Amplification Based Assays in the Nanoliter Volume Range

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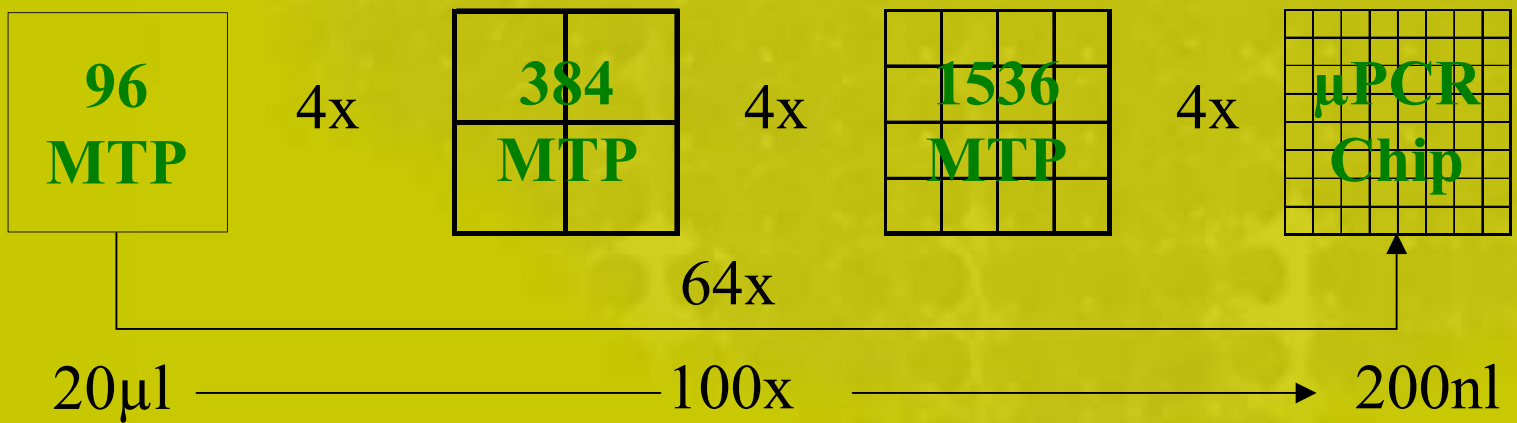
Overview

- Miniaturisation
- Platform properties
- qPCR assay implementation
- Tissue specific expression study

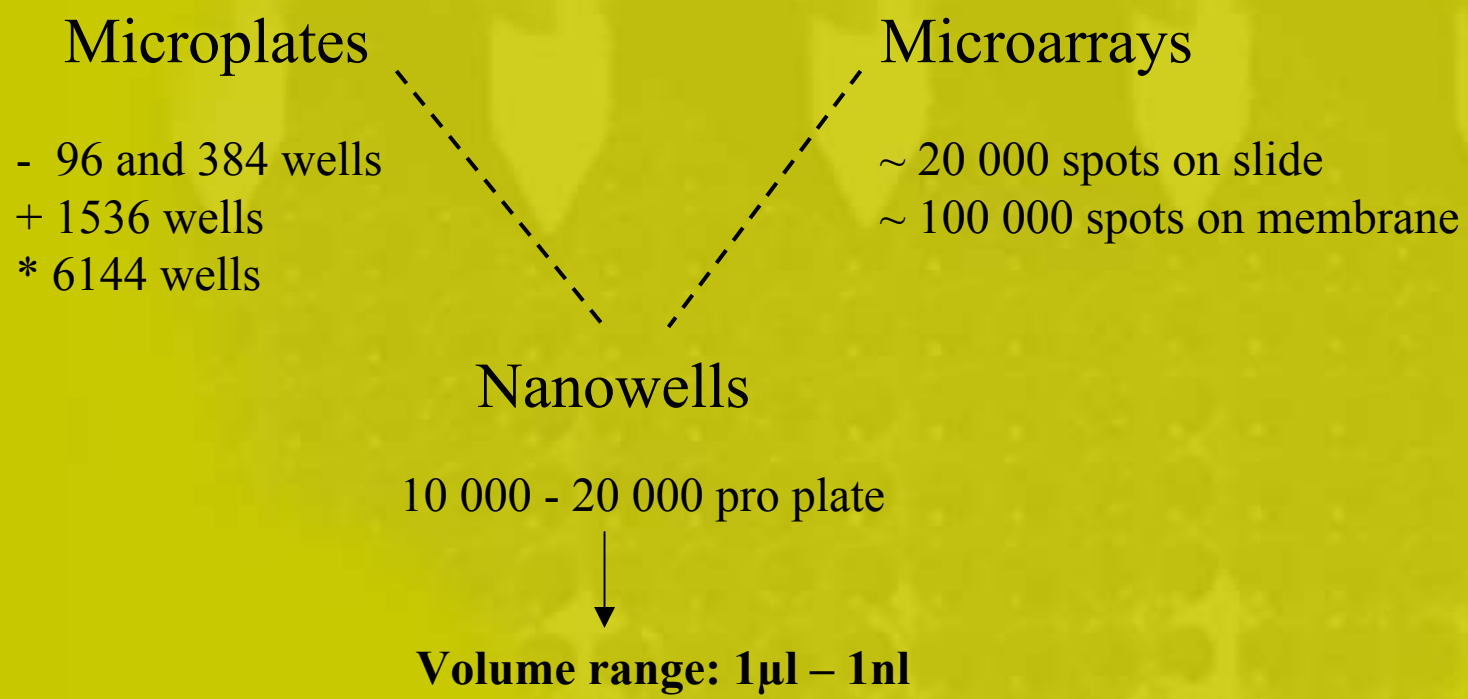
Motives for Miniaturisation

- Reduction of needed biological material
- Reduction of costs
- Shorter processing time
- Higher data density



Microstructured Titerplates in Miniaturisation

Technologies in arrayed formats



Challenges for Miniaturisation of Liquid Assays

- Surface-to-volume ratio → wall-reactant interactions
- Liquid handling → nanodispensing
- Detection sensitivity (at reasonable cost & equipment effort)
- Connectivity to other platforms → Macro-to-micro interface
- Flexibility, combinatory aspects & assay diversity

*Sauer, S., Lange, B.M., Gobom, J., Nyarsik, L., Seitz, H. and Lehrach, H. (2005) Miniaturization in functional genomics and proteomics. Nat Rev Genet, **6**, 465-476.*

The Open Well Approach

+ large number of individual reactors
+ random access to individual wells
+ samples kept in confined space

- contamination risk from environment (dusk)
- volume transfer demands rather complex systems
- evaporation

Litborn, E. and Roeraade, J. (2000) *J Chromatogr B Biomed Sci Appl*, **745**, 137-147.

