

Making Biological Sense of QPCR Data from Small Samples

From Process Considerations of Sample Preparation to Normalized Gene Expression Profiling

ABRF 2005

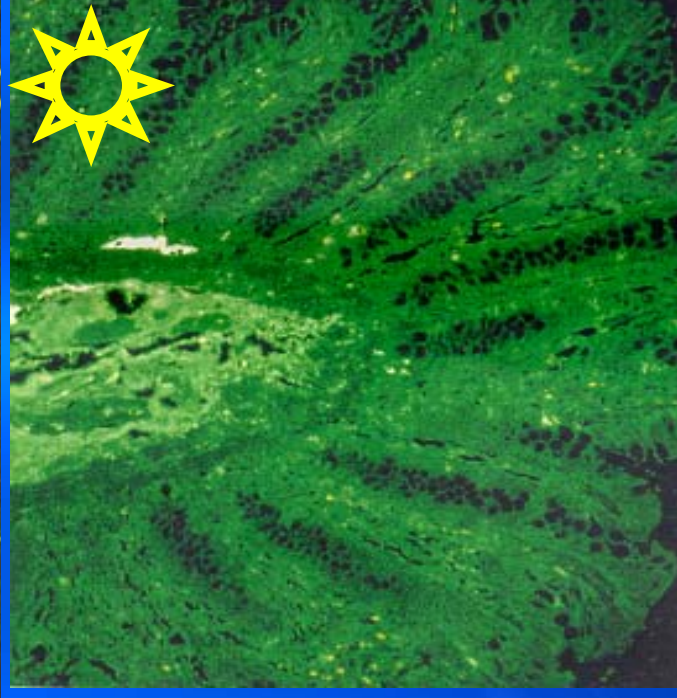
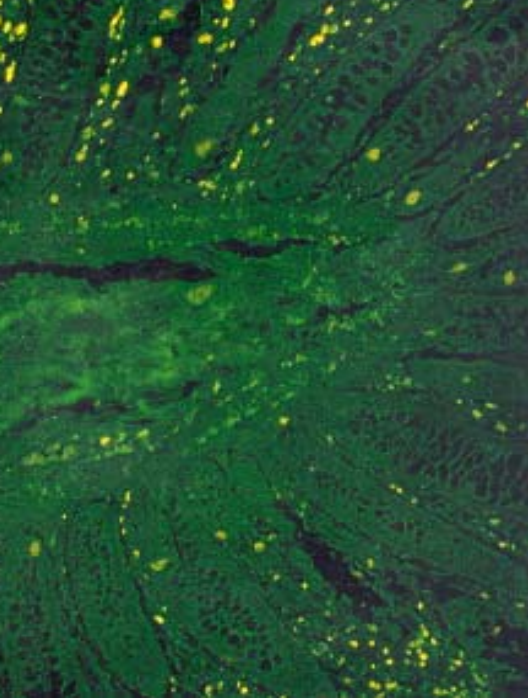
Tania Nolan & Reinhold Mueller

Presentation Overview

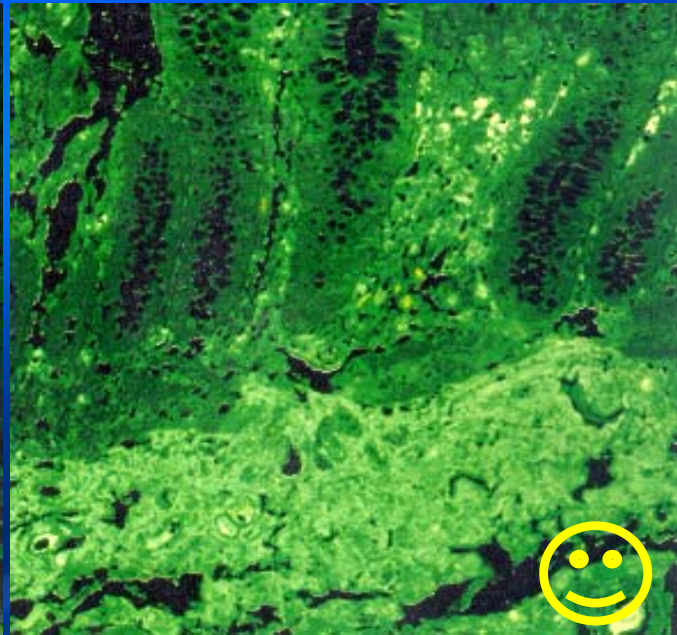
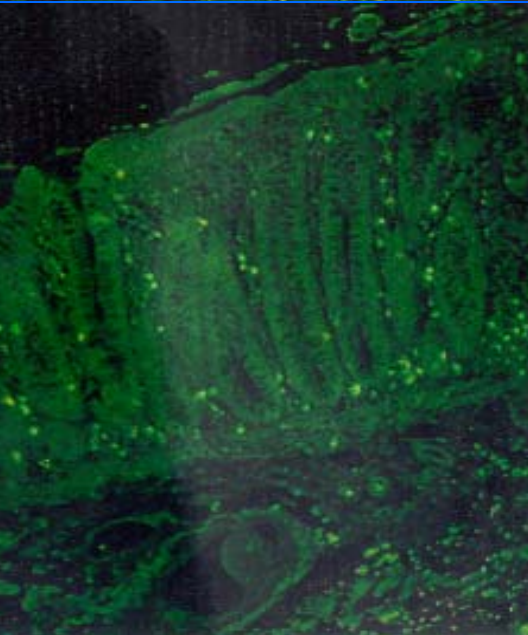
- Big issues with small samples
- Nucleic acid extraction
- Reverse transcription
- QPCR Assay design and optimisation
- Normalisation
- Data analysis and presentation

Examples of small sample applications

- Laser capture microscopy samples (Fresh or frozen samples or Formalin fixed samples)
- Limiting clinical biopsy samples
- Single cell assays
- Animal models (small organs)



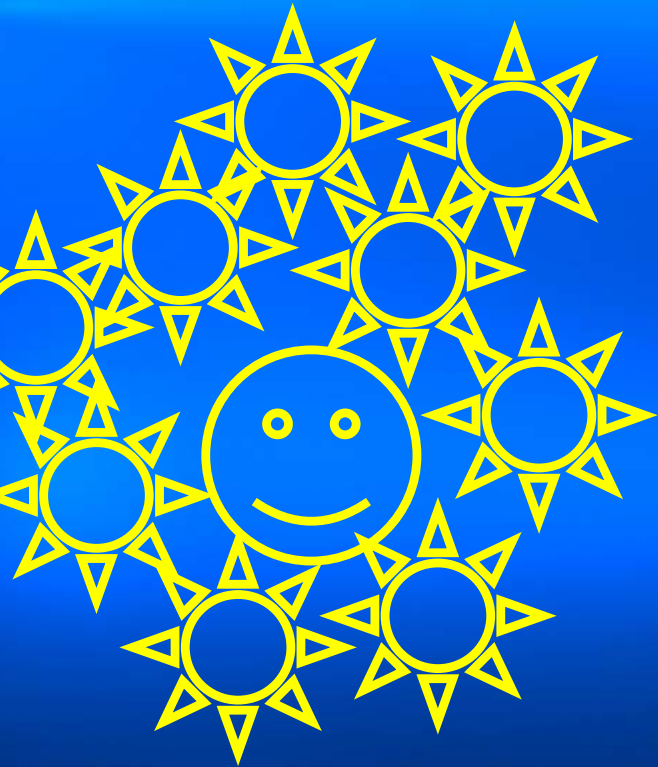
Normal colon tissue



Colon tumour tissue

Sample complexity distorts expression data

Smiley gene is expressed in 1/10 of cells:

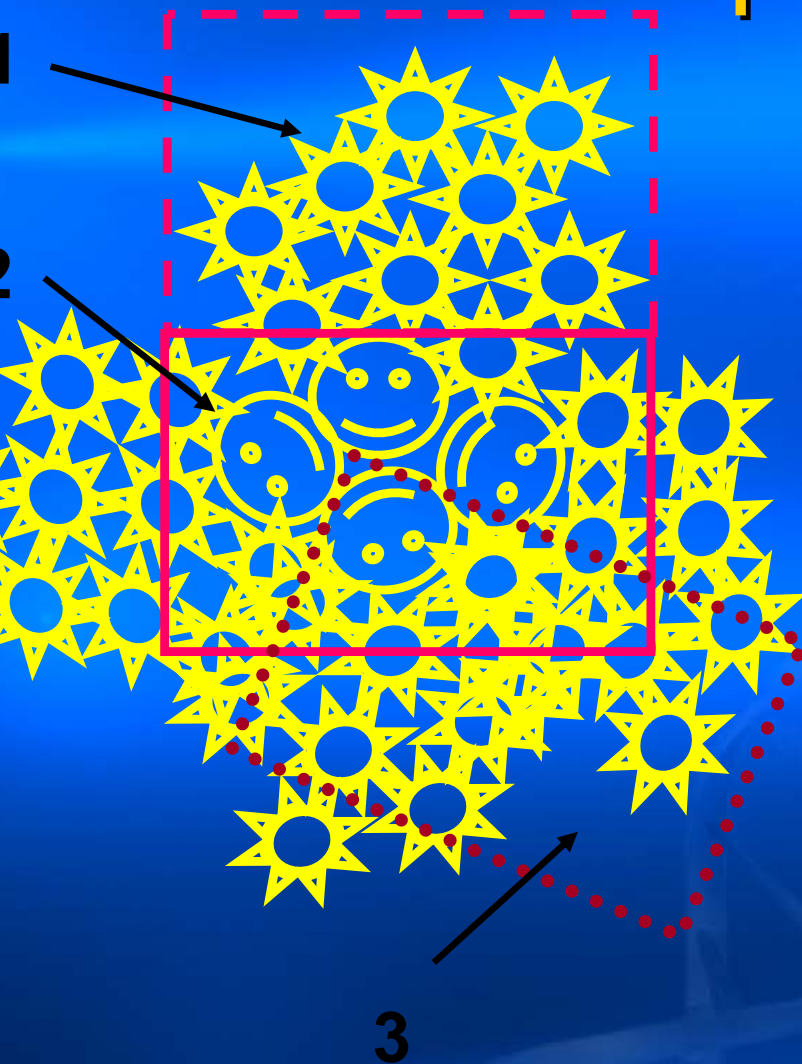


If SMILEY is present at 10^6 copies per + cell
QPCR of 10 mixed cells would give a result of
 10^5 copies per cell

If SMILEY is present at 10 copies per + cell
QPCR of 10 mixed cells would give a result of
1 copy per cell (and be challenging to detect)
Possibly giving a negative result

Tissue complexity results in an
UNDERESTIMATE of gene quantification

Sample complexity distorts expression data



Smiley gene is expressed in 1/10 of cells:

Cut around position 1 –

No SMILEY gene detected

Cut around position 2 –

SMILEY gene detected in 4/8 cells
reported at 1/5 real quantity

Cut around position 3 –

SMILEY gene detected in 1/10 cells
reported at 1/10 real quantity

POSITIONAL effect of cell type sampled
effects gene quantification results

3

Sample complexity distorts expression data



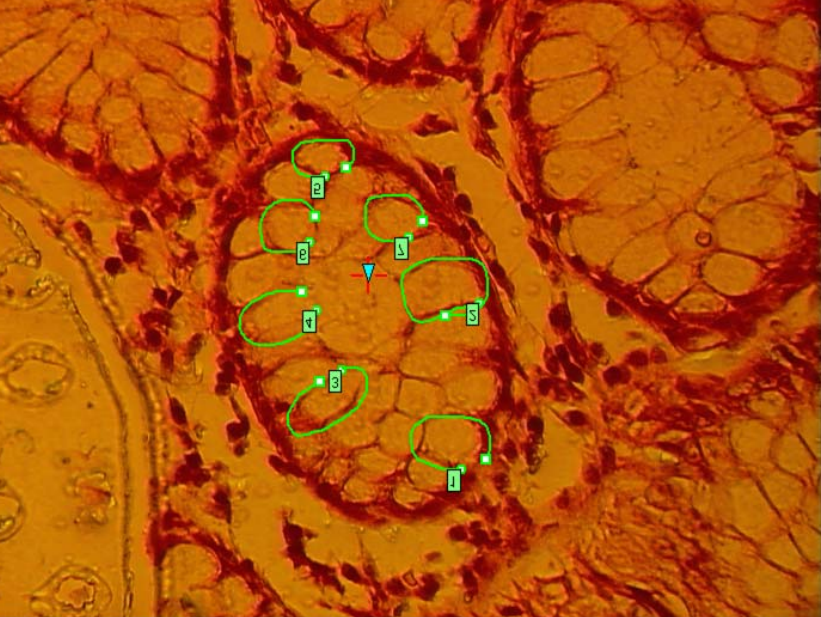
Tissue complexity results in an **UNDERESTIMATE** of gene quantification

POSITIONAL effect of cell type sampled effects gene quantification results

Isolation of specific cell type or tissue of interest reveals gene quantity changes that are masked in heterogeneous cell samples

Methods of purifying cells/separation techniques

- LCM
- Microdissection
- FACs
- Magnetic cell sorting
- Ficoll gradients



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Element List

Show figures only

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Summary for colors :

	Figures	Areas (μm^2)
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Total : 7 2112 μm^2

Laser function

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Case Study

Scientist:

Prof Colin Sibley and Dr Helen Lacey
(Manchester UK)

Research question:

What are the genetic changes in
prevalence of ion channel transcripts
through gestation?

