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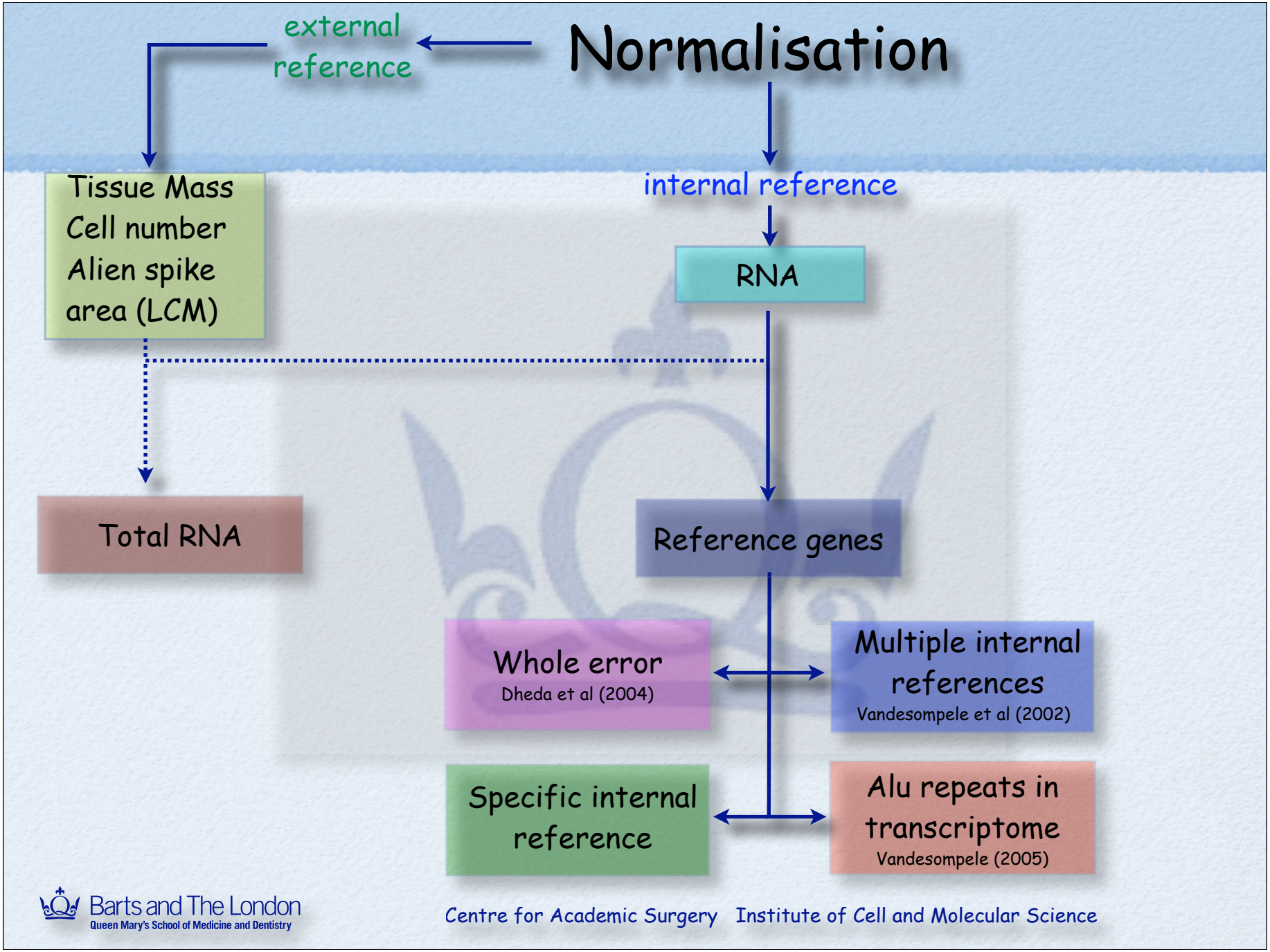
2nd qPCR meeting Freising, Germany 6th September 2005

Normalisation of mRNA against gDNA

Professor Stephen A Bustin

Centre for Academic Surgery
Institute of Cell and Molecular Science
s.a.bustin@qmul.ac.uk

Queen Mary
University of London

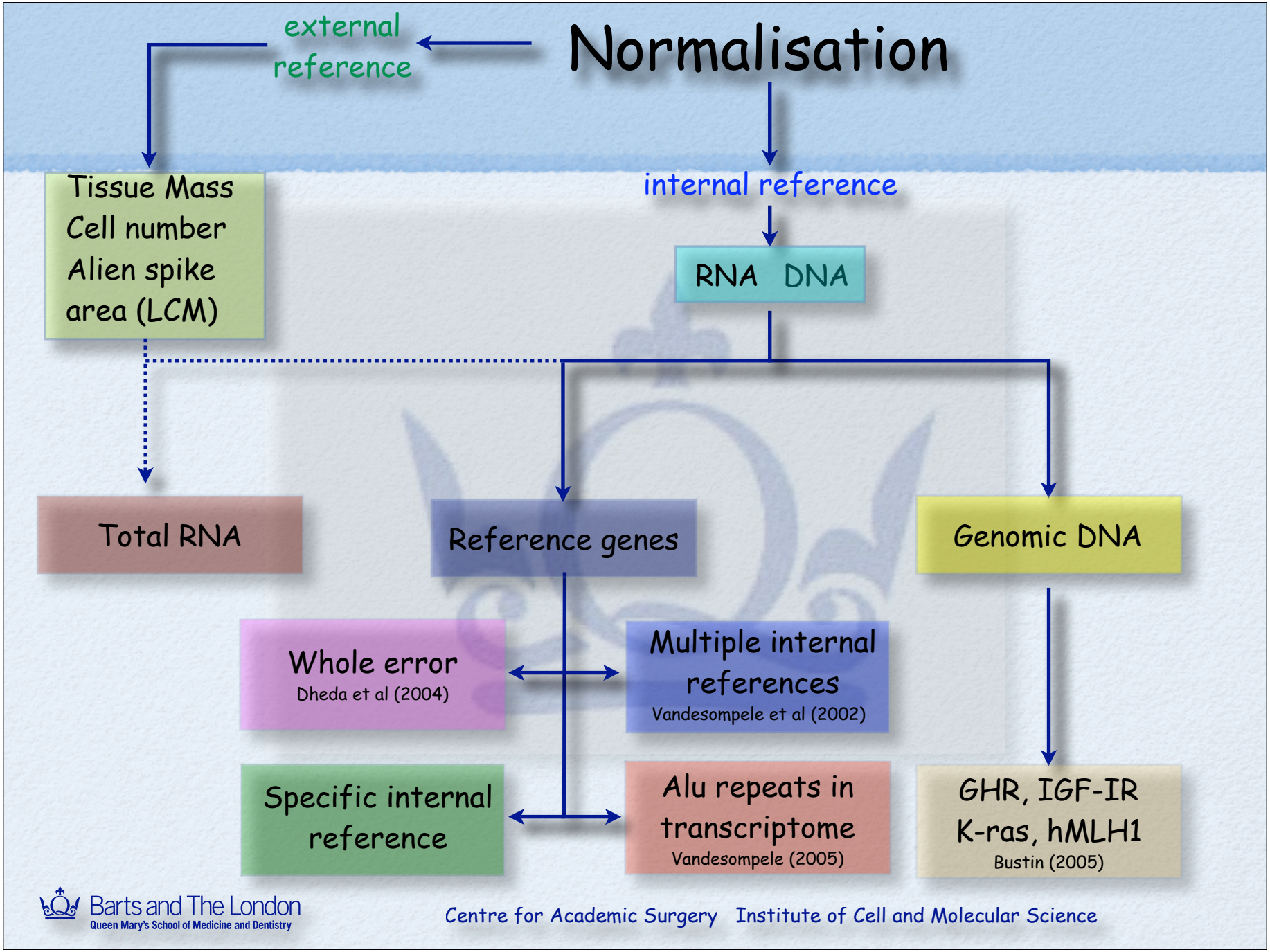

Normalisation
external reference
internal reference
Tissue Mass
Cell number
Alien spike area (LCM)
RNA
Total RNA
Reference genes
Whole error
Dheda et al (2004)
Multiple internal references
Vandesompele et al (2002)
Specific internal reference
Alu repeats in transcriptome
Vandesompele (2005)
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Centre for Academic Surgery
Institute of Cell and Molecular Science

Aim

- Development of a simple & universal normalisation method for qRT-PCR

Why?

- Unable to quantitate RNA
  - rules out normalisation against total RNA
- Extremely limited amounts of target mRNA
  - Unable to identify appropriate reference genes
- External references too inaccurate


Normalisation
external reference
internal reference
Tissue Mass
Cell number
Alien spike area (LCM)
RNA DNA
Total RNA
Reference genes
Genomic DNA
Whole error
Dheda et al (2004)
Multiple internal references
Vandesompele et al (2002)
Specific internal reference
Alu repeats in transcriptome
Vandesompele (2005)
GHR, IGF-IR
K-ras, hMLH1
Bustin (2005)
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Genomic DNA

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Nucleic Acids Research, 2002, Vol. 30 No. 20 e104

Genomic DNA standards for gene expression profiling in *Mycobacterium tuberculosis*

Adel M. Talaa
Stephen Albe

Center for Biome
Texas–Southwes
[1]Department of In
Albuquerque, NM

the predicted mycobacterial ORFs. Evaluation of expression levels by real time, quantitative PCR revealed a higher percentage of agreement (90%) with the genomic normalization protocol for microarray analysis compared to RNA

Research Report

Use of RNA and Genomic DNA References for Inferred Comparisons in DNA Microarray Analyses

BioTechniques 33:924-930 (October 2002)

H. Kim, B. Zhao, E.C. Snesrud, B.J. Haas, C.D. Town, and J. Quackenbush
The Institute for Genomic Research, Rockville, MD, USA

ence. Our analysis demonstrates that while genomic DNA can serve as a reasonable reference source for microarray assays, a much greater correlation with direct measurements can be achieved using an RNA-based reference sample.

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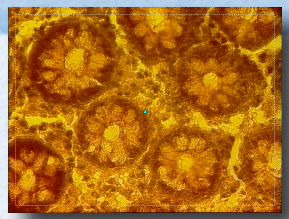
Why not use DNA ?

- May inhibit reverse transcription and/or PCR
- RNA & DNA extraction efficiencies may vary
- May generate false positive results
- Does not account for variable RT efficiency
- Cannot be (easily) used for intron-less genes
- RNA extraction kits designed to remove DNA
- Genetic instability (LOH, amplification)
- Hyperproliferation

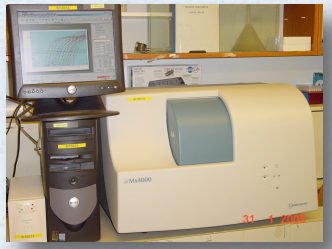
Why use DNA ?