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Goals of Development

- Enabling of PCR based assays below the 500nl volume threshold
  - real-time detection
  - substrate material issues & chip design
  - robust biochemical assays
  - critical concentrations for reaction partners
- Applicability in a setup for high throughput screening
  - Efficient, robust and accurate liquid handling
  - flexibility, combinatory aspects & assay diversity

Goals of Development

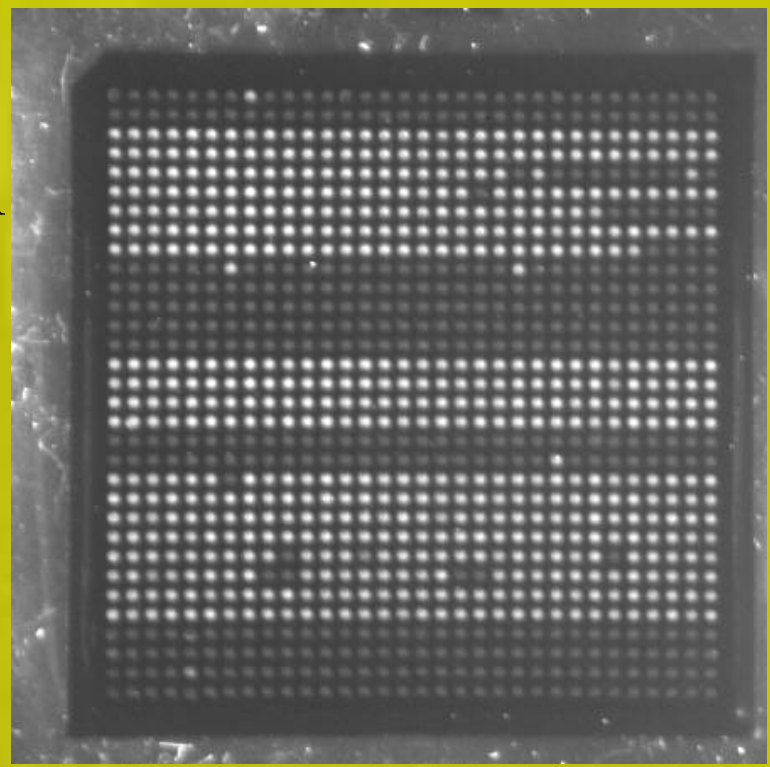
- Platform for functional genomics:
  - Needs application of variety of assays
  - High data density
  - Large number of targets and/or samples
  - Combination with other technologies

Applications of Miniaturised Amplification Based Assays

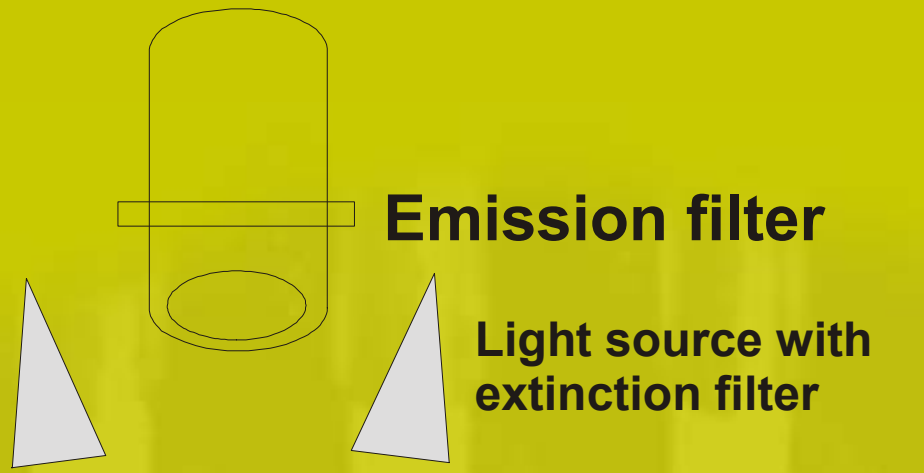
- Real-time quantification
  - Expression profiling
  - Immuno PCR
- Endpoint measurement
  - Genotyping
- Combination with other systems
  - Re-Sequencing
  - Mutation screening

