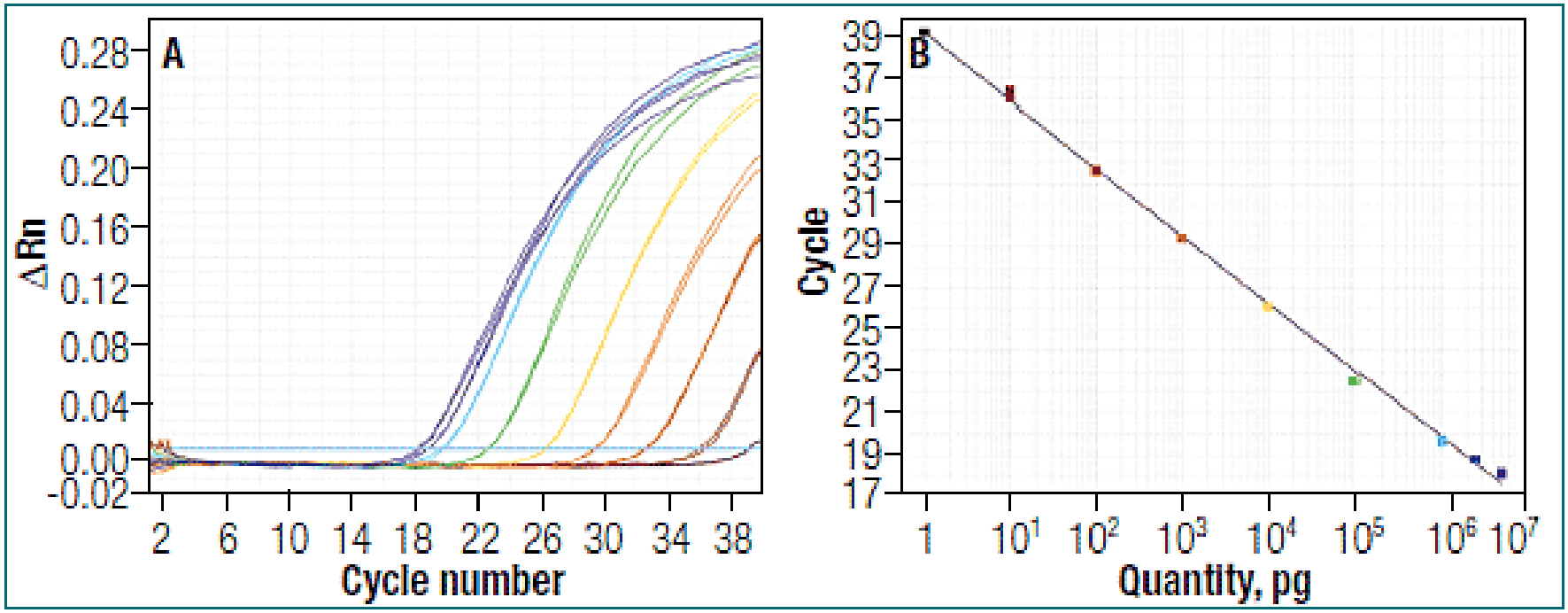


qPCR Experiment and Analysis

Optimizing and Analyzing qPCR

Standard Curve

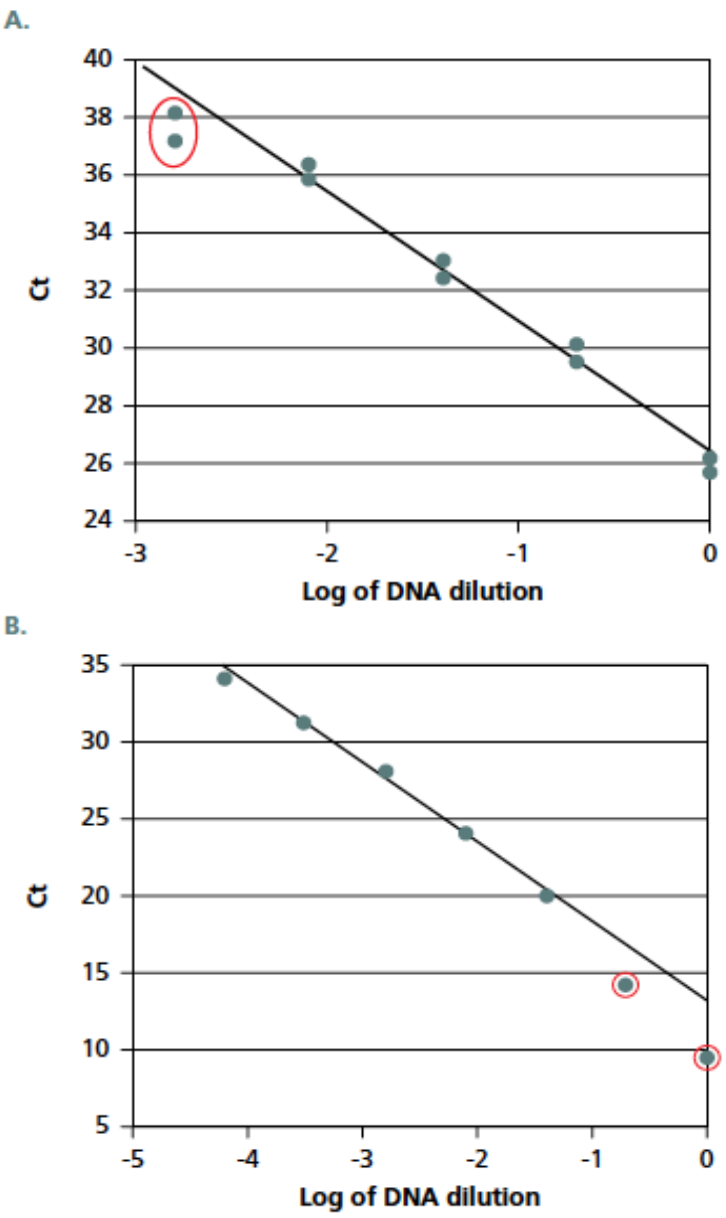


$2^x = 10 \quad x = 3.32$

