Accurate Gene Expression Analysis with High Flexibility: Concepts and Developments

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Gene Expression Analysis

Goals and Requirements

To quantitatively detect subtle changes in amounts of mRNA

Preliminary Requirements:

- Accurate and reproducible measurements
- Steady quantitative analysis of data
- To obtain reliable data that can be compared over a long period against a complex background
- To quantitatively detect subtle changes in amounts of mRNA

Diagnostics
Influencing Factors

RT-PCR Quantification

Diagnostics
Newly developed, high-throughput system for real-time PCR.

Specifically developed optical system for maximized sensitivity and the uniform collection of signals across the plate.

Optimized heating and cooling technology for increased speed and maximized temperature uniformity.

384-microwell plates.

Compact benchtop instrument for 96- and/or 480-microwell plates.

Summary

LightCycler® 480 System
Standard Block Cycler

Ramping up Temperature
- Cool location: condensation with heat release
- Hot location: evaporation into vacuum
- Wick structure lining inside walls
- Vacuum chamber saturated with working fluid

*Therm-a-Base® 480 Blockcycler*
Roche Applied Science

LightCycler® 480 System

Thermocycler

96 wells in < 1 hour and 384 wells in < 40 min.

Includes Therm-a-Base™ for optimized heat exchange.

Includes Therm-a-Base™ for

Heat sink

MWP Mount

Heated lids

Thermocycler

LightCycler® 480 System
Homogeneous illumination and imaging due to long object-distance.

- Pinhole to mask lateral portions.
- Large field lens to efficiently collect emitted light and focus on central, perpendicular portions.
- Long object-image distance.

Optical System

LightCycler® 480 Instrument
Optical Properties

LightCycler® 480 Instrument

- Filters for Excitation
  - 450, 483, 523, 558, 615 nm

- Filters for Emission
  - 500, 533, 568, 610, 640, 670 nm

Lifetime: approx. 500-1000 h

Light source: high intensity xenon lamp
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

<table>
<thead>
<tr>
<th>SNP Analysis</th>
<th>Fluorescein</th>
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<th>Simplicable Probes</th>
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<tbody>
<tr>
<td>Quantification</td>
<td>Cy5</td>
<td>670 (+/-10)</td>
<td>615 (+/-15)</td>
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<tr>
<td>LightCycler® Red 640</td>
<td>640 (+/-10)</td>
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<td>FAM</td>
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<td>Hydrolysis Probes</td>
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<td>Fluo - LightCycler® Red 640</td>
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<td>Hydrolysis Probes</td>
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<th>Qualitative Detection Product - Assay Format</th>
<th>SYBR Green I</th>
<th>530</th>
<th>483</th>
<th>SYBR Green I</th>
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<td>530</td>
<td>483</td>
<td>SYBR Green I</td>
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### Assay Formats, Dyes, and Application

**LightCycler® 480 System**
### Intra-Instrument Reproducibility

<table>
<thead>
<tr>
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<th>( T_m(1) )°C</th>
<th>( T_m(2) )°C</th>
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<tbody>
<tr>
<td>Standard deviation</td>
<td>0.1612</td>
<td>0.1801</td>
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<tr>
<td>Maximum</td>
<td>66.4</td>
<td>65.4</td>
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<tr>
<td>Minimum</td>
<td>56.4</td>
<td>56.14</td>
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<tr>
<td>Average</td>
<td>56.4</td>
<td>56.47</td>
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</table>

Ninety-six replicates for each of the 3 different genotypes.