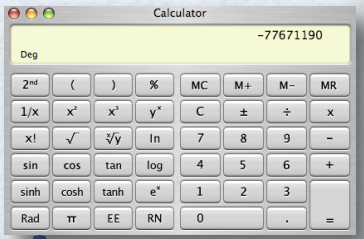
- Conceptual simplicity
  - usually two target copies/cell
- Internal reference
  - most accurate and relevant normaliser
- No inter-tissue, inter-patient variability
  - Facilitates comparison of expression data between different groups or multiple conditions

Protocol outline









Sample acquisition

Lysis → DNA, RNA Extraction

Multiplex assay:

qPCR -gDNA target(s)

qRT-PCR - mRNA targets

mRNA copy number calculation:

x copies/genome equivalent

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gDNA markers

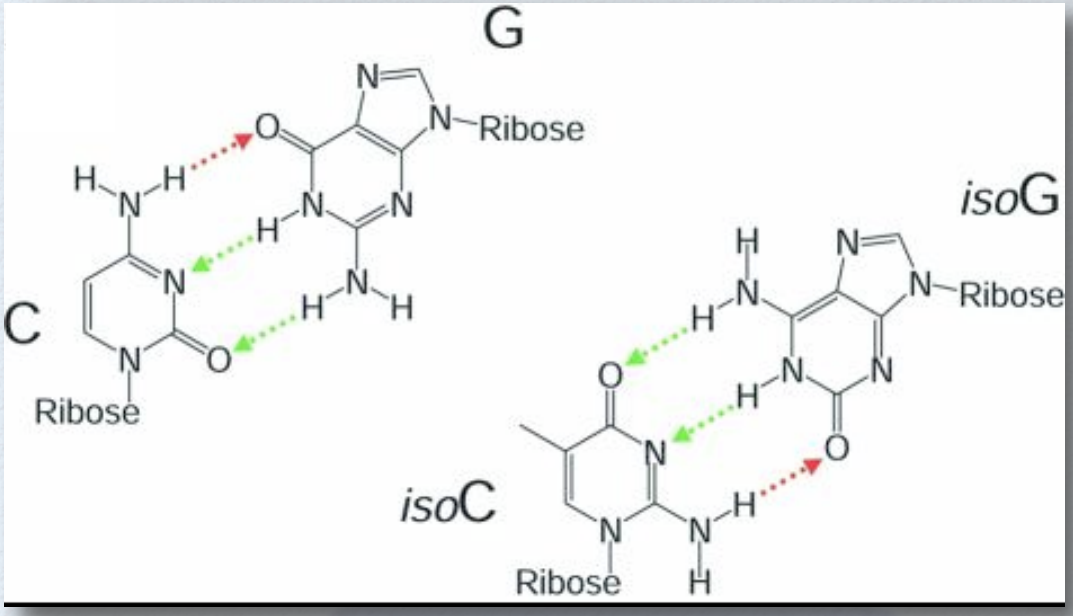
- Must not be amplified
  - c-myc, c-erbB-2
- Must not be lost
  - LOH hotspots
- No polymorphisms/mutations

DNA targets

Gene	Accession no	Location	sense (targets intron)	antisense (targets exon)	amplicon size
hMLH-1	NM_00249	3p21.3	FAM-isoC-GCCCGTTAAGTCGTAGCCCTT	GCCAGCTAATGCTATCAAAG AGATGAT	86 bp
GHR	NM_000163	5p13-p12	Quasar 670-isoC-TGCTATGTAAAACTTGCATCTTTTAATA AAATCATCCAAA	CAGGATCAAGTGATGCTTTT TCTGGAA	147 bp
IGF-IR	NM_000875	15q26.3	CAL Fluor Red 610-isoC-CTCGGGTTTCGCAAACAGGG	TCTGGCCGACGAGTGGAGAA	99 bp
K-Ras	NM_004985	12p12.1	CAL Fluor Orange 560-isoC-GGGTGGTCCGCTCCGTAC	CATTTCGGACTGGGAGCGAG	104 bp

Chemistry

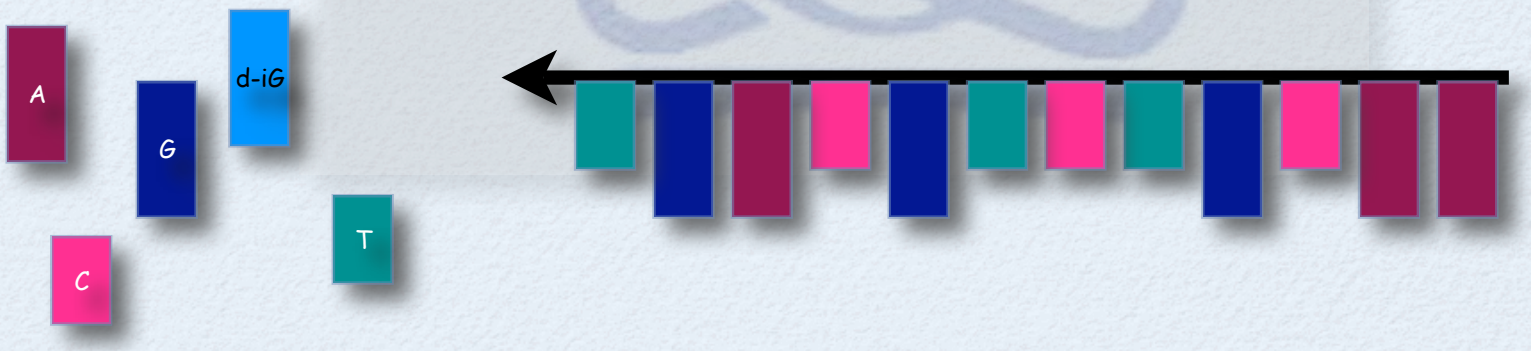
Plexor (1)



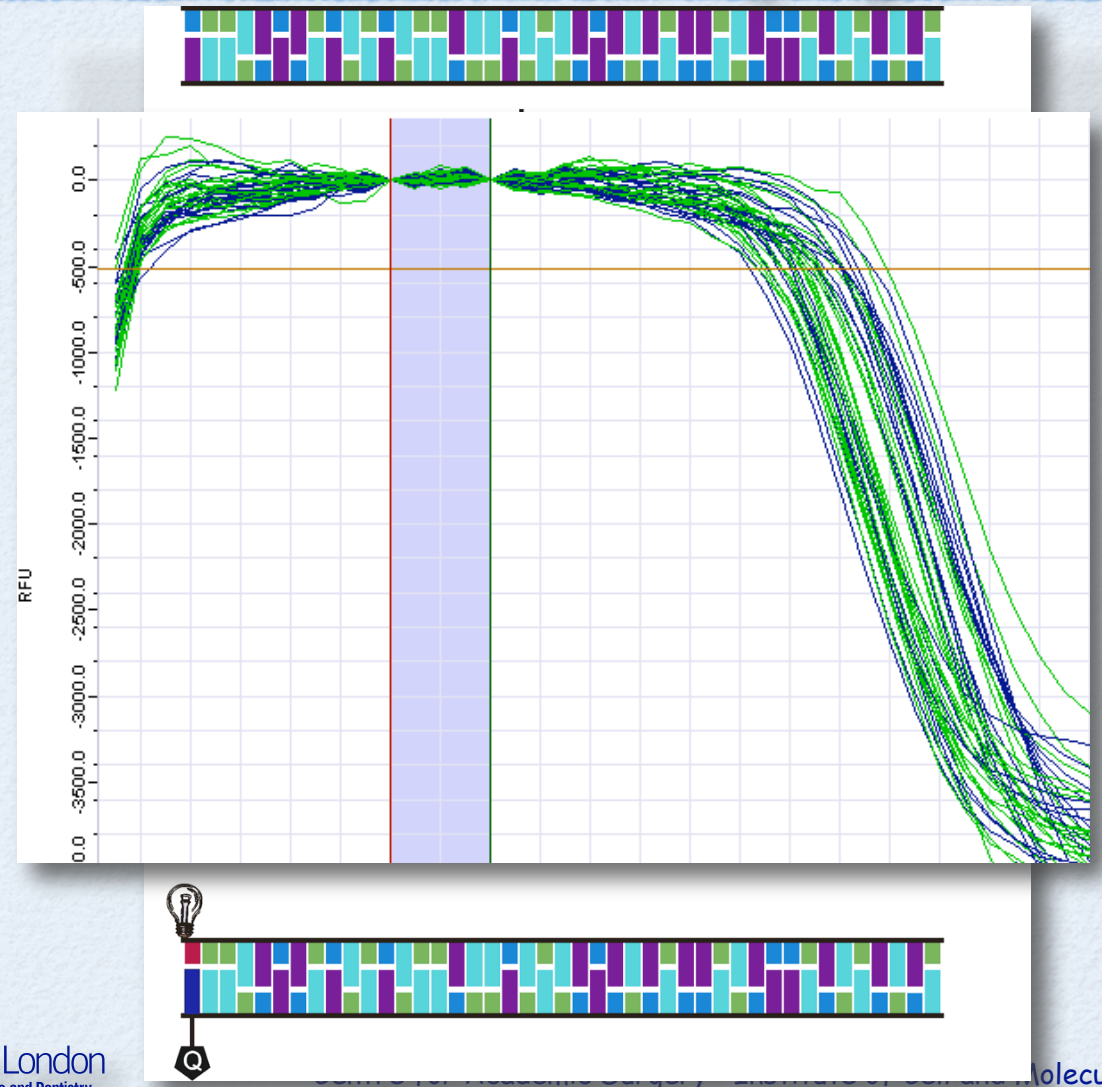
Moser & Prudent, Nucl Acids Res 31:5048-5053

Reagents

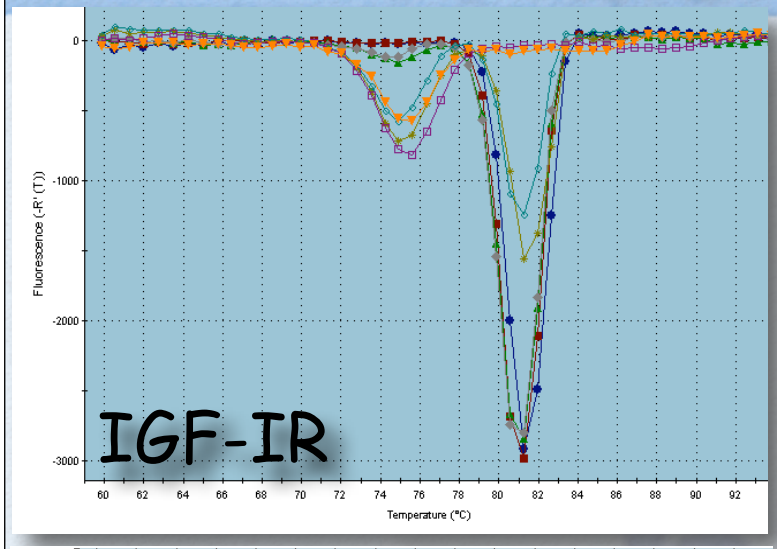


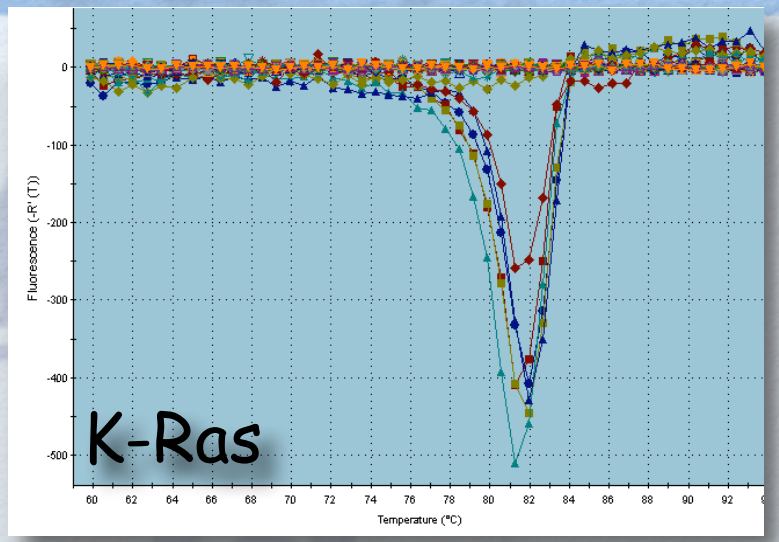


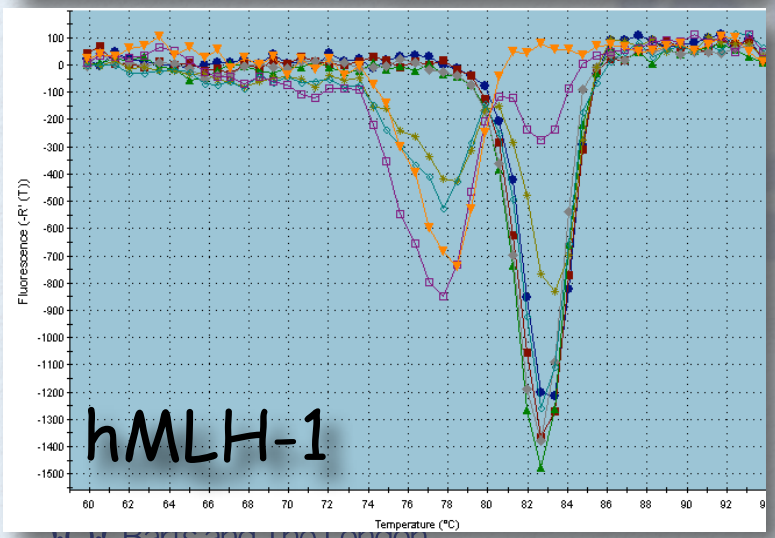
Plexor (2)