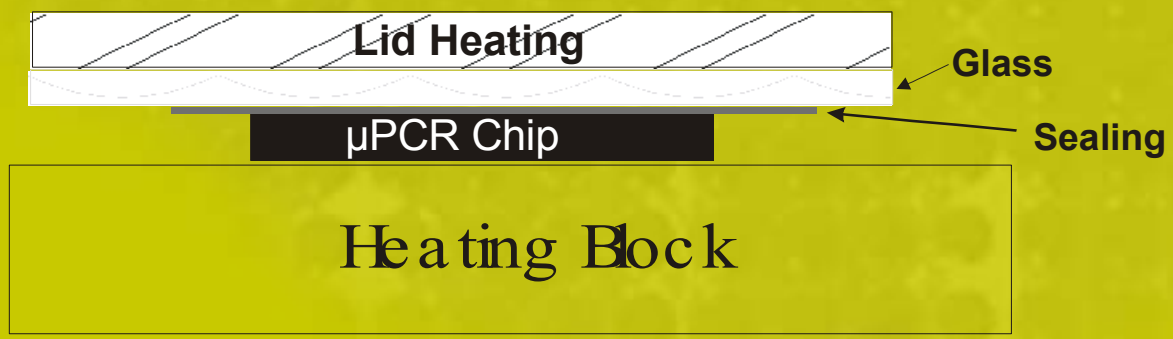
The µPCR Chip

- 32x32 wells
- Working volume 200nl/well
- Sealing with adhesive films

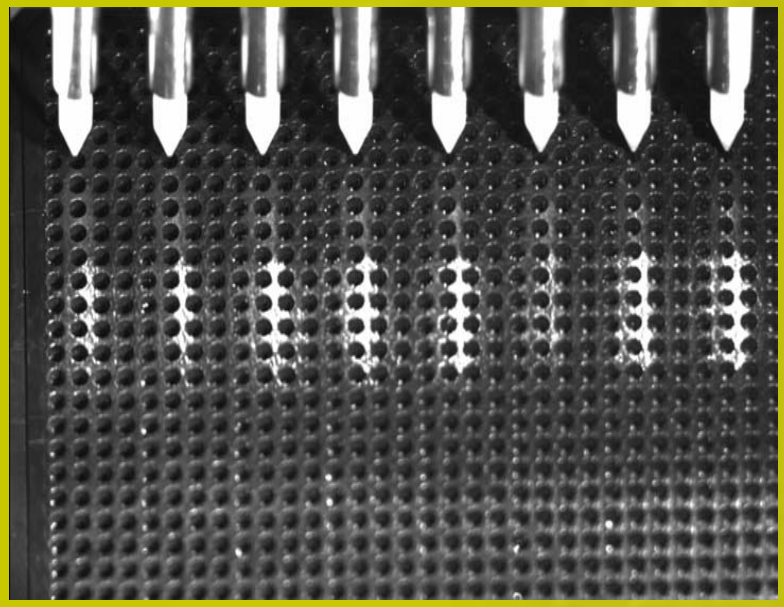


Processing Unit





Liquid Handling in Nanoliter Volume Range



µPCR chip with valve dispensing nozzles

Non contact dispensing

- Piezo dispensing
  - Scienion, Berlin
- Valve dispensing

⇦ – Seyonic, Switzerland

  - Caliper, US

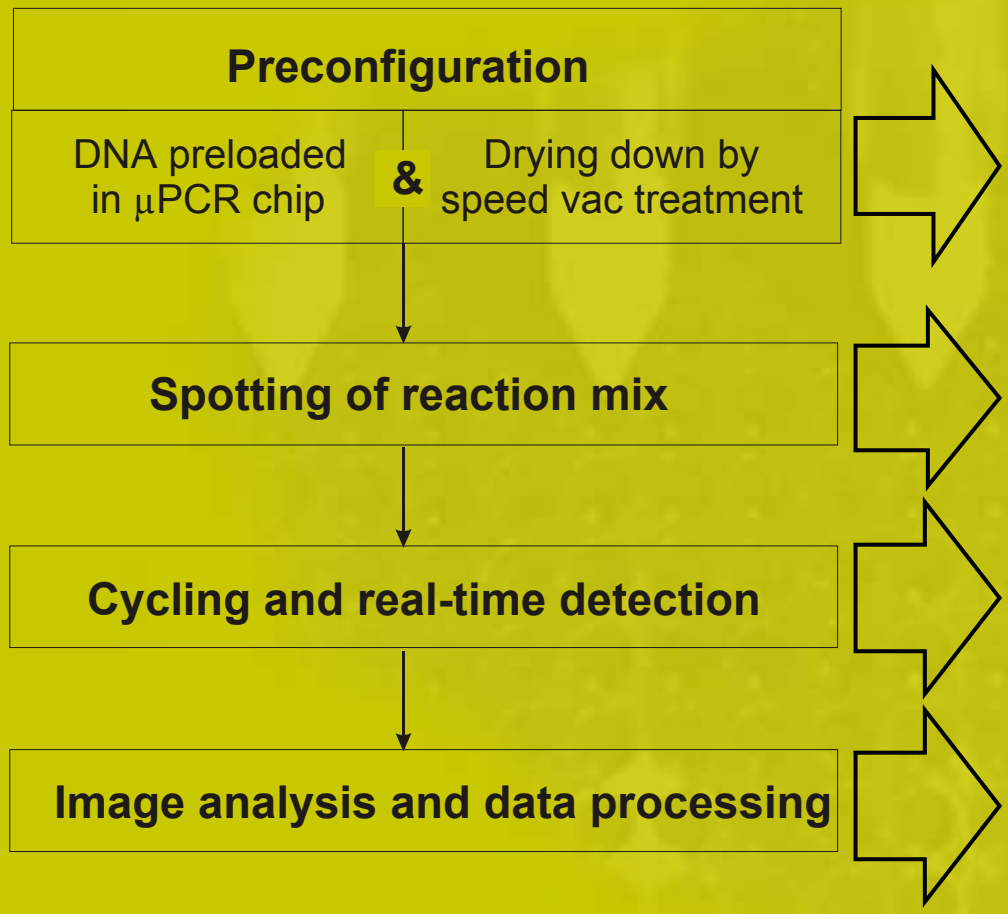
Valve Dispensing

- Resolution of 25nl
- Parallelisation of dispensing (8-Nozzle bank ➔ 96-nozzle-array)
- Separation liquid ➔ reduction of dilution/mixing effects in nozzle

Piezo Dispensing

- 400pl resolution
- 8 nozzles in parallel
- Highly flexible positioning

Workflow



Focus on:

Flexibility, avoiding cross contaminations, storage afterwards

Fast finalisation, avoiding evaporation

Tight sealing, directed condensation, sensitivity

Standardised data handling, data normalisation

Summary I – Platform Properties

- Combination of high reactor density with real-time based quantification
- 200nl working volume in µPCR chip
- Image/filter based detection system
- Workflow allows flexibility and throughput
- Parallelised non contact liquid handling

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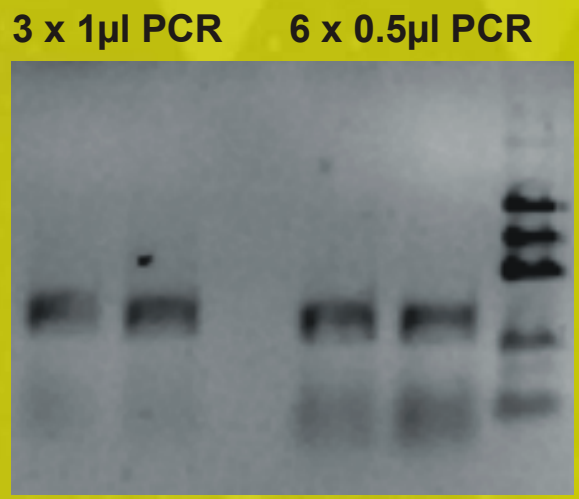
The Appropriate qPCR Assay

- **SybrGreen assays**
  - False positives due to primer dimerisation
  - Increased volume-to-surface ratio seems to enhance this effect

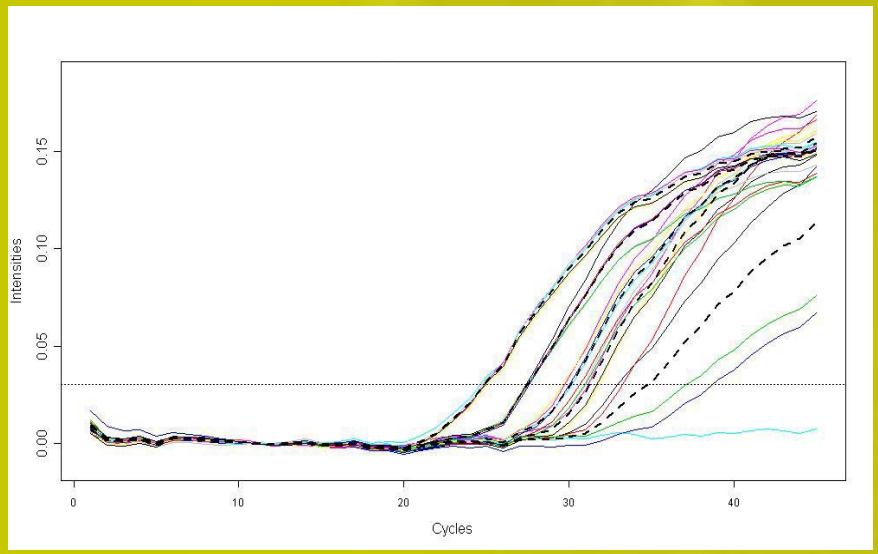


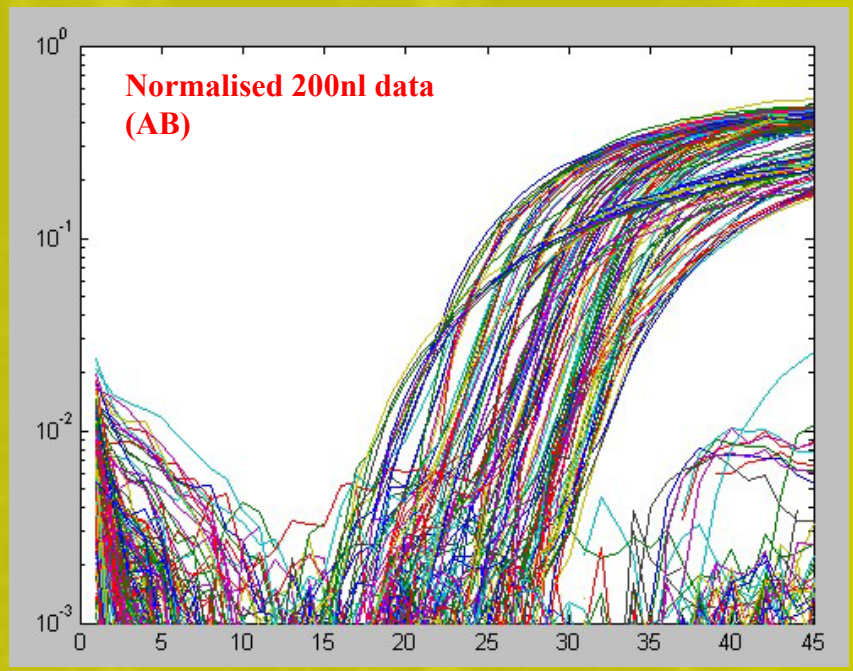
- **Probe based assays**
  - No signal from primer dimers
  - Robust

Be aware of:
Straight forward system, no possibility of post investigation (gel)

