g., pipetting precision.  
-Reduce effect of system precision on the final results.  
Duplicates and triplicates are used very commonly.



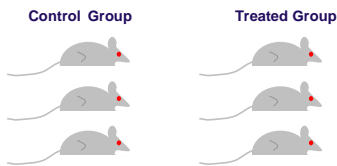
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## Biological Replicates

Biological replicates are needed in gene expression experiments to determine if the observed change in expression could be explained by biological variation: natural variation individual-to-individual.

A minimum of 3 biological replicates per group is generally recommended, but 3 may not be sufficient.



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## Controls

No Template Controls (NTC) consist of master mix, but no template.  
NTC's test for template contamination.

No Reverse Transcription Controls (No RT) consist of PCR master mix and RNA, with no reverse transcription.

No RT Controls measure genomic DNA contamination for gene expression experiments.

Contamination of 1% quantity or less is considered mathematically insignificant.

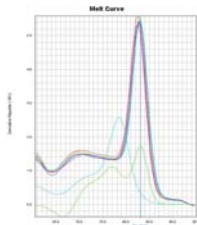
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## Basic Analysis Steps

Step 1. For SYBR® Green assays, evaluate the melt data for each well. This step is not done for TaqMan® probe-based assays.

Wells with melt anomalies should be omitted.



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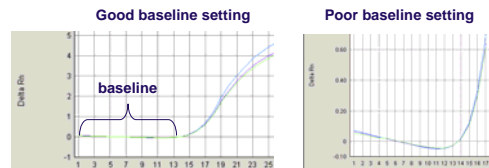
## Setting Baseline

Step 2. On the amplification plot using a linear y-axis scale, evaluate whether the baseline is flat or tilted.

If tilted, the baseline needs adjustment.

The baseline may be set automatically or manually.

Manual baseline defaults to 3-15; reduce 15 if needed.

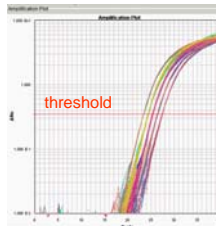


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## Setting Threshold

Step 3. On the amplification plot using a log y-axis scale, evaluate the threshold position and adjust if needed. The threshold should intersect the geometric phase, but not too close to the baseline.

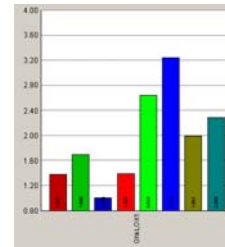


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## Relative Quantity Plot

The  $\Delta\Delta Ct$  software plots the Relative Quantity values. The results may be exported for any final analyses.

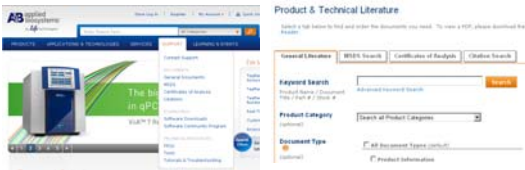


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## Support Documents

For further reading, the Applied Biosystems website offers many documents free to download: [www.appliedbiosystems.com](http://www.appliedbiosystems.com)



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## Survey

As part of our effort to continually improve training, we would like you to take a quick survey, consisting of 12 short questions.

<http://tinyurl.com/LIFEqPCR>



We Want to Hear From  
Your needs are important to us! Please take a few minutes to tell us about your training experience with Applied Biosystems. There is a space for comments after each section, so we encourage you to offer suggestions on how we can improve your experience. Your feedback is confidential. (Fields marked with an \* are required.)

Thank you!

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