Case Study Method

- Samples:
 - Fresh tissue
 - Human placenta tissue biopsies
 - Provided one sample at a time by surgeon
 - Early gestation samples from pregnancy termination and term samples at delivery

Case study Method

- Protocol:
 - Extract total RNA
 - Reverse transcribe using random primers
 - Quantify each gene of interest relative to a standard curve using QPCR
 - Express data normalised to input RNA and relative to control sample

Sample extraction and treatment

- Efficient RNA extraction is critical
- Quality and quantity controls
- Avoiding inhibitors
- Avoid gDNA contamination
- Long and short term RNA storage

Extra precautions for working with RNA

- DNA is robust and survives centuries of hardship
- RNA is delicate and requires care and respect
- Dedicated working area
- Bake all glassware and metal equipment
- Maintain RNA-only reagents
- Always wear gloves
- Use Molecular Biology grade water (or treat solutions with DEPC, not Tris)
- Use RNase inhibitors in RNA samples

Efficient RNA extraction

- Tissue may be “fresh” (or stored in RNAlater), snap frozen or FFPE (as sections)
- Efficient extraction requires effective tissue homogenisation (do not allow frozen samples to defrost)
- Work quickly with an easy protocol
- Column based techniques effectively remove contaminants and gDNA
- Using columns also avoids nasty guanidinium and phenol
- Precipitate with glycogen carrier
- Store working aliquot at -80°C with RNase inhibitor (long term under ethanol at -80°C)

RNA quantification and quality control

“Small” samples: (material from 100 cells typically 1-50pg total RNA)

- **Quantification:**

- Nanodrop (2ng)
- Ribogreen staining (read on Mx3000P) (5ng)

- **Quality Control and Quantification:**

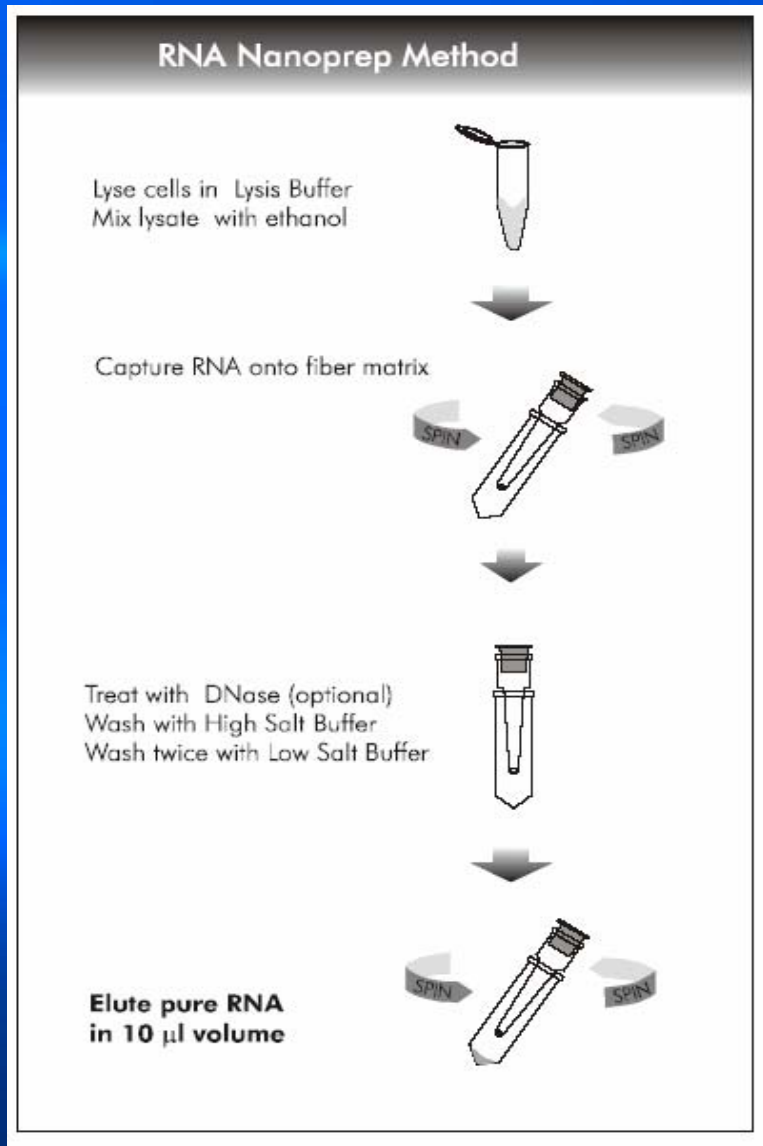
- Agilent (Caliper) BioAnalyser 2100 (10ng +)

- **Not suitable for small samples:**

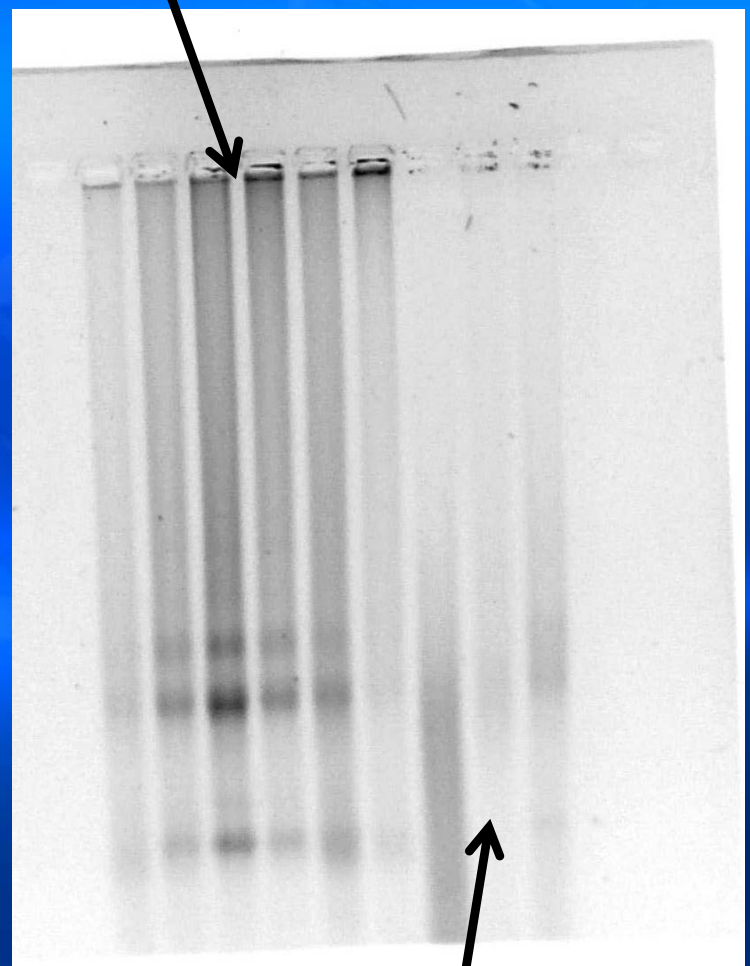
- UV spectrophotometry (150ng)

- Agarose Gel Electrophoresis (1 μ g)

Small Sample RNA purification (1-10⁴ cells)