µPCR Chip – real-time Measurement

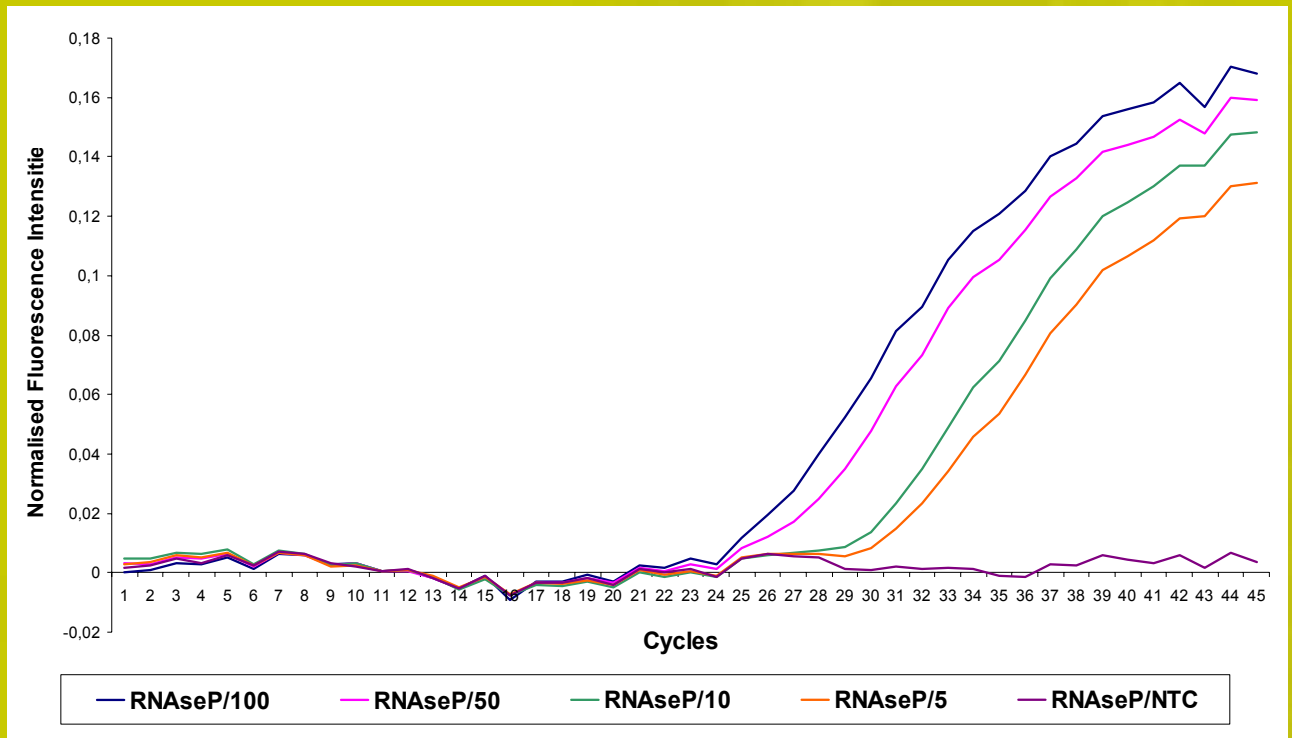
Image retrieval → Image analysis → Raw data → Data normalisation





Capability of Quantification

Mean values of normalised intensities

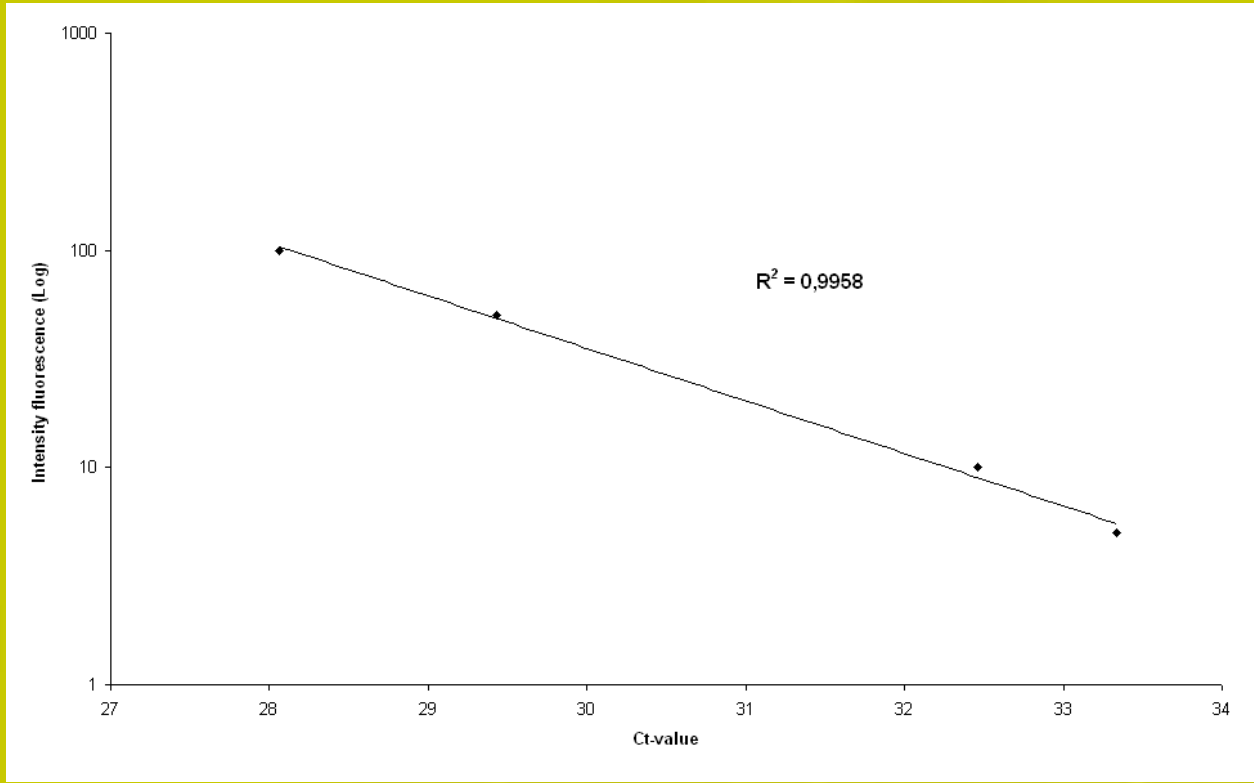


Number of reactions	15-fold	30-fold	45-fold	45-fold

200nl, target: hgDNA, RNAse P TaqMan assay,
number of initial molecules: 100, 50, 10, 5

Andreas Dahl, Max Planck Institute for Molecular Genetics, Berlin - qPCR 2005, Munich

