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in analytical science*



Measurements for Biotechnology

# The Data Comparability Challenge - Standards and Best Practices

Morten Andersen, LGC

qPCR 2005, Freising-Weihenstephan

# Overview



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- 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 coordinated and developed in harmony with those of other countries



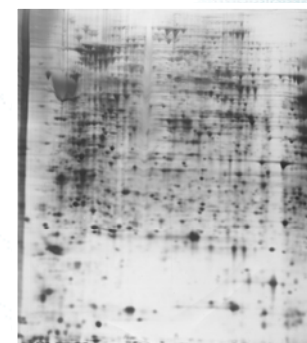
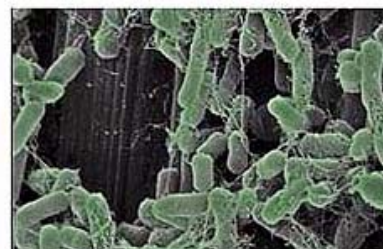
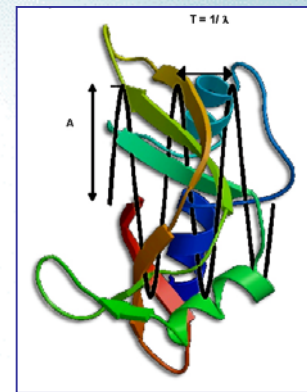
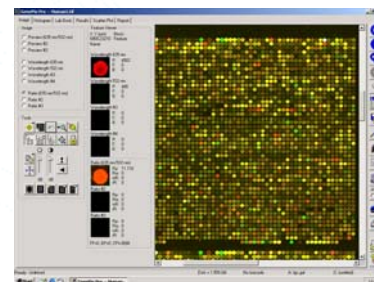
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# Measurements for Biotechnology ([mfbprog.org.uk](http://mfbprog.org.uk))



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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



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- 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



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# 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

