Accurate Gene Expression Analysis with High Flexibility: Concepts and Developments

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Gene Expression Analysis

Goals and Requirements

Roche Diagnostics

- 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

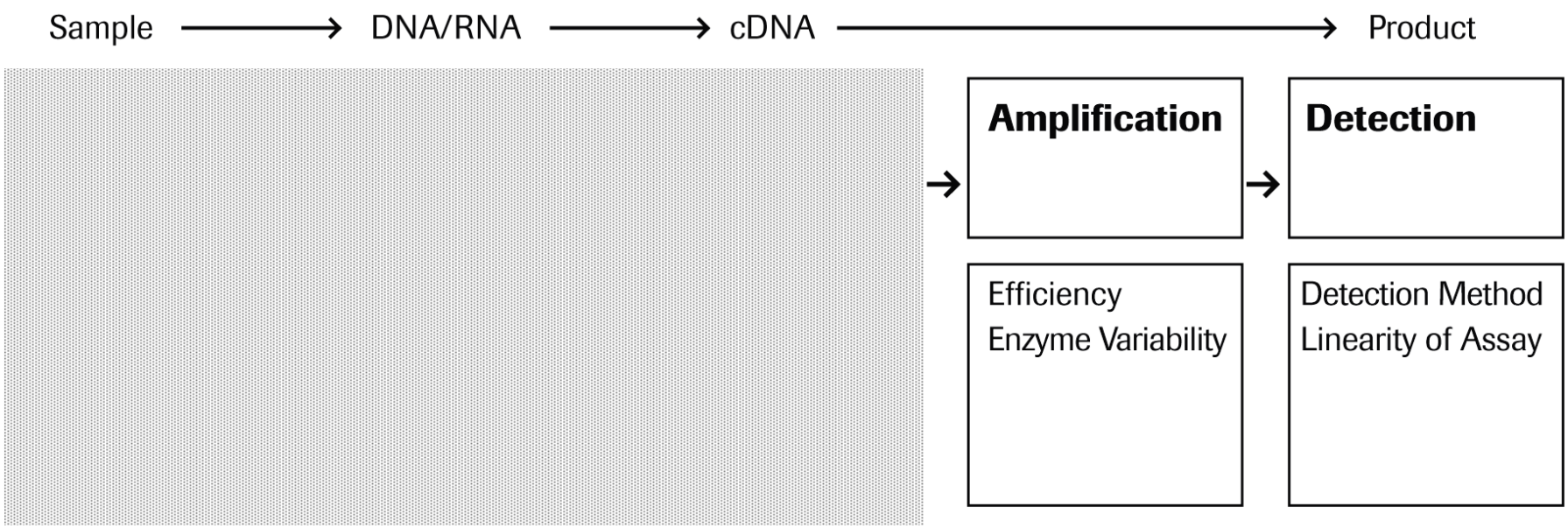
Preliminary Requirements:

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

RT-PCR Quantification

Influencing Factors

Roche Diagnostics



LightCycler® Systems

Technological (r)evolutions

Roche Diagnostics





LightCycler® 480 System

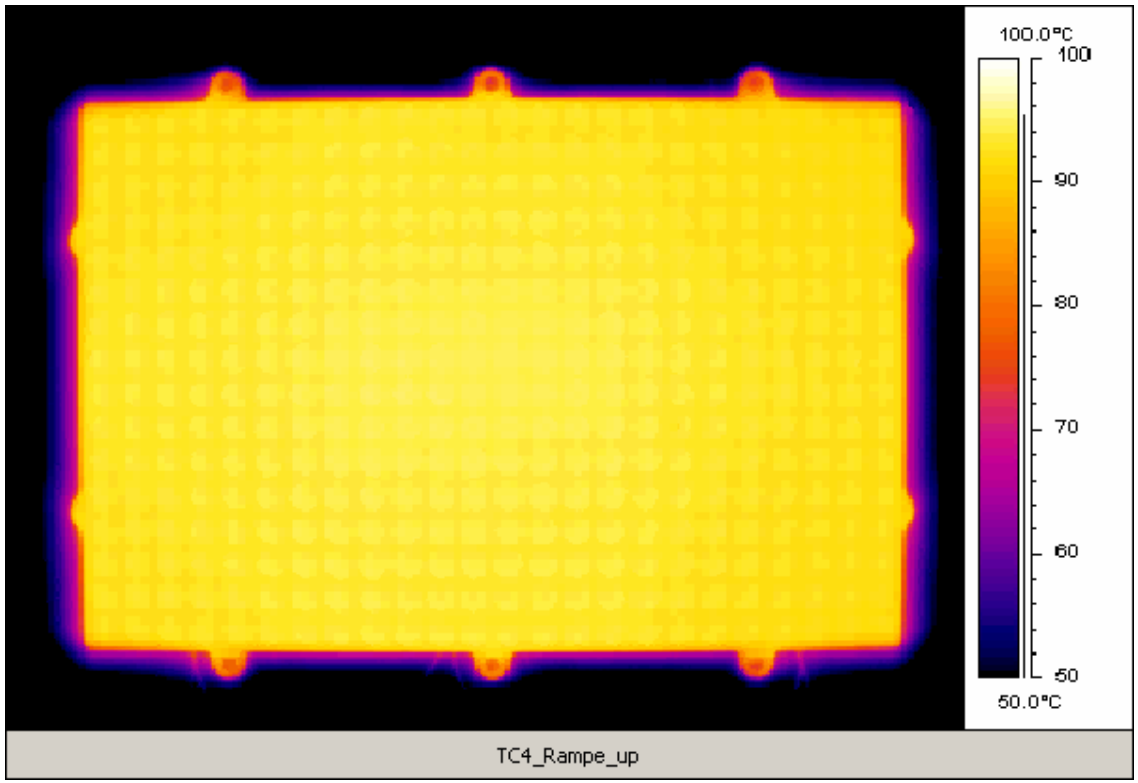
Summary

Roche Diagnostics

- 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.

