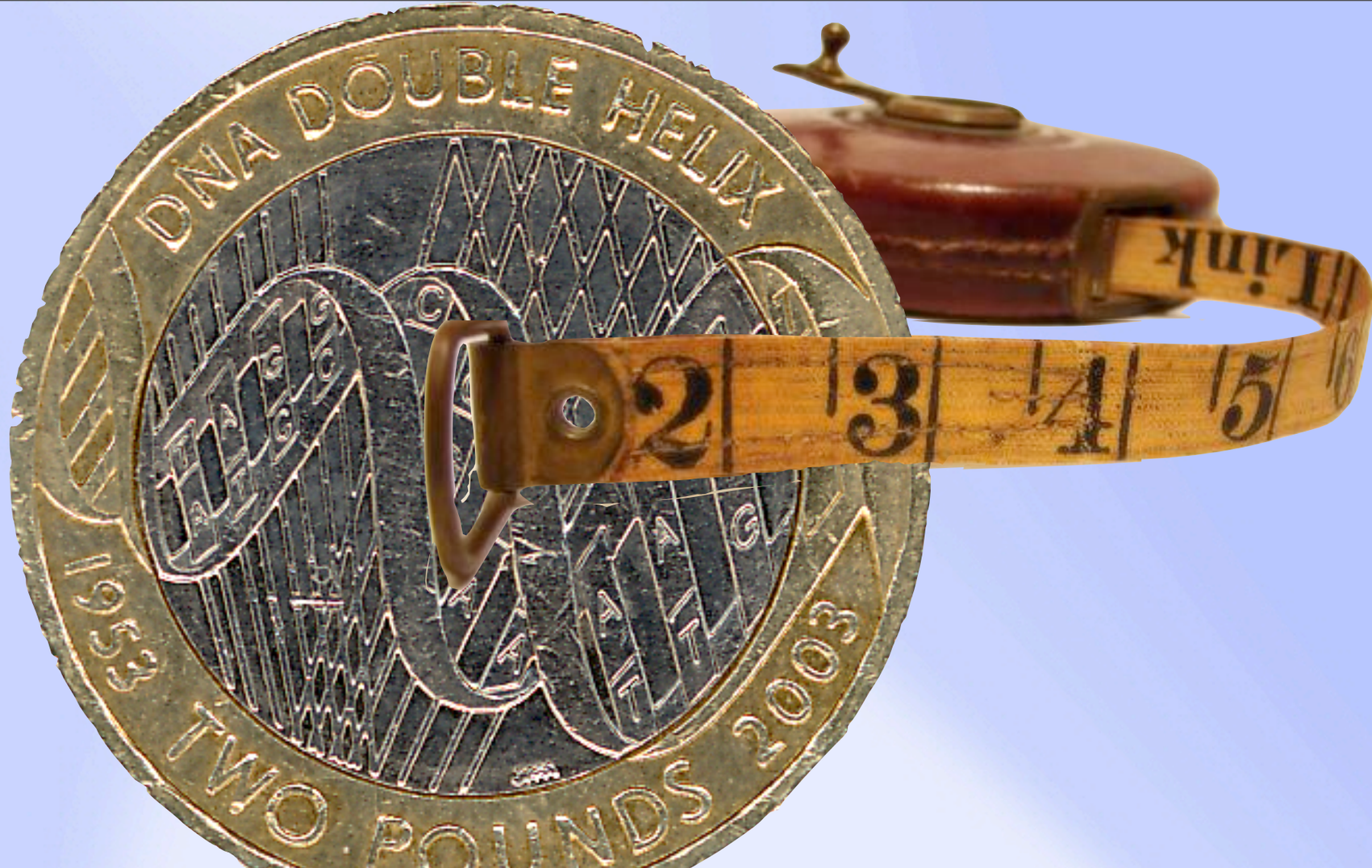
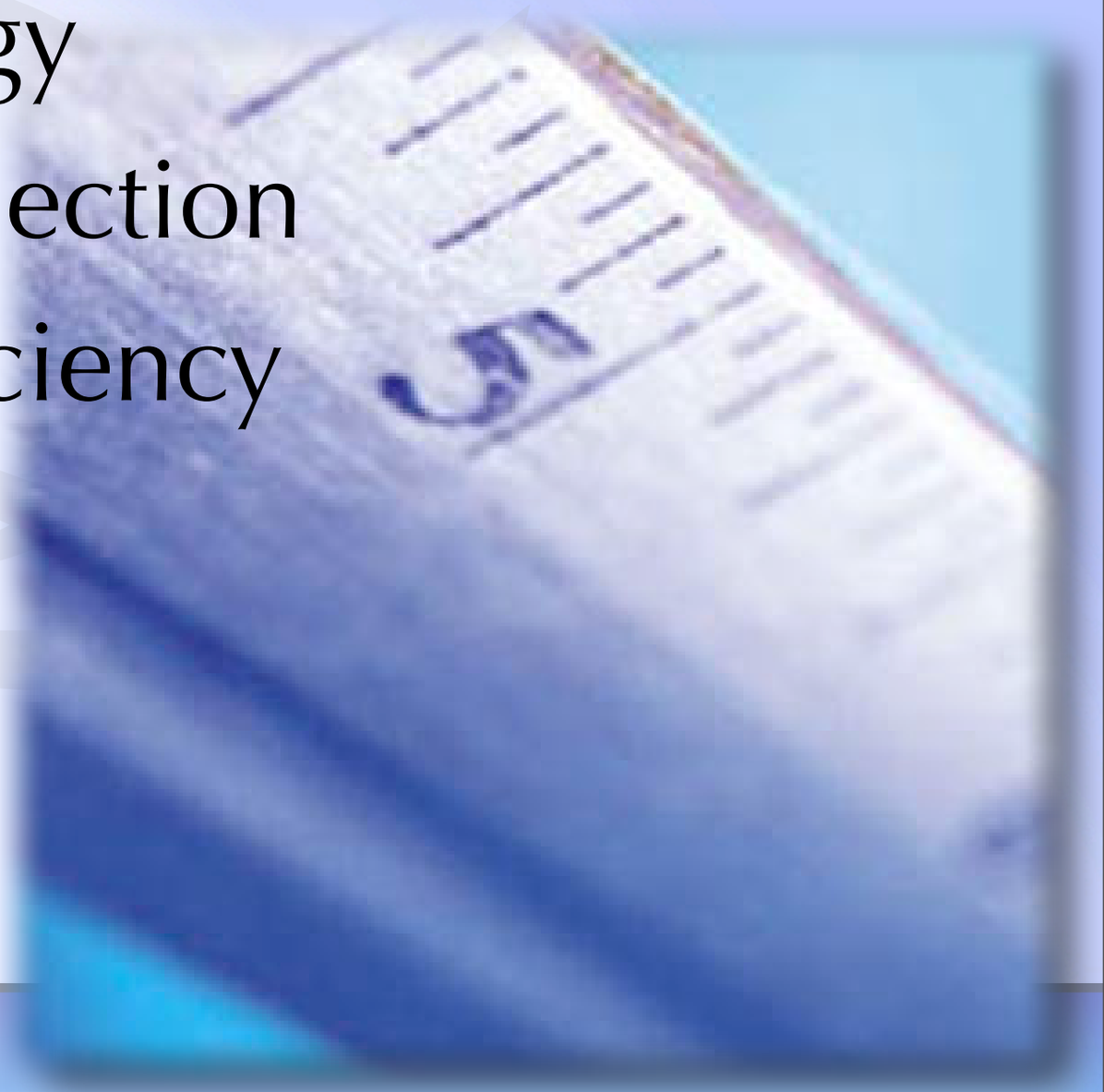


Essential considerations for generating reliable RT-qPC data

Stephen A Bustin BA (Mod) PhD
Professor of Molecular Science

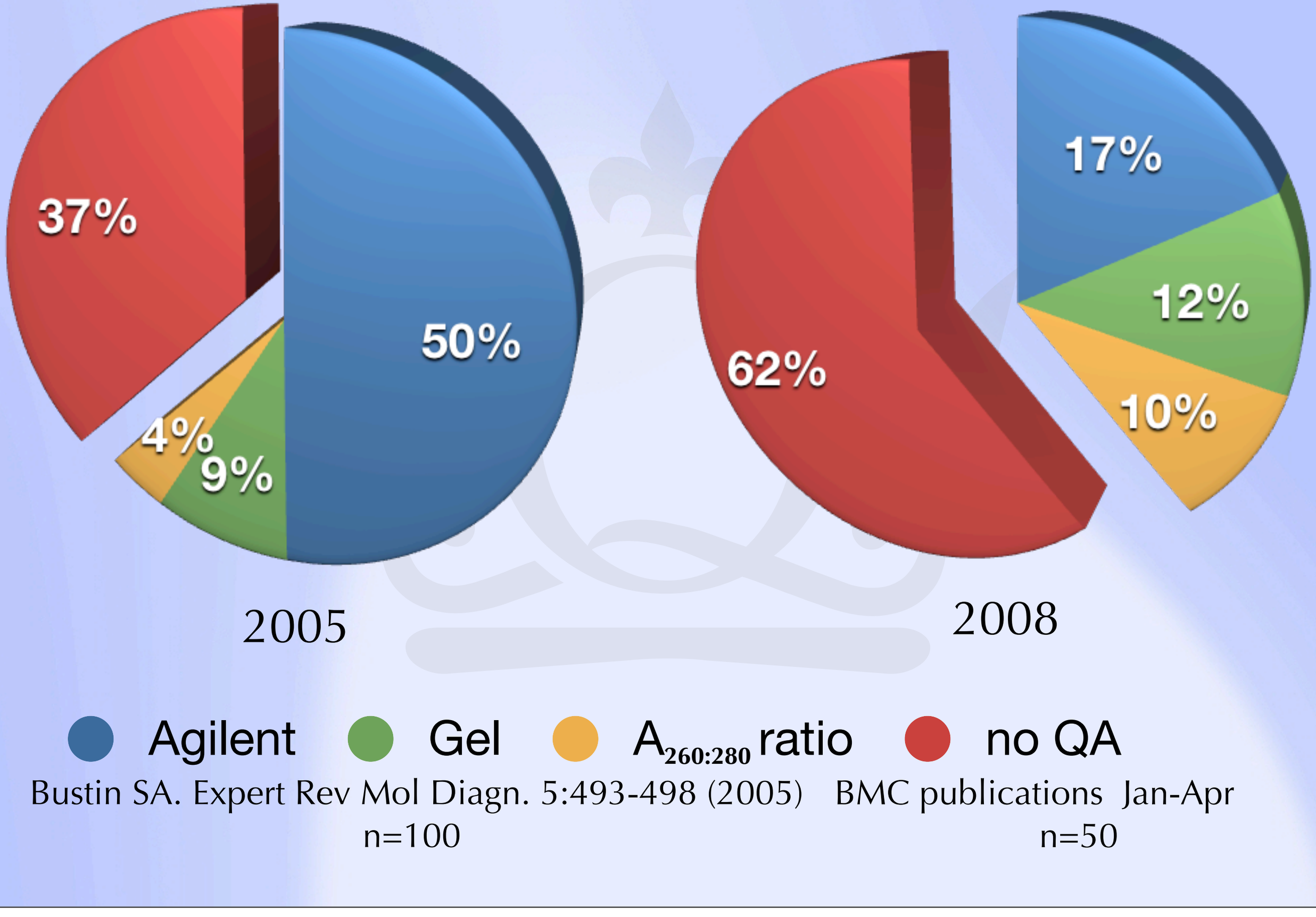
Barts and The London
School of Medicine and Dentistry

- Sample selection and handling
- RNA quality assessment
- Reverse transcription
- cDNA synthesis strategy
- RT and PCR primer selection
- PCR amplification efficiency
- Data analysis
- Data reporting



RT-qPCR problems

How do you quality assess your RNA?



Bustin SA. Expert Rev Mol Diagn. 5:493-498 (2005) n=100

BMC publications Jan-Apr n=50

Pancreas • Volume 36, Number 1, January 2008

Stem Cell Marker Prominin-1/AC133 Is Expressed in Duct Cells of the Adult Human Pancreas

Jessy Lardon, PhD, Denis Corbeil, PhD,† Wieland B. Huttner, PhD, MD,‡ Zhidong Ling, PhD,§ and Luc Bouwens, PhD**

Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was performed to quantify the expression level of prominin-1 transcripts. Total RNA was isolated from cultured exocrine cells using the GenElute Mammalian Total RNA Miniprep kit (Sigma, St Louis, Mo). Complementary DNA was prepared from 500 ng of total RNA after DNase treatment and 10 ng of RNA equivalent used for PCR with specific primers (see below) in the presence of SYBR Green I. Polymerase chain reaction reagents were from Abgene (Epsom, UK). A melt curve analysis was performed at the end of each reaction. Values (mean ± SEM) are from 4 independent experiments. Expression levels were normalized to individual glyceralde-hyde-3-phosphate dehydrogenase (GAPDH, internal control).

This month:
TheScientist
MAGAZINE OF THE LIFE SCIENCES

April 2007

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By Edyta Zielinska

NEWS

Science retracts major *Arabidopsis* paper

Scientist acknowledges omitting data, but denies any impropriety

[Published 20th April 2007 03:47 PM GMT]

Four out of five authors of a *Science* paper that the journal called a "breakthrough of the year" in 2005 have retracted it, saying that the data it was based on could not be replicated.

The study, which described the migration of mRNA to initiate flowering, was based on real-time PCR data, which researchers in the *Umeå* Plant Science Center lab where it had been performed found impossible to replicate. According to principle investigator Ove Nilsson, first author Tao Huang had manipulated data, removing certain points and giving increased weight to others.

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 22, Number 12, 2006, pp. 1253–1259

Quantitative Analysis of Human Endogenous Retrovirus-W *env* in Neuroinflammatory Diseases

JOSEPH M. ANTONY,[1] MARYAM IZAD,[1,2] AMIT BAR-OR,[3] KENNETH G. WARREN,[4] MOHAMMED VODJGANI,[2] FRANCOIS MALLET,[5] and C. POWER[1,4]

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 23, Number 10, 2007, pp. 1251–1256

DOI: 10.1089/aid.2006.0274

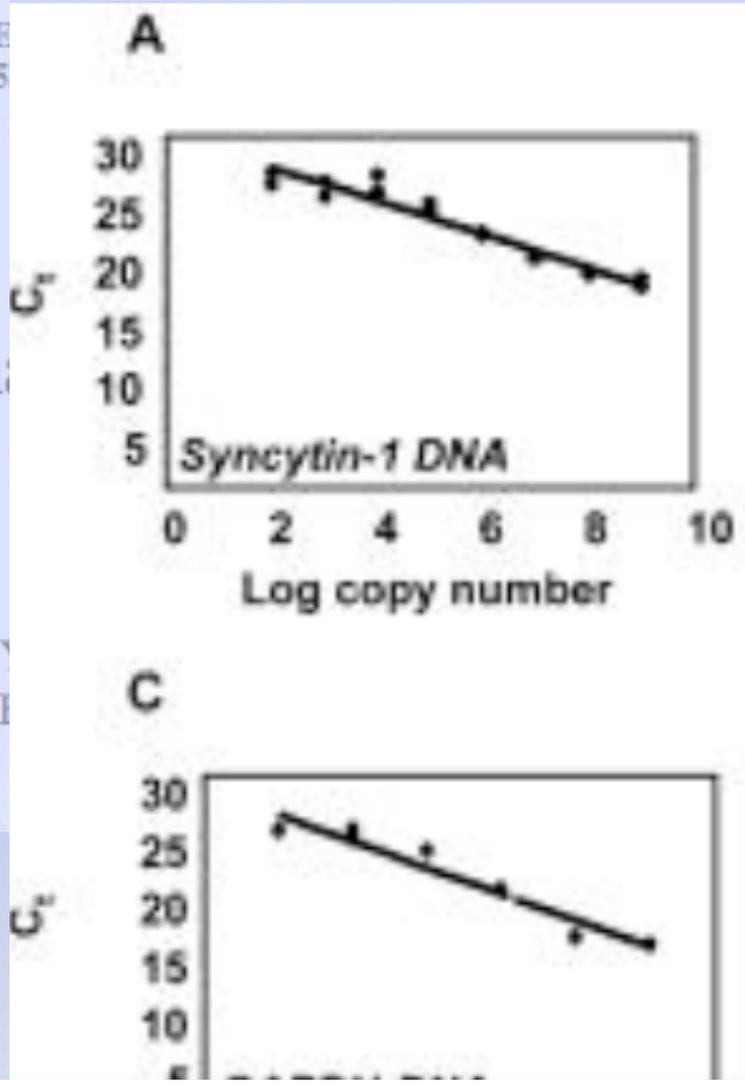
Comparative Expression of Human Endogenous Retrovirus-W Genes in Multiple Sclerosis

JOSEPH M. ANTONY,[1,*] YU ZHU,[2,*] MARYAM IZAD,[3] KENNETH G. WARREN,[2] MOHAMMED VODJGANI,[3] FRANCOIS MALLET,[4] and CHRISTOPHER POWER[1,2]

AIDS RESEARCH AND HUMAN RE
Volume 22, Number 12, 2006, pp. 125

Quantitative An genous Retrovirus-W *env* Diseases

JOSEPH M. ANTON R,[3] KENNETH G. WARREN,[4] MOHAMM ',[5] and C. POWER[1,4]



slope: −1.365

slope: −2.276

FIG. relatic copie curve A copy number (cor- number of viral DNA g_{10}) (**B**). A standard 1) was derived (**C**).