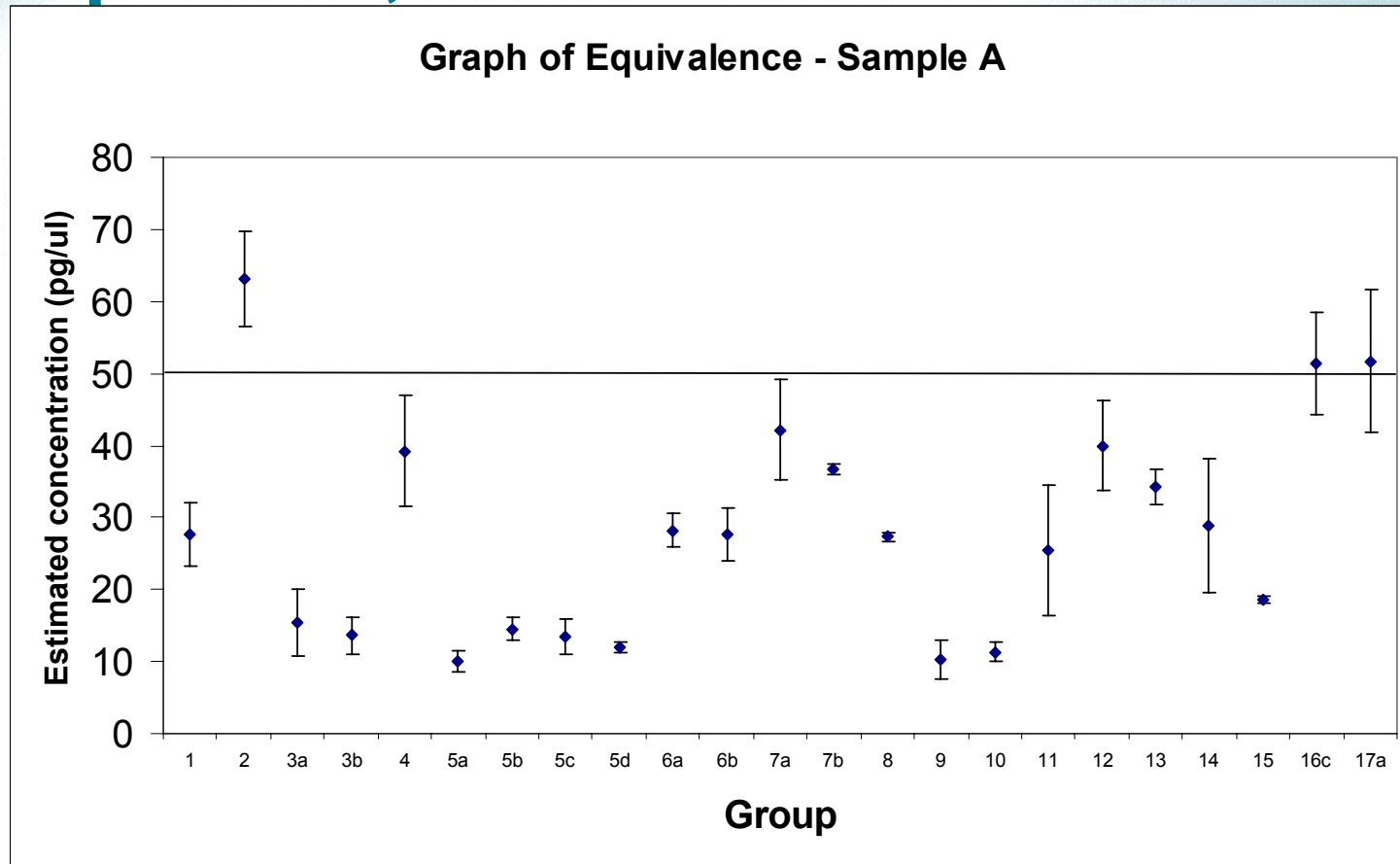


- Bio-analysis Working Group
  - The International Bureau of Weights and Measures
- Organised by NIST and LGC
- Aims:
  - To verify the ability of laboratories to quantify a specific DNA sequence
  - To determine factors and practices that contribute to accurate quantification
- Plasmid-based DNA calibration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

