Quantification of mRNA using Real-Time RT-PCR

Tania Nolan, Rebecca Hands and Stephen Bustin

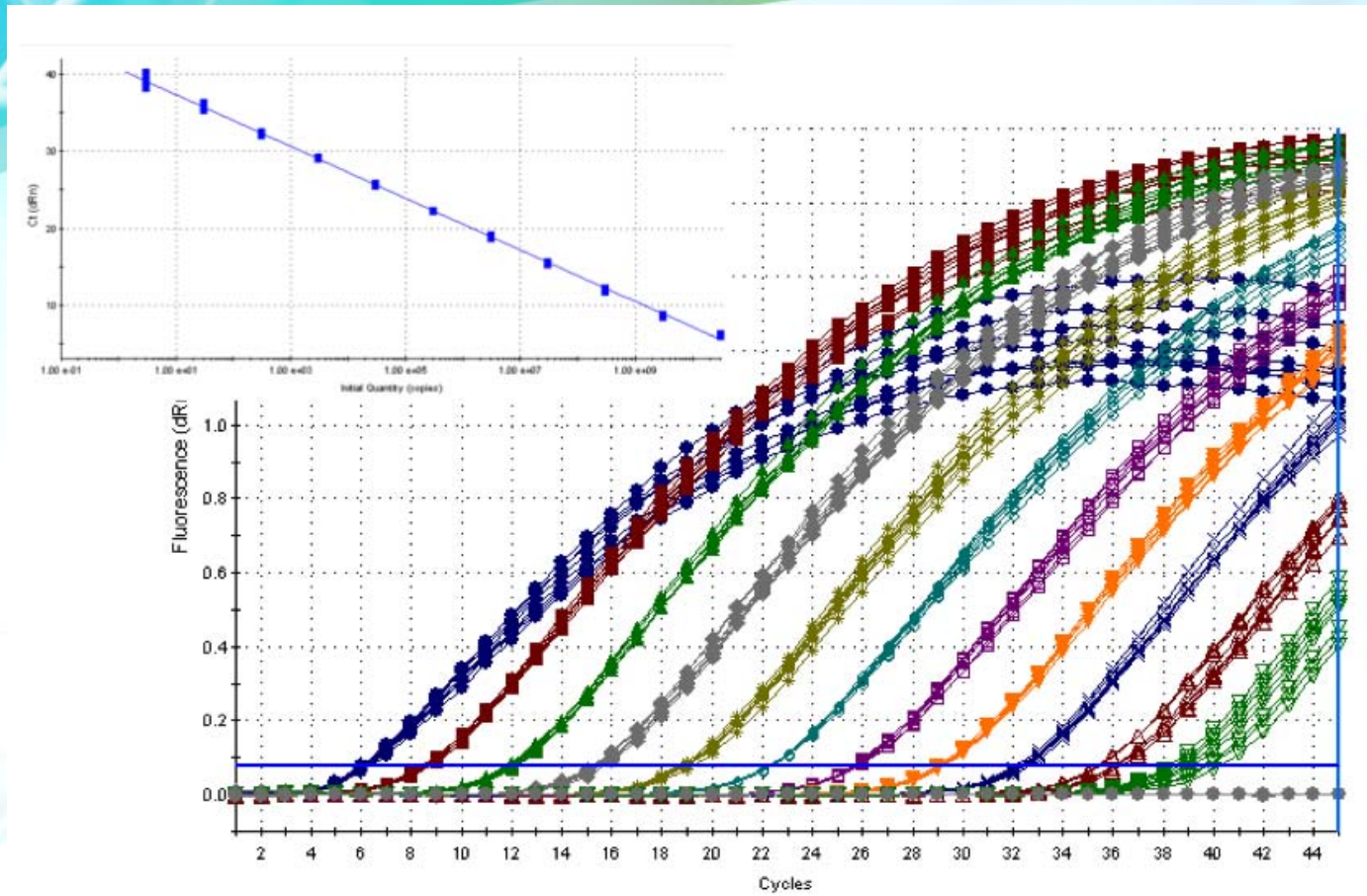
Tania.Nolan@.sial.com

SIGMA-ALDRICH

Normalisation, Optimisation and Standardisation

1. Assay design and optimisation
2. Template quality
3. Normalisation considerations

Using the standard curve for quality control



Amplification plots:

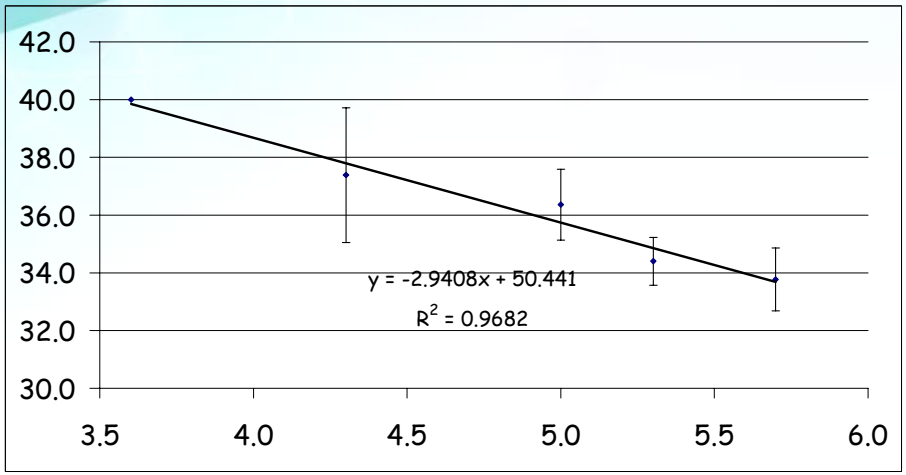
- Baseline is horizontal
- Threshold is in LOG region of curve
- Curves are parallel

y=mx+c

- Slope = -3.323
- RSqu = 0.98

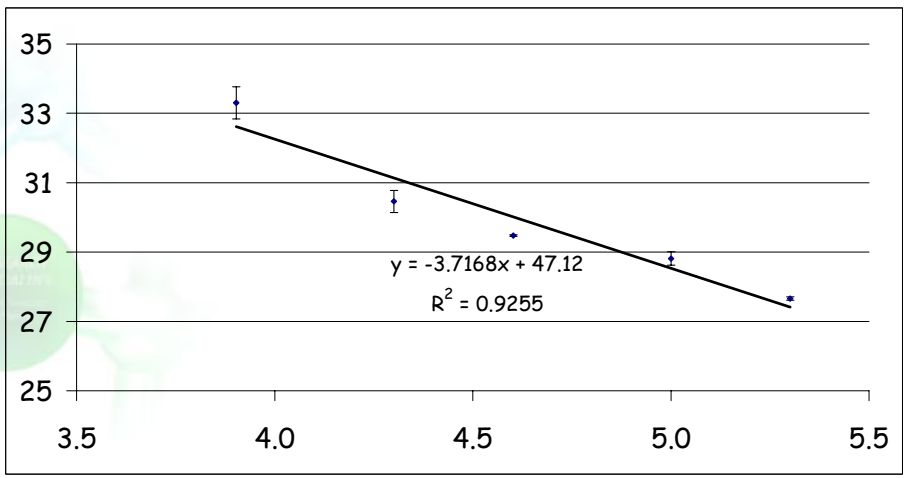
Intercept on y gives a measure of sensitivity

IL-15 Assay



One tube assay (RNA dilutions) - Specific primers

Slope = -2.9



Two tube assay (cDNA dilutions) - Random primers

Slope = -3.7

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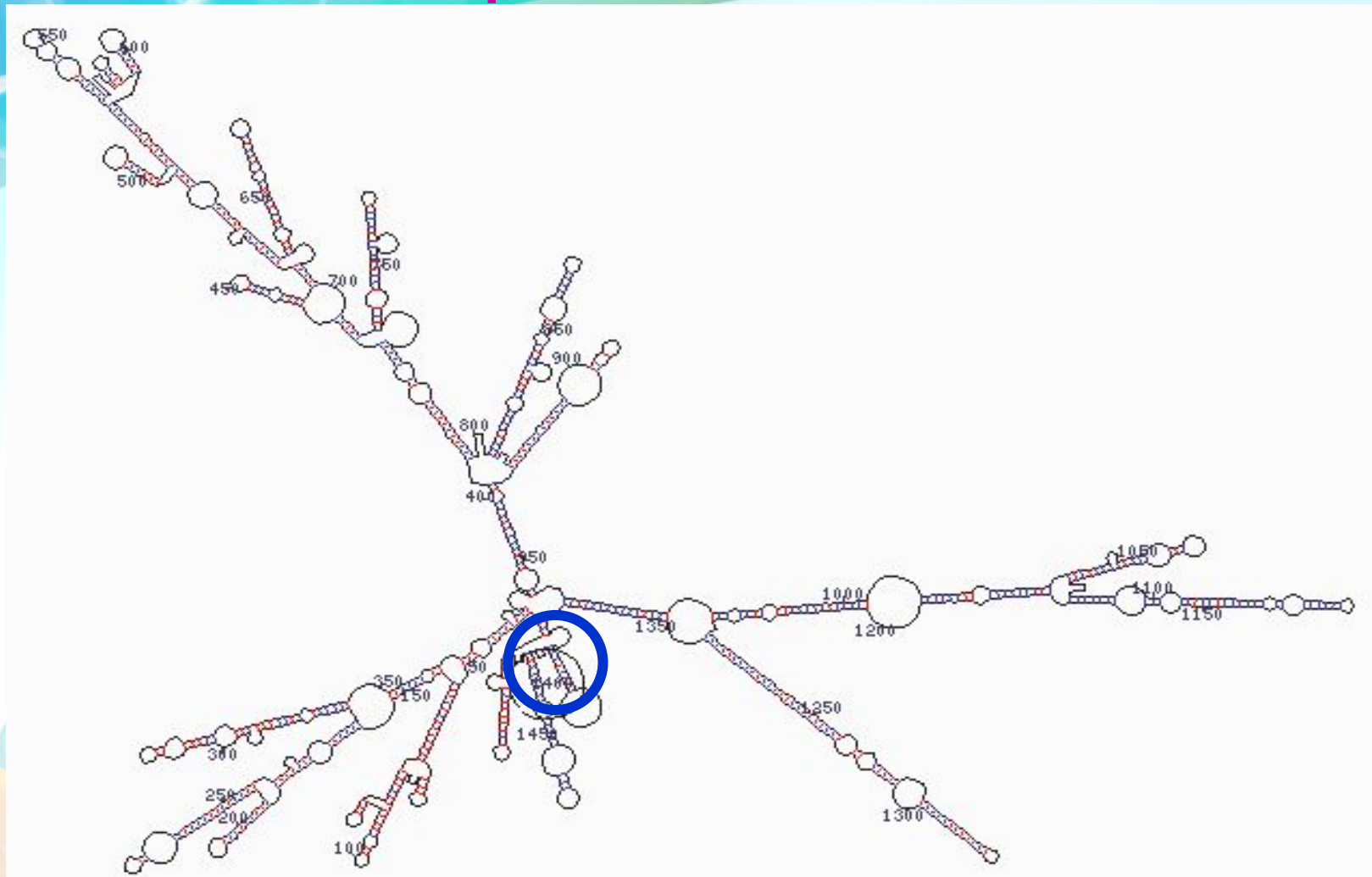
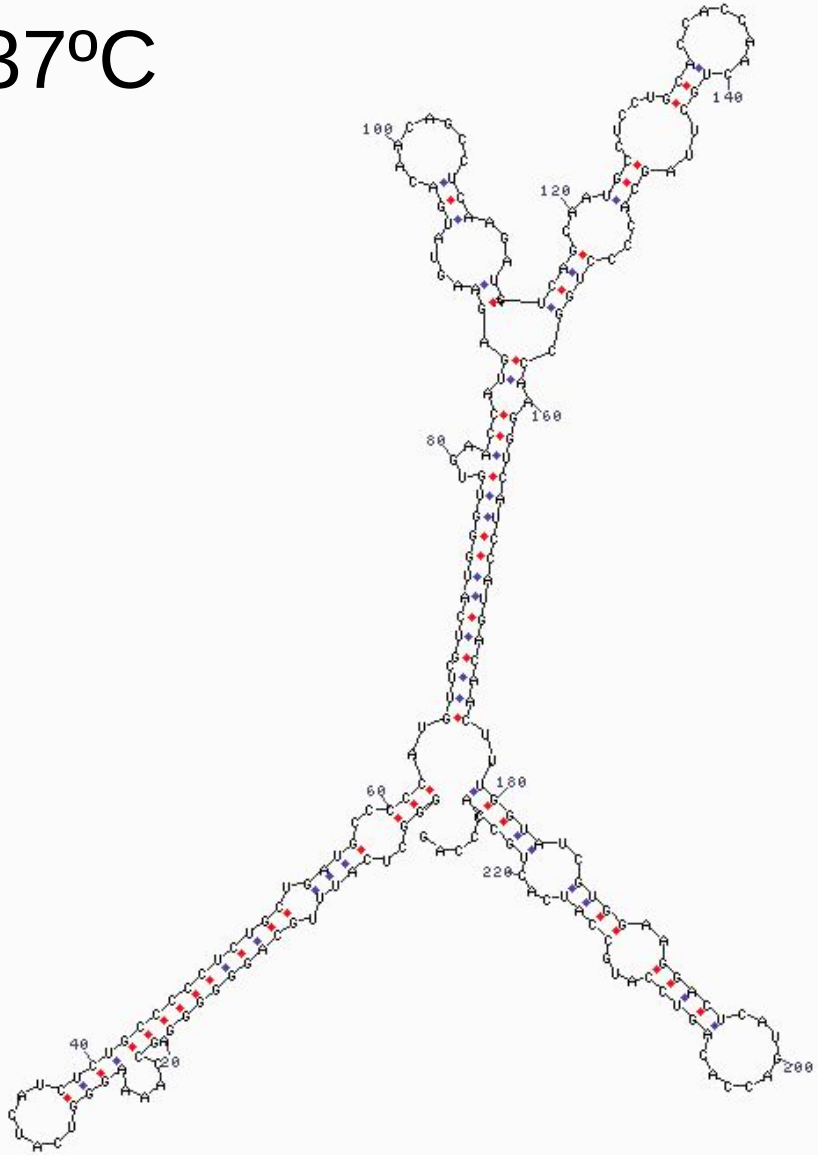
IL-15 predicted structure

