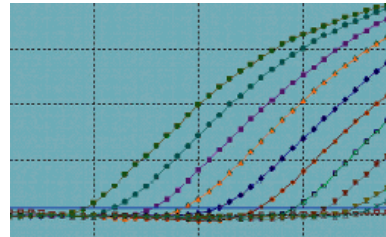


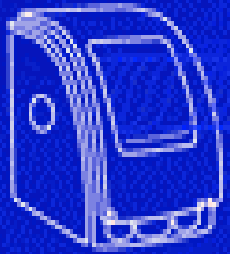
# News from the Field

## A Summary of the 3rd QPCR symposium Freising/Germany



**Dr. Steffen Müller**

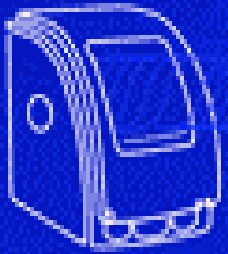
Field Application Scientist



# Summary of Conference

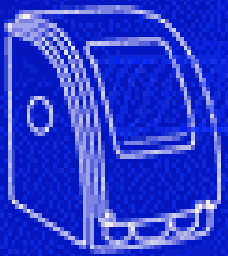
Main discussion topics in Freising:

- Data analysis
  - Statistics and bioinformatics
  - Inter-run comparison and calibration
- Single cell and limiting samples
  - Preamplification
  - LATE-PCR
- miRNA expression
  - diagnostic use of miRNA expression or ratios of miRNAs
- ImmunoPCR



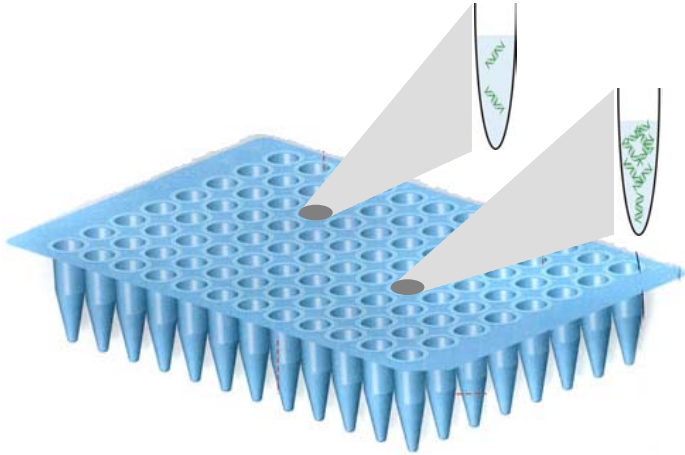
# Data Analysis

- Choosing the right data for statistical data analysis is important (Ct vs quantity/RQ vs log transformed Q/RQ)
- Parametric or non-parametric significance testing
- Applying clustering algorithms to identify coregulated genes
- Mathematical models for outlier detection and removal  
Nordgård et al., Anal. Biochem. 356 (2006)  
Bar et al. NAR 31 (2003)
- New software dedicated to QPCR statistical analysis to appear from various companies



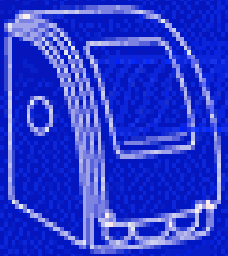
# Data Analysis

## Adressing experimental variability



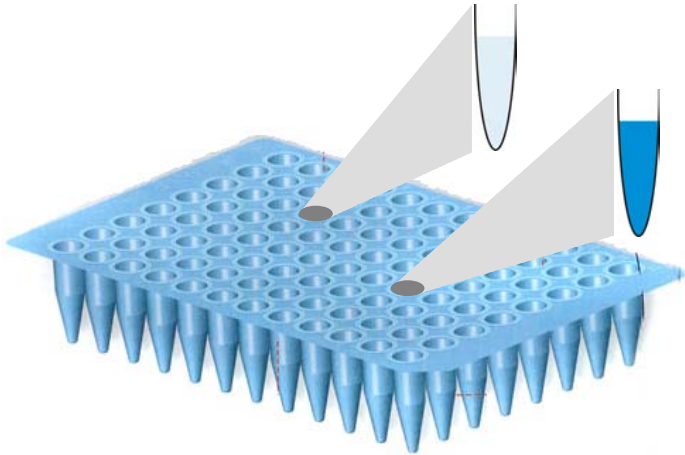
Differences in RQ due to:

- different gene expression level  
= variation of interest



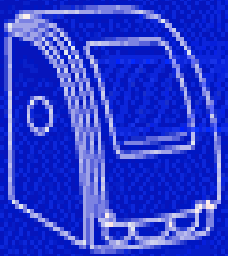
# Data Analysis

## Adressing experimental variability



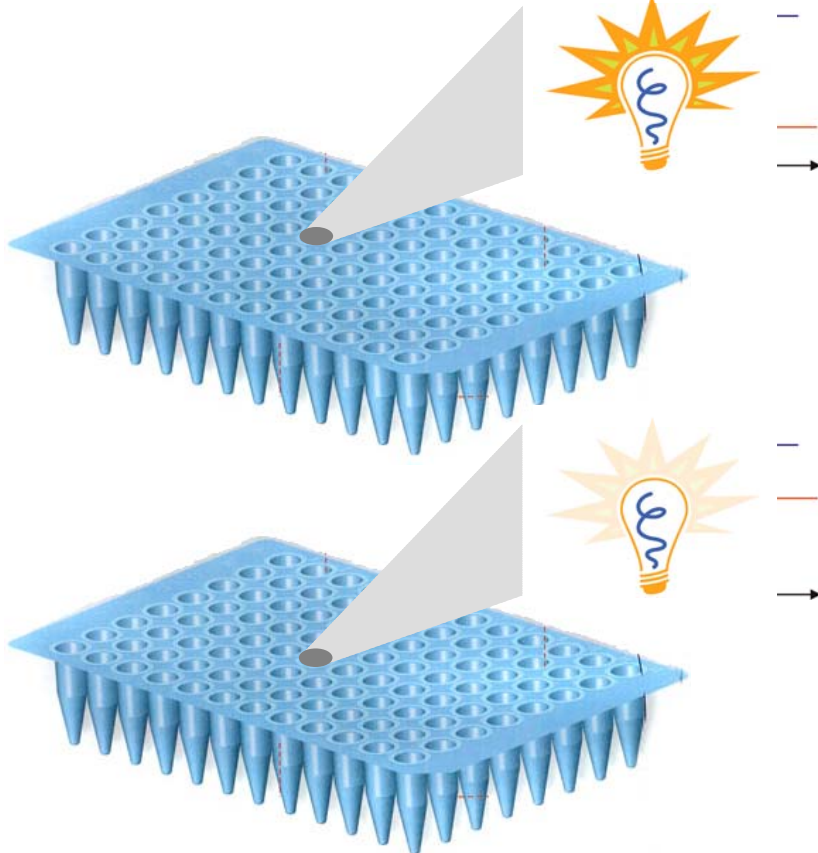
Differences in RQ due to:

- different gene expression level
- different total starting amount
  - cell numbers
  - RNA isolation
  - RNA degradation
  - cDNA synthesis



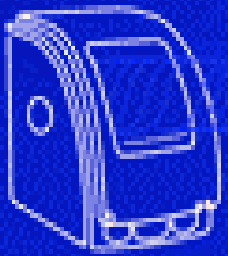
# Data Analysis

## Adressing experimental variability



### Differences in RQ due to:

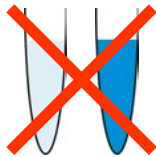
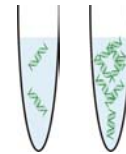
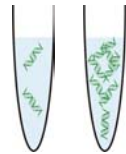
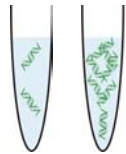
- different gene expression level
- different total starting amount
- run dependent differences
  - data analysis settings
  - instrument related variation
  - reagents
  - optical properties of plastics



# Data Analysis

## Addressing experimental variability

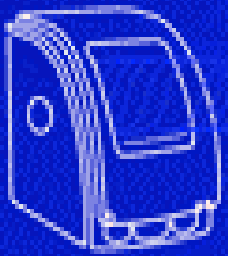
Ct → RQ → NRQ → CNRQ



Normalization Inter-run  
calibration

Avoid variation: Maximize Samples per assay not assays per plate!  
If you have variability → Minimize & correct for variation