Capability of Quantification



200nl, target: hgDNA, RNAse P TaqMan assay,
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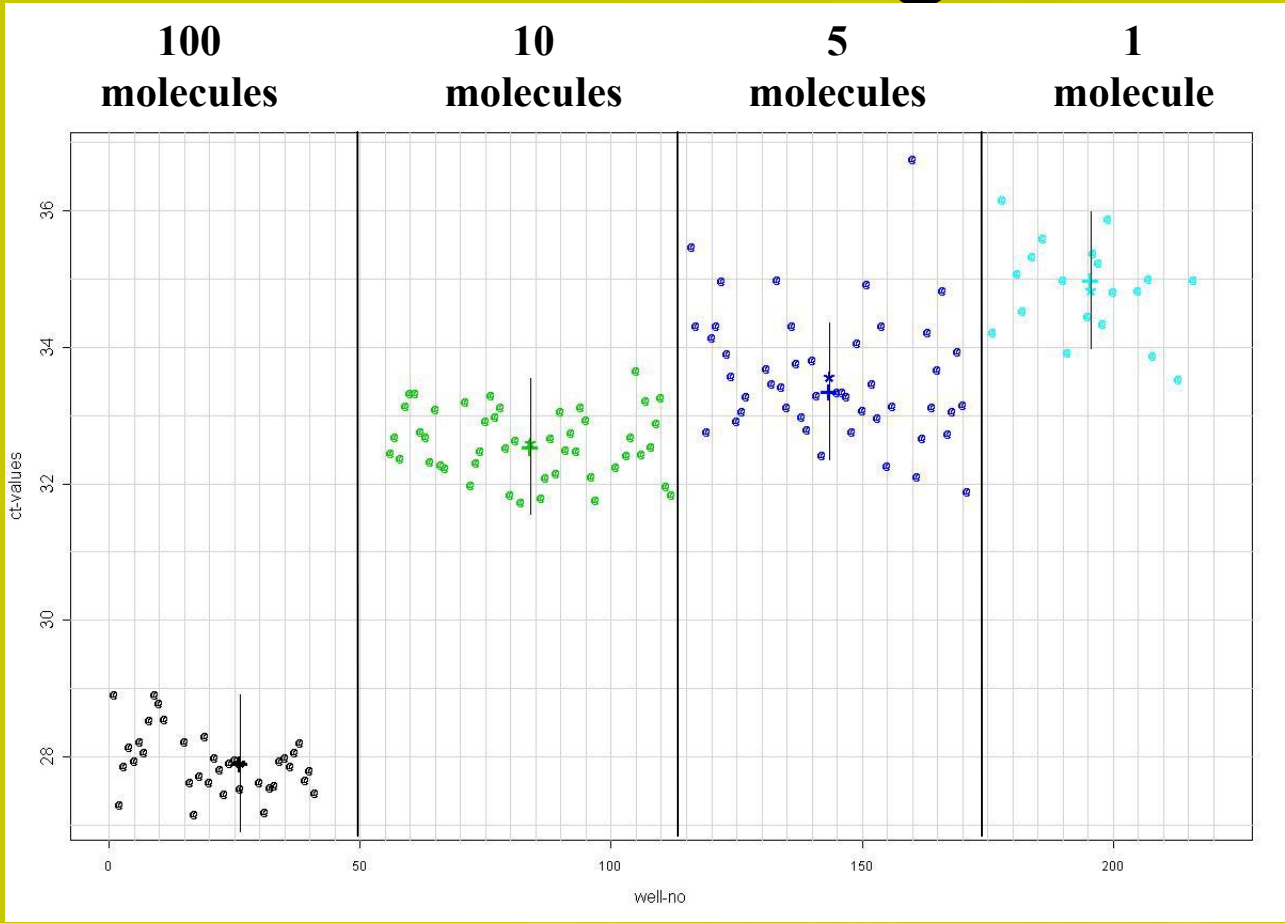
Variation of Ct-values Correlates with Number of Initial Target Molecules



200nl, Target: hgDNA, RNAse P TaqMan Assay, 36-fold

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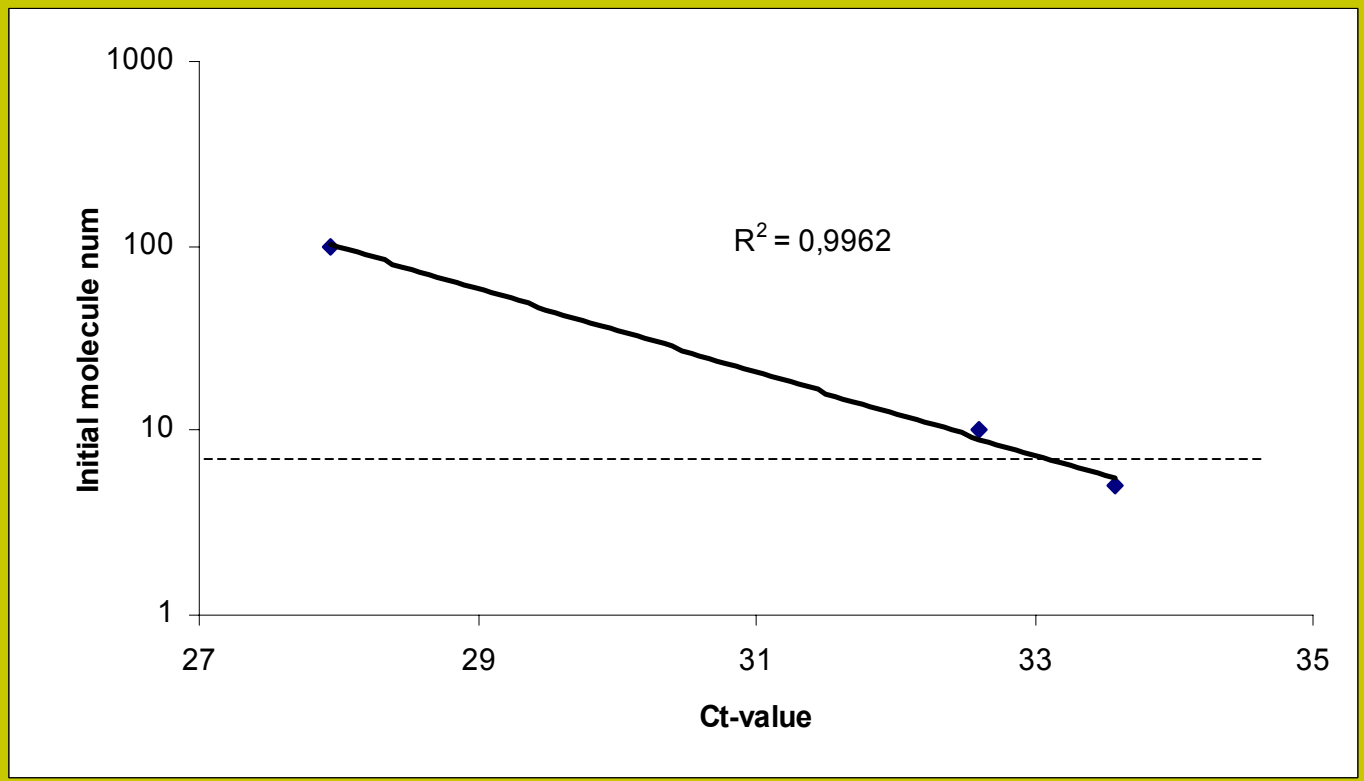
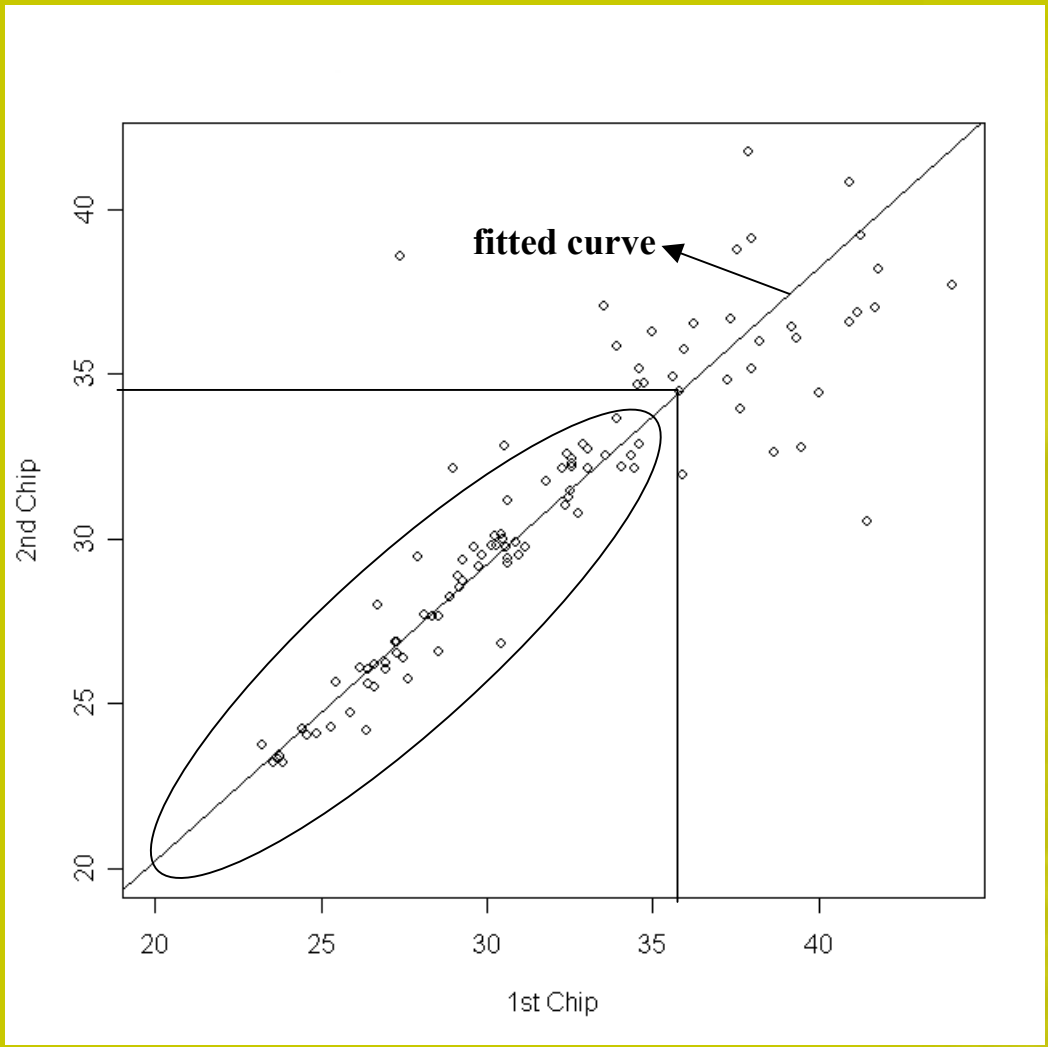
Impact of Increased Variation on Quantification