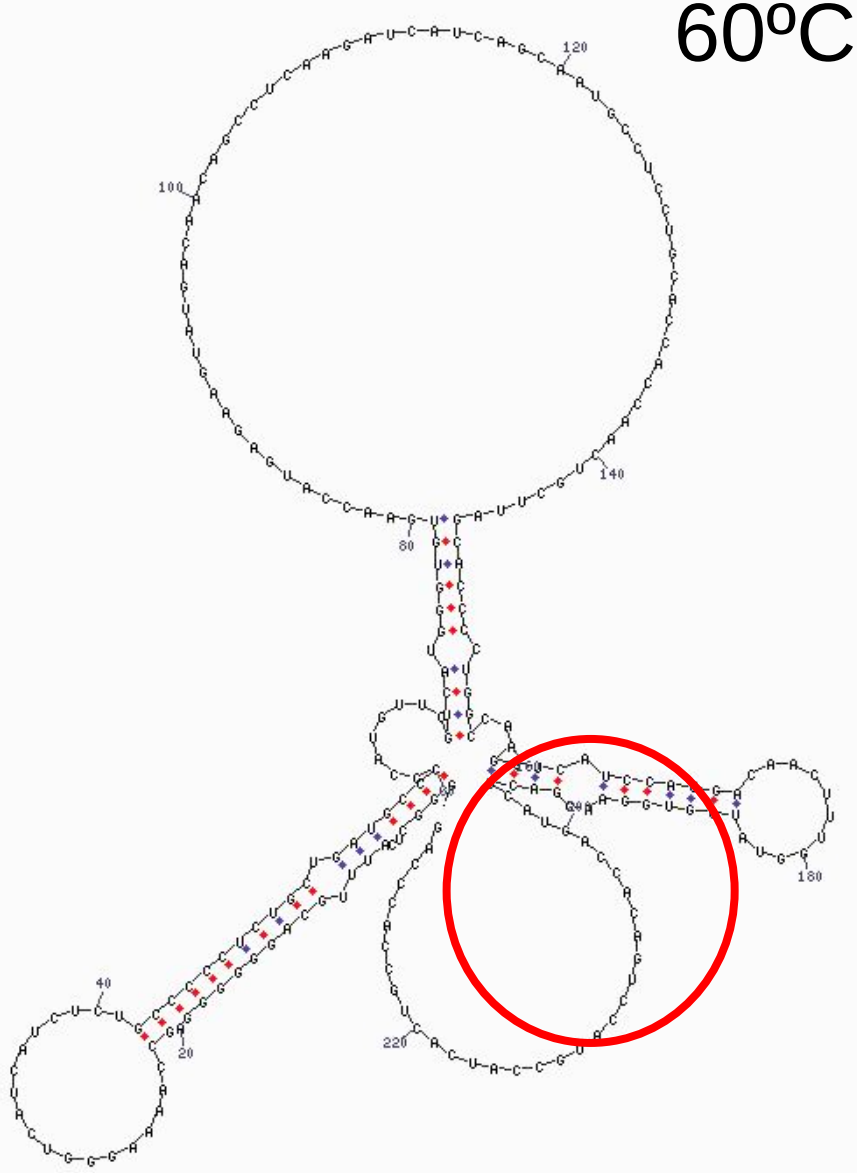


GAPDH 5’

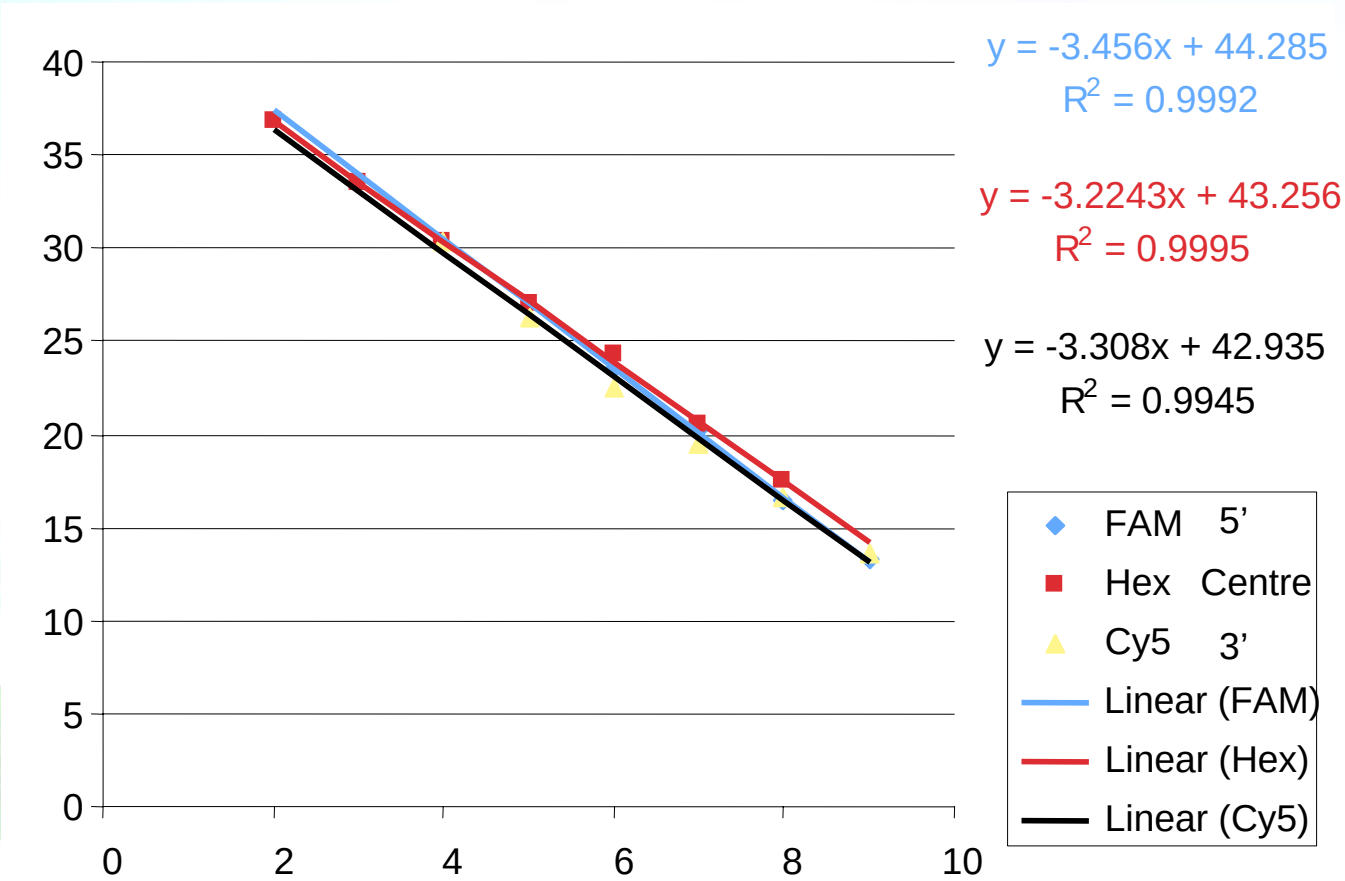
37ºC



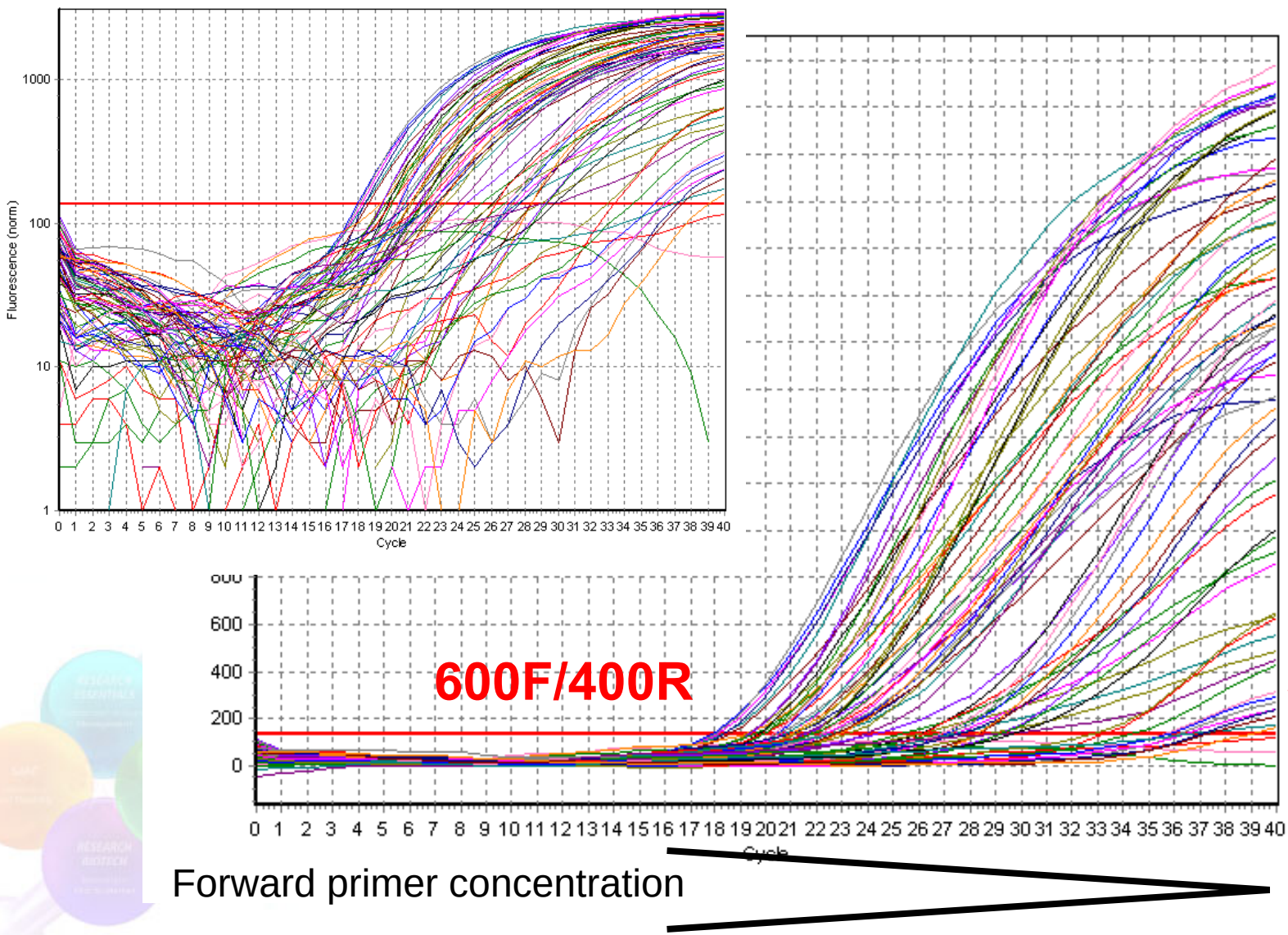
60ºC



Total RNA target
GAPDH specific primed dilution series



Optimisation can improve assay sensitivity



Forward primer concentration

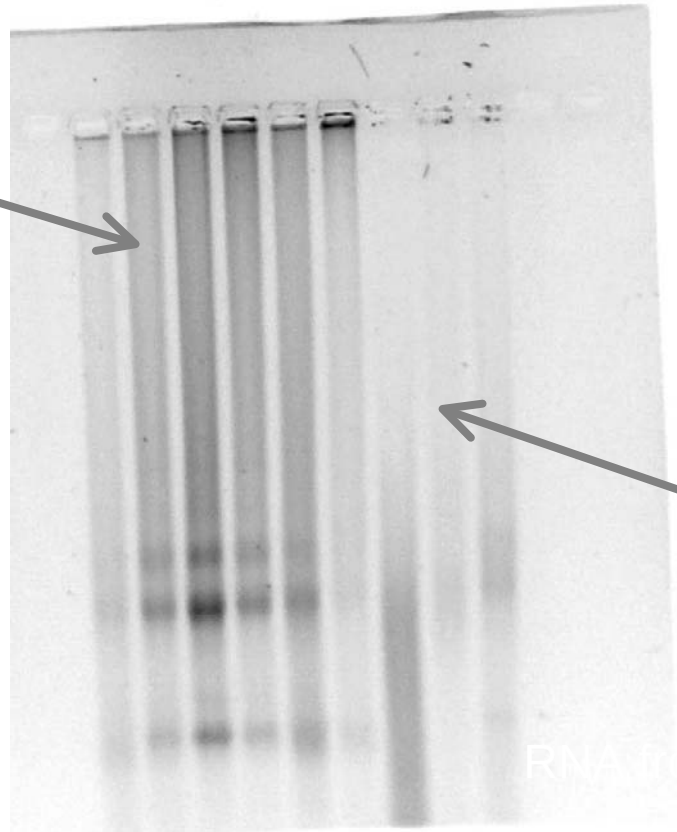
Mouse cDNA was amplified using PCR primers specific to Hepcadin 1 (Nemeth *et al*, 2004). All combinations of primer concentrations ranging between 50nM and 600nM were used (Nolan et al 2006; Nils Gerke, Eppendorf and Jens Stolte, EMBL Heidelberg qRT-PCR workshop, 2006).