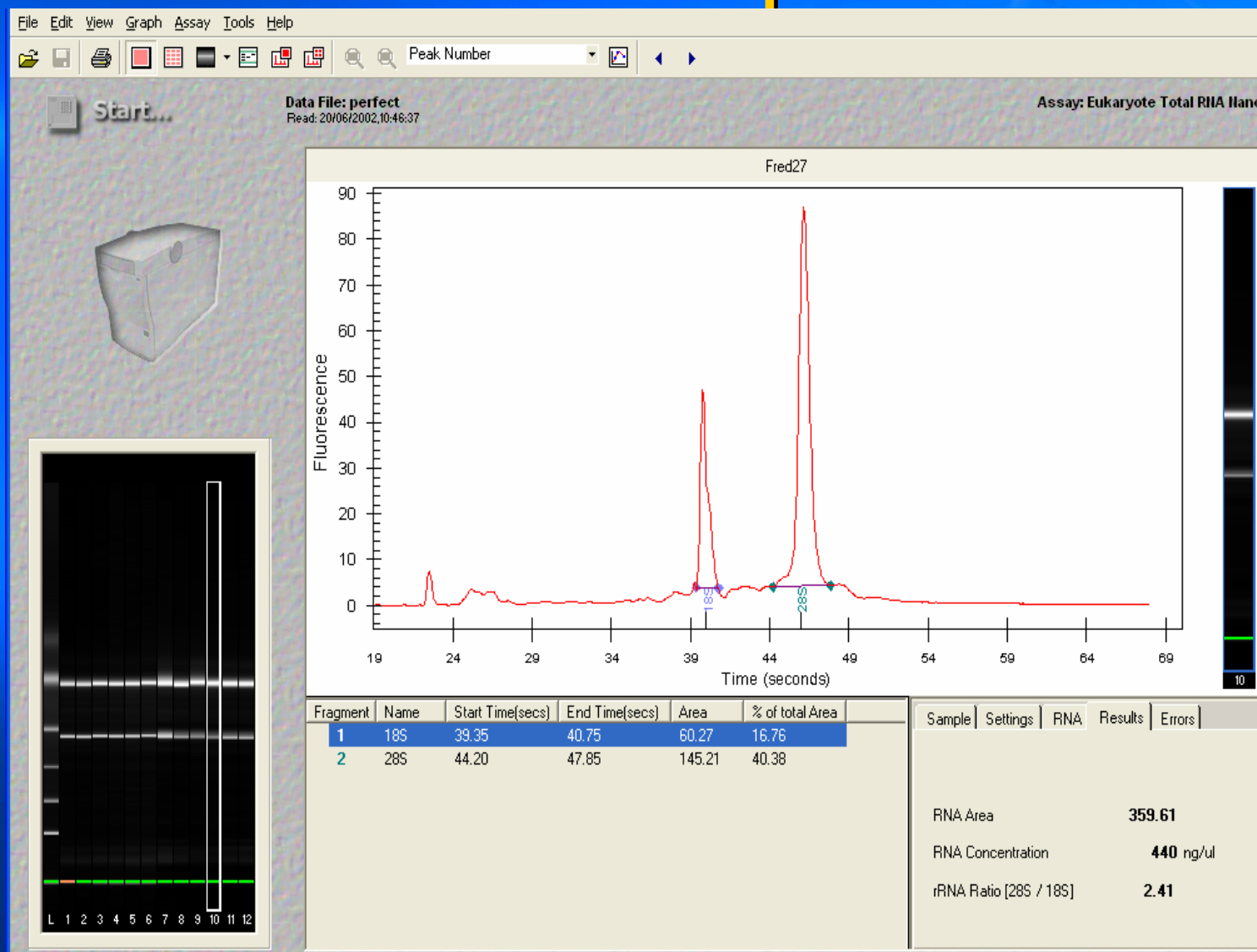


RNA From frozen tissue sections

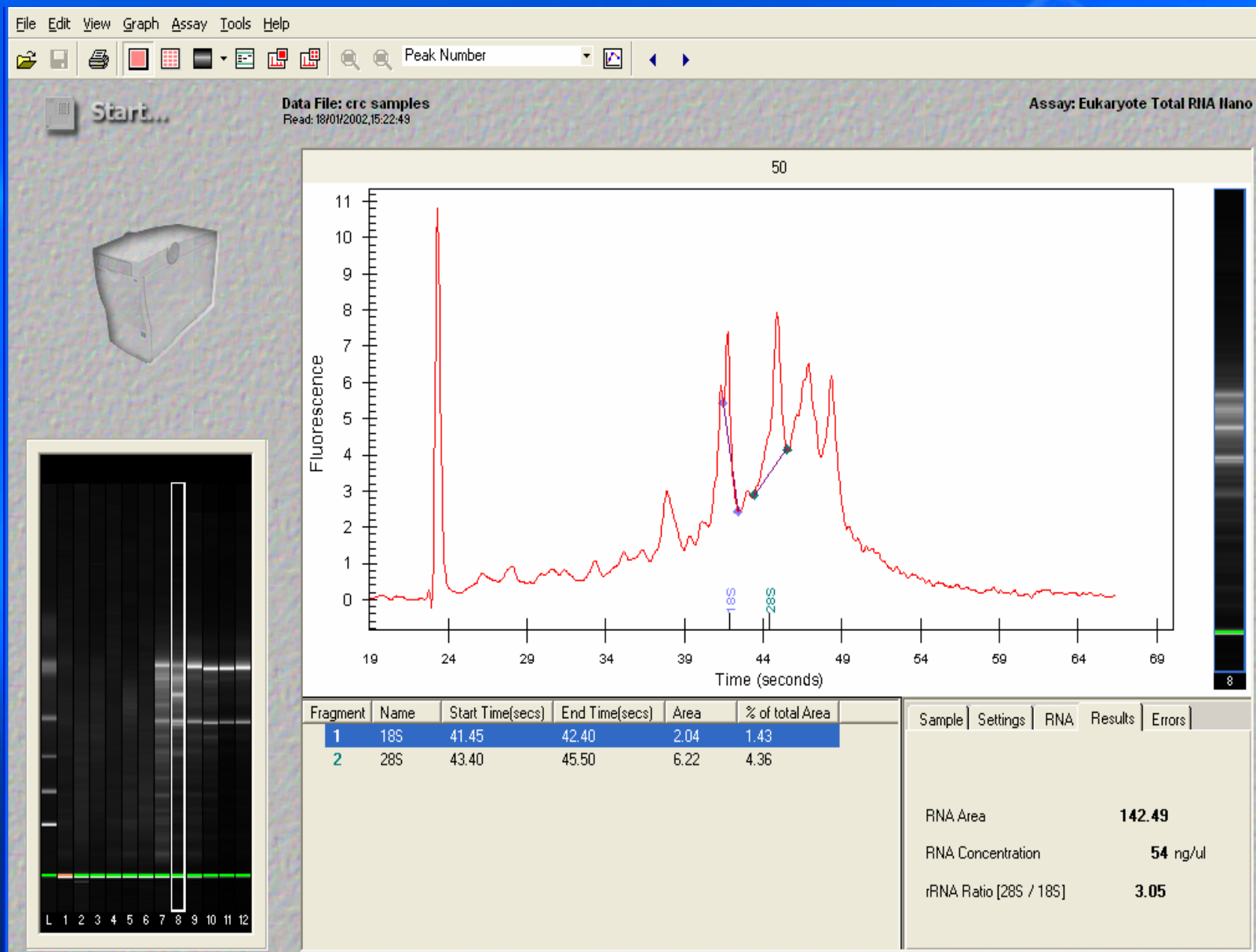


RNA from FFPE tissue sections

High quality RNA extracted from cell samples

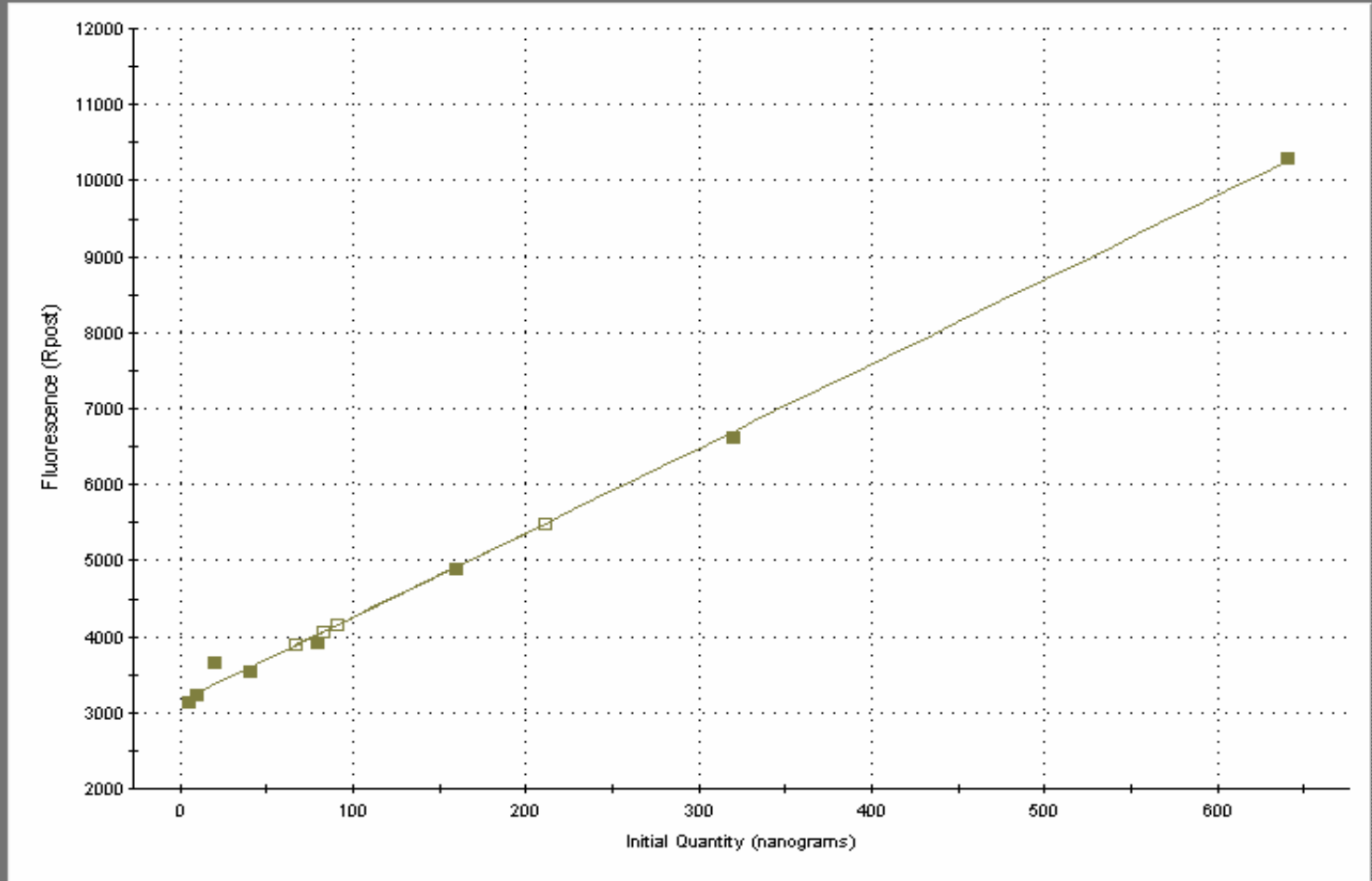


Some extractions result in degraded RNA



Ribogreen plate-read assay for RNA quantification

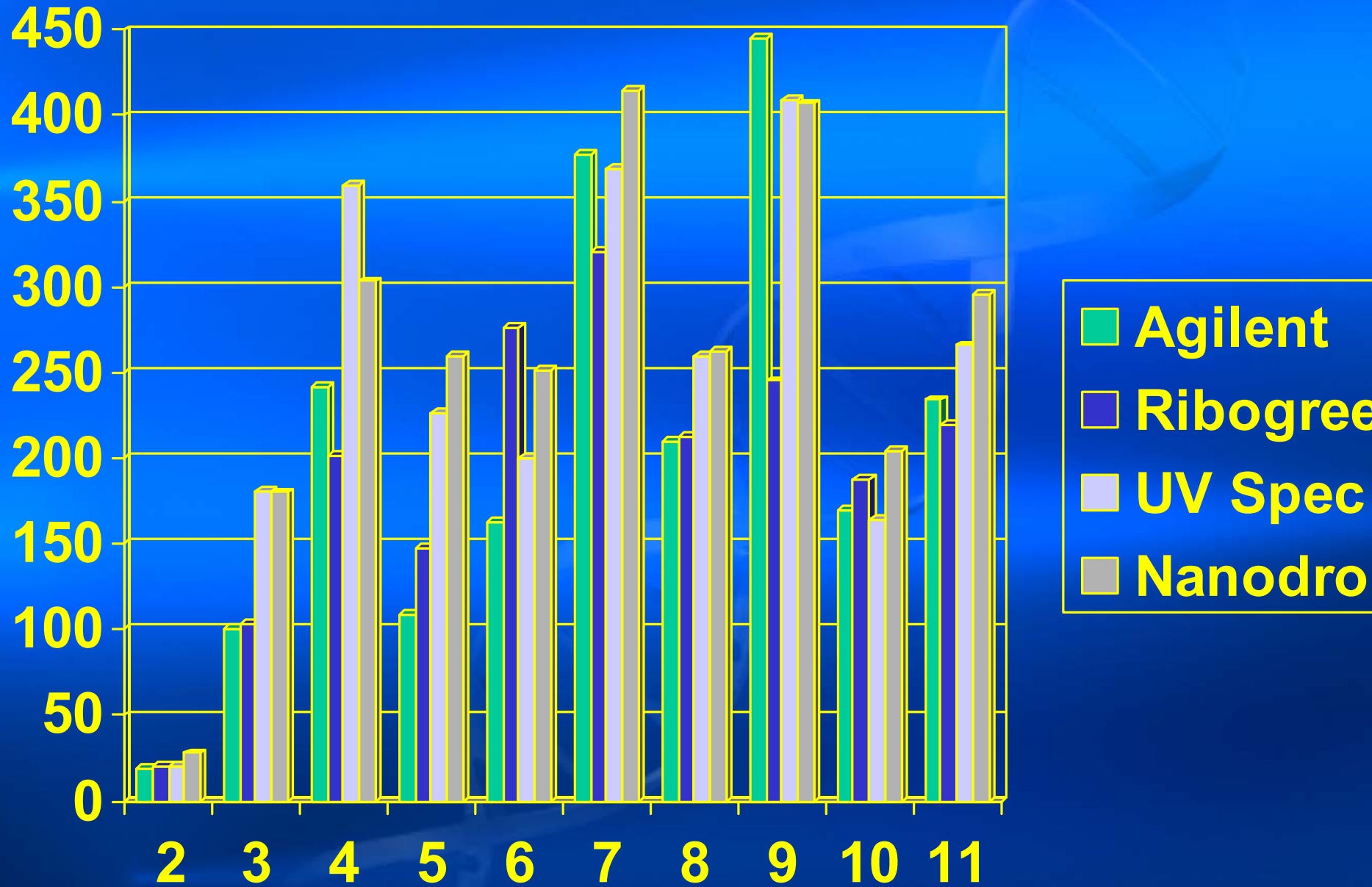
Ribogreen Quantification Standard Curve



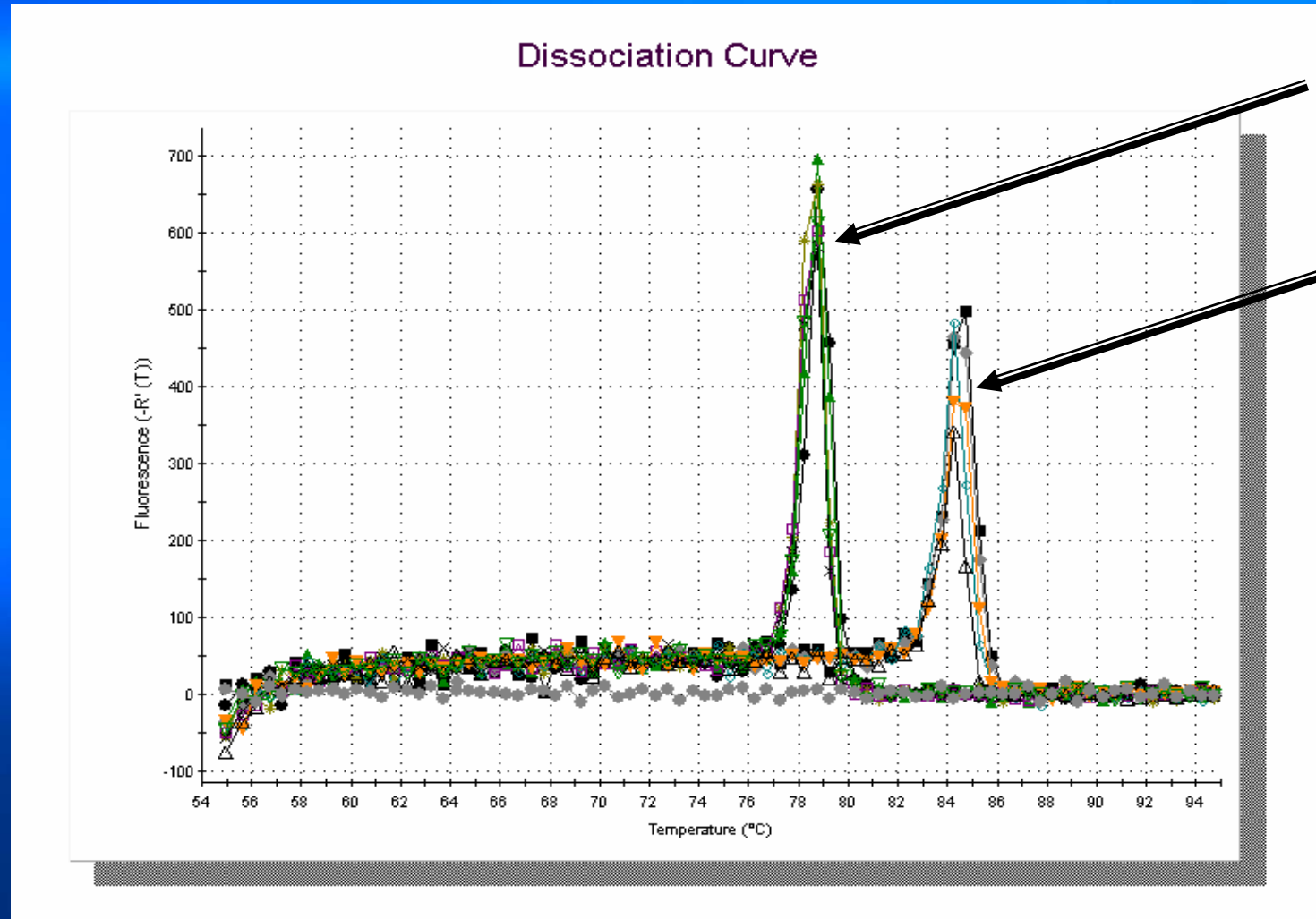
A Quantification Comparison

- EMBL QPCR training course Heidelberg
- 11 groups extracted RNA from frozen cell pellets
- Each RNA sample was quantified using 4 techniques