# QPCR - inter-laboratory comparison, round 1



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

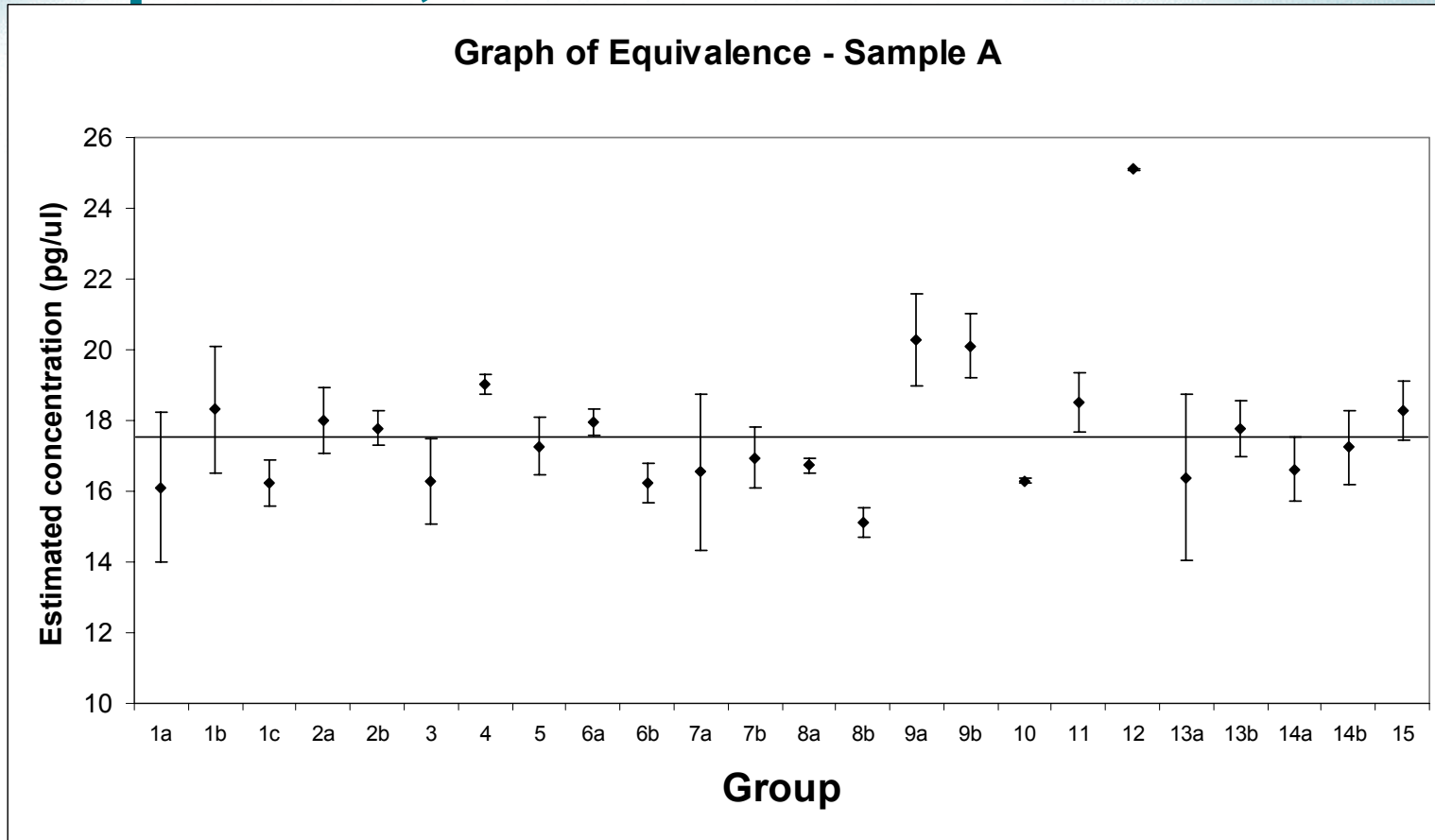
CCQM P44 - real-time PCR pilot study



# QPCR - inter-laboratory comparison, round 2



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

CCQM P44 - real-time PCR pilot study

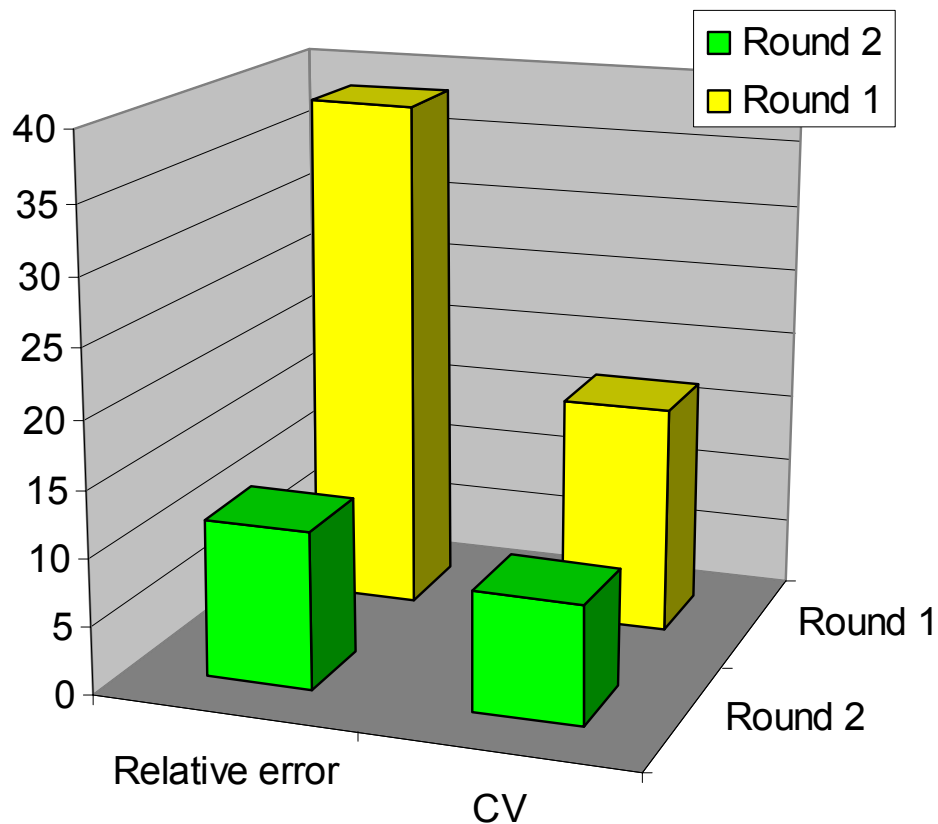


# Comparison between rounds



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Comparisons between rounds - values based on averages



# Factors affecting performance



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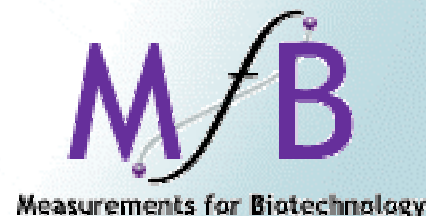
- List of changes since Round 1:
  - Difference in concentrations of two sample unknowns
