



Diagnostics

# **Accurate Gene Expression Analysis with High Flexibility: Concepts and Developments**

**Dr. Oliver Geulen**

**Roche Applied Science**

**Research & Development, Penzberg**



# Gene Expression Analysis

## *Goals and Requirements*

- To quantitatively detect subtle changes in amounts of mRNA against a complex background
- To obtain reliable data that can be compared over a long period of time or between different experimental systems

### **Preliminary Requirements:**

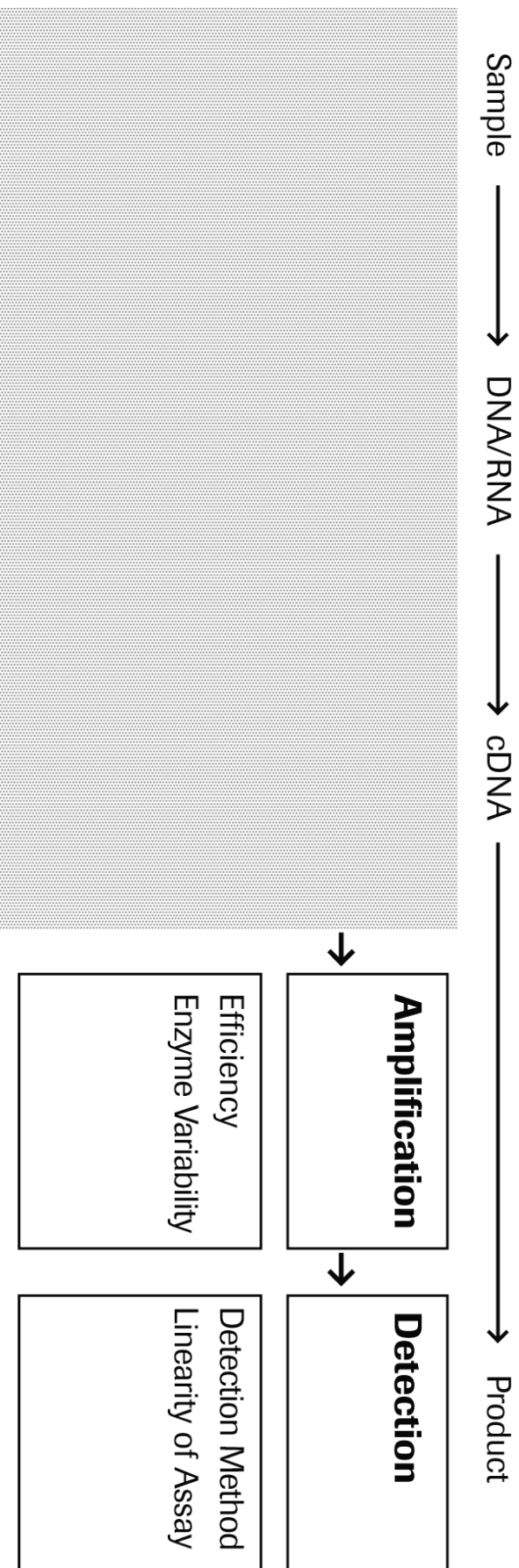
- **Accurate and reproducible measurements**
- **Steady quantitative analysis of data**