RNA quantification



SYBR Green I dissociation curve: cDNA and gDNA target



cDNA

gDNA

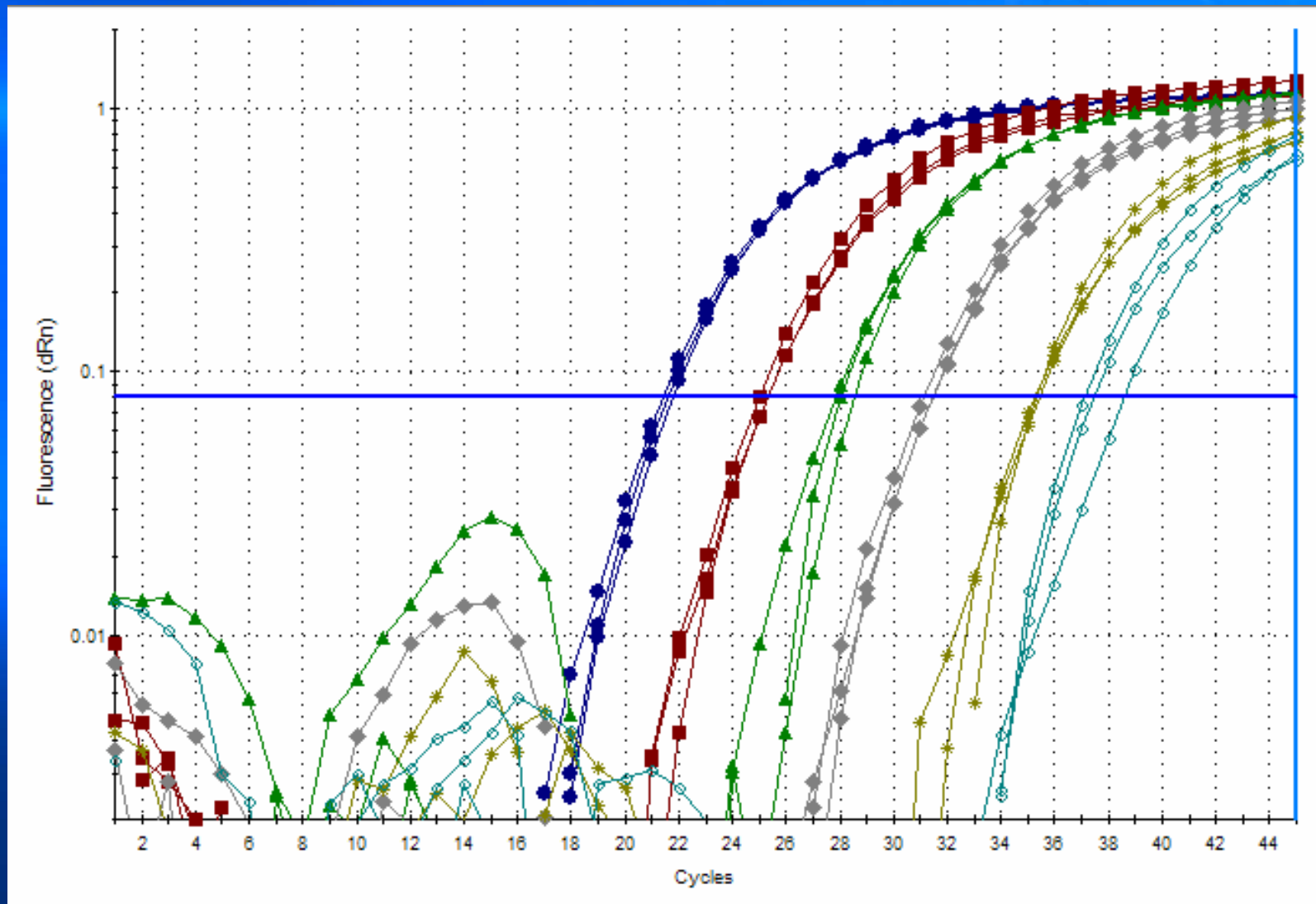
Reverse Transcription

- Enzyme choice
 - RNase H-
- Variations between samples
- Variations between sample batch
- One step or two step protocol
- Primer considerations

Priming RT: One-step protocols

- Gene specific primers capture single target
- Usually higher sensitivity for low copy number target
- Must return to RNA sample for each quantification
- Restricted in the number of genes to be quantified and multiplexing requires multiple “single “ RT reactions
- Individual target RT variability may confound interpretation

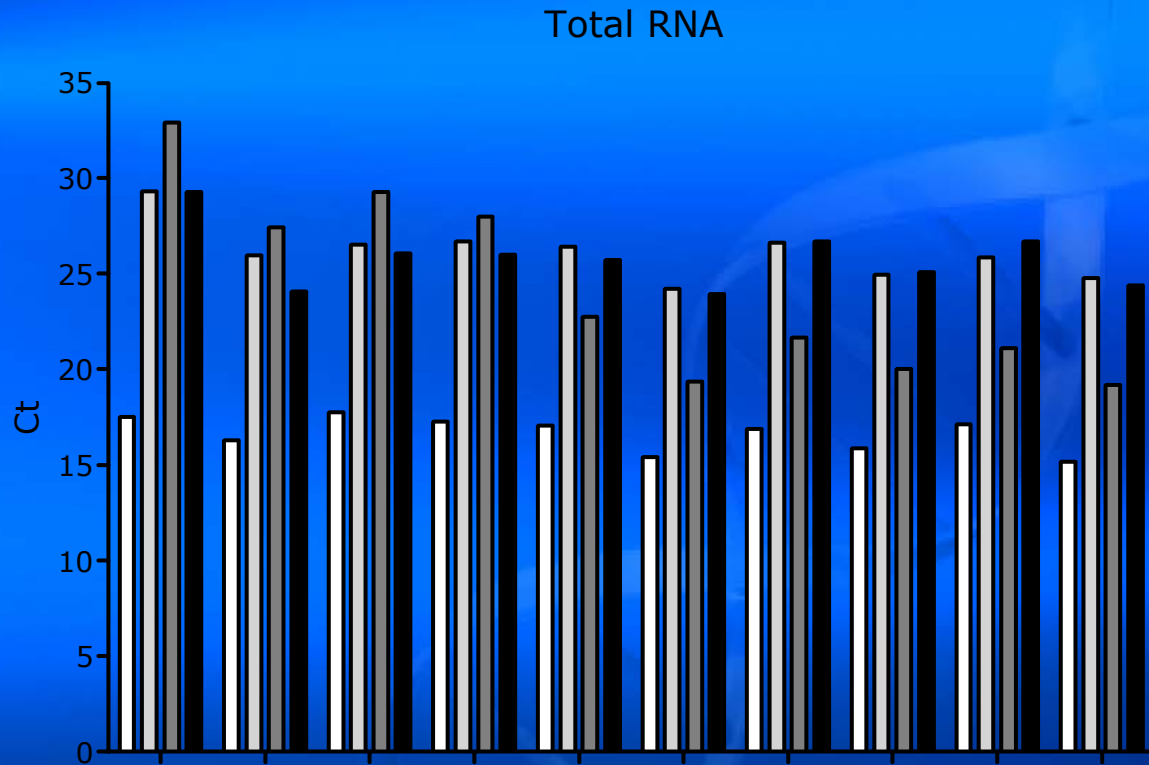
Gene specific RT and QPCR (10-fold dilutions)



Priming RT: Two-step protocols

- Random primers capture all targets
 - May miss low expressed targets
 - Usually better for structured sequences
- Oligo dT captures all PolyA+ transcripts but not ribosomal RNA
 - Not all mRNA has polyA tail (e.g. histones)
 - QPCR must be designed towards the 3' of gene sequence
- Usual approach for analysis of multiple genes and for multiplexing

Reverse Transcription - 1



10 colon samples, 100ng total RNA, 3ng mRNA

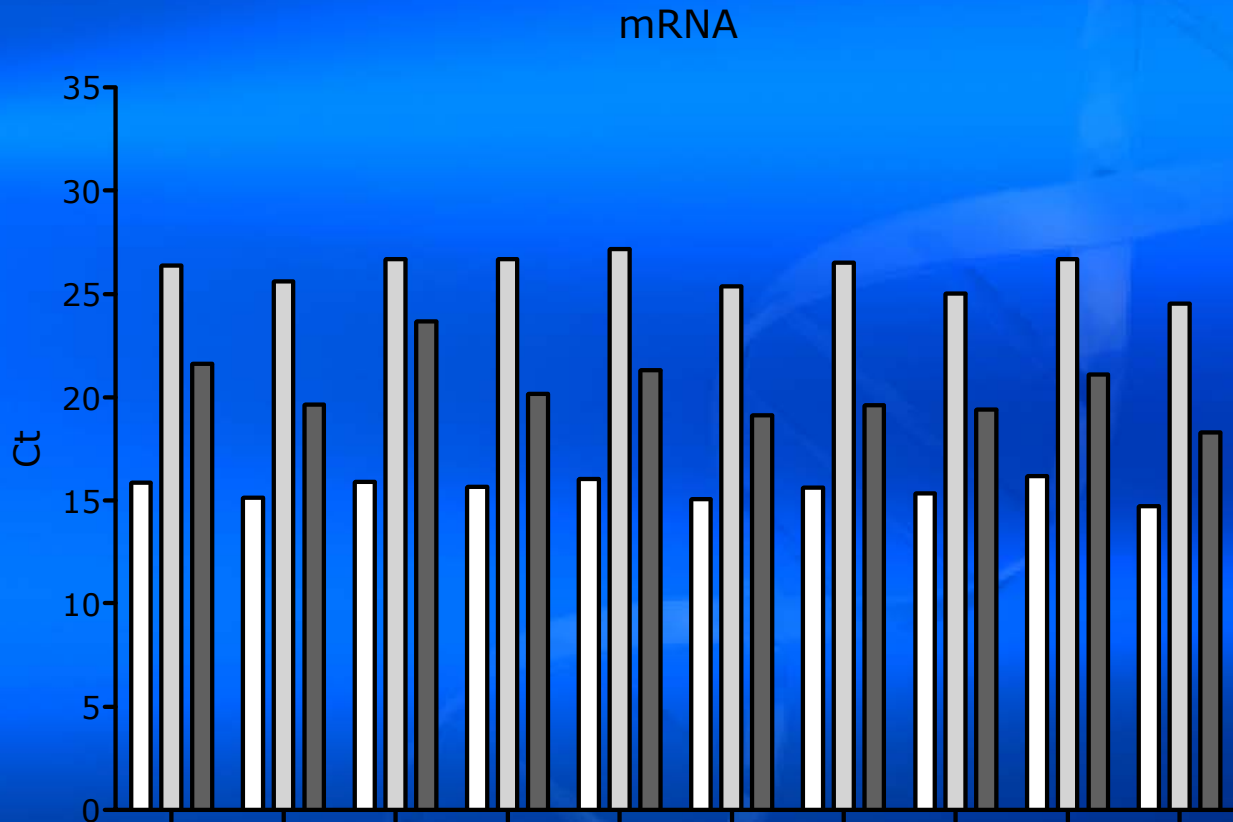
White bar: specific GAPDH

Grey 1: random

Grey 2: oligo-dT(15)

Grey 3: random + oligo-dT(15)

Reverse Transcription - 2



10 colon samples, 100ng total RNA,
3ng mRNA

White bar: specific GAPDH

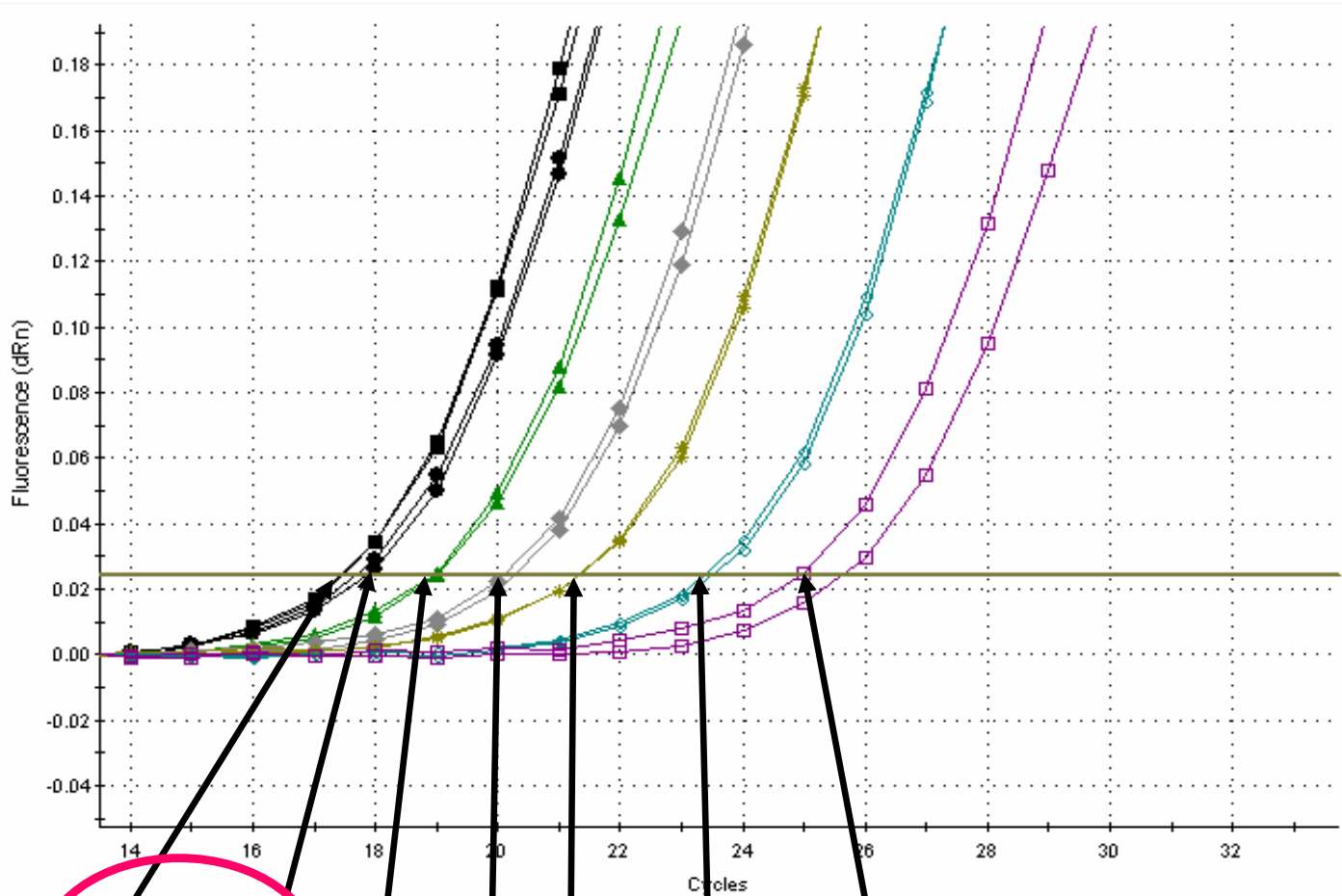
Grey 1: random

Grey 2: oligo-dT(15)