Standard curve – 3 points



200nl, Target: hgDNA, RNAse P TaqMan Assay, 36-fold

Reproducibility



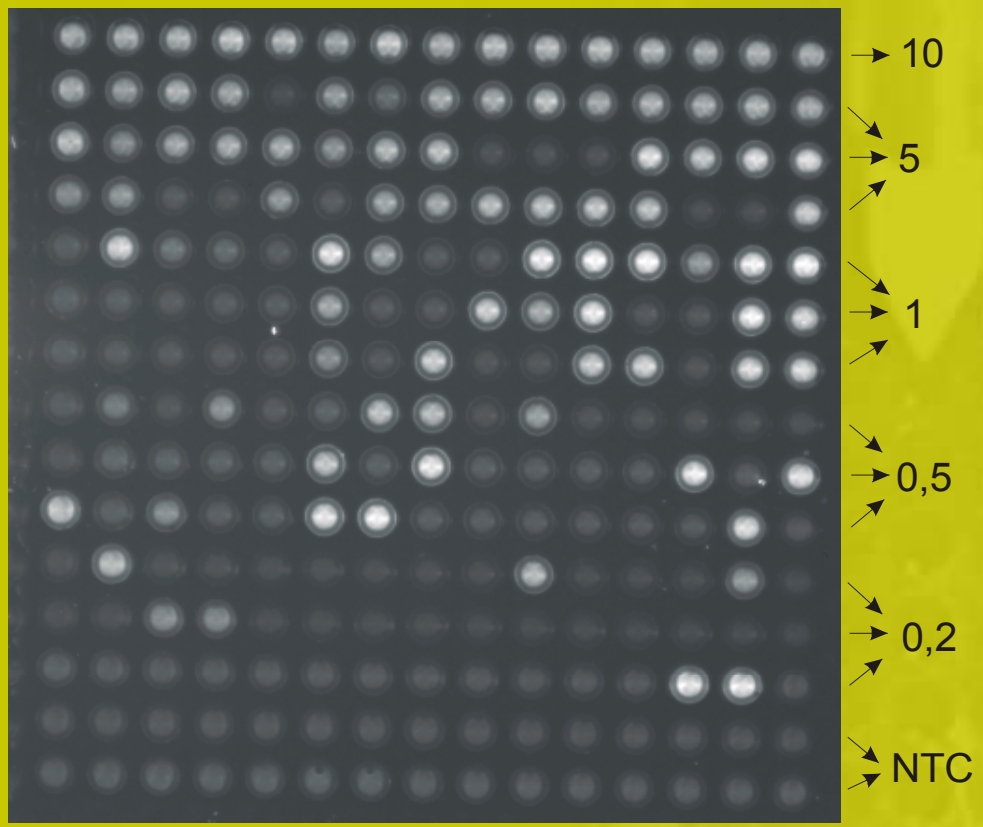
Chip to Chip comparison

-2 identically pre-configured and processed µPCR chips

-Variation increases dramatically with ct-value above 35

-Ct-value of 35 seems to be limiting threshold for reliable quantification

Limiting Target Concentrations for Detection



- 200nl PCR, RNAse P TaqMan assay
- target: hgDNA,ultra low numbers of initial molecules
- endpoint measurement

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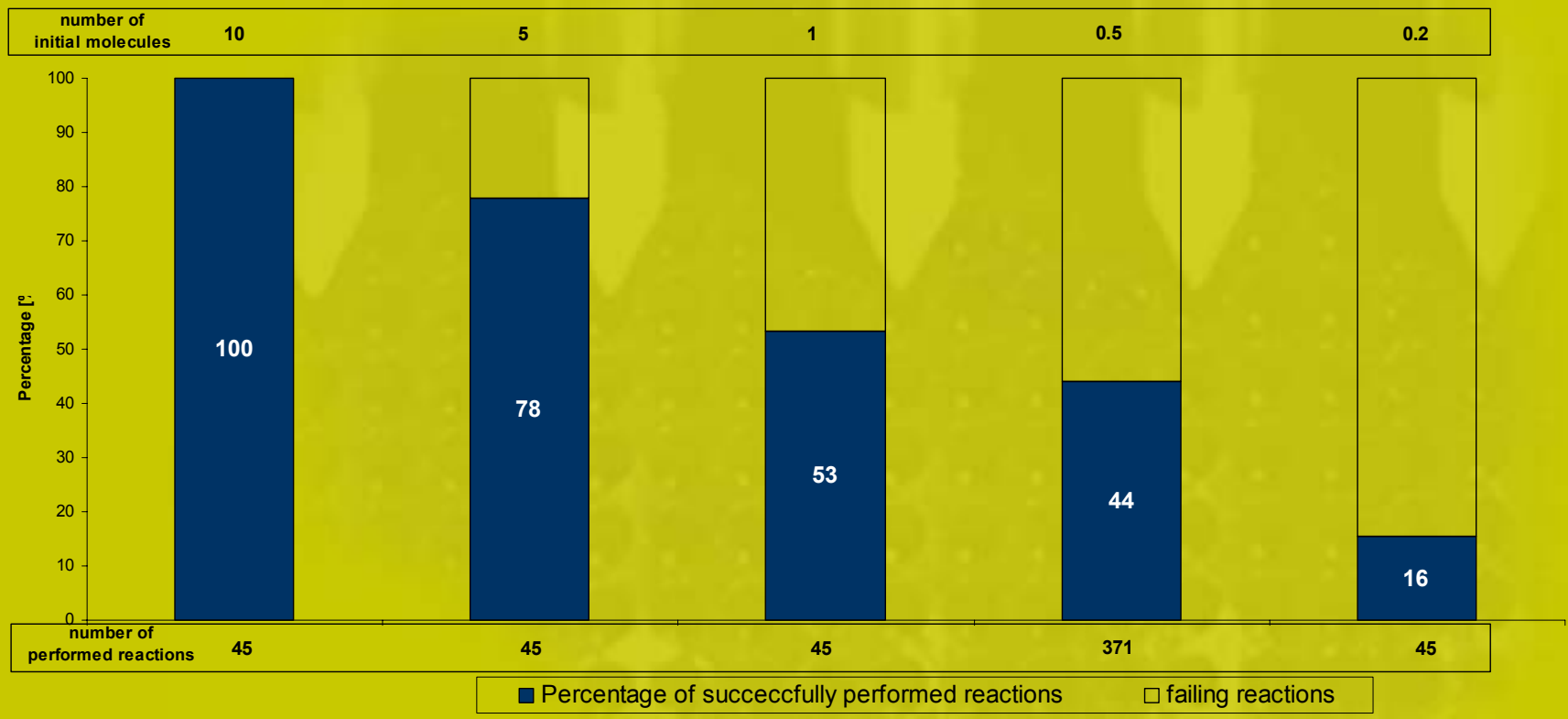
Stochastics Below 10 initial Target Molecules