Normalisation, Optimisation and Standardisation

1. Assay design and optimisation
2. Template quality
3. Normalisation considerations

Total RNA purification – agarose gel visualisation

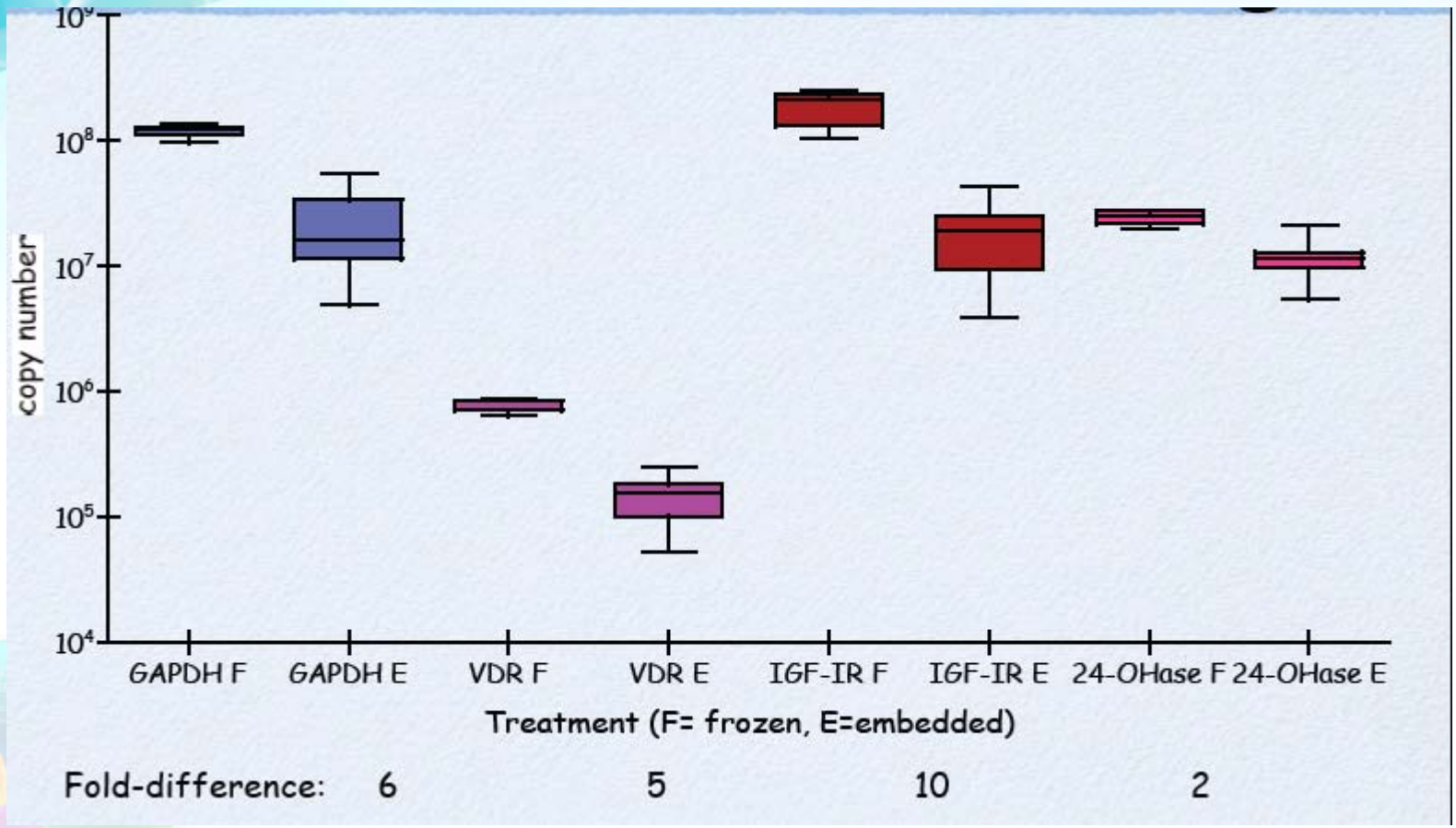
Extracted from frozen tissue sample



Extracted from FFPE tissue sample

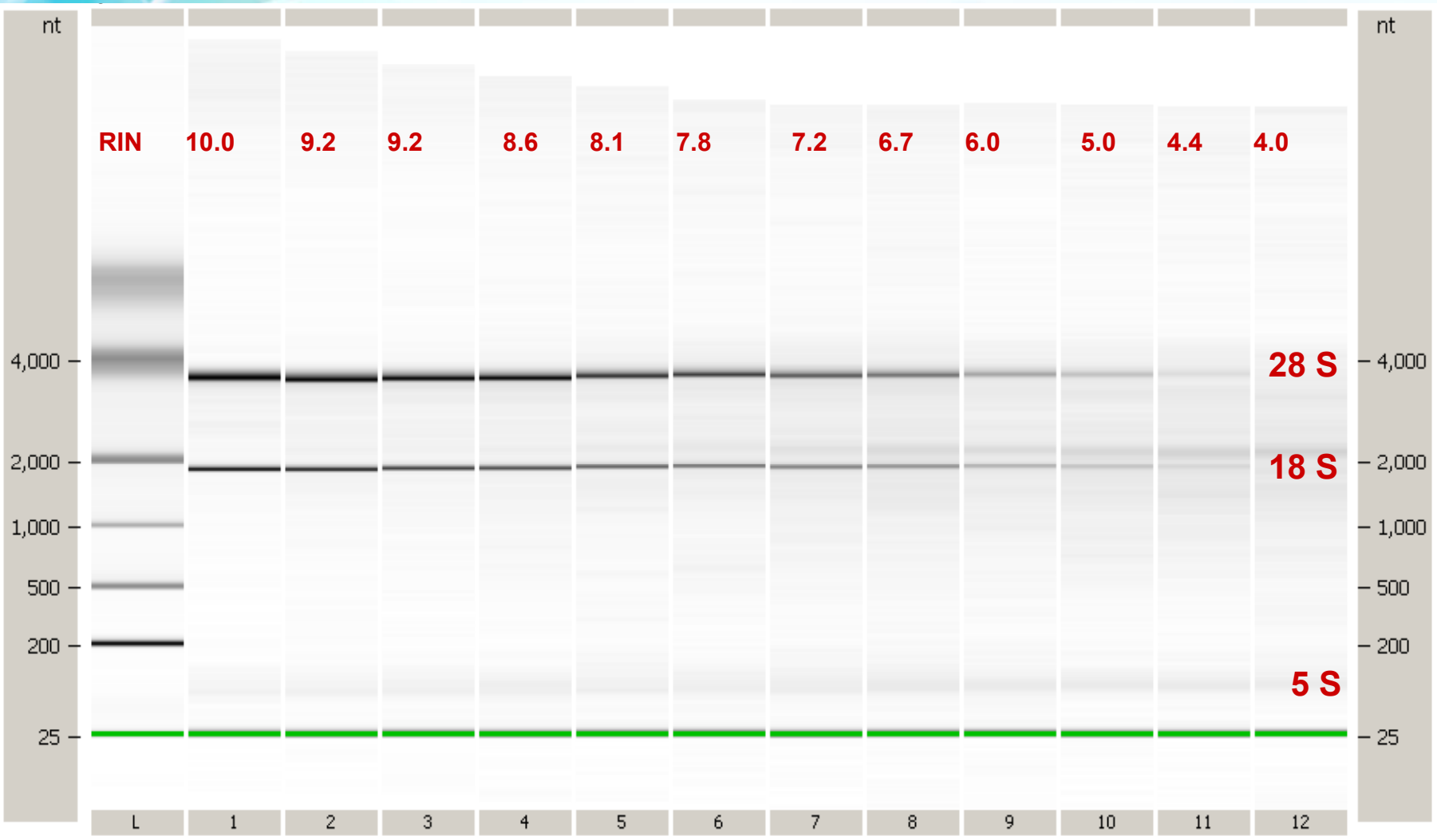
From: Anna Antonacopoulou, Patras, Greece

Treatment of samples affects data



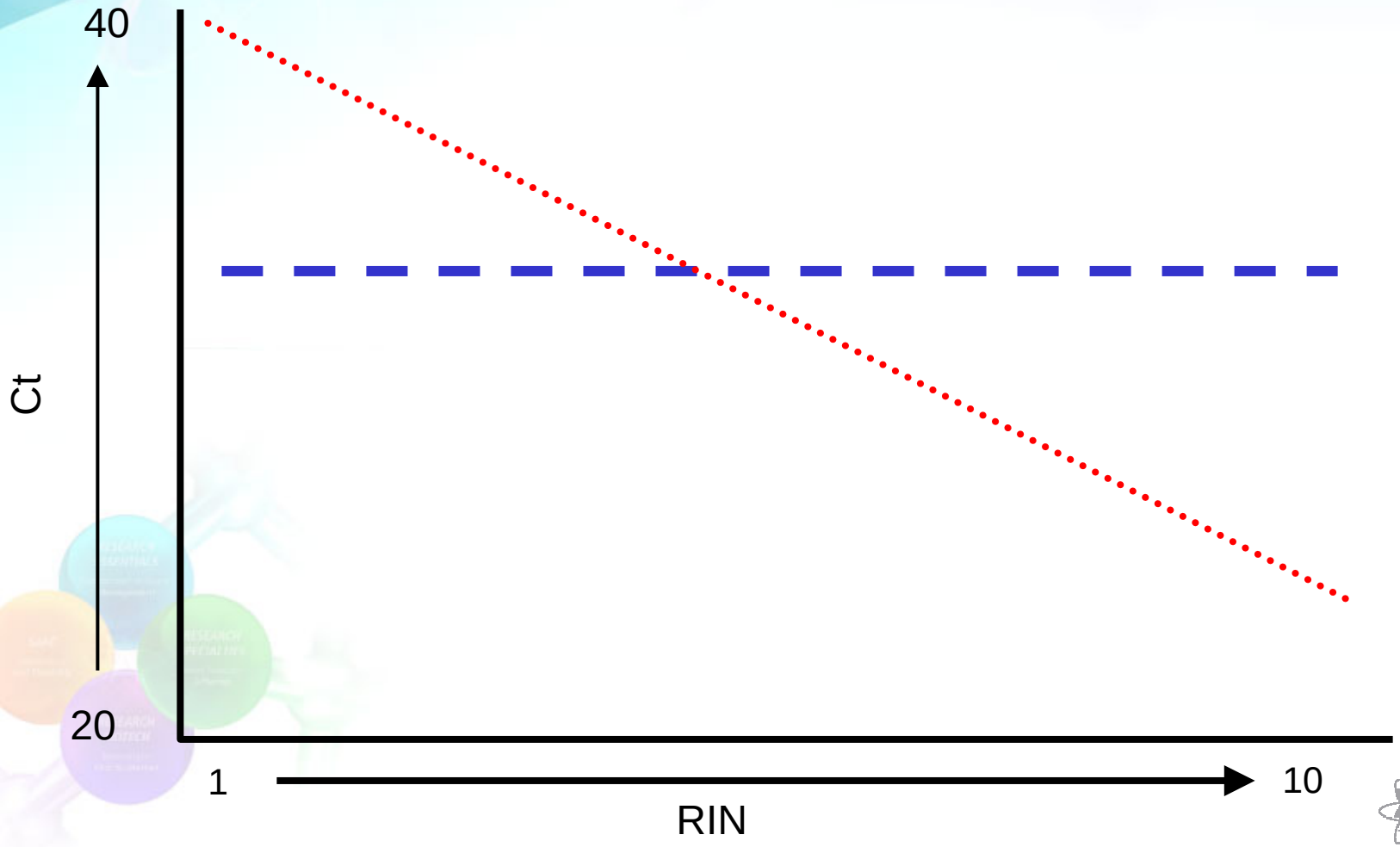
Data source: Prof Stephen Bustin, London

Degradation of tissue extracted total RNA



Data source: Michael Pfaffl and Simone Fleige, Freising

Thought Experiment:
Effect of RNA degradation (RIN) on Ct



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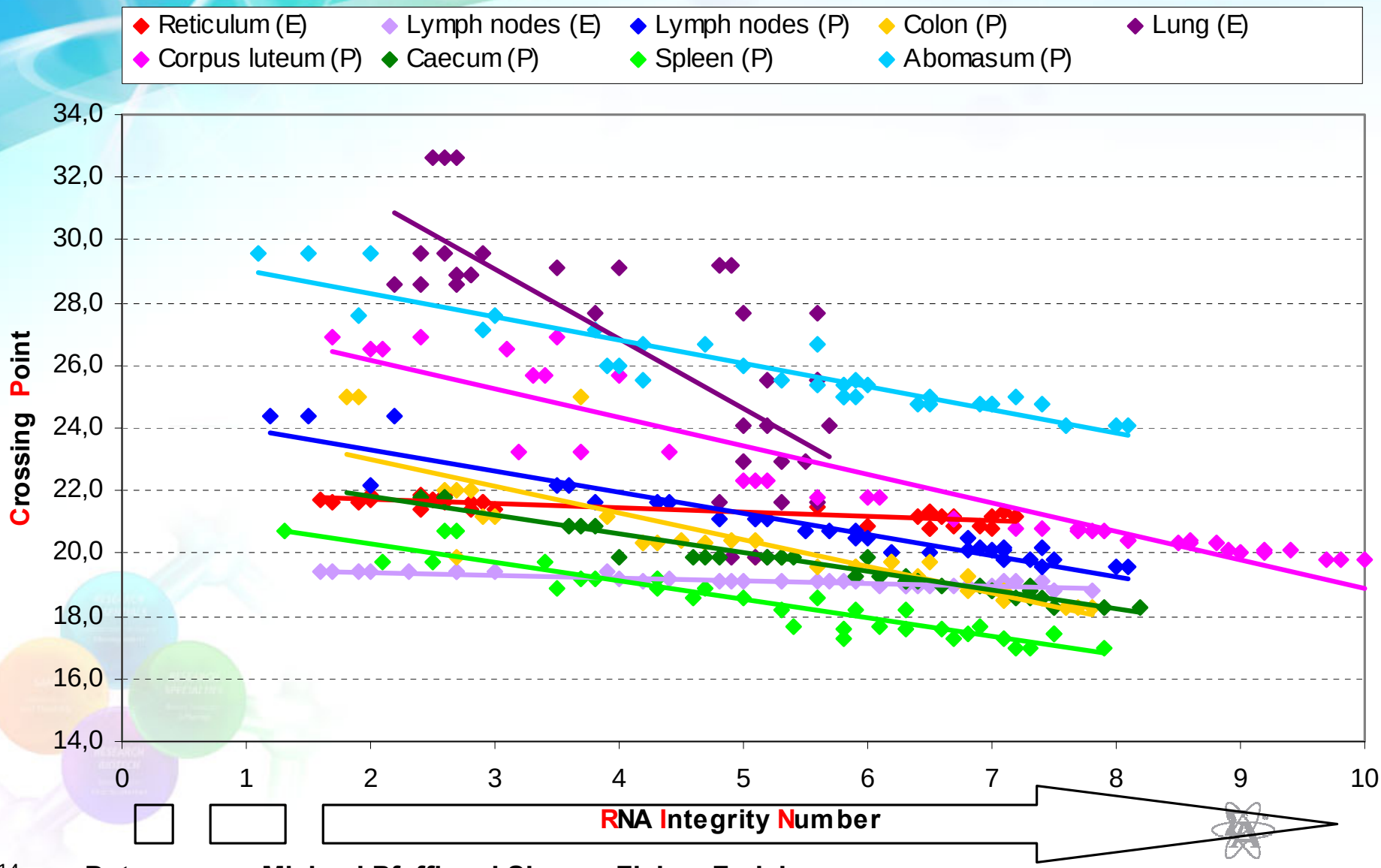
Influence of total RNA quality on qRT-PCR

IL-1: *Crossing Point*



Data source: Michael Pfaffl and Simone Fleige, Freising

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Molecular Aspects of Medicine 27 (2006) 126–139
MOLECULAR ASPECTS OF MEDICINE
www.elsevier.com/locate/mam

Review

RNA integrity and the effect on the real-time qRT-PCR performance

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[a] *Physiology Weihenstephan, Center of Life and Food Sciences (ZIEL), Technical University of Munich, 85350 Freising, Germany*
[b] *TATAA Biocenter Germany, Freising-Weihenstephan, Germany*

PCR performance as well as on PCR efficiency is described. On the basis of the derived results we can argue that qRT-PCR performance is affected by the RNA integrity and PCR efficiency in general is not affected by the RNA integrity. We can recommend a RIN higher than five as good total RNA quality and higher than eight as perfect total RNA for downstream application.