Grey 3: random + oligo-dT(15)

Reverse transcribed RNA dilution series (β -actin)

RNA serial dilution (B actin)



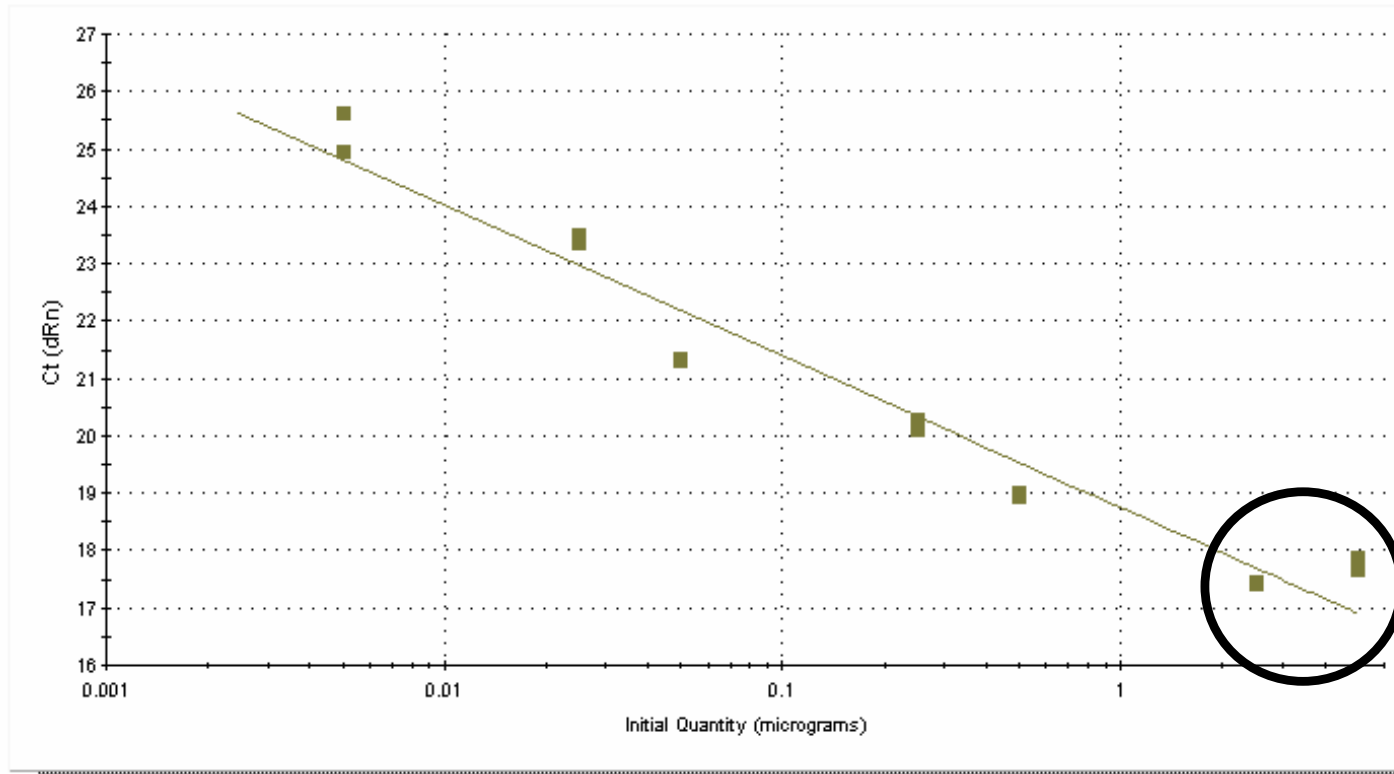
2.5 5 0.5 0.25 0.05 0.025 0.005

Reverse transcribed RNA dilution series (β -actin)

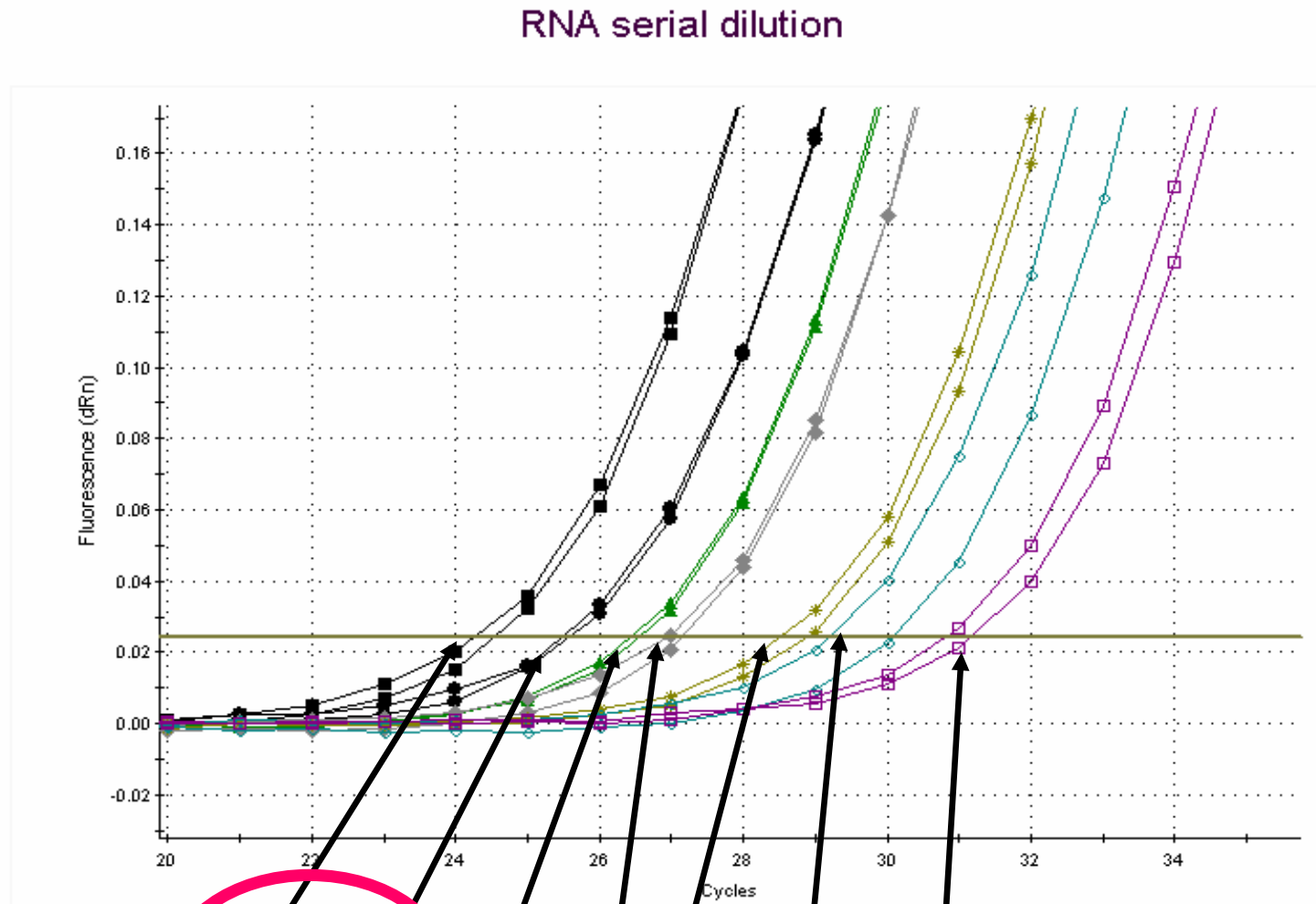
Standard Curve

Log fit values

- SYBR Standards, RSq:0.953
- SYBR, $Y = -2.632 * \text{LOG}(X) + 18.75$, Eff. = 139.8%



Reverse transcribed RNA (2X) dilution series (Target: NHE1)



2.5

5

0.5

0.25

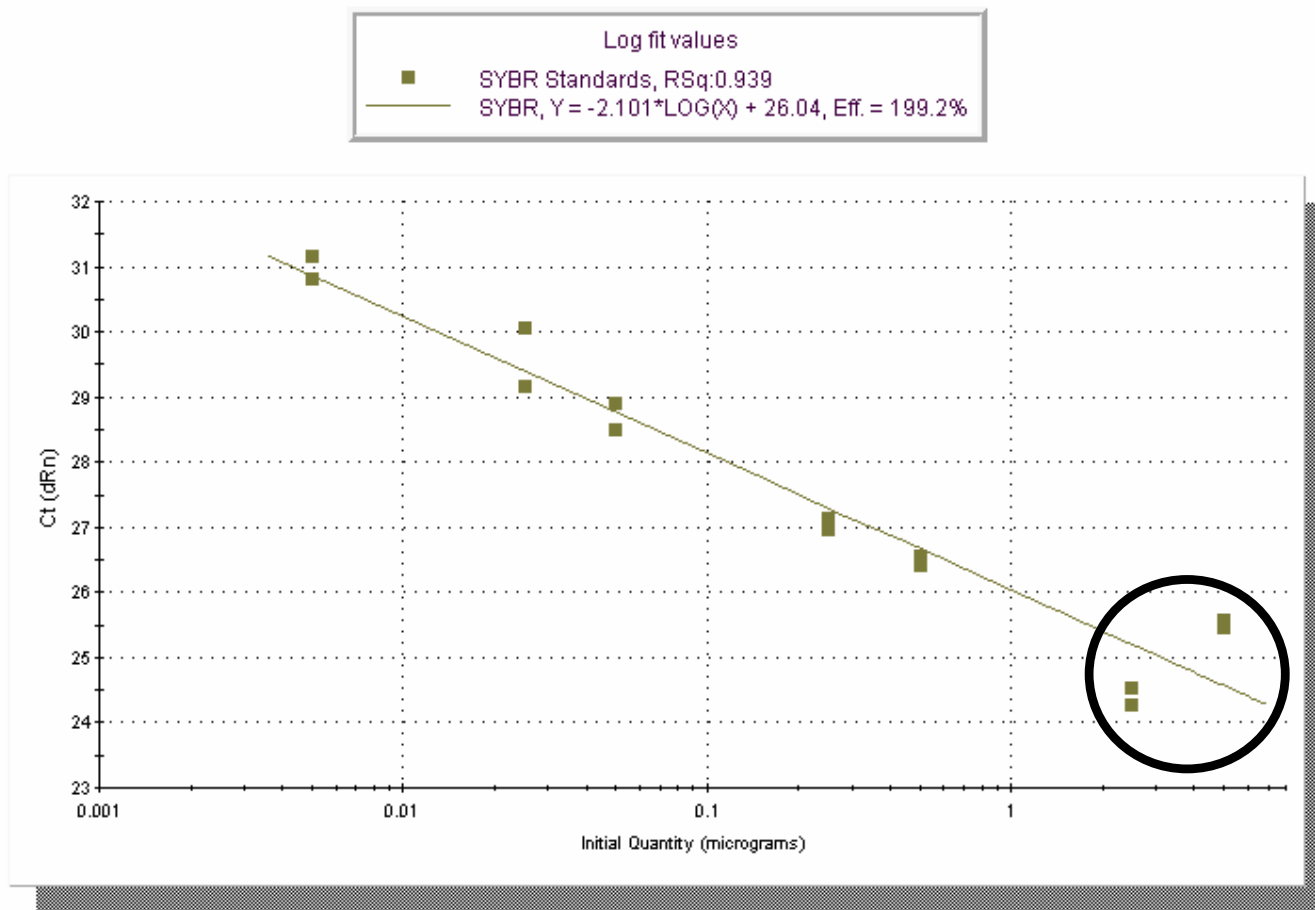
0.05

0.025

0.005

Reverse transcribed RNA (2x) dilution series (Target: NHE1)

Standard Curve



Case Study: 2-tube RT protocol

- Stratascript (RNase H-) enzyme
- Random Nonamers
- 2.5 μ g RNA/reaction (based on Ribogreen quantification)
- Positive control sample (Human QPCR Ref RNA) included with each batch of test samples
- -RT negative control

QPCR Design and Optimisation

- Gene sequences acquired from Genbank
- Design using Beacon Designer software
- All primer pairs tested using SYBR green on Human ref RNA
- Primers and dual-labeled hydrolysis probes from PrOligo

Primer Design Software Options

– Beacon Designer 3 (Premier Biosoft Intl)

- Designs primers, Taqman-type probes, molecular beacons, and includes multiplexing, amplicon structure, and BLAST analysis tools

– Free web-based software

- Primer3: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
- IDT: <http://scitools.idtdna.com/Primerquest/>

– Other commercial software

- Visual OMP (DNA Software)

– Design services

- BioSearch Technologies: <http://www.biosearchtech.com>
- Proligo: <http://www.proligo.com>
 - Offer custom design of LNA (Locked nucleic acids) primers/probes

– Primer databases

- RTPPrimerDB: <http://medgen.ugent.be/rtpprimerdb/>
- QPPD: <http://web.ncifcrf.gov/rtp/gel/primerdb/>

Primer Optimization

- Primer Optimization

- Thermal profile

- Design all primers with similar T_m (using same software) in order to use same thermal profile
 - Since primer T_m is an estimate forward and reverse primers probably have different actual T_m and therefore different annealing kinetics

- Optimize primer concentrations

- Titrate forward and reverse concentrations down to increase sensitivity and specificity (no primer-dimer)

