The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

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www.sial.com/designmyprobe

Here It Begins

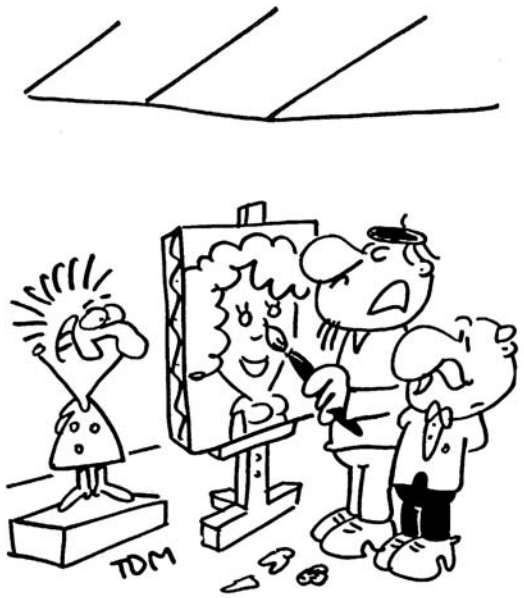
Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

Why Do We Need MIQE?



"I paint what I see . . . in this case a commission worth eight hundred guineas."

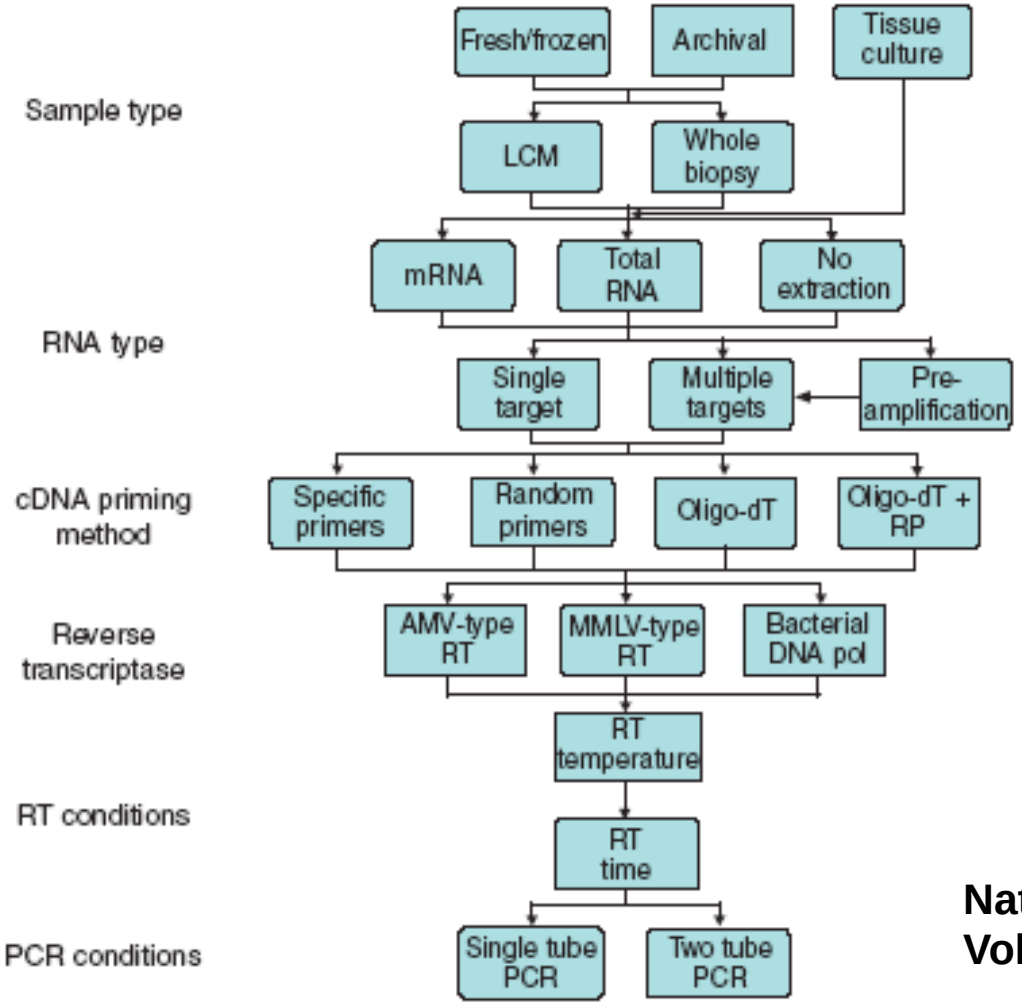
Help scientists to design and report valuable qPCR experiments

Allow publishers to understand the way experiments were conducted

Allow other scientists to repeat the same experiment

Work Flow



Nature Protocols
Vol.1 NO.3/ 2006/ 1559

Proposing MIQE:

- Primer/probe/amplicon sequence information
- Sample quantity and quality
- Detailed reverse transcription conditions
- Detailed PCR conditions (including controls)
- Efficiency of PCR and error
- Methods of analysis

Guidelines are being adopted by major journal groups and adherence will be required for publication

A MIQE Based Workflow

Assay design

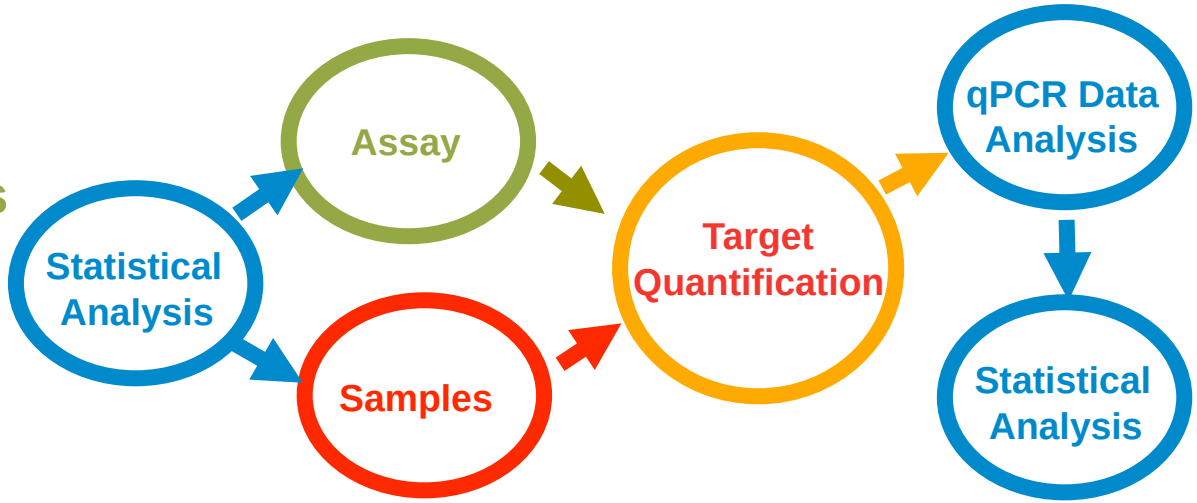
Reaction components

Optimization

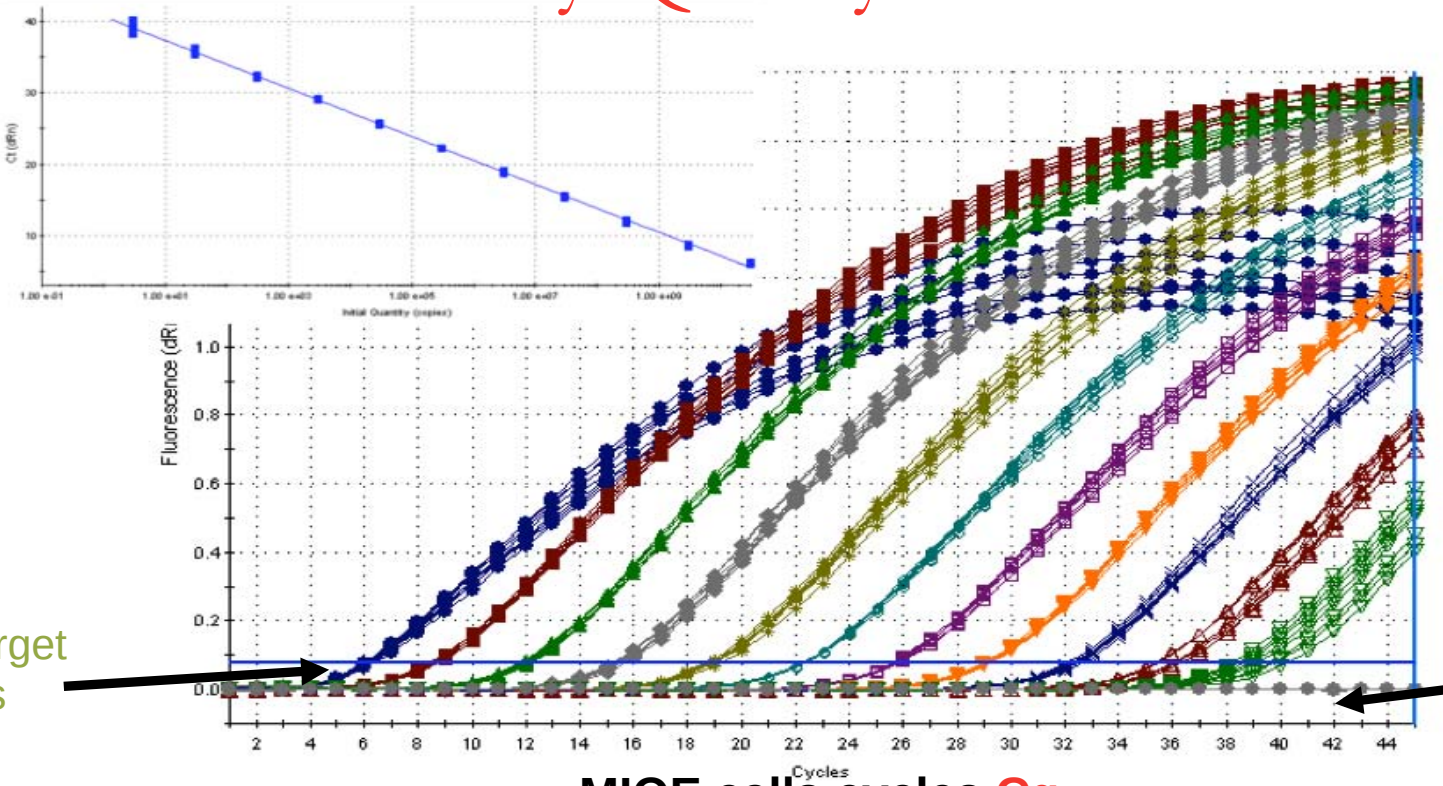
Sample QC

Sample QC and reaction components

Methods of analysis



Determine Assay Quality



$3x10^{10}$ target copies

3 target copies

MIQE calls cycles Cq

The serial dilution provides a measure of:

- Efficiency and reproducibility
- Theoretical sensitivity
- Practical dynamic range

y=mx+c

- Slope = -3.323 (between -3.5 and -3.2)
- RSqu > 0.98
- Intercept on y gives a theoretical measure of sensitivity

Assay Design

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).

Published online 9 November 2006; doi:10.1038/nprot.2006.236

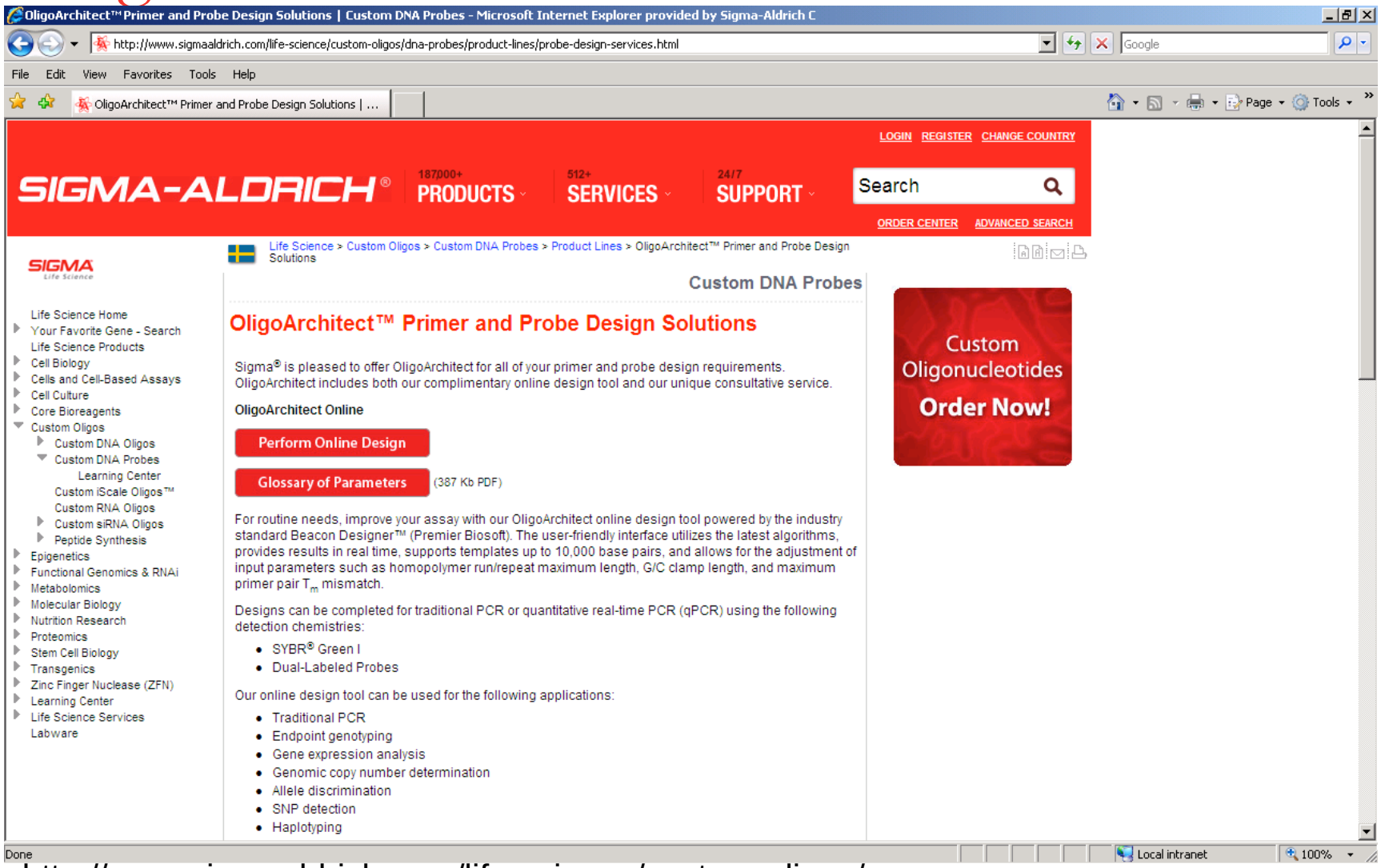
Nature Protocols

- Follow usual guidance for design of PCR primers and also consider:
  - Keep amplicon length to <150bases
- Constraints on region of target sequences:
  - SNPs / splice variants / intron exon boundaries
- Folding of target regions
- Homology to similar sequences:
  - Same species and other species
- Avoid repetitive sequences
- Avoid 3' clamping in primers
- Aim for primer Tm 60ºC and probes 67 ºC -70 ºC

Tools for Design

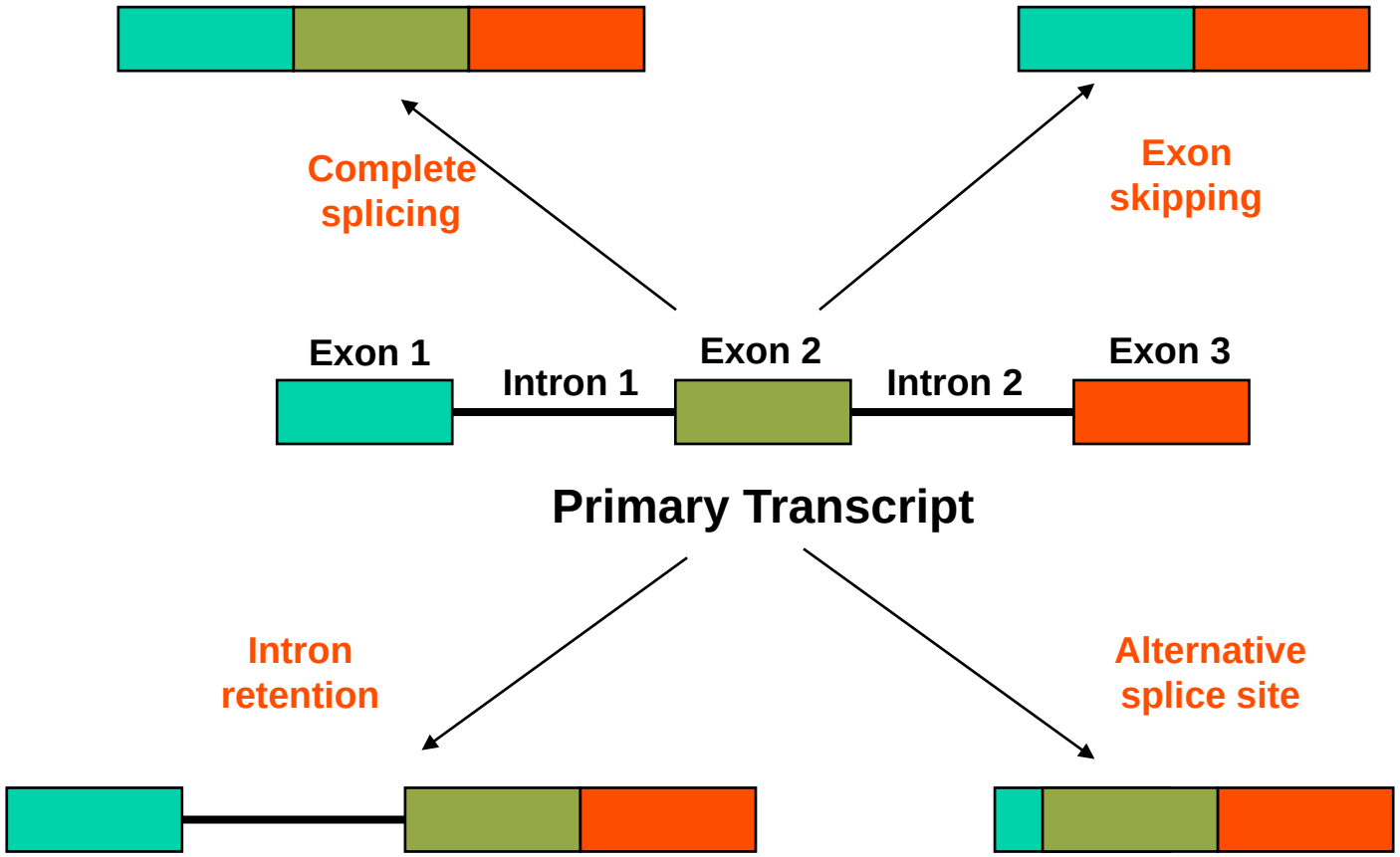
- OligoArchitect
  http://www.sigmaaldrich.com/life-science/custom-oligos/dna-probes/product-lines/probe-design-services.html
- Beacon Designer
- NCBI GenBank http://www.ncbi.nlm.nih.gov/nuccore
- Primer3 http://frodo.wi.mit.edu/primer3/
- RTPrimerDB http://medgen.ugent.be/rtprimerdb/
- PrimerBank http://pga.mgh.harvard.edu/primerbank/
- NCBI Primer Design Tool http://www.ncbi.nlm.nih.gov/tools/primer-blast/
- Mfold webserver http://mfold.rna.albany.edu/
- Other