Table 2. Analysis of Primer Efficiency

Gene	Regression equation
HERV-W_{deg}	$-4.665x + 43.941$
MSRV	$-3.456x + 36.961$
ERVWE1	$-1.365x + 29.435$

Table 3. Linear Regression Equations[a]

	CV from different samples in the same run (intraassay) (%)				CV across separate PCR runs (interassay) (%)			
	PBMC		Brain		PBMC		Brain	
Gene	Non-MS	MS	Non-MS	MS	Non-MS	MS	Non-MS	MS
HERV-W_{deg}	3.75	5.92	10.03	9.52	2.52	5.91	13.39	9.77
MSRV	ND	6.74	9.68	7.92	ND	12.73	2.07	9.85
ERVWE1	5.39	4.50	10.73	8.49	3.87	3.62	9.77	8.60
	Overall CV				Overall CV			
	4.57	5.72	10.15	8.64	3.20	7.42	8.41	9.41

[a]Coefficient of variance for duplicate readings for intraassay and interassay variability using raw C_t values for all samples. ND, not detected.

HomeNews

Top Irish pathologist criticised in US court

Dr Muiris Houston
Medical Correspondent

The work of a leading Irish pathologist which formed a key element in the purported link between autism and the MMR vaccine has been heavily criticised in a US court.

The criticism emerged as Dr Andrew Wakefield, the doctor who first proposed a link between autism and the MMR vaccine, begins his defence against allegations of professional misconduct in Britain.

Prof John O'Leary, professor of pathology at Trinity College Dublin, carried out research in a laboratory at the Coombe hospital confirming the presence of the measles virus in gut biopsies of children with autism.

However, last month, the US Court of Federal Claims in Washington was told by an expert witness, Prof Stephen Bustin: "I do not believe there is any measles virus in any of the cases they [Prof O'Leary's research team] have looked at".

Prof Bustin, a professor of molecular science at Queen Mary's School of Medicine and Dentistry, University of London, and a world expert in the technology of polymerase chain reaction (PCR) – the basis for many medical diagnostic tests – reached his conclusion after visiting the laboratory at the Coombe hospital in 2004 and following a series of studies which failed to replicate Prof O'Leary's results. He was giving evidence in the first test case brought by the families of more than 4,800 US children claiming damages from a fund set up to compensate people harmed by vaccination.

Prof Bustin told the court, it was "a scientific certainty" that the Unigenetics laboratory at the Coombe has failed to identify measles virus RNA (genetic material) in the children it had tested.

Doctor defends research into MMR vaccine link to autism

In February 1998, Dr Andrew Wakefield, a British gastroenterologist then working at the Royal Free Hospital in north London, claimed there could be a link between rising levels of autism and the measles, mu

may have overwhelmed the immune system, leaving some children prone to bowel disease and autism.

In a press conference on February 26th, 1998, Wakefield said he would advise par-

Coombe hospital in Dublin and they began a research collaboration in February 1999. Published in the journal *Molecular Pathology* in 2002, the results described an association between mea-

levelled against the three doctors is that they undertook the research without full approval from the hospital ethics committee and that they did not treat the young children in accordance with the oval they had received.

l is also accused of taking esearch purposes from chilirthday party after offering y. If found guilty of profesnduct, Wakefield, who now the US, could be struck off.

Muiris Houston

virus in any of the cases they have looked at".

Prof Bustin's opinion is widely regarded as final proof that the theory put forward by British gastroenterologist, Dr Andrew Wakefield, that a distinctive inflammatory bowel condition – autistic enterocolitis – was the link between the MMR vaccine and increasing levels of autism, lacks credibility.

Dr Wakefield published research in the *Lancet* medical journal in 1998 describing how he had detected measles virus in the

practise hearings into the prosional conduct of Dr Wakeld and two of his co-authors. Prof Bustin told the court that e assay used was not specific measles and it was not propy carried out". He said that the sitive result were positive for

DNA and as measles i virus, "if it's DNA it can sles". The expert sai pected that the unigene ratory has been contam DNA from another sour netics, a private con reported to have been stg£800,000 by the UK fund.

Dr Michael Fitzp London GP and autho *and Autism: What parer know* told *The Irish T* "Bustin's evidence blo the water the only singl evidence which seemec a link between MMR an

Prof O'Leary was u for comment yesterda strongly disputed the contamination migl occurred in his labor has said he never set ou that MMR caused autis tion, he has publicly u ents to vaccinate chil the combined MMR vac

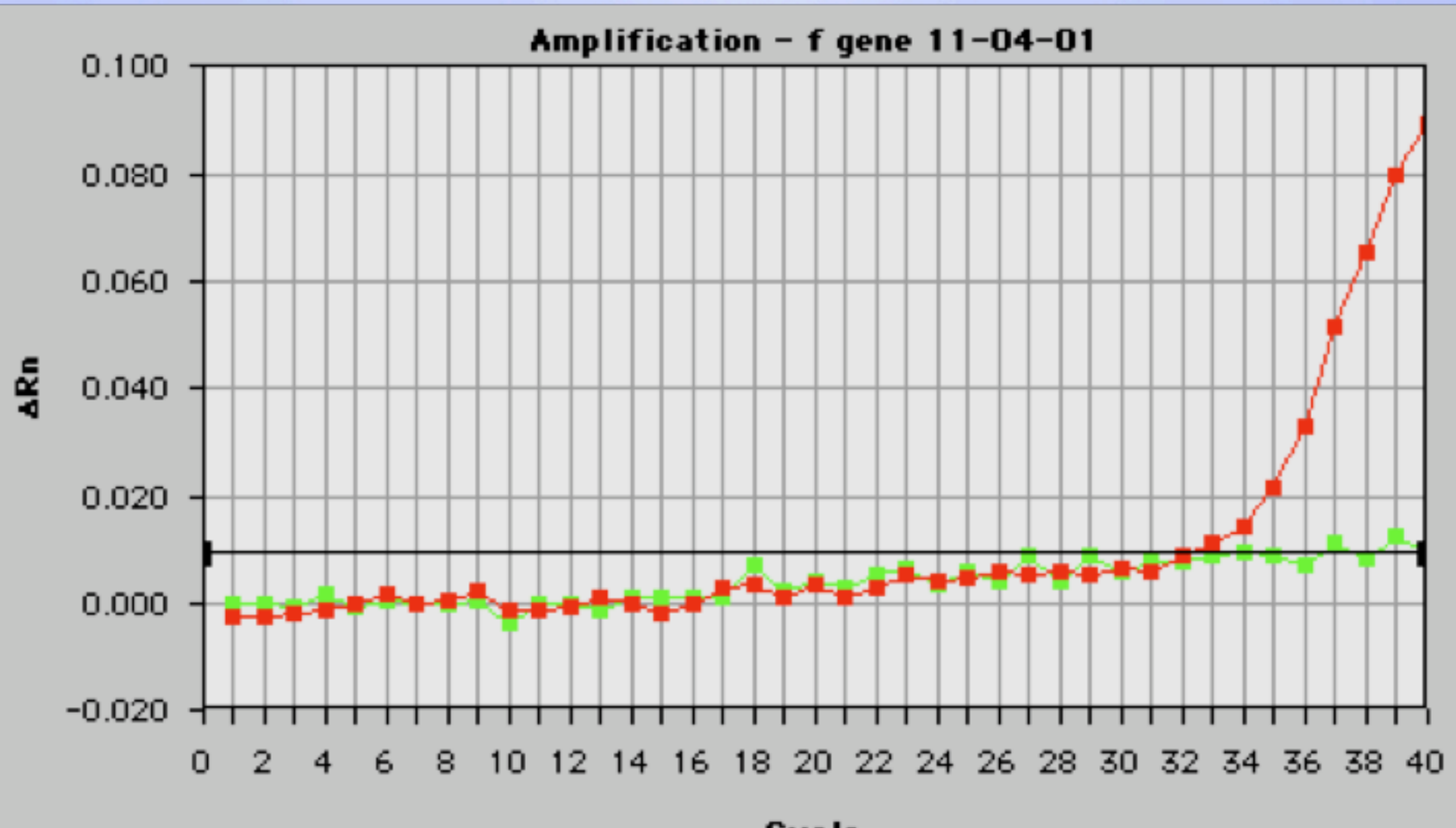
Table 1 Measles virus primer and probe sequences

Primer/Probe	Sequence 5′–3′	Amplicon size
N1 forward	5′ TCA GTA GAG CGG TTG GAC CC 3′	
N1 reverse	5′ GGC CCG GTT TCT CTG TAG CT 3′	150 bp
N2 forward	5′ GAG TCG AGG AGA AGC CAG GG 3′	
N2 reverse	5′ GCT GGA CTC CGA TGC AGT GT 3′	120 bp
H1 forward	5′ TTC ATC GGG CAG CCA TCT AC 3′	
H1 reverse	5′ CTC TGA GGT GTC CTC AGG CC 3′	150 bp
H2 forward	5′ TGG GCA CCA TTG AAG GAT AA 3′	
H2 reverse	5′ AAC CGT GTG TGA TCA ATG GC 3′	120 bp
		0 bp
		0 bp
GAPDH 2	5′ GAA GAT GGT GAT GGG ATT TC 3′	226 bp
N1 probe	5′ CAA ACA GAG TCG AGG AGA AGC CAG GGA 3′	
H1 probe	5′ CCG CAG AGA TCC ATA AAA CCC TCA GCA C 3′	
F1 probe	5′ CTG CAC GAG GGT AGA GAT CGC AGA ATA CAG 3′	

```
                CTG CAC GAG GGT AGA GAT CGC AGA ATA CAG
                *** *** *** *** *** ***  ** *** *** ***
Consensus       CTG CAC GAG GGT AGA GAT TGC AGA ATA CAG
```

llowing Genbank sequence entries
1999, U03661, U03658, and
on the following GenBank
71, U03667, Z80793,
bes were designed based on the
48, U03662, U08146, U03657,

```
AJ133108    CTGCACGAGGGTAGAGATTGCAGAATACAG
U03648      CTGCACGAGGGTAGAGATTGCAGAATACAG
U03651      CTGCACGAGGGTAGAGATTGCAGAATACAG
U03655      CTGCACGAGGGTAGAGATTGCAGAATACAG
U03657      CTGCACGAGGGTAGAGATTGCAGAATACAG
U03659      CTGCACGAGGGTAGAGATTGCAGAATACAG
U03662      CTGCACGAGGGTAGAGATTGCAGAATACAG
U03666      CTGCACGAGGGTAGAGATTGCAGAATACAG
x16567      CTGCACGAGGGTAGAGATTGCAGAATACAG
x16565      CTGCACGAGGGTAGAGATTGCAGAATACAG
Consensus   CTGCACGAGGGTAGAGATTGCAGAATACAG
```



Samples

- FAM - F4
- FAM - H2

Viewer: ΔRn (B...