A 1:10 dilution will take 3.32 cycles longer to reach C_t assuming ideal replication efficiency (doubling once every cycle)

Ideal dilution range

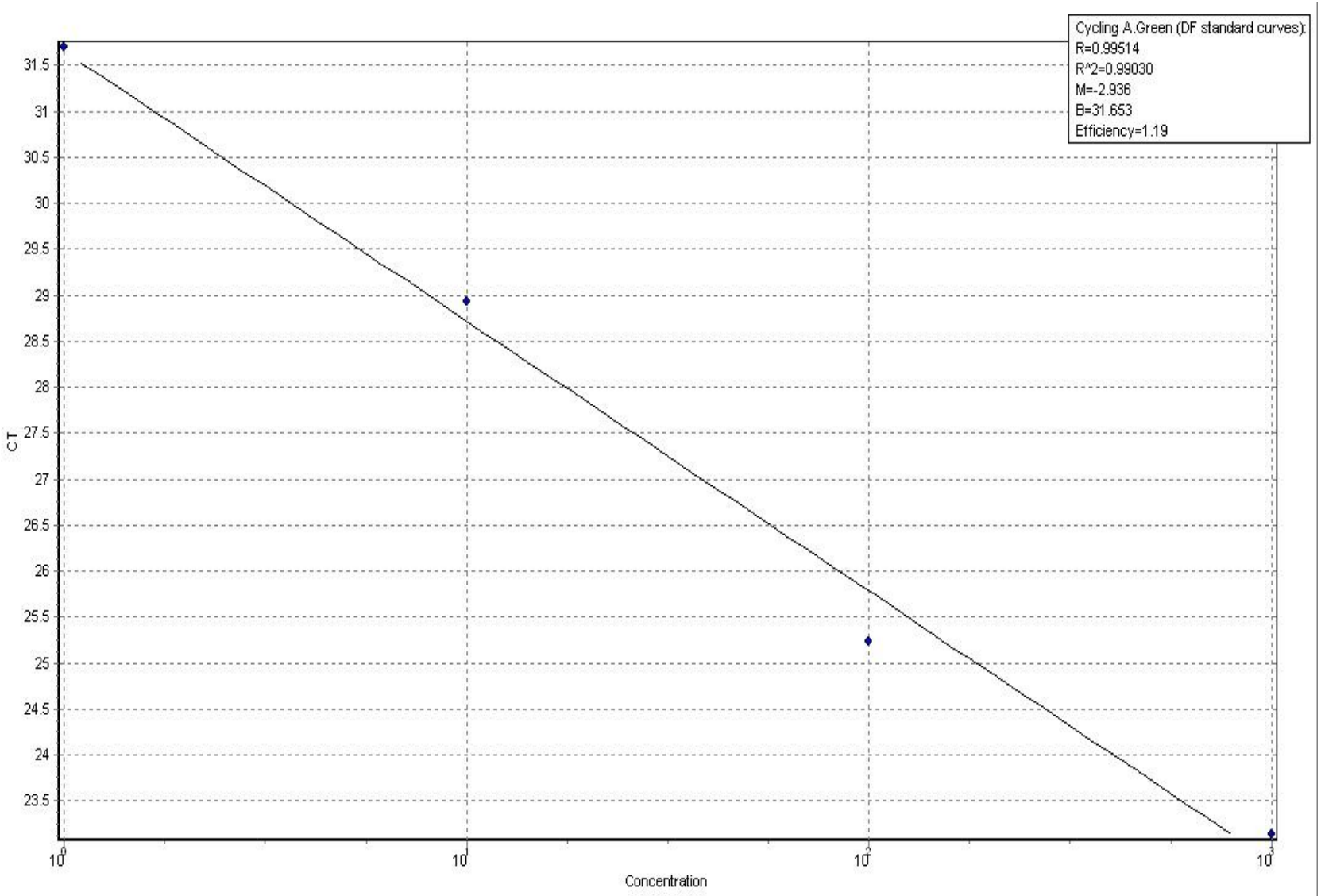
Figure 11. Use of Standard Curves to Evaluate qPCR Optimization



