Setting standards in analytical science

Measurements for Biotechnology

The Data Comparability Challenge - Standards and Best Practices

Morten Andersen, LGC

qPCR 2005, Freising-Weihenstephan

Overview

Setting standards in analytical science

- Measurements for Biotechnology Programme
- Our aims
- Approach



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UK Measurements for Biotechnology (MfB) programme

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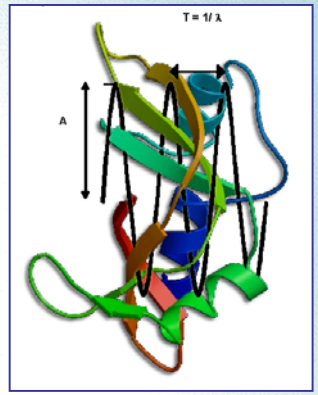
- LGC is the designated UK Measurement Institute for Chemical and Biochemical measurements
- Our role is underpinned by funding from the Government Funded 'Measurements for Biotechnology' Programme aimed at:
  - Improving the accuracy and reliability of biomeasurement, working closely with industry
  - Strengthening the measurement science underpinning the regulation of biotechnology
  - Ensuring that the UK biomeasurement system is co-ordinated and developed in harmony with those of other countries

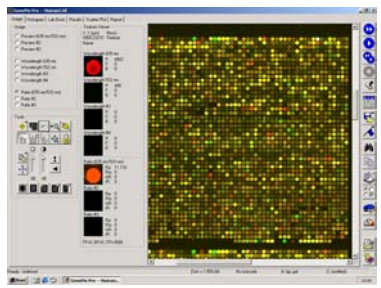
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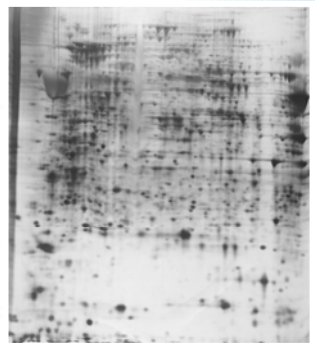
Measurements for Biotechnology *(mfbprog.org.uk)*

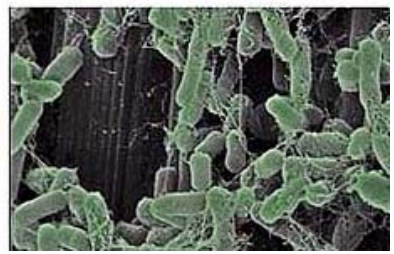
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- High priority technical themes identified where there are rapid developments in measurement technology critical to their robust application and commercial exploitation:
  - Biological product characterisation
  - Gene measurements & DNA microarray standardisation
  - Complex protein measurement
  - Cell based technology
- Knowledge transfer - MfB “hub”, UK Centre for Biomeasurement (UKCB - LGC+NPL) & UK Bioindustry Association









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Development of standard units to measure gene expression

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- Working towards improved standardisation of gene expression measurements
- Help to reach consensus and acceptance on the issue of standardisation

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The idea

- To identify methods which, when applied to an experimental procedure, will help to improve confidence in the gene expression data generated
- Ideas from the literature, conferences and discussion groups
- Aim
  - To put these ideas into practice and evaluate their impact on accuracy, inclusive of reproducibility and comparability of gene expression measurements
  - eventually, a ‘best practice’ may be developed

The approach

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- QRT-PCR technology is providing benchmark results in the field of gene expression measurements
- We will be investigating the effects of
  - experimental design, especially replication
  - using prescriptive protocols or SOPs
  - RNA quality
  - using spike-in controls at RNA and DNA level
  - choosing appropriate reference genes; selection process
  - instrumentation
  - approach to data analysis
  - training

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Quantitative real-time PCR study