# RT-PCR Quantification



Diagnostics

## *Influencing Factors*





Diagnostics

# LightCycler® Systems

## *Technological (r)evolutions*



# LightCycler® 480 System



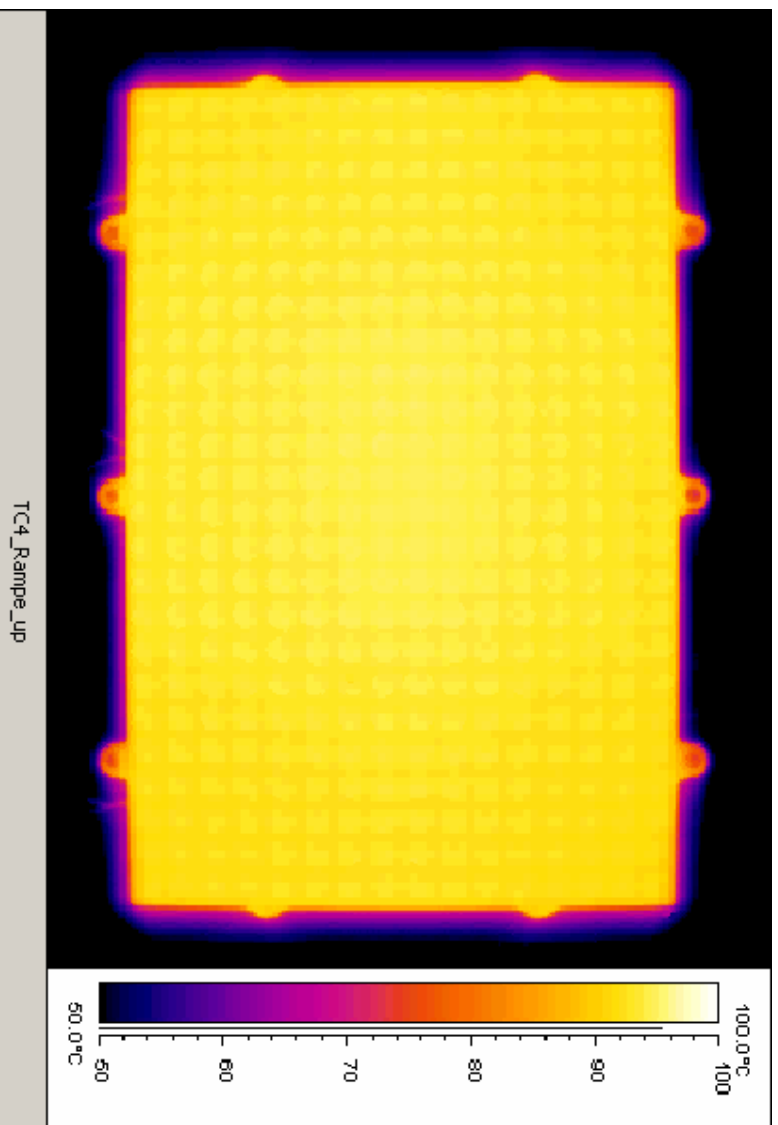
Diagnostics

## *Summary*

- Newly developed, high-throughput system for real-time PCR.
- Compact benchtop instrument for 96- and/or 384-microwell plates.
- Optimized heating and cooling technology for increased speed and maximized temperature uniformity.
- Specifically developed optical system for maximized sensitivity and the uniform collection of signals across the plate.

# Standard Block Cycler

## *Ramping up Temperature*



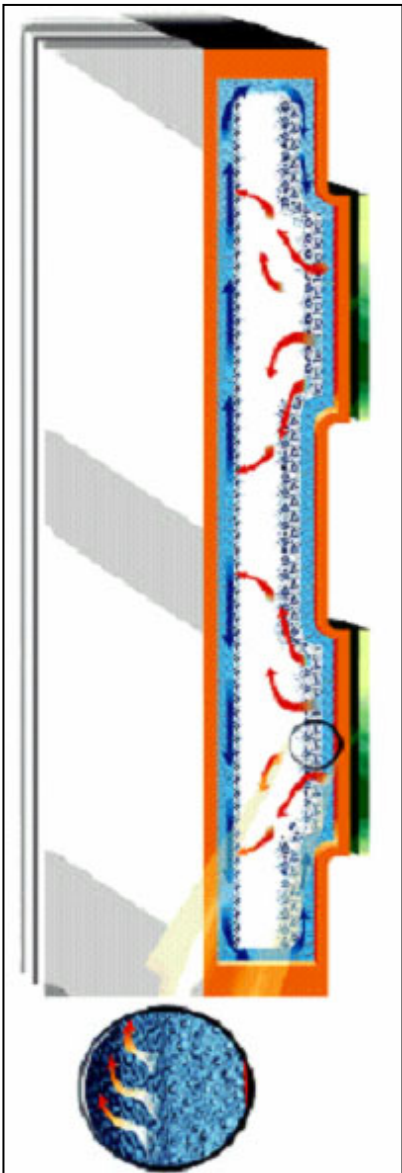
Diagnostics

# LightCycler® 480 Blockcycler

*Therma-Base™*



Diagnostics

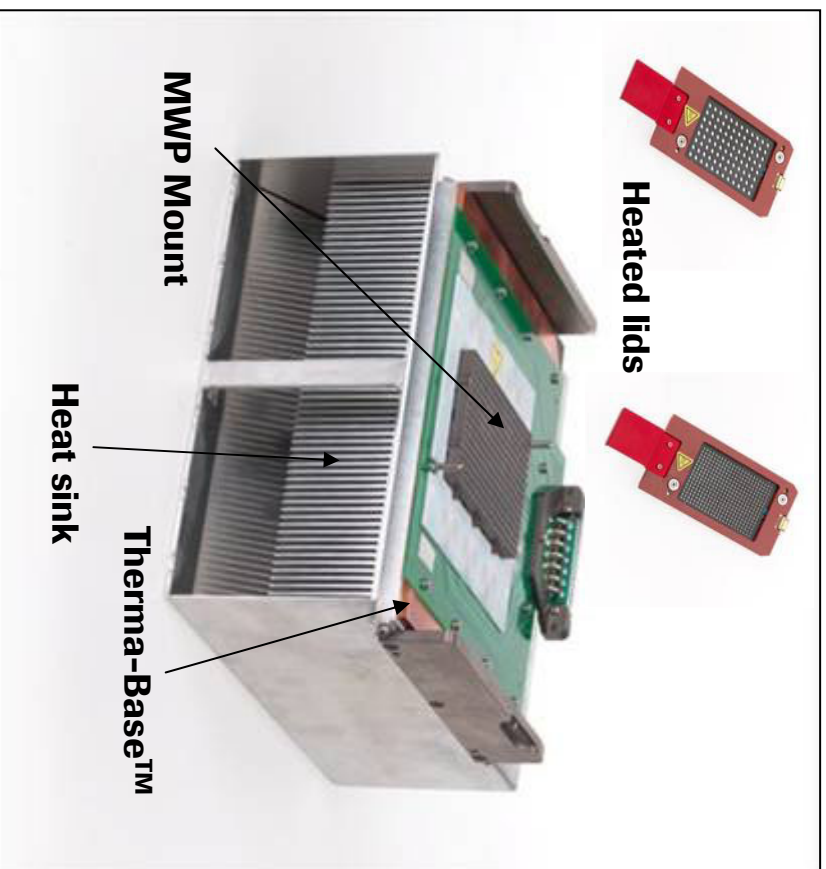


- vacuum chamber saturated with working fluid
- wick structure lining inside walls
- hot location: evaporation into vacuum
- cool location: condensation with heat release