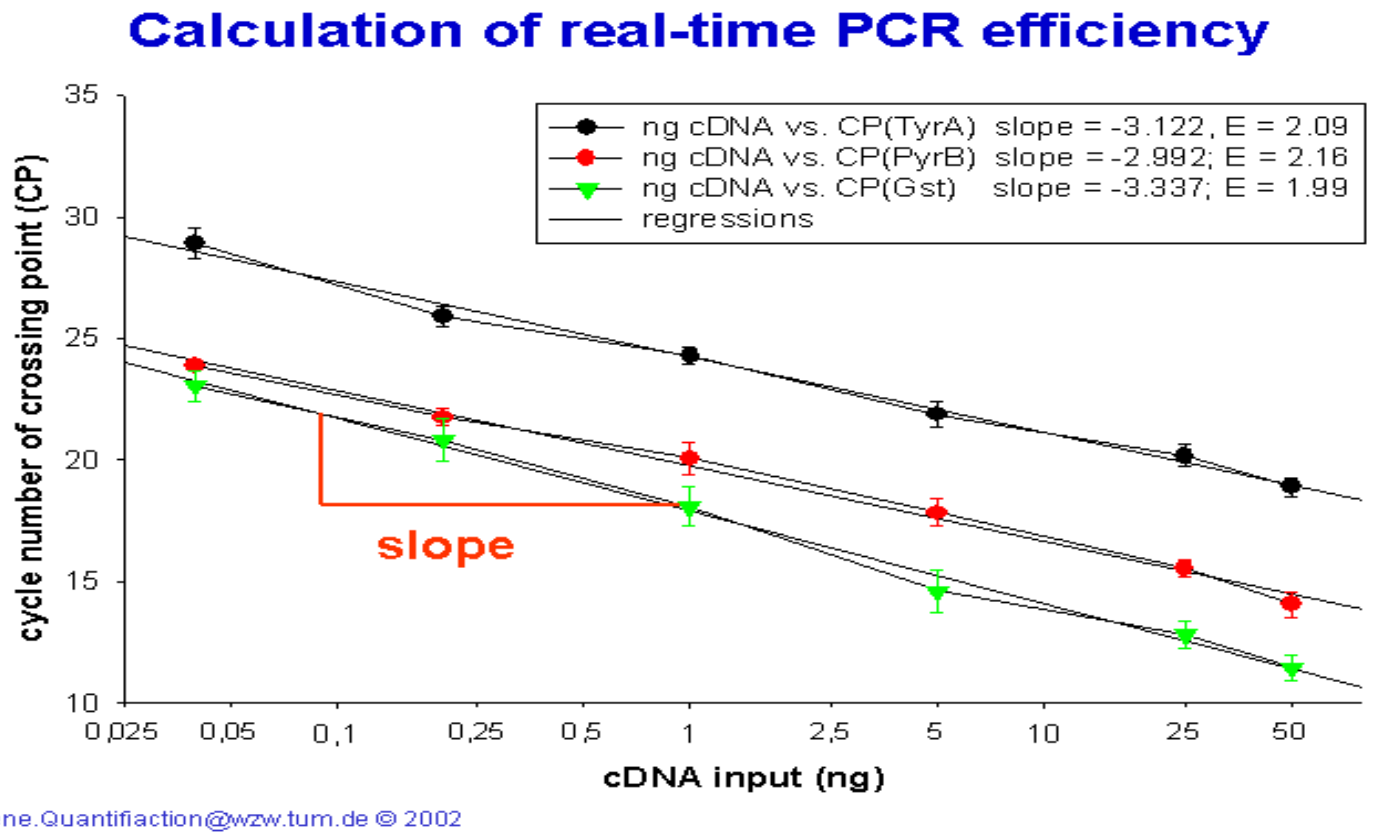
Exceeded assay sensitivity

Reaction is saturated (entering linear phase)

Coefficient of Determination (R^2) measures how well the data fits a straight line