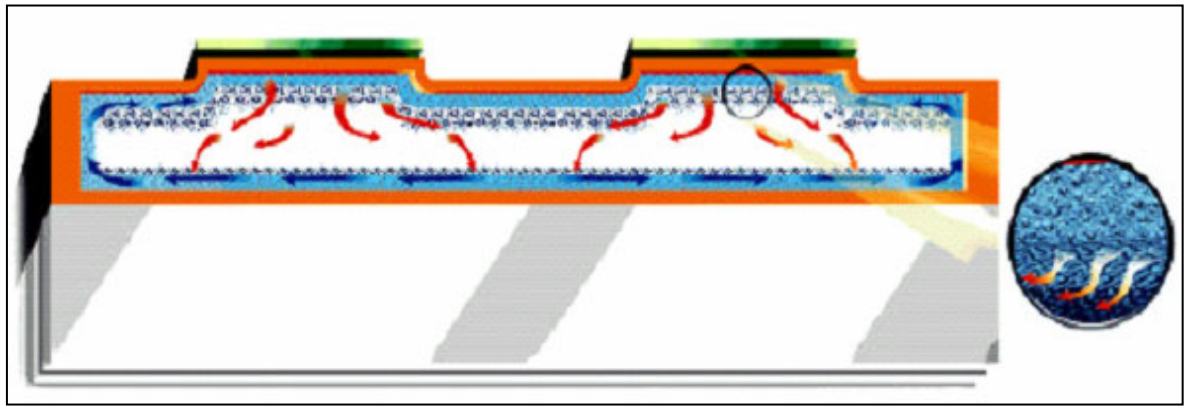
Standard Block Cycler

Ramping up Temperature



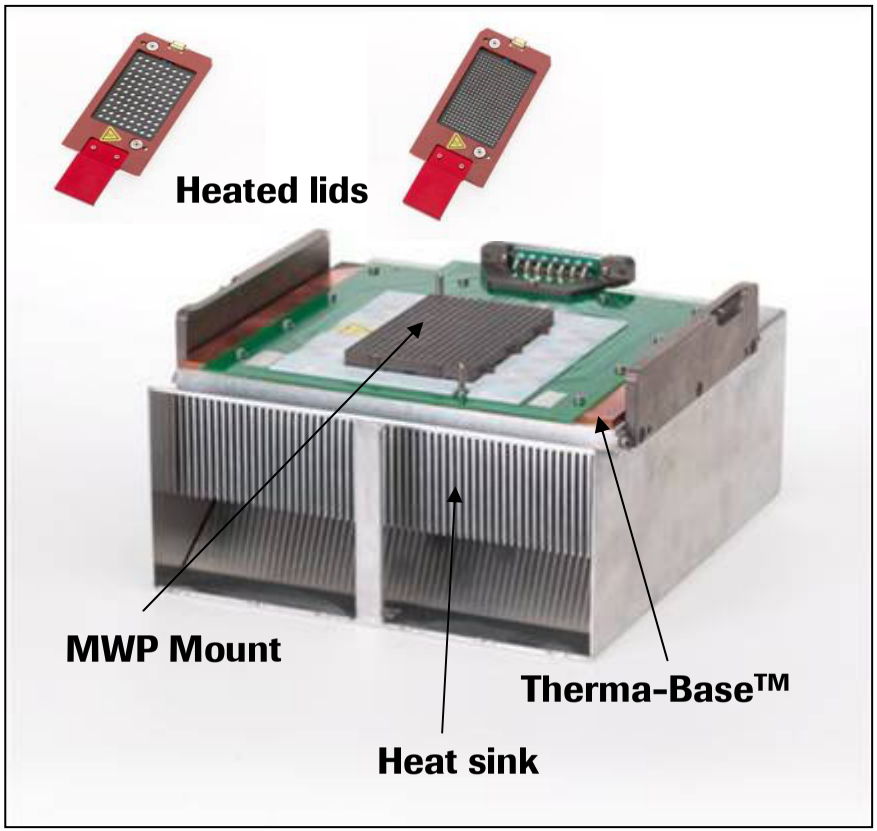
LightCycler® 480 Blockcycler

Therma-Base™



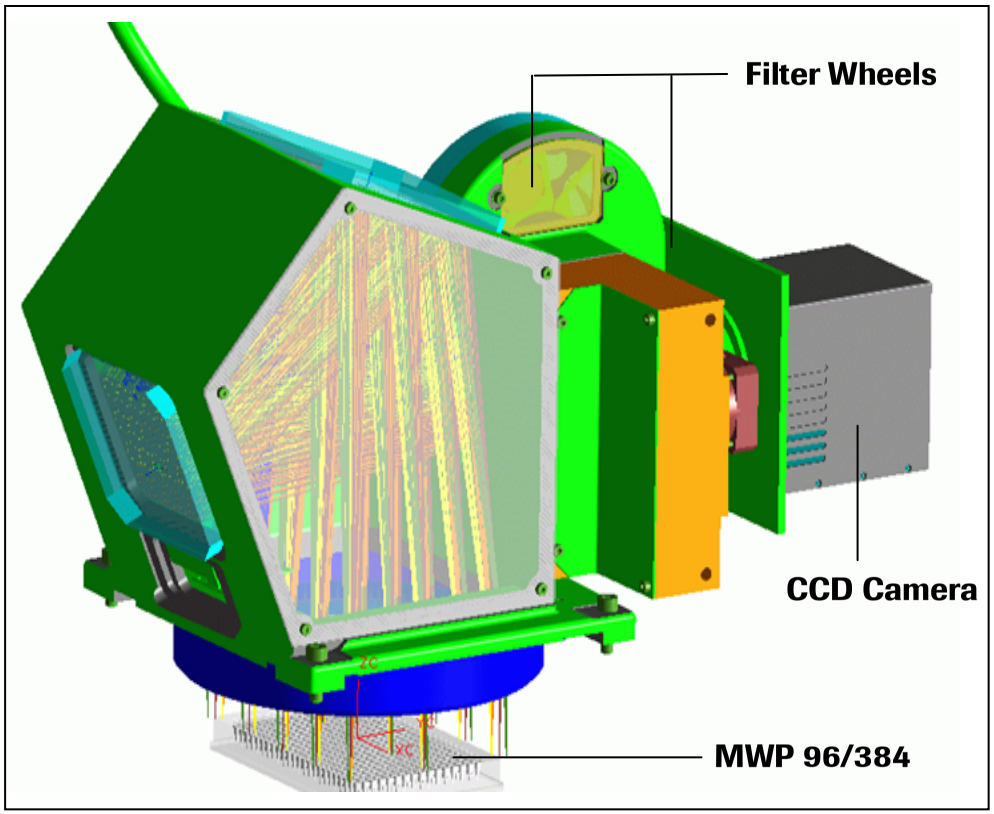
- vacuum chamber saturated with working fluid
- wick structure lining inside walls
- hot location: evaporation into vacuum
- cool location: condensation with heat release