OligoArchitect



http://www.sigmaaldrich.com/life-science/custom-oligos/dna-probes/product-lines/probe-design-services.html

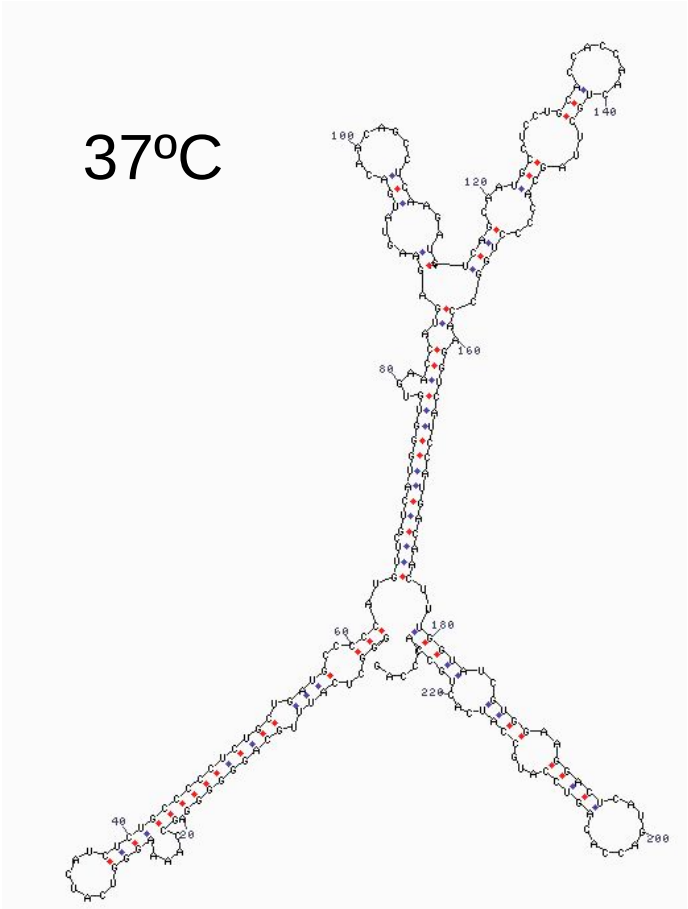
Consider Splice Variants



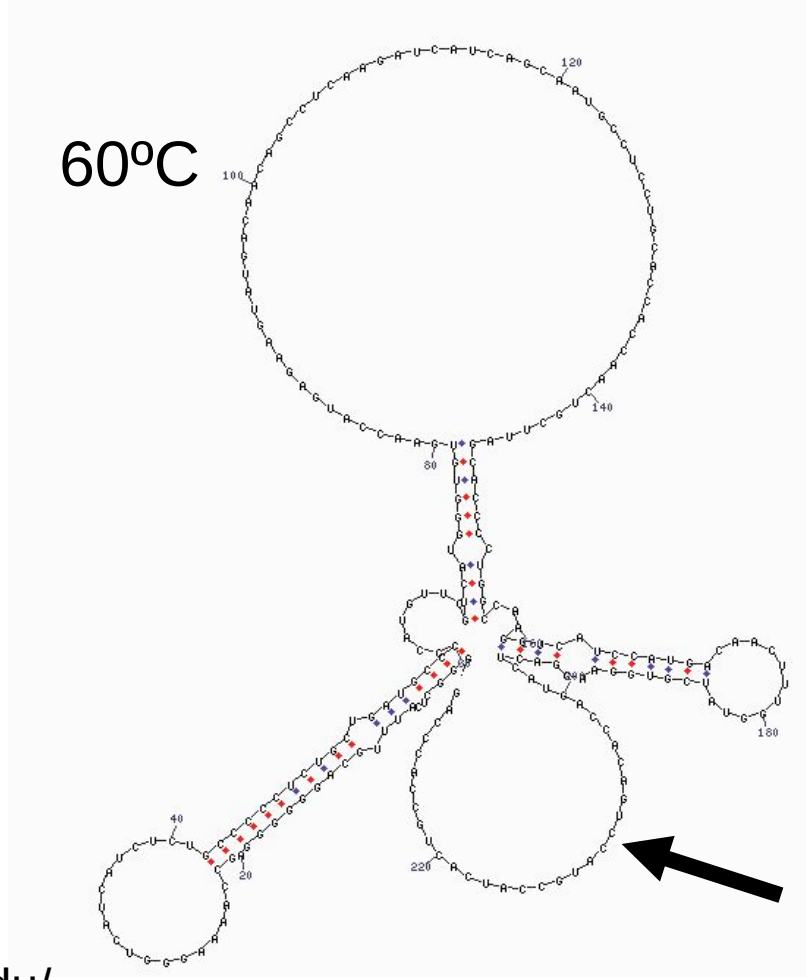
Folding of Target Region

GAPDH 5’

37ºC

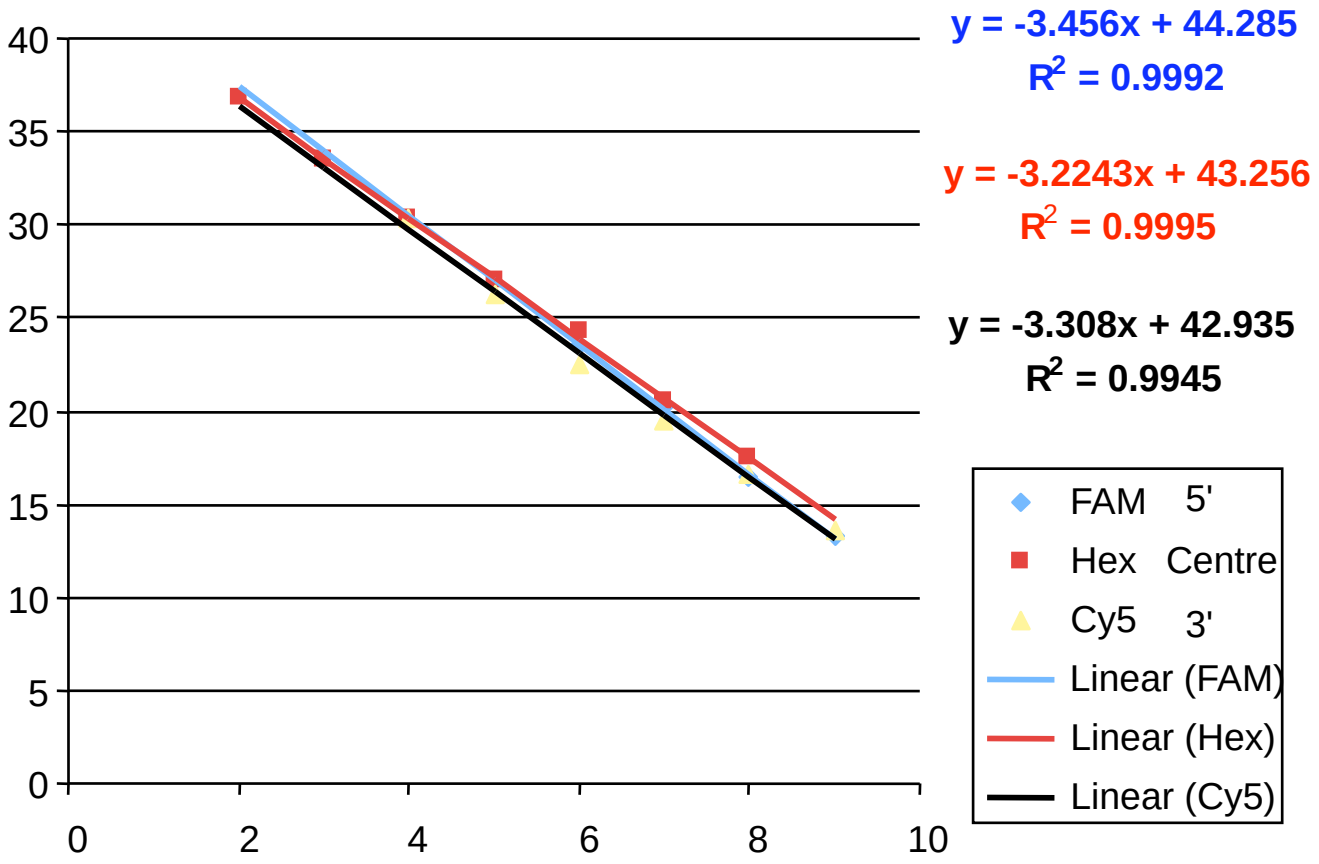


60ºC



MFOLD - http://mfold.bioinfo.rpi.edu/

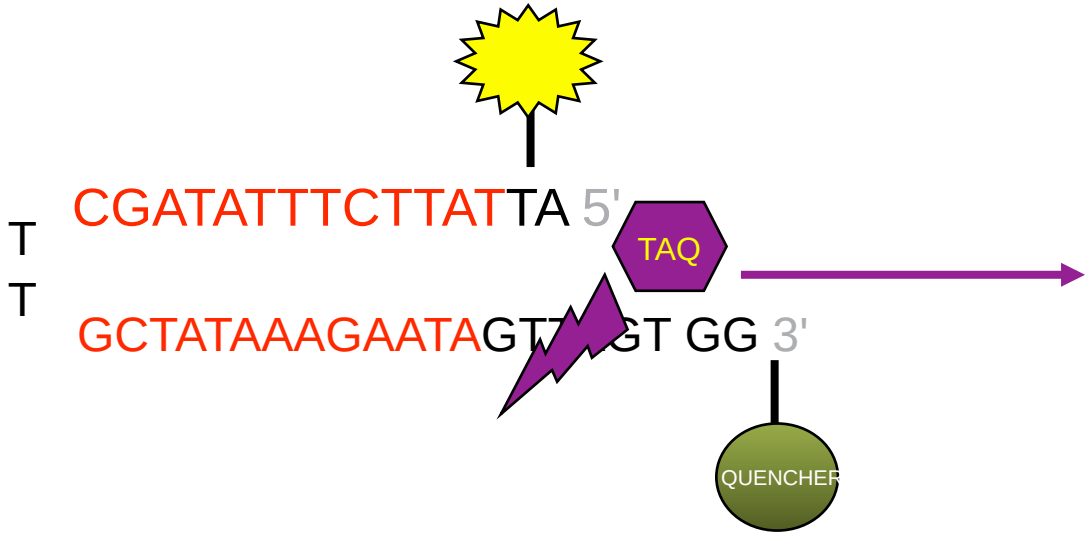
Quality assessment of 3 assays to the same transcript

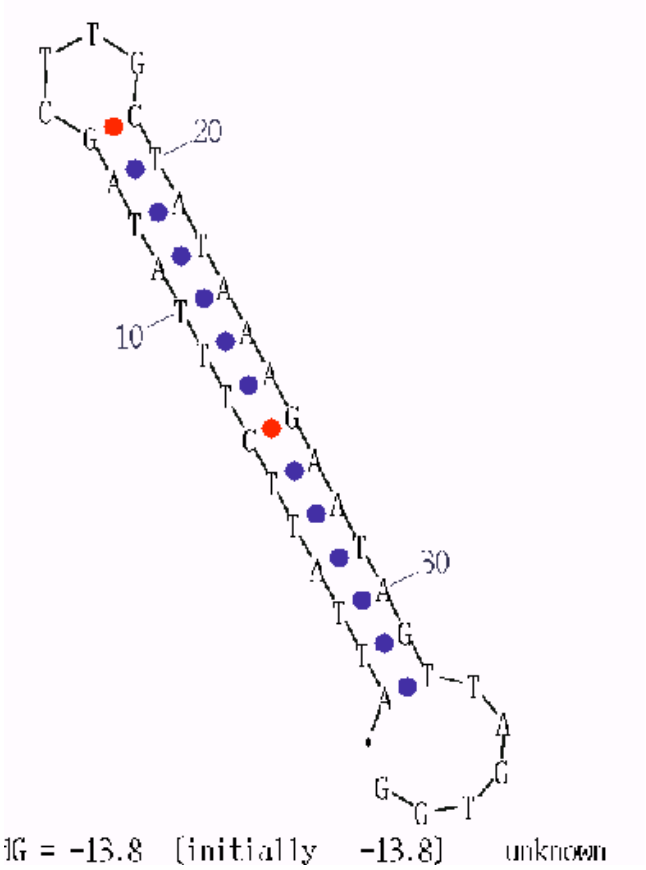


Total RNA target GAPDH specific primed dilution series

Influence of Folding on Assay Quality

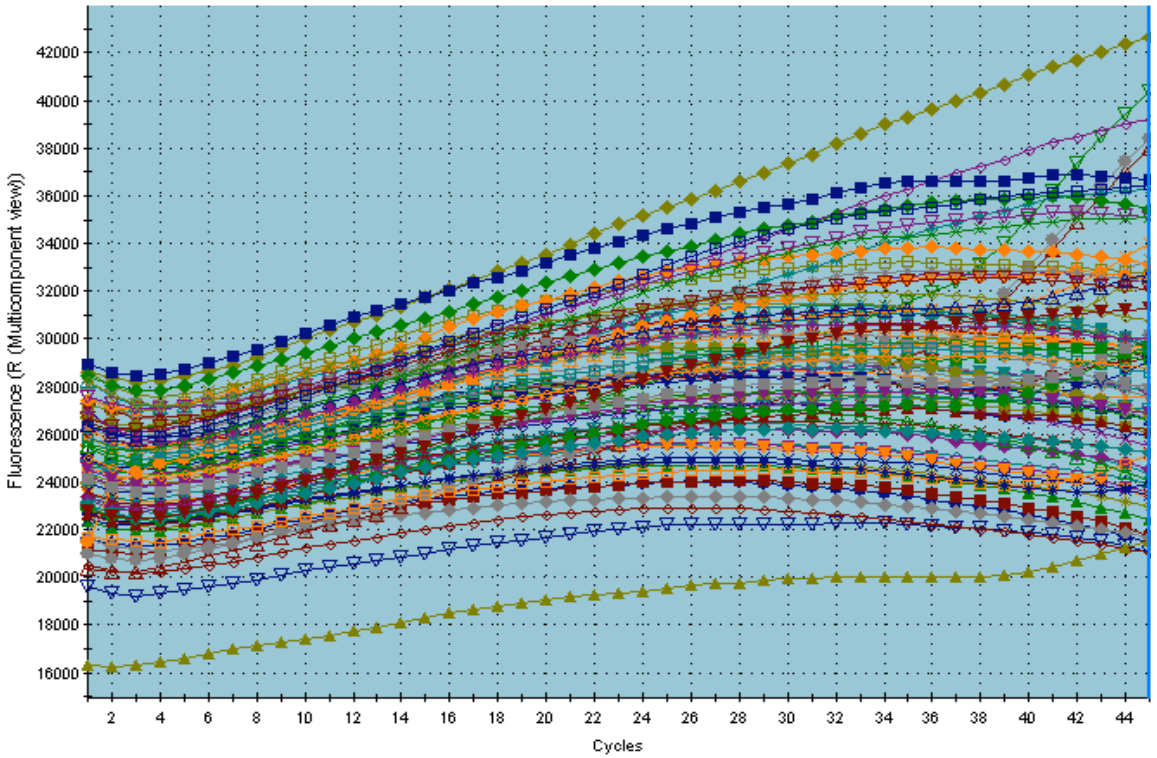
5' (Texas red) ATTATTCTTTATAGCTTGCTATAAAGAATAGTTAGT GG (BHQ2) 3'



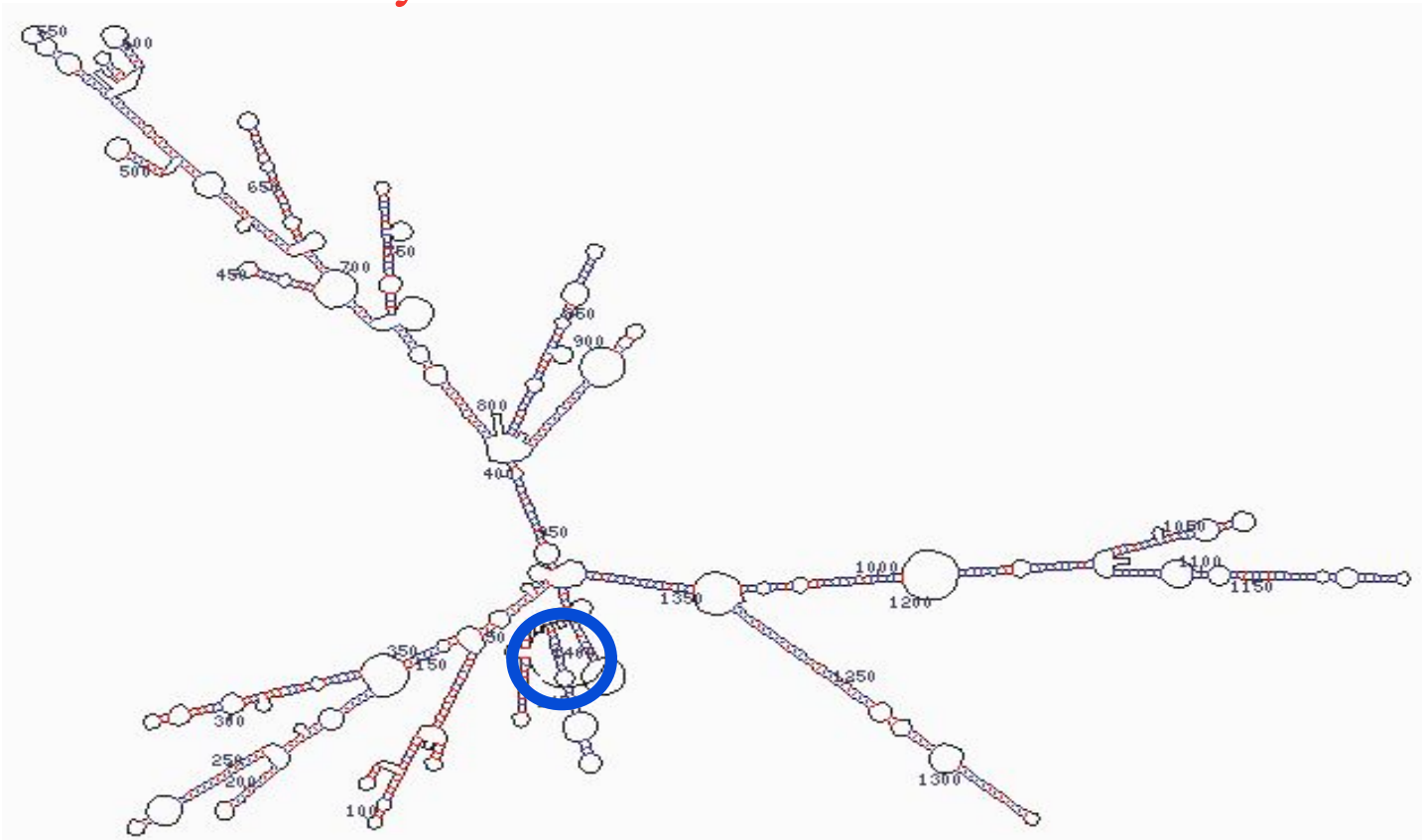


Probe Folding Causes Assay Failure

Small increase in fluorescence as some of the probes are cleaved – independent of target



A Predesigned - Ready to Run Commercially Available Assay



dG = -400.05 (initially –424.73)