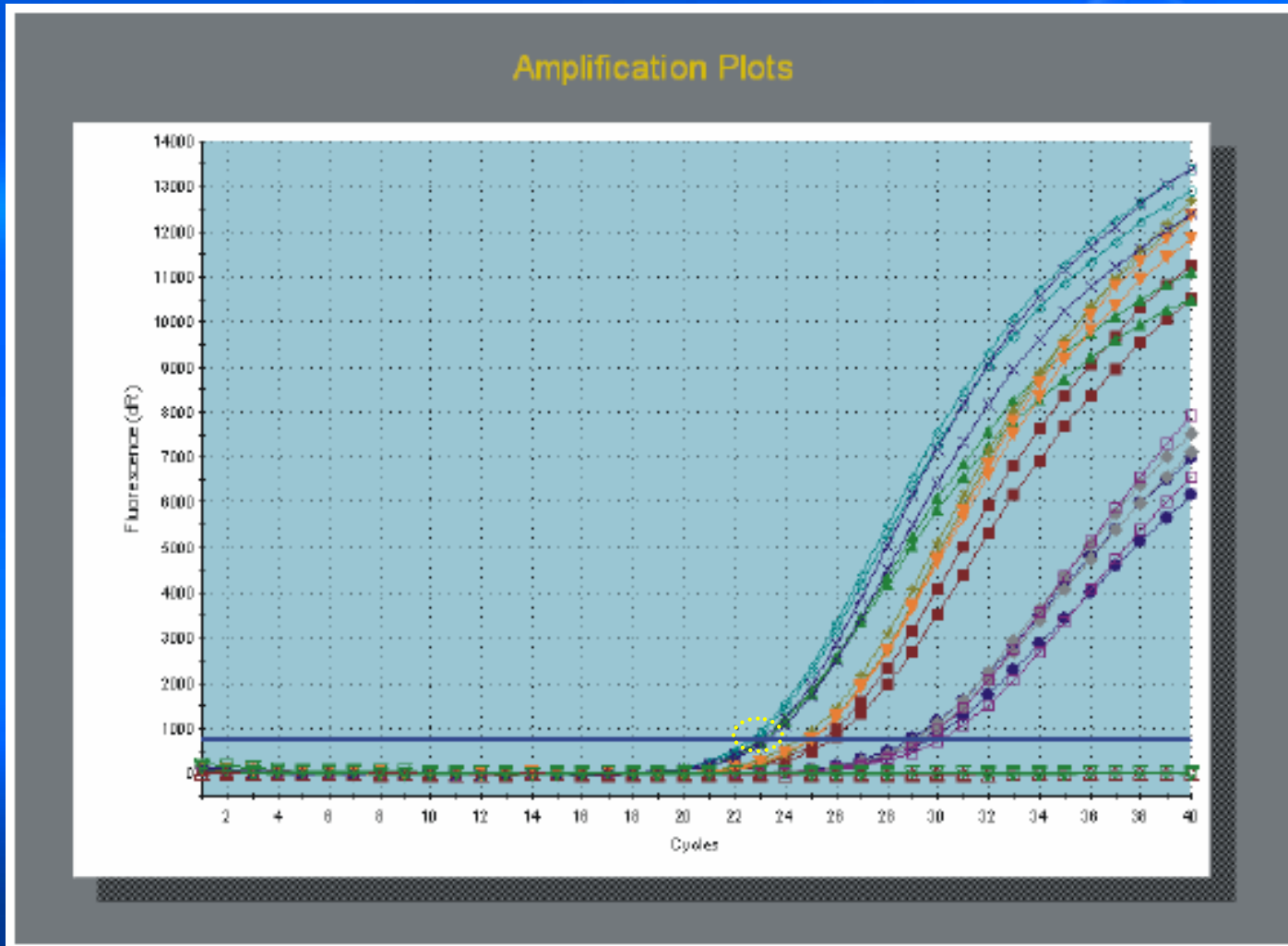
Primer Titration Assays

Probe based assay primer titration

- for 3-4 target multiplex may want to expand the matrix to include additional concentrations
- for multiplex, select combination generates best data at lowest overall primer concentration

	100nM	300nM	600nM	900nM
100nM				
300nM				
600nM				
900nM				

100nM – 900nM Primer Titration



300nM forward and 900nM reverse chosen, several primer combinations yielded similar Ct values. Overall Ct range form ~22.5 to 25.

Multiplex Assay – Reagent Optimization

- Multiplex assays (particularly 3-4 target) require increasing reagent concentration
- Use Stratagene's Brilliant Multiplex QPCR Master Mix
- General guidelines for adjusting reagents
 - Increase Taq concentration (50-100%)
 - Increase dNTP concentration (50-100%)
 - Increase Mg⁺⁺ concentration (0.25-0.50 mM)
 - In some cases, increase buffer concentration to 1.5x

Fluorogenic Probe Titration

	100nM	200nM	300nM	400nM
Optimal [primer]				

Dual-labeled probe titration

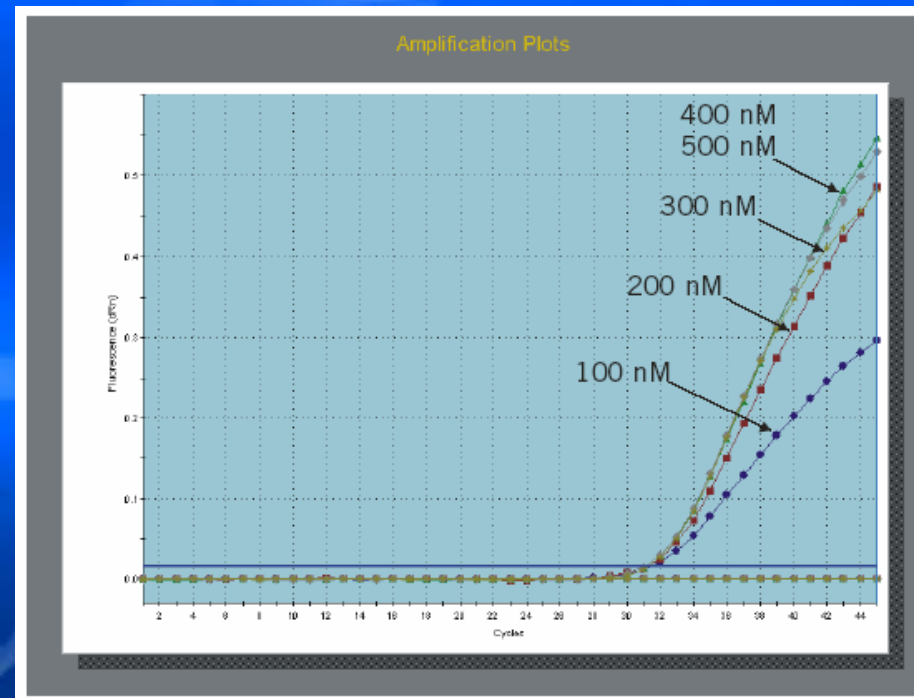
usually not necessary unless multiplexing, typically more important for 3-target multiplex

single target use 100nM

two target use 100 or 200nM (slightly higher [probe] if late Ct)

3-4 target run probe titration

Look for lower Ct value, minimal triplicate variability, and higher overall fluorescence



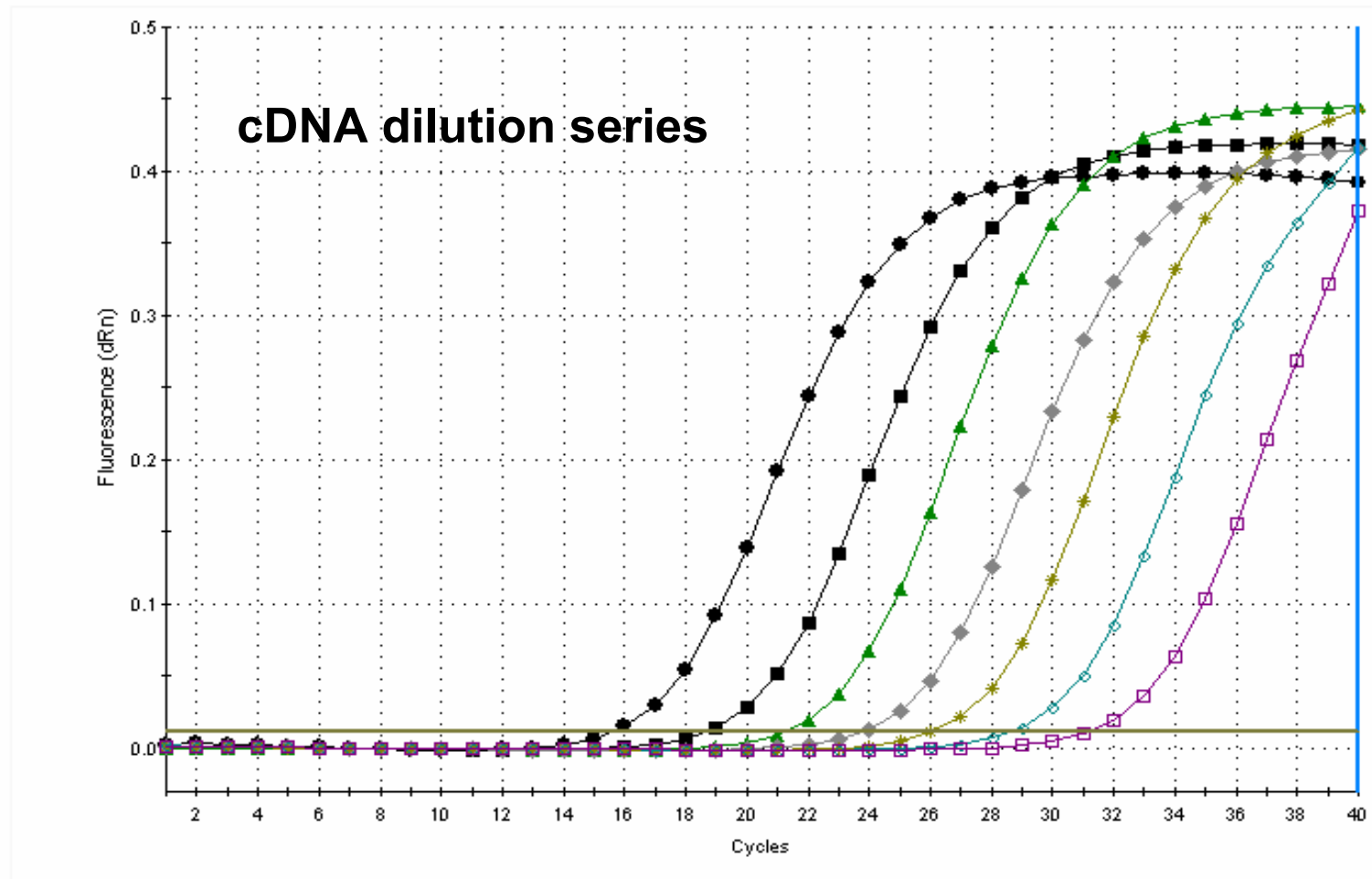
In this example 200-500nM probe all generate approximately the same data.

Standard Curve

- Synthetic oligonucleotide
- T7 transcribed RNA template
- Linearised plasmid
- QPCR Human reference RNA
 - Requires target genes to be present
 - (used in this case study)

Dilution series (5 fold) of cDNA from RT of 2.5ng RNA (β -actin)

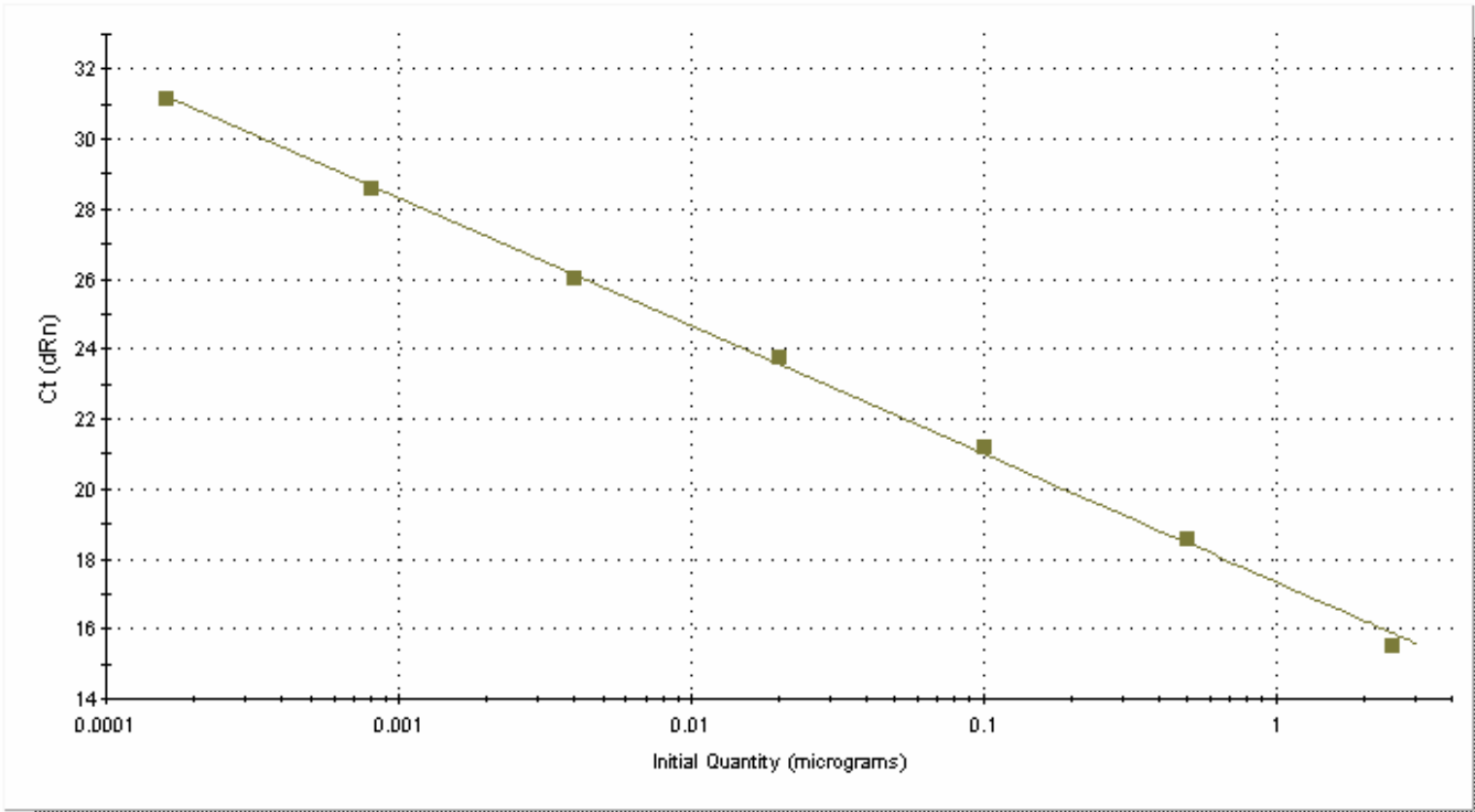
Amplification Plots



Standard Curve

Log fit values

- SYBR Standards, RSq:0.999
- SYBR, $Y = -3.665 \cdot \text{LOG}(X) + 17.34$, Eff. = 87.4%