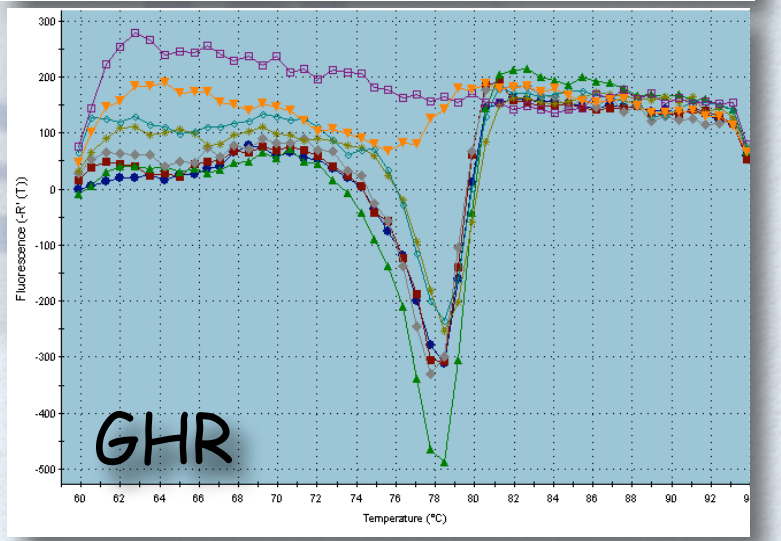


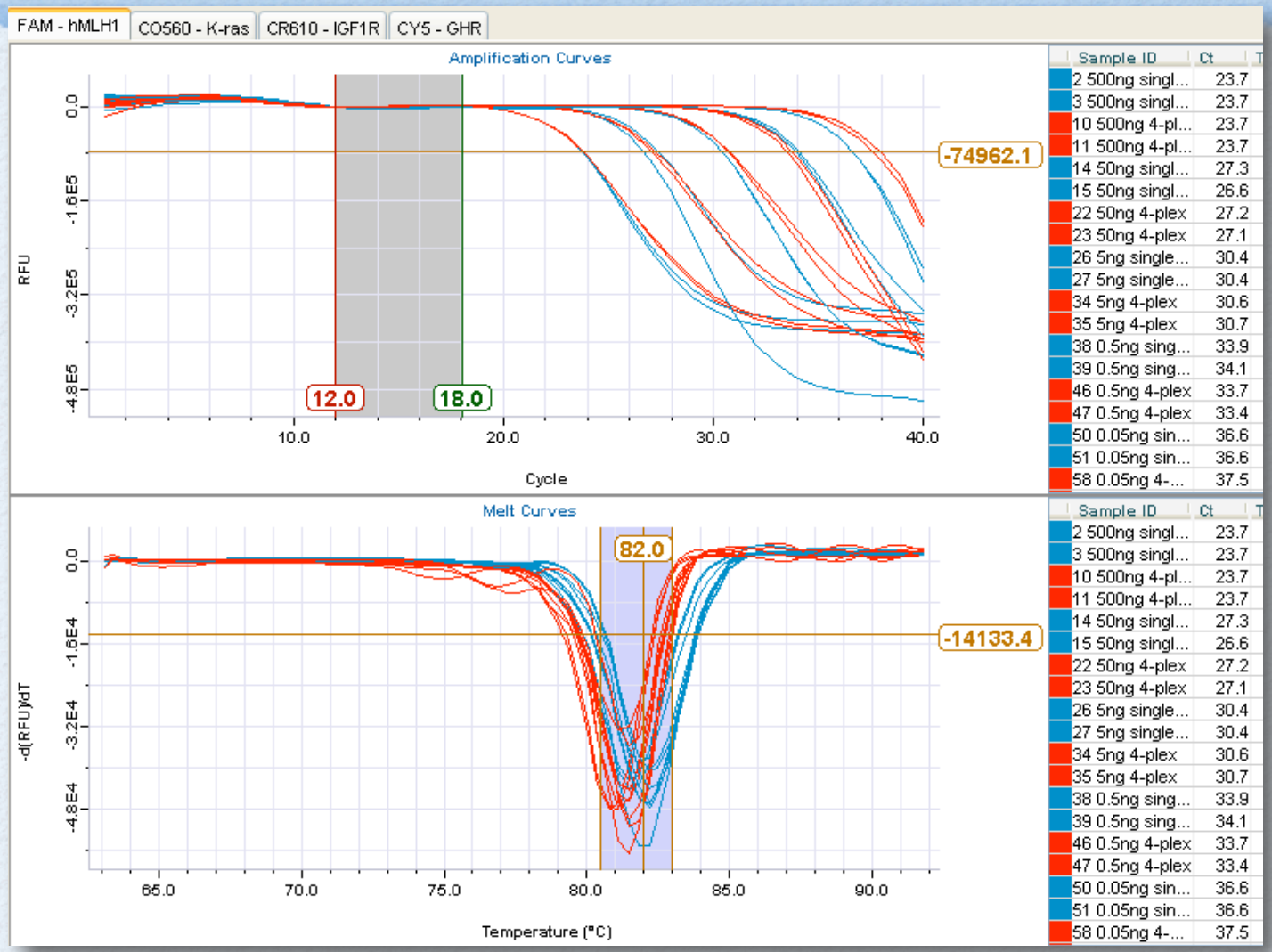
Primer evaluation









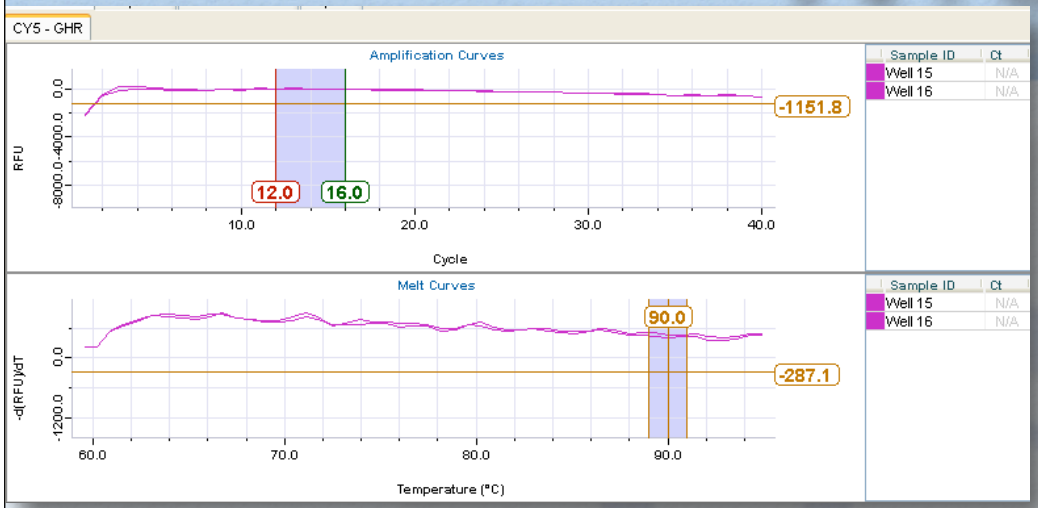

FAM - hMLH1
CO560 - K-ras
CR610 - IGF1R
CY5 - GHR
Amplification Curves
RFU
0.0
-1.6E5
-3.2E5
-4.8E5
-74962.1
12.0
18.0
10.0
20.0
30.0
40.0
Cycle
Sample ID
Ct
2 500ng singl... 23.7
3 500ng singl... 23.7
10 500ng 4-pl... 23.7
11 500ng 4-pl... 23.7
14 50ng singl... 27.3
15 50ng singl... 26.6
22 50ng 4-plex 27.2
23 50ng 4-plex 27.1
26 5ng single... 30.4
27 5ng single... 30.4
34 5ng 4-plex 30.6
35 5ng 4-plex 30.7
38 0.5ng sing... 33.9
39 0.5ng sing... 34.1
46 0.5ng 4-plex 33.7
47 0.5ng 4-plex 33.4
50 0.05ng sin... 36.6
51 0.05ng sin... 36.6
58 0.05ng 4-... 37.5
Melt Curves
-d(RFU)/dT
0.0
-1.6E4
-3.2E4
-4.8E4
82.0
-14133.4
65.0
70.0
75.0
80.0
85.0
90.0
Temperature (°C)
Sample ID
Ct
2 500ng singl... 23.7
3 500ng singl... 23.7
10 500ng 4-pl... 23.7
11 500ng 4-pl... 23.7
14 50ng singl... 27.3
15 50ng singl... 26.6
22 50ng 4-plex 27.2
23 50ng 4-plex 27.1
26 5ng single... 30.4
27 5ng single... 30.4
34 5ng 4-plex 30.6
35 5ng 4-plex 30.7
38 0.5ng sing... 33.9
39 0.5ng sing... 34.1
46 0.5ng 4-plex 33.7
47 0.5ng 4-plex 33.4
50 0.05ng sin... 36.6
51 0.05ng sin... 36.6
58 0.05ng 4-... 37.5