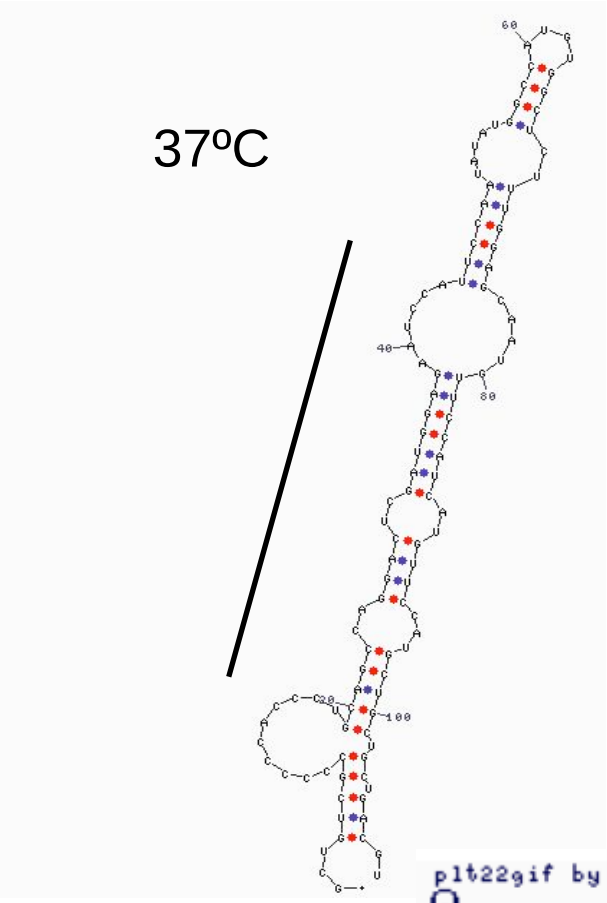
IL-15

Assay on demand – unknown sequences

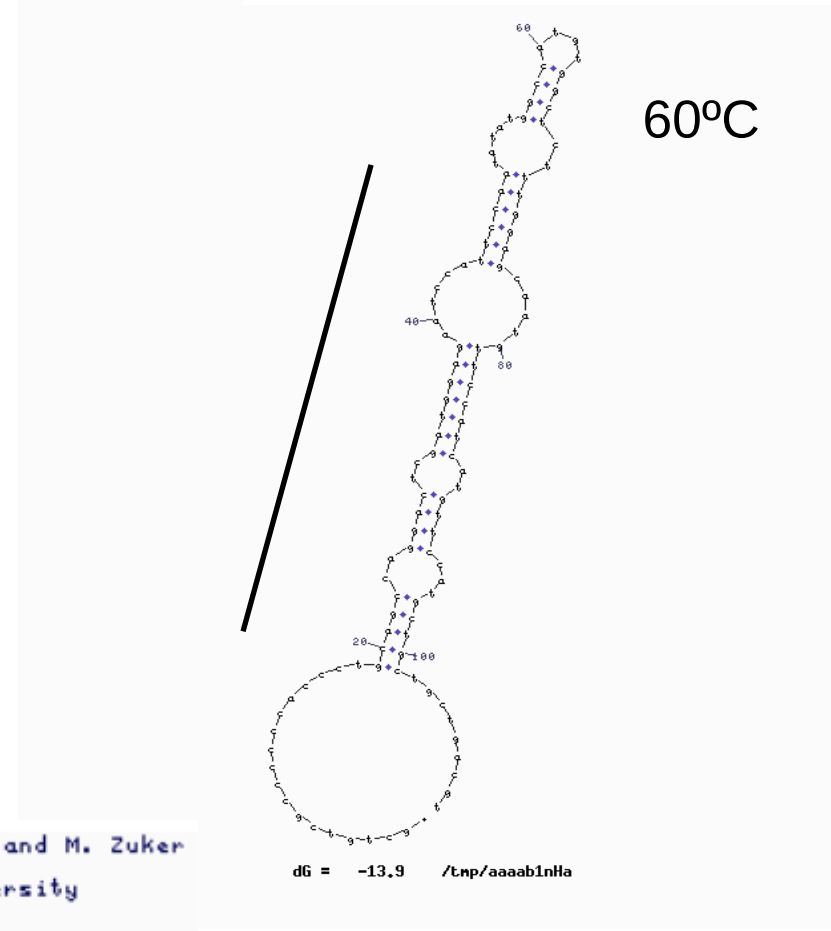
Analysis of Template Folding – IL-15

IL-15: Folding analysis

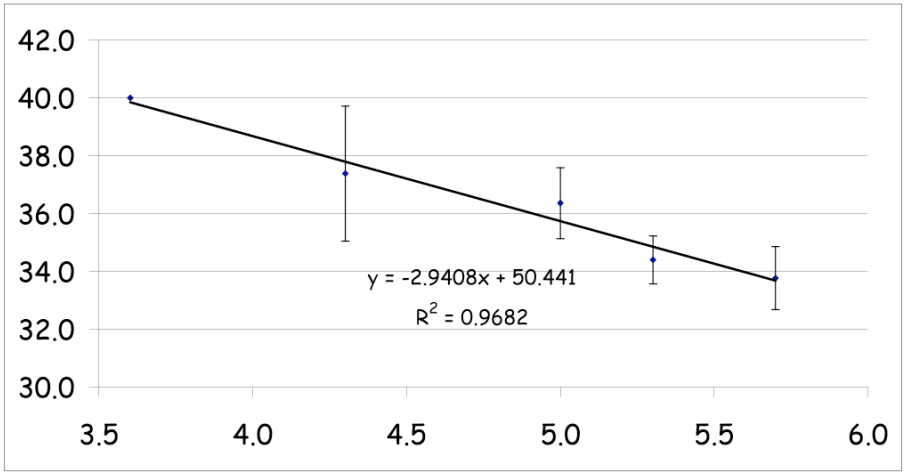
37ºC



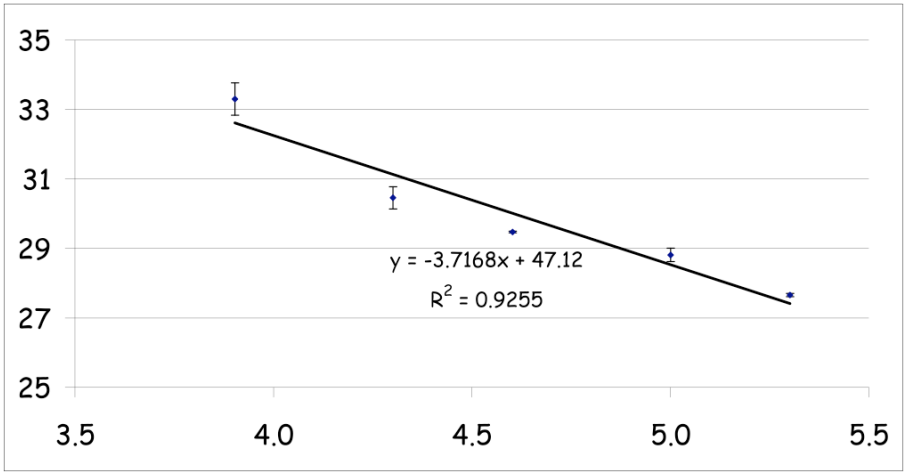
60ºC



Folded Template Causes Commercially Available Assay to Fail in RTqPCR



One tube assay
Gene specific reverse transcription priming



Two tube assay
Random primed reverse transcription

Assay on demand – unknown sequences

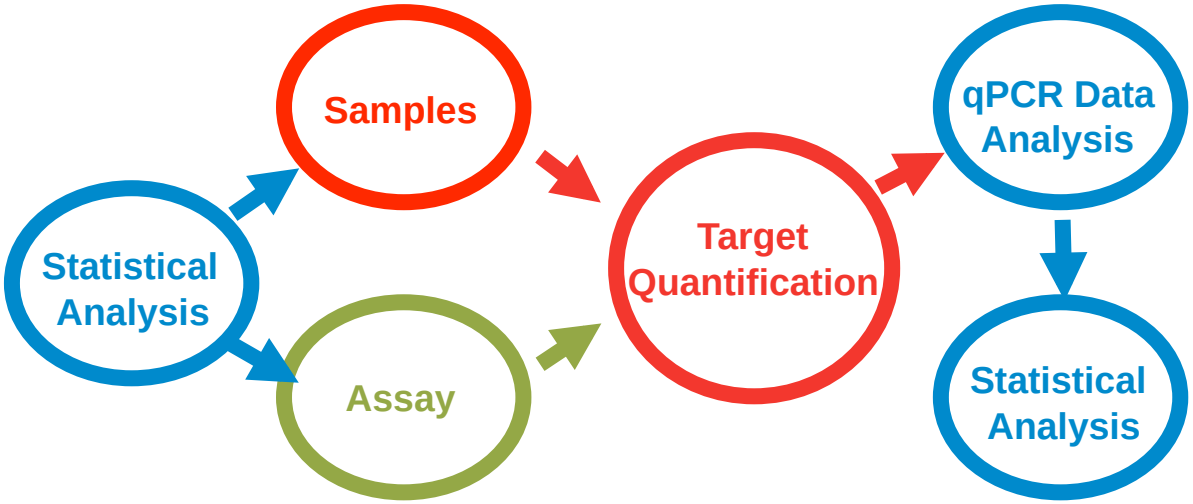
A MIQE Based Workflow

Assay design

Reaction components

Optimization

Sample QC



Sample QC and reaction components

Methods of analysis

Primer Concentration Optimisation

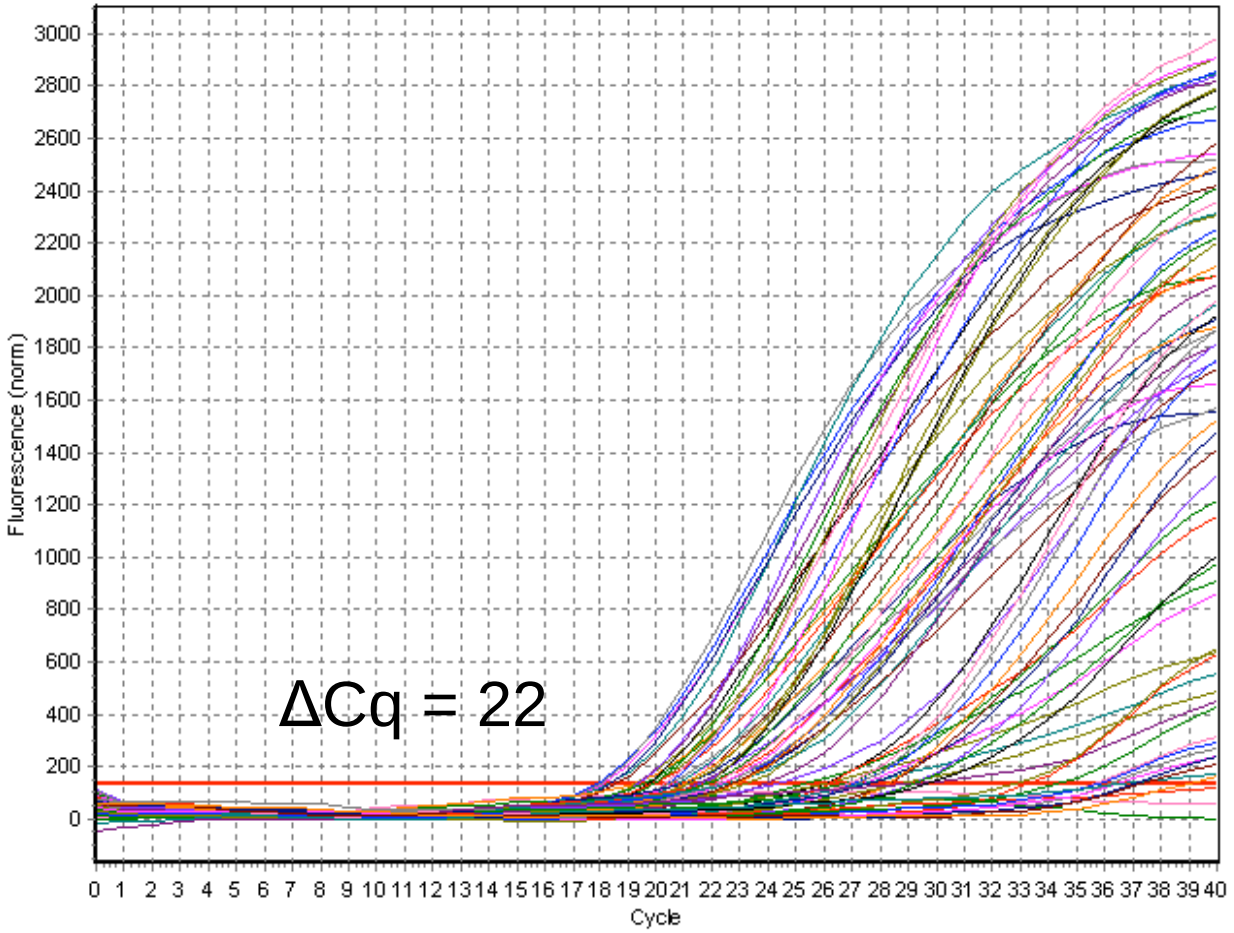
Primer matrix

- 100-600 nM (start with 300 nM) for probes
- 50-300 nM (start with150 nM) for SYBR Green I

Cq Values		Fwd (nM) 100	300	500
Rev (nM)	100			
	300			
	500			

Primer Optimization Matrix:

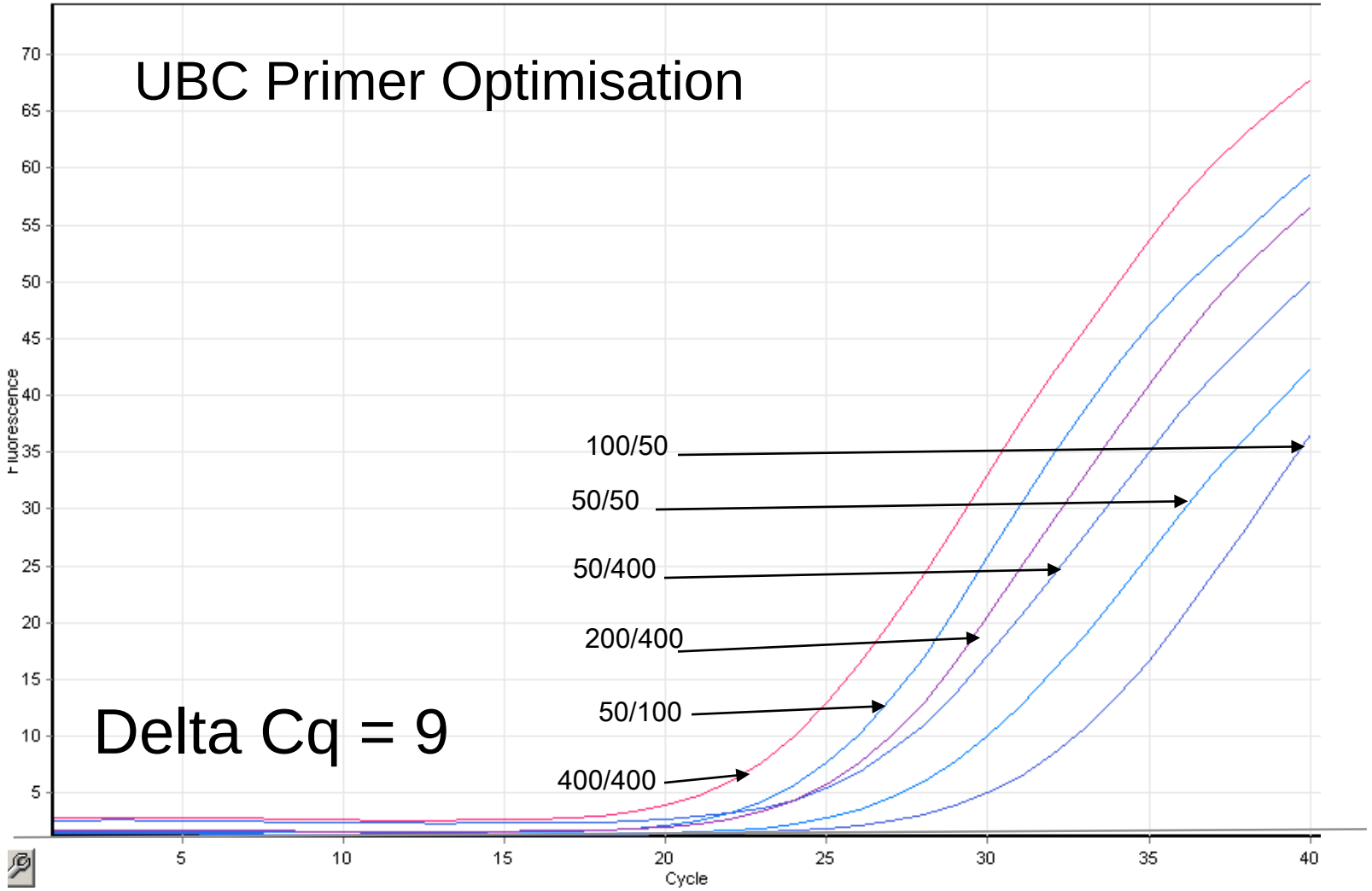
All Primer Concentrations 600 nM to 50 nM – Same Target Concentration



Optimisation:

- Check all primer concentrations with same target concentrations
- Select conditions with lowest Cq and no primer dimers (check with NTC)
- Check with serial dilution of target

Optimisation of a Well Designed Primer Pair



A MIQE Based Workflow

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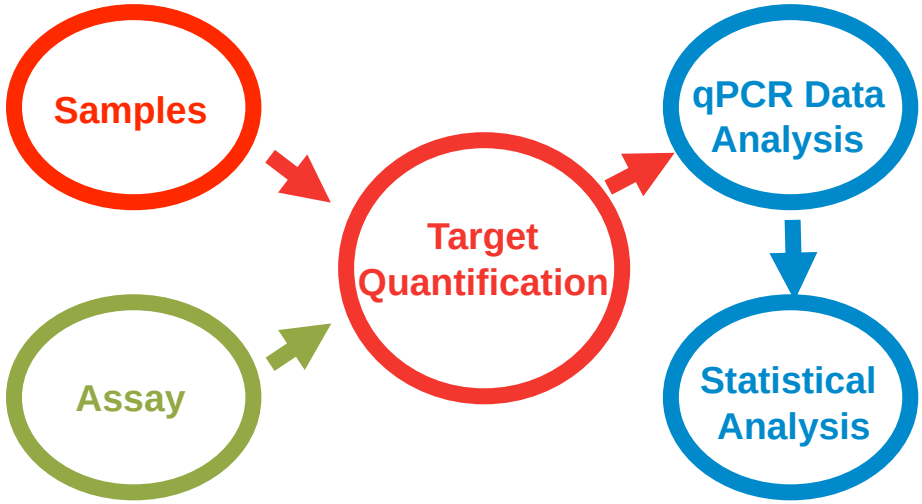
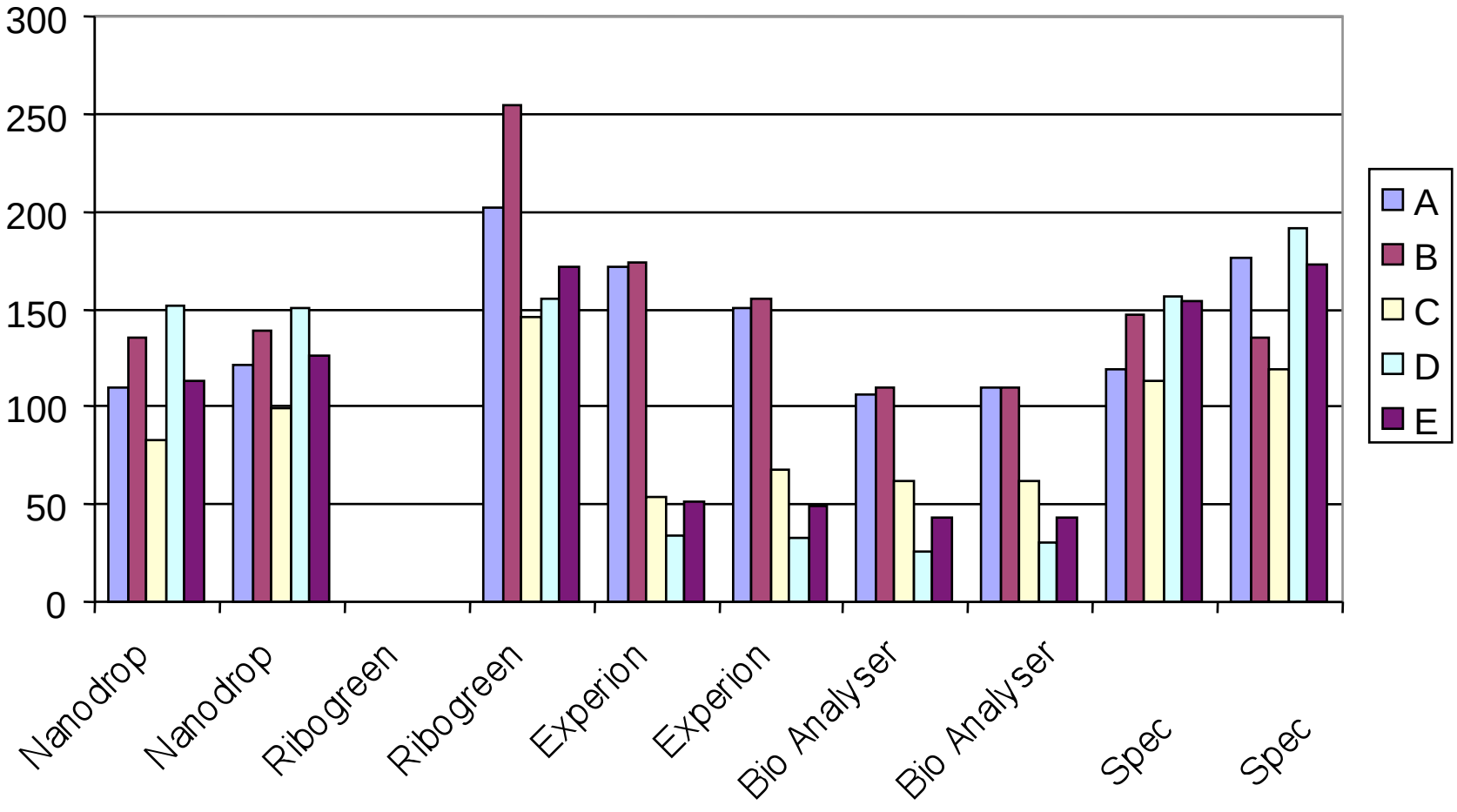