Poster 26

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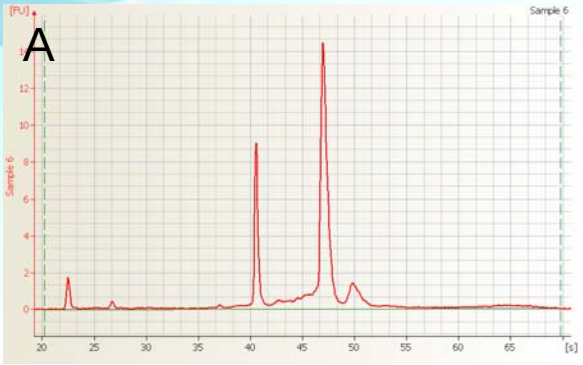
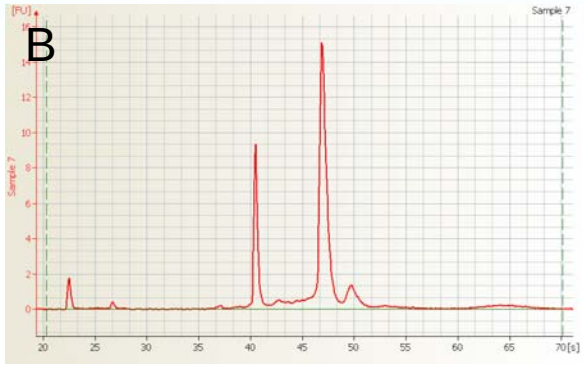
Agilent 2100 Bioanalyzer analysis of 5 RNA samples

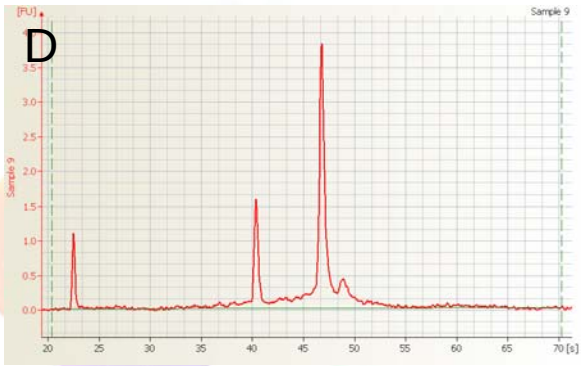


Conc. 110 ng/ul
Ratio: 2.5
RIN: 10

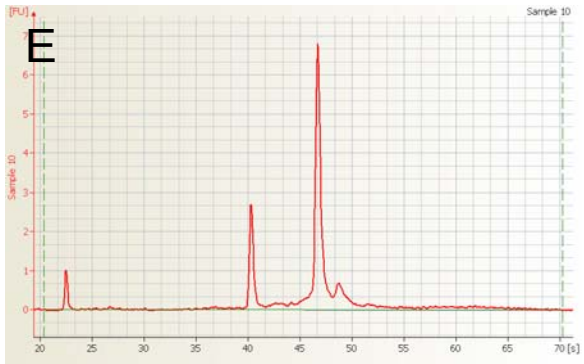


Conc. 110 ng/ul
Ratio: 2.5
RIN: 10

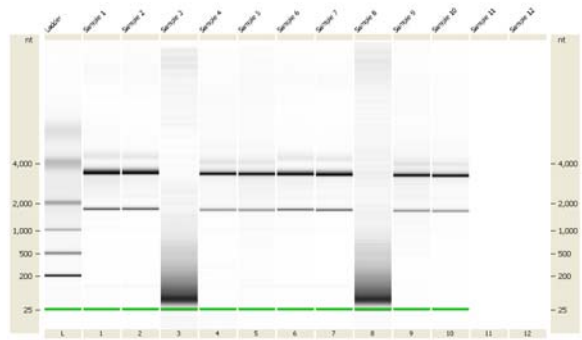
Conc. 62 ng/ul
Ratio: 0.0
RIN: 2.4



Conc. 30 ng/ul
Ratio: 2.7
RIN: 9.1



Conc. 43 ng/ul
Ratio: 2.6
RIN: 9.5



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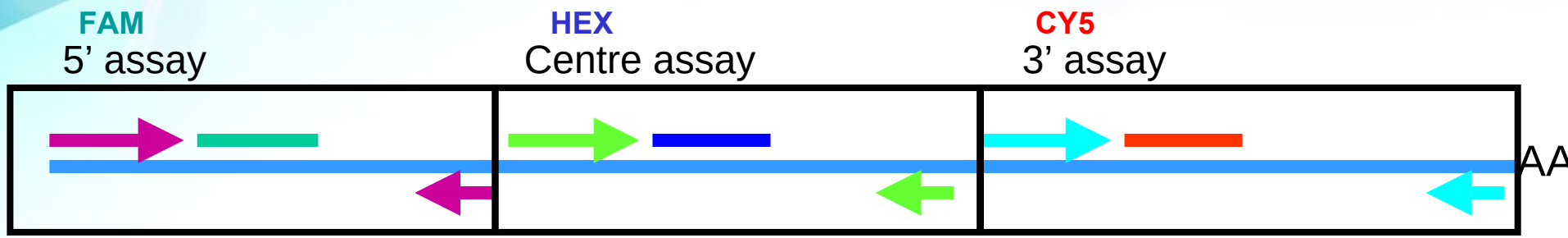
5’ / 3’ integrity assay



- Perform RT using oligo dT
- If RNA is intact detection of 5’, centre and 3’ should be equal
- If RNA is degraded detection of 3’ > 5’

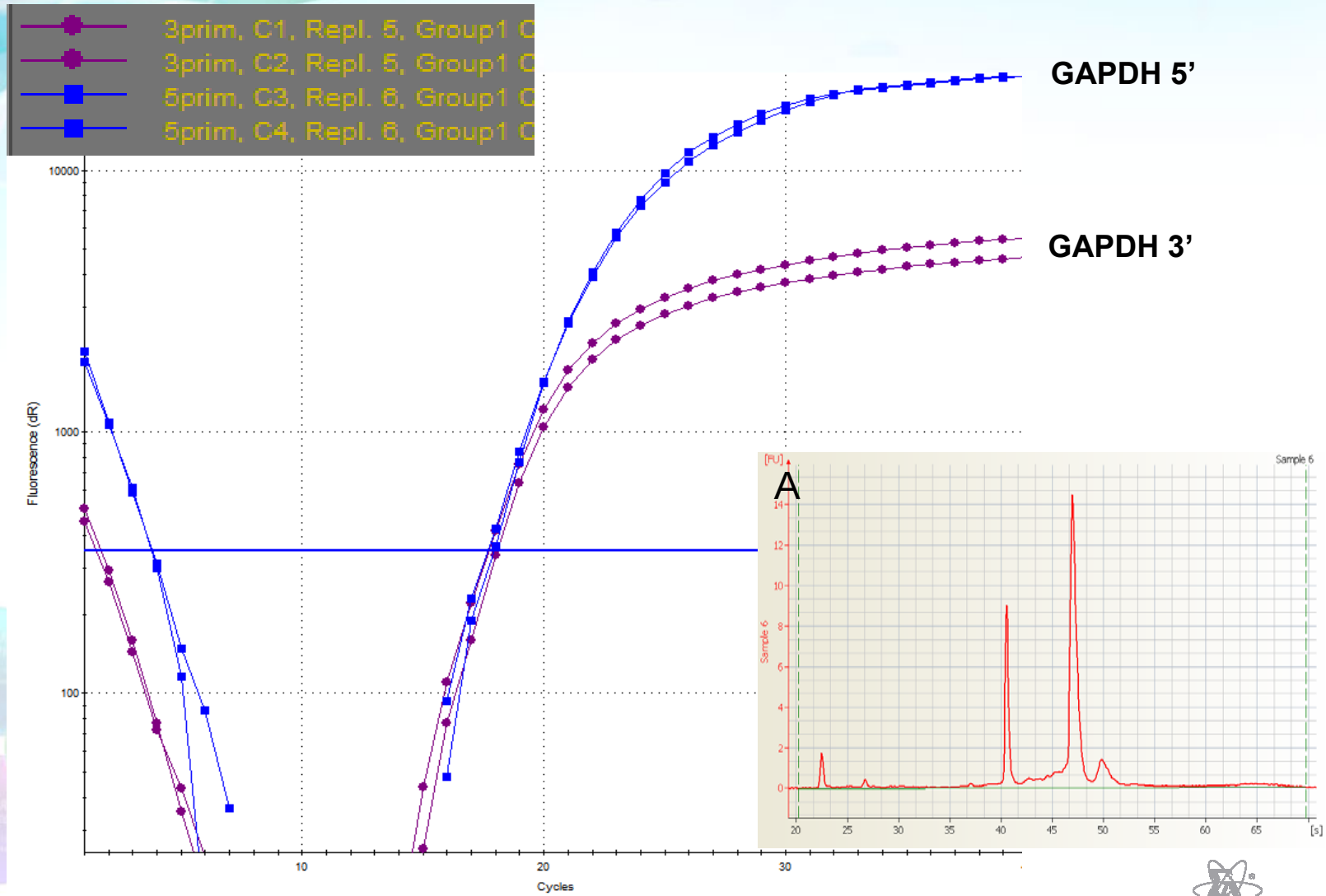
PROTOCOL

Quantification of mRNA using real-time RT-PCR

Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

[1]Sigma-Aldrich, Homefield Road, Haverhill, UK. [2]Institute of Cell and Molecular Science, Barts and the London Queen Mary’s School of Medicine and Dentistry, University of London, Whitechapel, London E1 1BB, UK. Correspondence should be addressed to S.A.B. (s.a.bustin@qmul.ac.uk).

GAPDH 5’/3’ Multiplex Assay – Intact RNA



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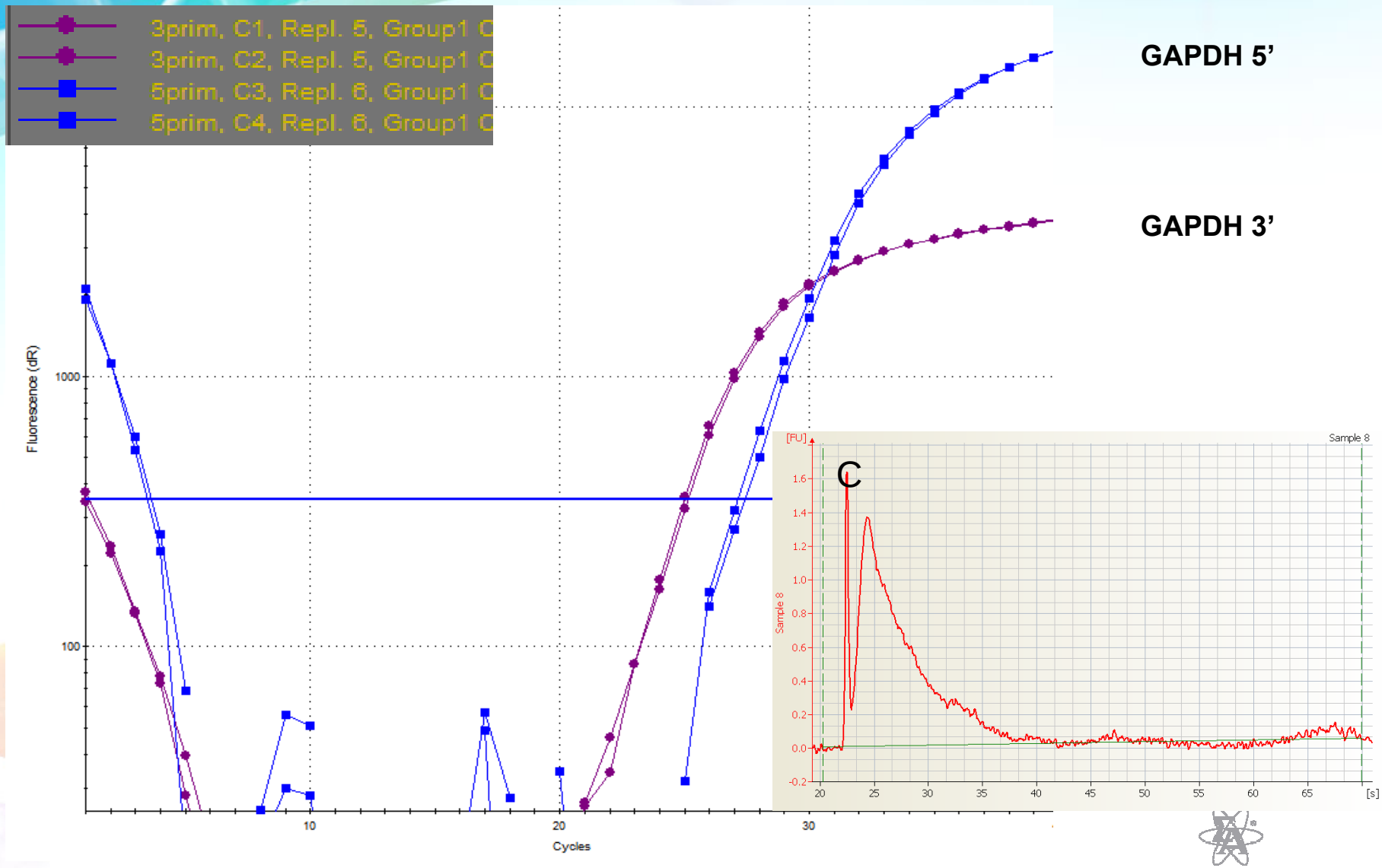
GAPDH 5’/3’ Multiplex Assay – Degraded RNA