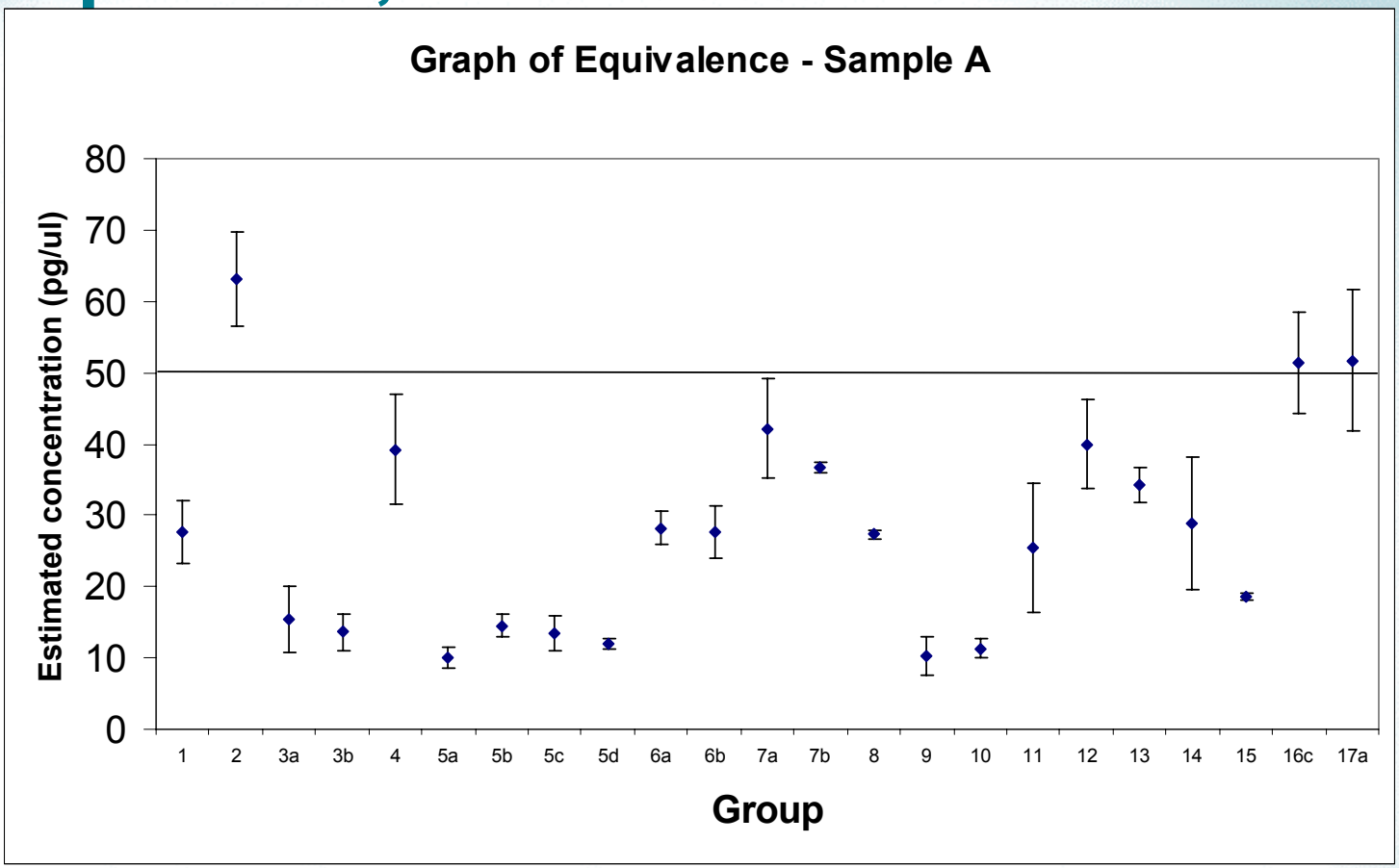
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- Bio-analysis Working Group
  - The International Bureau of Weights and Measures
- Organised by NIST and LGC
- Aims:
  - To verify the ability of laboratories to quantify a specific DNA sequence
  - To determine factors and practices that contribute to accurate quantification
- Plasmid-based DNA calibration and testing materials

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QPCR - inter-laboratory comparison, round 1