# LightCycler® 480 System Thermocycler



Diagnostics

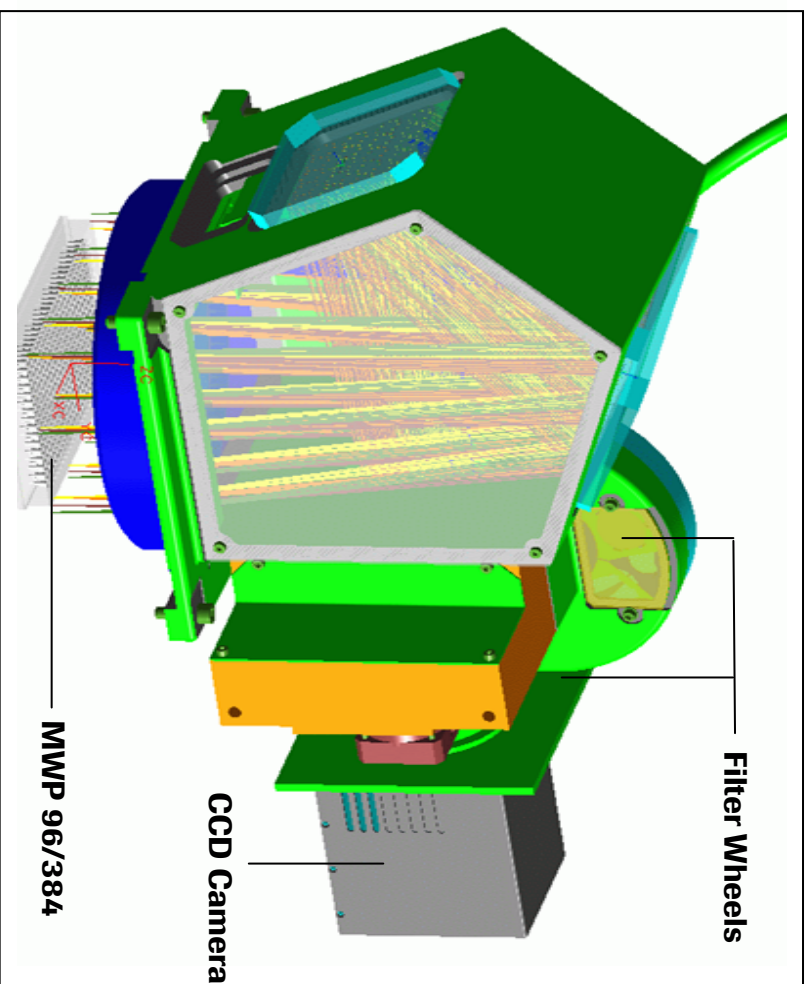


- Includes Thermo-Base™ for optimized heat exchange.
- Allows to finish a PCR run for 96 wells in < 1 hour and 384 wells in < 40 min.

# LightCycler® 480 Instrument Optical System



Diagnostics



- Homogeneous illumination and imaging due to long object-image distance.
- Pinhole to mask lateral portions of emitted light and focus on central, perpendicular portions.
- Large field lens to efficiently collect rays also from lateral wells.

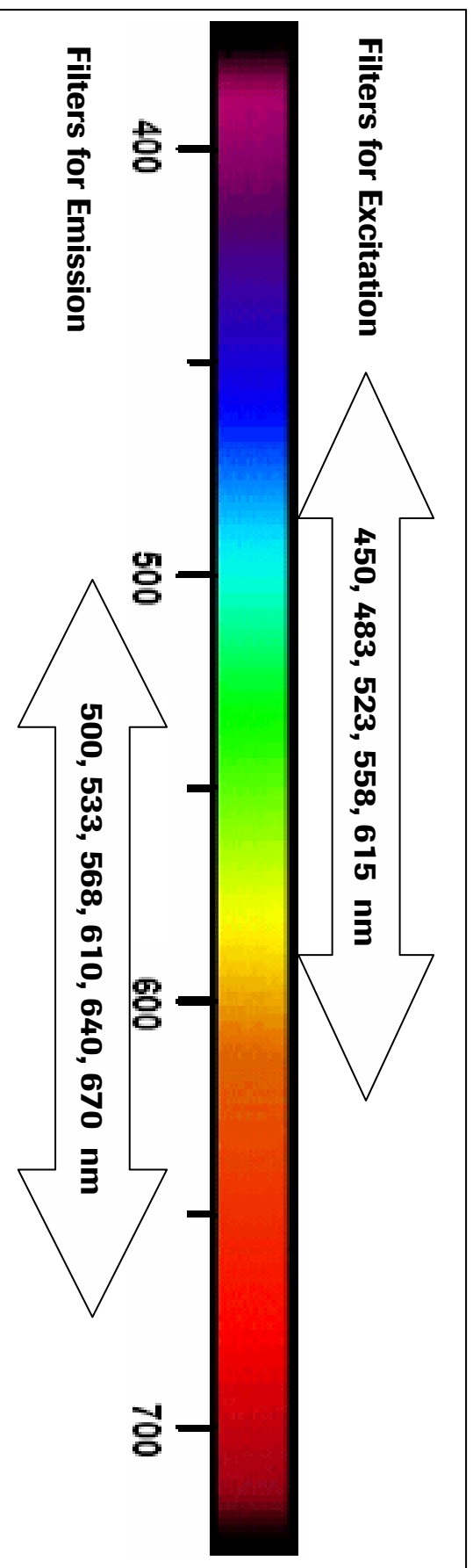
# LightCycler® 480 Instrument

## *Optical Properties*



Diagnostics

- **Lightsources: high intensity xenon lamp**
- **Lifetime: approx. 500-1000 h**



# Internal Reference Dye



Diagnostics

## **Internal reference dye is not required**

due to

- accurate data generation (thermoblock)
- data detection (optical system)
- data analysis (software)