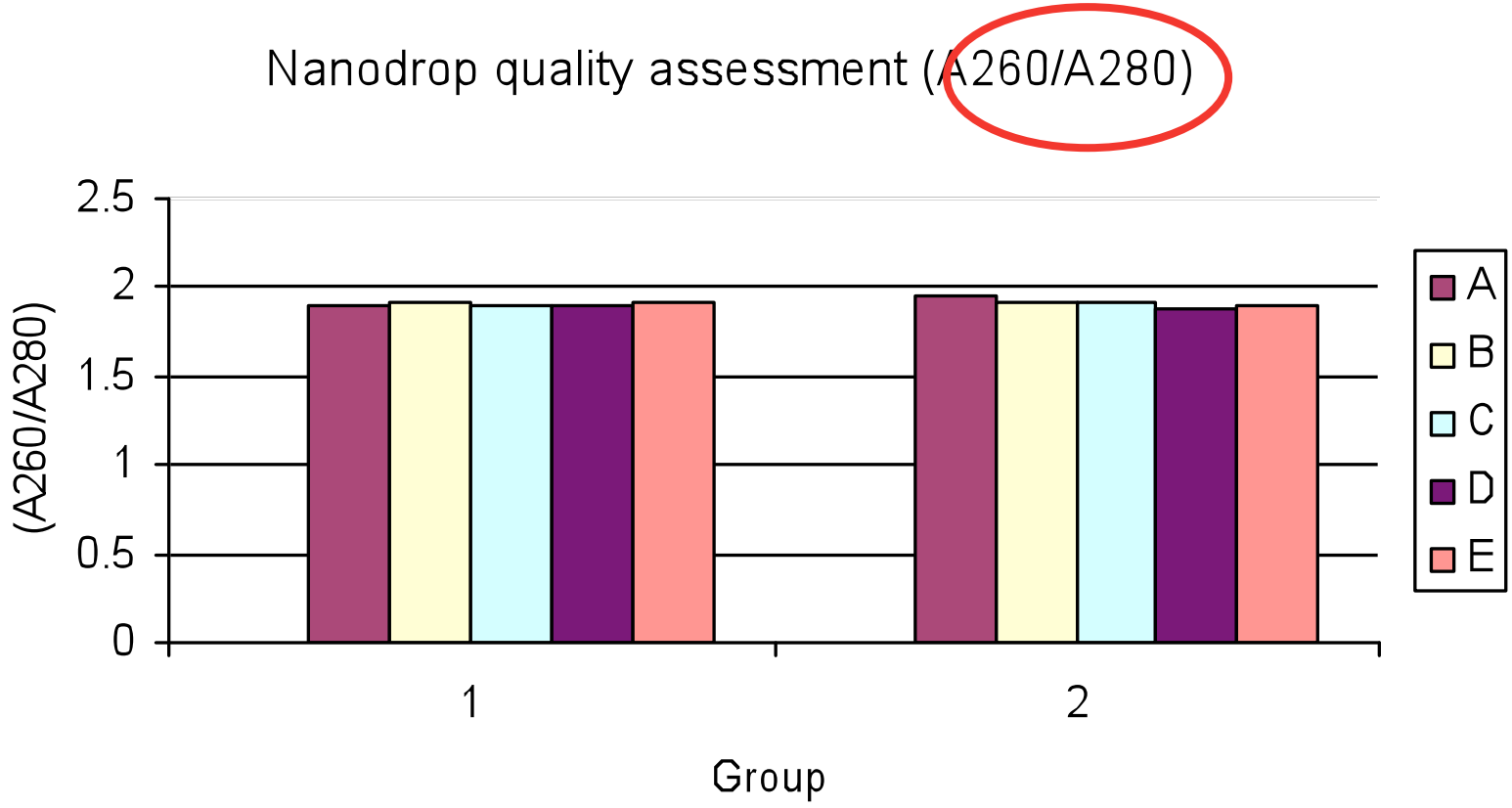
Sample QC and reaction components

Methods of analysis

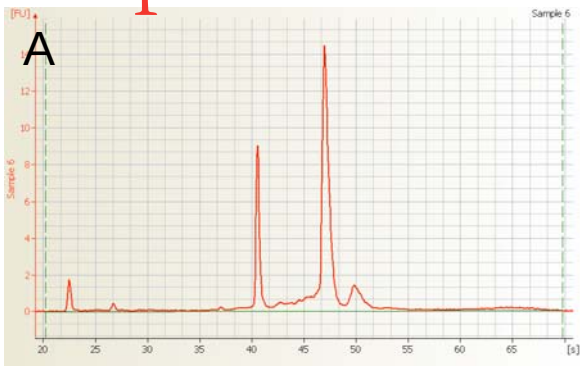
Quantification of Identical RNA Samples



RNA quality control

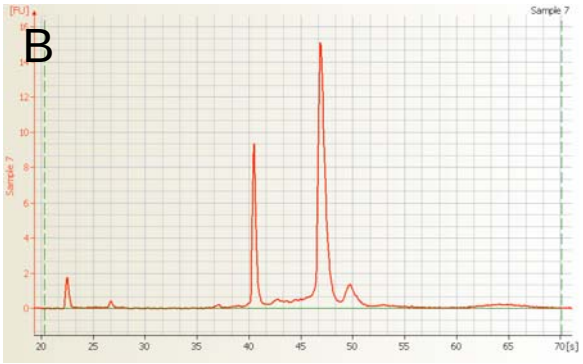


Agilent Bioanalyzer 2100 Analysis of 5 RNA Samples



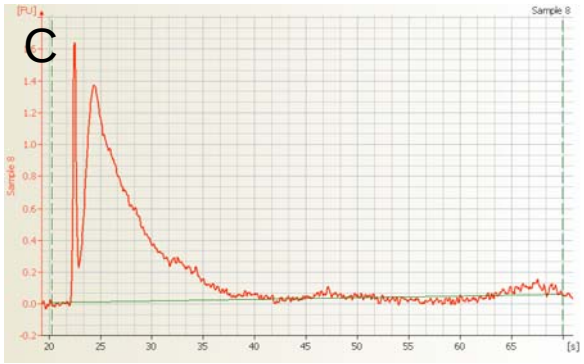
Conc. 110 ng/µl

RIN: 10



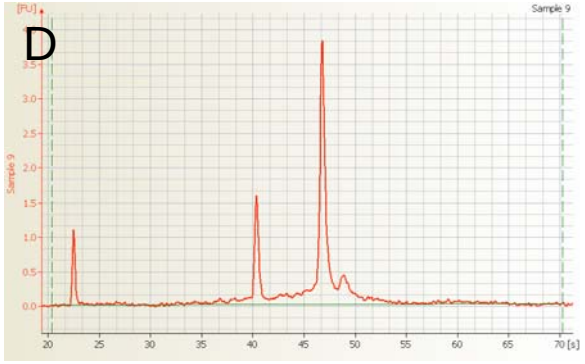
Conc. 110 ng/µl

RIN: 10



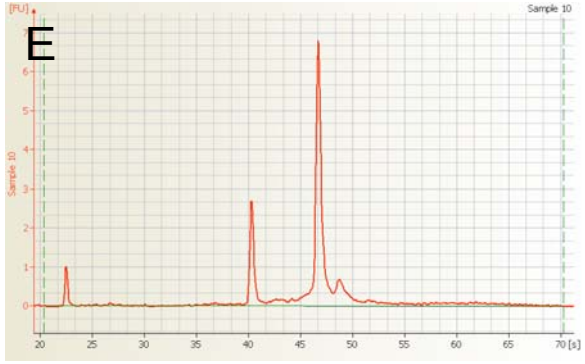
Conc. 62 ng/µl

RIN: 2.4



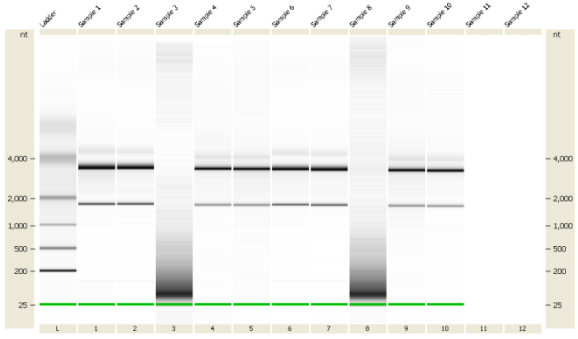
Conc. 30 ng/µl

RIN: 9.1

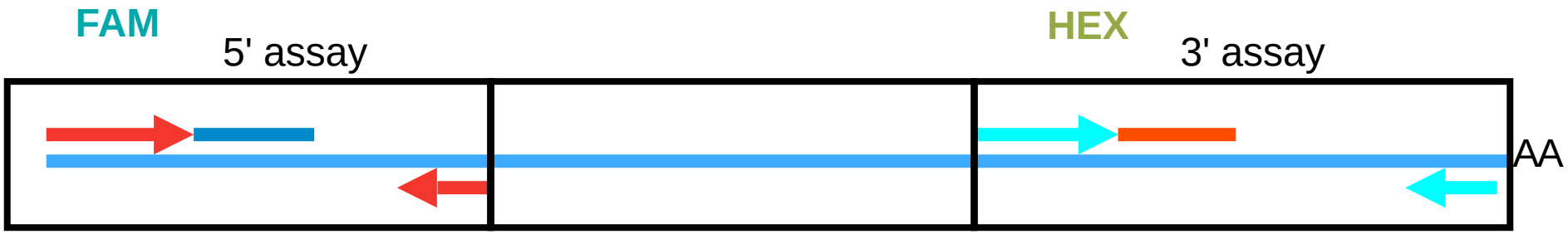


Conc. 43 ng/µl

RIN: 9.5



3'/5' Integrity Assay to Detect Degraded RNA



Perform RT using oligo-dT

If RNA is intact detection of 5‘ and 3' should be equal

If RNA is degraded detection of 3' > 5'

PROTOCOL

Quantification of mRNA using real-time RT-PCR

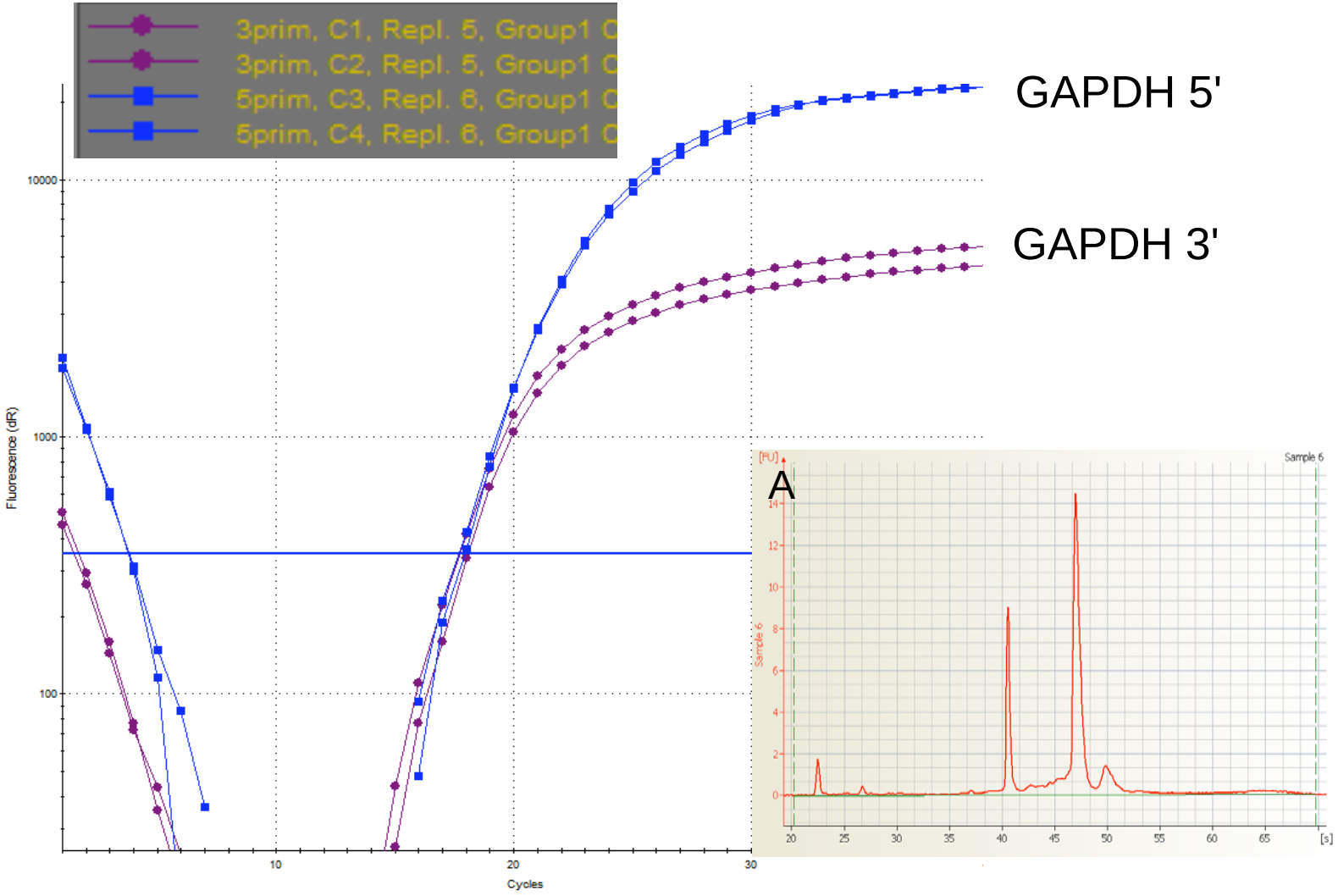
Tania Nolan[1], Rebecca E Hands[2] & Stephen A Bustin[2]

Nature Protocols

[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).