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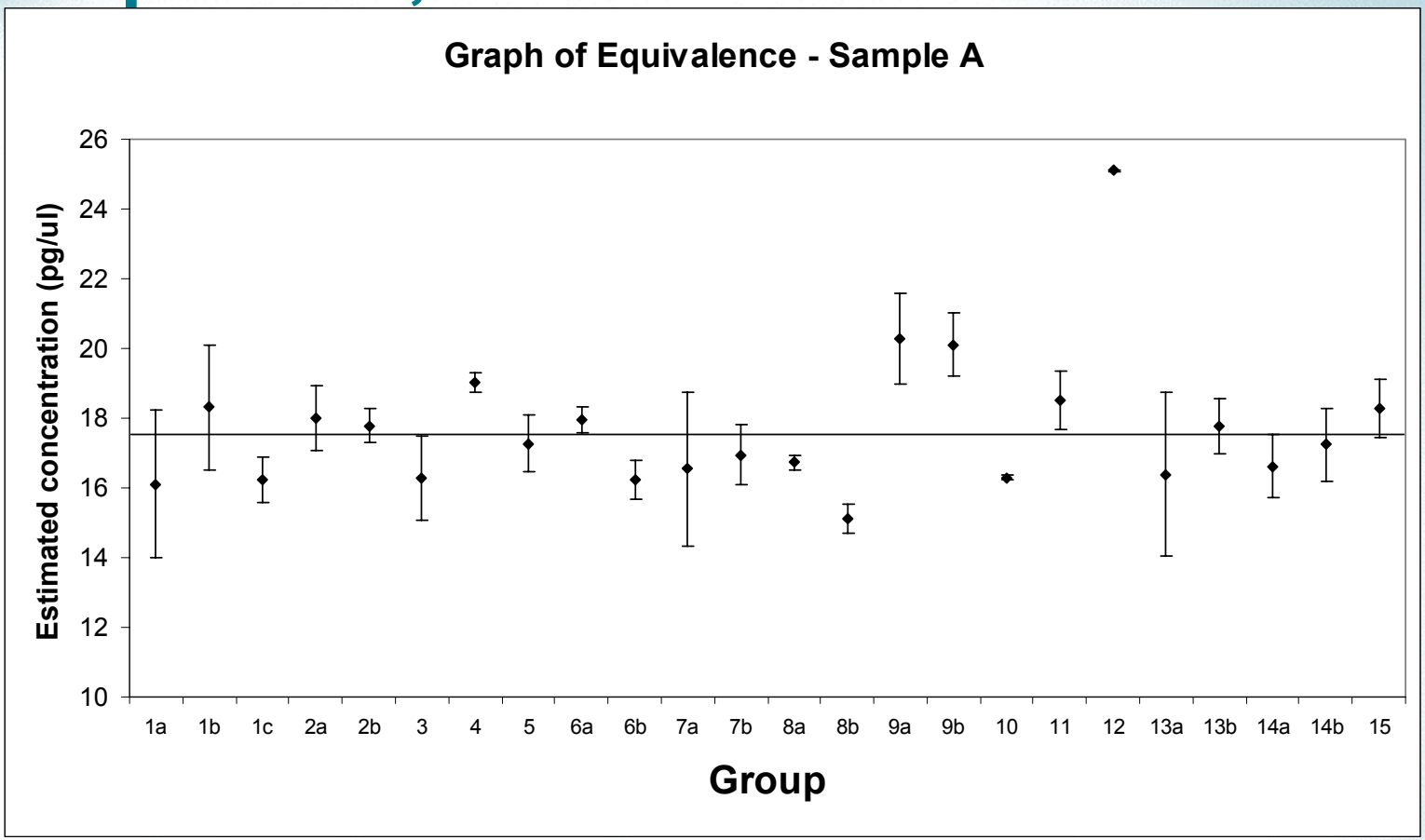
Group means and 95% confidence intervals are displayed on the graph

CCQM P44 - real-time PCR pilot study

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QPCR - inter-laboratory comparison, round 2