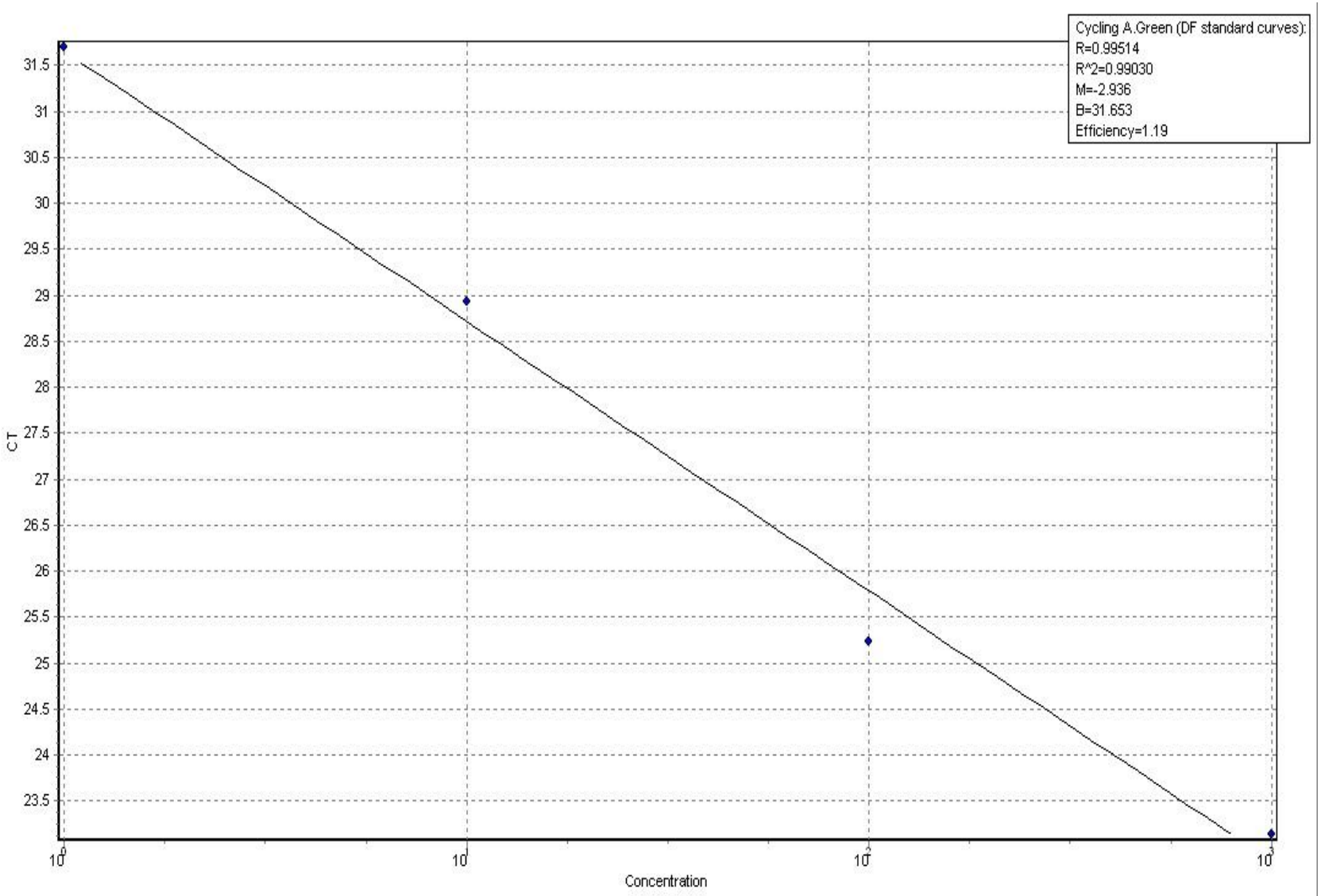
qPCR efficiency



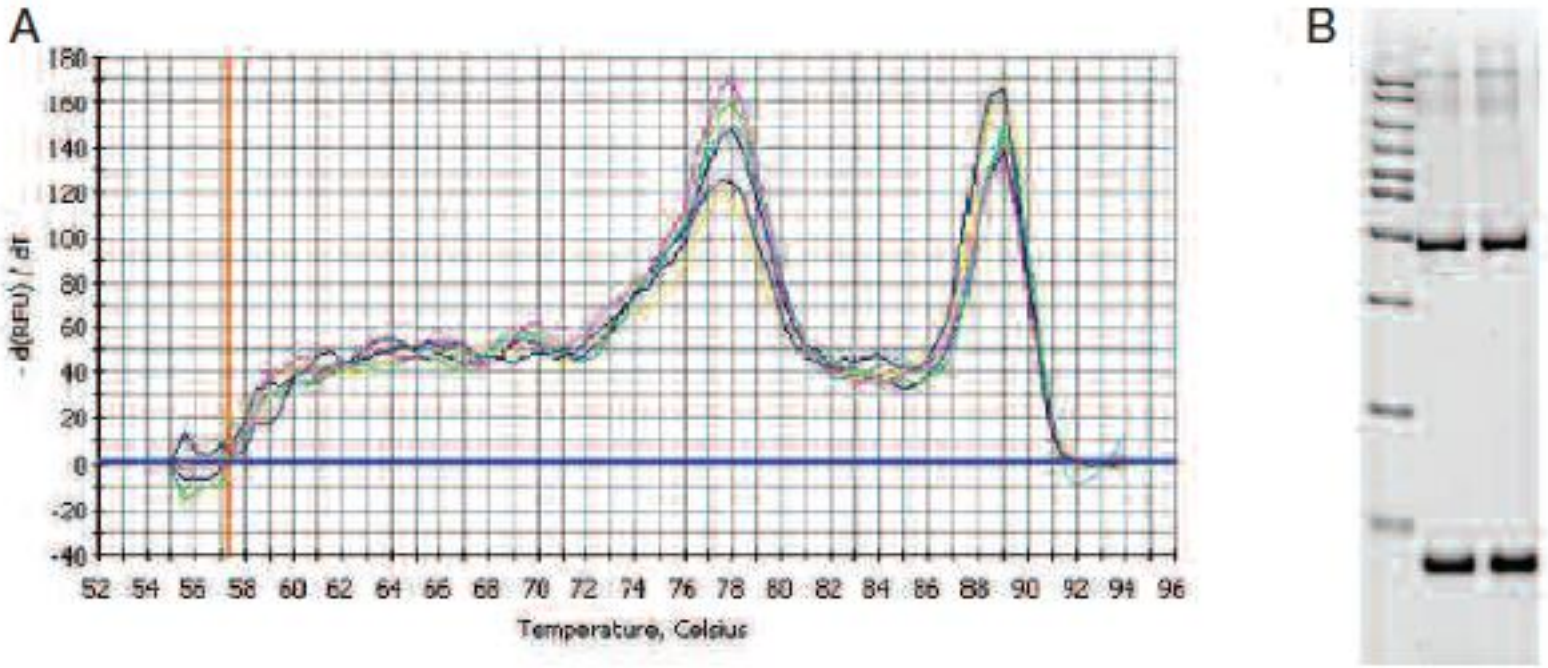
$$E = 10^{-1/slope}$$

$$\% \text{ efficiency} = (E - 1) \times 100\%$$

Efficiency


Cycling A.Green (DF standard curves):
R=0.99514
R^2=0.99030
M=-2.936
B=31.653
Efficiency=1.19
CT
31.5
31
30.5
30
29.5
29
28.5
28
27.5
27
26.5
26
25.5
25
24.5
24
23.5
10^0
10^1
10^2
10^3
Concentration