MDR-1 C3435T polymorphism; SimpleProbe Format.
LightCycler® 480 qPCR Performance

Intra-Run Reproducibility

Viral target detected with HybProbe probes

<table>
<thead>
<tr>
<th>Copies</th>
<th>Mean</th>
<th>SD</th>
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<tbody>
<tr>
<td>1.0E+01</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>1.0E+01</td>
<td>0.85</td>
<td>0.1092</td>
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<tr>
<td>1.0E+01</td>
<td>27.31</td>
<td>2.12E-01</td>
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<tr>
<td>1.0E+02</td>
<td>0.0899</td>
<td>2.41E-01</td>
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<tr>
<td>1.0E+03</td>
<td>20.85</td>
<td>0.0315</td>
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<tr>
<td>1.0E+04</td>
<td>17.77</td>
<td>0.0549</td>
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</table>
Accuracy of LightCycler® 480 System

Discrimination of 500 and 1000 copies

Hydrolysis Probe Format

196 bp amplicon

7 replicates of human genomic DNA distributed over MWP

50 000 25 000 10 000 1 000 500
Goals and Requirements

To quantitatively detect subtle changes in amounts of mRNA

Preliminary Requirements:

- Accurate and reproducible measurements
- Steady quantitative analysis of data
- To obtain reliable data that can be compared over a long period against a complex background
- To quantify and reproducibility of experimental systems at time or between different experimental systems
Types of Quantification

- Absolute Quantification
  - Method with Internal Control (dual color)
  - External Standards (monocolor)

- Relative Quantification
  - Calibration Normalized
  - Correction (with internal control) (external standards)
  - Correction (without efficiency) (external standards)
Relative Quantification

Relative Ratio

\[ N = N_0 \times 2^n \]

For calculation:

\[ \frac{\text{Amount of target RNA in a sample}}{\text{Amount of housekeeping RNA in a sample}} \]
From Block Cyclers to Real-Time PCR

Monitoring of PCR Reactions

Displayed amplification curve is influenced by:
- Detection format
- Reaction conditions, e.g., pH
- Fluorescence dye

Generation of Cq/CT is influenced by:
- Algorithm used

Influenced by:
- Displayed amplification curve
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

Fluorescence

Regen [Eq. 1]

Cp  Δn
Calculation of PCR Efficiency

Derived from Amplification Curve

Phenomenological descriptions of efficiency depending on individual amplification curves of samples

So far only estimation of efficiency possible

Quality depending on user's ability / algorithms used

So far no full automation possible

Standardization critical
Calculation of PCR Efficiency

Derived from Standards

Efficiency based on dilution series

\[ \text{Efficiency} = \frac{\text{Efficiency}_{\text{Standard}}}{\text{Efficiency}_{\text{Sample}}} \]

Prerequisite for Standards: Efficiency_{Standard} = Efficiency_{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
Diagnostics

Linear vs. Non-Linear PCR Efficiency

Curve Fit Depends on Data

Linear Fit

Non-Linear Fit
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 corrects for differences in PCR efficiency between the target and reference gene. A calibrator does not correct for differences in PCR efficiency between the target and reference gene.
Relative Quantification (1)

**Known as ΔΔCt Method**

Calibrator Normalization without Efficiency Correction

- Not every PCR-System is even following a constant PCR efficiency
- \( E = 2 \)
- Not every PCR-System is running with optimal/identical PCR efficiency
- **Calculation errors, because** an efficiency correction would significantly reduce
- This method assumes that the PCR efficiency of both genes is 2
- This method assumes that Reference gene and Target gene are amplified with the same efficiency

Diagnostics
Relative Quantification Methods (2)

Calibrator Normalization with Efficiency Consideration

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

- Efficiency is generated via linear/polynomial standard curves
- Uses the individual PCR efficiency in the calculation
Comparison of Methods

Validity of Calculated Values

Predefined GMO standards of known concentrations (in %) are analyzed with relative quantification with or without efficiency correction.

Sample preparation greatly affects results.

Efficiency correction

<table>
<thead>
<tr>
<th>GMO Content</th>
<th>Result without Efficiency Correction</th>
<th>Result with Efficiency Correction</th>
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</thead>
<tbody>
<tr>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
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<tr>
<td>0.4%</td>
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<td>0.0%</td>
</tr>
<tr>
<td>0.3%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>0.4%</td>
<td>0.0%</td>
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<tr>
<td>0.3%</td>
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<td>0.0%</td>
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<tr>
<td>0.6%</td>
<td>0.0%</td>
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Diagnostics

Valdity of Calculated Values

Comparison of Methods
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

Try to minimize technical variation