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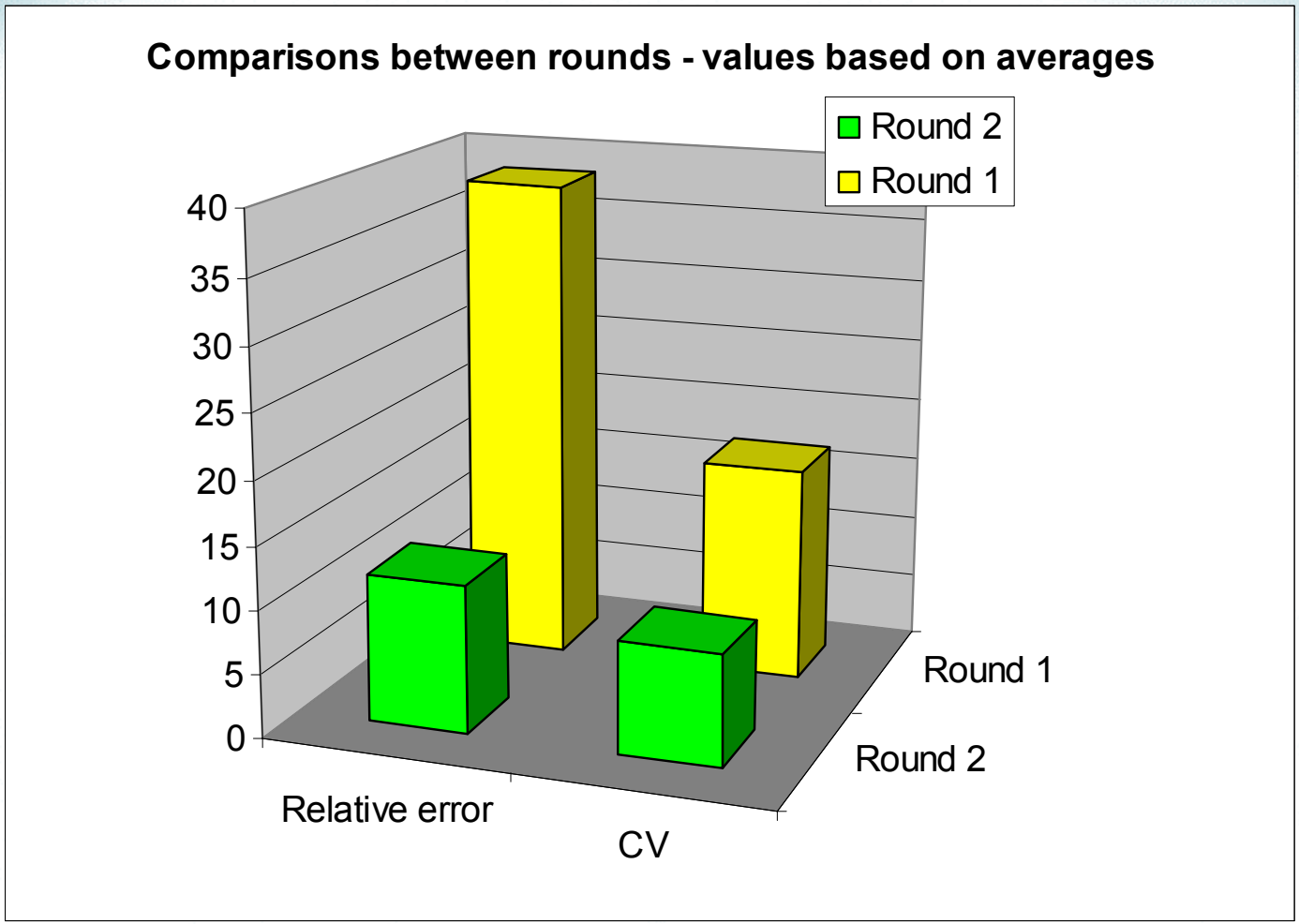
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CCQM P44 - real-time PCR pilot study

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Comparison between rounds

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CCQM P44 - real-time PCR pilot study

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Factors affecting performance

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- List of changes since Round 1:
  - Difference in concentrations of two sample unknowns
    - Theoretically decrease performance
  - Experience of participants (potential large effect)
  - Form of target analyte (solution vs. dried-down DNA)
  - Prescriptive protocol
  - Submission of all raw data - negate potential bias
  - Set number of replicates
  - Data handling
  - Verification of data collation

Initial focus

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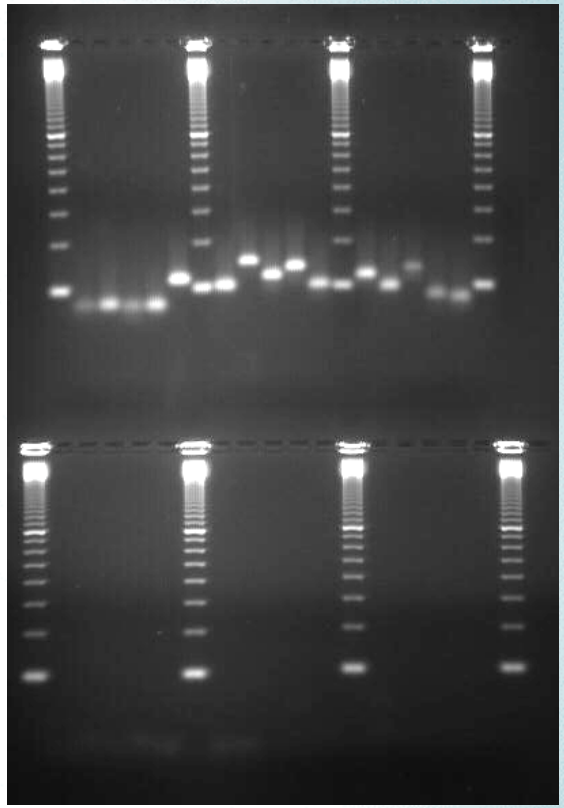
- CCQM study indicates that QPCR results can be comparable between laboratories
- Striving to achieve similar confidence in every step of the QRT-PCR experimental procedure
- Initially focussing on
  - RNA quality control
  - designing exogenous RNA spike-in controls
  - designing QPCR assays for the current and future work