    - Theoretically decrease performance
  - Experience of participants (potential large effect)
  - Form of target analyte (solution vs. dried-down DNA)
  - Prescriptive protocol
  - Submission of all raw data -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

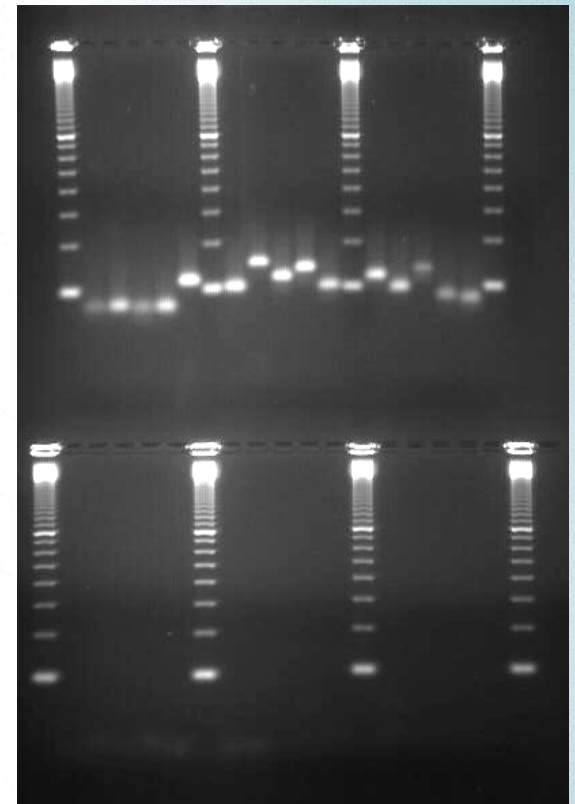


# QPCR assay design



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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



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- 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<sub>260/280</sub> and OD<sub>260/230</sub>



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# 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