Reporter: FAM

Threshold Cycle Calculation

Threshold

Use Threshold: .01 Suggest

Mult. * Stddev: 10.0 * .001

Omit Threshold: 2.0

Baseline

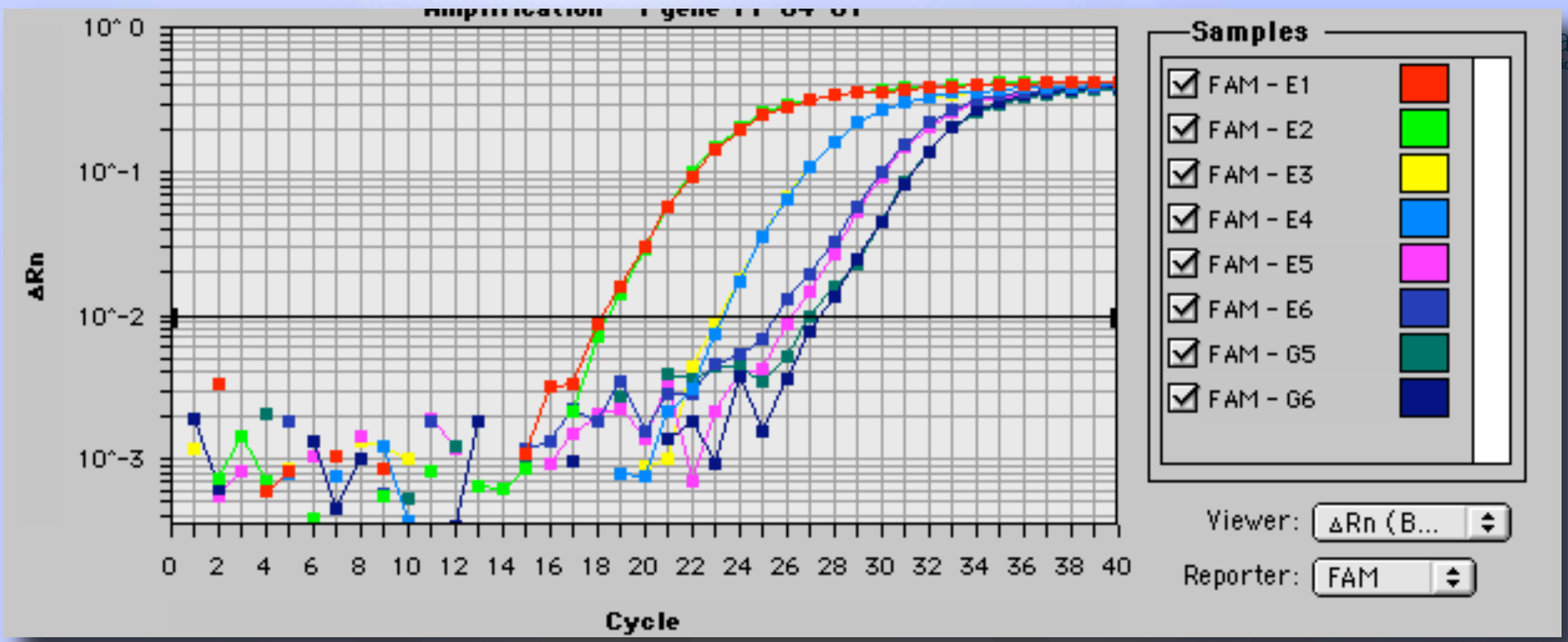
Start: 3 Stop: 15

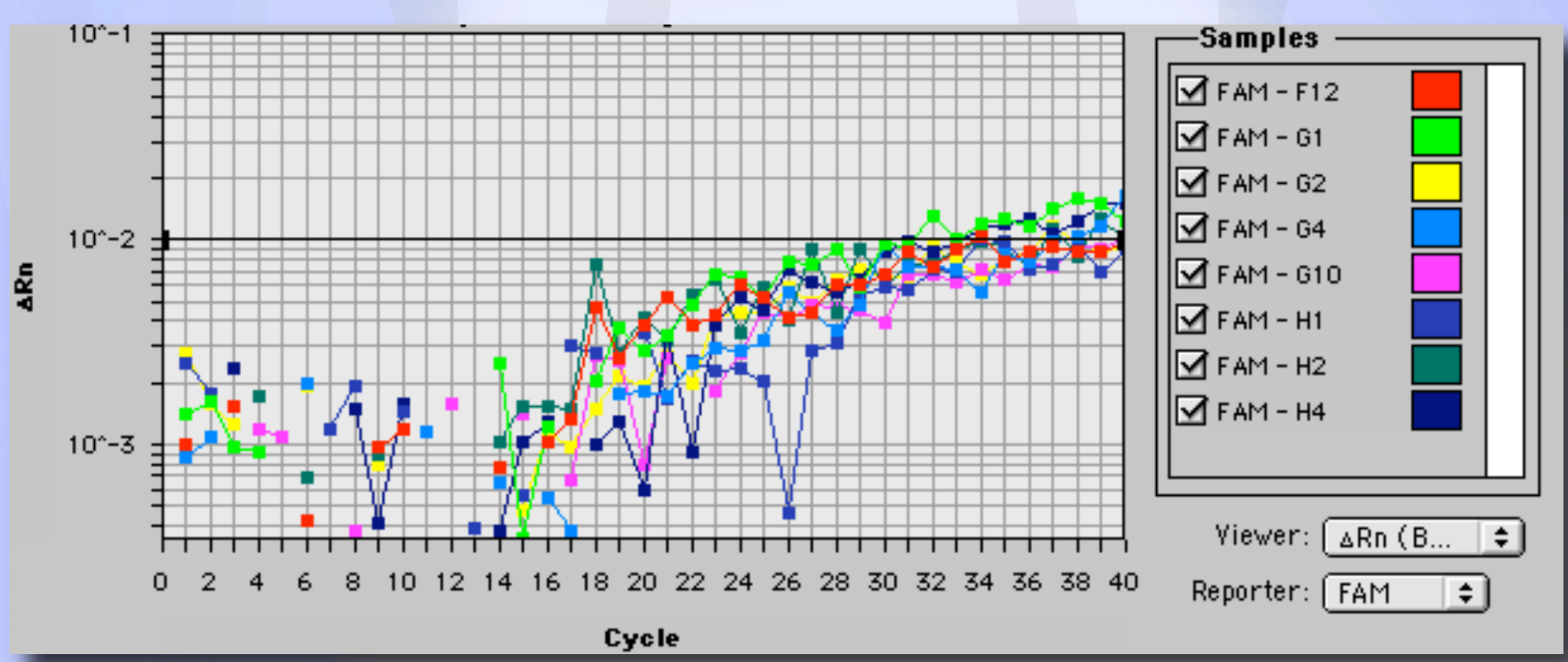
Update Calculations

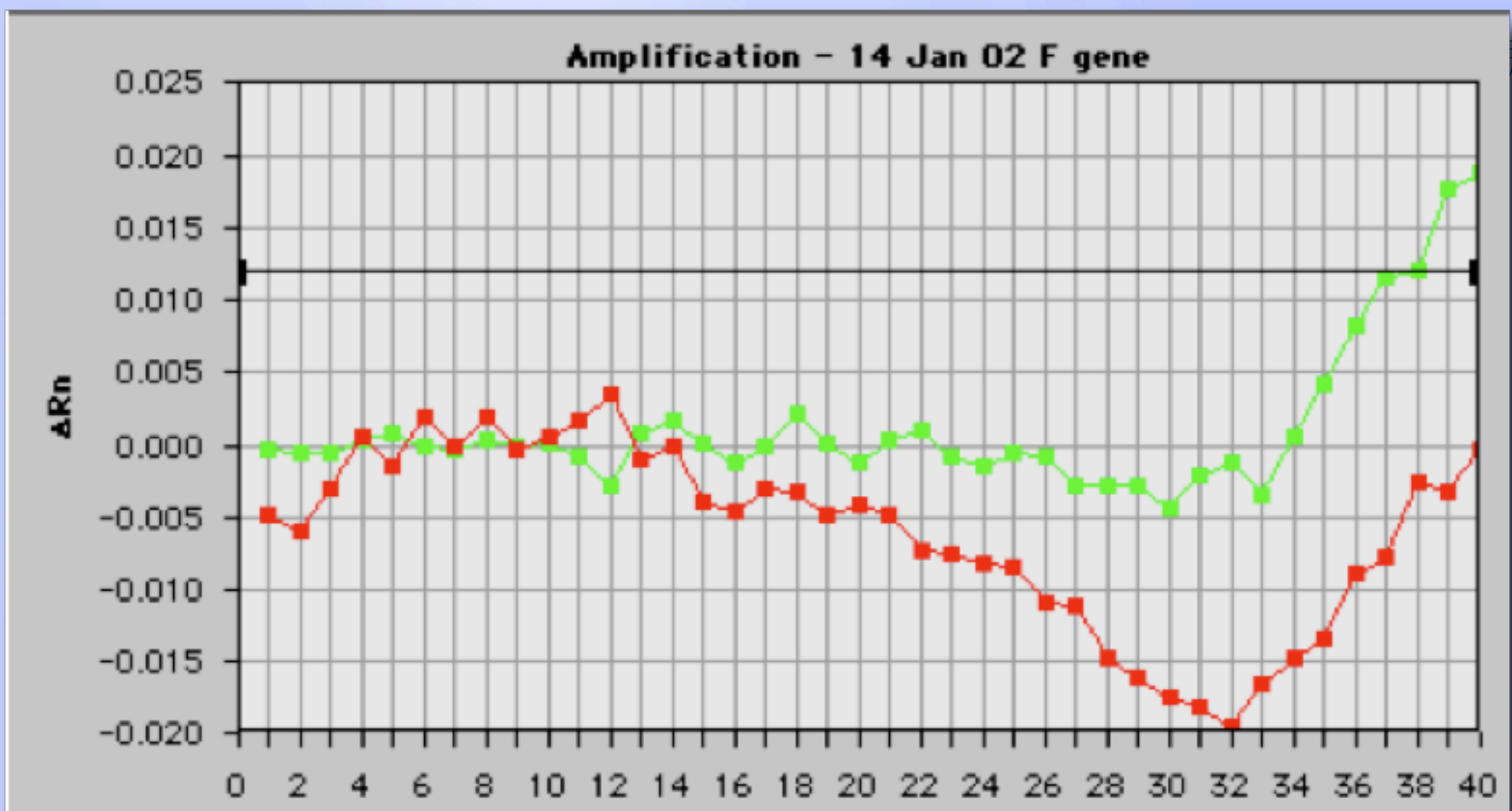
	Ct	Std Dev
FAM - F4	32.319	0.001
FAM - H2	36.667	0.001

F1	UNKN	323		36.03	6.0e+01	0.00	60.37
F2	UNKN	323		40.00		0.00	0.00
F3	UNKN	15		32.71	5.4e+02	0.00	539.40
F4	UNKN	15		32.32	7.0e+02	0.00	698.57
F5	UNKN	47		28.96	6.4e+03	0.00	6383.89
F6	UNKN	47		30.56	2.2e+03	0.00	2221.60
F7	UNKN	49		30.47	2.4e+03	0.00	2369.26
F8	UNKN	49		32.70	5.4e+02	0.00	543.23
F9	UNKN	59		30.27	2.7e+03	0.00	2700.91
F10	UNKN	59		31.02	1.6e+03	0.00	1641.64
F11	UNKN	88		40.00		0.00	0.00
F12	UNKN	88		33.70	2.8e+02	0.00	281.24
G1	UNKN	276		31.22	1.4e+03	0.00	1444.24
G2	UNKN	276		36.47	4.5e+01	0.00	45.10
G3	UNKN	277		35.20	1.0e+02	0.00	104.47
G4	UNKN	277		37.18	2.8e+01	0.00	28.33
G5	UNKN	100		27.03	2.3e+04	0.00	22778.29
G6	UNKN	100		27.39	1.8e+04	0.00	17971.48
G7	UNKN	44		30.48	2.3e+03	0.00	2346.93
G8	UNKN	44		31.06	1.6e+03	0.00	1604.67
G9	UNKN	99		26.59	3.0e+04	0.00	30436.46
G10	UNKN	99		39.74	5.2e+00	0.00	5.24
G11	UNKN	92		30.81	1.9e+03	0.00	1888.68
G12	UNKN	92		31.37	1.3e+03	0.00	1308.76
H1	UNKN	223		33.98	2.3e+02	0.00	233.57
H2	UNKN	223		36.67	4.0e+01	0.00	39.74
H3	NTC	H3	NTC	40.00		0.00	0.00
H5	NTC	H5	NTC	40.00		0.00	0.00

E11	STND	14.1 260 cRNA	40.00	5.0e+01	0.00	0.00
E12	STND	14.1 260 cRNA	40.00	5.0e+01	0.00	0.00


10^ 0
10^-1
10^-2
10^-3
ΔRn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
Samples
FAM - E1
FAM - E2
FAM - E3
FAM - E4
FAM - E5
FAM - E6
FAM - G5
FAM - G6
Viewer: ΔRn (B...
Reporter: FAM


10^-1
10^-2
10^-3
ΔRn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
Samples
FAM - F12
FAM - G1
FAM - G2
FAM - G4
FAM - G10
FAM - H1
FAM - H2
FAM - H4
Viewer: ΔRn (B...
Reporter: FAM