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QPCR assay design

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- Using guidelines
  - manufacturers
  - users
- Dual-labelled fluorogenic probes
  - PrimerExpress 2.0
  - spanning exon/exon boundaries
  - multiple primers where possible
  - primer combination; SYBR Green
  - primer concentration
  - probe concentration
  - linearity
  - PCR efficiency



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RNA quality control

- Gel electrophoresis
  - requires relatively large quantities of RNA
- Agilent Bioanalyzer
  - small amounts of RNA; RIN value
  - expensive
- QRT-PCR based assay
  - 3':5' assay suggested in the discussion forum



  - measure relative abundance
- How well do RIN values and 3':5' data correlate?
- $OD_{260/280}$ and $OD_{260/230}$

Controlled RNA degradation

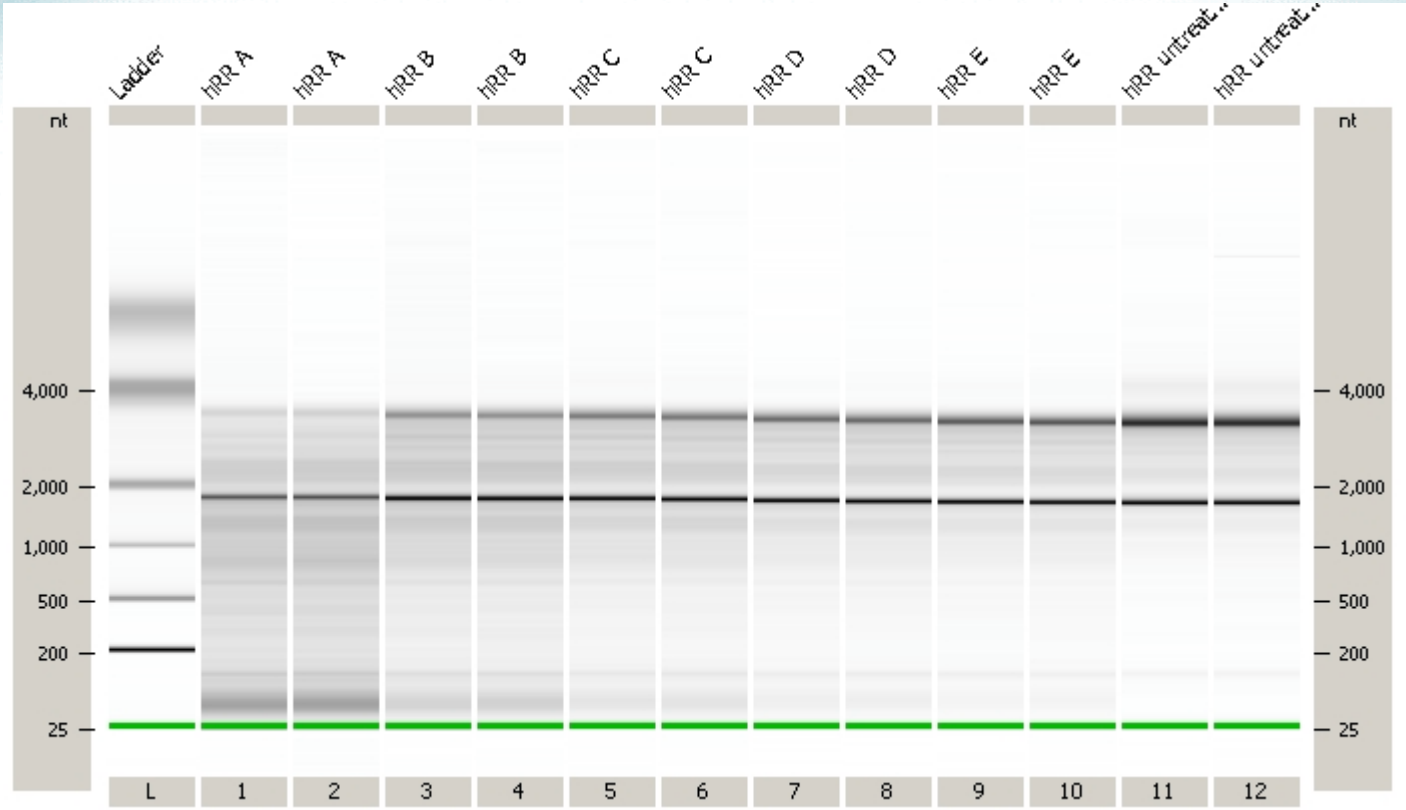
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- QPCR Human Reference RNA
- RNase A at different concentrations
- 37 degC for 5 min
- Analysed on RNA 6000 Nano LabChip kit, 2100 Bioanalyzer