Normalisation

RT normalisation

- Total RNA input (used for case study) easiest approach (but mRNA may vary independently from total RNA)

If RNA cannot be measured:

- Single normaliser gene – multiplex gives best data, low confidence
- Multiple normaliser genes – multiplex gives best data, high confidence

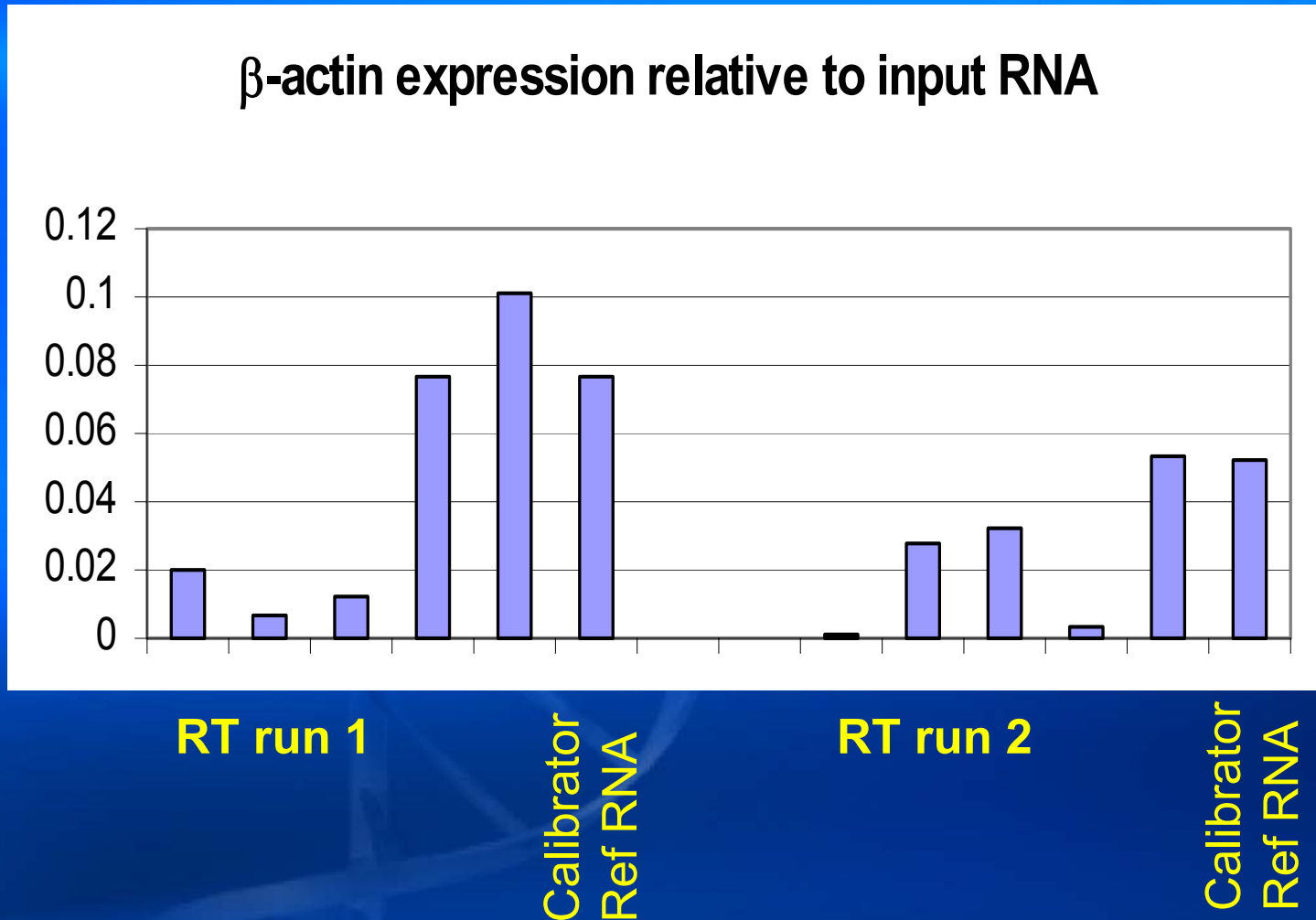
Alternative normalisation

- Ribosomal RNA (Independent of mRNA variation, requires Random priming)
- Tissue area (RNA extractions will vary)
- Cell number (RNA extractions will vary)

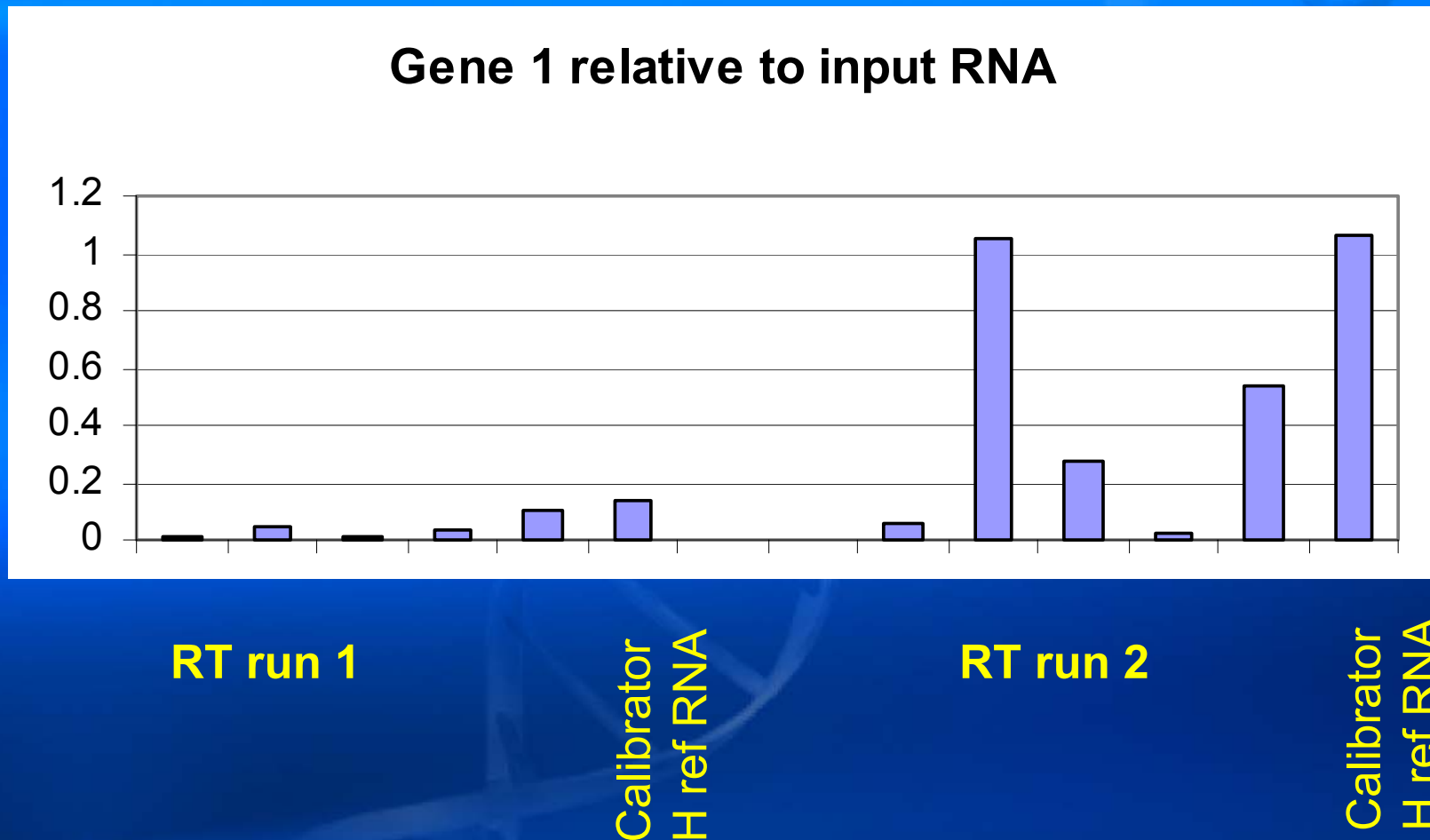
Case Study - Results

- 5 samples (per RT run) + calibrator sample (hu-ref RNA) were quantified relative to ref RNA standard curve
- Quantities expressed relative to (constant) input RNA quantity

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



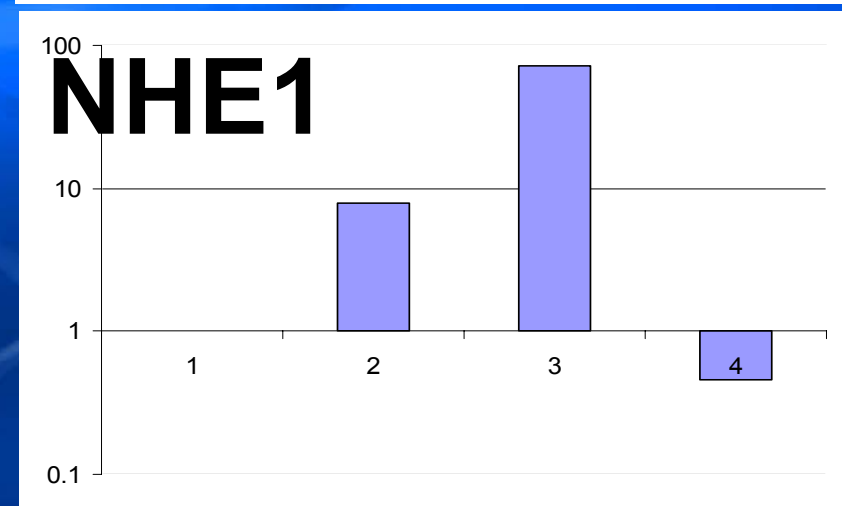
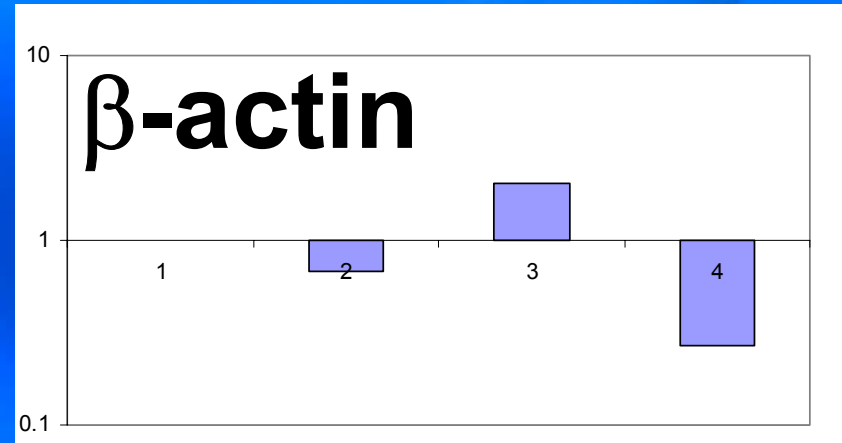
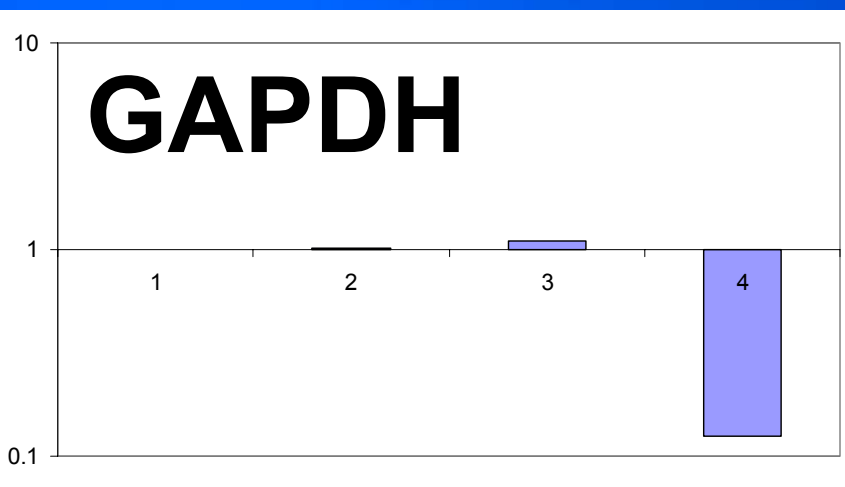
Independent reverse transcription reactions relative to input RNA (Low expressed gene: NHE1)



RT Run-to-Run Variation

- Differences in gene quantities in calibrator samples revealed batch to batch RT variations
- Greater deviations are noted when genes are expressed at lower level