Threshold Cycle Calculation

Threshold

Use Threshold: .012 Suggest

Mult. * Stddev: 10.0 * .001

Omit Threshold: 2.0

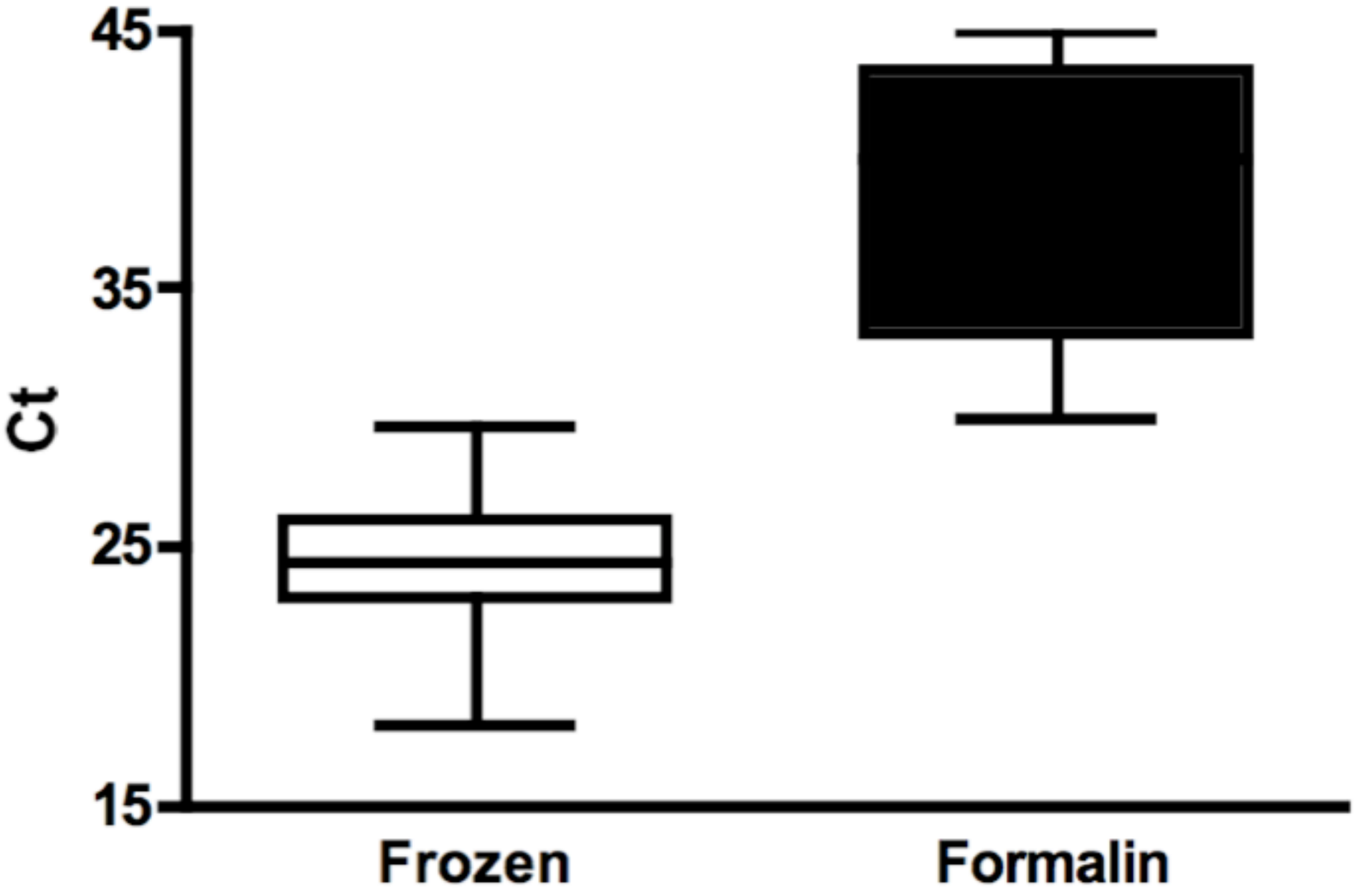
Baseline

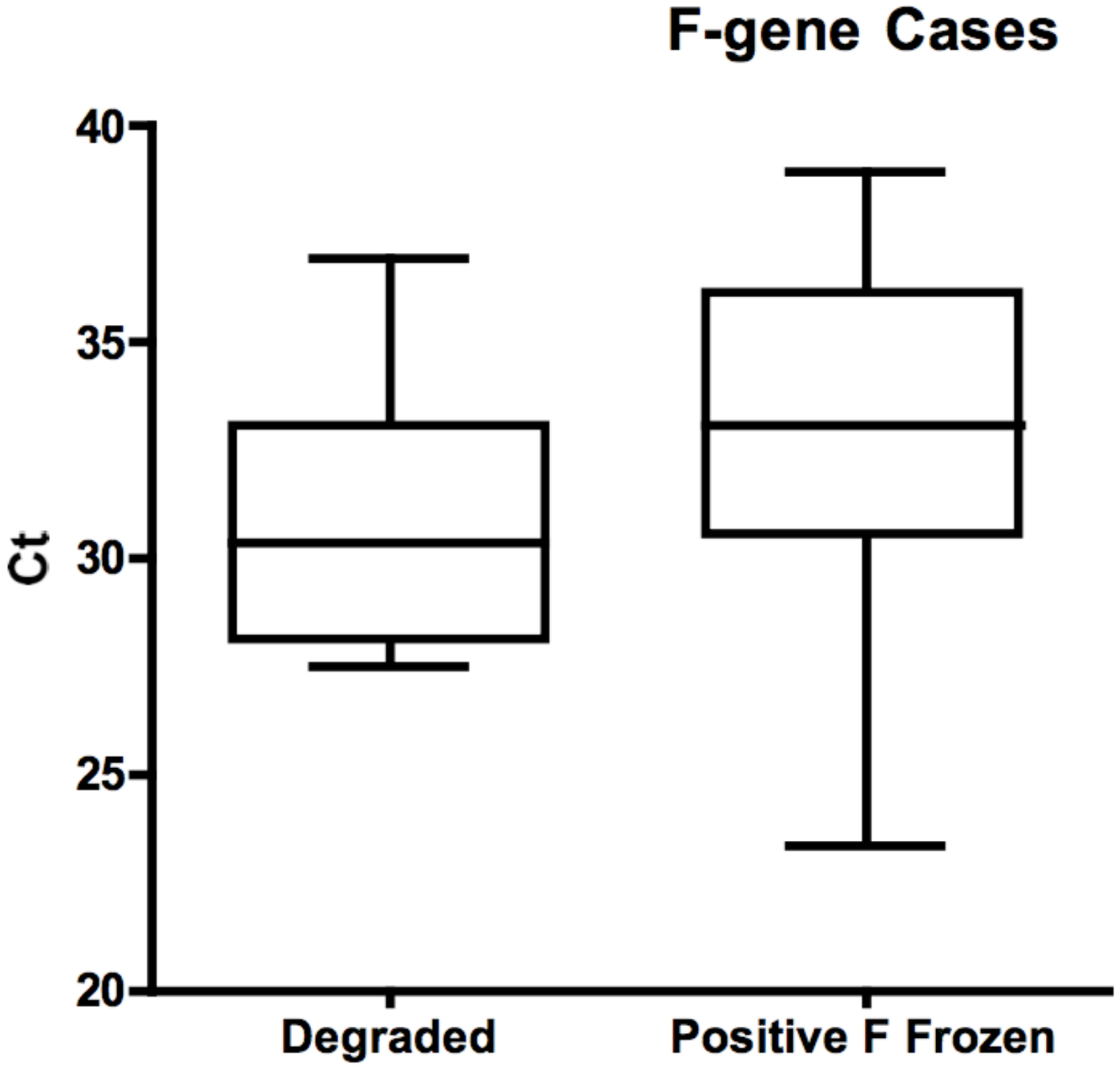
Start: 3 Stop: 15

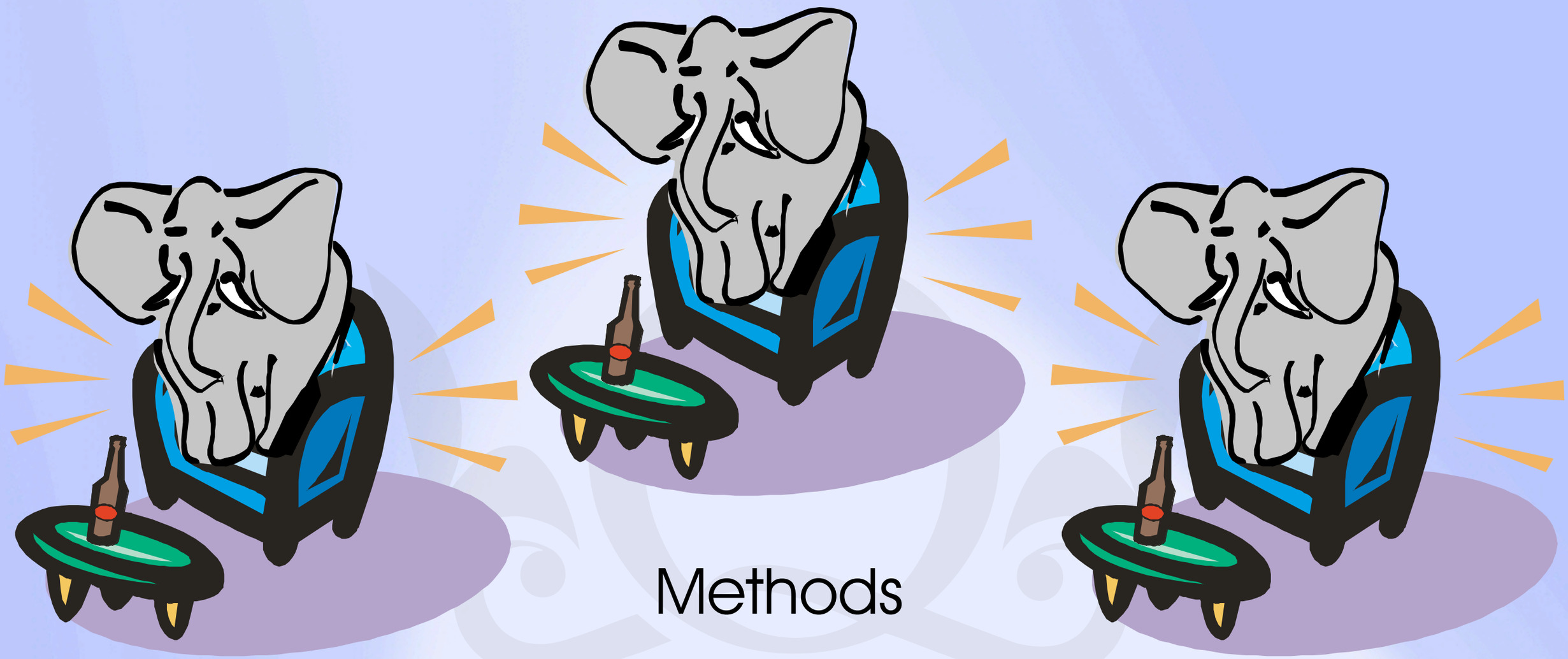
Update Calculations

	Ct	Std Dev
FAM - C4	40.000	0.002
FAM - E7	37.836	0.001

Effects of fixation on GAPDH-Cases




F-gene Cases
40
35
30
25
20
Ct
Degraded
Positive F Frozen

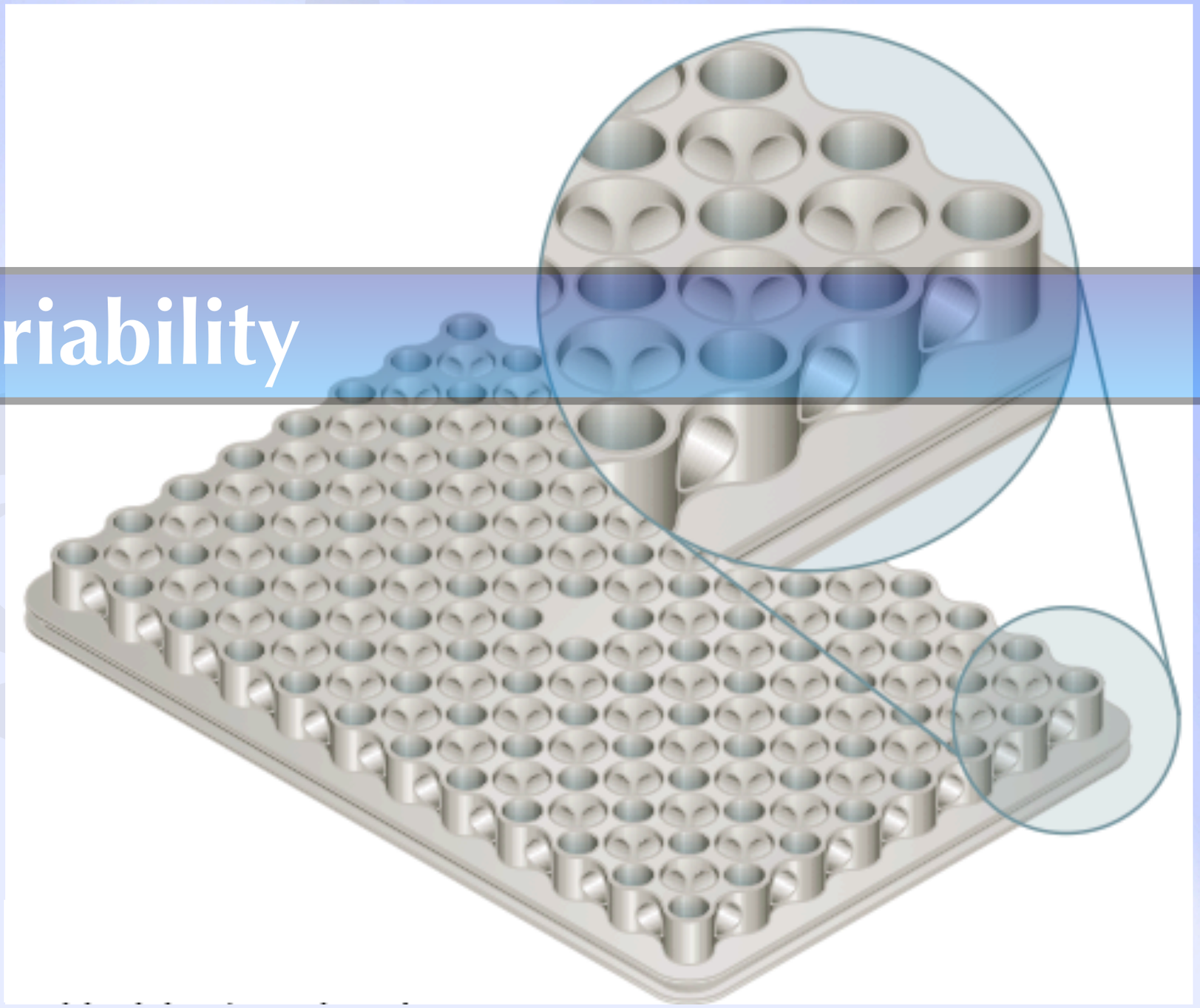


Biological/clinical relevance

Methods

Analysis/ interpretation

Instrument variability



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C	129%	87%	90%	75%	88%	75%	83%	74%	91%	89%	97%	91%
D	112%	96%	85%	81%	74%	75%	88%	84%	88%	90%	87%	91%
E	114%	100%	94%	84%	91%	87%	90%	91%	102%	104%	104%	103%
F	129%	114%	112%	106%	102%	99%	107%	109%	111%	137%	121%	105%
G	134%	133%	110%	107%	107%	110%	93%	101%	109%	113%	106%	104%
H	139%	104%	130%	104%	104%	85%	79%	94%	96%	90%	98%	100%

	1	2	3	4	5	6	7	8	9	10	11	12
A	100%	101%	106%	111%	106%	107%	98%	103%	122%	98%	120%	97%
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C	118%	88%	96%	103%	118%	100%	111%	96%	107%	103%	102%	99%
D	99%	99%	93%	93%	102%	102%	108%	104%	98%	110%	99%	102%
E	100%	99%	117%	106%	98%	105%	125%	107%	114%	116%	134%	134%
F	115%	118%	122%	117%	121%	136%	140%	119%	125%	131%	125%	108%
G	120%	143%	123%	133%	124%	139%	142%	125%	140%	141%	142%	133%

	1	2	3	4	5	6	7	8	9	10	11	12
A	100%	108%	110%	121%	110%	117%	117%	100%	104%	130%	115%	112%
B	115%	111%	122%	133%	117%	118%	109%	113%	110%	127%	115%	127%
C	120%	107%	115%	126%	117%	134%	125%	116%	104%	120%	117%	112%
D	118%	137%	116%	125%	126%	121%	107%	112%	107%	120%	106%	102%
E	108%	133%	134%	120%	110%	126%	105%	117%	109%	118%	97%	107%
F	123%	120%	114%	114%	108%	124%	111%	119%	111%	105%	100%	122%
G	118%	130%	125%	129%	120%	115%	130%	119%	124%	114%	104%	113%
H	121%	134%	130%	136%	123%	125%	126%	120%	113%	119%	96%	108%

	1	2	3	4	5	6
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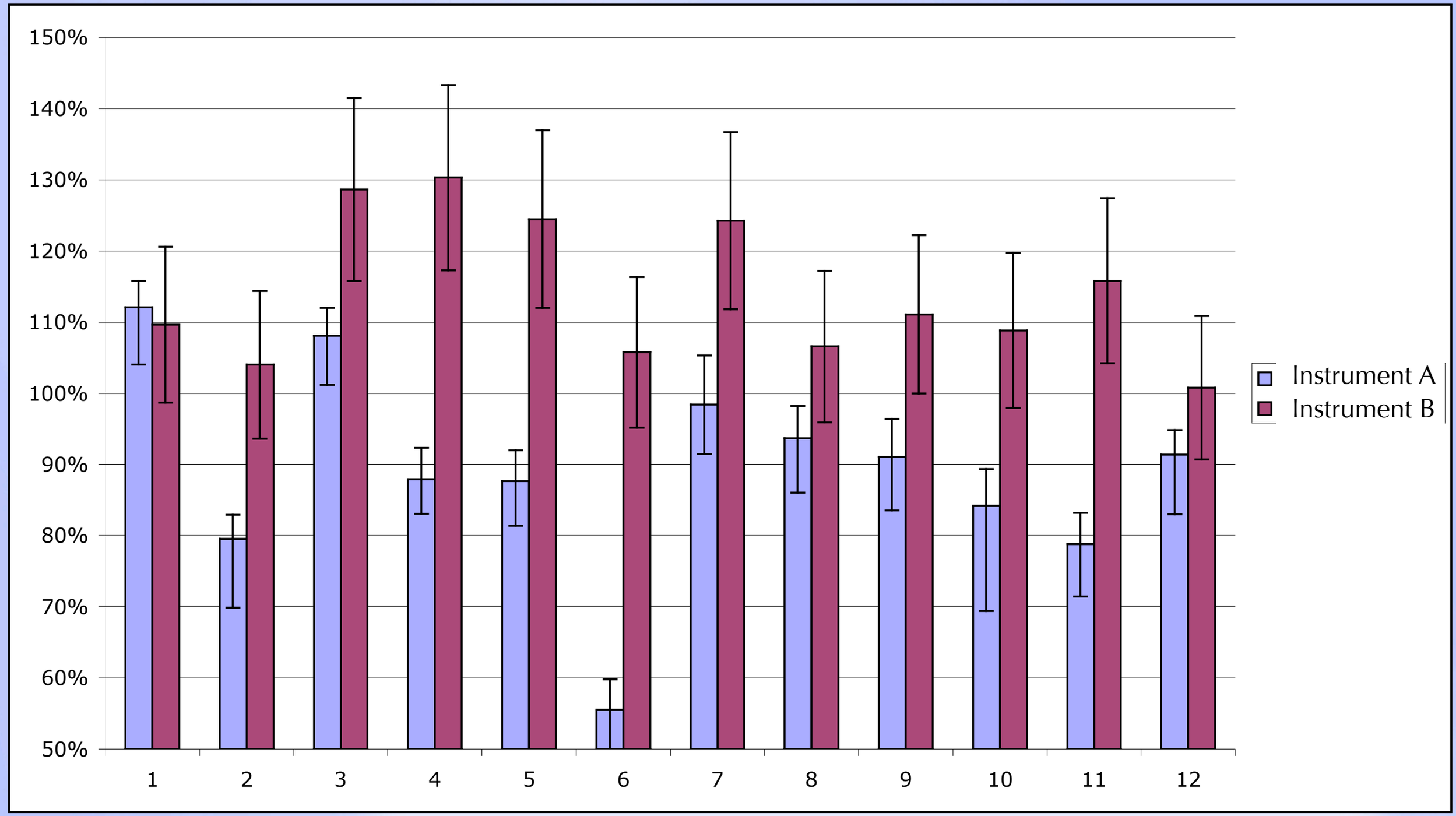
B	106%	111%	120%	119%	111%	99%
C	101%	108%	128%	121%	125%	123%
D	98%	110%	98%	103%	91%	110%
E	82%	90%	92%	91%	90%	88%
F	81%	96%	94%	92%	88%	96%
G	86%	96%	99%	105%	88%	96%
H	81%	73%	94%	106%	110%	102%

>125%
115-125%
105-115%
95-105%
85-95%
75-85%
<75%

ABI 7700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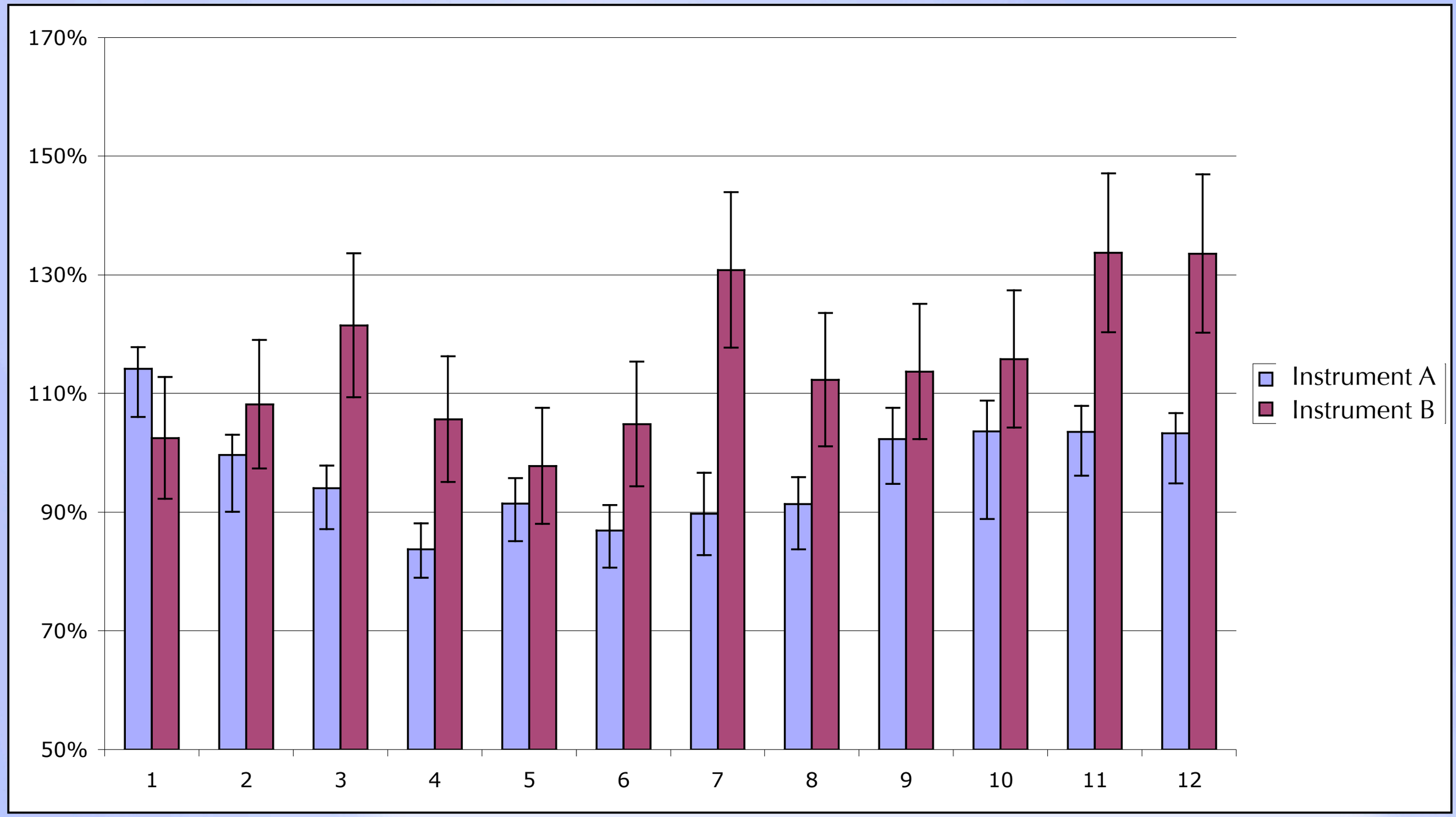
Instrument A
Instrument B