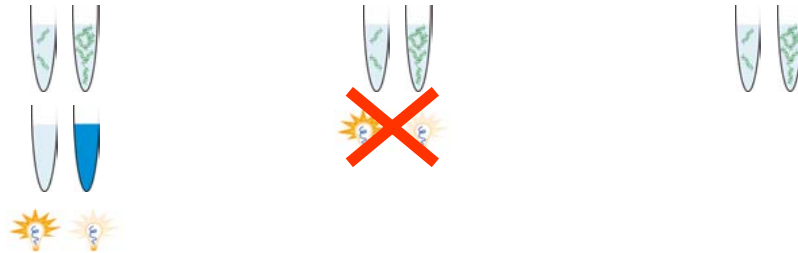




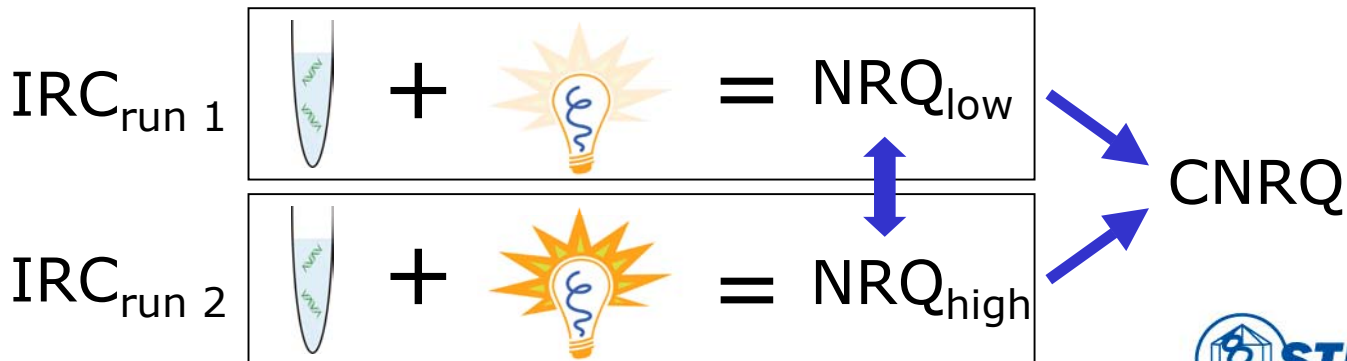
# Data Analysis

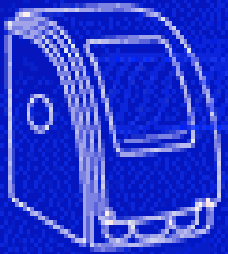
## Adressing experimental variability

Ct → RQ → NRQ → CNRQ



- Inter-run differences are generally ignored
- Inter-run differences can be corrected for with IRCs  
inter-run calibrator = identical sample measured for the same gene in different runs

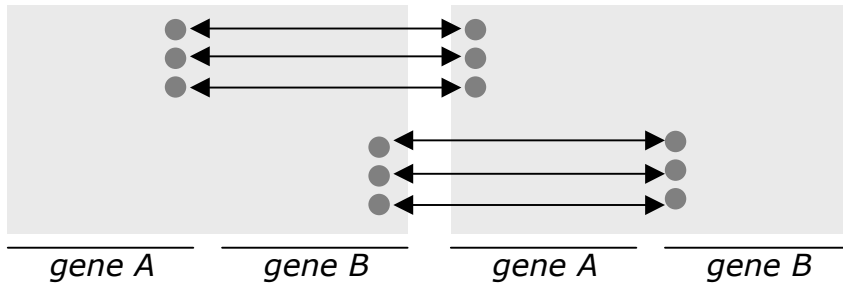




# Data Analysis

## Addressing experimental variability

- Inter-run calibration needs to be performed for each gene separately

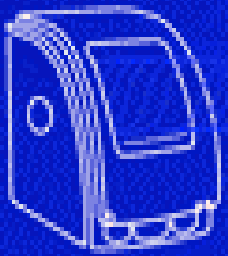


- Inter-run calibration on the level of

– Ct values  $( \text{V} = \text{V} )$

– NRQ values  $( \text{V} \text{ or } \text{V} )$

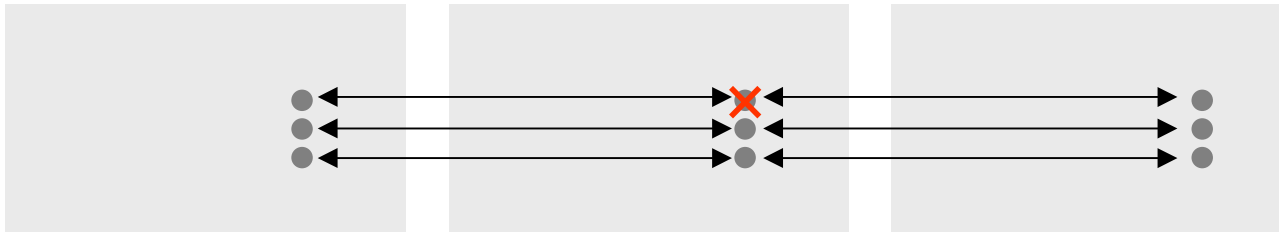
➔ Enables comparison with an IRC from a fresh cDNA synthesis