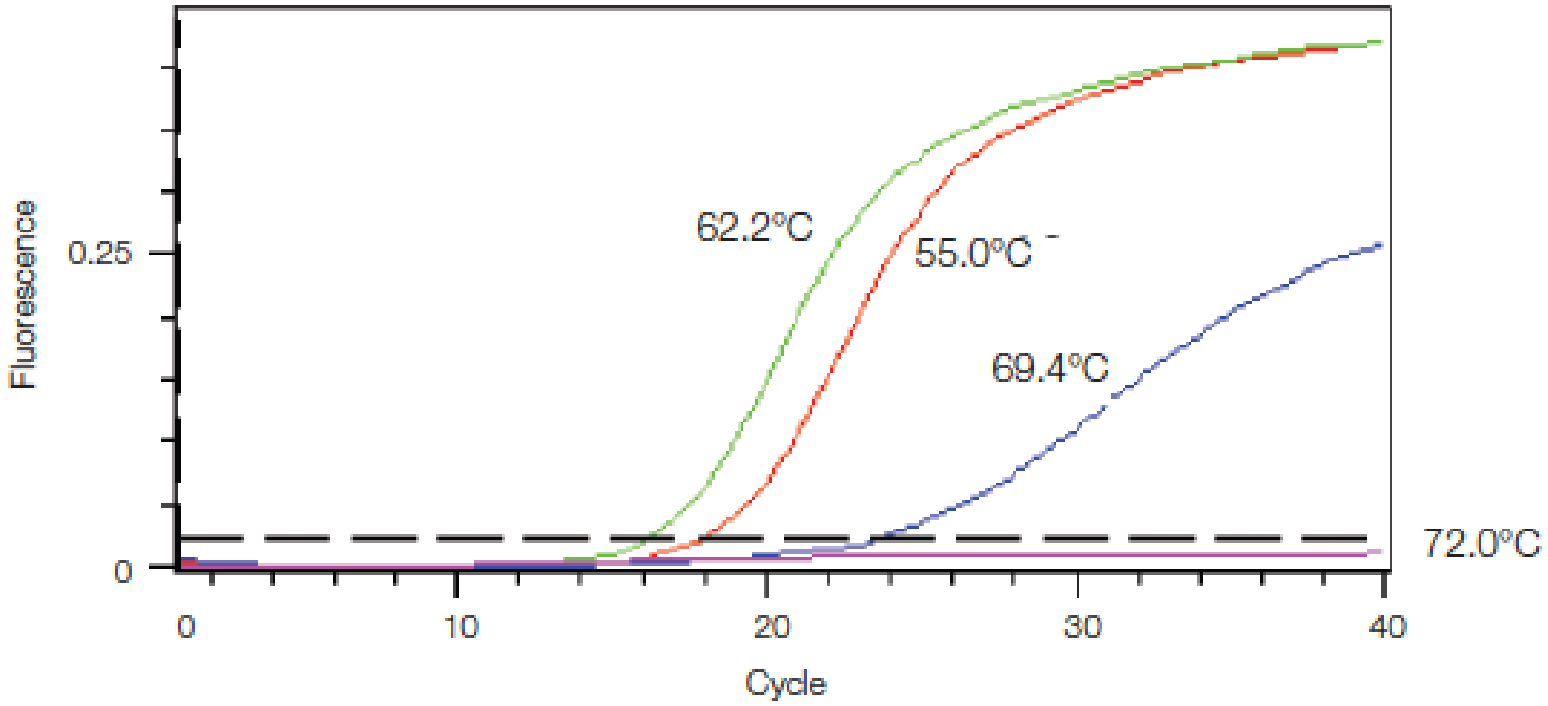
Melting curve



A melting curve tests whether the PCR produces only the desired product.

An assay with high specificity will give a single peak at the temperature it takes to release the double stranded product.

Annealing temperature optimization



One more precaution that can be taken to identify the optimum annealing temperature for your primers.