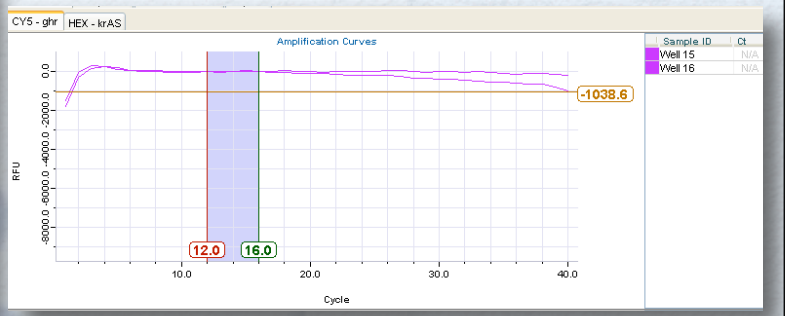
Results

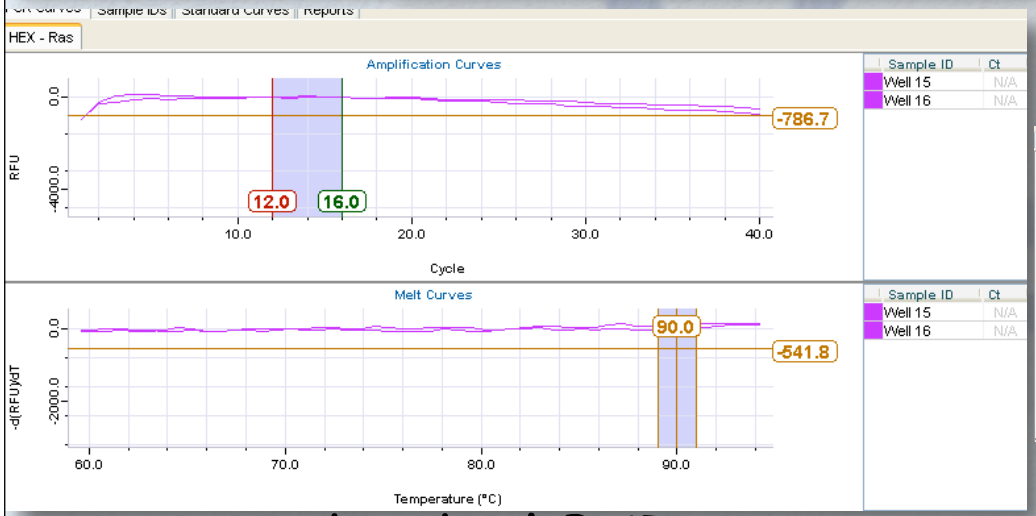
Experimental setup

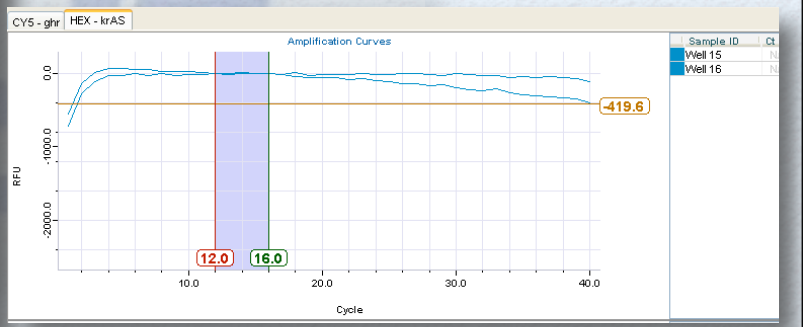
- Fresh colonic biopsies
  - cancers
  - adjacent normal tissue
- gDNA targets
  - K-Ras, GHR
- mRNA targets:
  - c-myc, VDR, GAPDH
  - Taqman™ chemistry