Summary II – Miniaturised Assay

- Probe assays verified for real-time detection assays in nanoliter volume range
- Ct-value variation correlates with number of initial target molecules
- Chip to chip variation decreases with lowering the target concentration
- Capability of quantifications down to low molecule level with RNAse P TaqMan assay
- Below 10 initial molecules strong impact of stochastics

Overview

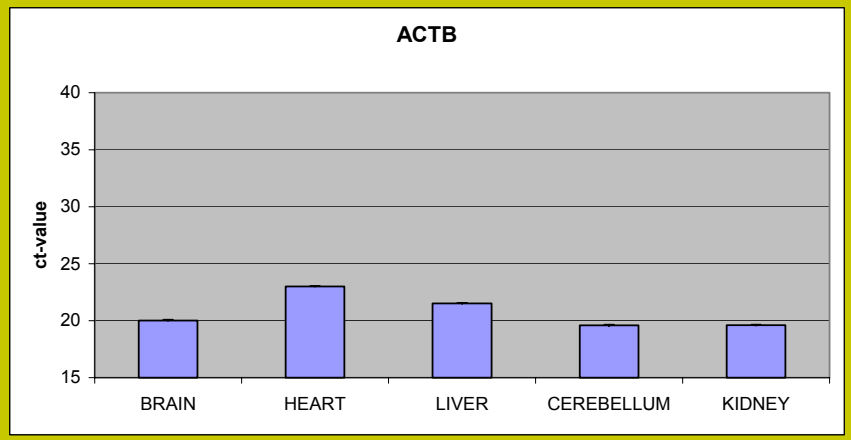
- Miniaturisation
- Platform properties
- qPCR assay implementation
- Tissue specific expression study

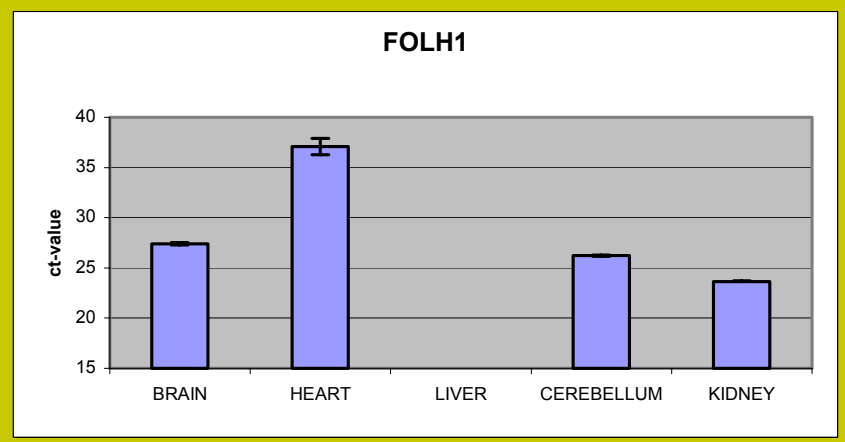
Tissue Specific Expression Profiling (Mouse)

- set of 19 genes, which are known to be tissue specific expressed
- samples from 5 tissues (brain, liver, kidney, cerebellum and heart)
- each reaction has been done 5-fold, each µPCR chip was processed in duplicate
- 10µl volume as standard assay, 4-fold

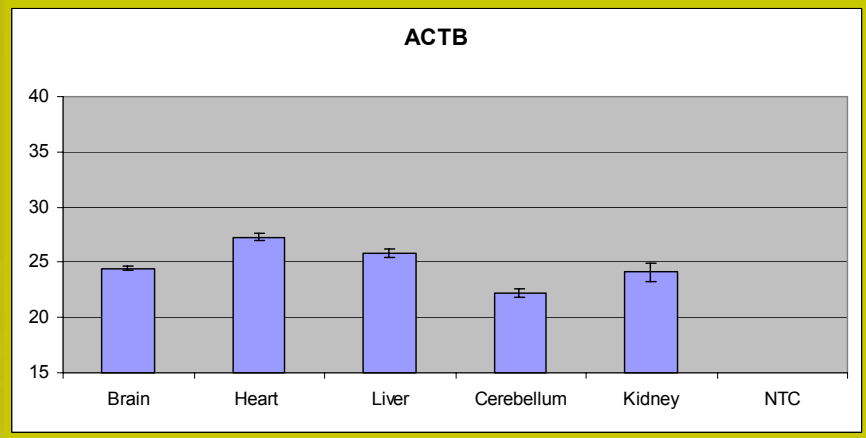
Comparison of Absolute Ct-values I

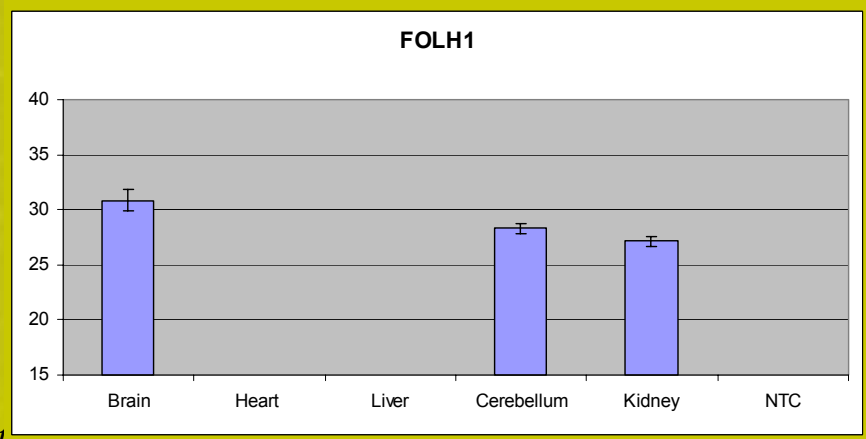
Standard(10µl)