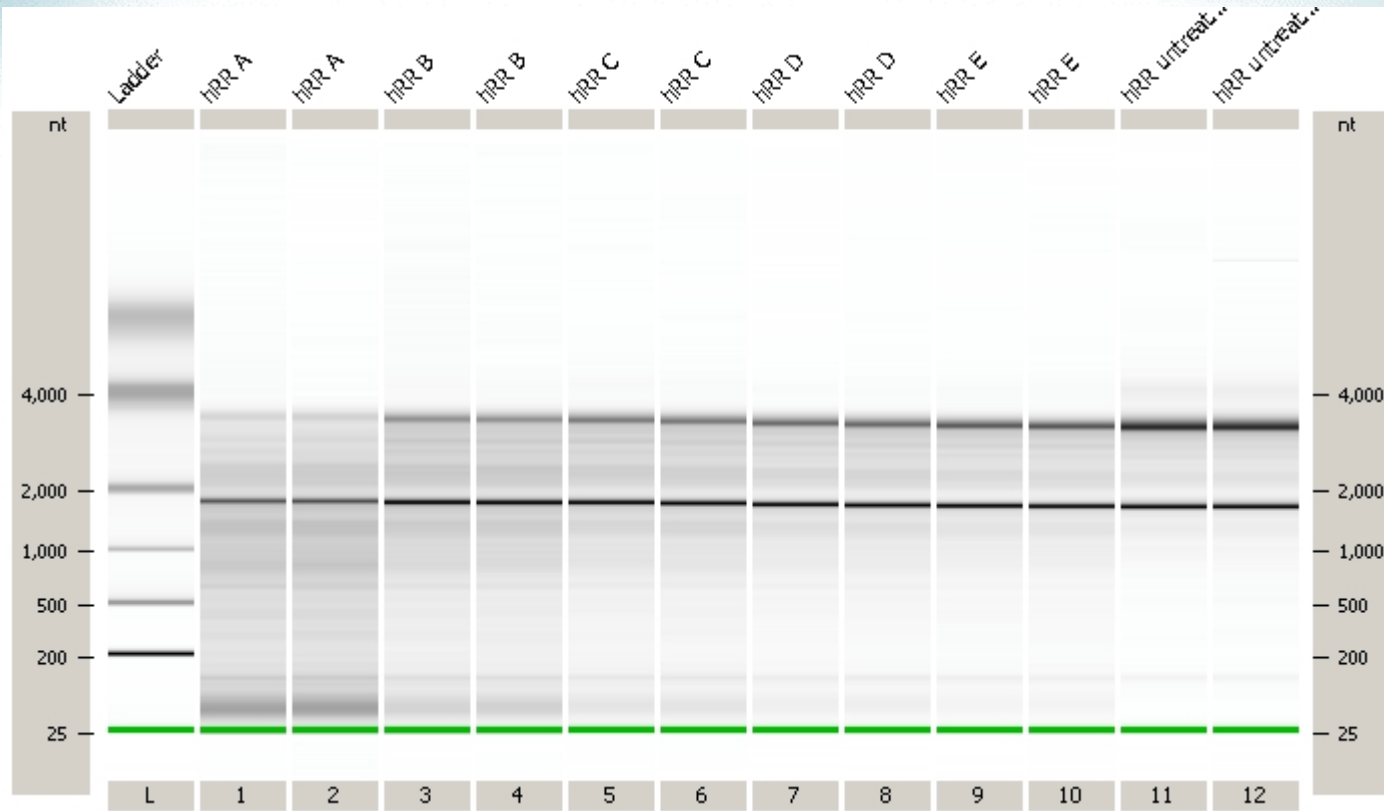


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RIN            4.2   4.4   6.0   5.9   6.8   6.6   7.3   7.3   7.6   7.7   8.8   8.7