GAPDH 5’

GAPDH 3’

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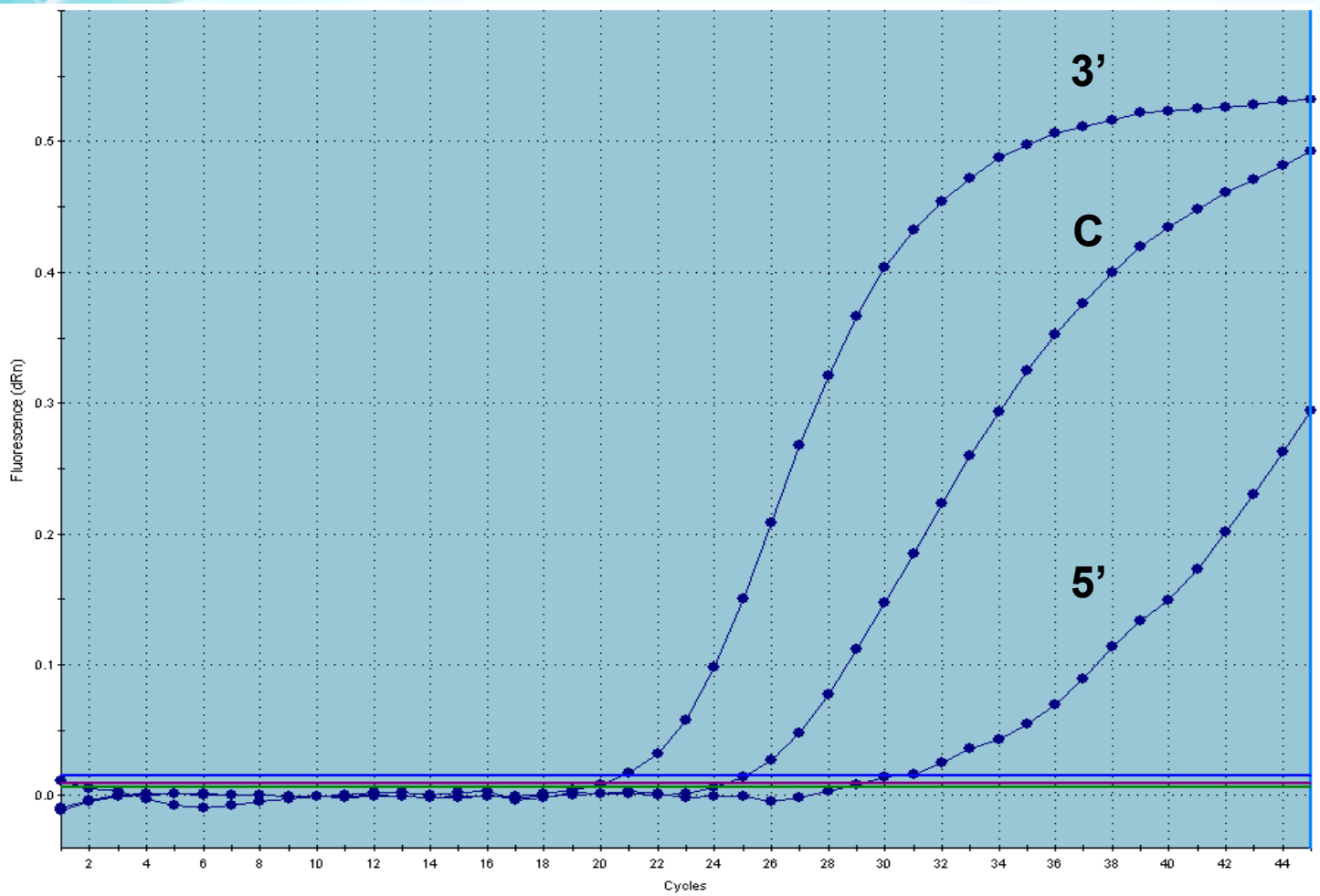
GAPDH 5’ 3’ Multiplex Assay - FFPE RNA



Seq	Ct
5’ :	30.5
3’ :	20.2
Ce :	24.1

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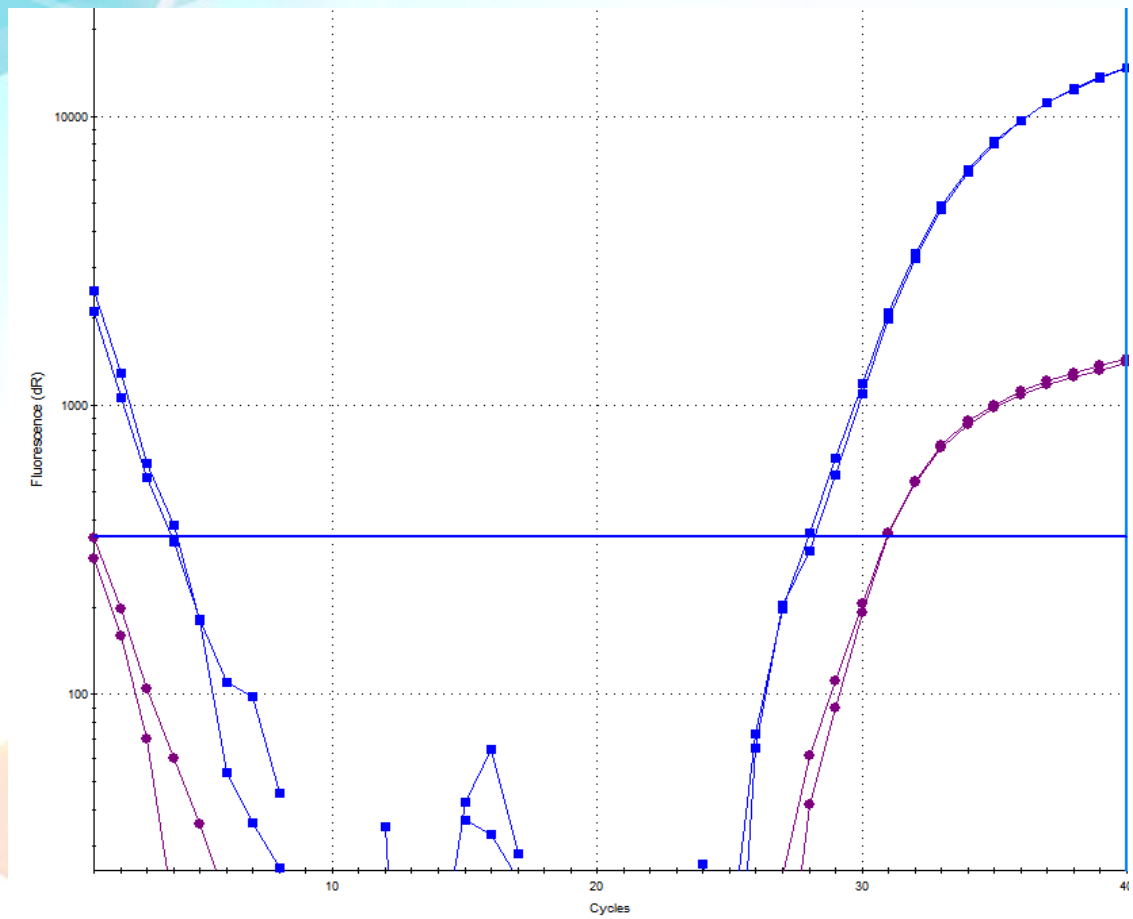
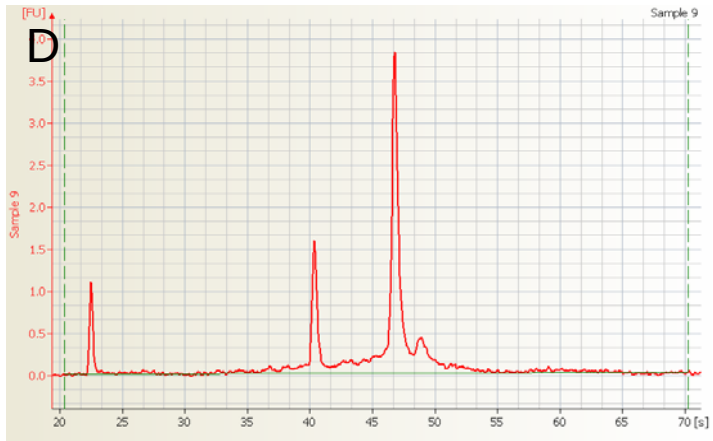
Inhibitors are not created equal





Conc. 30 ng/ul

Ratio: 2.7

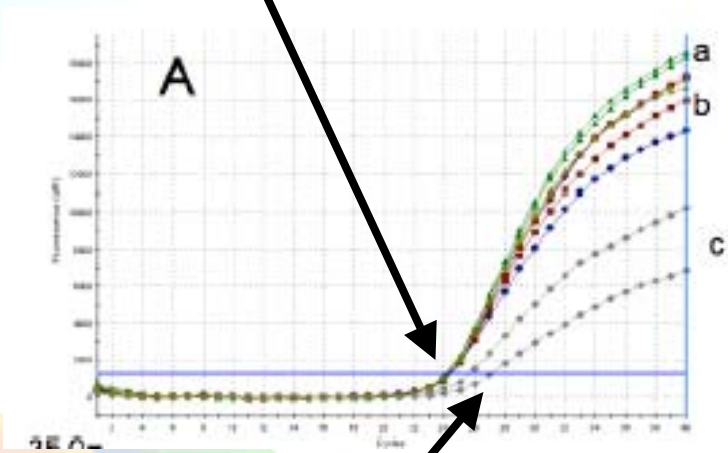
RIN: 9.1

(125mM EDTA)

SPUD: for detection of inhibitors

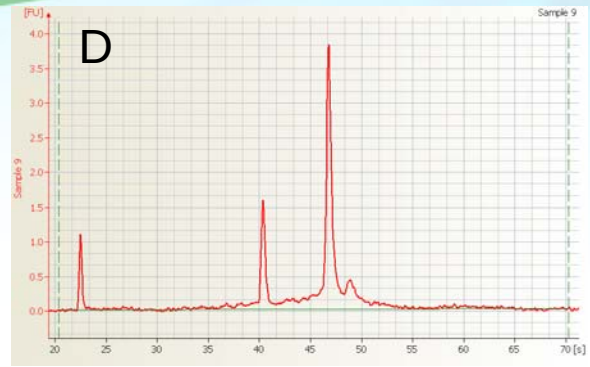
Reaction 1

Ct (SPUD + Water) = 24



Reaction 2

Ct (SPUD + sample) = 26
(Phenol from extraction reagent)



D 125mM EDTA

Conc. 30 ng/ul

Ratio: 2.7

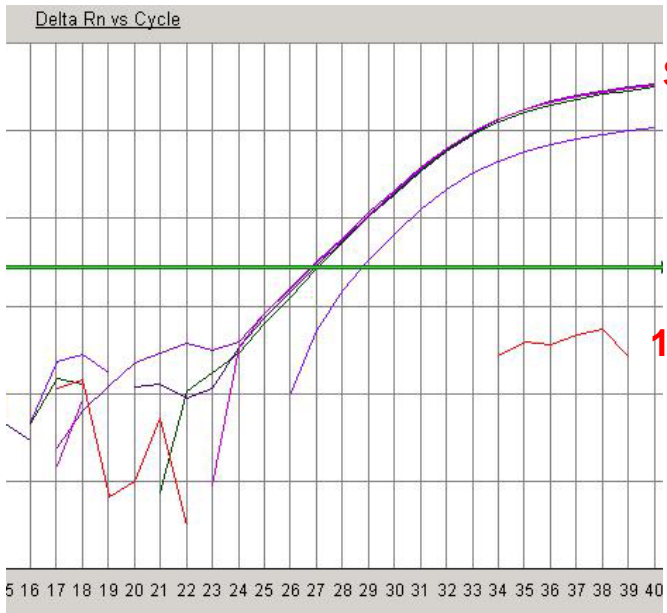
RIN: 9.1

E 62.5mM EDTA

Conc. 43 ng/ul

Ratio: 2.6

RIN: 9.5



Samples A,B,C

62.5mM EDTA

125mM EDTA

Notes & Tips

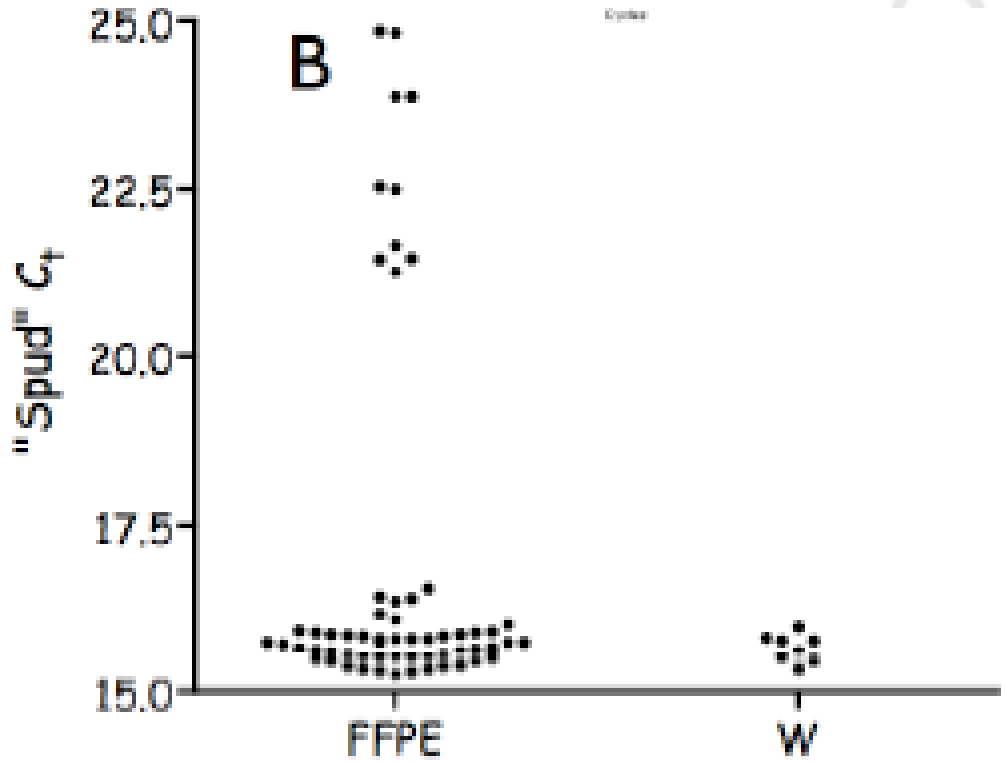
SPUD: A quantitative PCR assay for the detection of inhibitors in nucleic acid preparations