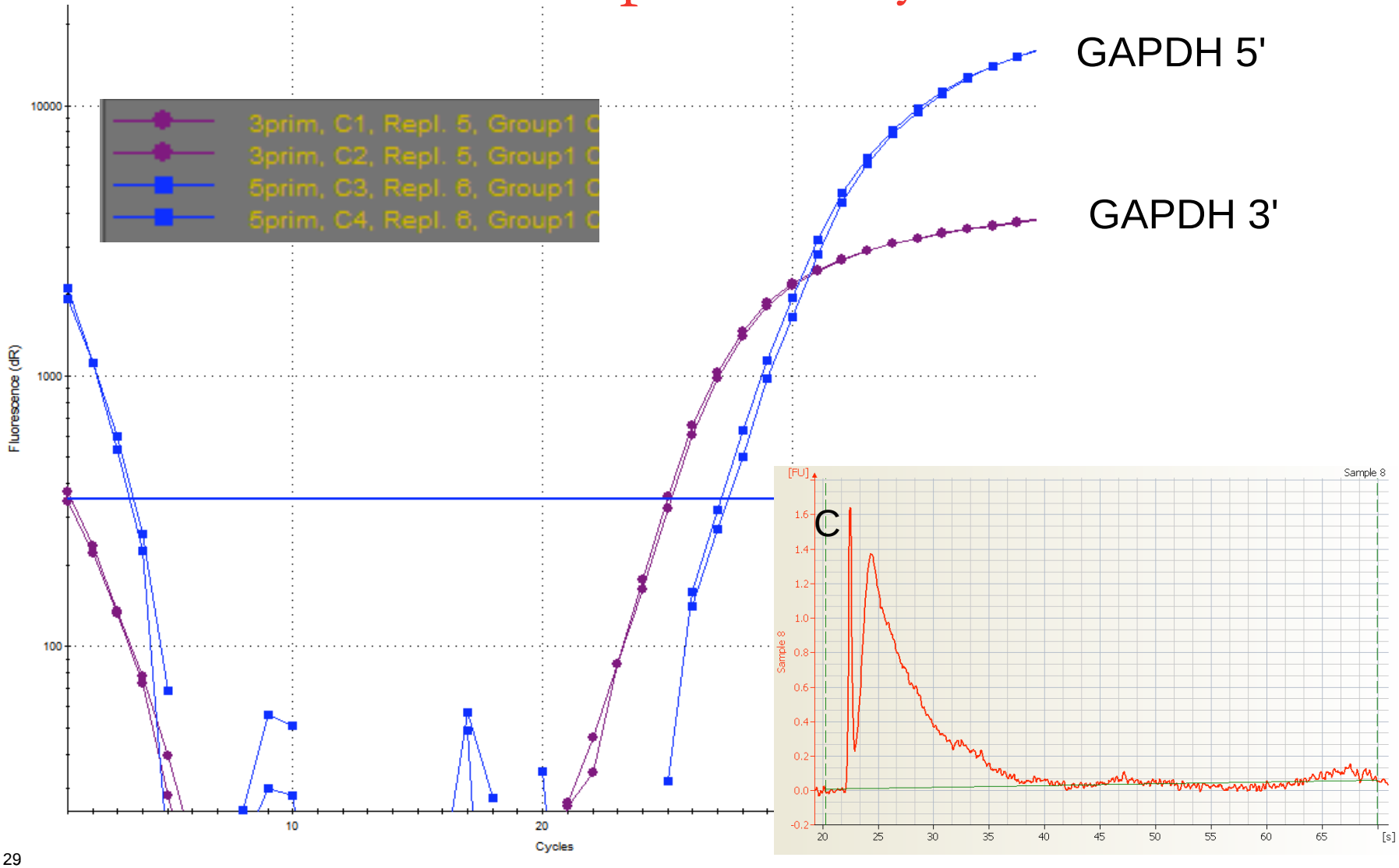
GAPDH 3'/5' Multiplex Assay

Intact RNA

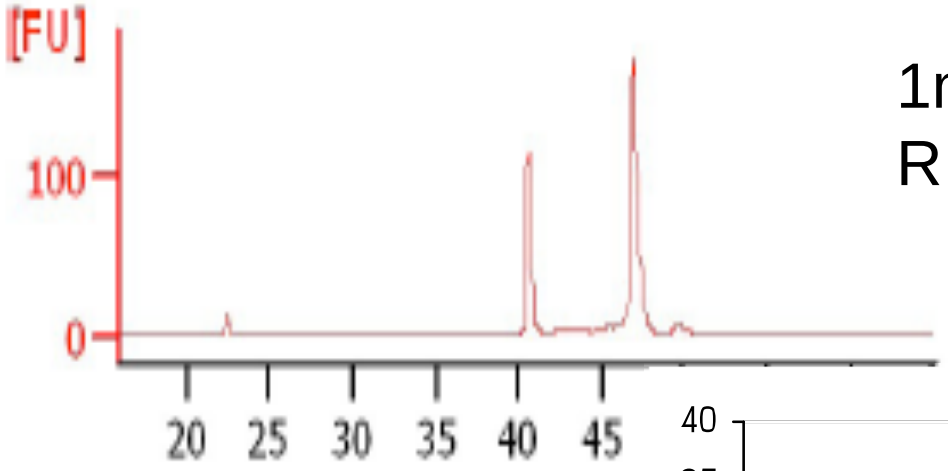


GAPDH 3'/5' Multiplex Assay

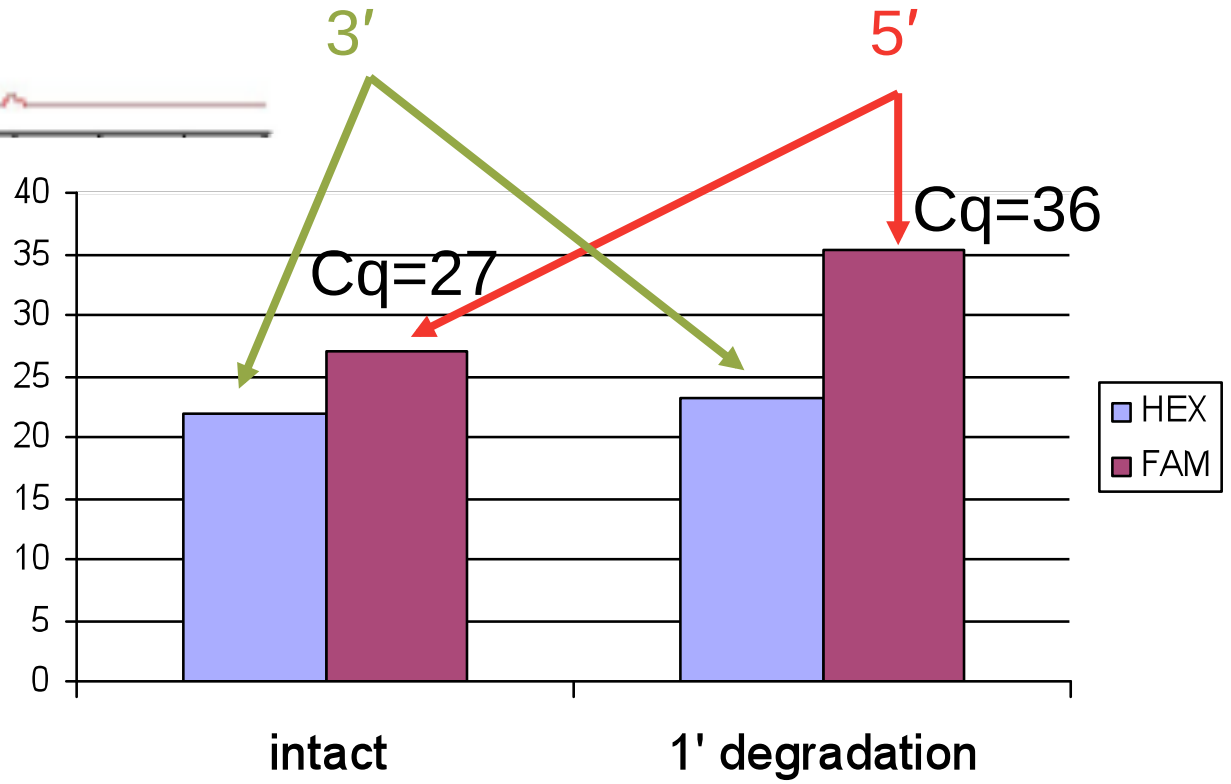
Degraded RNA



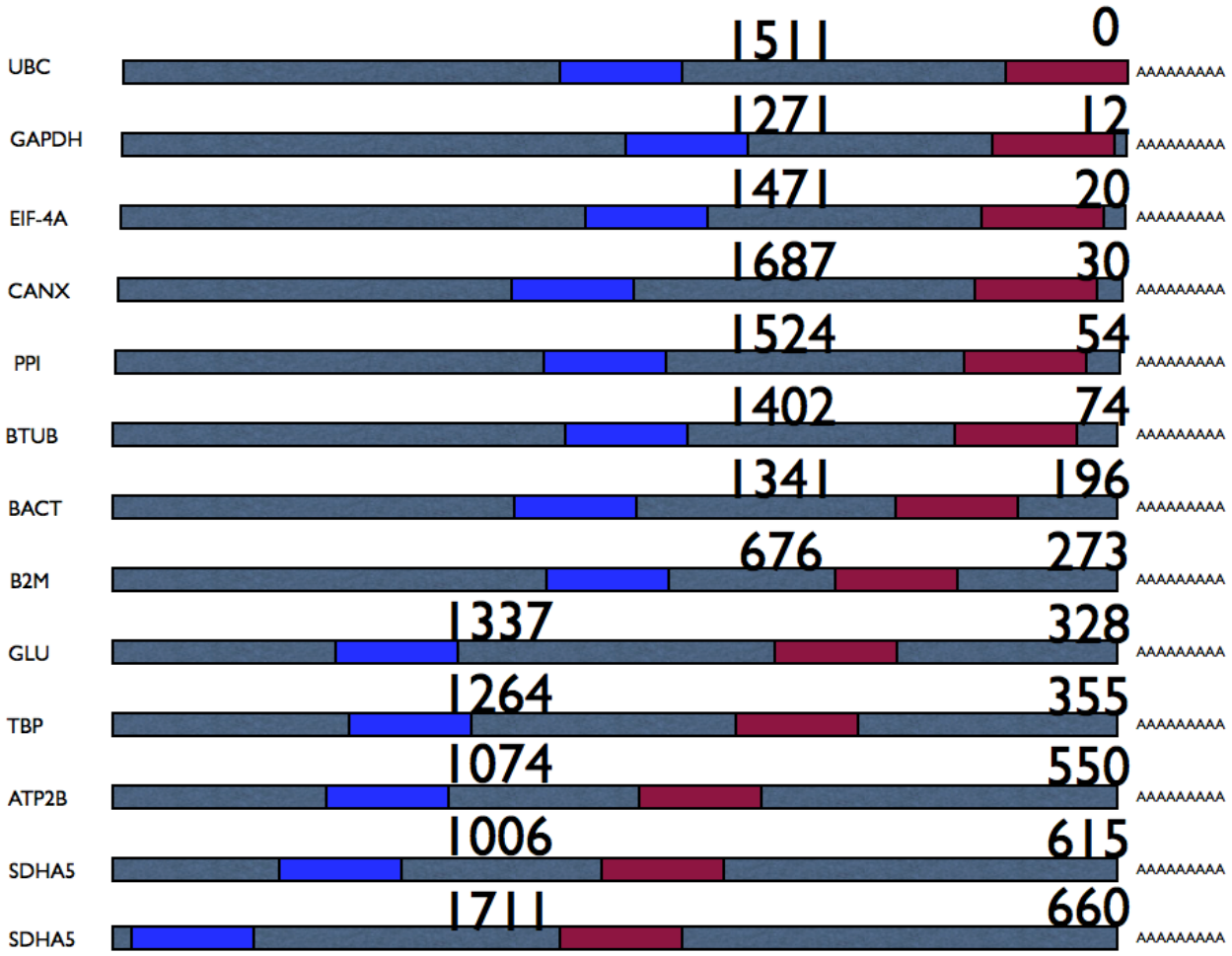
……..And Sample B?



1minute on Betsy's hand!
RIN 10



RNA Quality Determination



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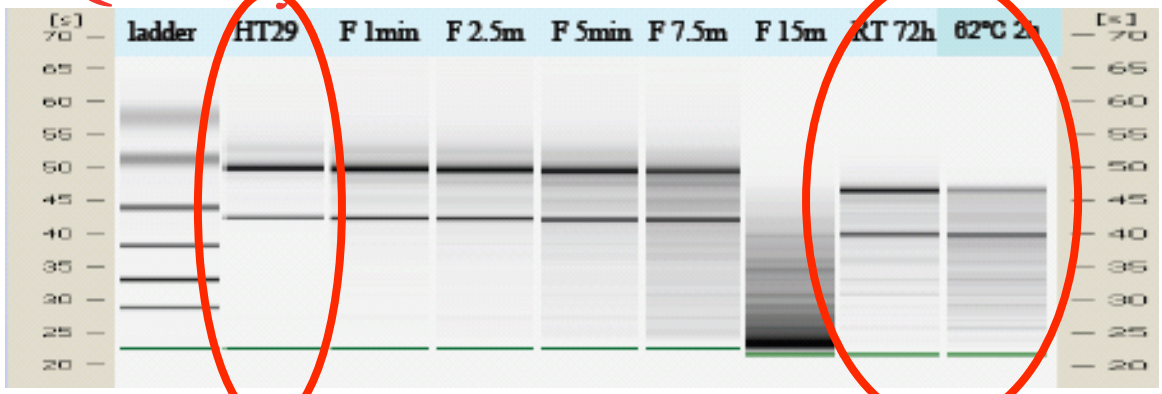

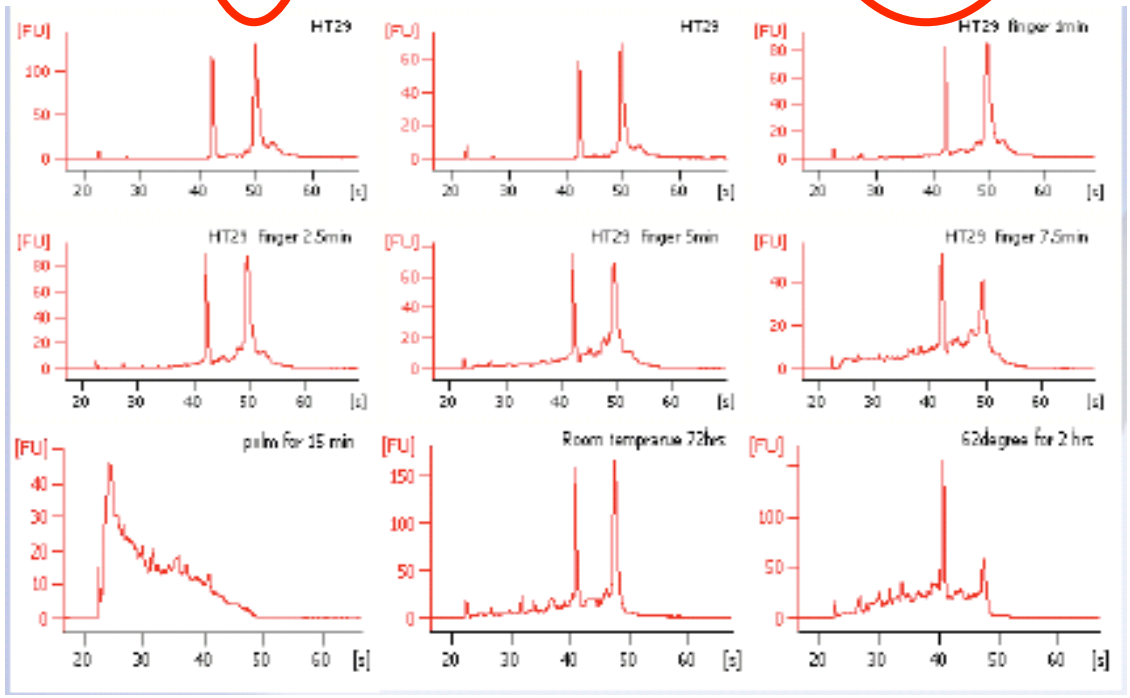

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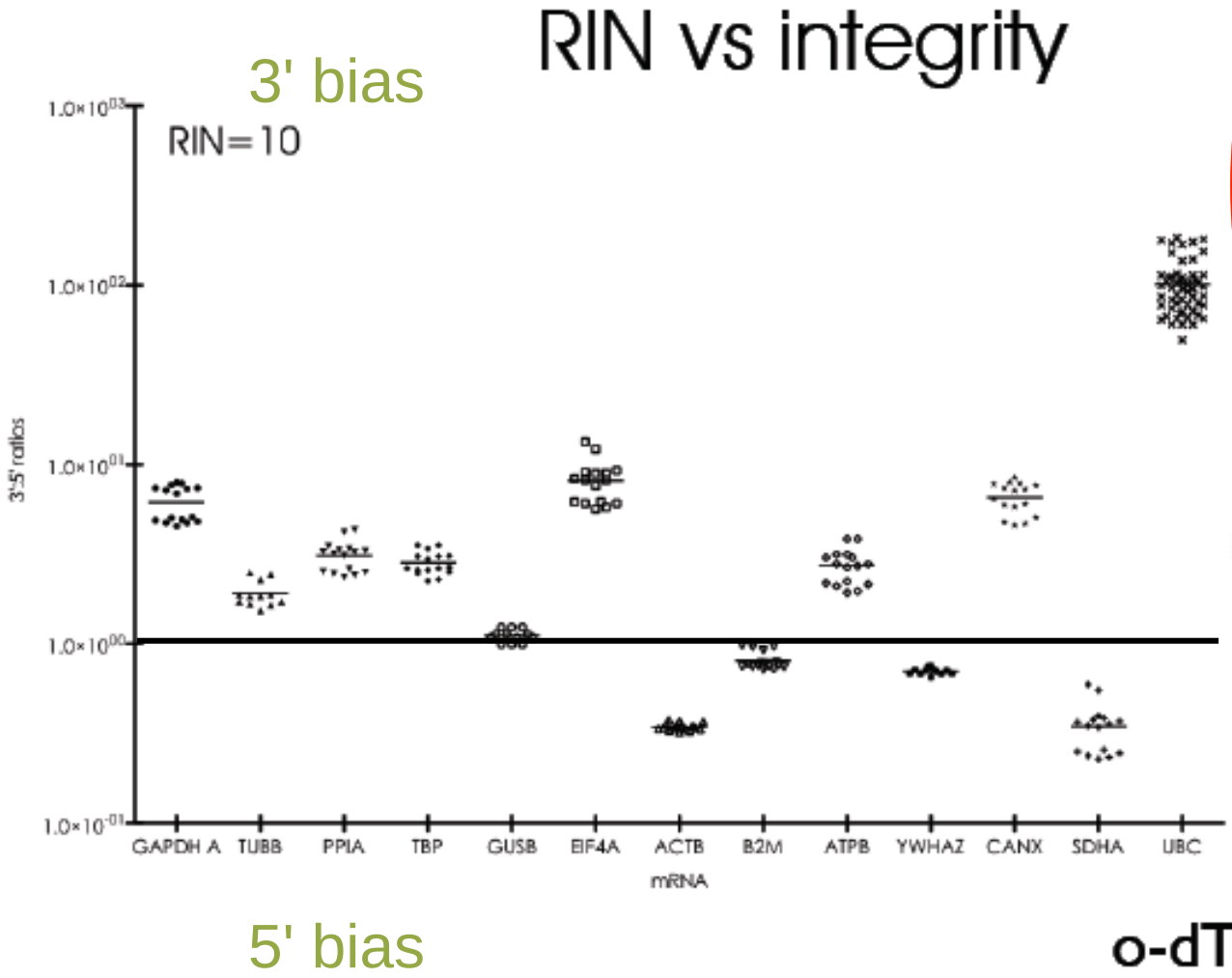
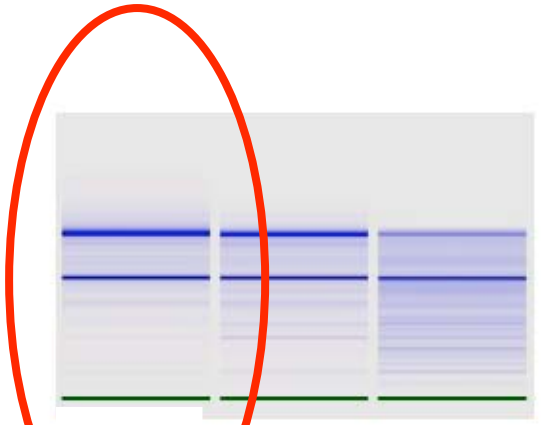
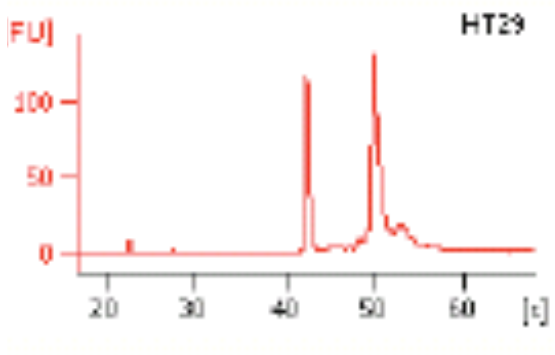




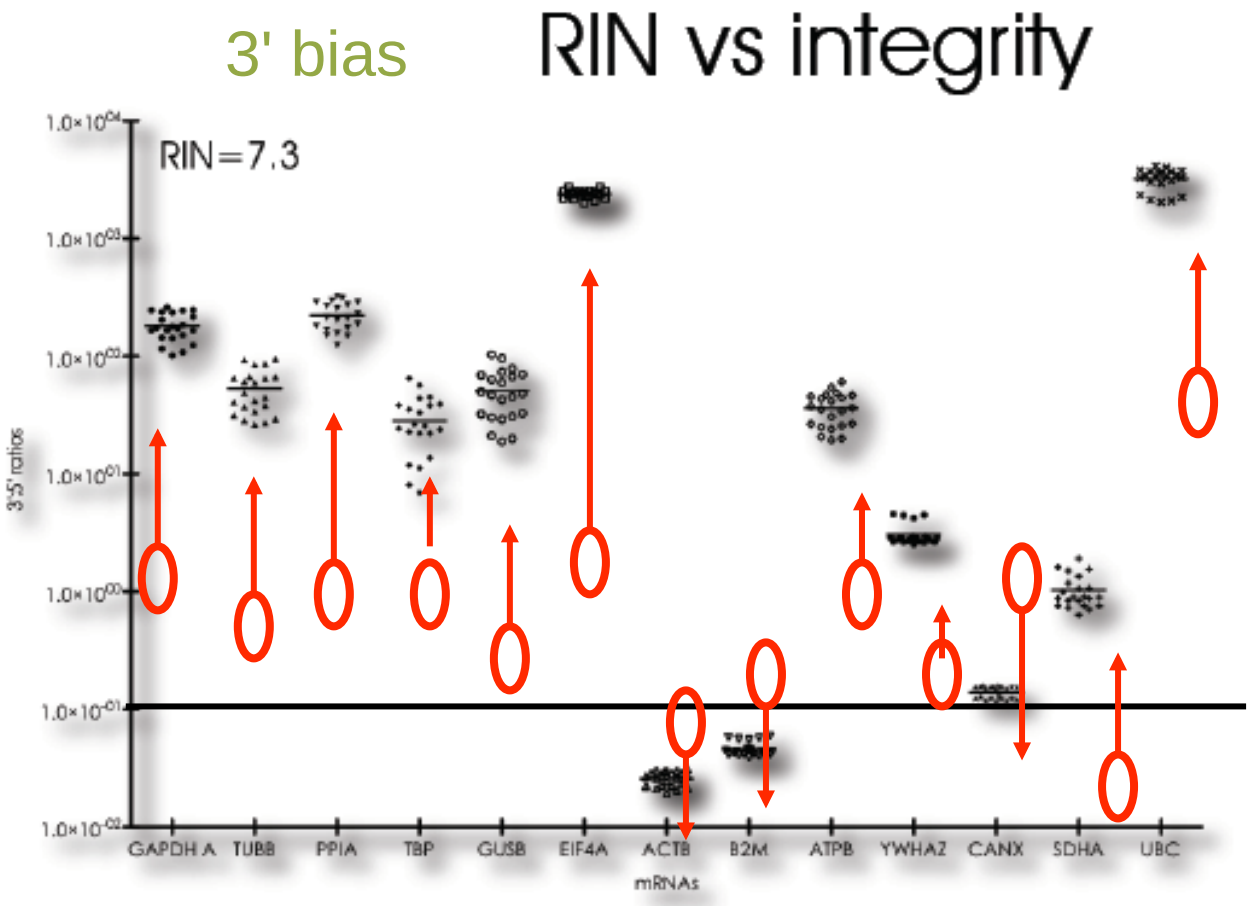
quantitate 14 target mRNAs
o-dT cDNA synthesis & 5' and 3' SP