Diagnostics

# LightCycler® 480 System

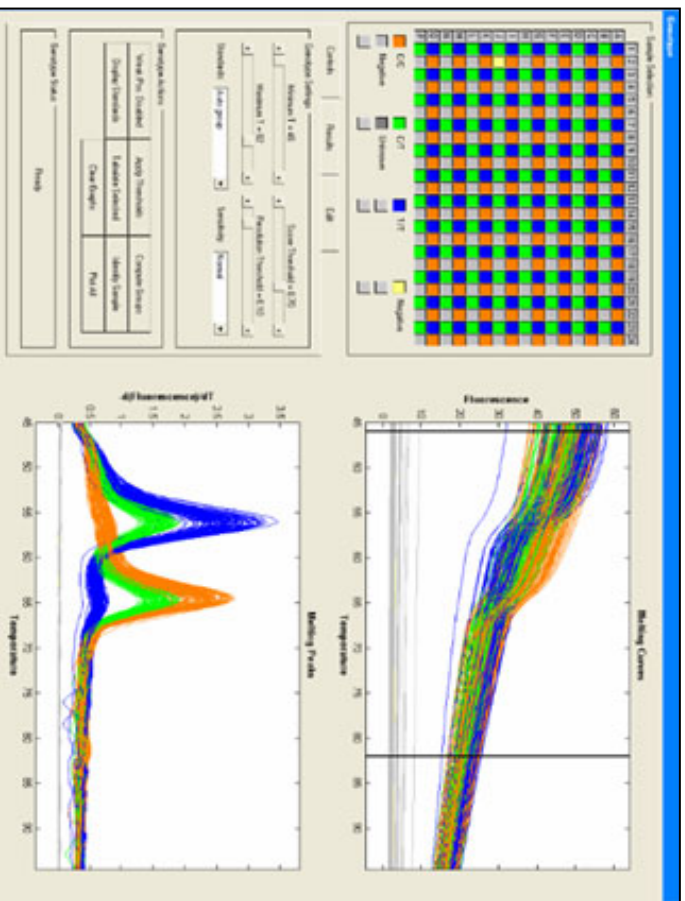
## Assay Formats, Dyes, and Application

Assay Format	Excitation (nm)	Detection (nm)	Dyes	Application
SYBR Green I	483	530	SYBR Green I	Qualitative Detection Product - Characterization Quantification
HybProbe Probes	483	533/ 610 533/ 640 533/ 670	Fluo - LightCycler® RED 610 Fluo - LightCycler® RED 640 Fluo - Cy5	Quantification SNP Analysis
Hydrolysis Probes	450 (+/-15) 483 (+/-17.5) 523 (+/-10) 558 (+/-15) 558 (+/-15) 615 (+/-15)	500 (+/-10) 533 (+/-10) 568 (+/-10) 610 (+/-10) 640 (+/-10) 670 (+/-10)	LightCycler® CYAN 500 FAM VIC/HEX LightCycler® RED 610 LightCycler® RED 640 Cy5	Quantification
SimpleProbe Probes	483	533	Fluorescein	SNP Analysis

# LightCycler® 480 Genotyping

## *Intra-Instrument Reproducibility*

MDR-1 C3435T polymorphism; SimpleProbe Format.  
 Ninety-six replicates for each of the 3 different genotypes.



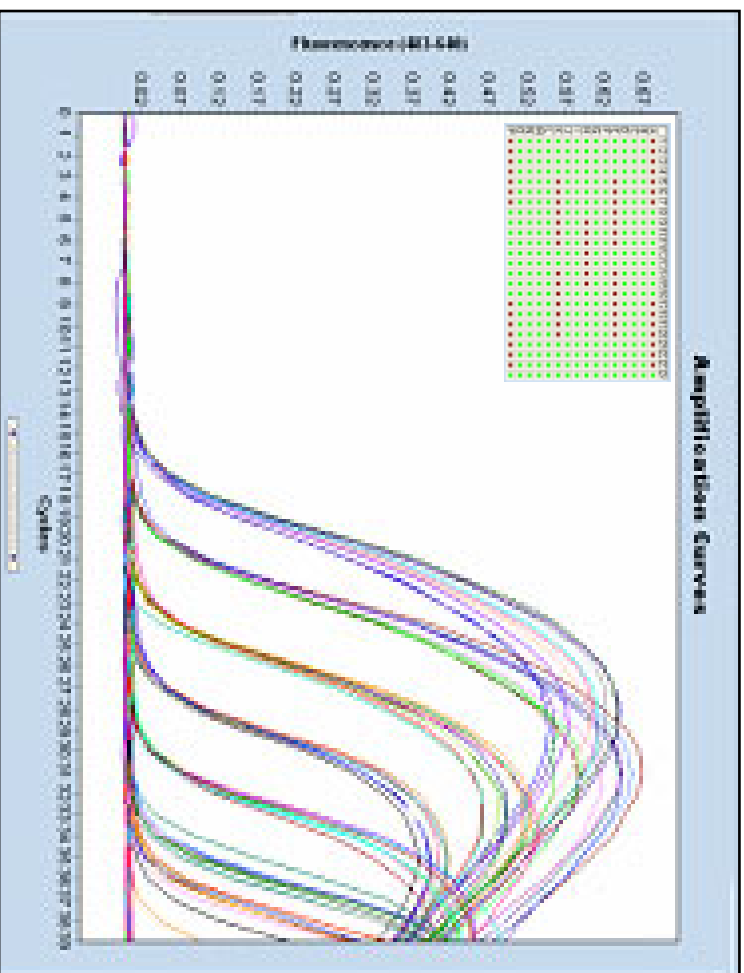
	$T_m(1)^{\circ}\text{C}$	$T_m(2)^{\circ}\text{C}$
<b>Average</b>	56.47	64.88
<b>Minimum</b>	56.14	64.67
<b>Maximum</b>	56.85	65.4
<b>Standard deviation</b>	0.1612	0.1801