Tania Nolan [a], Rebecca E. Hands [b], William Ogunkolade [b], Stephen A. Bustin [b,*]

[a] *Sigma–Aldrich, Haverhill CB9 8QP, UK*

[b] *Institute of Cell and Molecular Science, Barts and The London, Queen Mary's School of Medicine and Dentistry, University of London, London E1 1BB, UK*

Received 3 November 2005



Also; see poster 62 (Tanya Novak and Jim Huggett) for further developments and clinical applications

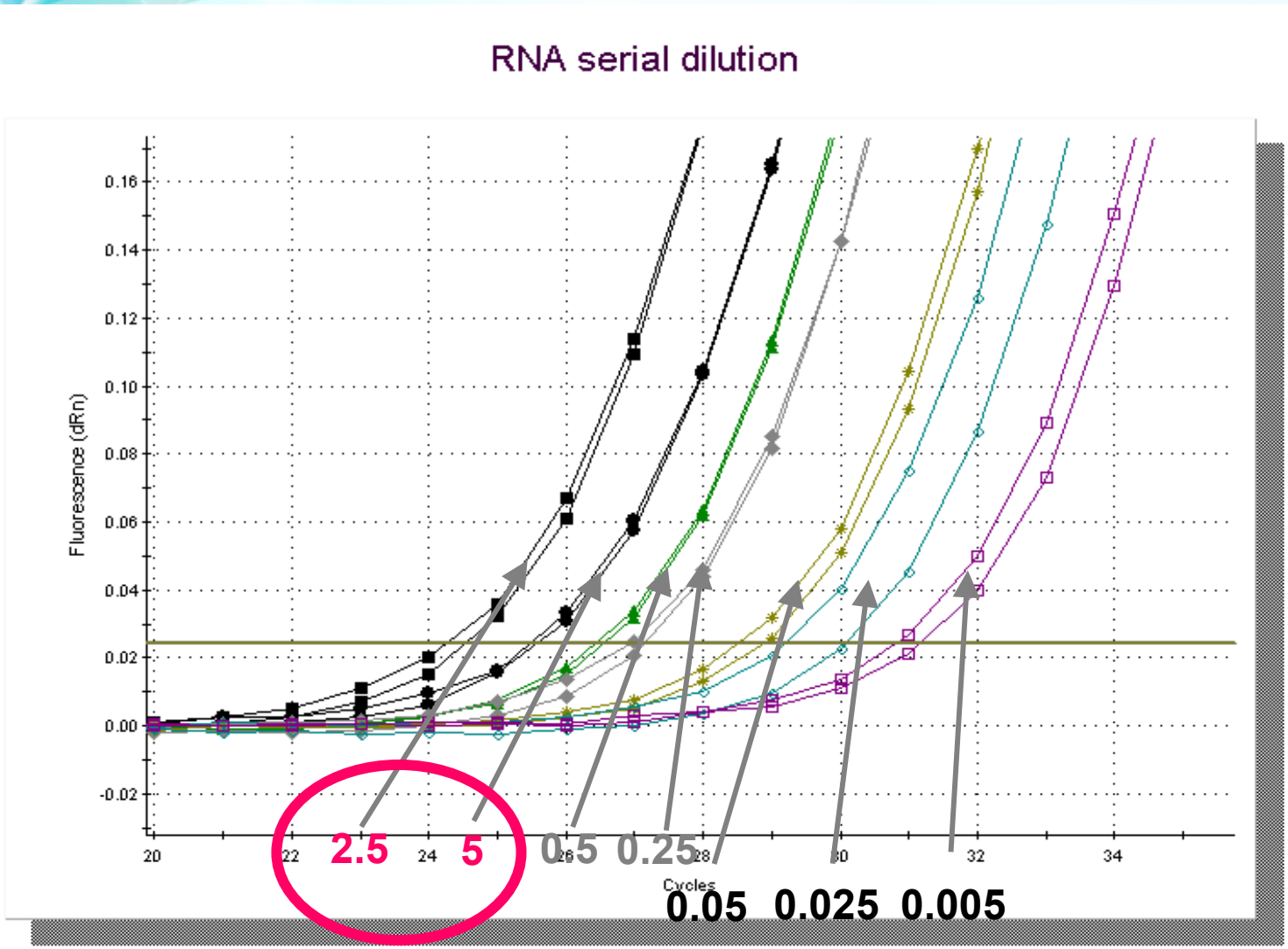
Normalisation, Optimisation and Standardisation

1. Assay design and optimisation
2. Template quality
3. Normalisation considerations

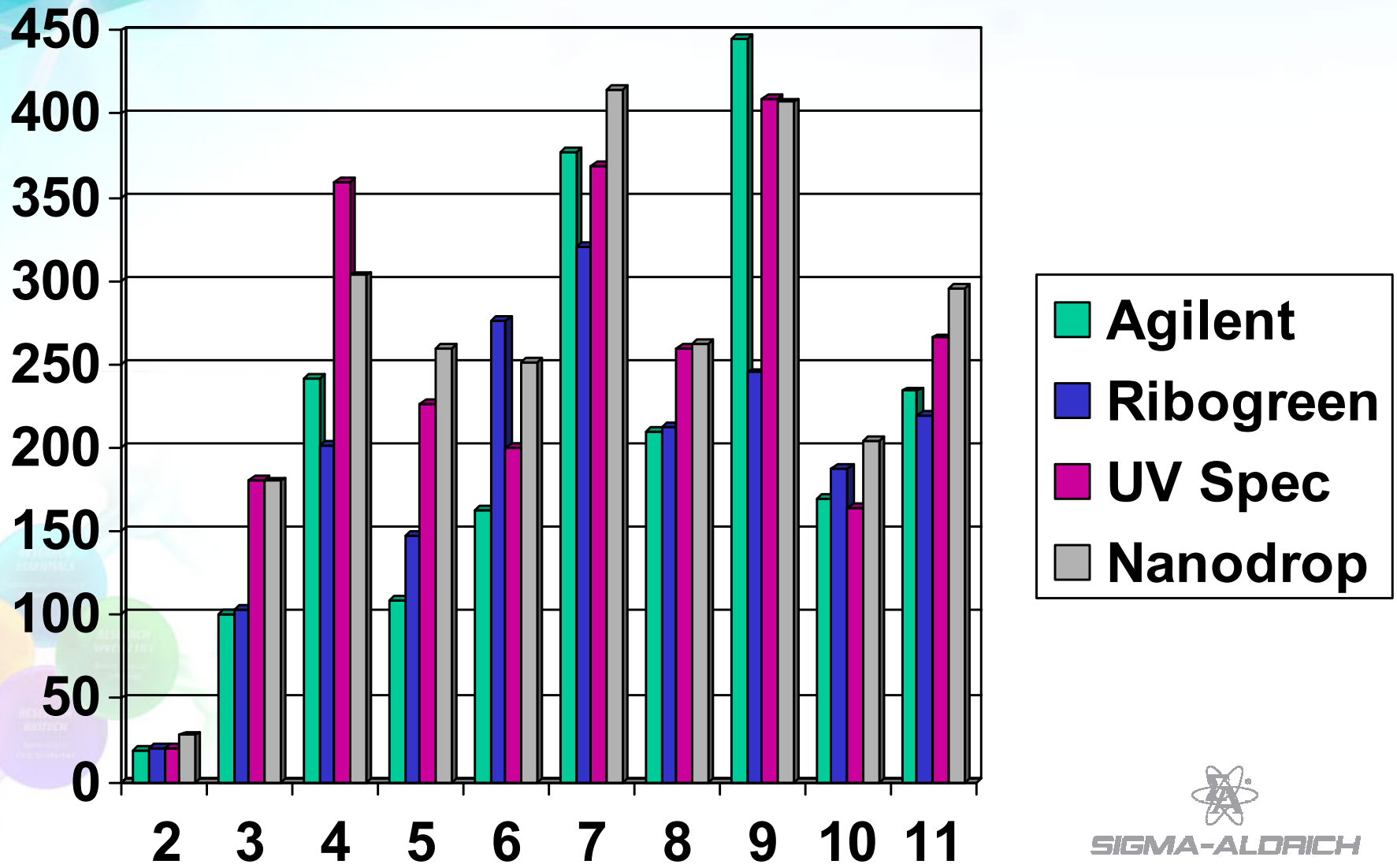
Normalisation

- Correct for different amounts of input target
- Correct for RT differences
- Express data relative to;
  - *total RNA*
  - *a stable reference gene or multiple genes*
  - DNA
  - number of cells
  - cDNA
  - a relevant gene (SIR) (Stephen Bustin, London, UK)
  - Alu repeats (J.Vandesompele, Uni Ghent, Belgium)
  - a spiked target (Gilsbach, Weinstephan, Germany)

Random primed RNA (2x) dilution series (QPCR NHE1)