LightCycler® 480 System
Thermocycler



- Includes Thema-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

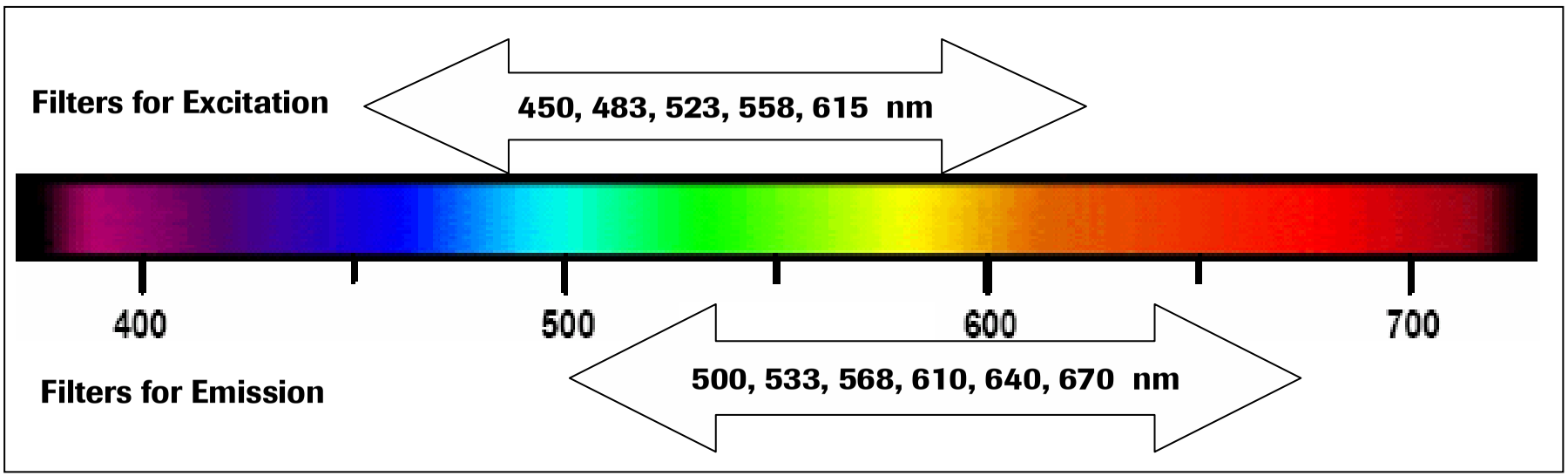


- Homogeneous illumination and imaging due to long object-image distance.
- Pinhole to mask lateral portions of emitted light and focus on central, perpendicular portions.
- Large field lense to efficiently collect rays also from lateral wells.

LightCycler® 480 Instrument

Optical Properties

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



Internal Reference Dye

Internal reference dye is not required

due to

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

LightCycler® 480 System

Assay Formats, Dyes, and Application

Roche Diagnostics

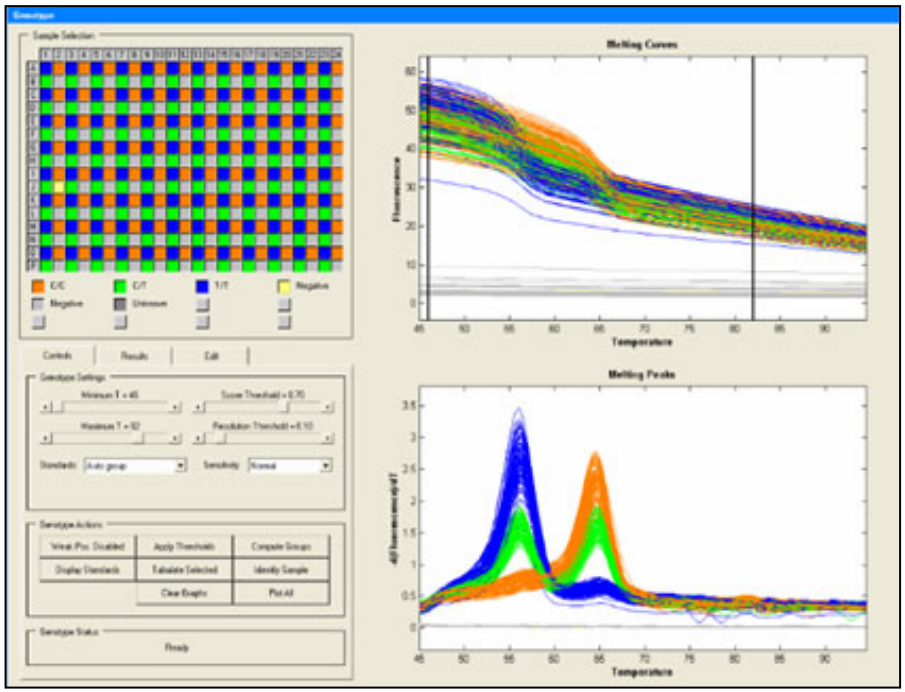
Assay Format	Excitation (nm)	Detection (nm)	Dyes	Application
SYBR Green I	483	530	SYBR Green I	Qualitative Detection Product - Characterization Quantification
HybProbe Probes	483	533/ 610 533/ 640 533/ 670	Fluo - LightCycler® RED 610 Fluo - LightCycler® RED 640 Fluo - Cy5	Quantification SNP Analysis
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