Row B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130%
110%
90%
70%
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5
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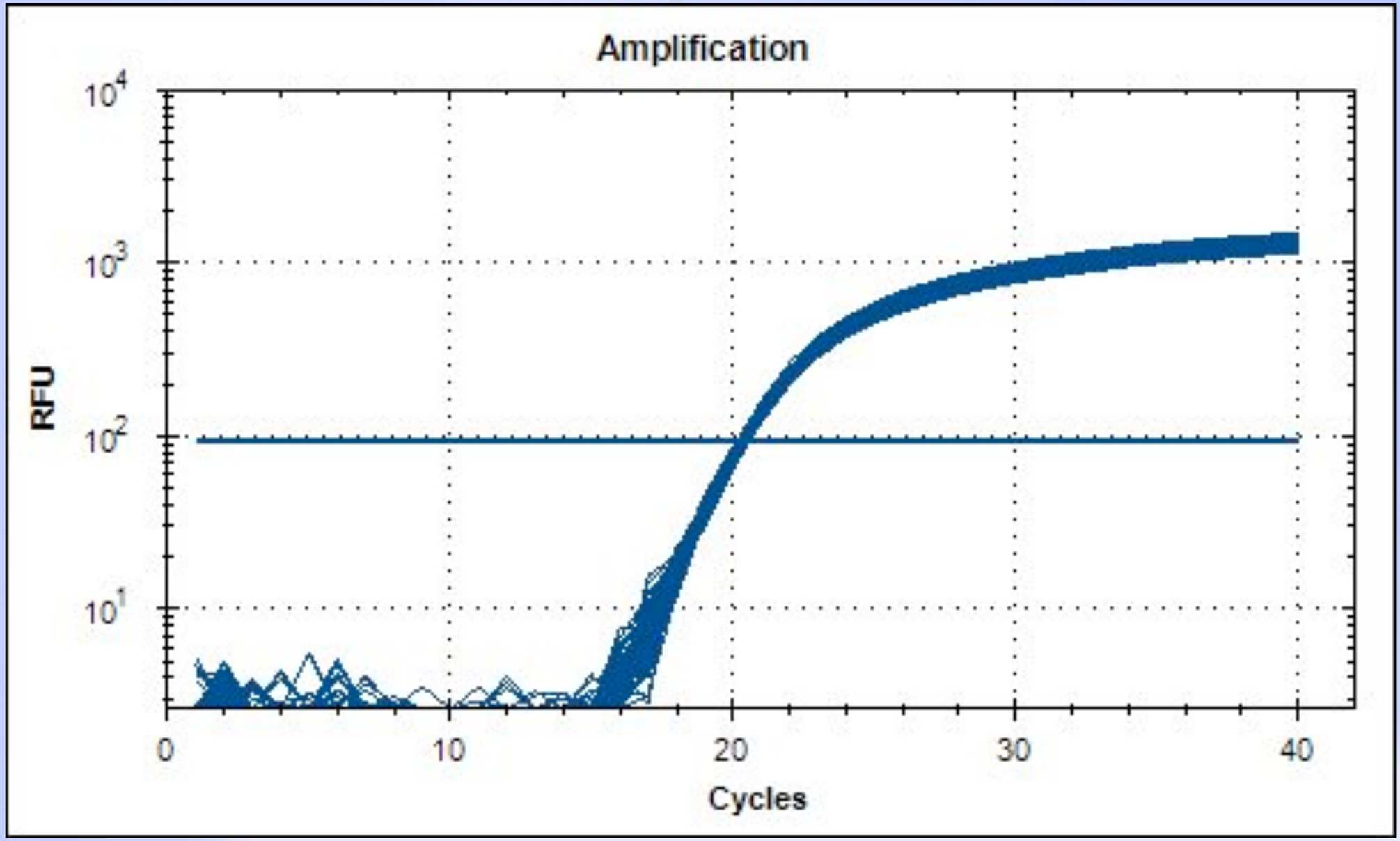
9
10
11
12
Instrument A
Instrument B

Row C


Amplification
RFU
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10^2
10^1
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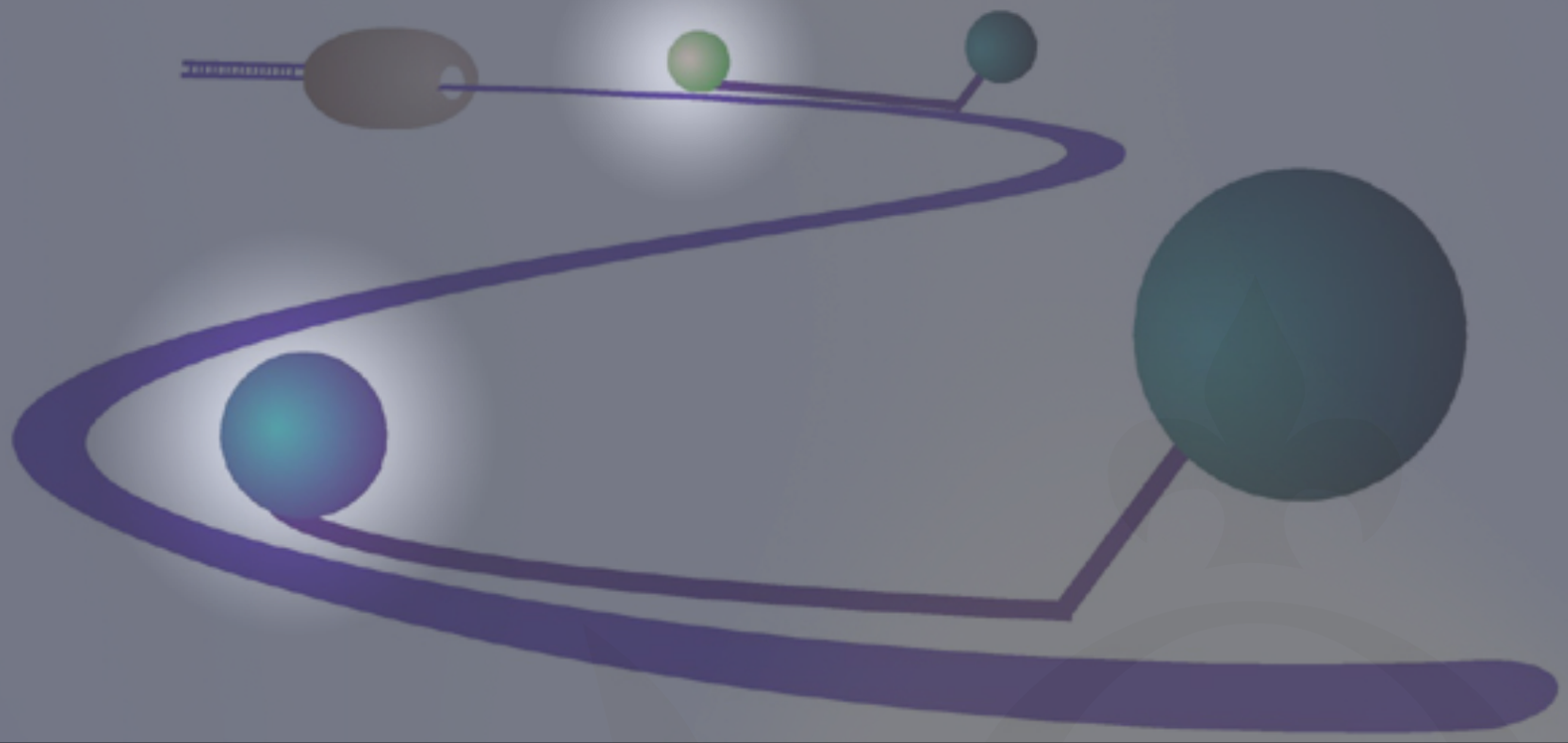
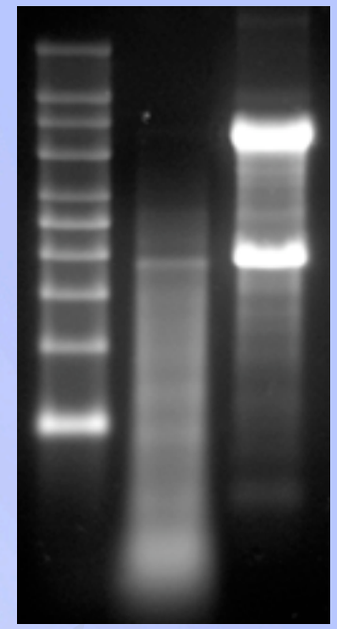
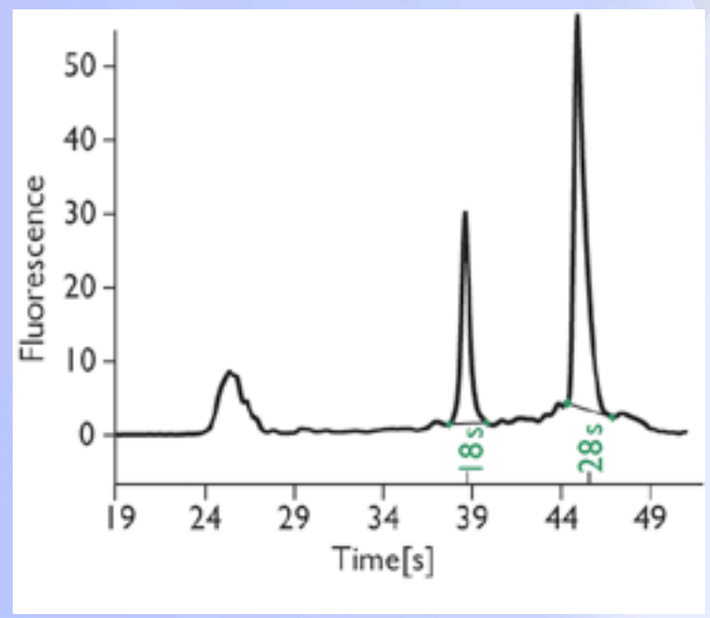
20
30
40
Cycles

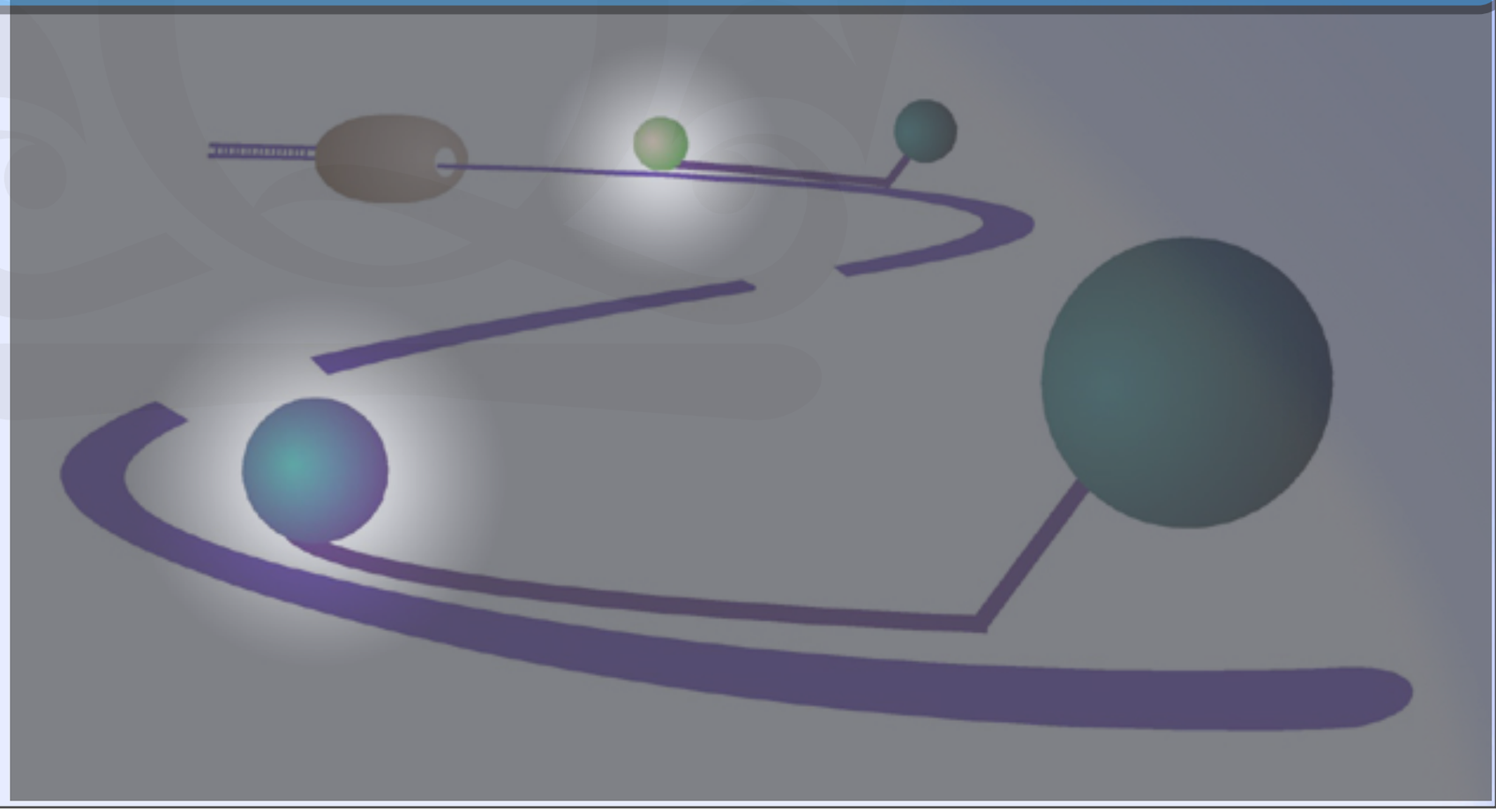


Barts and The London
School of Medicine and Dentistry



RNA integrity



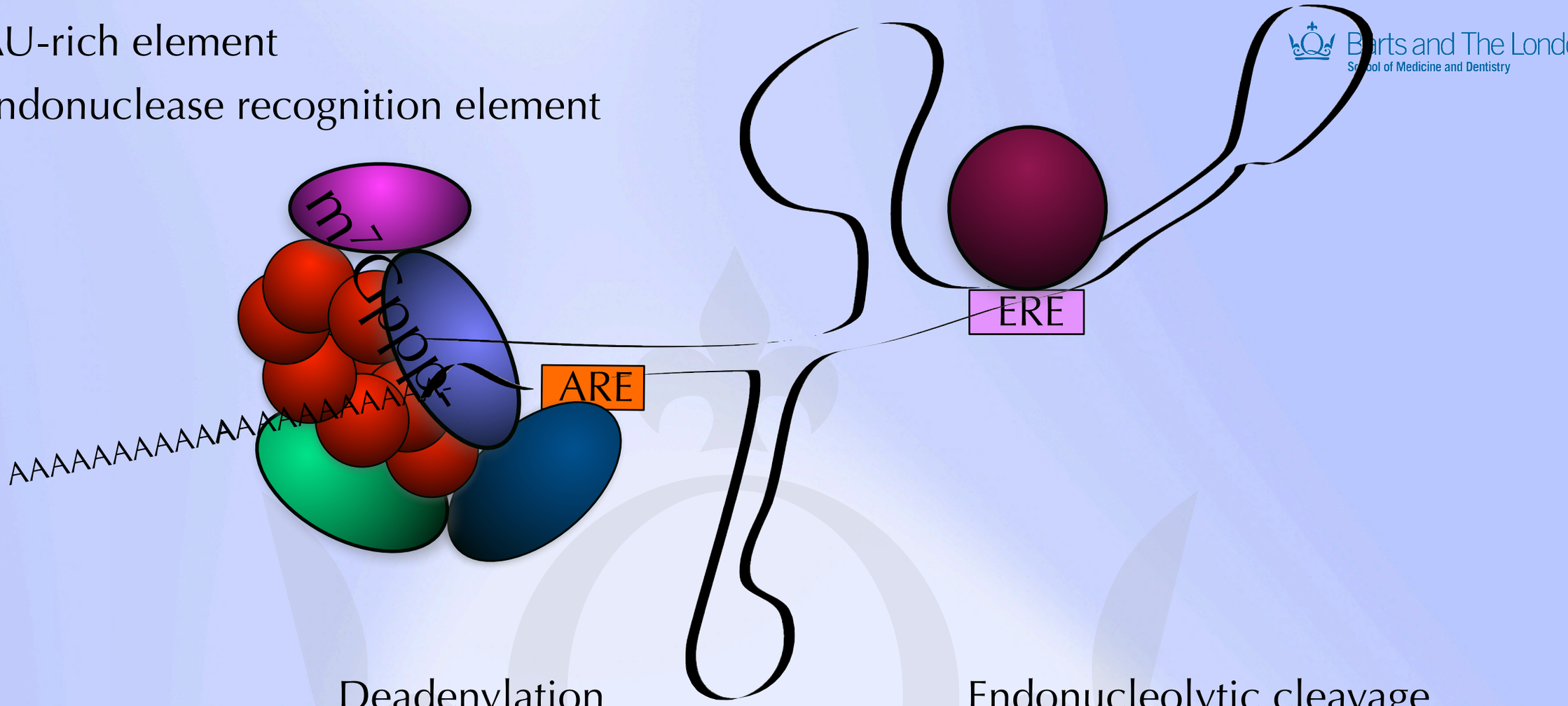


- *in vivo*
  - natural biological variability
  - not linked to RNA extraction
- *in vitro*
  - experimentally-induced
  - dependent on RNA extraction