Extraction and quantification of RNA



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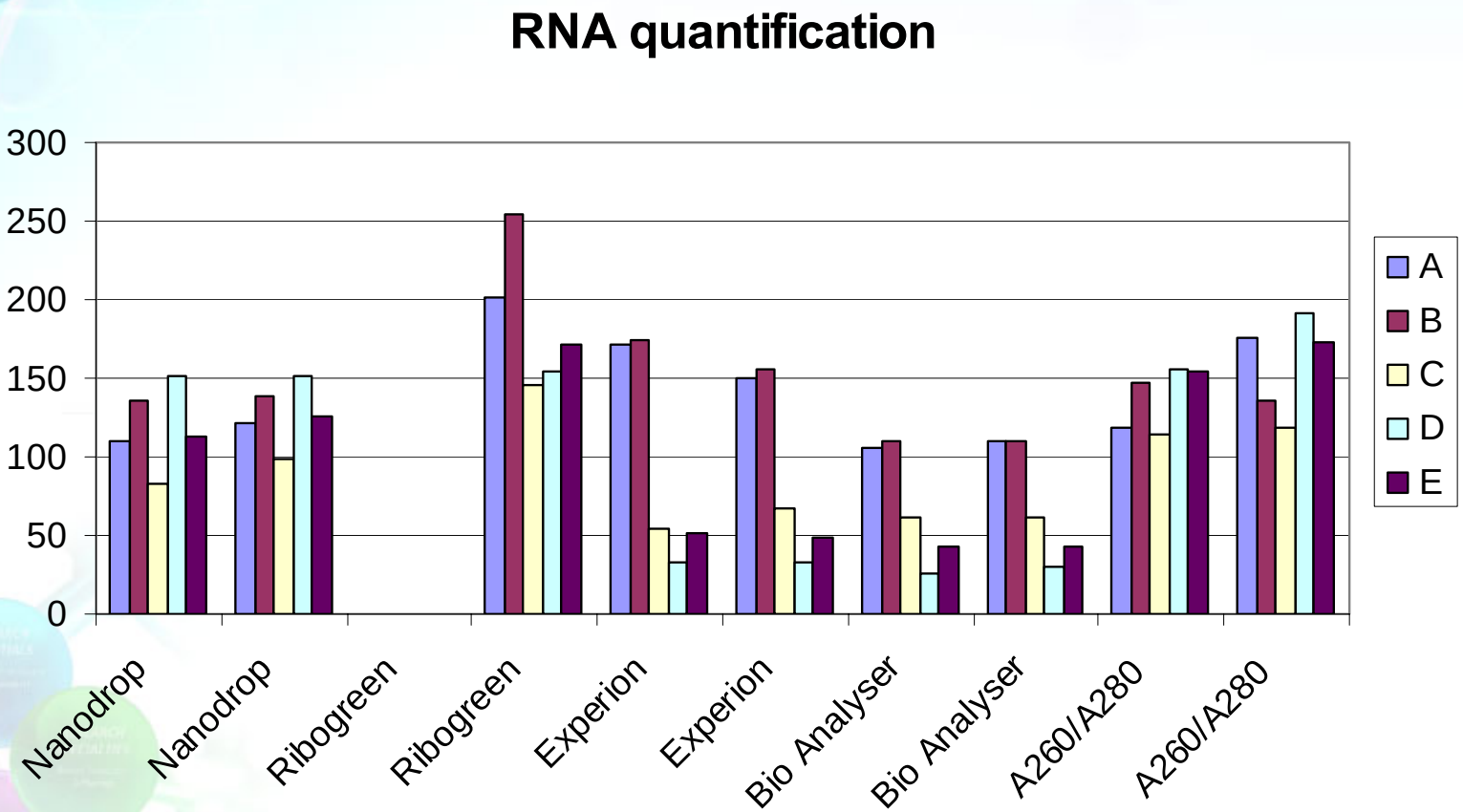
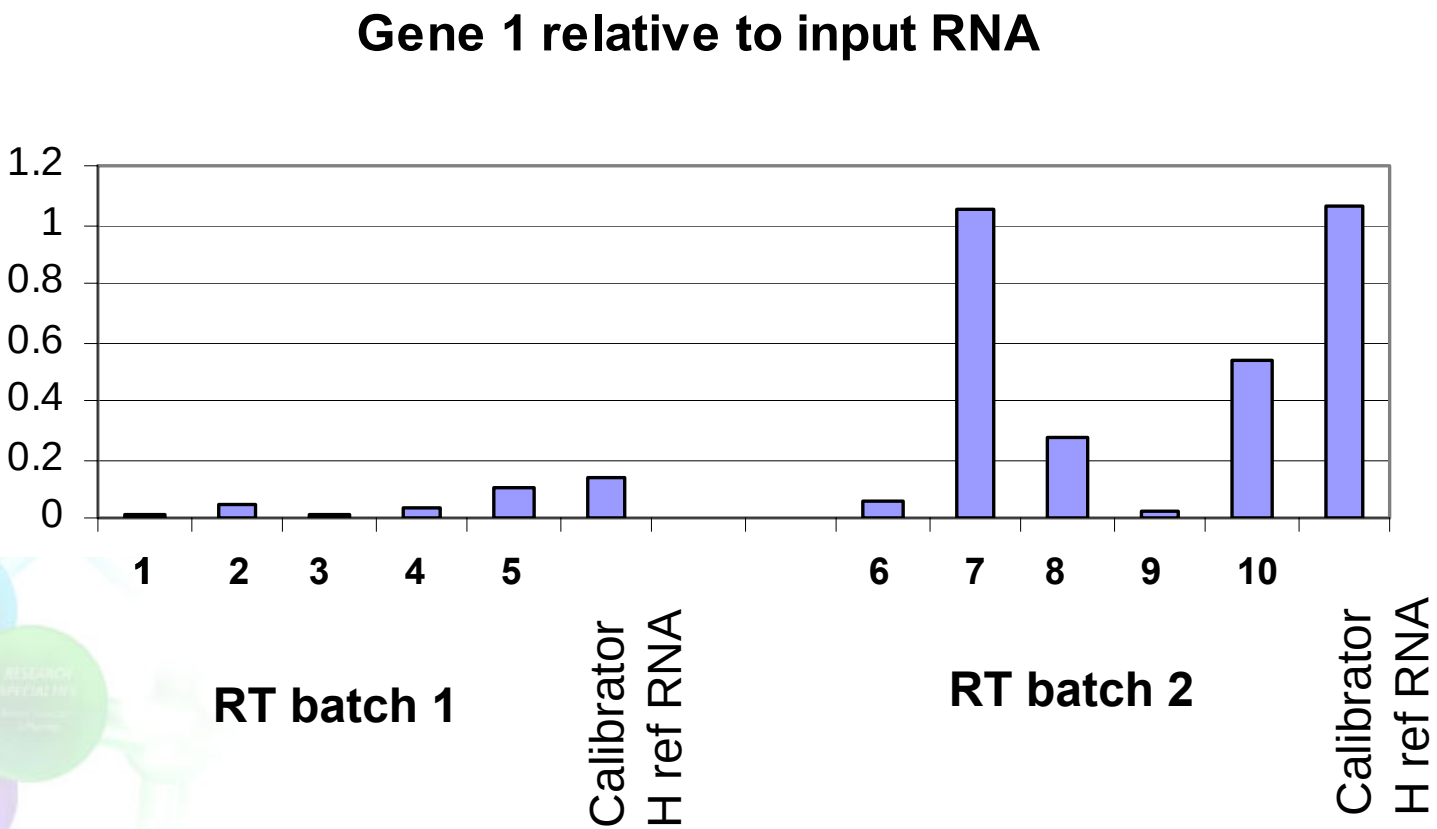
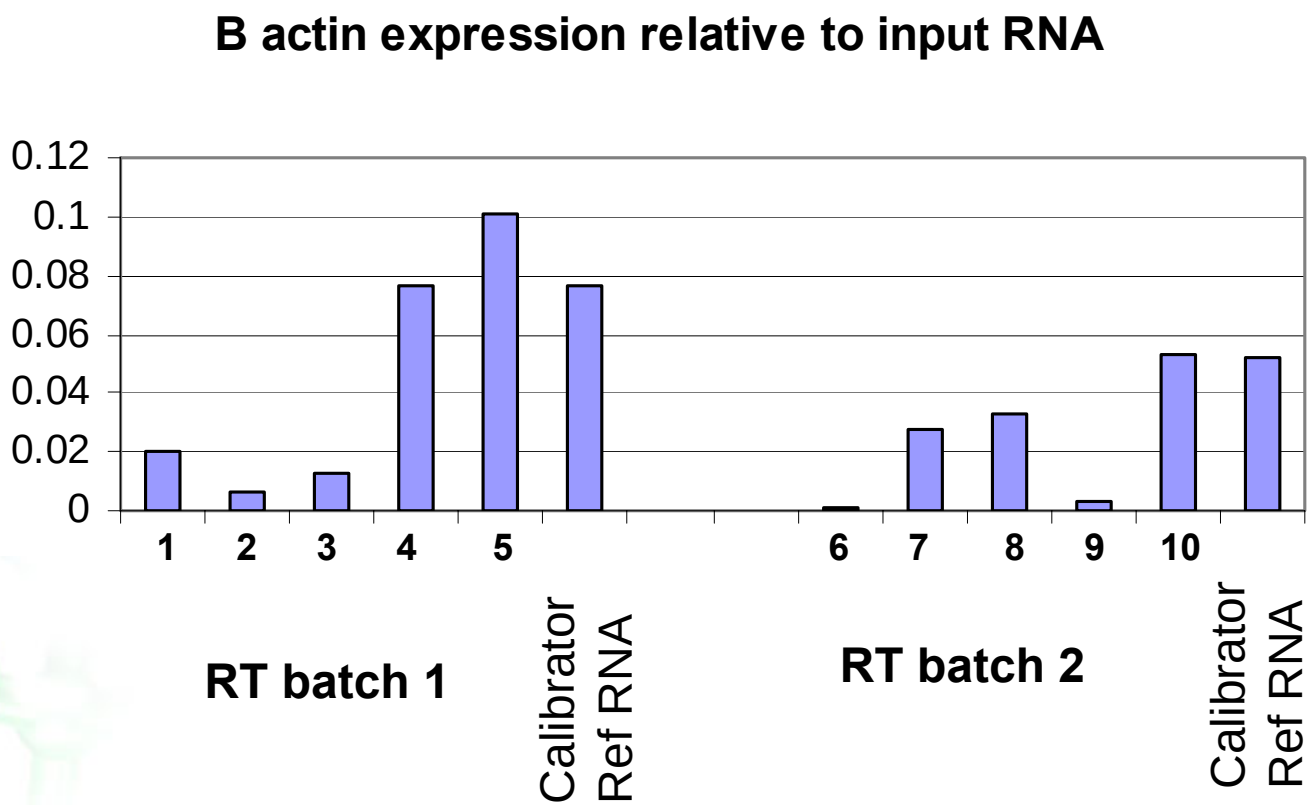
RNA Quantification



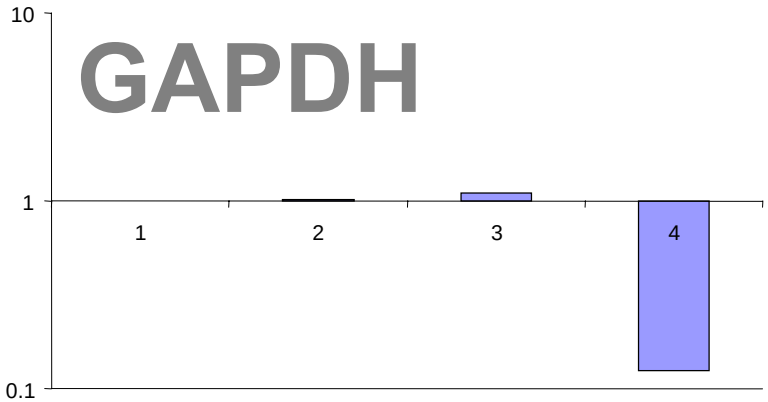
Independent reverse transcription reactions relative to input RNA (Medium/Low expressed gene, NHE1)

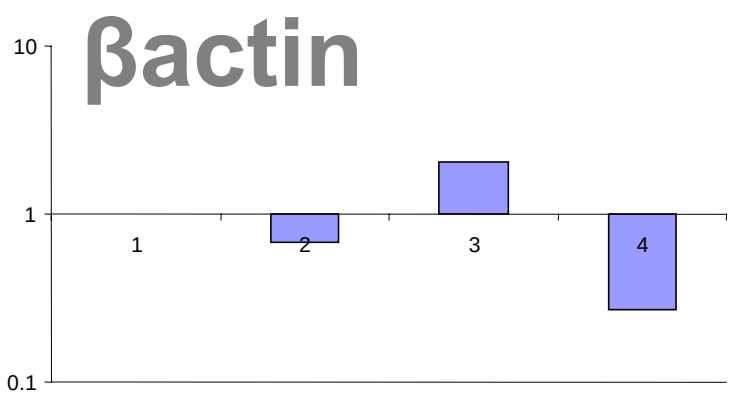


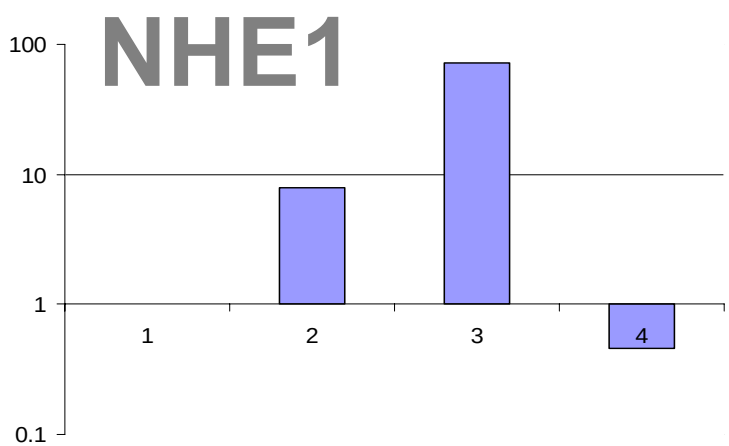
Reverse transcription reactions normalised to constant input RNA value (β actin)



Gene quantification is not reproducible between different RT reactions



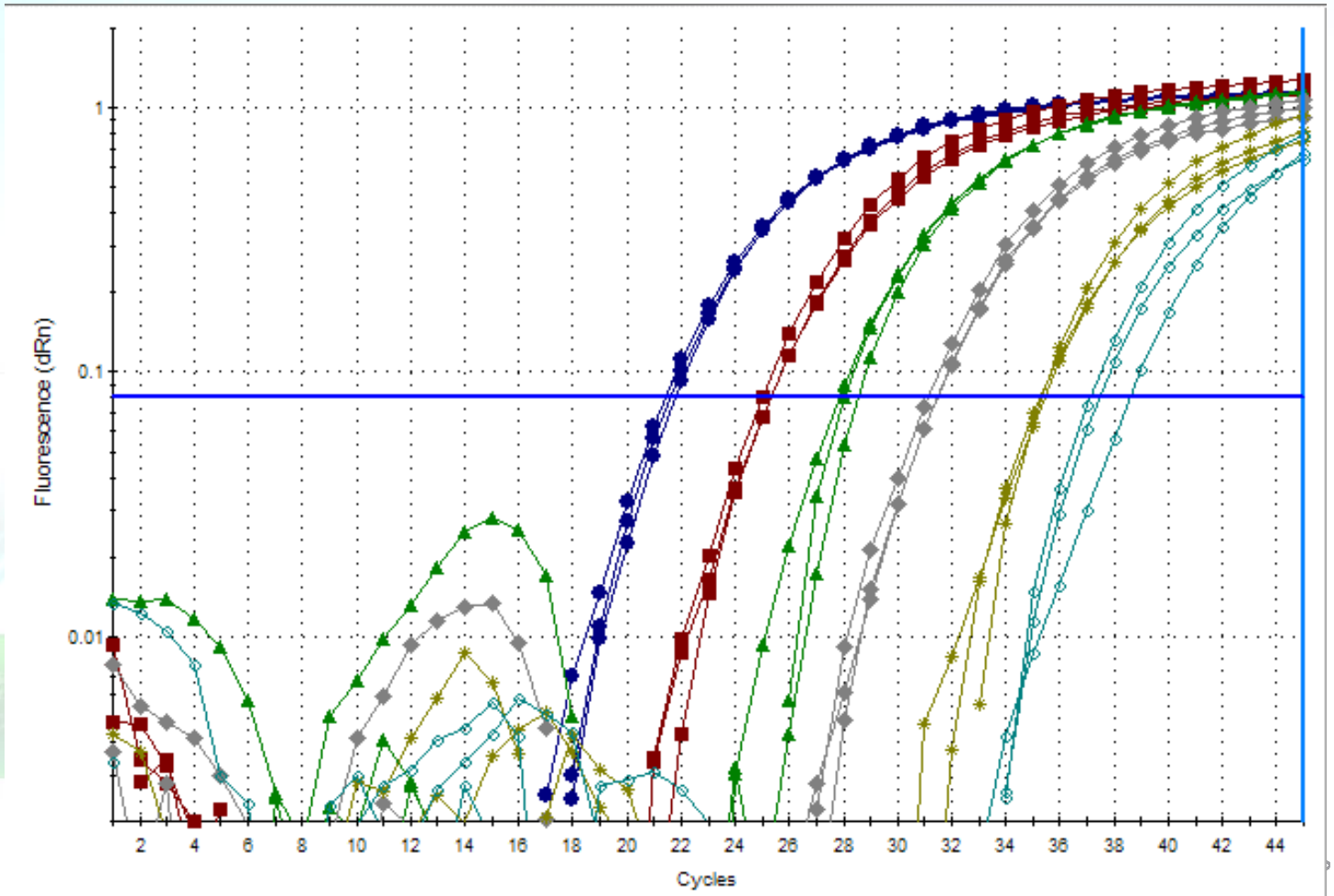




Correcting for batch to batch variations

- Assumption: The gene quantity in the calibrator represents the RT reaction efficiency for that gene in that sample batch
- Definition: Gene quantity in calibrator is 100% (for each batch)
- Quantities of the gene in the sample are expressed relative to gene quantity in calibrator (processed in same batch)