NTC







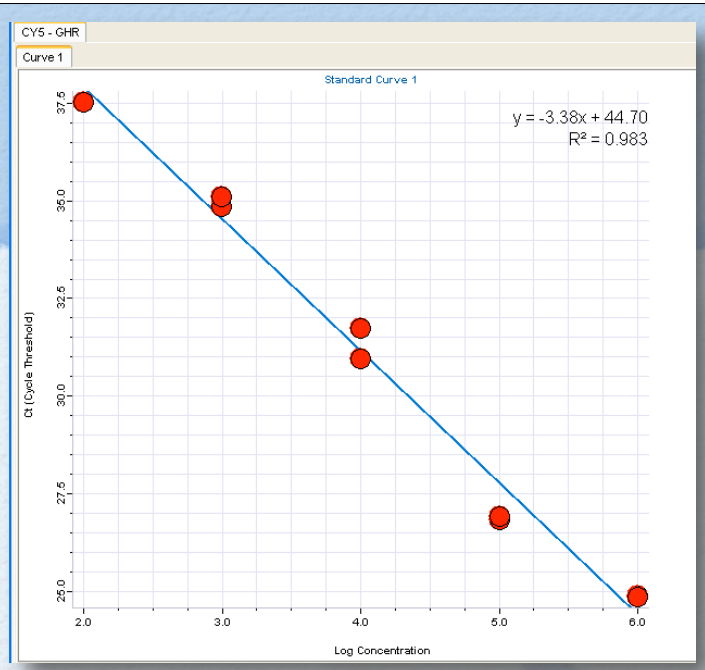


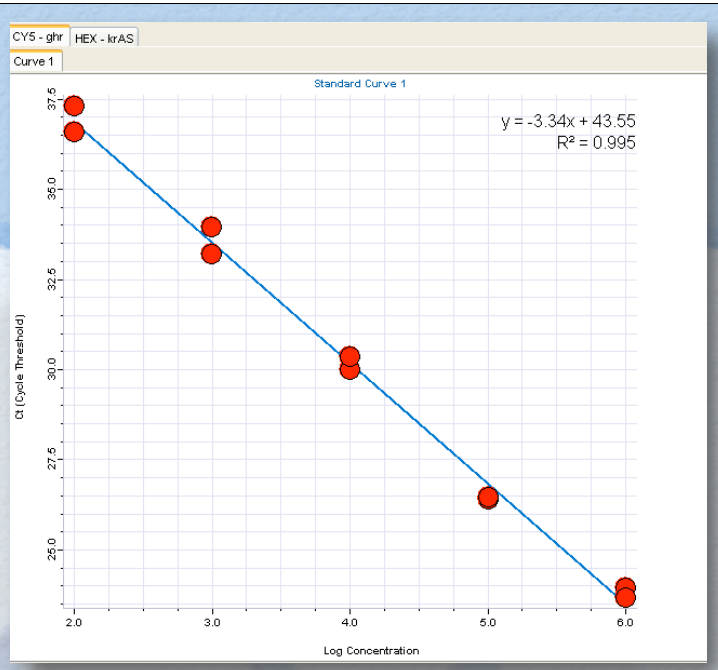
Individual PCR

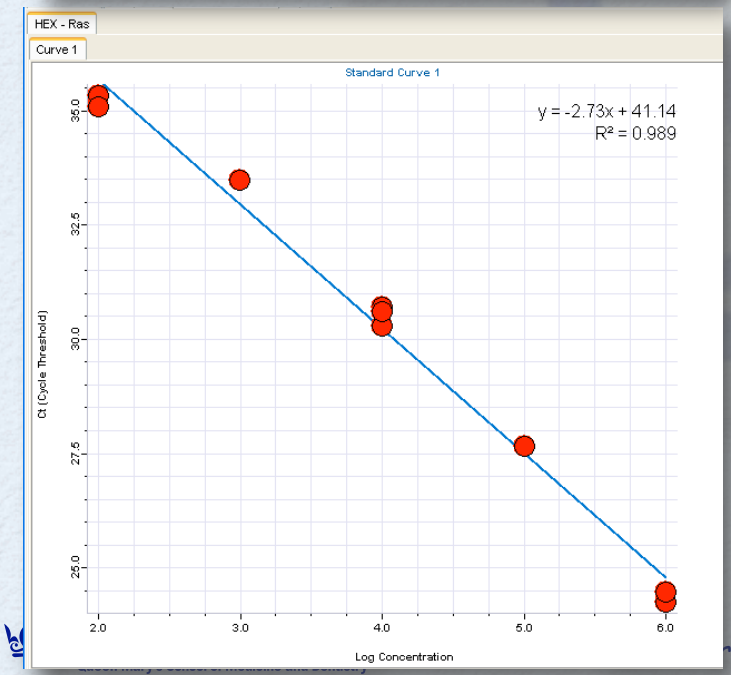
Multiplex PCR

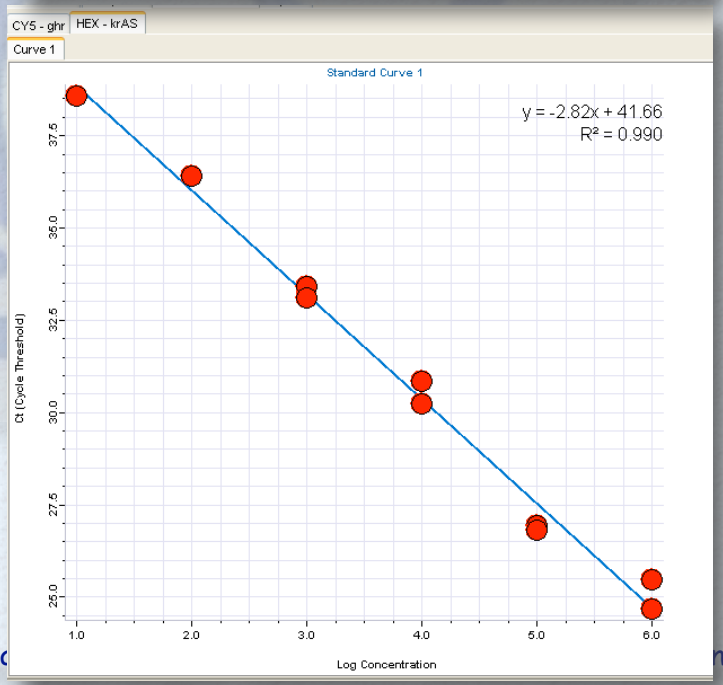
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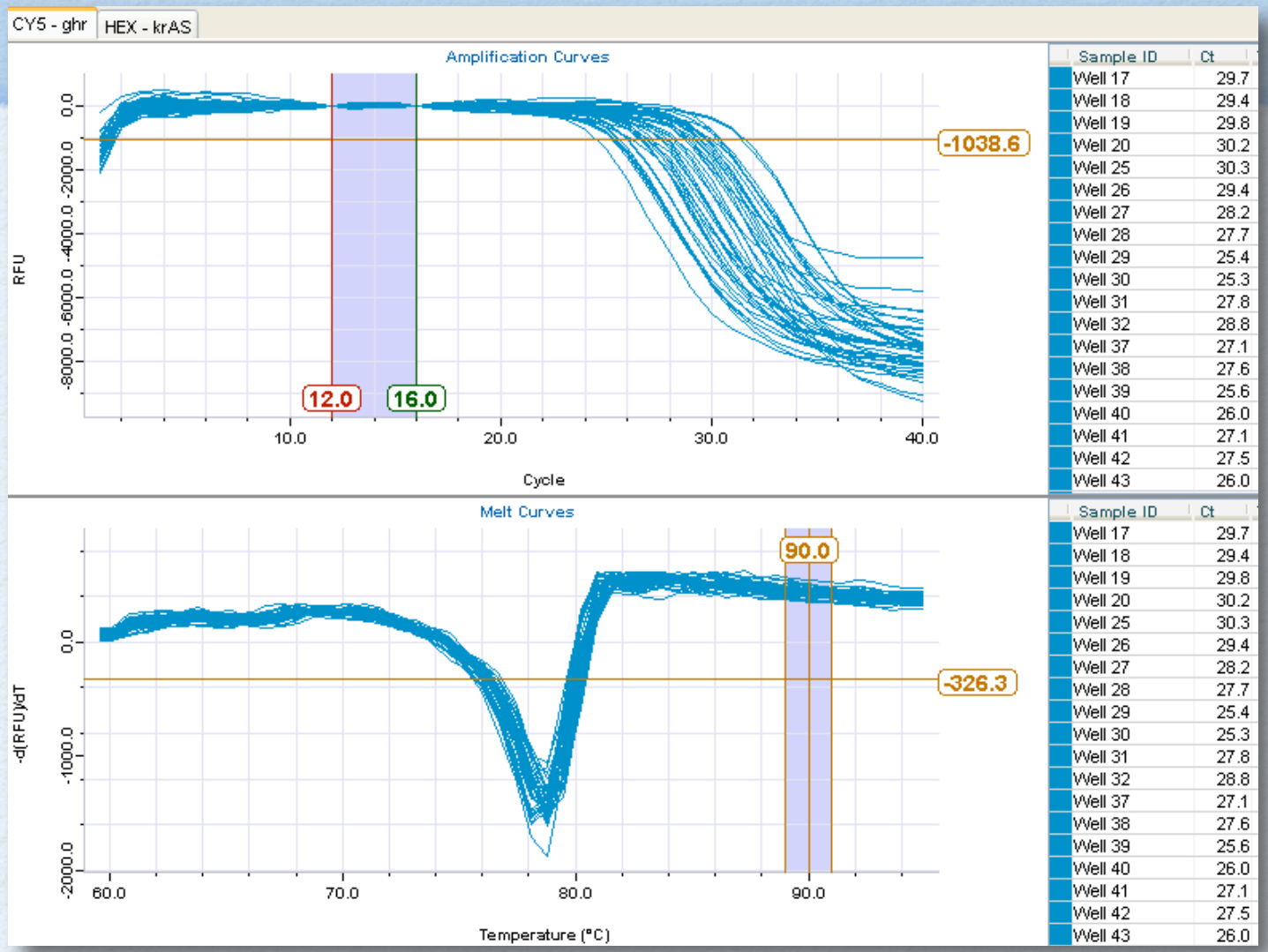
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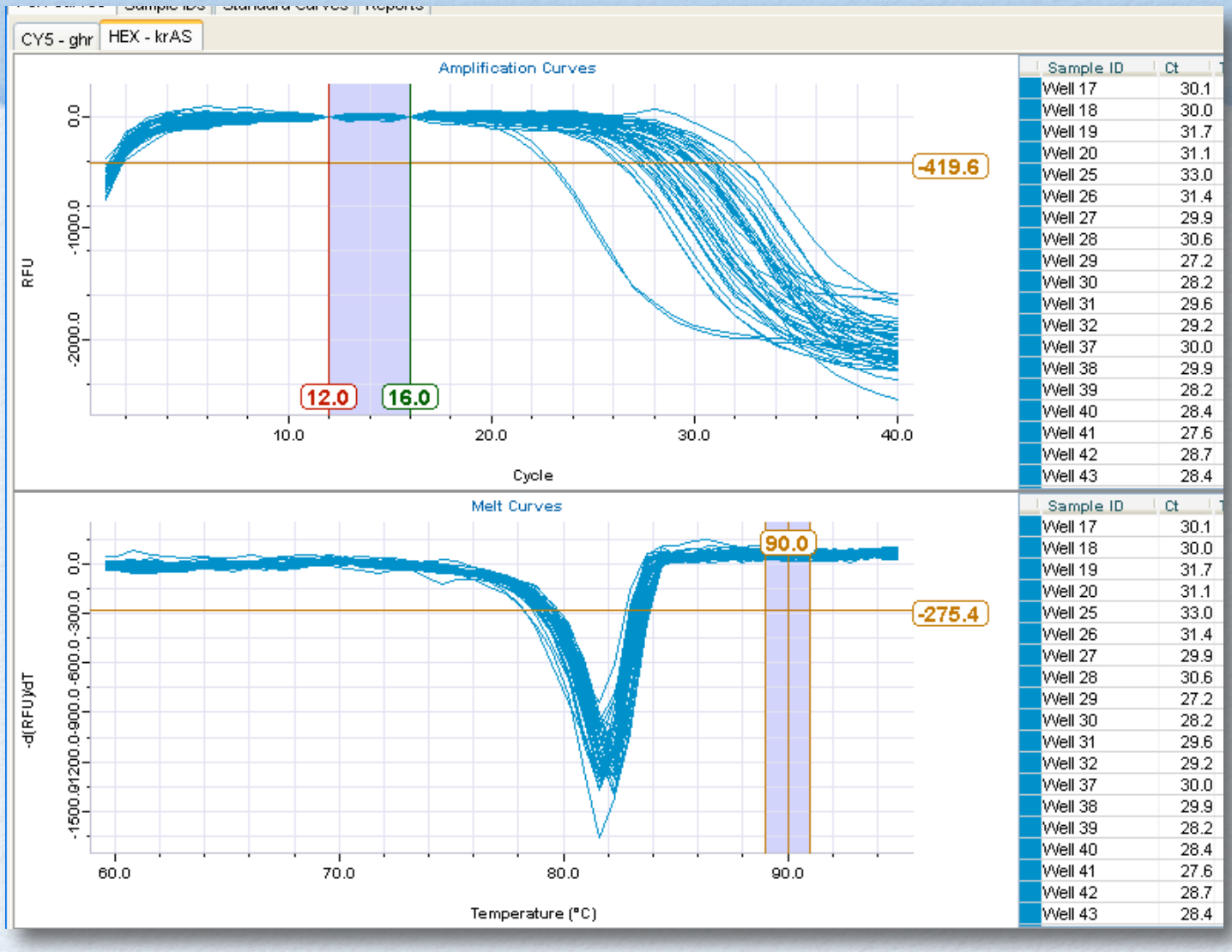

CY5 - GHR
Curve 1
Standard Curve 1
y = -3.38x + 44.70
R² = 0.983
Ct (Cycle Threshold)
Log Concentration


CY5 - ghr
HEX - krAS
Curve 1
Standard Curve 1
y = -3.34x + 43.55
R² = 0.995
Ct (Cycle Threshold)
Log Concentration

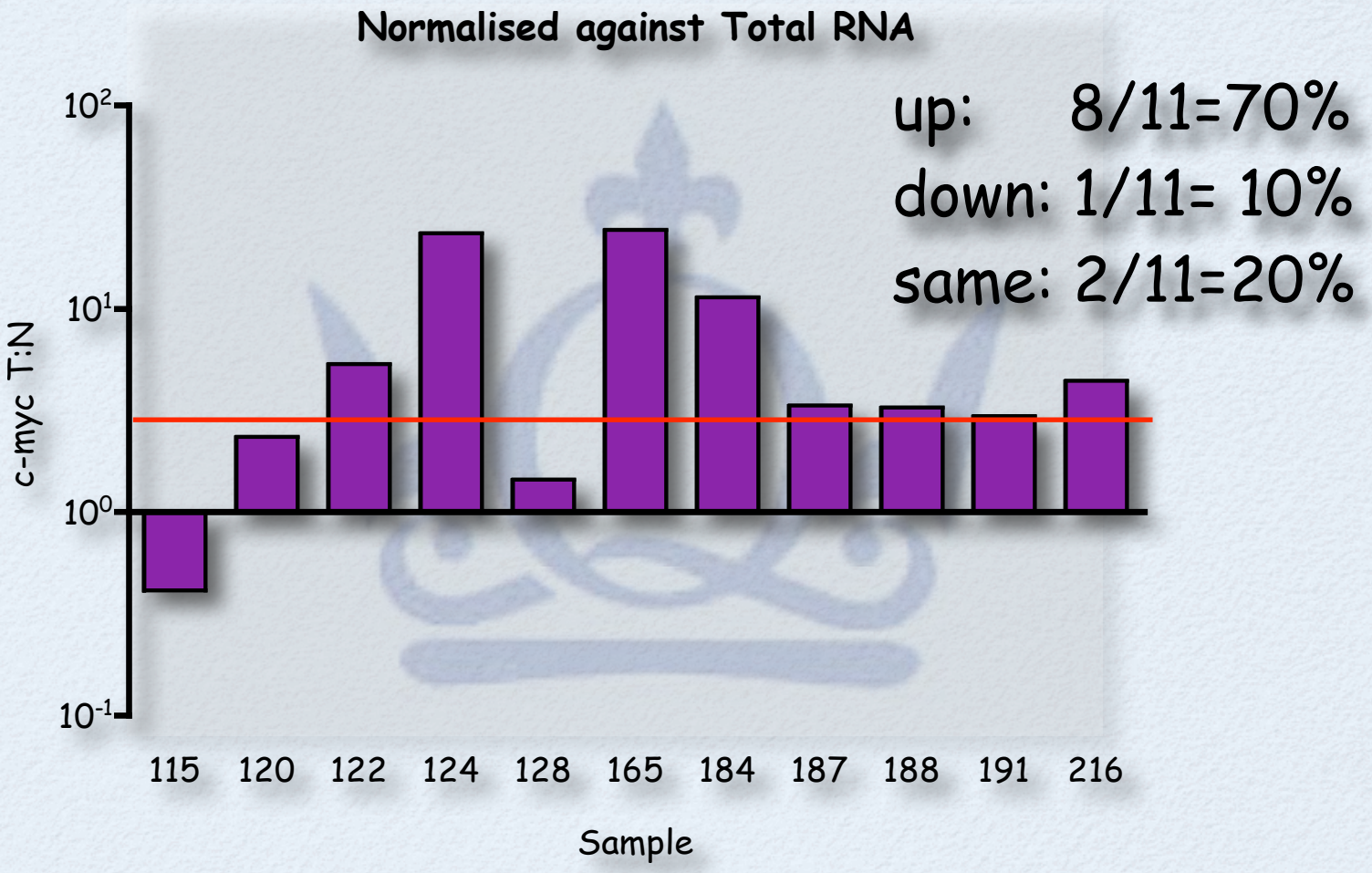

HEX - Ras
Curve 1
Standard Curve 1
y = -2.73x + 41.14
R² = 0.989
Ct (Cycle Threshold)
Log Concentration


CY5 - ghr
HEX - krAS
Curve 1
Standard Curve 1
y = -2.82x + 41.66
R² = 0.990
Ct (Cycle Threshold)
Log Concentration


CY5 - ghr
HEX - krAS
Amplification Curves
RFU
0.0
-2000.0
-4000.0
-6000.0
-8000.0
-1038.6
12.0
16.0
10.0
20.0
30.0
40.0
Cycle
Melt Curves
-d(RFU)/dT
0.0
-1000.0
-2000.0
90.0
-326.3
60.0
70.0
80.0
90.0
Temperature (°C)
Sample ID
Ct
Well 17 29.7
Well 18 29.4
Well 19 29.8
Well 20 30.2
Well 25 30.3
Well 26 29.4
Well 27 28.2
Well 28 27.7
Well 29 25.4
Well 30 25.3
Well 31 27.8
Well 32 28.8
Well 37 27.1
Well 38 27.6
Well 39 25.6
Well 40 26.0
Well 41 27.1
Well 42 27.5
Well 43 26.0


CY5 - ghr
HEX - krAS
Amplification Curves
RFU
0.0
-1000.0
-2000.0
-419.6
12.0
16.0
10.0
20.0
30.0
40.0
Cycle
Sample ID
Ct
Well 17 30.1
Well 18 30.0
Well 19 31.7
Well 20 31.1
Well 25 33.0
Well 26 31.4
Well 27 29.9
Well 28 30.6
Well 29 27.2
Well 30 28.2
Well 31 29.6
Well 32 29.2
Well 37 30.0
Well 38 29.9
Well 39 28.2
Well 40 28.4
Well 41 27.6
Well 42 28.7
Well 43 28.4
Melt Curves
-d(RFU)/dT
0.0
-300.0
-600.0
-900.0
-1200.0
-1500.0
90.0
-275.4
60.0
70.0
80.0
90.0
Temperature (°C)
Sample ID
Ct
Well 17 30.1
Well 18 30.0
Well 19 31.7
Well 20 31.1
Well 25 33.0
Well 26 31.4
Well 27 29.9
Well 28 30.6
Well 29 27.2
Well 30 28.2
Well 31 29.6
Well 32 29.2
Well 37 30.0
Well 38 29.9
Well 39 28.2
Well 40 28.4
Well 41 27.6
Well 42 28.7
Well 43 28.4

c-myc expression in CRC



c-myc upregulation in CRC

Biochemical and Biophysical Research Communications 271, 537–543 (2000)
doi:10.1006/bbrc.2000.2666, available online at http://www.idealibrary.com on IDEAL®