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RIN	**4.2**	**4.4**	**6.0**	**5.9**	**6.8**	**6.6**	**7.3**	**7.3**	**7.6**	**7.7**	**8.8**	**8.7**



RNase A, ng/mL	**10**	**10**	**8**	**8**	**6**	**6**	**4**	**4**	**2**	**2**	**Un-treated**	**Un-treated**

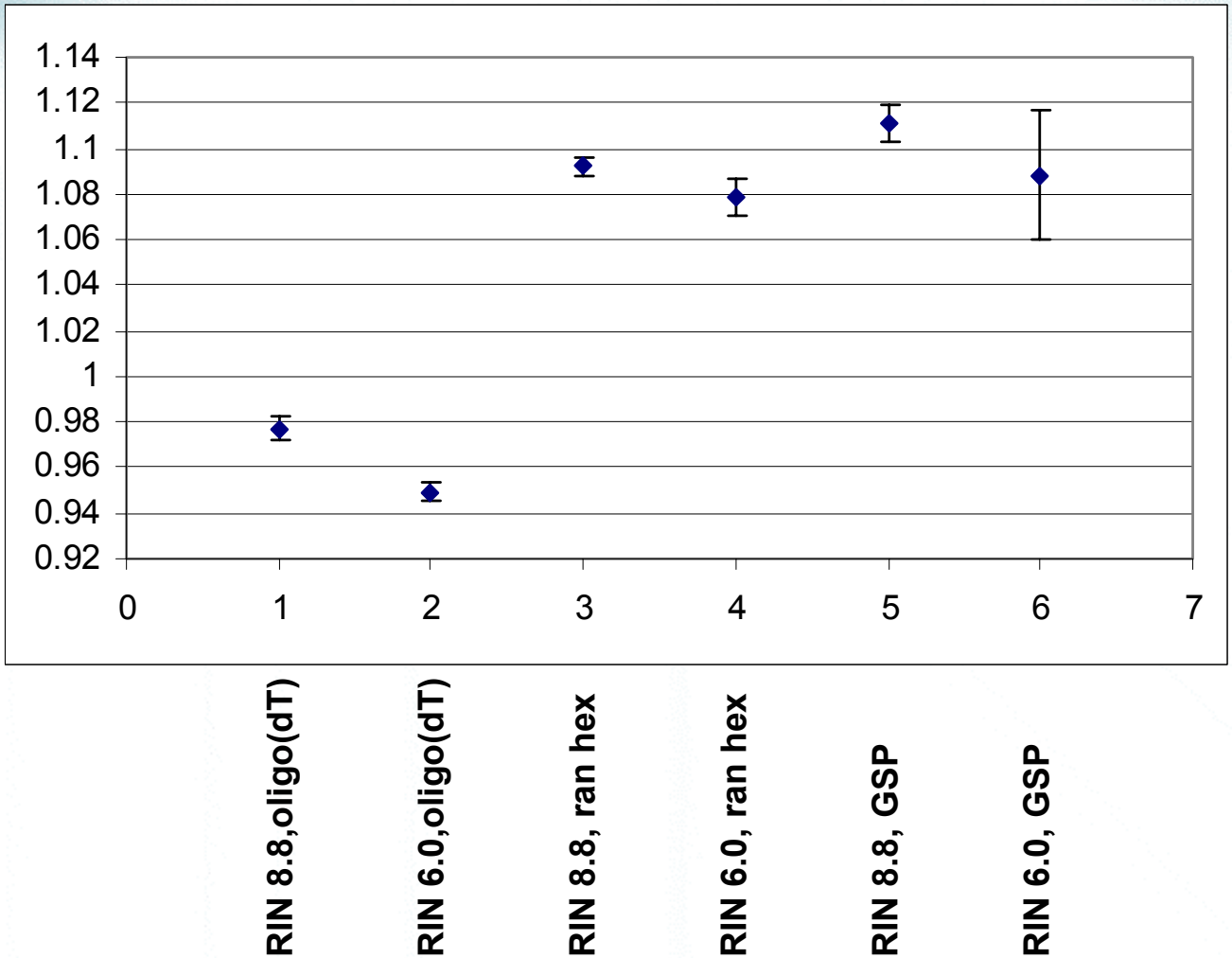
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Measuring 3':5' ratio