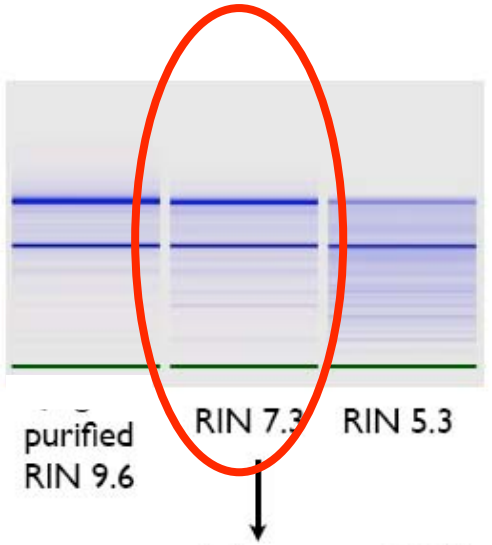
o-dT

RNA Quality Determination

3' bias



5' bias



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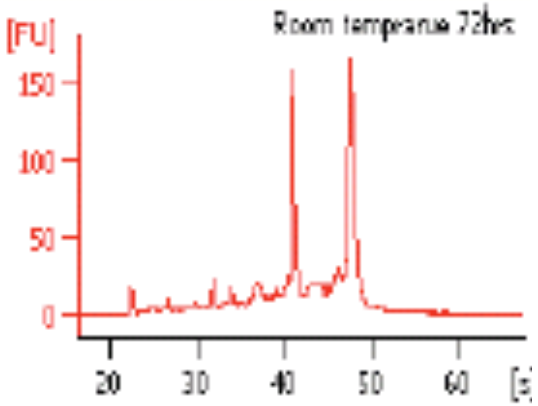
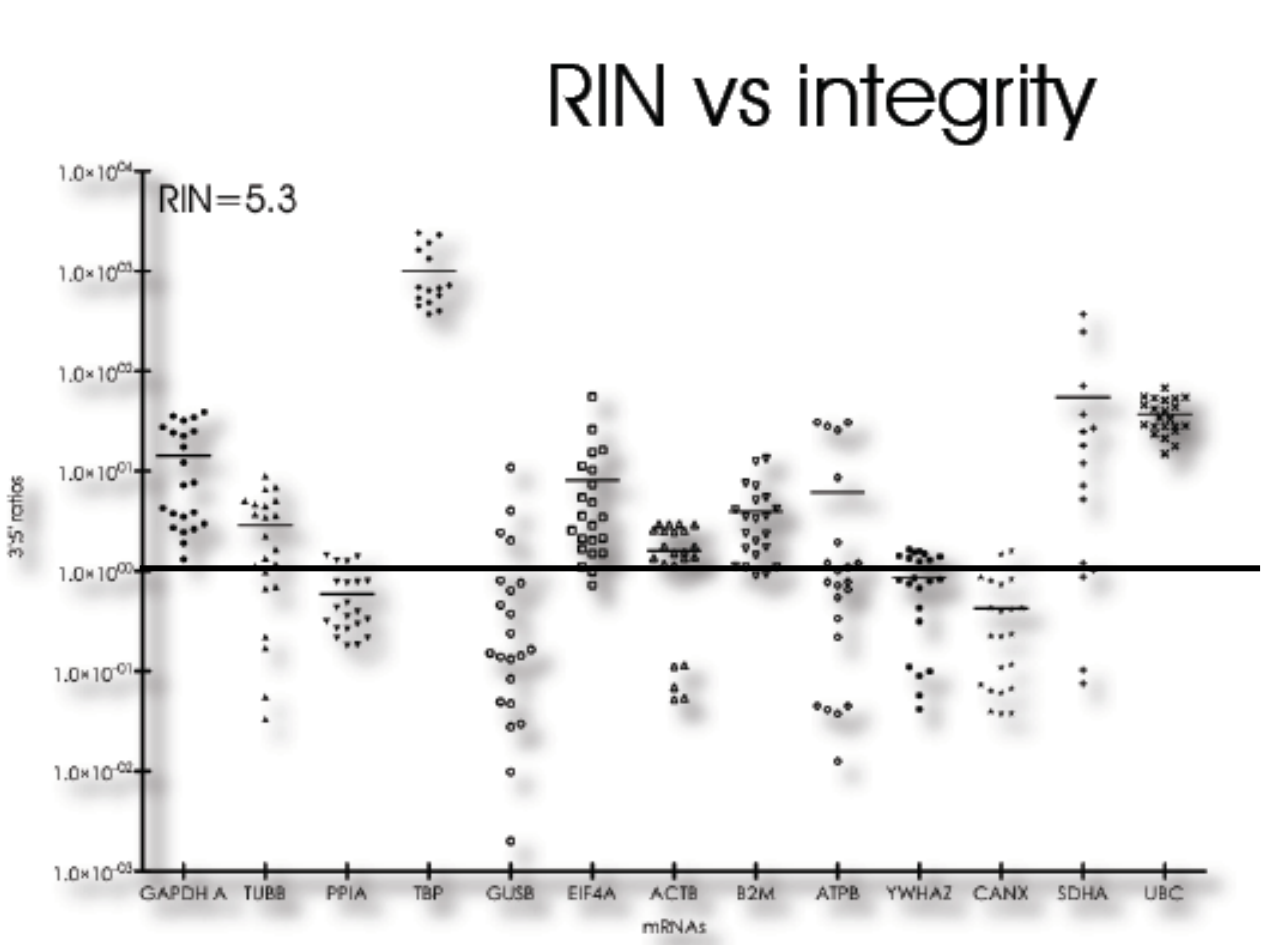
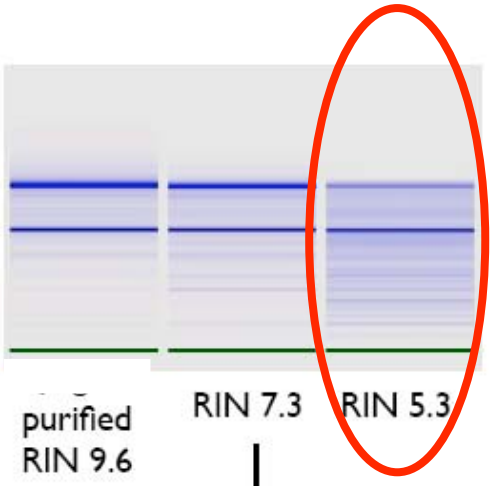