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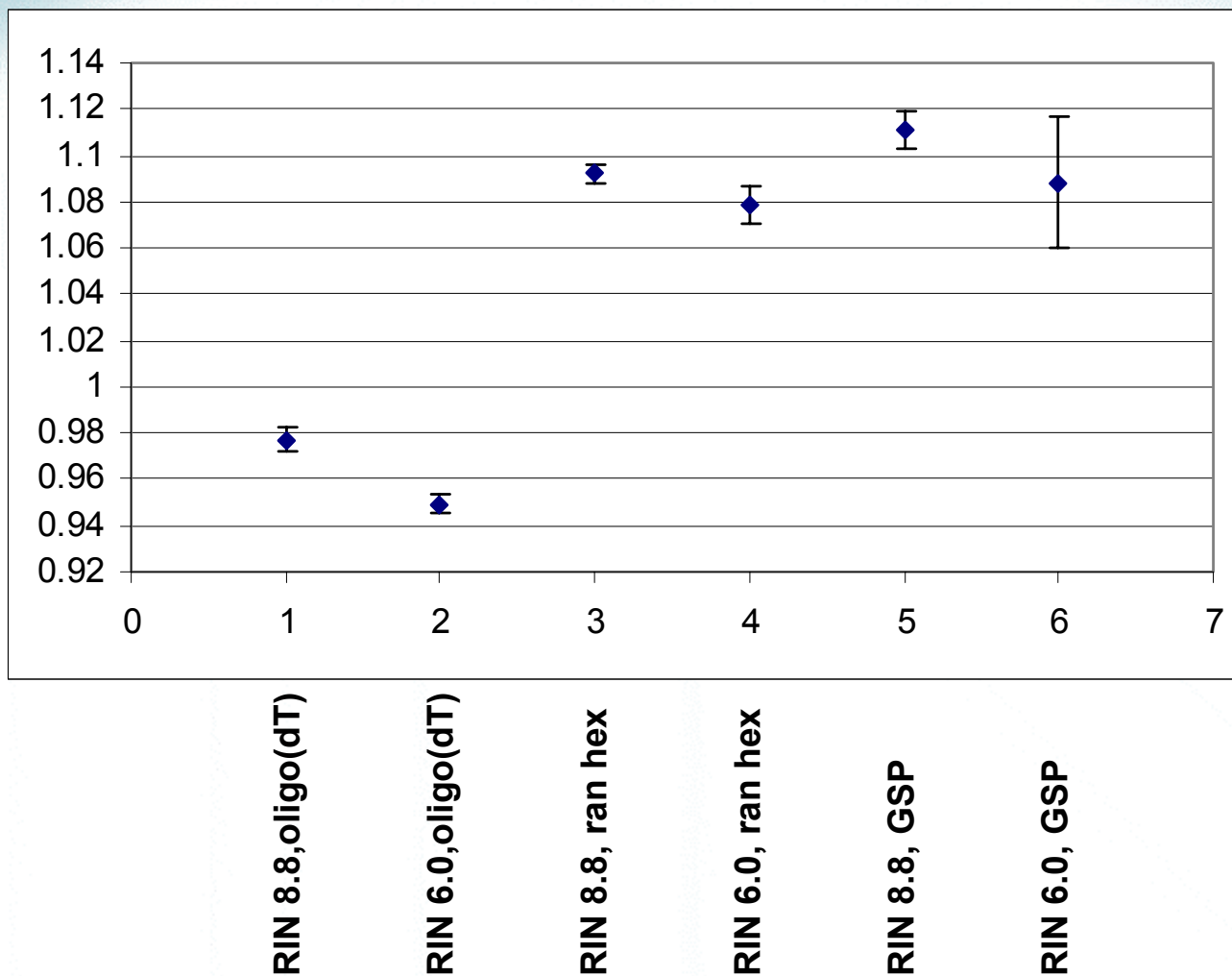
- MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli) (MLH1)
  - mRNA: 2524
- Assays
  - 5' position: 256
  - 3' position: 2154
- Untreated (RIN: 8.8) and degraded sample (RIN: 6)
- Reverse transcription in duplicate
  - oligo(dT), random hexamers and gene-specific primers (GSPs)
- QPCR reactions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



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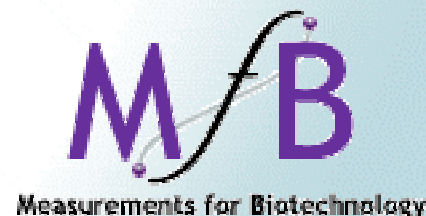


# Preliminary results



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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



# 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



# Web-based forum



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**Standard units for measuring gene expression | Format of standard unit**

Author: [Redacted] | Topic: [Redacted]

Posted - 10 June, 2004 at 11:41

Currently, the choice of units applied to gene expression measurements depends largely on the circumstances under which the individual assay is performed (e.g. transcripts per cell, transcripts per ml blood etc.). A universally accepted standard unit should report results in a way that is applicable to all types of measurements.

Would anyone like to comment on the most sensible format for a standard unit for measuring gene expression?

Posted - 06 July, 2004 at 12:24

This is a laudable concept and in theory it might be possible to achieve this. However, in practice it is more difficult to see how a single standard unit could be agreed upon and implemented.