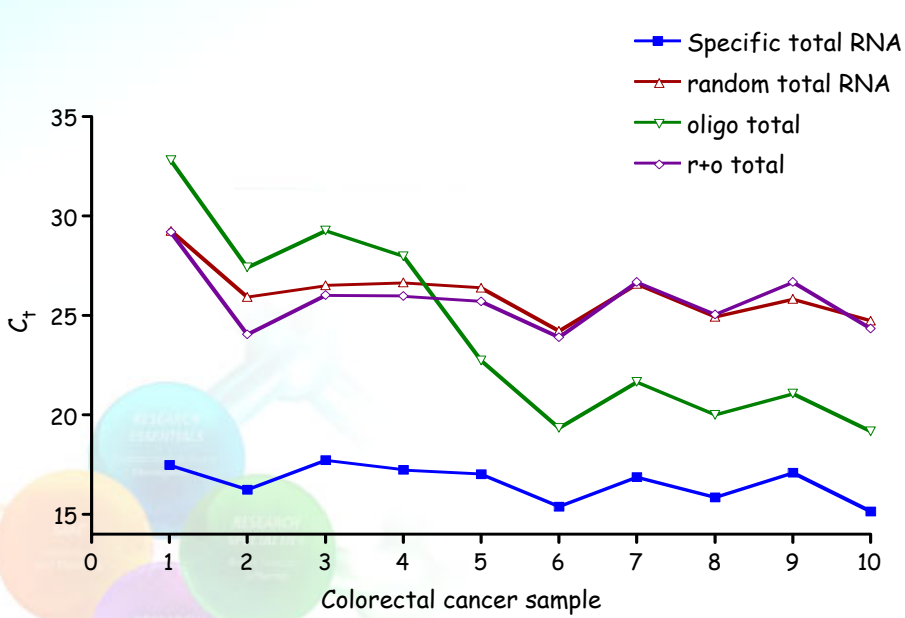
Gene specific priming RT and QPCR (10 fold dilutions, GAPDH)

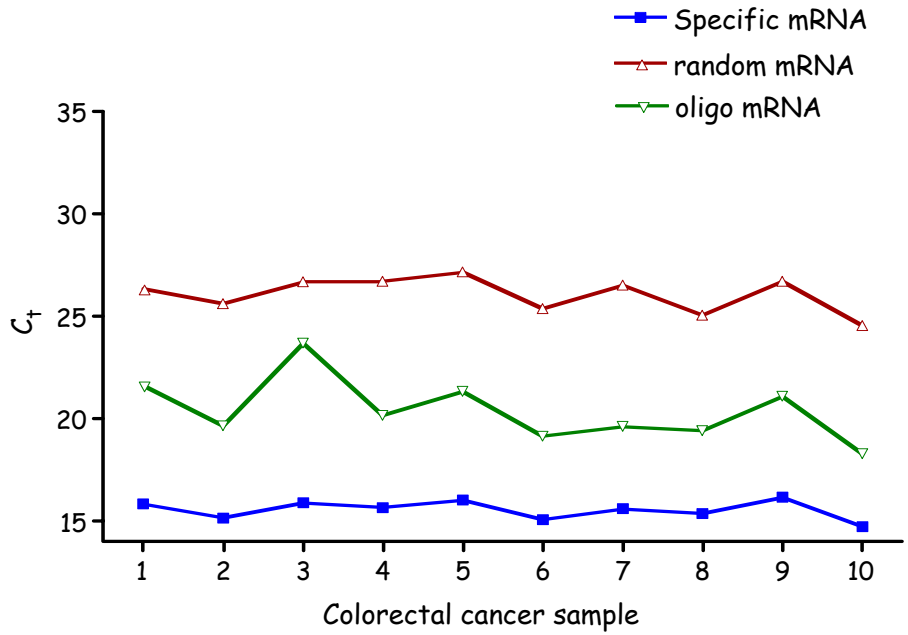


Comparing RT Priming Strategies

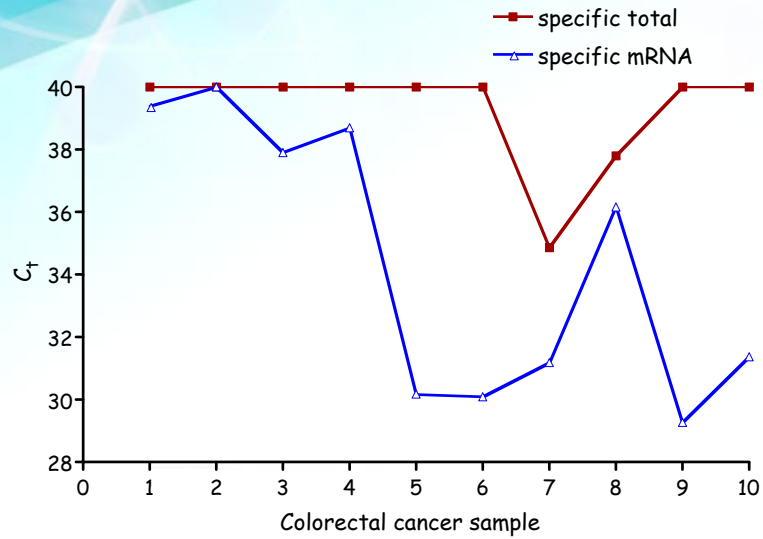
GAPDH

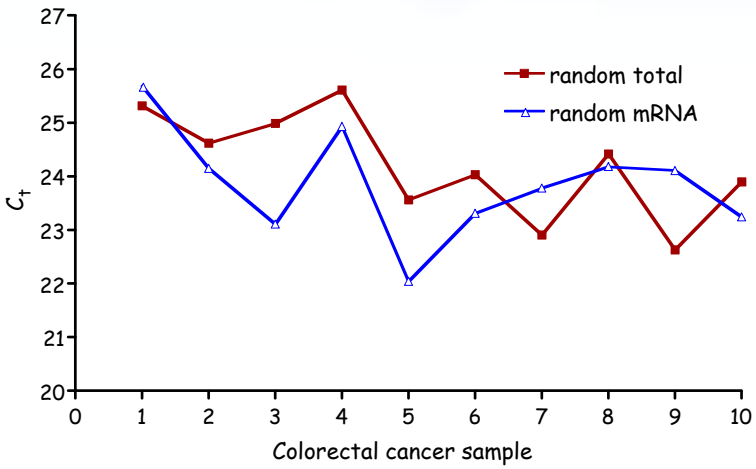
Constant RNA input concentration

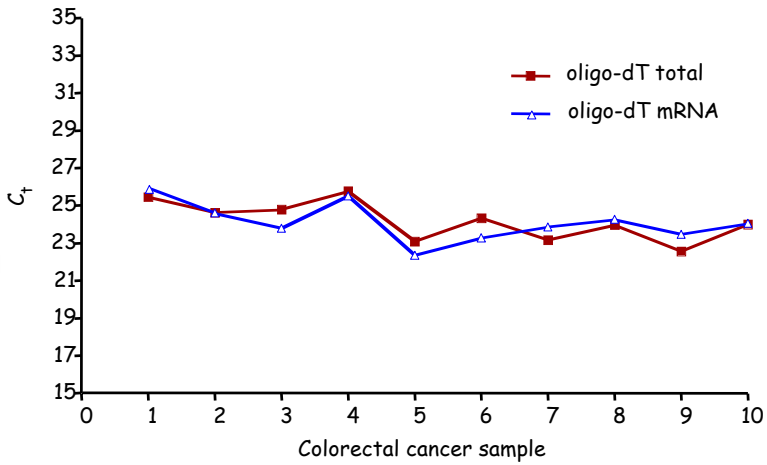




Total RNA vs mRNA - IGF-I







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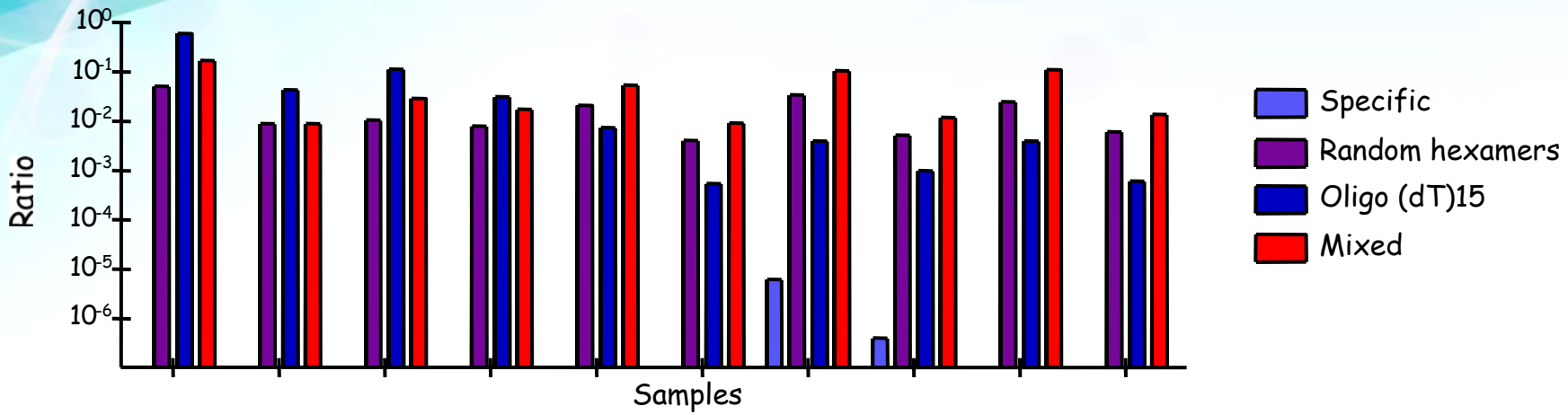
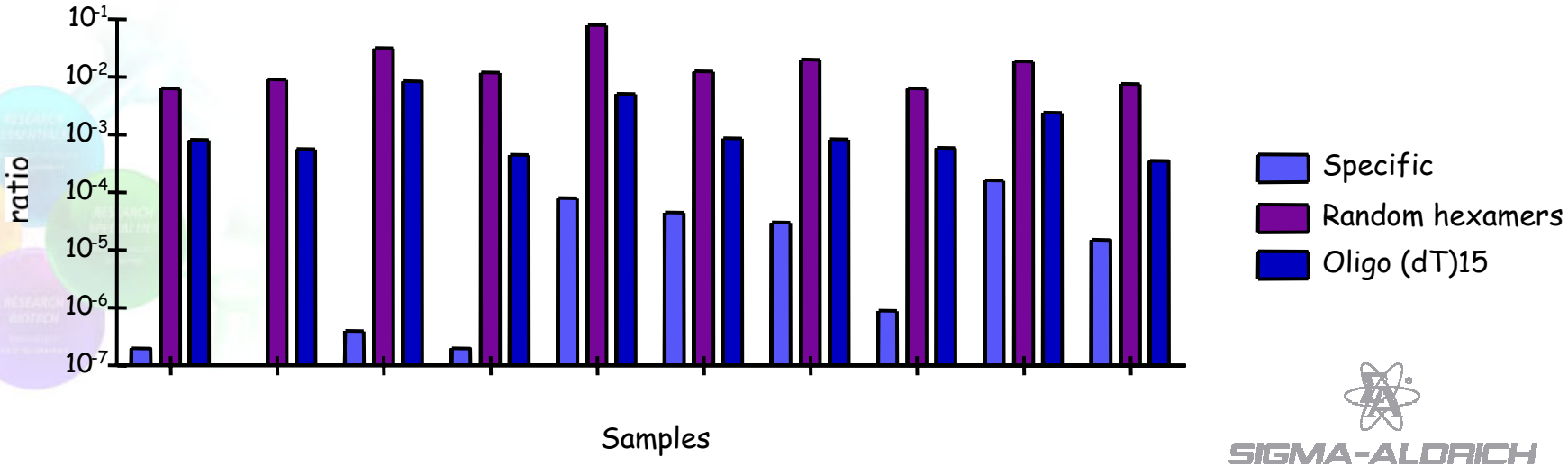
IGF-I/GAPDH total RNA



IGF-I/GAPDH mRNA



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Summary:

- Use best quality RNA/DNA possible
- QC everything

When using RT and random/oligo dT primers:

- Use same RNA quantity in each RT reaction
- Minimise RT batches and correct for differences

When using RT and specific primers:

- Design in open regions of transcript (mfold)

Assistance throughout the process:

ask the qPCR team at www.designmyprobe.com

Many Thanks to:

- Prof Stephen Bustin (QMUL, London, UK)

- Dr Michael Pfaffl (Freising, Germany)
- Dr Jo Vandesompele (University Ghent, Belgium)
- Dr Reinhold Mueller and Gothami Padmabandu (Formerly Stratagene, La Jolla, USA)
- Dr Anna Antonacopoulou, Patras, Greece
- Dr Helen Lacey and Prof Colin Sibley (Manchester University Medical School, UK)

- Dr Natalie Simpson (Formerly Sigma-Genosys, UK)
- Dr Steffen Mueller (Stratagene, Germany)
- Tanya Novak and Dr Jim Huggett (UCL, UK)
- Dr Vladimir Benes and the EMBL Team Heidelberg

www.designmyprobe.com