# Data Analysis

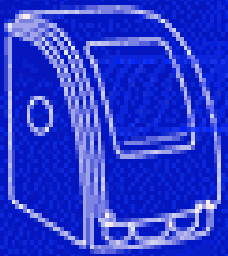
## Addressing experimental variability

- Limitation: same set of IRCs required in all runs



### Solutions:

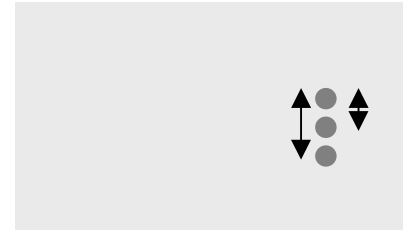
- No calibration for that gene in that run
- Exclude this IRC in all runs
- Complex inter-run calibration



# Data Analysis

## Adressing experimental variability

Complex inter-run calibration:  
based on multiple imputation

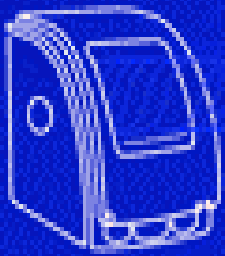


- Same NRQ ratio between identical samples in different runs
- Use this ratio to impute missing data (in several ways)
- Calculate calibration factor (multiple times)
- Average all instances of the calibration factor

### Multiple inter-run calibrators

- ➔ more accurate results
- ➔ less increase in error
- ➔ allows quality control





# Data Analysis

Open Access

Method

## **qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data**

Jan Hellemans, Geert Mortier, Anne De Paepe, Frank Speleman and Jo Vandesompele

Address: Center for Medical Genetics, Ghent University Hospital, De Pintelaan, B-9000 Ghent, Belgium.

Correspondence: Jo Vandesompele. Email: [Joke.Vandesompele@UGent.be](mailto:Joke.Vandesompele@UGent.be)

Published: 9 February 2007

*Genome Biology* 2007, **8**:R19 (doi:10.1186/gb-2007-8-2-r19)

The electronic version of this article is the complete one and can be found online at <http://genomebiology.com/2007/8/2/R19>

Received: 31 August 2006

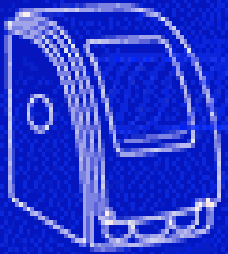
Revised: 7 December 2006

Accepted: 9 February 2007

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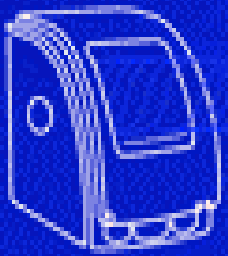
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# Single Cell and Limiting Samples

- Single cell PCR as well as limiting samples PCR are challenging
- Often sequence is below LOD or very late Cts are observed
- 2 approaches to increase sensitivity:
  - ➔ LATE-PCR (linear after the exponential PCR)
  - ➔ Pre-amplification of samples



# Single Cell and Limiting Samples

## LATE-PCR

Sanchez et al. PNAS 101 (2004)

Linear-After-The-Exponential (LATE)-PCR: An advanced method of asymmetric PCR and its uses in quantitative real-time analysis

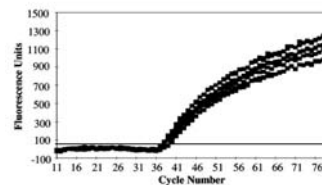
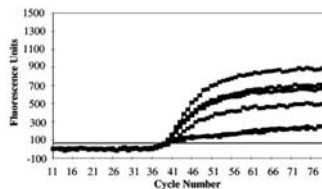
J. Aquilas Sanchez, Kenneth E. Pierce, John E. Rice, and Lawrence J. Wangh\*

Department of Biology, MS 008, Brandeis University, 415 South Street, Waltham, MA 02454-9110

PNAS | February 17, 2004 | vol. 101 | no. 7 | 1922-1926

- Highly asymmetric primer concentrations  
→ 1000 nM vs 25 nM  
→ limiting primer needs adjusted design to account for  $T_m$  difference