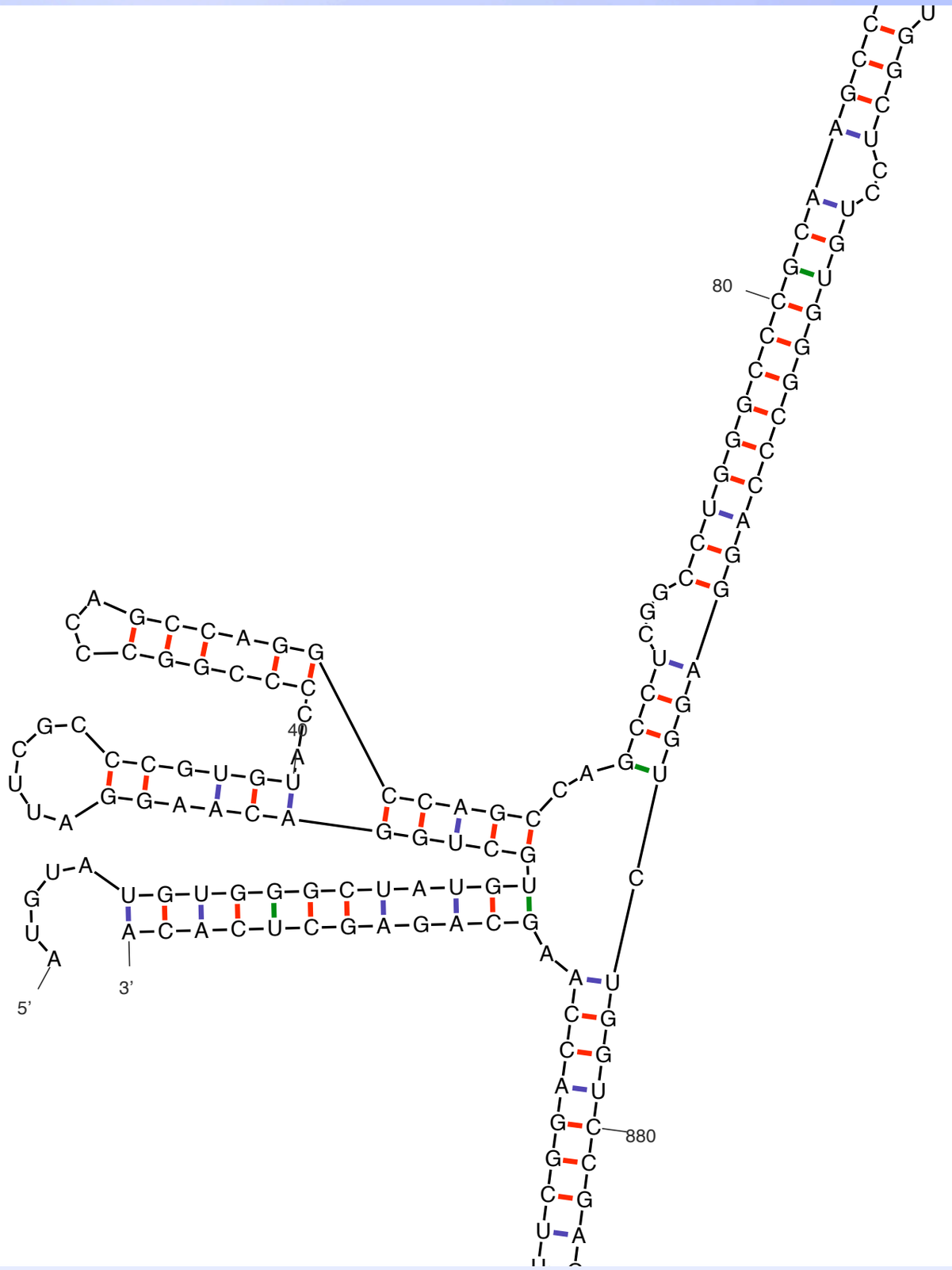
RNA degradation

ARE AU-rich element

ERE endonuclease recognition element



In vivo degradation



mRNA folding

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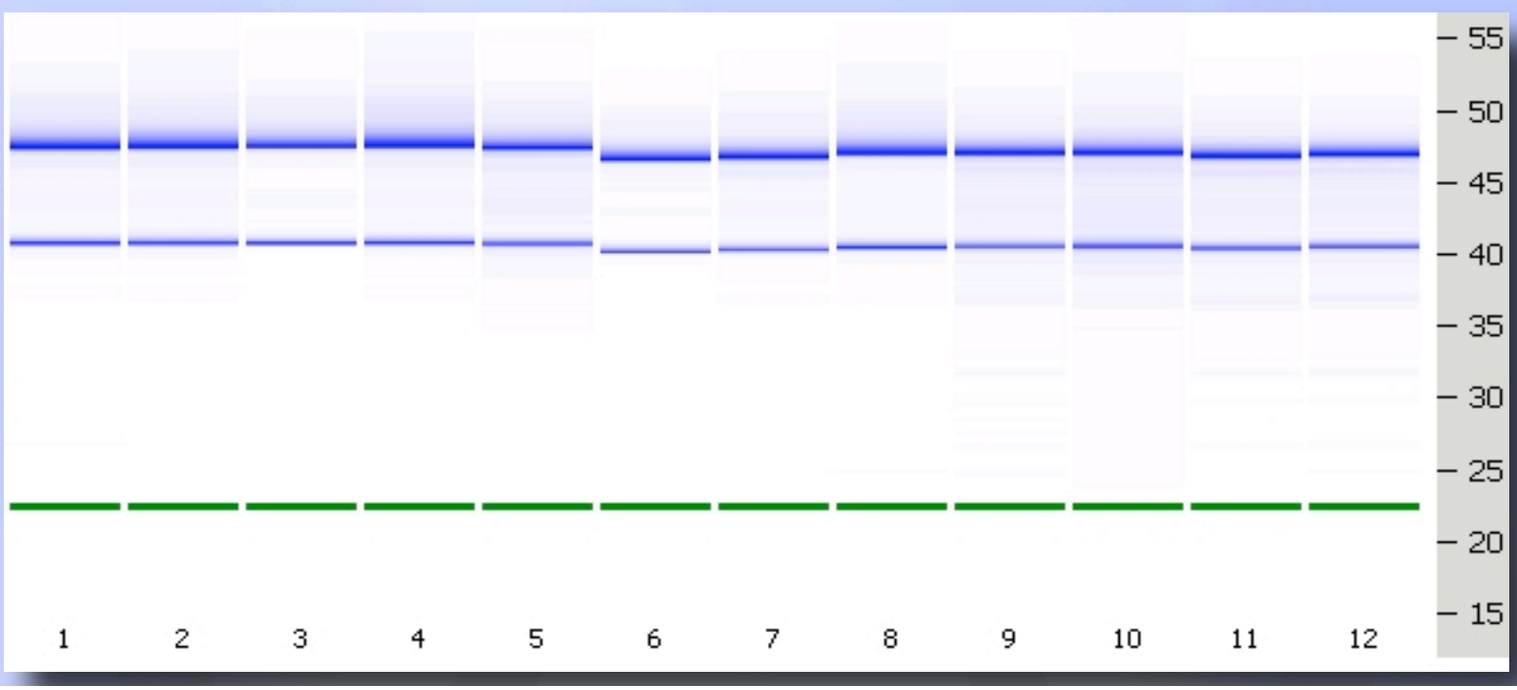
In vivo RNA degradation


55
50
45
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35
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25
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1
2
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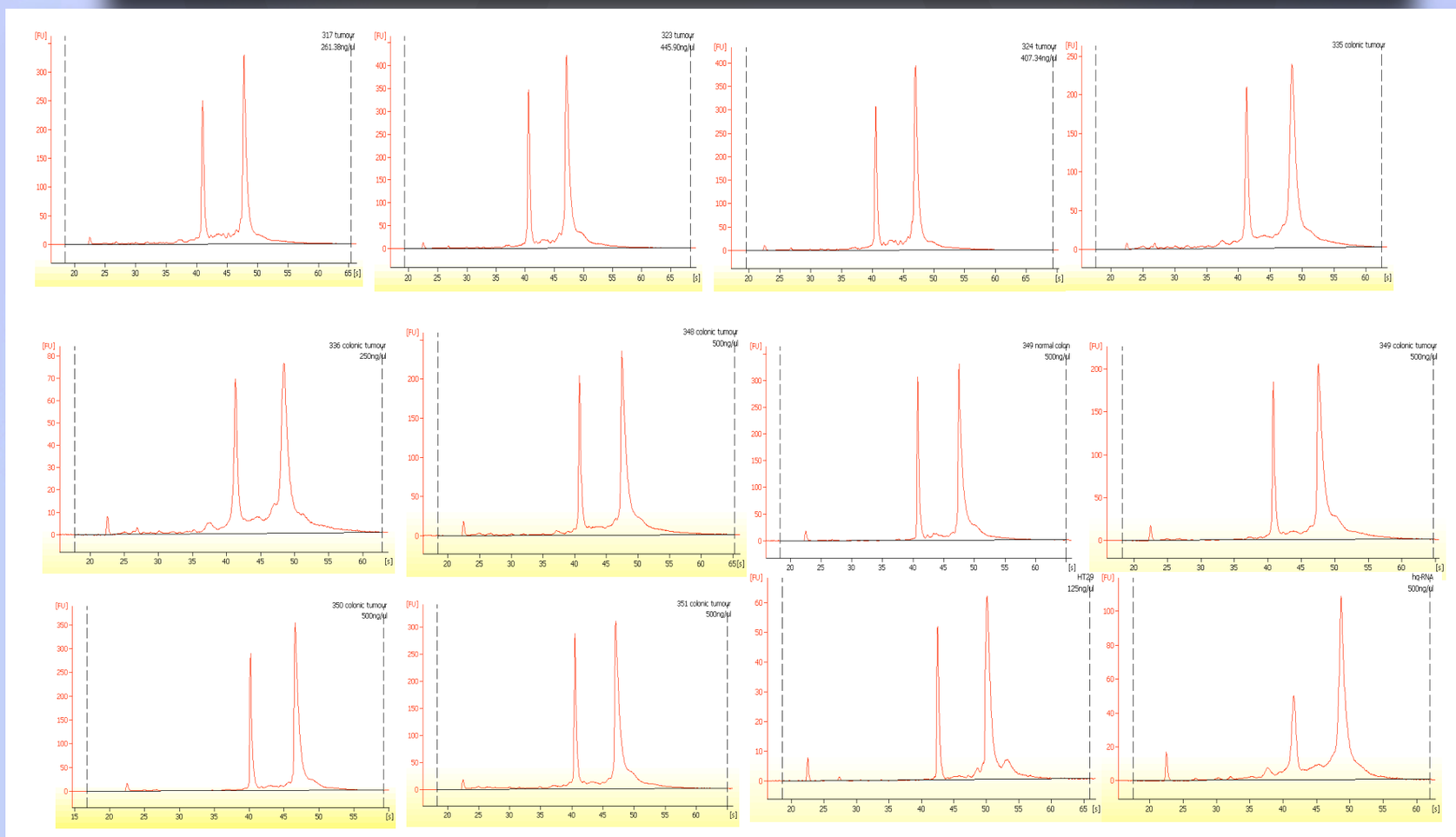
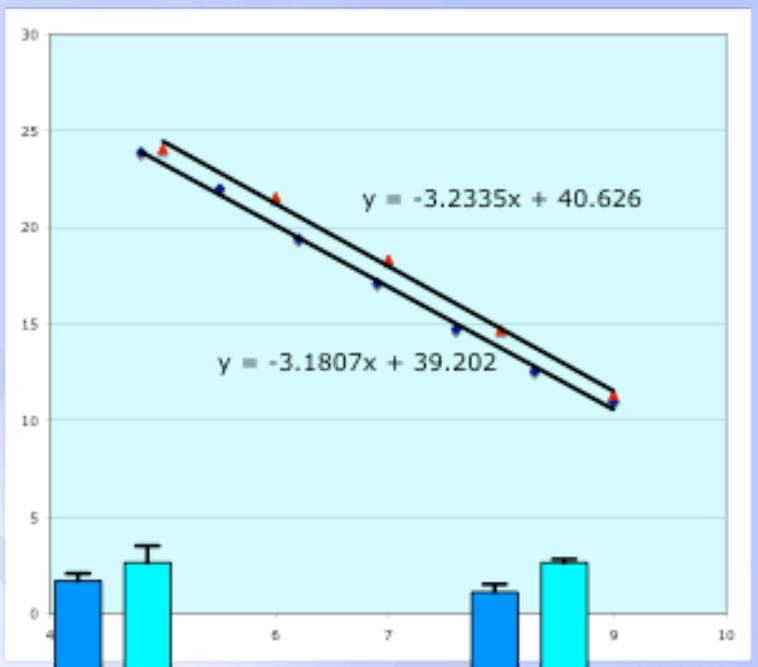
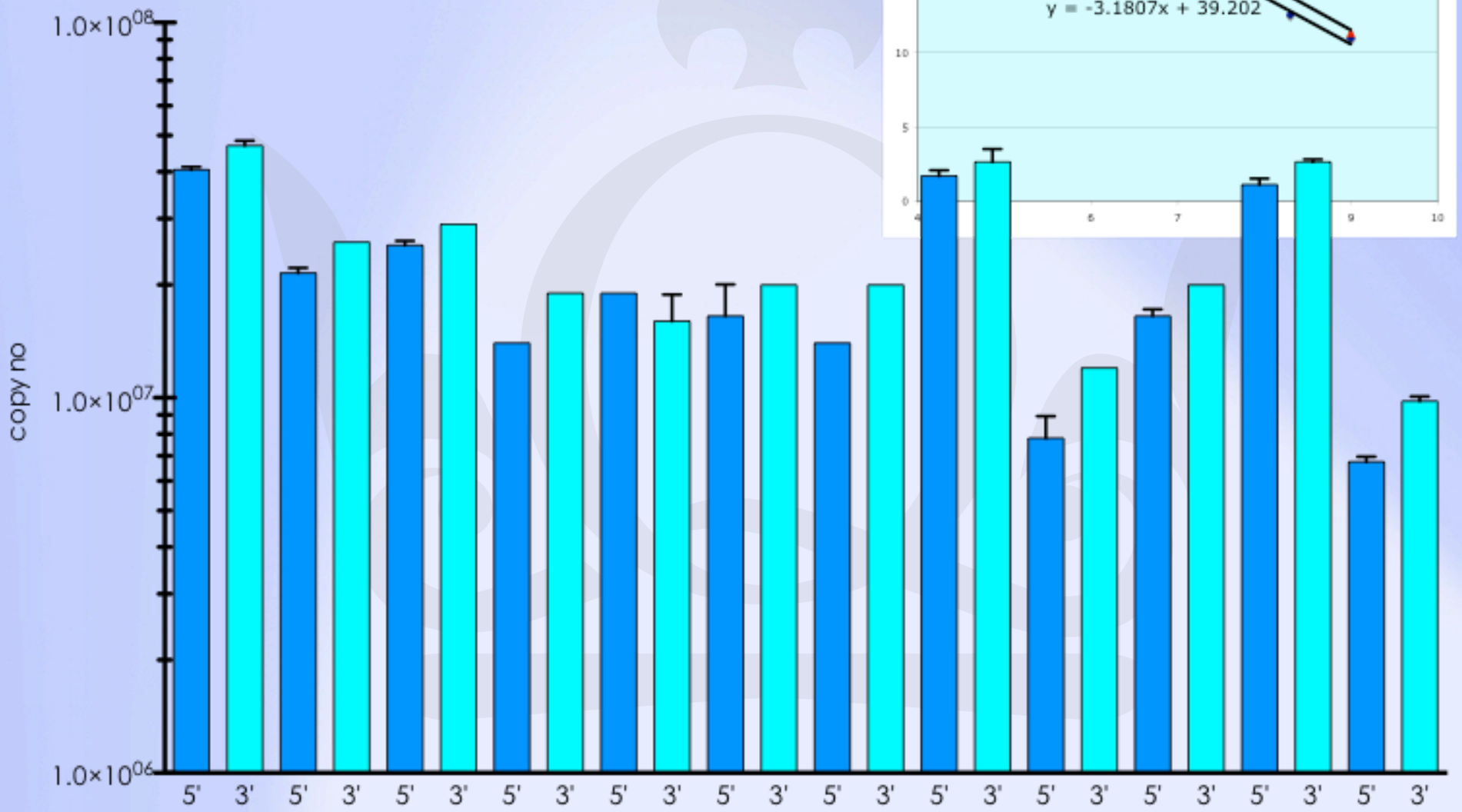
11
12