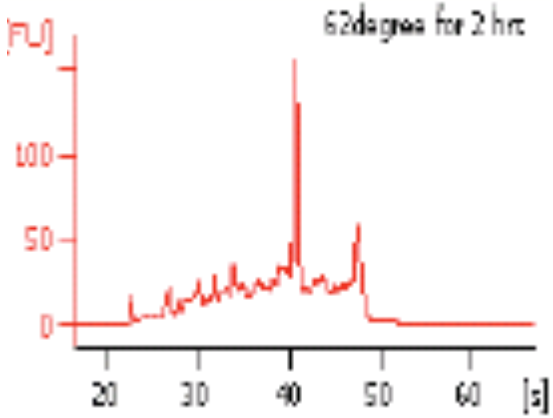
o-dT

RNA Quality Determination





quantitate 14 target mRNAs
o-dT cDNA synthesis & 5' and 3' SP



o-dT

A MIQE Based Workflow

Assay design

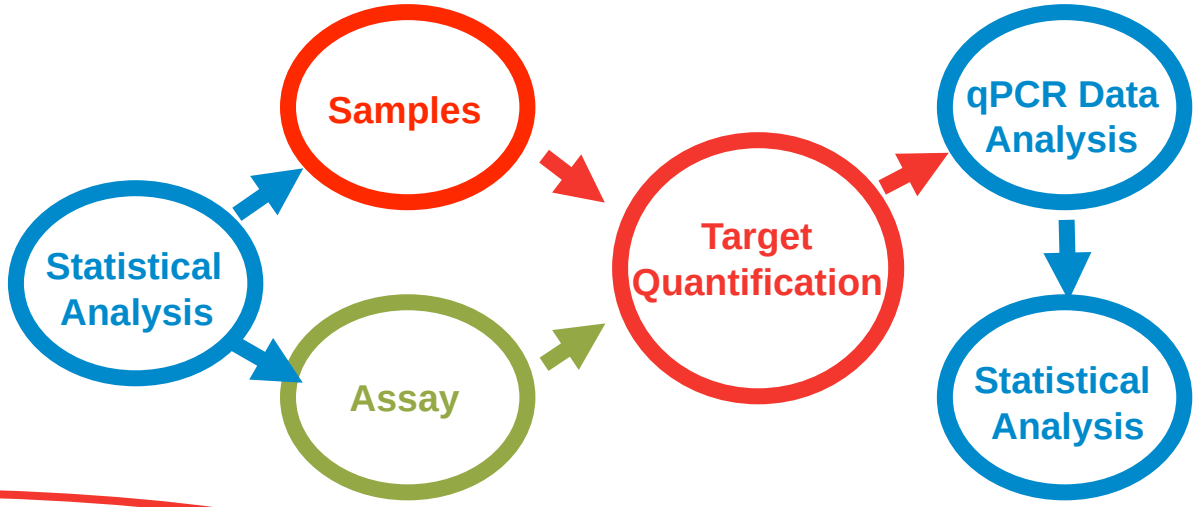
Reaction components

Optimization

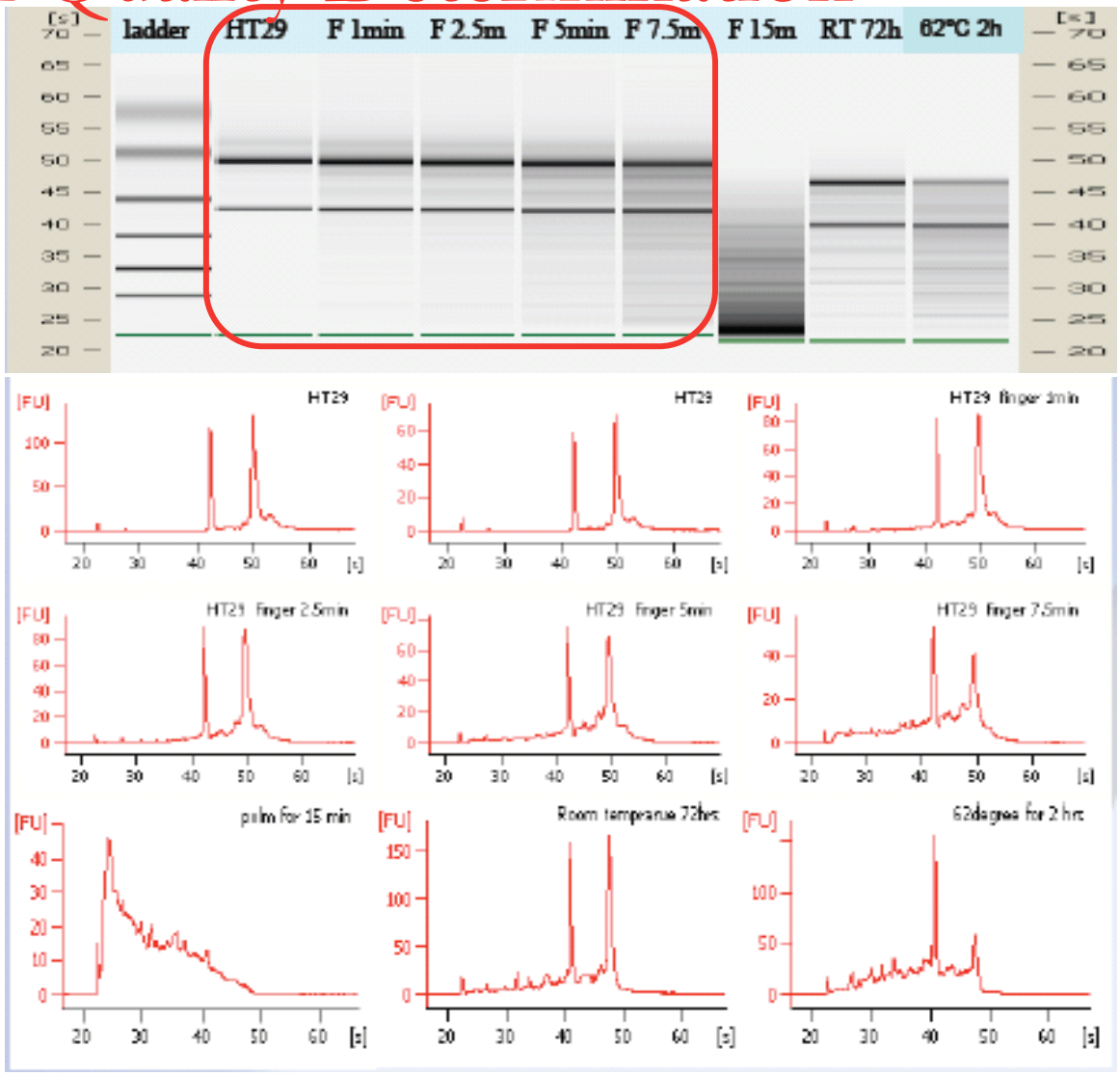
Sample QC

Sample QC and reaction components

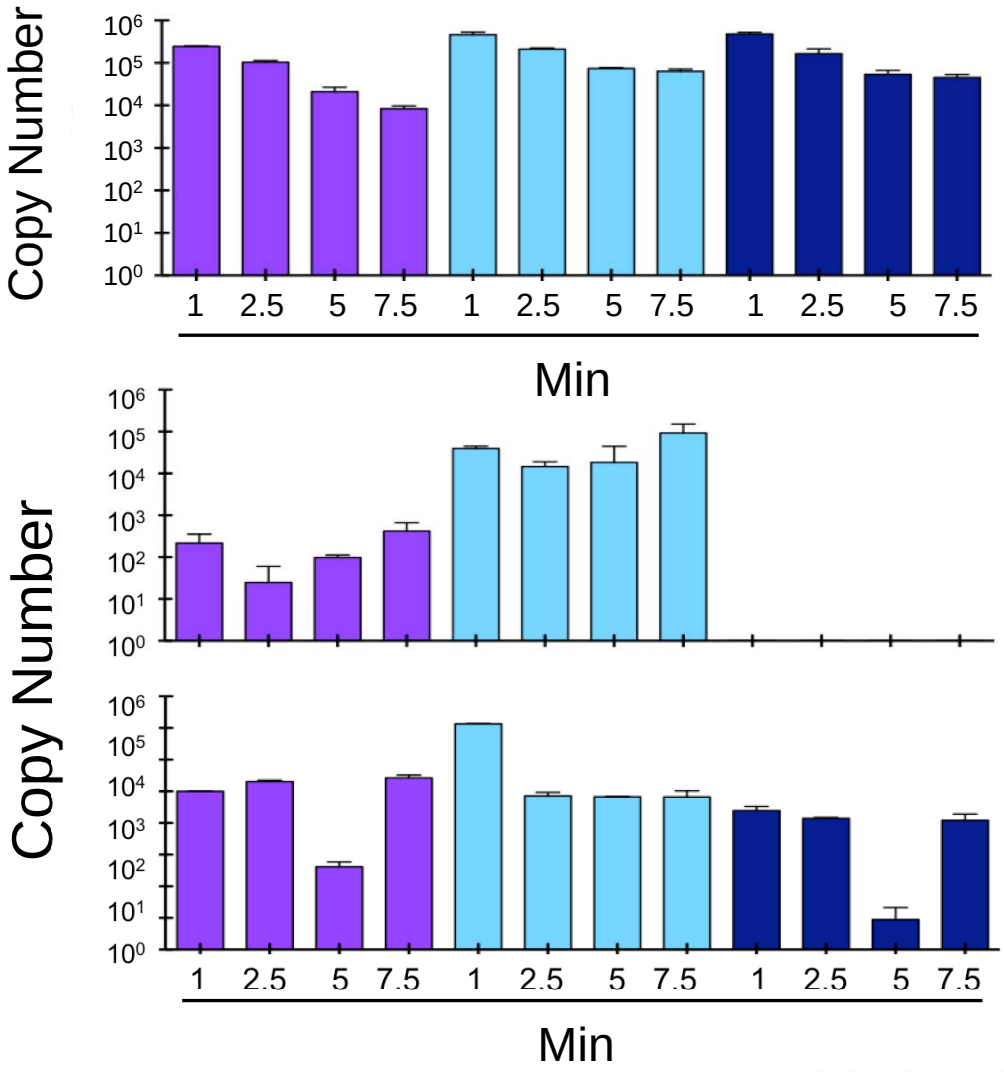
Methods of analysis



RNA Quality Determination



Effect of RT Priming and RNA Quality

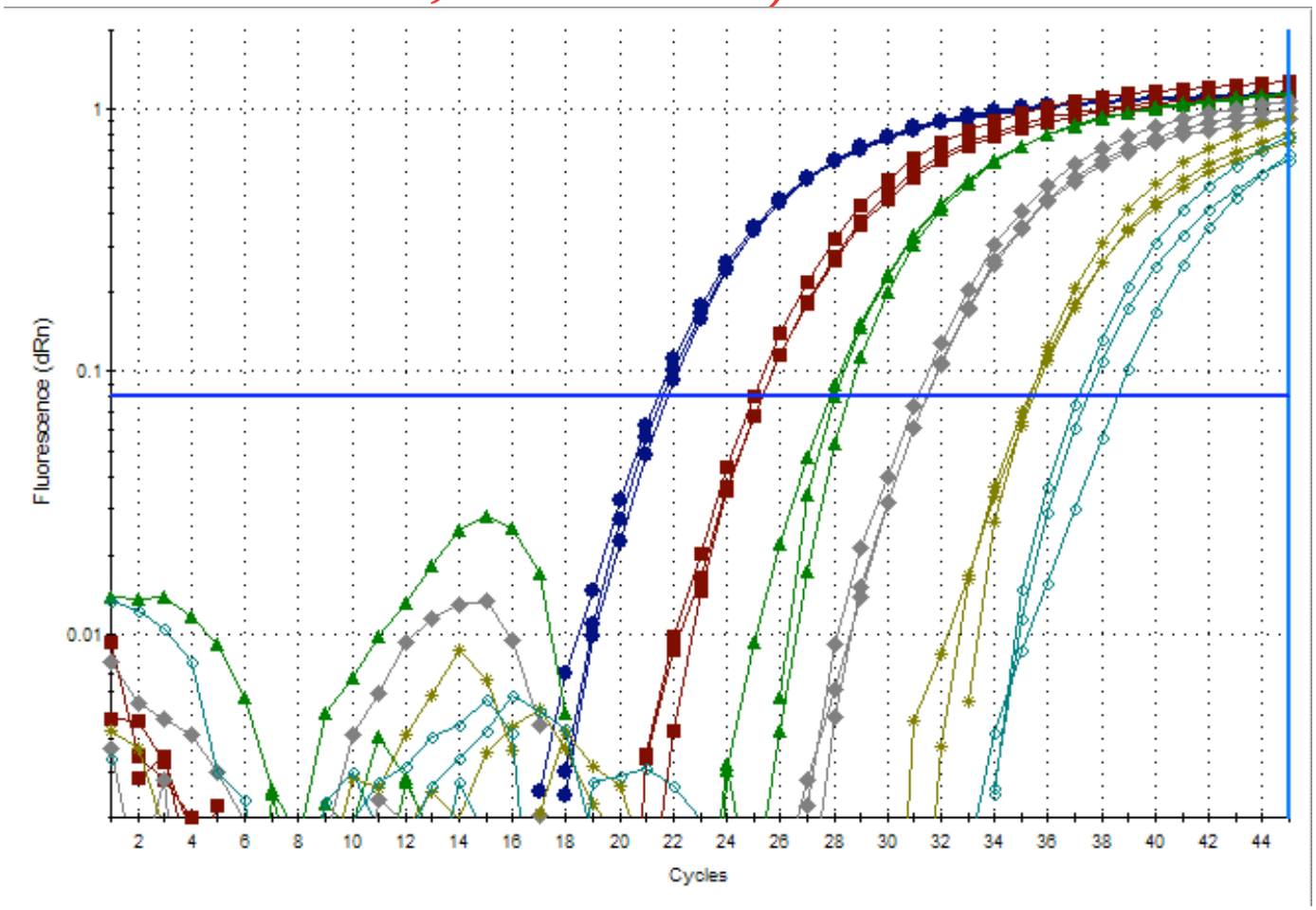


Gene-specific priming

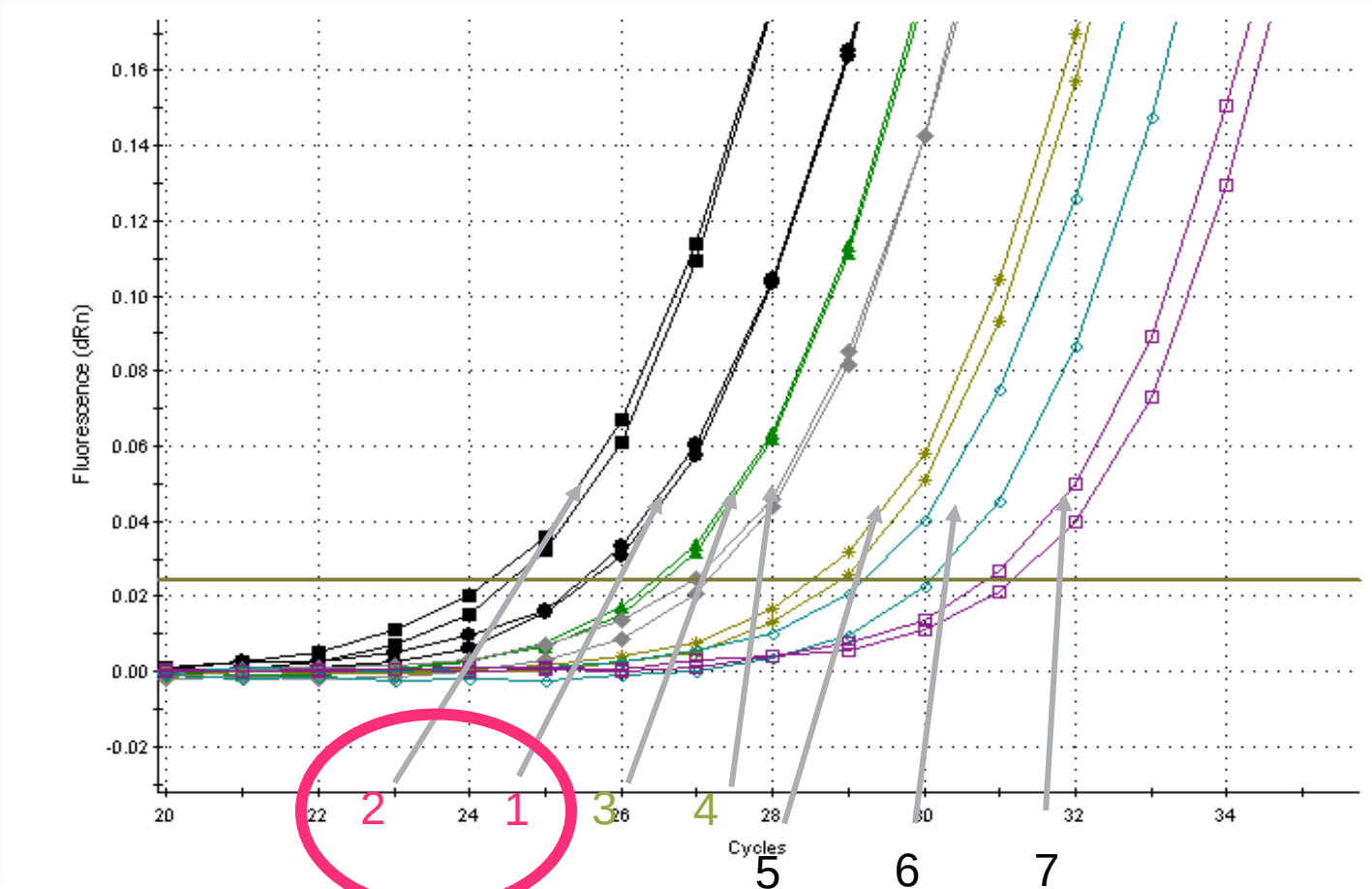
oligo-dT priming

Random priming

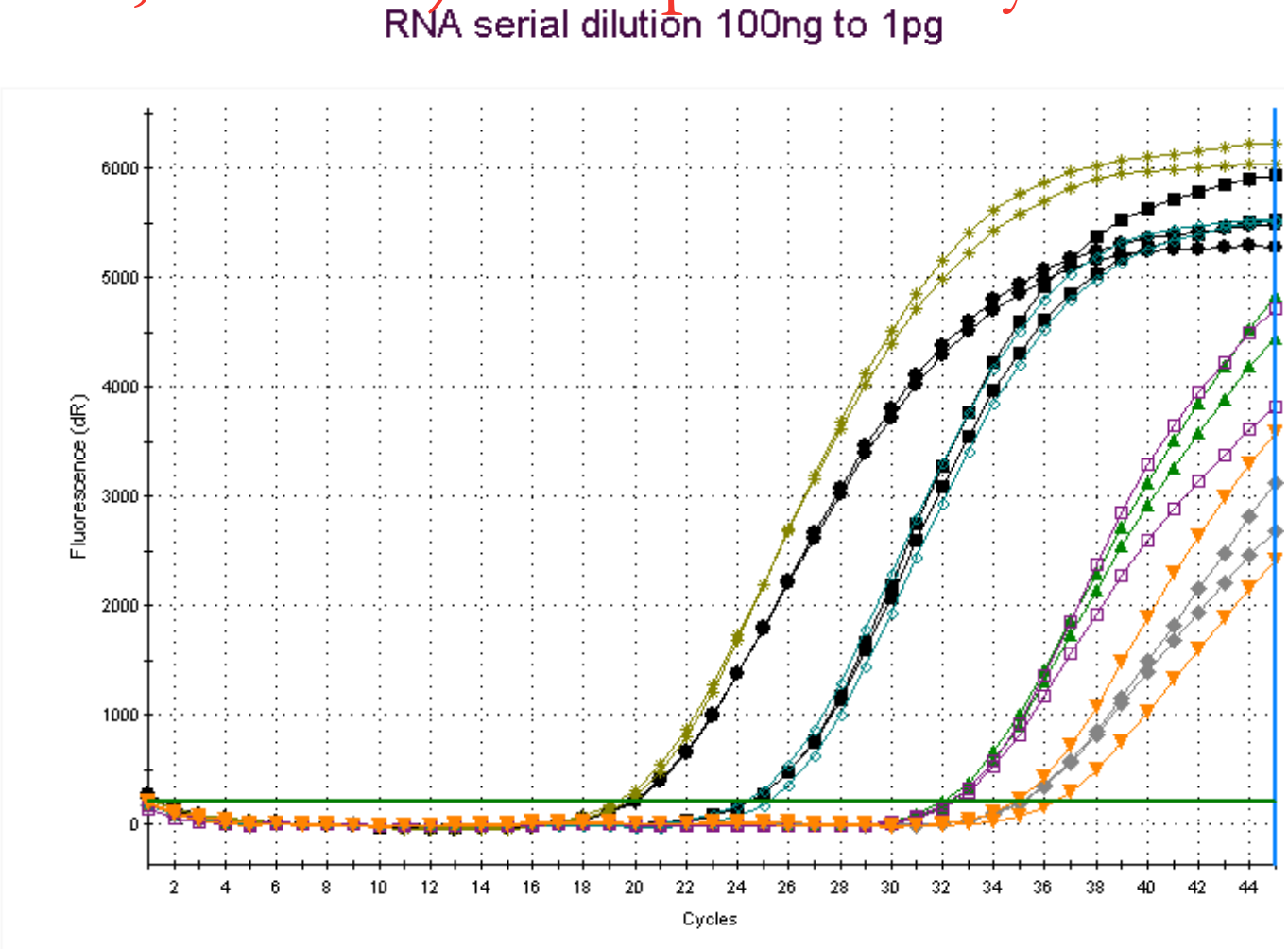
Gene Specific Priming RT and QPCR (10 Fold Dilutions,GAPDH)



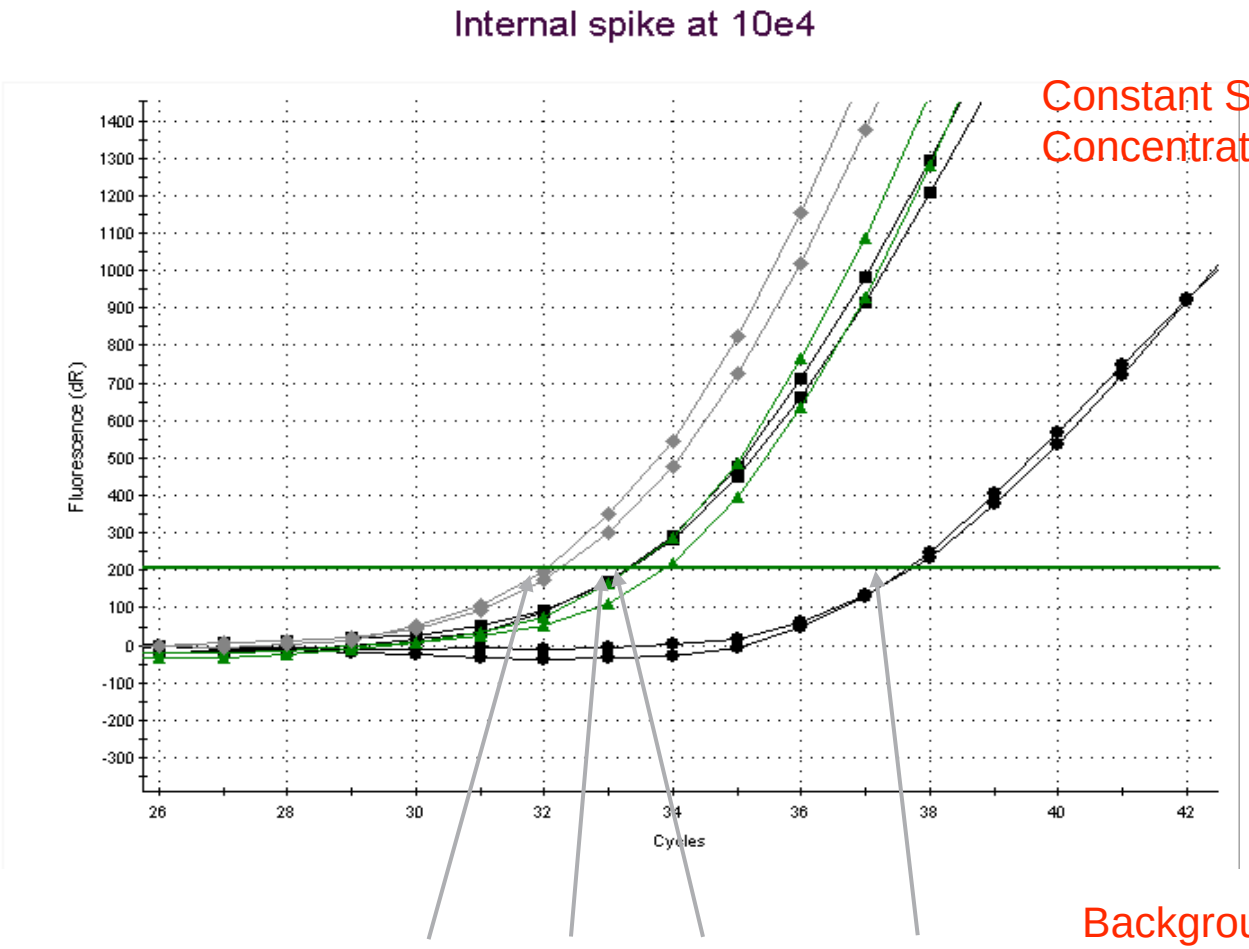
Random Primed RNA Dilution Series (QPCR NHE1)



Random Priming RT and QPCR (100 Fold Dilutions, B-actin) Is Reproducibly Non Linear



Constant Input SPIKE Quantification Varies in Presence of RNA Serial Dilution

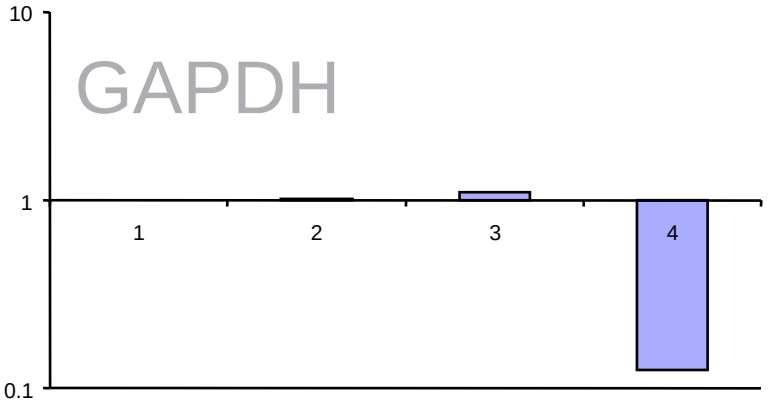


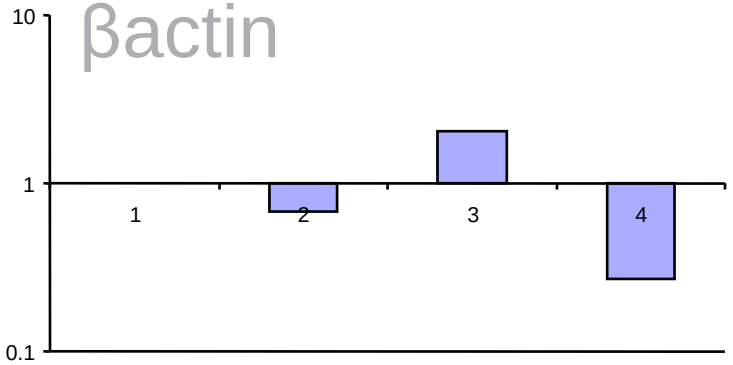
Constant SPIKE
Concentration 10^4 copies

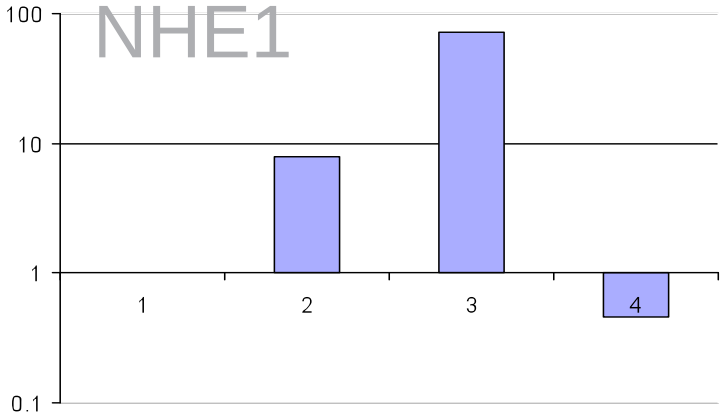
Background
RNA concentration

1pg 100pg 1ng 100ng

Gene Quantification is not Reproducible Between Independent RT Reactions







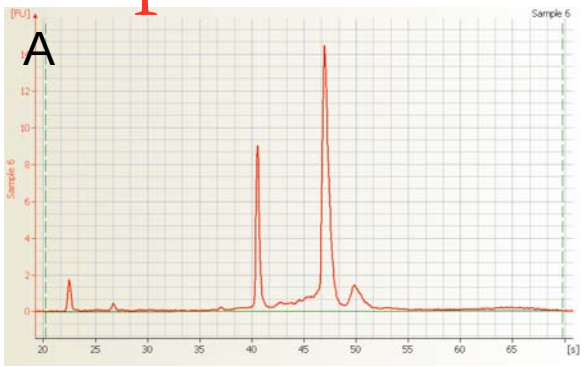
Correcting for Batch to Batch Variations

Assumption: the gene quantity in the calibrator samples (eg universal reference RNA) represents the RT reaction efficiency for that gene in that batch of RT reactions

Definition: gene quantity in calibrator is 100% (for each batch)

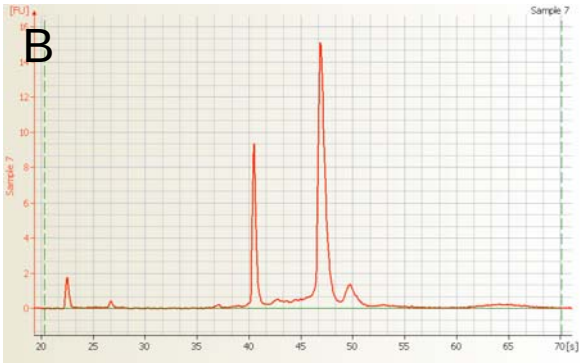
Quantities of the gene in the sample are expressed relative to gene quantity in calibrator (processed in same batch)