Data Analysis in qPCR

Absolute Quantification

Compare test sample to a standard curve of **known quantity**. Results give the quantity of nucleic acid (copy number, ug) per given amount of sample (per cell or per ug of RNA). Normalizes against a **standard curve of known quantity**.

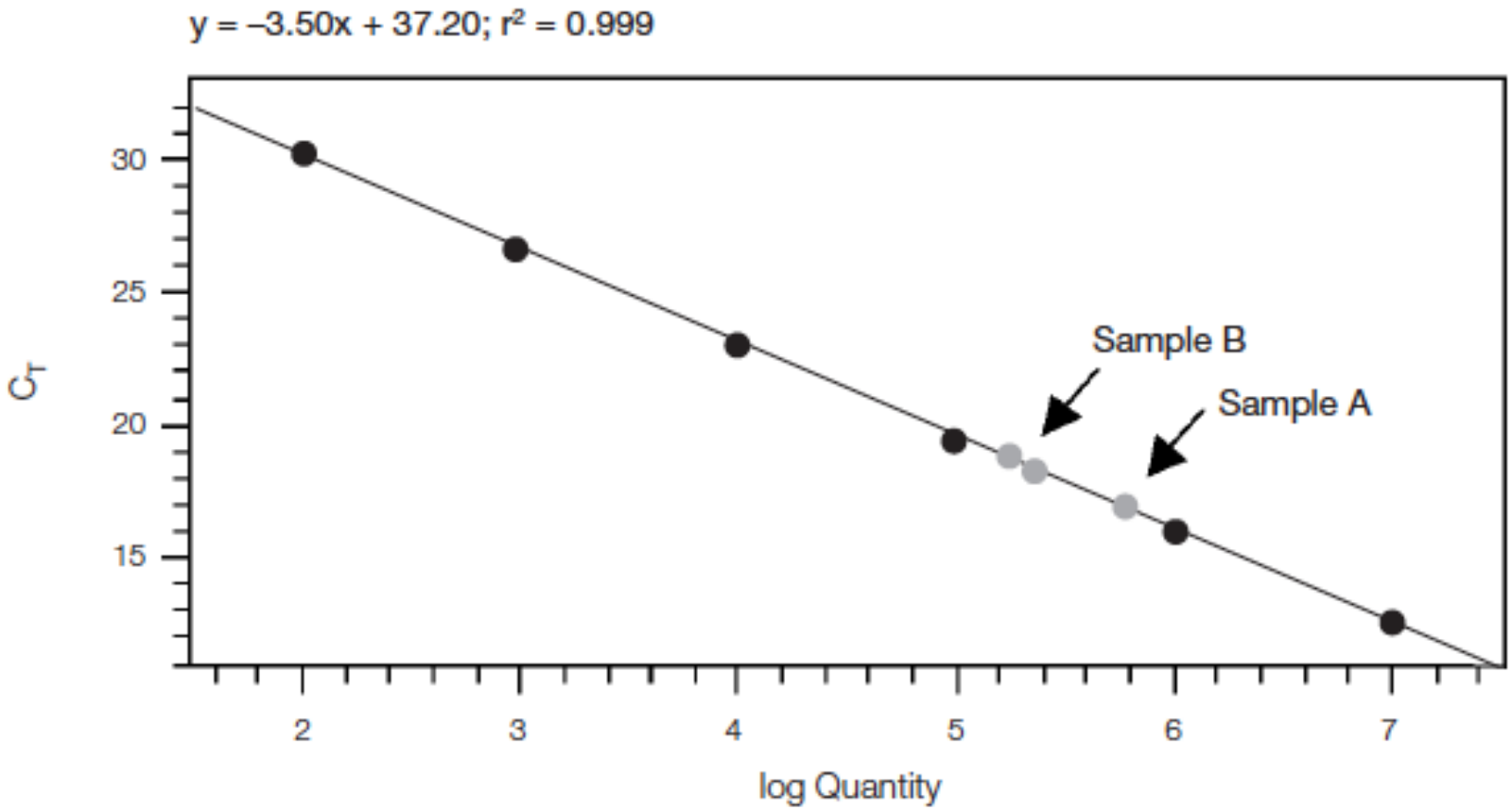
e.g. virus particles per ul of blood sampled