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



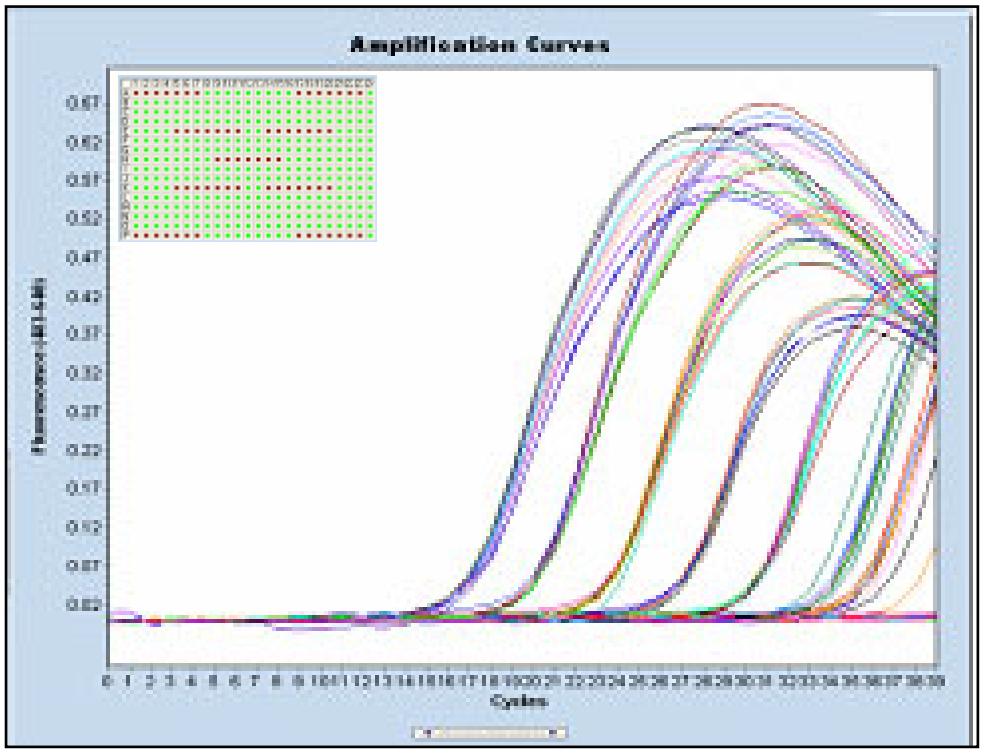
	$T_m(1)^oC$	$T_m(2)^oC$
Average	56.47	64.88
Minimum	56.14	64.67
Maximum	56.85	65.4
Standard deviation	0.1612	0.1801

LightCycler® 480 qPCR Performance

Intra-Run Reproducibility

Diagnostics

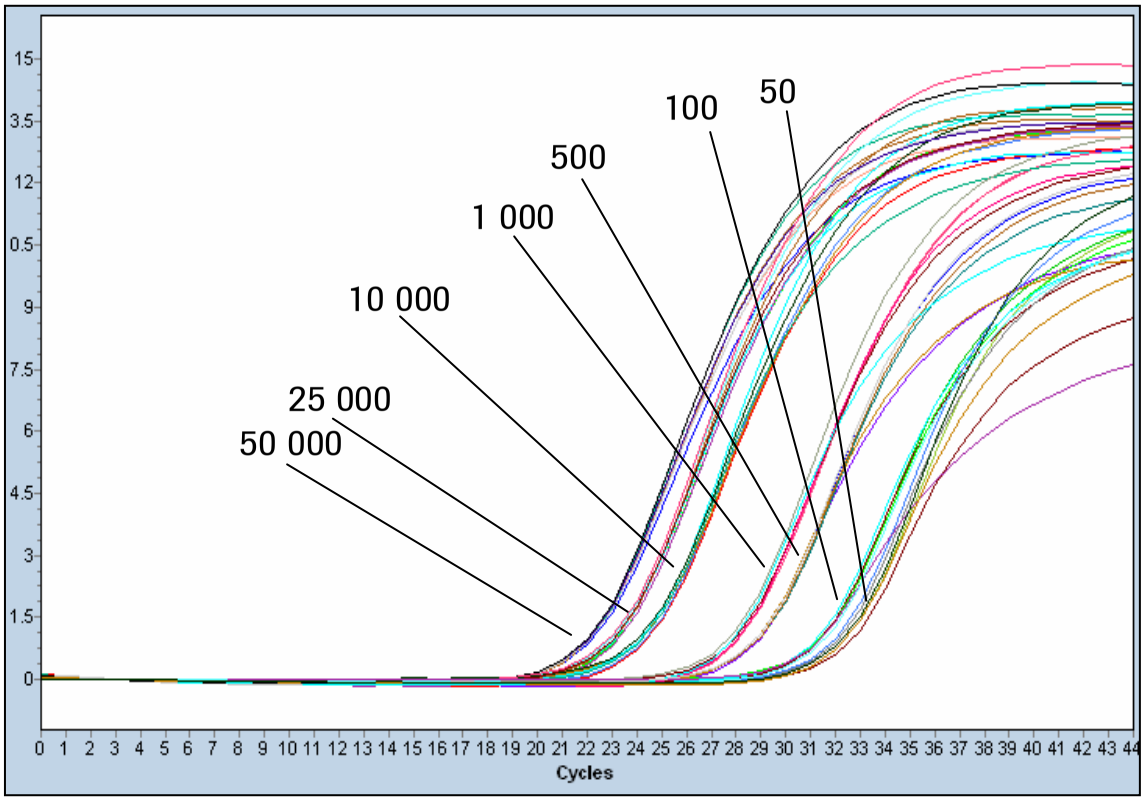
Viral target detected with HybProbe probes