- Wider dynamic range

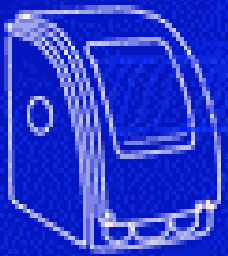


## Preamplification

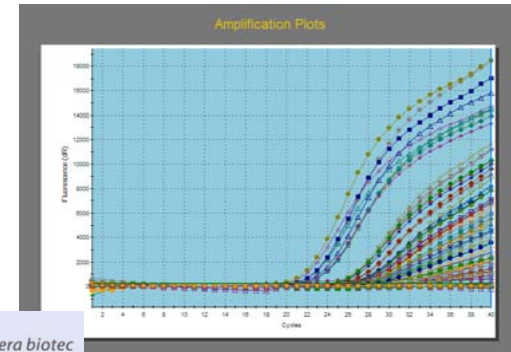
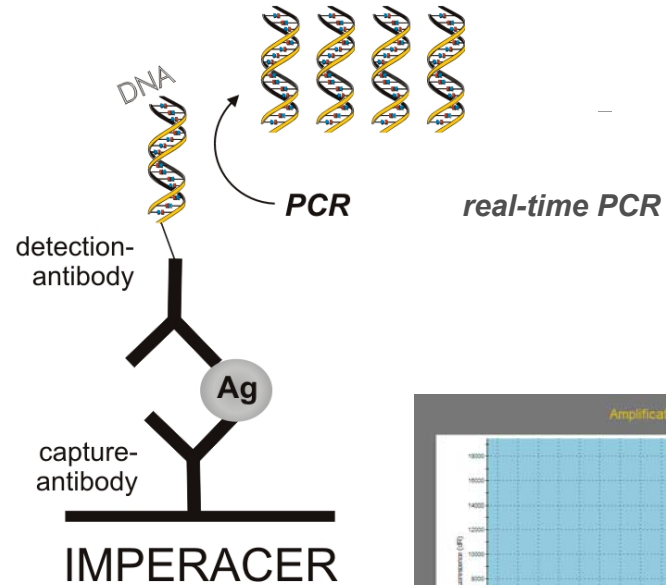
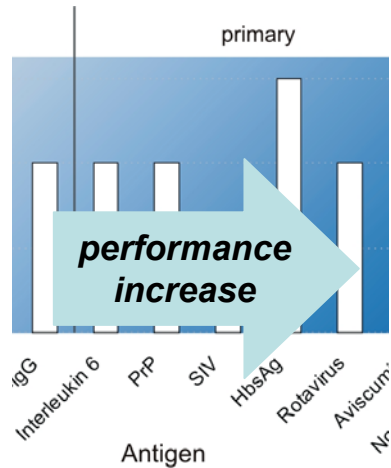
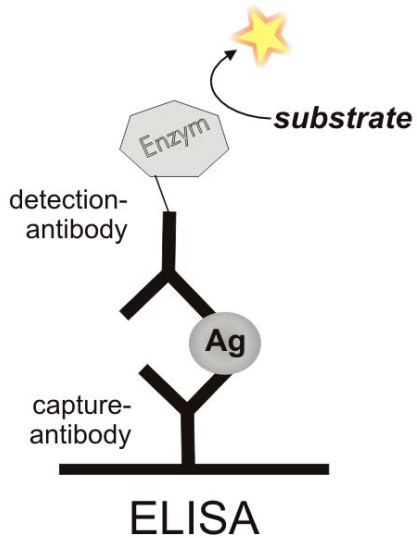
Stanley and Szewczuk NAR 33 (2005)

Crawford et al. BBRC 293 (2002)

- 2 rounds of amplification
- highly multiplexed 1<sup>st</sup> round for template enrichment (up to ~70 primer pairs)
- 1<sup>st</sup> round for 5-10 cycles where normally the 'baseline' would be observed
- Dilution of 1<sup>st</sup> round PCR into individual 2<sup>nd</sup> 'detection'-PCR

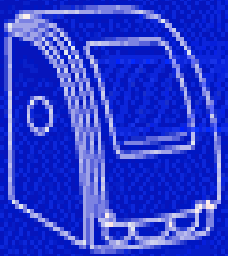


# ImmunoPCR



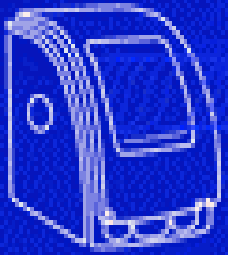
chimera biotec

Typically 100-1000 fold increase in sensitivity



# Summary

- Technology itself is not developing just moving towards higher throughput and lower volumes  
→ fast PCR, 384 and more, nanoliter PCR
- Focus currently is on proper data analysis applying methods already used in microarray analysis
- Single cell experiments are regarded as critical to understand biology as analysis of cell populations or tissue sometimes can be misleading and only shows the averaged effect in an organism



# Real Time Quantitative PCR

## Fast Track – QPCR Education

Thanks for your attention!

Educational material can be found at  
<http://www.stratagene.com/fasttrack/>