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- MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli) (MLH1)
  - mRNA: 2524
- Assays
  - 5' position: 256
  - 3' position: 2154
- Untreated (RIN: 8.8) and degraded sample (RIN: 6)
- Reverse transcription in duplicate
  - oligo(dT), random hexamers and gene-specific primers (GSPs)
- QPCR reactions in triplicate
- 3':5' ratio based on Ct values

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Preliminary results

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- Conclusion
  - Significant difference between 3':5' values obtained for the untreated and partially degraded MLH1 transcripts primed with oligo(dT) and random hexamers
  - Large uncertainty associated with the GSP strategy on partially degraded RNA may be caused by variation between duplicate RT reactions
- Is RNase A degradation a good approach?
  - cell extract instead?
  - freeze/thawing cycles?
- More targets to establish correlation

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RNA spike-in control

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- *Arabidopsis thaliana*
- Cloned in pSP64 Poly(A) Vector
  - SP6 promoter
  - poly(A) sequence
  - allow for use of main RT priming strategies
- Quantification using RiboGreen assay
- Currently to be sequenced

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Ongoing and future work

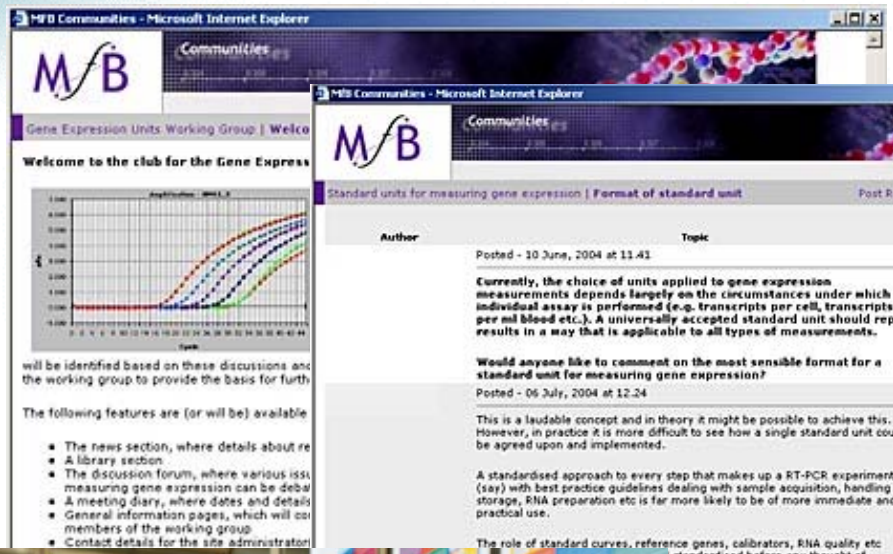
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- Develop panel of 3':5' assays for representative transcripts
  - stable, unstable, abundant, rare
- Spike-in controls
  - RT and QPCR level
- Format of standard curves
  - cDNA, *in vitro* transcripts, synthetic amplicons…
- Experimental design
  - *how well can you trust your data…?*
- Inter-laboratory evaluations of methodologies