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 MWP
- Hydrolysis Probe Format
- 196 bp amplicon

Gene Expression Analysis

Goals and Requirements

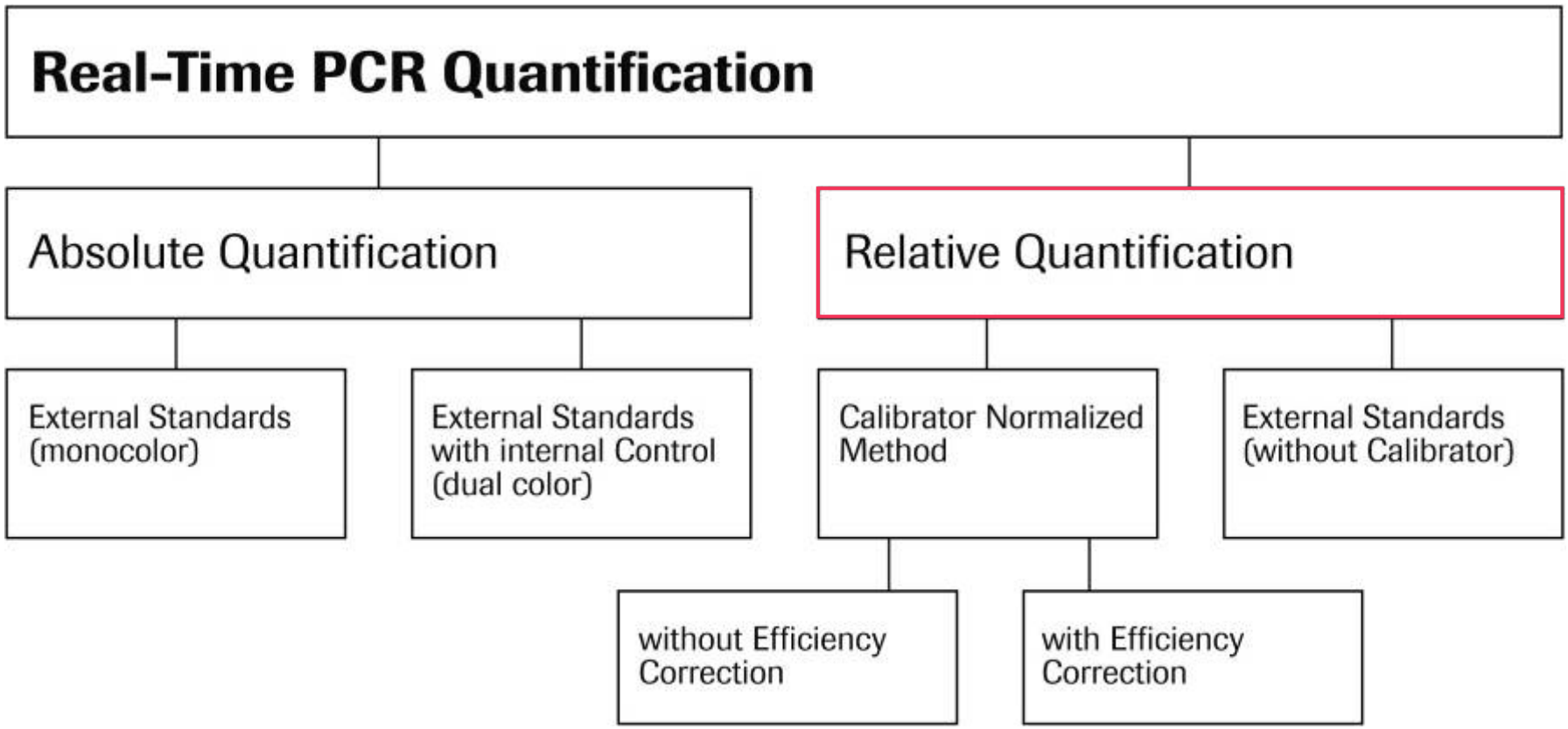
Roche Diagnostics

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Preliminary Requirements:

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Types of Quantification



Relative Quantification

Relative Ratio

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

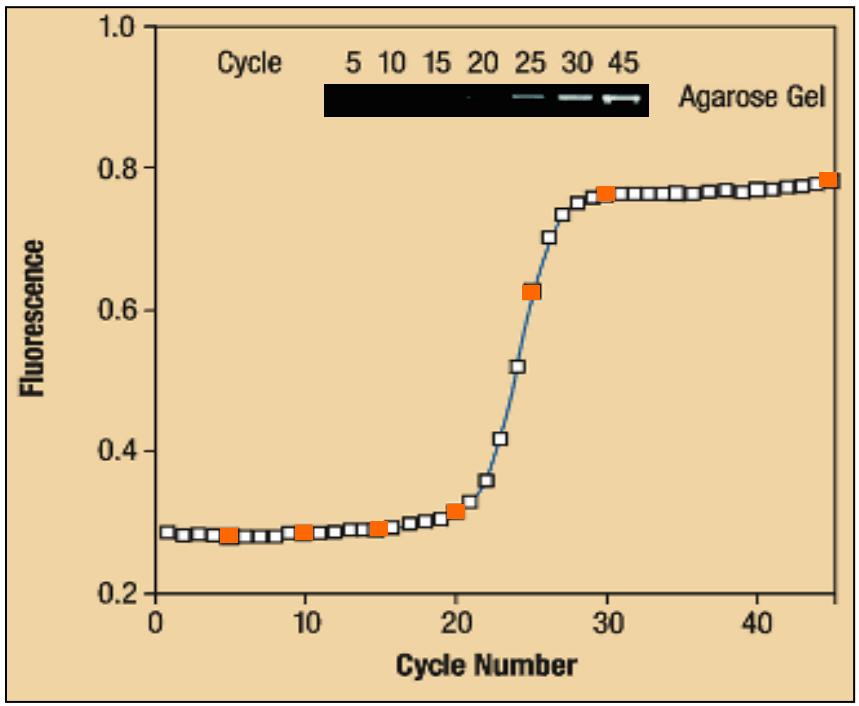
For calculation:

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

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

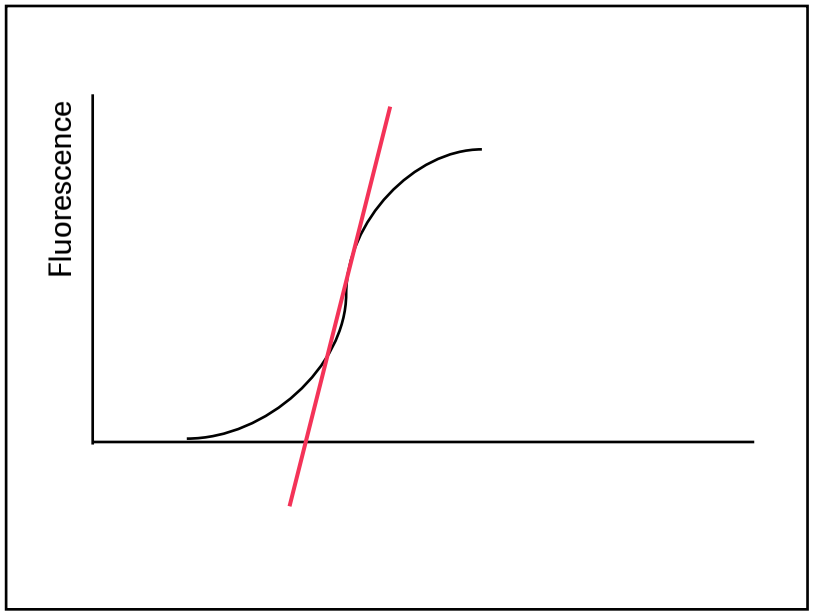
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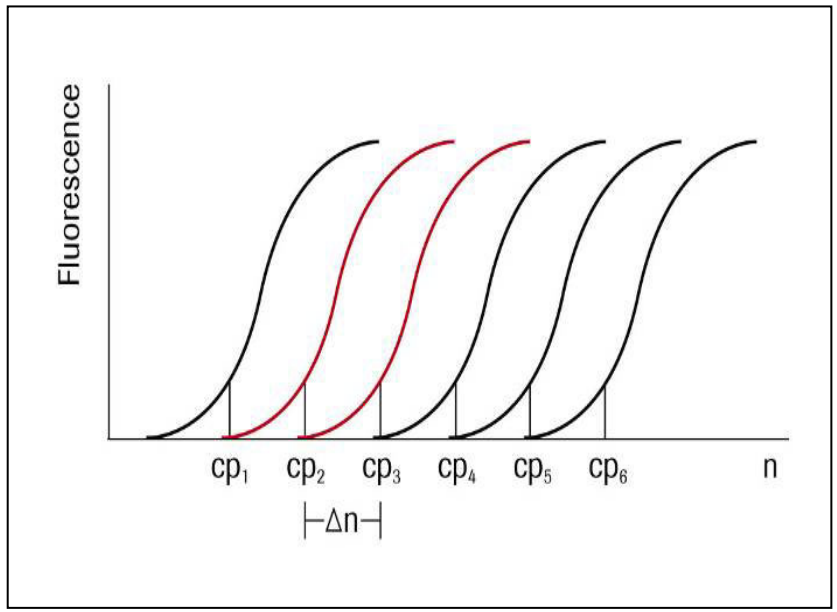
Efficiency is a synonym for quality of PCR.