# LightCycler® 480 qPCR Performance

## *Intra-Run Reproducibility*

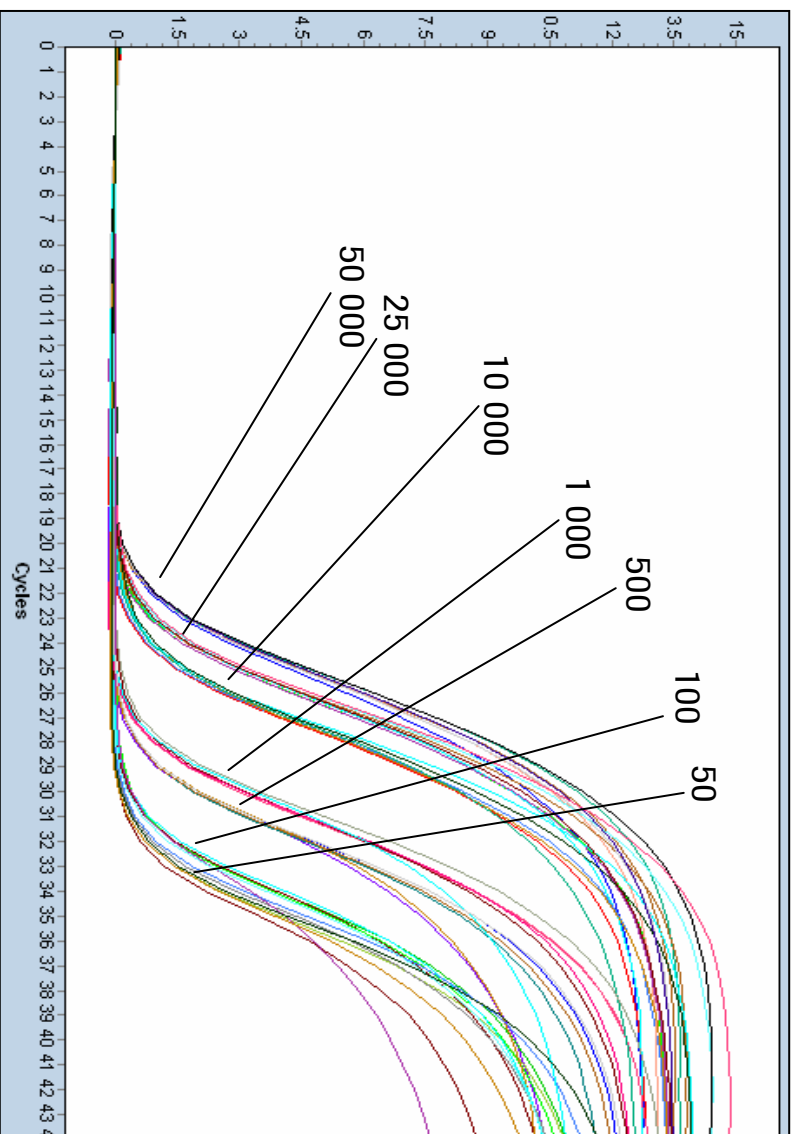
Viral target detected with HybProbe probes



Copies	Mean	SD
1.00E+06	17.77	0.0549
1.00E+05	20.85	0.0315
1.00E+04	24.13	0.0899
1.00E+03	27.31	0.0921
1.00E+02	30.85	0.1092
1.00E+01	n.a.	n.a.
1	n.a.	n.a.