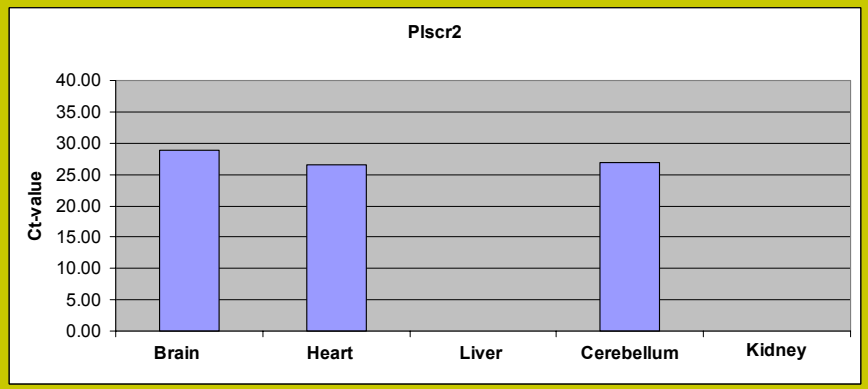
µPCR Chip (200nl)



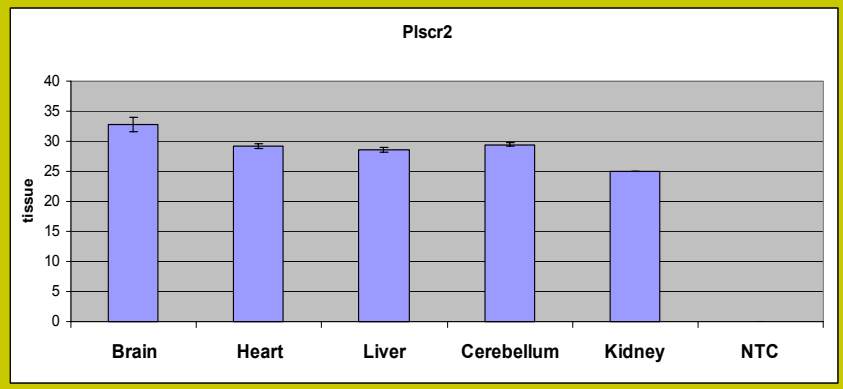


Comparison of Absolute Ct-values II

Standard(10µl)



µPCR Chip (200nl)



- Comparability of 10µl and 200nl reactions heterogeneous
  - Higher variation in 200nl
  - Stringent definition of outliers needed
  - Data normalisation

Summary III – Mouse Study

- Workflow verified
- Selected assays show comparable ct-value patterns in both volumes
- Impact factors on variability
  - Reliable & reproducible preloading with sample DNA
  - Wall-reactant-interactions
  - Data normalisation & evaluation

Outlook

- Reduction of variation in 200nl
  - Optimisation of dispensing accuracy
  - Optimisation of assay performance
- Large scale experiments on platform
- Further applications
  - Genotyping, ImmunoPCR
- Further reduction of the volume
- Surface modification

Acknowledgement

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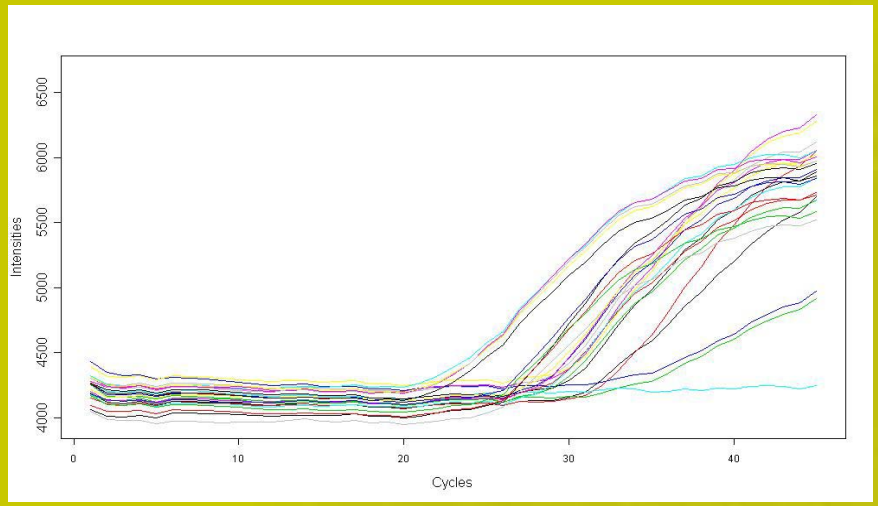
Ken Livak
Stephen Gunstream

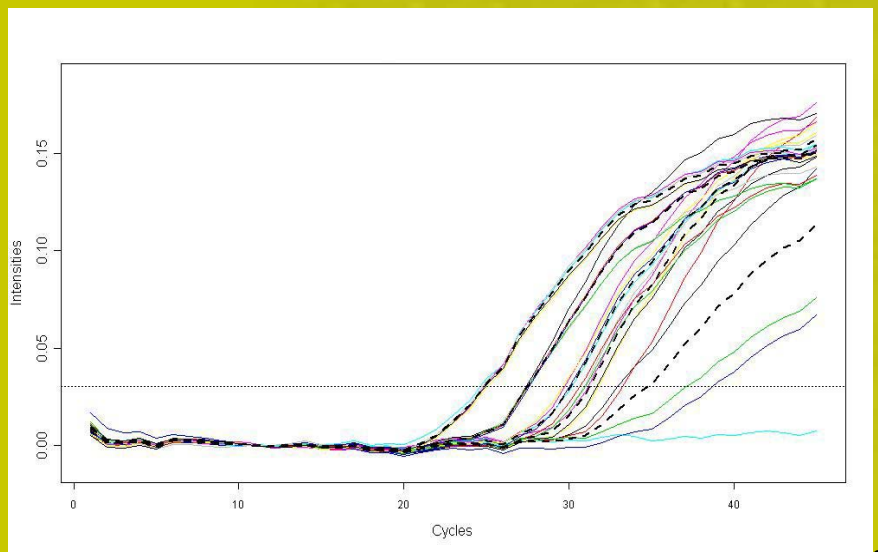
Thank You!

µPCR Chip – real-time Measurement



Raw data plot

(fluorescence intensities of each well over time, one image per cycle, data achieved by image analysis)



Normalised data plot

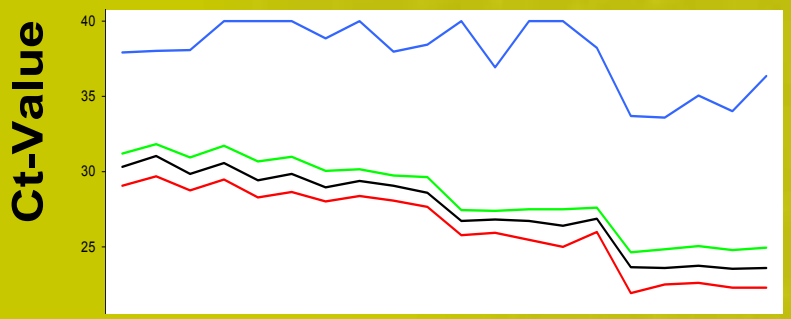
(the dotted lines are the averages of the 5-fold measurement per concentration)

Data Normalisation

- Removing noise of the system
- Normalisation of differences in reaction volumes

Image retrieval → Image analysis → Raw data → Data normalisation

Comparison with AB mathematics



Raw data (Berlin)

Background corrected (AB)

Normalised data (Berlin)

Normalised data (AB)