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

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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

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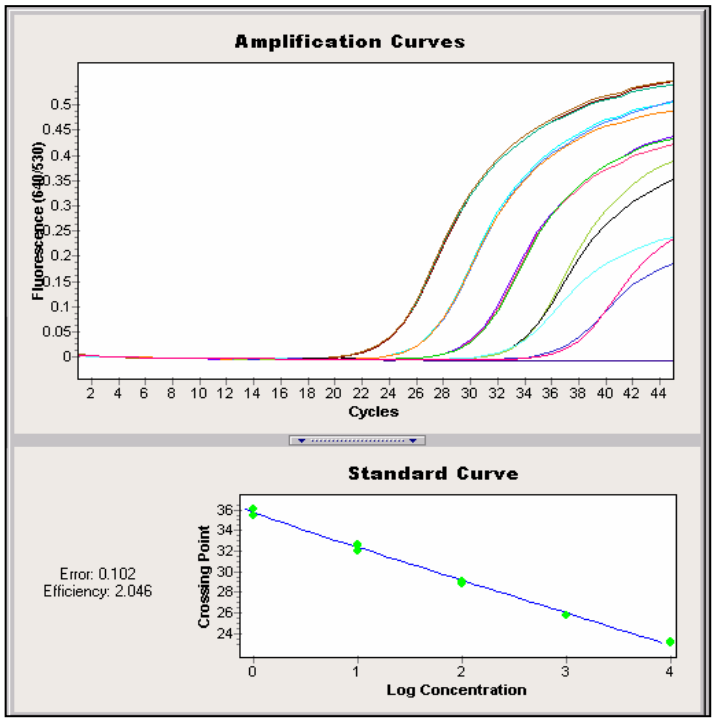
Efficiency based on dilution series

- Prerequisite for Standards: $\text{Efficiency}_{\text{Standard}} = \text{Efficiency}_{\text{Sample}}$
- Statistical approach (amount of standards)
- Laborious

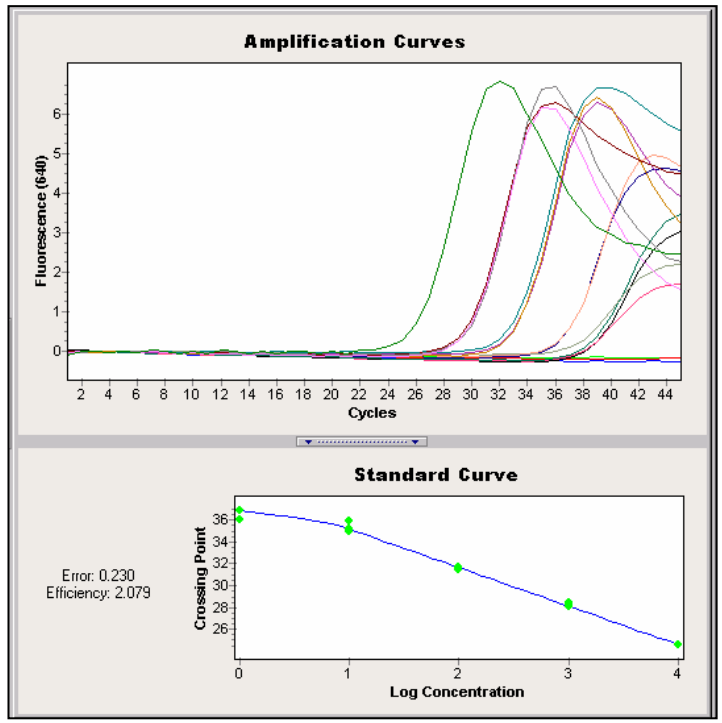
- **Standardization possible, but not reflecting the differences of amplification efficiencies of individual samples**
- **Reflects individual PCR´s behaviour**

Linear vs. Non-Linear PCR Efficiency

Curve Fit Depends on Data



linear fit



non-linear fit

Relative Quantification

With Calibrator Normalization

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- 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 ΔΔCt Method

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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)

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Calibrator Normalization with Efficiency Consideration

$$\text{Relative Ratio} = E_T^{\,CpT(C) - CpT(S)} \times E_R^{\,CpR(S) - CpR(C)}$$

- Uses the individual PCR efficiency in the calculaton
- 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 efficency correction
- Sample preparation greatly affects results

GMO Content	Result with Efficiency Correction	Result without Efficiency Correction (E=2)
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

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