# Accuracy of LightCycler® 480 System

## Discrimination of 500 and 1000 copies



- 7 replicates of human genomic DNA distributed over MWPs
- Hydrolysis Probe Format
- 196 bp amplicon

# Gene Expression Analysis

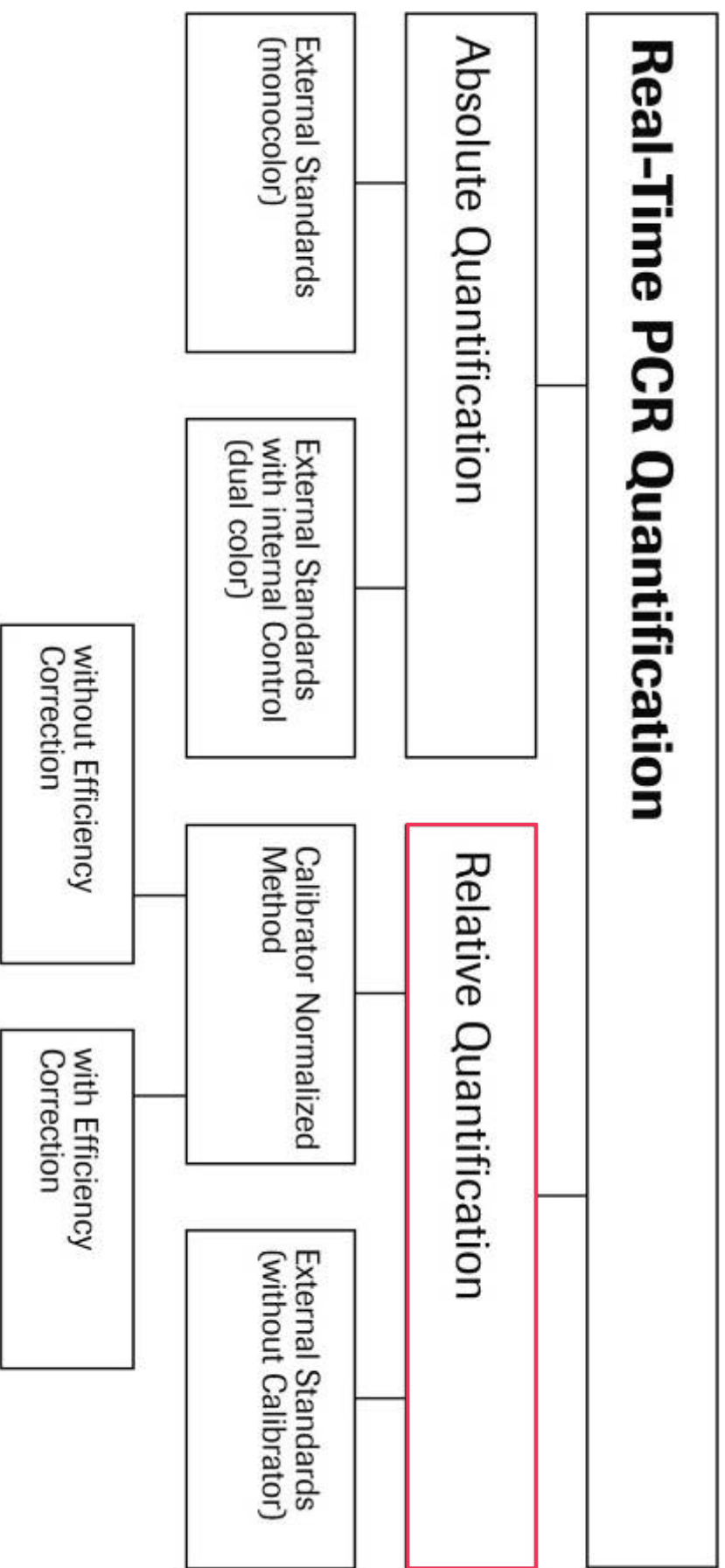
## *Goals and Requirements*

- To quantitatively detect subtle changes in amounts of mRNA against a complex background
- To obtain reliable data that can be compared over a long period of time or between different experimental systems

### **Preliminary Requirements:**

- **Accurate and reproducible measurements**
- **Steady quantitative analysis of data**

# Types of Quantification



# Relative Quantification

## *Relative Ratio*

$$\frac{\text{Amount of target RNA in a sample}}{\text{Amount of housekeeping RNA in a sample}}$$

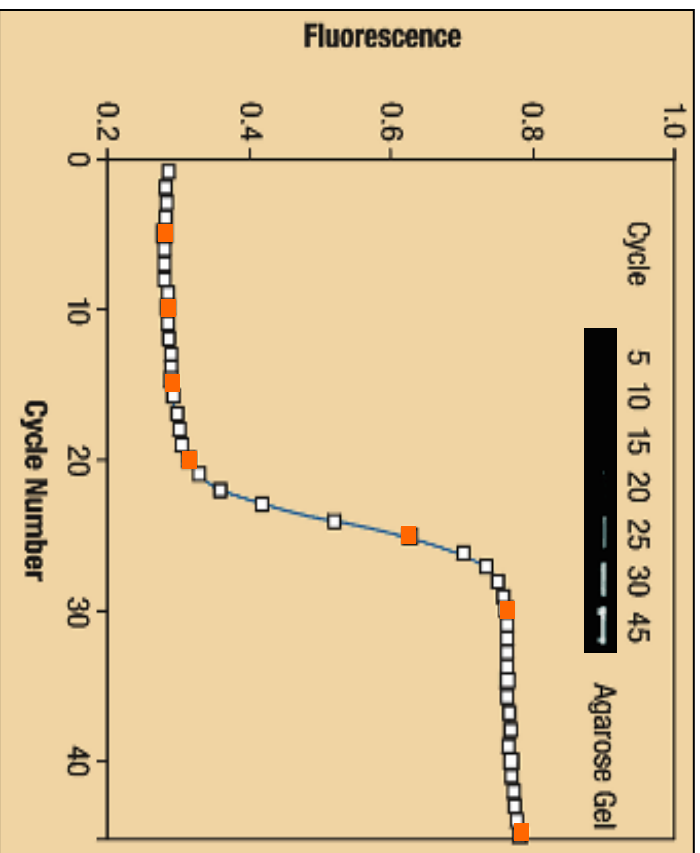
**For calculation:**

$$N = N_0 \times 2^n$$

- N number of amplified molecules
- $N_0$  initial number of molecule
- n number of amplification cycles