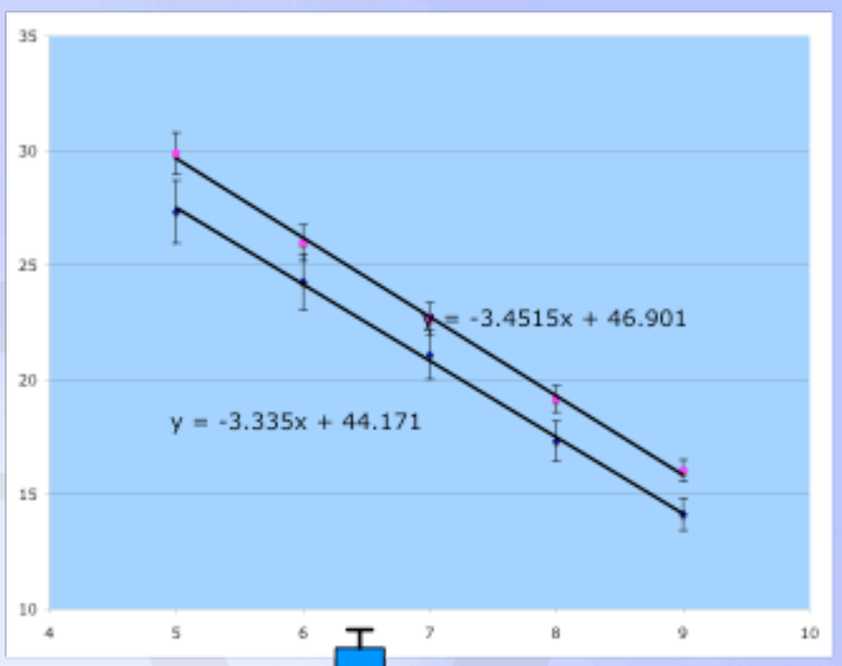

317 tumour
261.38ng/µl
323 tumour
445.90ng/µl
324 tumour
407.34ng/µl
335 colonic tumour
336 colonic tumour
250ng/µl
348 colonic tumour
500ng/µl
349 normal colon
500ng/µl
349 colonic tumour
500ng/µl
350 colonic tumour
500ng/µl
351 colonic tumour
500ng/µl
HT29
125ng/µl
hq-RNA
500ng/µl
[FU]
[s]





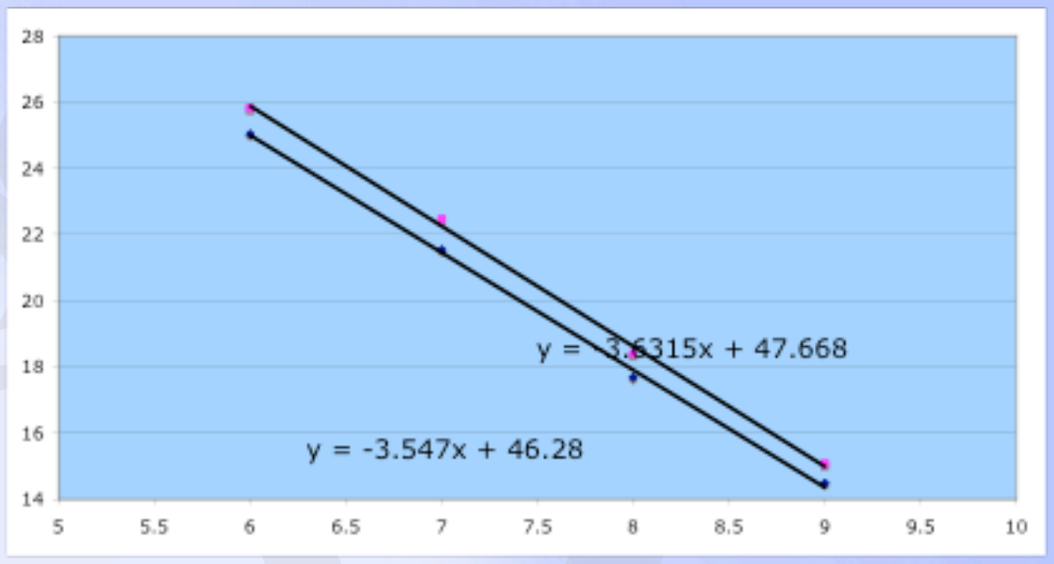
Individual high quality RNA samples gene-specific priming

In vivo degradation



Individual high quality RNA samples gene-specific priming

In vivo degradation



Individual high quality RNA samples gene-specific priming

In vivo degradation

- RNA integrity depends on
  - *in vivo* conditions
    - variation between genes within a sample
    - variation between samples
- This demarcates a basic variability intrinsic to and different for each sample

Summary (1)

- RNA integrity also depend on
  - In vitro handling
    - treatment-dependent
  - This variability may be minimised by appropriate handling and extraction protocols

Summary (2)

- Normalisation against reference genes must consider their differential stability
- cDNA priming strategies are influenced by RNA integrity

Summary (3)

- Introduction of a mRNA integrity assay
- Obligatory reporting of mRNA quality
- Realistic assessment of fold-change significance

Suggestions

cDNA priming

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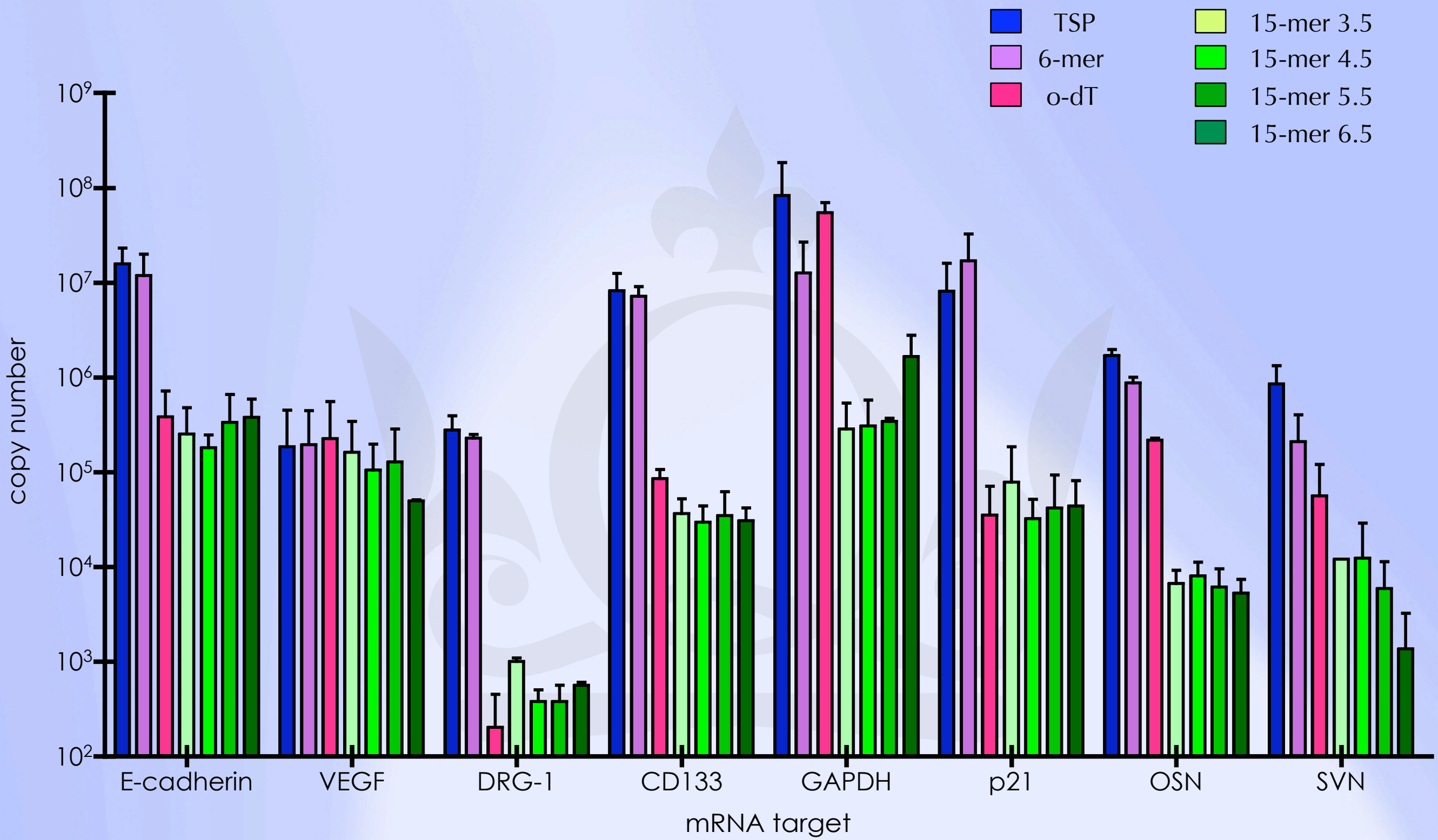
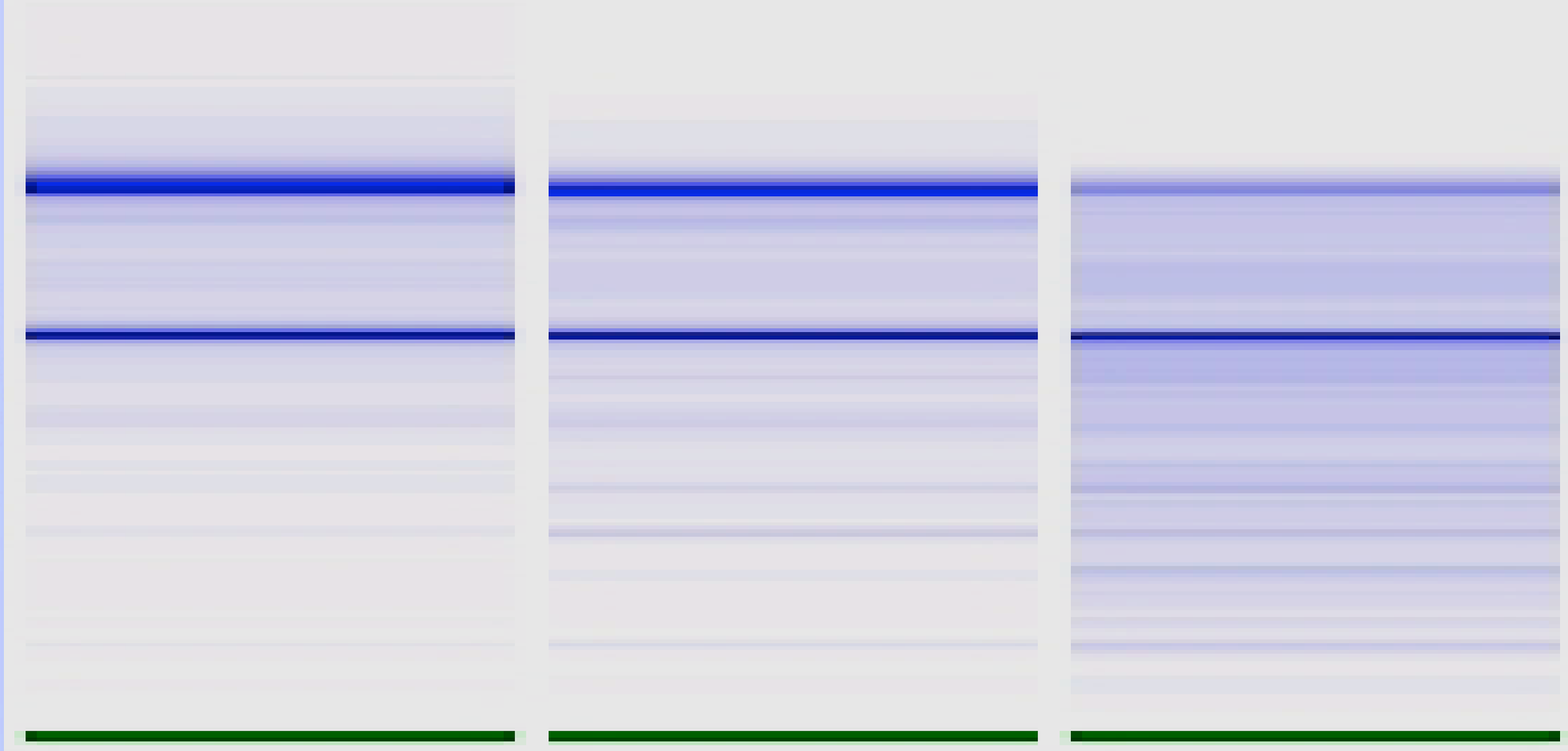
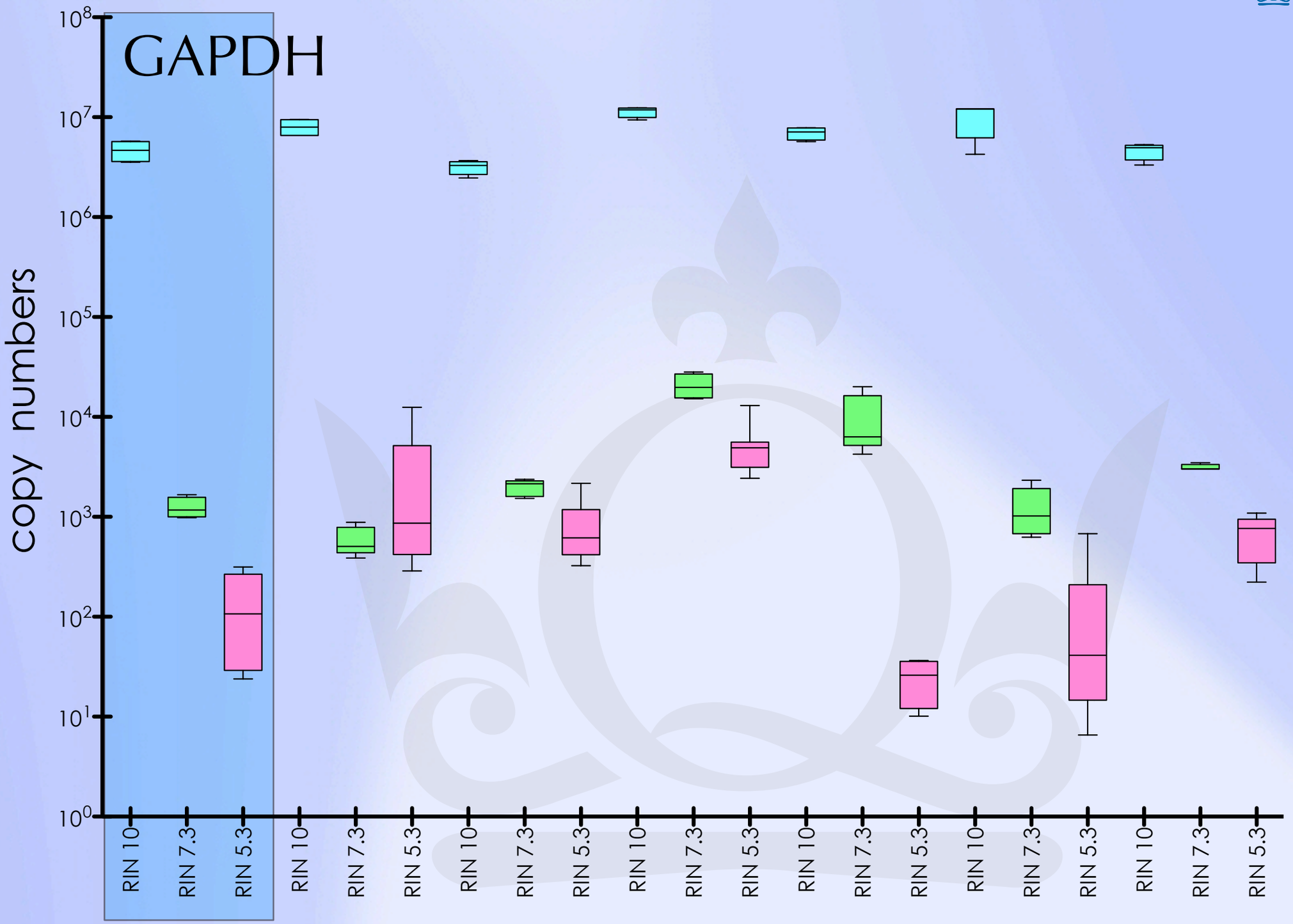
Output of sir_graph (®)
by D. Stewart and M. Zuker