Gene quantification in four independent but identical RT reactions



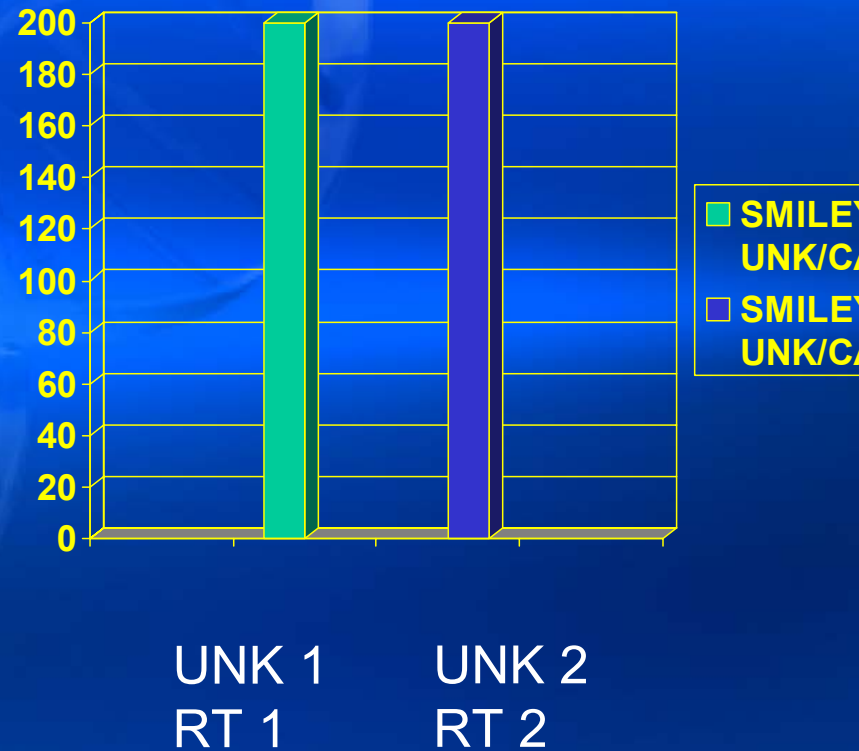
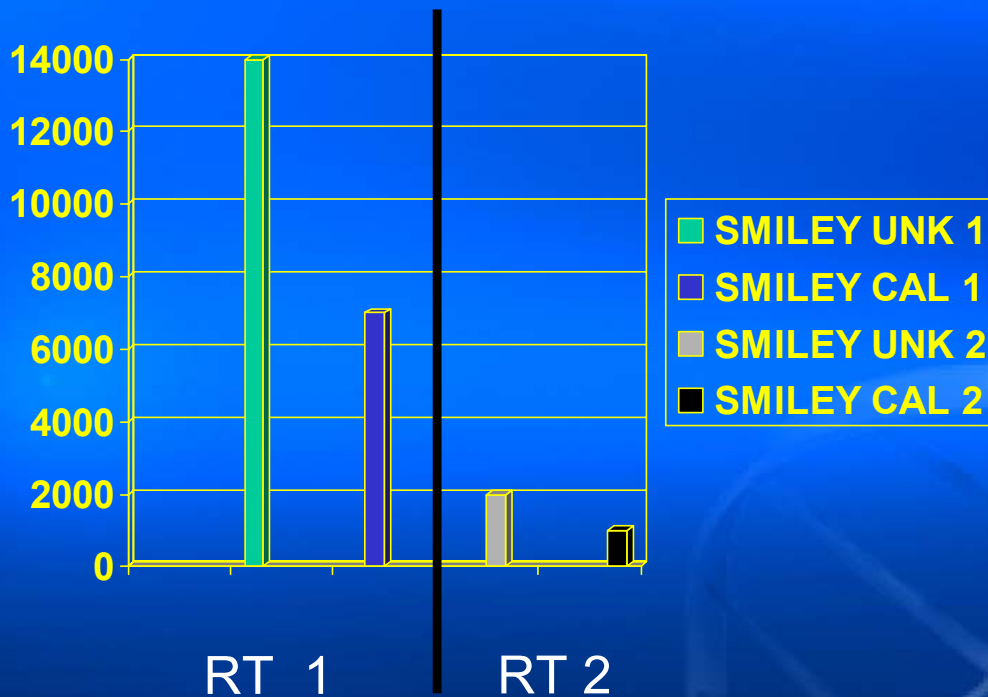
Normalising batch to batch variations

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

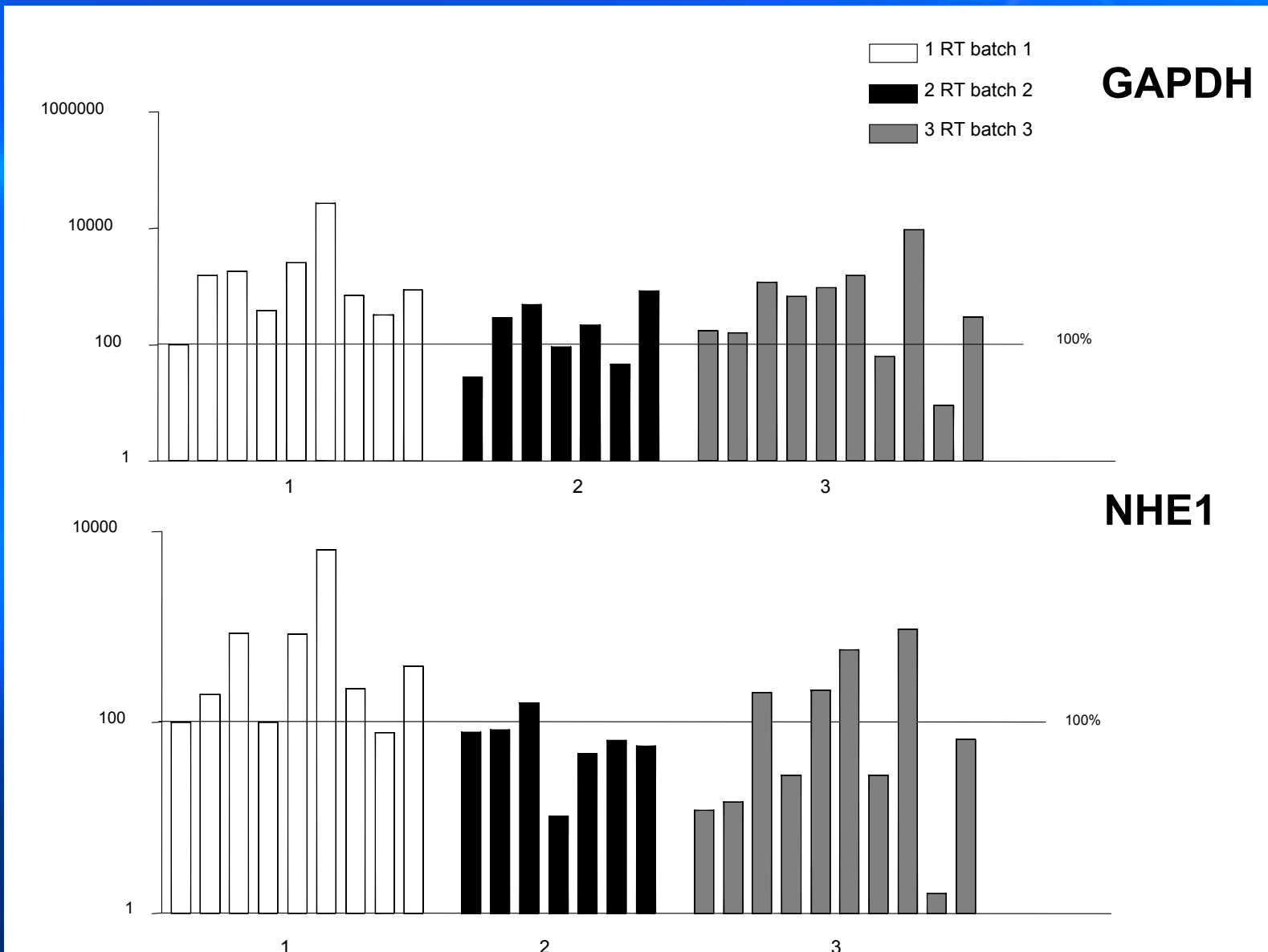
Normalising batch to batch variations

- SMILEY gene in calibrator batch 1 = 7000 measured against Standard curve
 - SMILEY 7000 = 100% (batch 1)
 - Batch 1 unknown sample is 14,000
 - Batch 1 unknown sample is 200%
-
- SMILEY gene in calibrator batch 2 = 2,000 measured against Standard curve
 - SMILEY 2000 = 100% (batch 2)
 - Batch 2 unknown sample is 4000
 - Batch 1 unknown sample is 200%

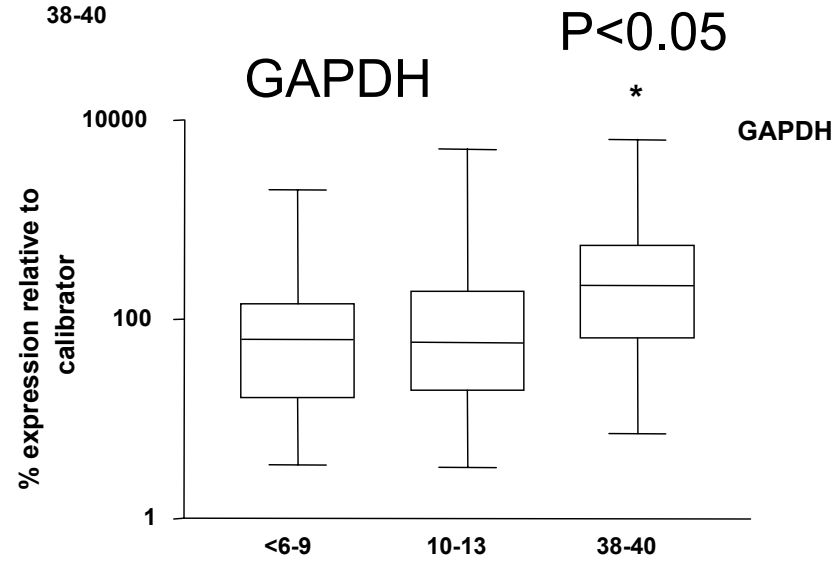
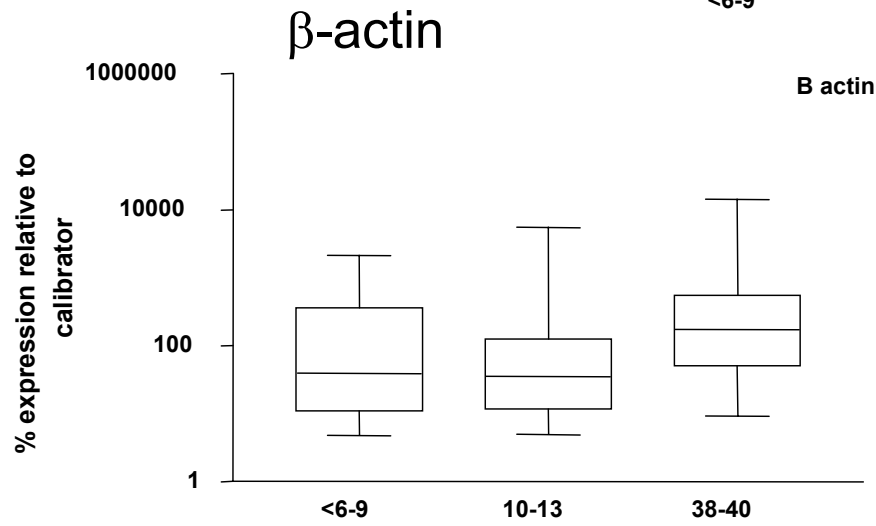
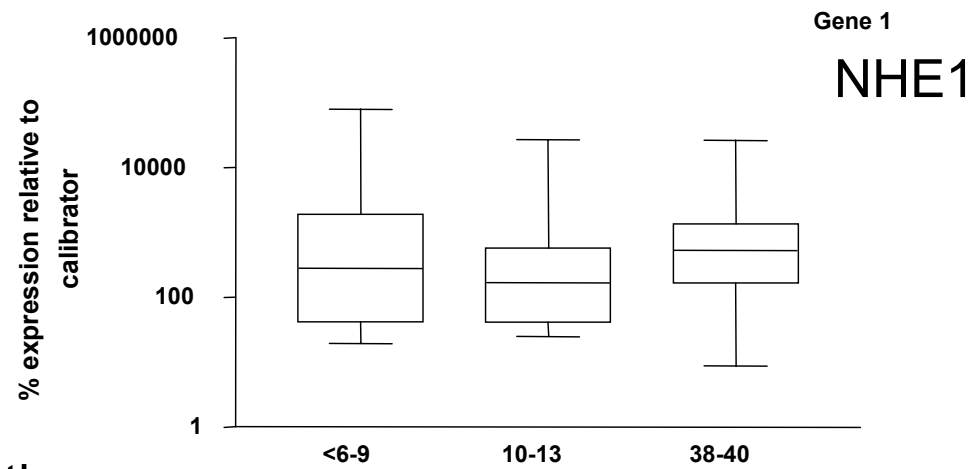
Normalising batch to batch variations



Independent reverse transcription reactions normalised to hu-ref RNA



Expression relative to calibrator to investigate transcript variation through gestation



Summary protocol

- Quantify RNA (if possible)
- Include a constant RNA amount into each RT
(2.5ng with Stratascript)
- Include a calibrator (constant) sample with each RT batch
- Measure transcript quantity for samples and calibrator relative to a DNA standard curve
- Normalise sample transcript quantity relative to calibrator quantity for each RT batch

Many Thanks to:

- Helen Lacey and Colin Sibley, St Mary's Hospital, Manchester, UK
- Dr William Ogunkolade and Stephen Bustin, Royal London Hospital, London, UK
- Gothami Padmabandu, Stratagene R & D, La Jolla, US