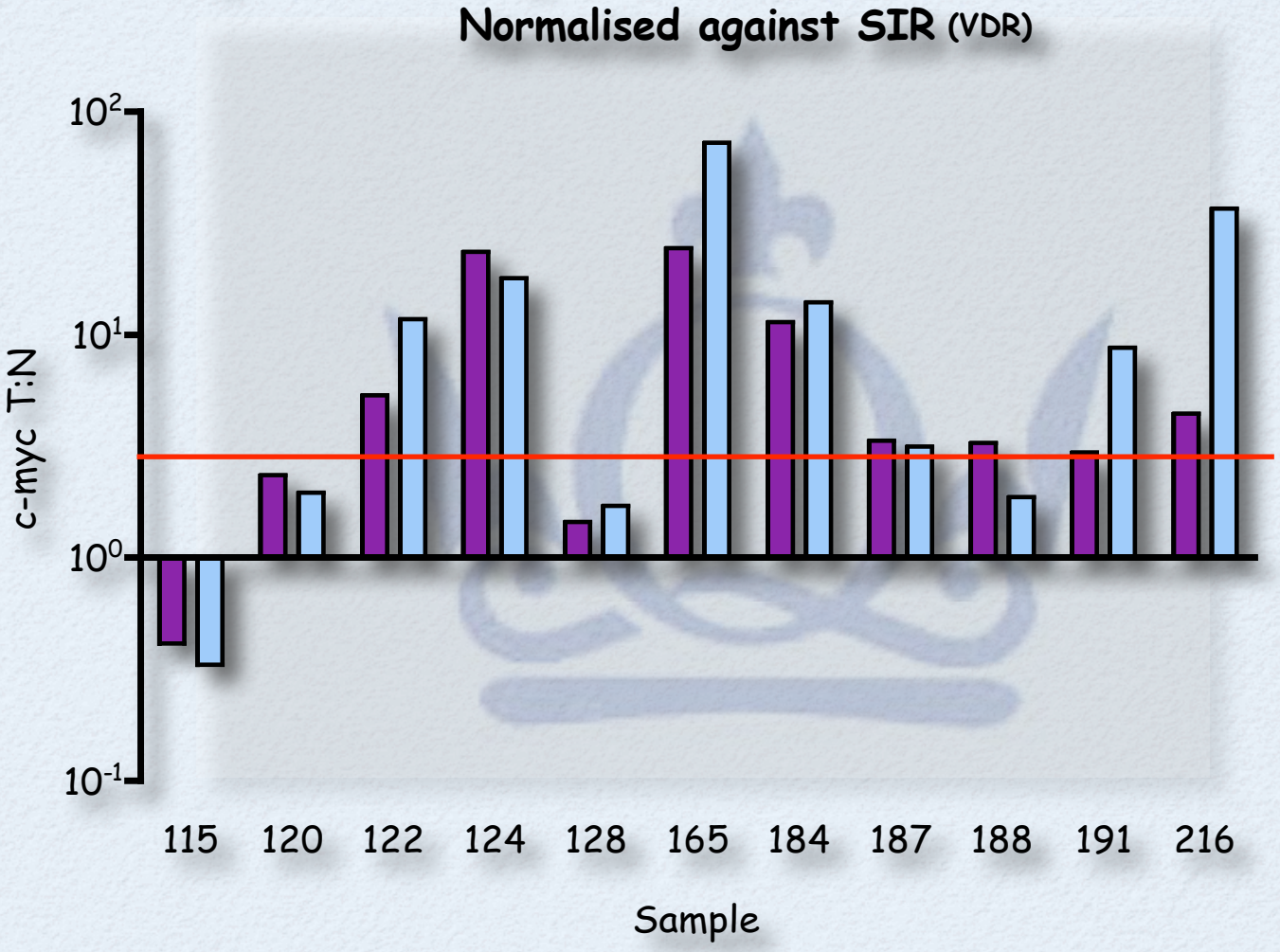
Elevated Levels of RanBP7 mRNA in Colorectal Carcinoma Are Associated with Increased Proliferation and Are Similar to the Transcription Pattern of the Proto-oncogene *c-myc*

Shu-Rui Li,[1] Valerie G. Gyselman, Sina Dorudi, and Stephen A. Bustin
Academic Department of Surgery, St Bartholomew's and the Royal London School of Medicine and Dentistry, London, United Kingdom

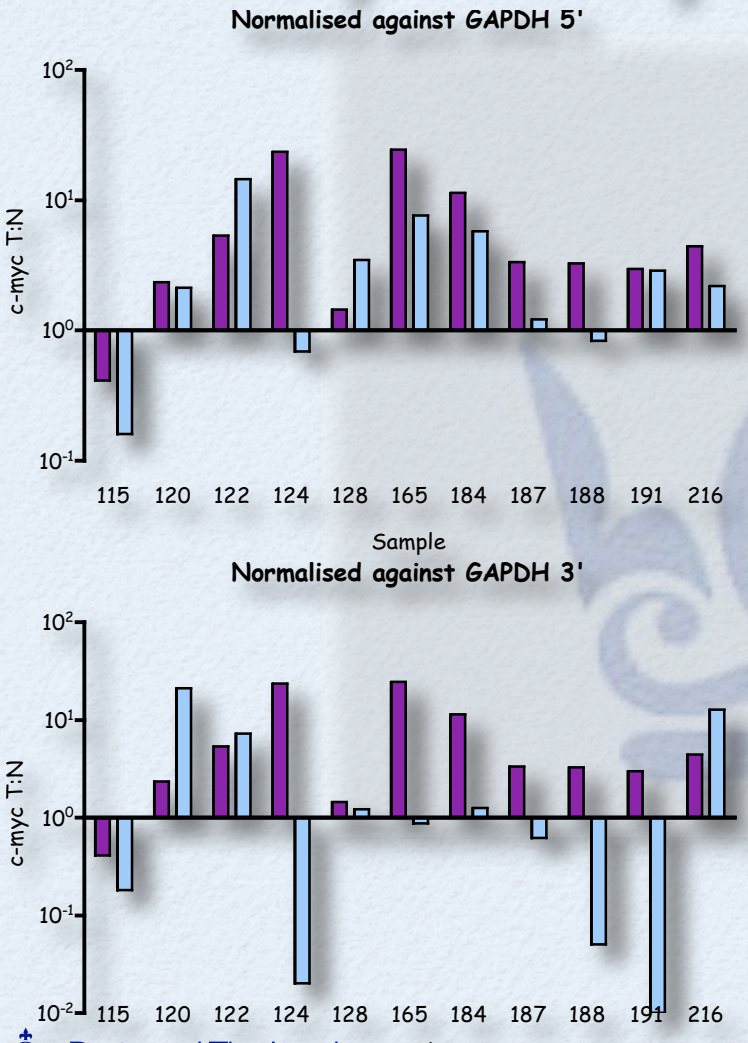
Received April 6, 2000

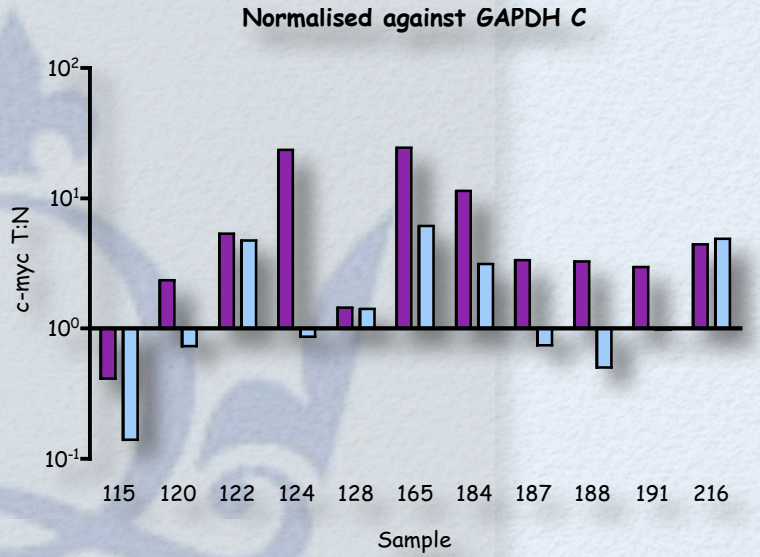
U test) and significant up-regulation of *c-myc* mRNA was observed in 52 tumours (69%). Lower levels of *c-myc* expression were observed in 10 tumours (13%) and levels were essentially the same in 13 tumours (17%) (Fig. 6A). However, when *c-myc* mRNA levels

c-myc expression in CRC



c-myc expression in CRC

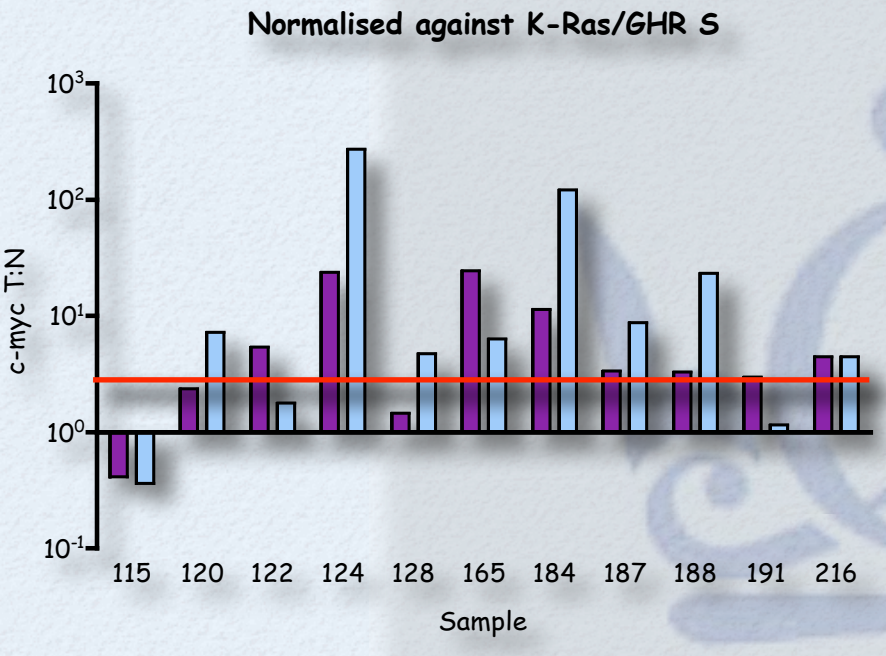


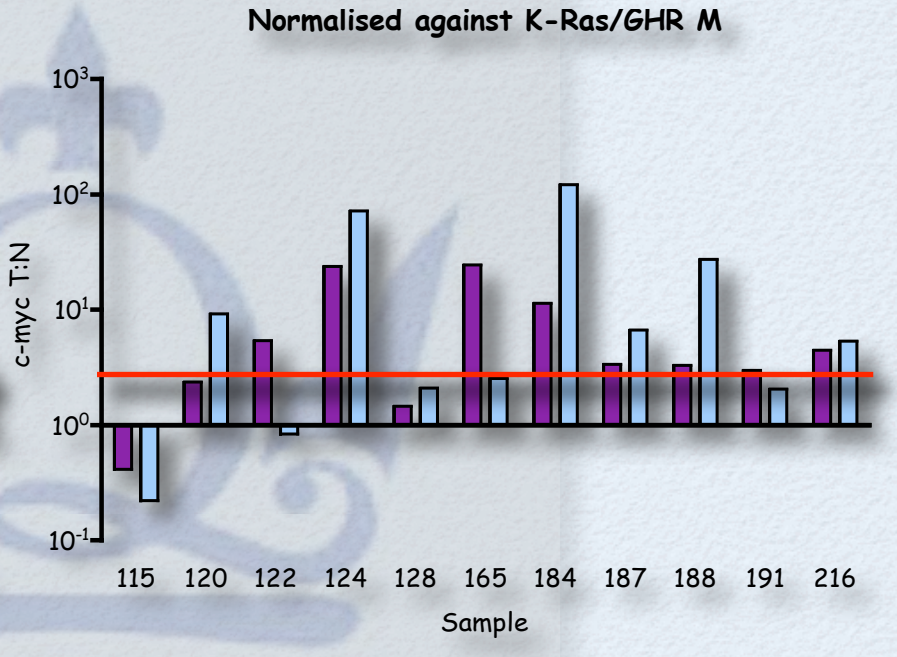


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c-myc expression in CRC





Microdissection

Sample

- Fresh colonic tissue
  - adjacent normal
  - cancer
- Embedded/frozen in OCT
- Microdissected using PALM system