Agilent Bioanalyzer 2100 Analysis of 5 RNA Samples



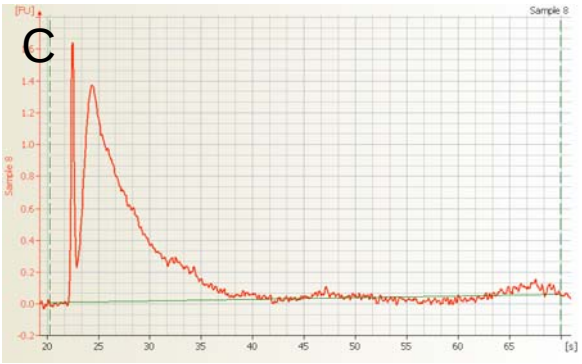
Conc. 110 ng/µl

RIN: 10



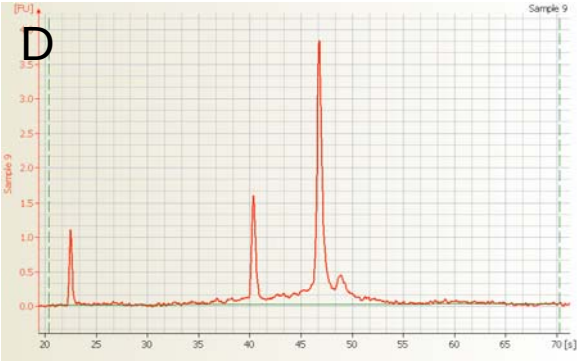
Conc. 110 ng/µl

RIN: 10



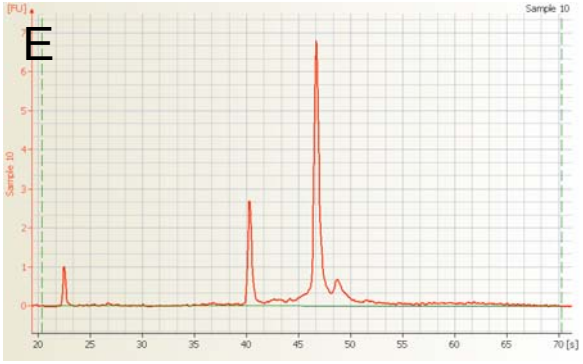
Conc. 62 ng/µl

RIN: 2.4



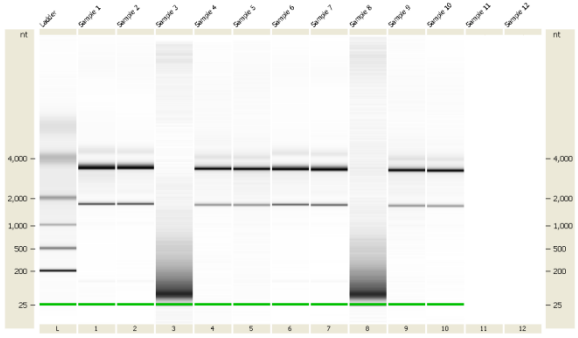
Conc. 30 ng/µl

RIN: 9.1

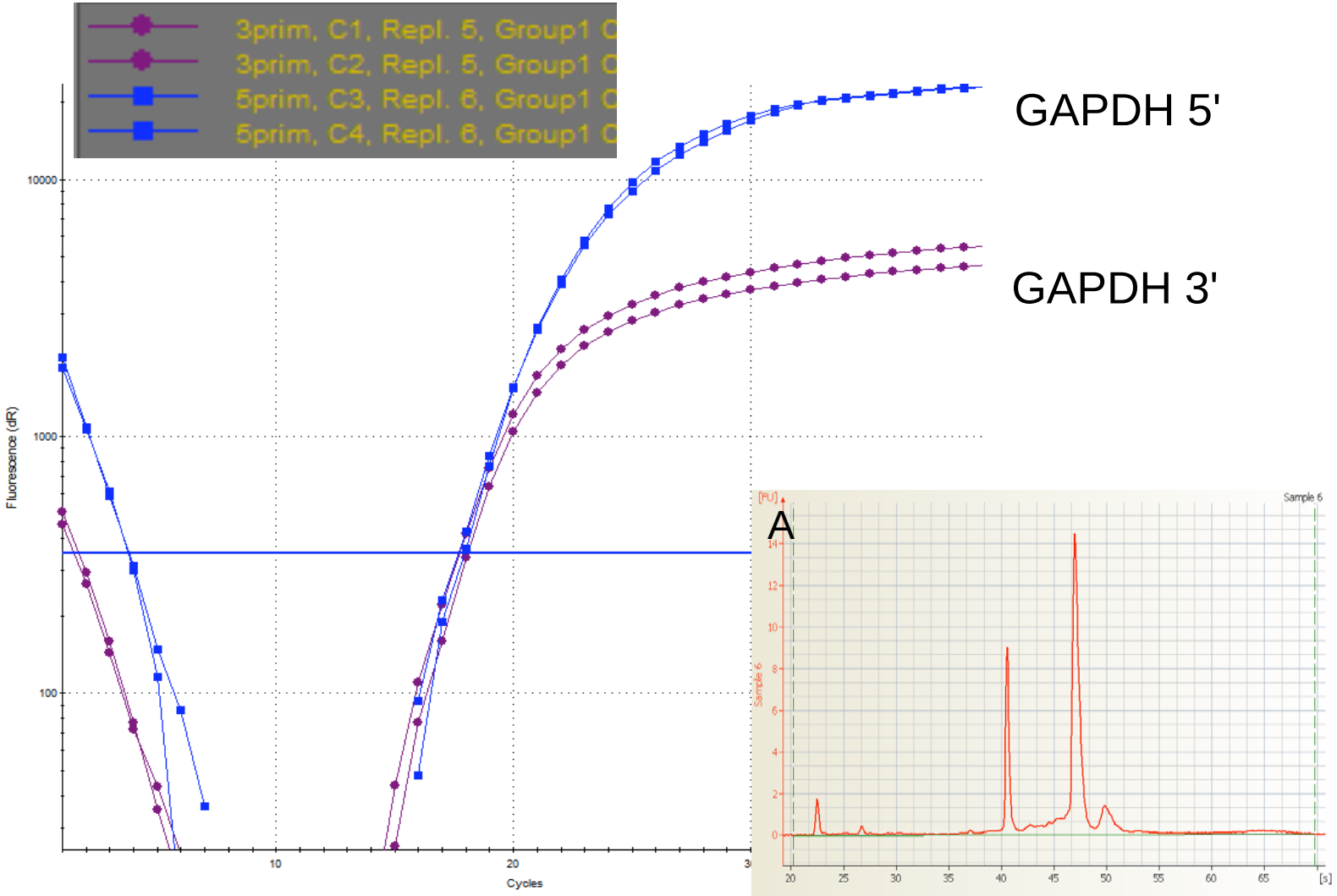


Conc. 43 ng/µl

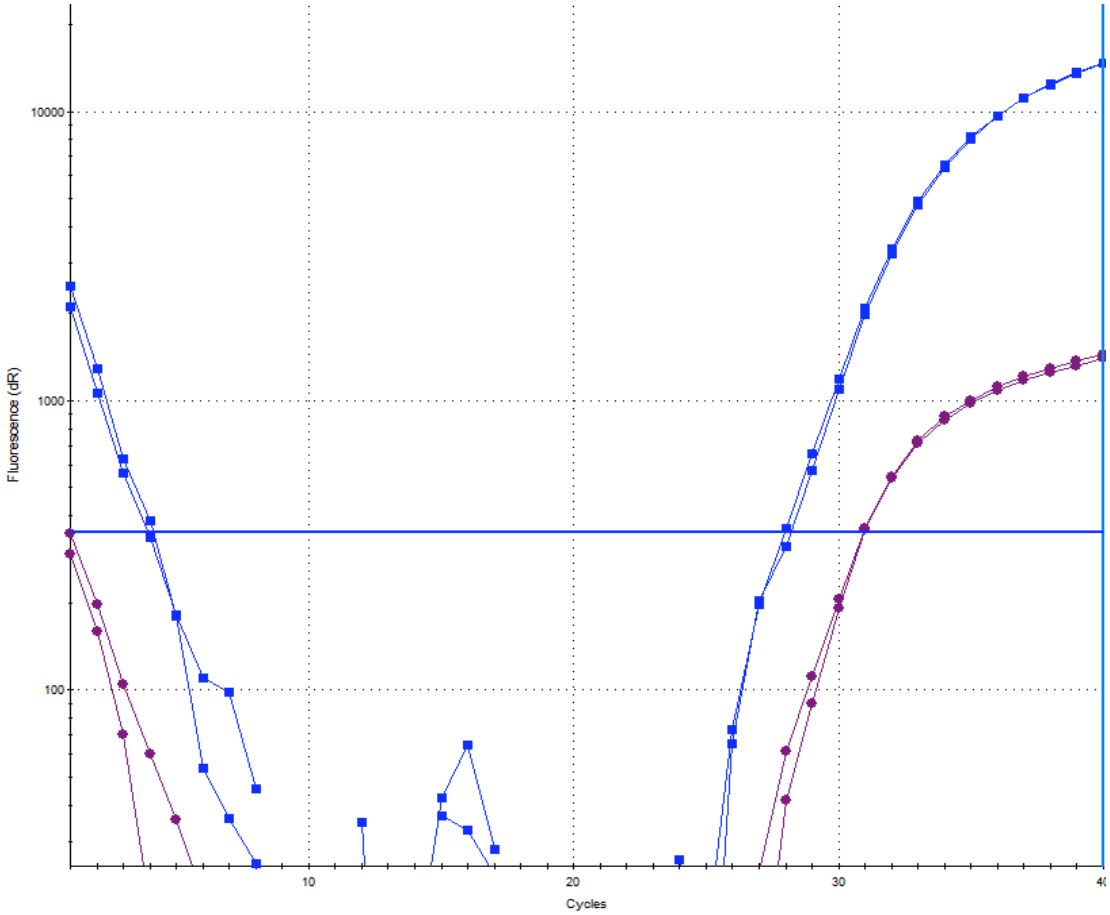
RIN: 9.5



GAPDH 3'/5' Multiplex Assay – Intact RNA

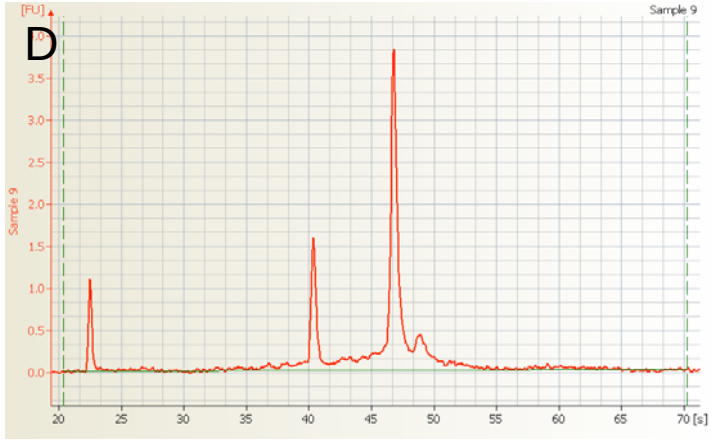


GAPDH More 5' Than 3'?



GAPDH 5'

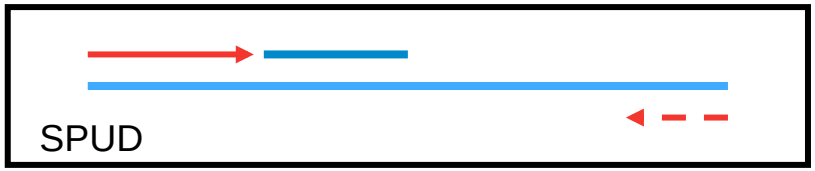
GAPDH 3'



Conc. 30 ng/µl

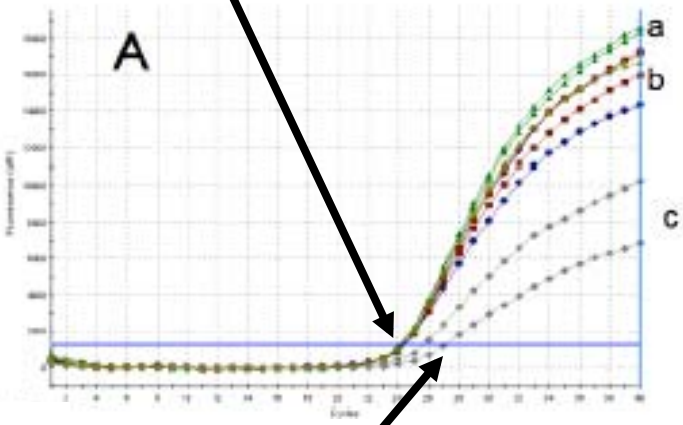
RIN: 9.1

SPUD: For Detection of Inhibitors



Reaction 1

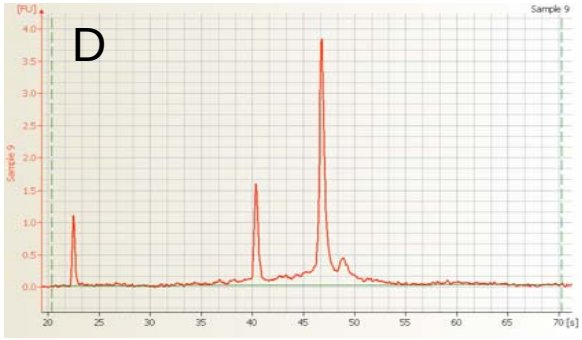
Cq (SPUD + Water) = 24



Reaction 2

Cq (SPUD + sample) = 26

(Phenol from extraction reagent)



D 12.5mM EDTA

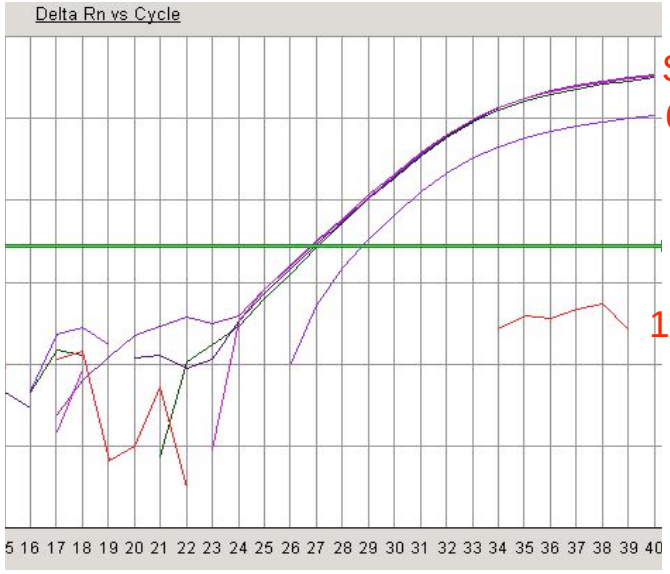
Conc. 30 ng/µl

RIN: 9.1

E 6.25mM EDTA

Conc. 43 ng/µl

RIN: 9.5

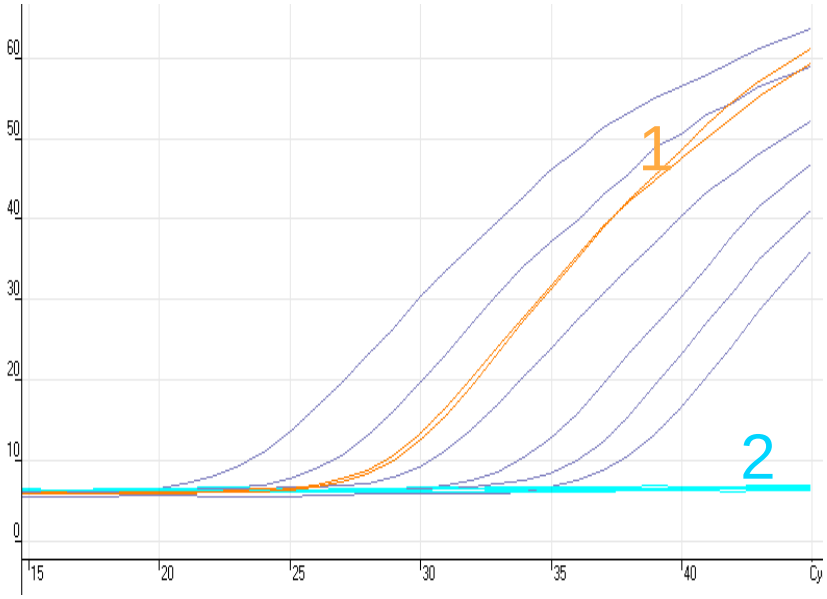


Samples A,B,C
6.25mM EDTA

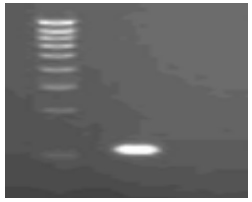
12.5mM EDTA

Using SPUD to avoid false negative results

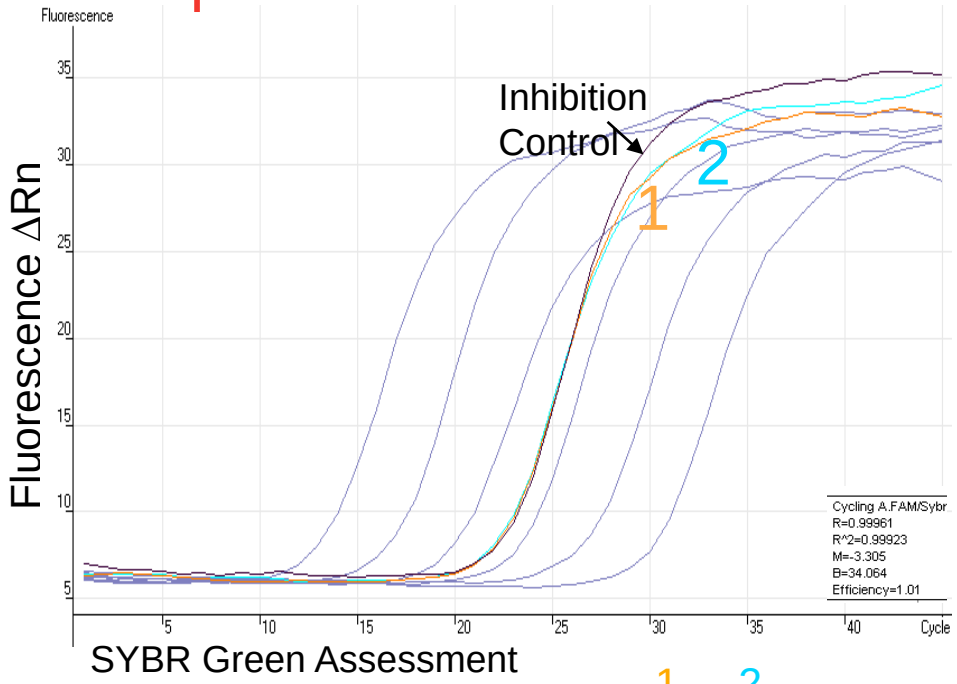
Hsp70 *P.jirovecii* assay



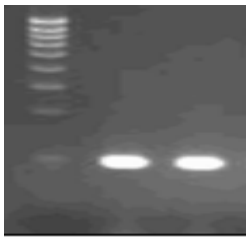
1 2



Spud Inhibition Test



1 2



Key:

1 = Clinical +

2 = Clinical –

Summary:

To reduce variability:

- Design assays in open regions of the template and ensure assays do not target regions containing SNPs. Consider splice variants
- When measuring small differences (eg <5 fold) compare 2 or more assays to the same target
- Optimise oligo conditions for greatest reproducibility and sensitivity
- Ensure samples are of the highest quality possible (3'/5' ratio) and free of inhibitors (SPUD or similar or dilute samples)
- If RNA concentration or degradation cannot be measured (eg FFPE samples) consider using gene specific priming for RT reactions
- Ensure RT reaction stability: prepare all cDNA in a single batch or control for variation
- QC everything and recognise compromises and therefore assay resolution. Report protocol details where possible

Further Information

- Assay design through
  www.sial.com/designmyprobe
  OligoArchitect™ Online Primer & Probe Designs for qPCR

- Request SPUD oligos, GAPDH 5'/ 3' assay sequences or make enquiries to
  Oligotechserv@sial.com

- EMBL Master Course
  Advanced qPCR Techniques for Publication Success:
  Following MIQE Recommendations
  EMBL, Heidelberg, Germany
  July 2012

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