# Monitoring of PCR Reactions

## *From Block Cyclers to Real-Time PCR*



- Displayed amplification curve is influenced by
  - Detection format
  - Fluorescence dye
  - Reaction conditions, e.g. pH
- Generation of Cp/Ct is influenced by algorithm used



# PCR Efficiency

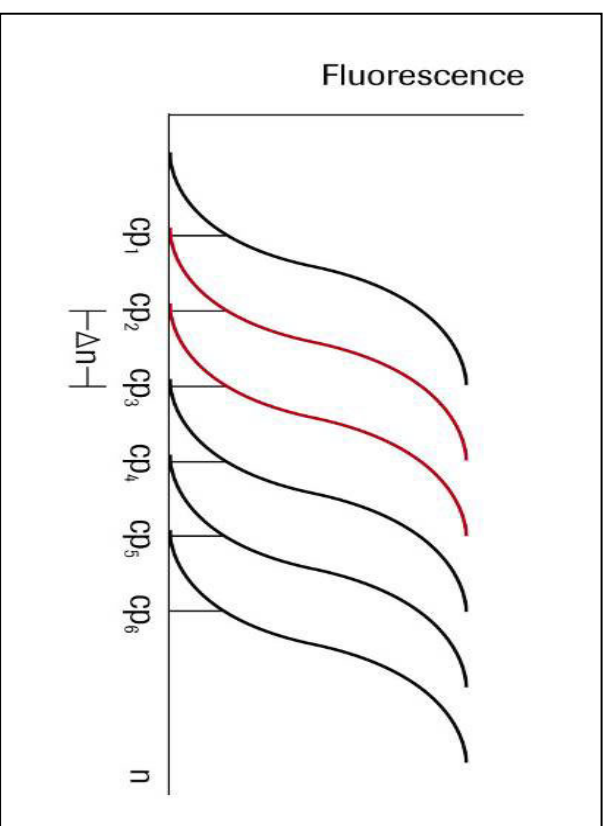
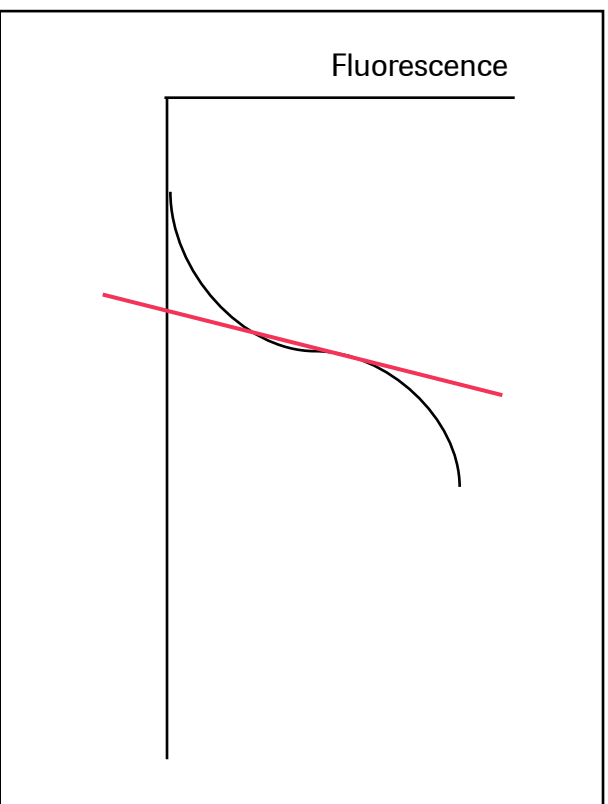
## *Amplification Curve vs Standards*

**Efficiency is a synonym for quality of PCR.**

**Is PCR efficiency reflected by standard curves or by individual amplification curves?**

# Determination of Amplification Efficiency

## *Amplification Curve vs Standards*



# Calculation of PCR Efficiency

## *Derived from Amplification Curve*

**Phenomenological descriptions of efficiency depending on individual amplification curve of samples**

- so far no full automation possible
- quality depending on users ability / algorithms used
- so far only estimation of efficiency