BMC Biotechnology

BioMed Central

Methodology article

Open Access

Rapid, single-tube method for quantitative preparation and analysis of RNA and DNA in samples as small as one cell

Cristina Hartshorn*[1], Aleksandra Anshelevich[1,2] and Lawrence J Wangh[1]

Address: [1]De
Medical Cen
Email: Cristi
Lawrence J W
* Correspon

Published: 13
BMC Biotechn

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gy, Beth Israel

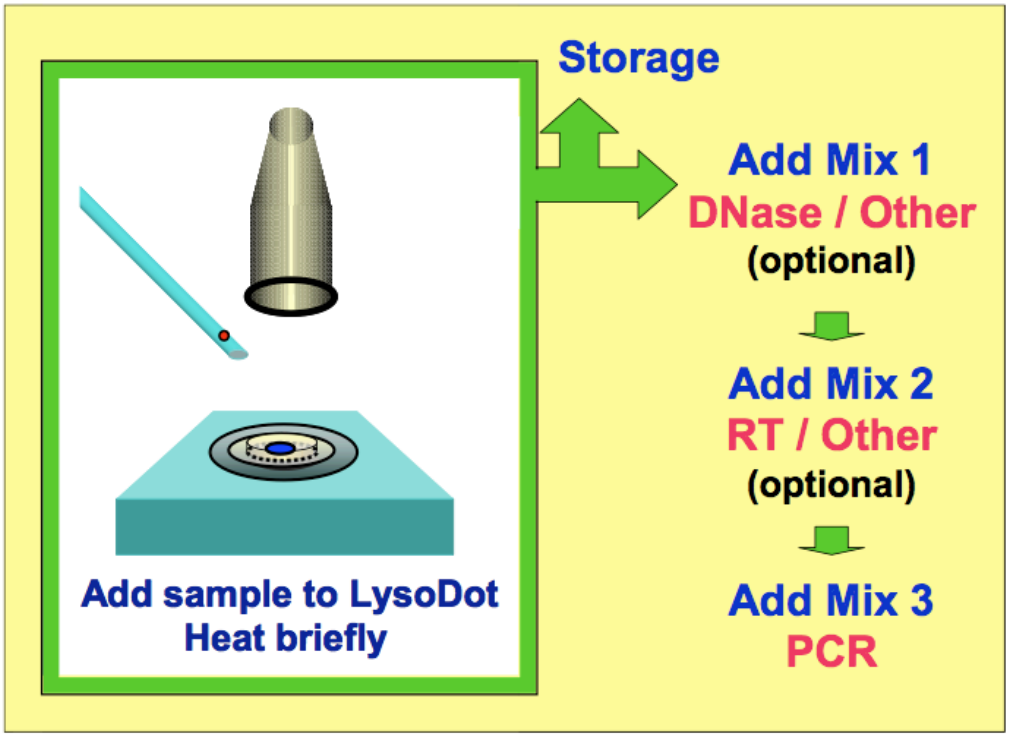
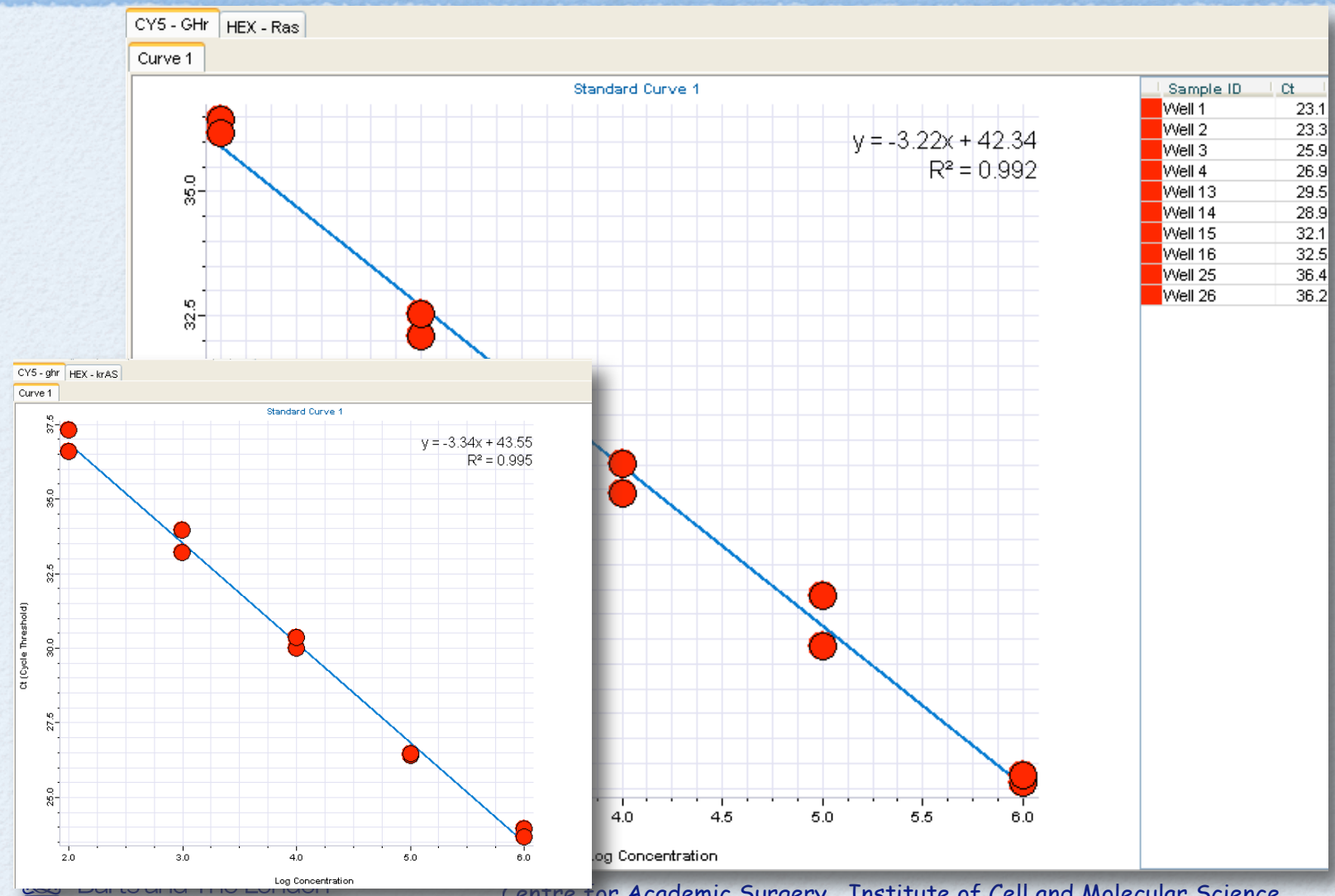


Figure 6
Easiness and versatility of the PurAmp method. In a streamlined version of the procedure, LysoDots (20 nl or less, see Methods for a definition) are dispensed to the lids of PCR tubes resting on a heating block. The LysoDots are quickly dried before adding the samples in a minimal volume of PBS. A second brief heating reduces the lysed samples to the semi-dry state, ready for storage or immediate processing, which may include a variable number and type of enzymatic reactions prior to the