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Web-based forum

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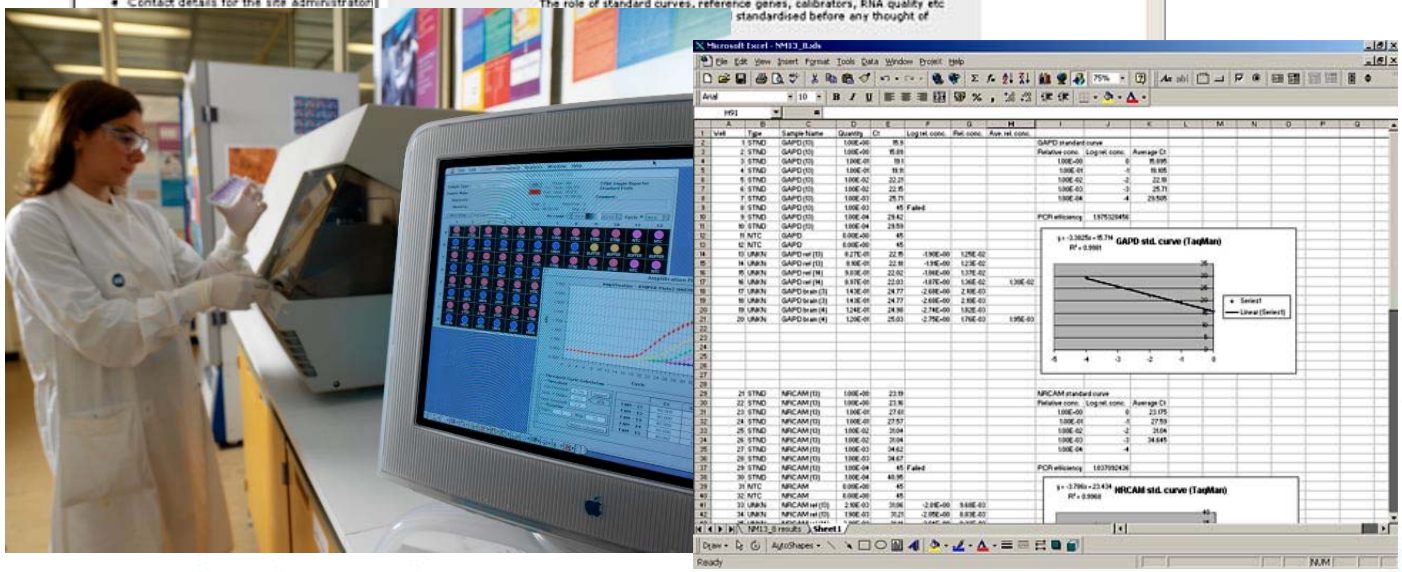


Possible approaches for the creation of a standard unit for measuring gene expression

Report number LGC/MFB/2004/018

Morten Andersen & Carole Foy

BioAnalytical Innovation Team, LGC, Queens Road, Teddington, Middlesex, TW11 0LY, UK



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Points for discussion

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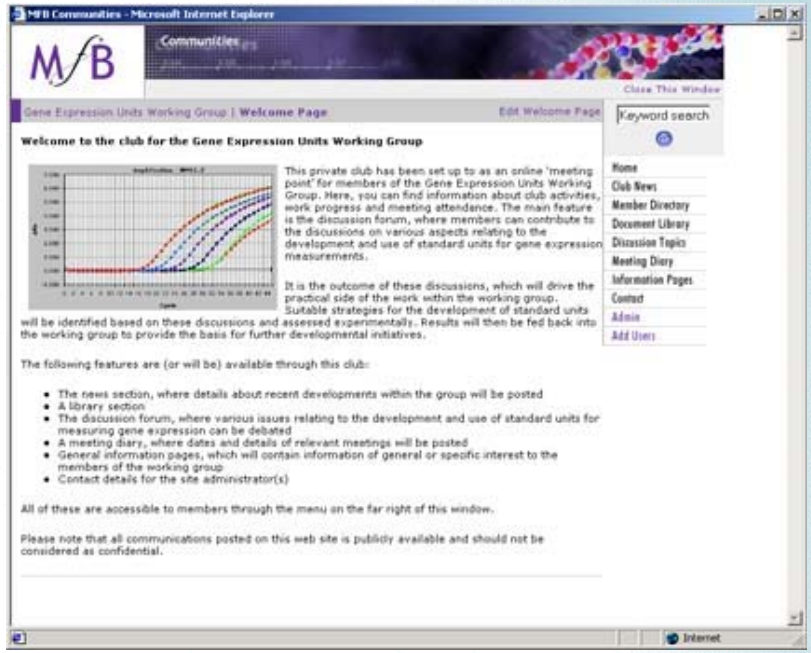
- 'MIAME'-style guidelines on information
- Experimental design
- Data analysis
  - standard curves; simple vs. weighted linear regression
- Appropriate reference materials
  - ERCC
- Additional strategies or new ideas?
- Potential for long-term development of standards units?

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www.mfbprog.org.uk

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- Web-based working group
  - discussion forum
- Open to everyone
  - end-users
  - manufacturers
  - regulatory agencies
- Ad hoc basis



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Conclusion

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- We are looking to identify ‘best practices’ in QRT-PCR
  - evaluation of current and novel strategies
- Need input from the users
- Let your views be known via the web-based forum

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Acknowledgements

- Members of the working group
- Dr. Carole Foy
- Dr. Malcolm Burns
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- DTI, NMS, MfB Programme