■ **Standardization critical**

# Calculation of PCR Efficiency

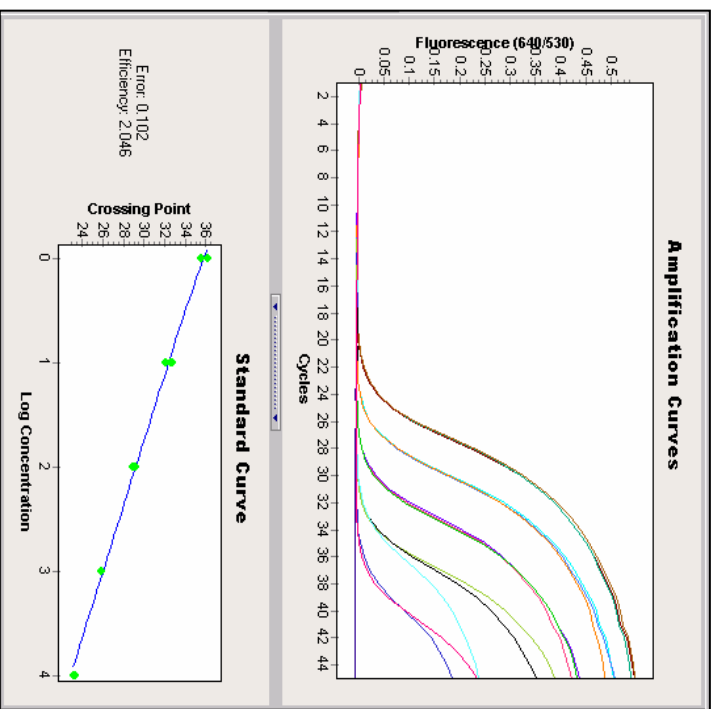
## *Derived from Standards*

### Efficiency based on dilution series

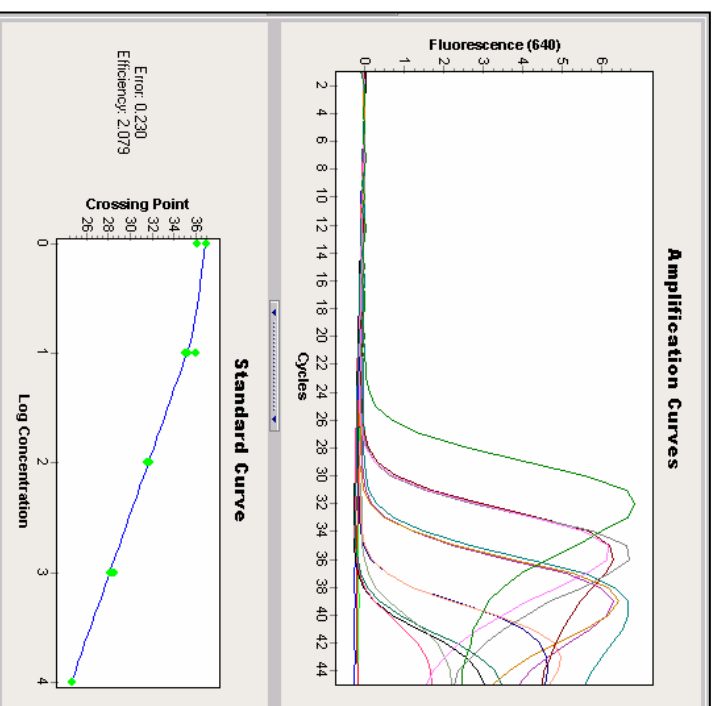
- Prerequisite for Standards:  $\text{Efficiency}_{\text{Standard}} = \text{Efficiency}_{\text{Sample}}$
- Statistical approach (amount of standards)
- Laborious
- **Standardization possible, but not reflecting the differences of amplification efficiencies of individual samples**
- **Reflects individual PCR's behaviour**

# Linear vs. Non-Linear PCR Efficiency

## Curve Fit Depends on Data



**linear fit**



**non-linear fit**

# Relative Quantification

## *With Calibrator Normalization*

- A calibrator provides comparison of many PCR experiments (used as a “positive control”)
- A calibrator corrects for differences in detection sensitivity between target and reference genes
- A calibrator does not correct for differences in PCR efficiency between the target and reference gene

# Relative Quantification Methods (1)

## *Known as $\Delta\Delta C_t$ Method*

### **Calibrator Normalization without Efficiency Correction**

- This method assumes that reference gene and target gene are amplified with the same efficiency
- This method assumes that the PCR efficiency of both genes is 2

### **An efficiency correction would significantly reduce calculation errors, because**

- Not every PCR-System is running with optimal/identical PCR efficiency (E = 2)
- Not every PCR-System is even following a constant PCR efficiency

# Relative Quantification Methods (2)



Diagnostics

## Calibrator Normalization with Efficiency Consideration

$$\text{Relative Ratio} = E_T^{C_{pT}(C) - C_{pT}(S)} \times E_R^{C_{pR}(S) - C_{pR}(C)}$$

- Uses the individual PCR efficiency in the calculator
- Efficiency is generated via linear/polynomial standard curves

# Comparison of Methods

## *Validity of Calculated Values*

- Predefined GMO standards of known concentrations (in %), are analysed with relative quantification with or without efficiency correction
- Sample preparation greatly affects results

<b>GMO Content</b>	<b>Result with Efficiency Correction</b>	<b>Result without Efficiency Correction (E=2)</b>
1.0%	1.06%	0.67%
0.5%	0.51%	0.31%
0.1%	0.08%	0.04%
1.0%	0.73%	0.45%
0.5%	0.49%	0.28%
0.1%	0.08%	0.04%



# Standardization in Gene Expression

## *Summary*

- **Try to minimize technical variation**
- **Use appropriate instrumentation with low variance and high reproducibility**
- **Use highly accurate software algorithms for qPCR**



# Thank You for Your Attention

[www.roche-applied-science.com](http://www.roche-applied-science.com)



**Diagnostics**

Roche Diagnostics GmbH  
Roche Applied Science  
68298 Mannheim  
Germany

LIGHTCYCLER is a trademark of Roche