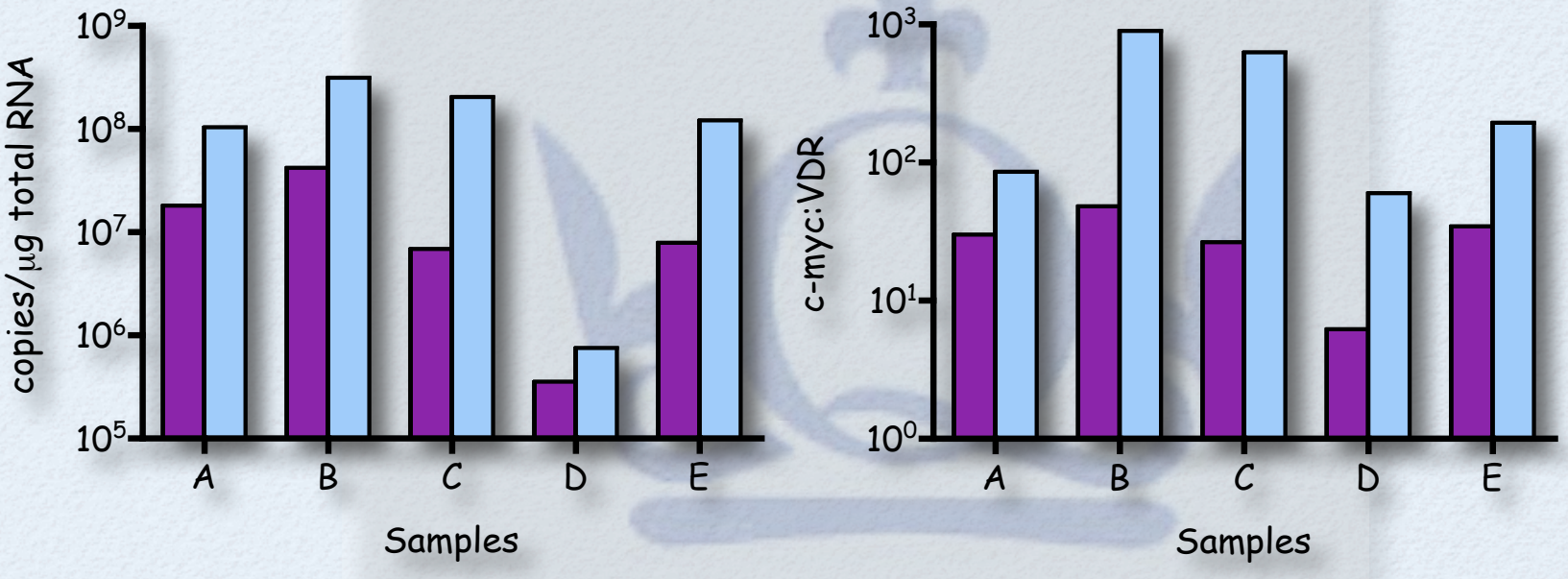
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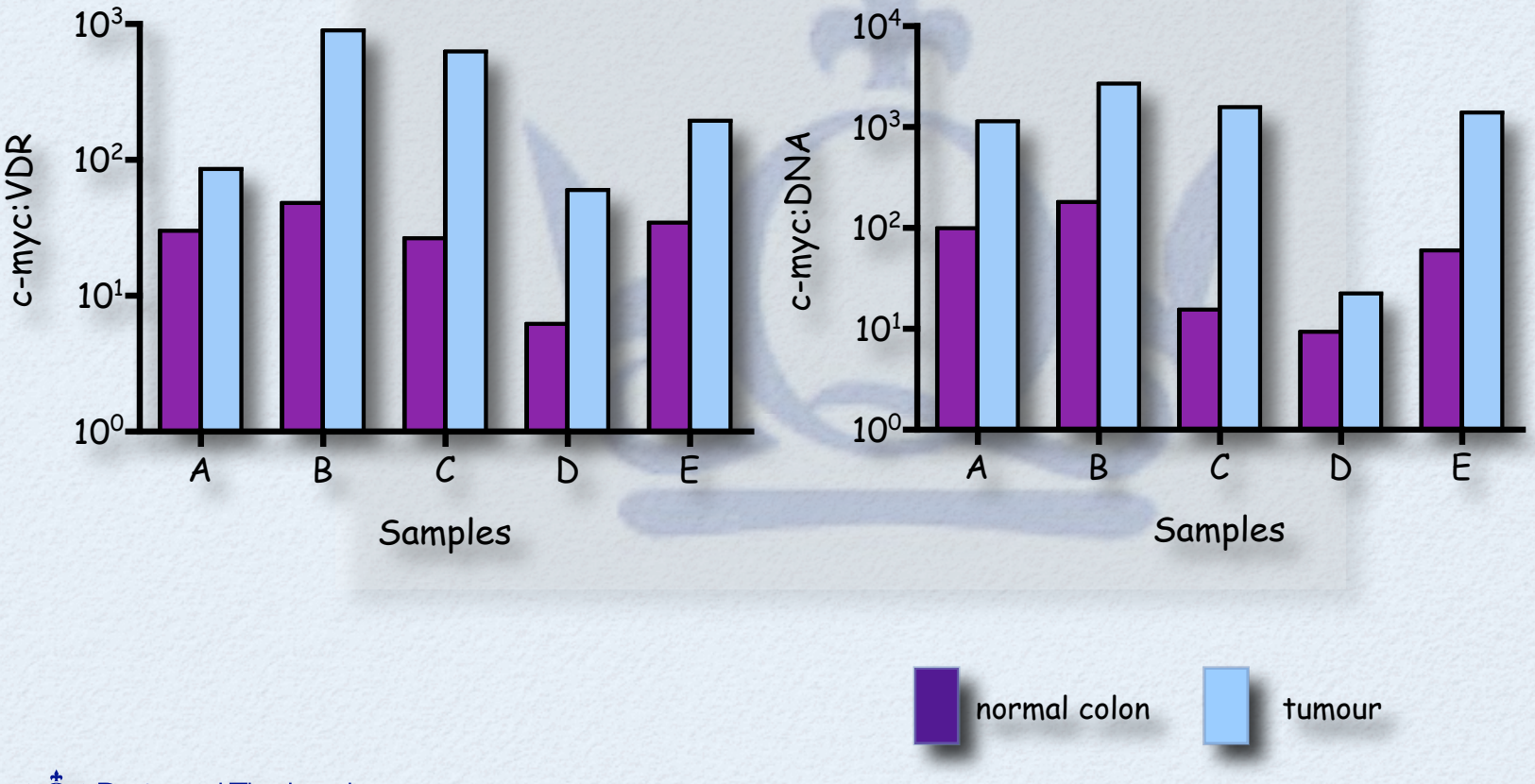
No need for extraction



c-myc expression N vs T



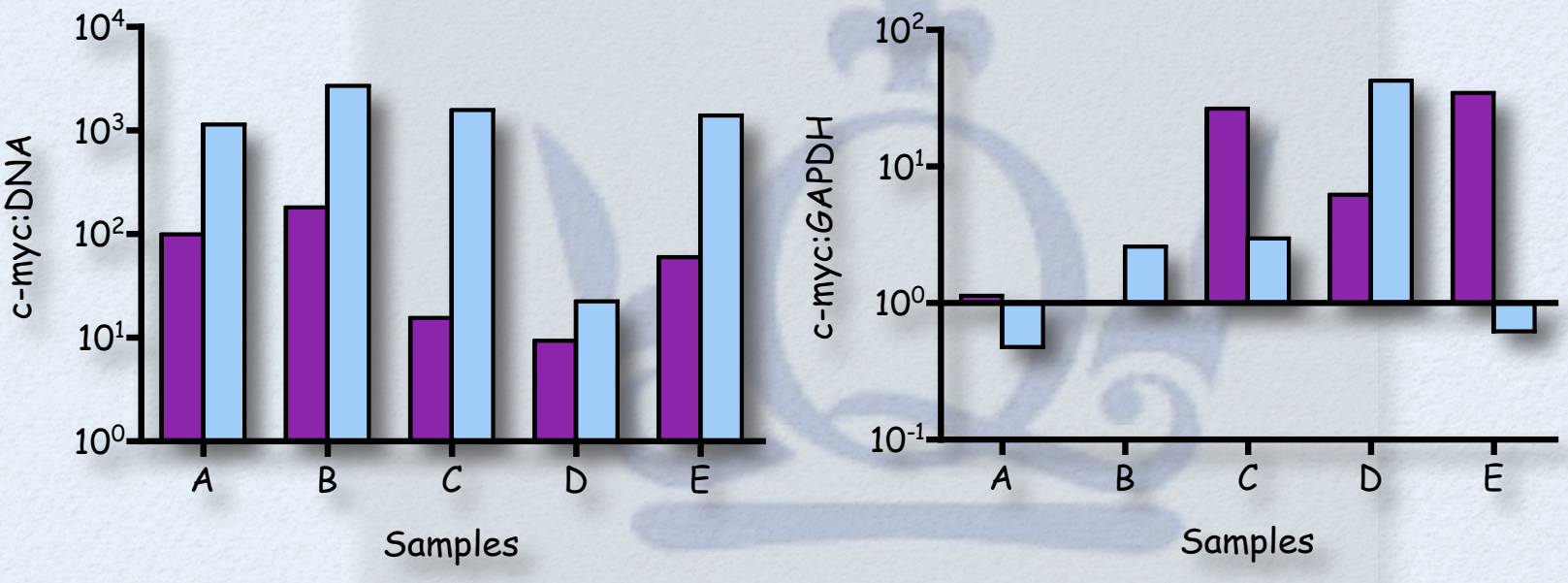
c-myc expression N vs T



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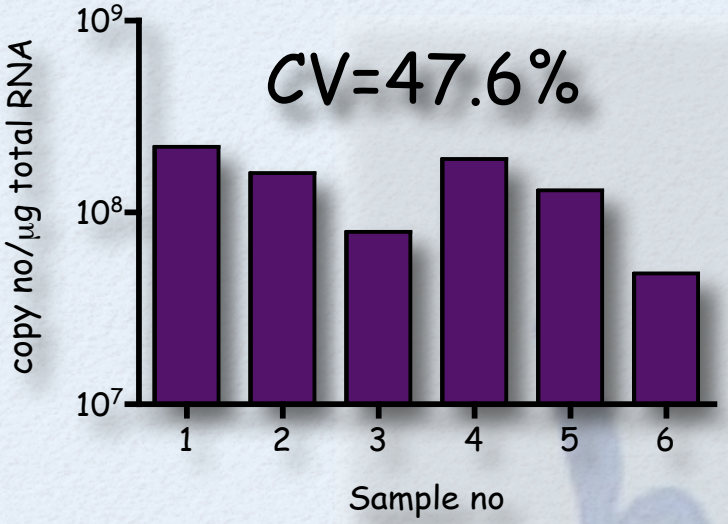
c-myc expression N vs T

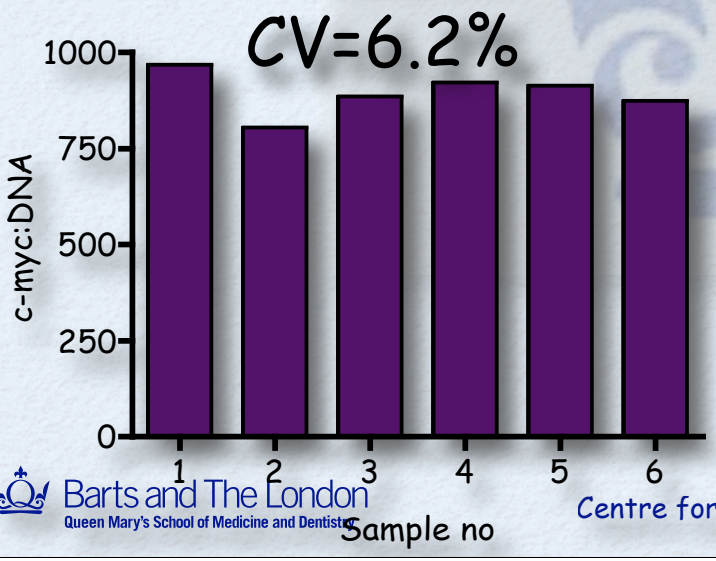


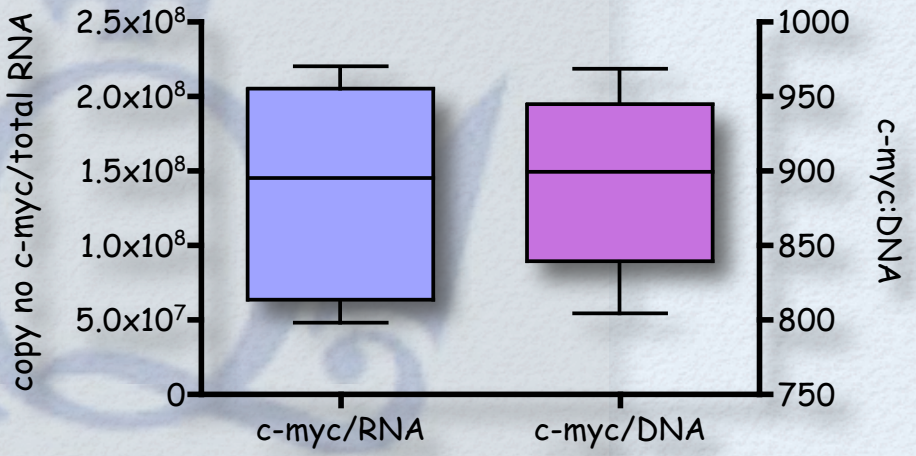
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Reproducibility







Disadvantages

- DNA normalisation does not address differential stability of DNA and RNA
  - not suitable for poor quality RNA
- Intronless genes not easily accommodated
  - requires assays pre- and post- DNase treatment

Summary

- Used Promega's Plexor chemistry for multiplexing of four DNA targets
- Normalisation against gDNA is feasible
- In colon, results similar to normalisation against
  - total RNA
  - SIR

Conclusions

- Simple, not quite universal normalisation method
- Suited to quantification from very small samples

Acknowledgements



Dr Shu-Rui Li

Promega

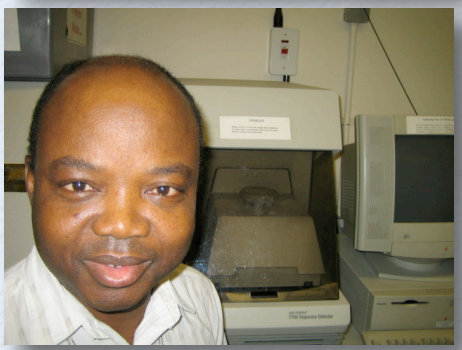
Dr Doug Storts
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Dr Ethan Strauss



Miss Becky Hands



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bowel & cancer research

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