A standardised approach to every step that makes up a RT-PCR experiment (say) with best practice guidelines dealing with sample acquisition, handling storage, RNA preparation etc is far more likely to be of more immediate and practical use.

The role of standard curves, reference genes, calibrators, RNA quality etc standardised before any thought of

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

Level	Type	Sample Name	Quantity	Cl	Log10 conc.	Avg. conc.	PCR efficiency
1	STND	GAPDH (0)	1.0E-00	95.9			
2	2 STND	GAPDH (0)	1.0E-00	95.91			
3	3 STND	GAPDH (0)	1.0E-00	95.91			
4	4 STND	GAPDH (0)	1.0E-00	95.91			
5	5 STND	GAPDH (0)	1.0E-00	95.91			
6	6 STND	GAPDH (0)	1.0E-00	95.91			
7	7 STND	GAPDH (0)	1.0E-00	95.91			
8	8 STND	GAPDH (0)	1.0E-00	95.91			
9	9 STND	GAPDH (0)	1.0E-00	95.91			
10	10 STND	GAPDH (0)	1.0E-00	95.91			
11	11 STND	GAPDH (0)	1.0E-00	95.91			
12	12 NTC	GAPDH	6.0E-00	45			
13	13 NTC	GAPDH	6.0E-00	45			
14	14 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
15	15 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
16	16 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
17	17 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
18	18 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
19	19 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
20	20 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
21	21 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
22	22 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
23	23 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
24	24 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
25	25 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
26	26 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
27	27 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
28	28 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
29	29 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
30	30 UARN	GAPDH (0)	8.17E-01	22.8	-1.9E-00	1.70E-02	
31	31 NTC	MPCAM	6.0E-00	45			
32	32 NTC	MPCAM	6.0E-00	45			
33	33 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
34	34 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
35	35 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
36	36 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
37	37 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
38	38 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
39	39 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
40	40 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
41	41 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
42	42 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
43	43 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
44	44 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	
45	45 UARN	MPCAM (0)	2.9E-00	30.6	-2.9E-00	3.68E-02	



# Points for discussion



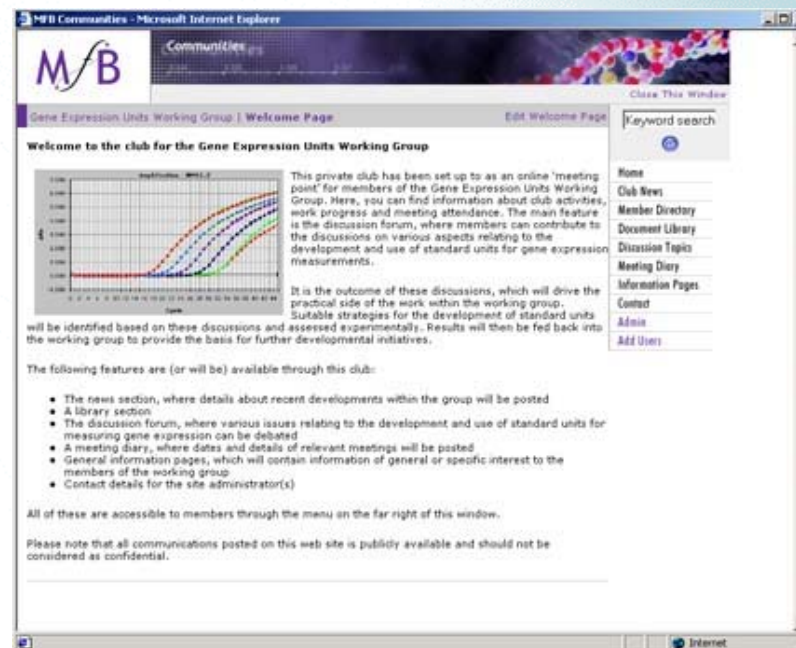
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- 'MIAME'-style guidelines on information
- Experimental design
- Data analysis
  - standard curves; simple vs. weighted linear regression
- Appropriate reference materials
  - ERCC
- Additional strategies or new ideas?
- Potential for long-term development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



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



# 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



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



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