Relative Quantification

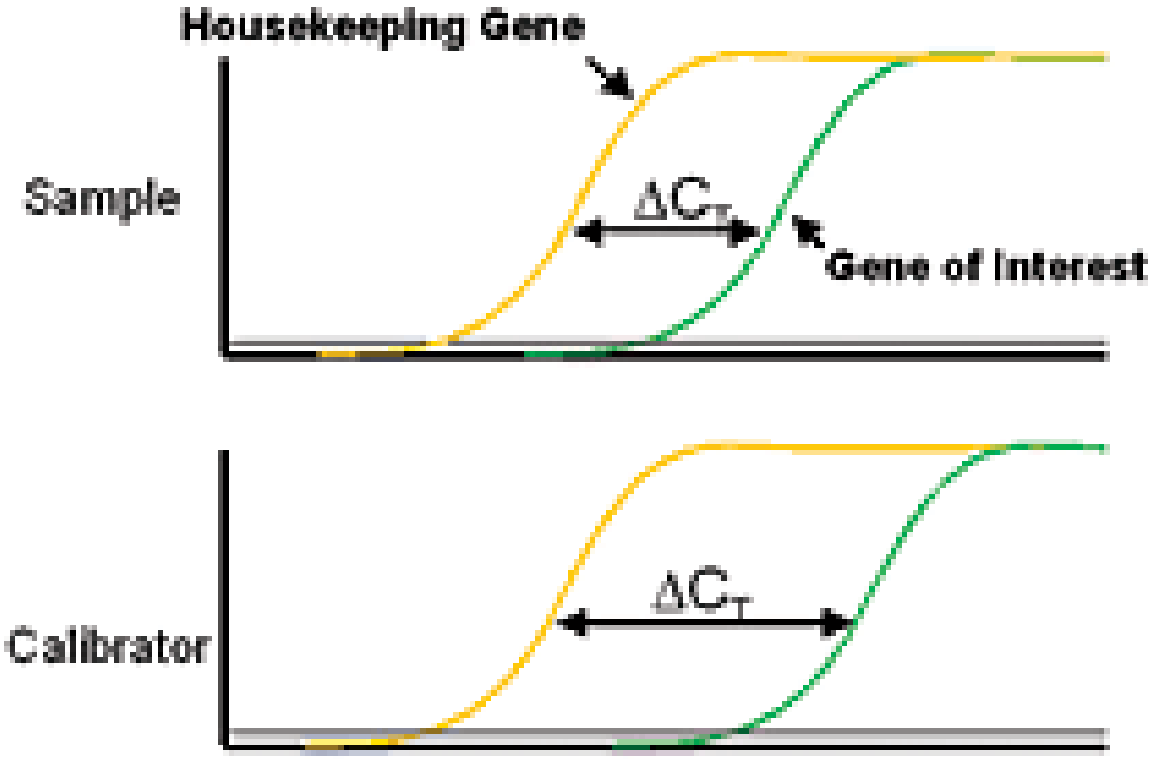
Compares the **relative amount** of nucleic acid between two samples of equal size. Results give the relative amount/expression of a gene between two samples. Normalizes against a **reference (housekeeper) gene**.

e.g. 3-fold decrease in p53 expression in cancer cell vs. normal cell

Absolute Quantification



Relative Quantification



Note: careful selection of an appropriate housekeeper gene is important!

For both absolute and relative quantification methods, quantities obtained from a qPCR experiment must be normalized in such a way that the data become biologically meaningful. This is done through the use of **normalizers**.

4.2.1 When Should Relative Quantification Be Used?

A researcher wants to know whether the expression level of p53 differs between cancerous and normal ovarian cells, and if so, by how much. He wants to find out the relative amount, sometimes called the “fold difference”, of p53 mRNA in equivalent amounts of the two tissues.

What can the researcher normalize his data too?

1) He can start with the same amount of material: **1,000 cancer cells and 1,000 noncancerous cells.**

2) He can normalize to a product that is unaffected by cancer: **GAPDH mRNA**

What are the advantages or disadvantages of each normalizer?

Relative Normalized Against Unit Mass

To determine the relative expression of p53 in cancerous vs. noncancerous. In this case total RNA would be prepared from an equal number of normal and cancerous cells.

$$\text{Ratio}_{(\text{test/calibrator})} = E^{C_T(\text{calibrator}) - C_T(\text{test})}$$

If E is ~80-100%, you can assume that with each cycle doubling occurs and the equation can be written this way:

$$\text{Ratio}_{(\text{test/calibrator})} = 2^{C_T(\text{calibrator}) - C_T(\text{test})}$$

Relative Normalized Against Unit Mass

Sample	C_T for p53 (target)
Normal (calibrator)	15.0
Tumor (test)	12.0

$$\text{Ratio}_{(\text{test/calibrator})} = 2^{C_T(\text{calibrator}) - C_T(\text{test})}$$

$$= 2^{(15 - 12)}$$

$$= 8$$

According to this data, the expression of p53 is 8-fold higher in tumor cells than in normal cells.

If we ignore our reference genes and assume that we took equal amounts of treated vs untreated cells we can calculate our fold difference this way.

Relative Normalized to a Reference Gene (ΔC_T)

In this case, the expression levels of both the target and the reference genes need to be determined.

Now we can compare the C_T values with the calibrator (normal cells) and a reference (GAPDH).

Sample	C_T p53 (target)	C_T GAPDH (reference)
Normal (calibrator)	15.0	16.5
Tumor (test)	12.0	15.9

Relative Normalized to a Reference Gene (ΔC_T)

Sample	C_T p53 (target)	C_T GAPDH (reference)
Normal (calibrator)	15.0	16.5
Tumor (test)	12.0	15.9

Step 1) Normalize your target gene to your reference in both the test and calibrator.

$$2^{(C_T(GAPDH) - C_T(p53))} = \text{Expression}$$

For normal cells, this yields $2^{(16.5 - 15)} = 2.8$

For tumor cells, this yields $2^{(15.9 - 12)} = 14.9$

Step 2) Evaluate the ratio of test expression to calibrator expression

Normal expression = Normal/Normal = 2.8/2.8 = 1

Tumor expression = Tumor/Normal = 14.9/2.8 = 5.3

5.3-fold higher expression

Your figures

- Standard Curves (Showing R and E)
- Melting Curves (label the peaks)
- A table summarizing your Cts and ratios.

Example Table

Table 6. Summary of RT-qPCR
This table shows the Ct values for the five selected genes. It is shown in both the treated and untreated samples. The fold difference was also calculated and demonstrated a downregulation in gene expression. There was a software error that could not be corrected and some values remain unknown.

Values	GSS	E2F2	CACNA1A	CD31	GJC1	MCU
Untreated Ct (get this from your report)	22.60	36.47	?	31.74	29.76	?
Treated Ct	21.46	33.20	33.83	29.78	28.31	?
Untreated Normalized Expression (use $2^{(\Delta Ct\ GSS\ and\ Gene)}$)		14972.21448	?	564.175	143.013	?
Treated Normalized Expression (use $2^{(\Delta Ct\ GSS\ and\ Gene)}$)		3420.5201	5293.4772	319.5726	115.3601	?
Fold difference (calculate the ratio Exp Treated/ Exp Untreated)		0.22845	?	0.5664	0.80664	?

Controls for qPCR

- standard curve of known quantity (absolute quantitation)
- control/unexposed sample (relative quantitation)
- reference gene (relative quantitation)
- no template control sample
- no RT control sample

qPCR Experimental Design

Exposed and Unexposed Samples (you will set up 11 reactions for each template).

1) HSP90, using a 1:10 dilution of your RT-PCR template

2) HSP90, using a 1:100 dilution of RT-PCR template

3) HSP90, using a 1:1000 dilution of RT-PCR template

4) HSP90, using a 1: 10,0000 dilution of RT-PCR template

5) GSS (a secondary housekeeping control), use the 1:10 dilution of your template

6-11) Genes 1-5, each reaction will have only one gene set and you will use the 1:10 dilution of your template.

NoT Control) In place of the template, you will add 2µl of PCR grade water.

qPCR Reaction Mixture

Prepare the following qPCR reaction:

7μl PCR grade water

2μl Template (appropriate dilution of cDNA, add this volume of water for your No template- control)

1.75μl Forward Primer solution (stock concentration is 1μM)

1.75μl Reverse Primer solution (stock concentration is 1μM)

12.5μl Absolute Blue qPCR SYBR Green ROX mix (contains SYBR green, DNA polymerase, dNTPs)

25μl

Final Thoughts

- Final Experiment Proposals
- Primer preparation
- Program for qPCR analysis (Rotor Gene Q-Qiagen)
  - https://www.qiagen.com/us/shop/automated-solutions/pcr-instruments/rotor-gene-q/#resources
  - Choose "Operating Software(2)"
  - The first download is compatible with Windows XP and 7
  - You will need our serial number: R120815