dG = -502.00 cdx

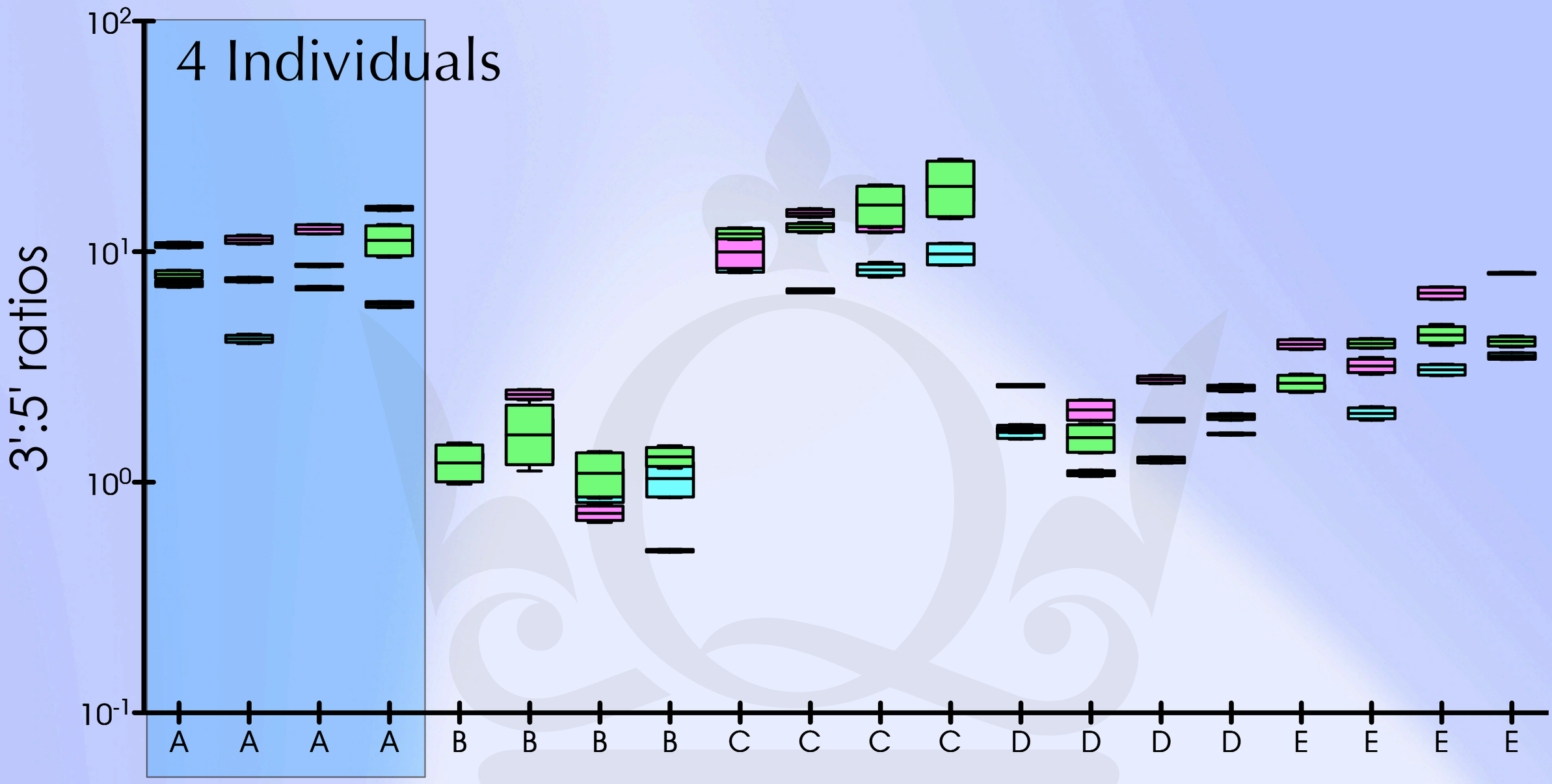

TSP
6-mer
o-dT
15-mer 3.5
15-mer 4.5
15-mer 5.5
15-mer 6.5
copy number
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
E-cadherin
VEGF
DRG-1
CD133
GAPDH
p21
OSN
SVN
mRNA target



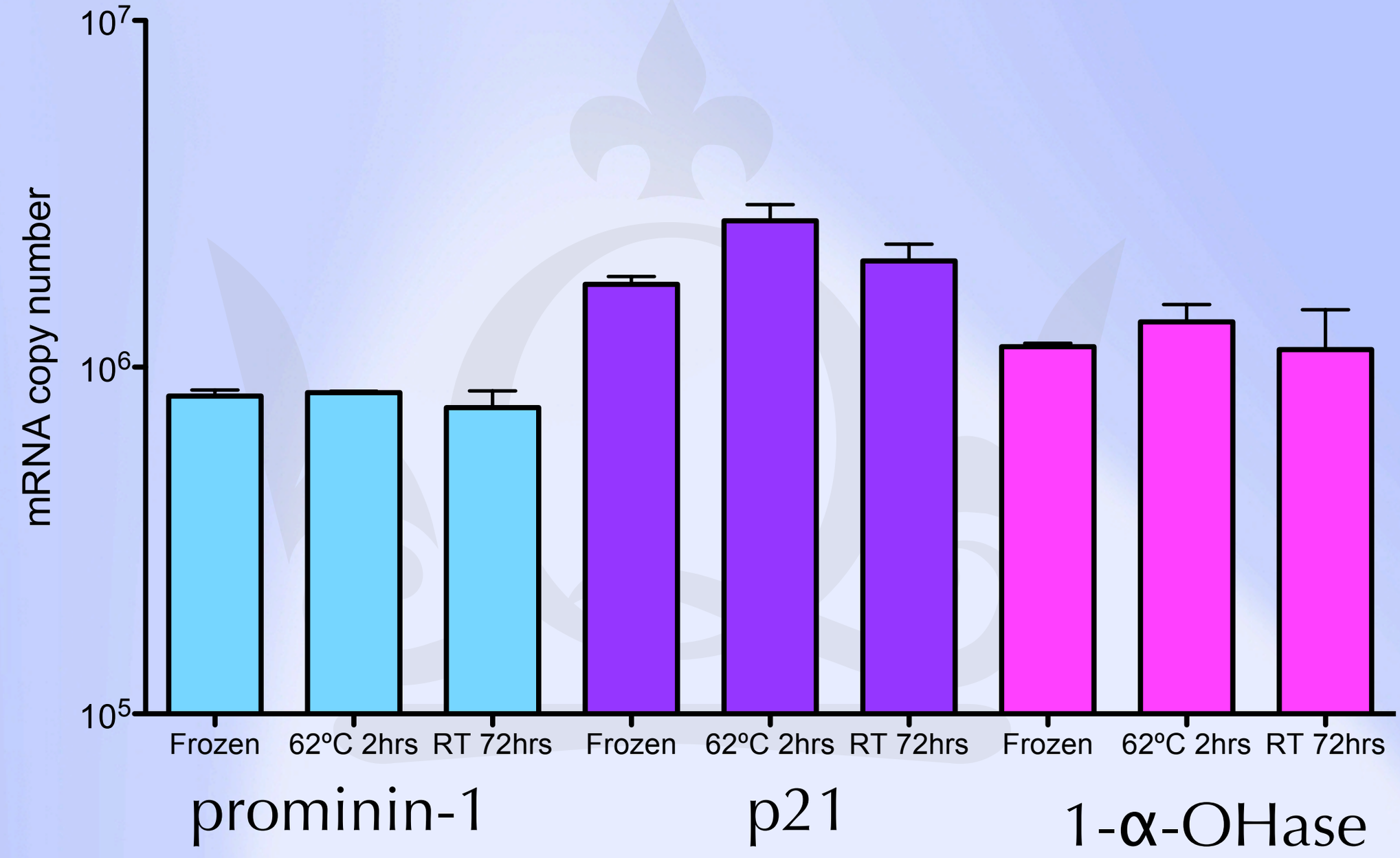
RNA quality and cDNA priming



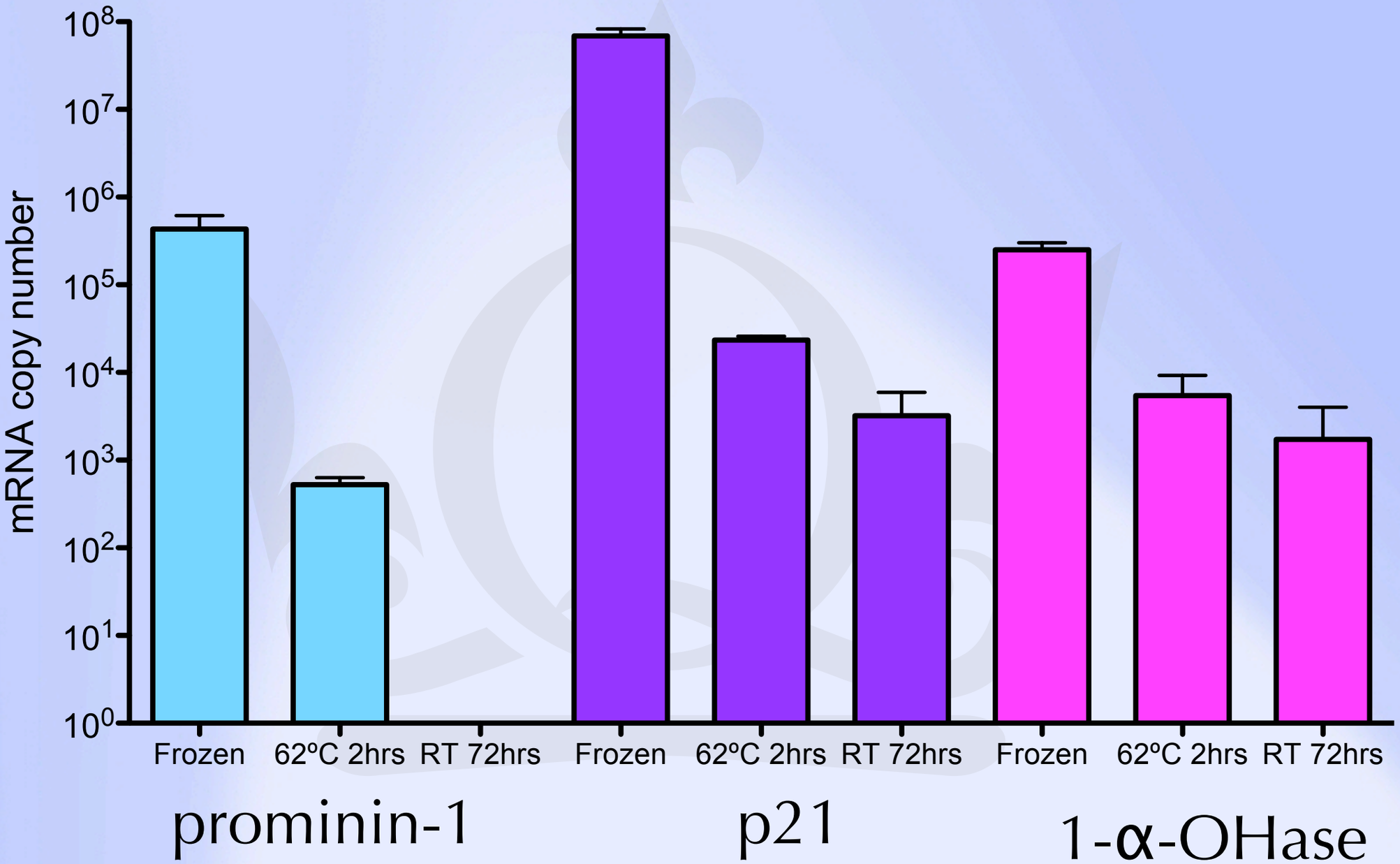
o-dT: lower RNA quality=lower copy no



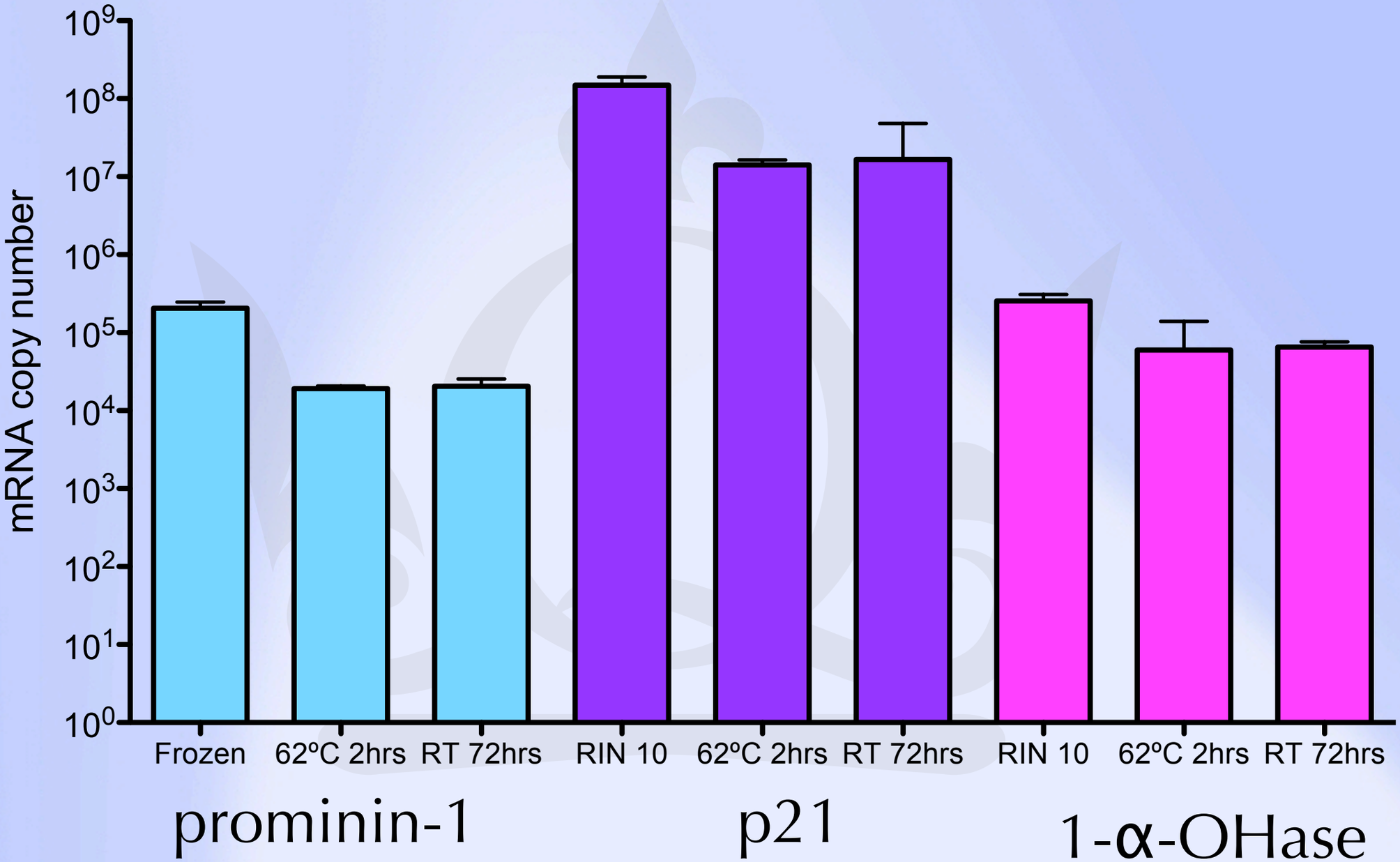
Gene-specific priming



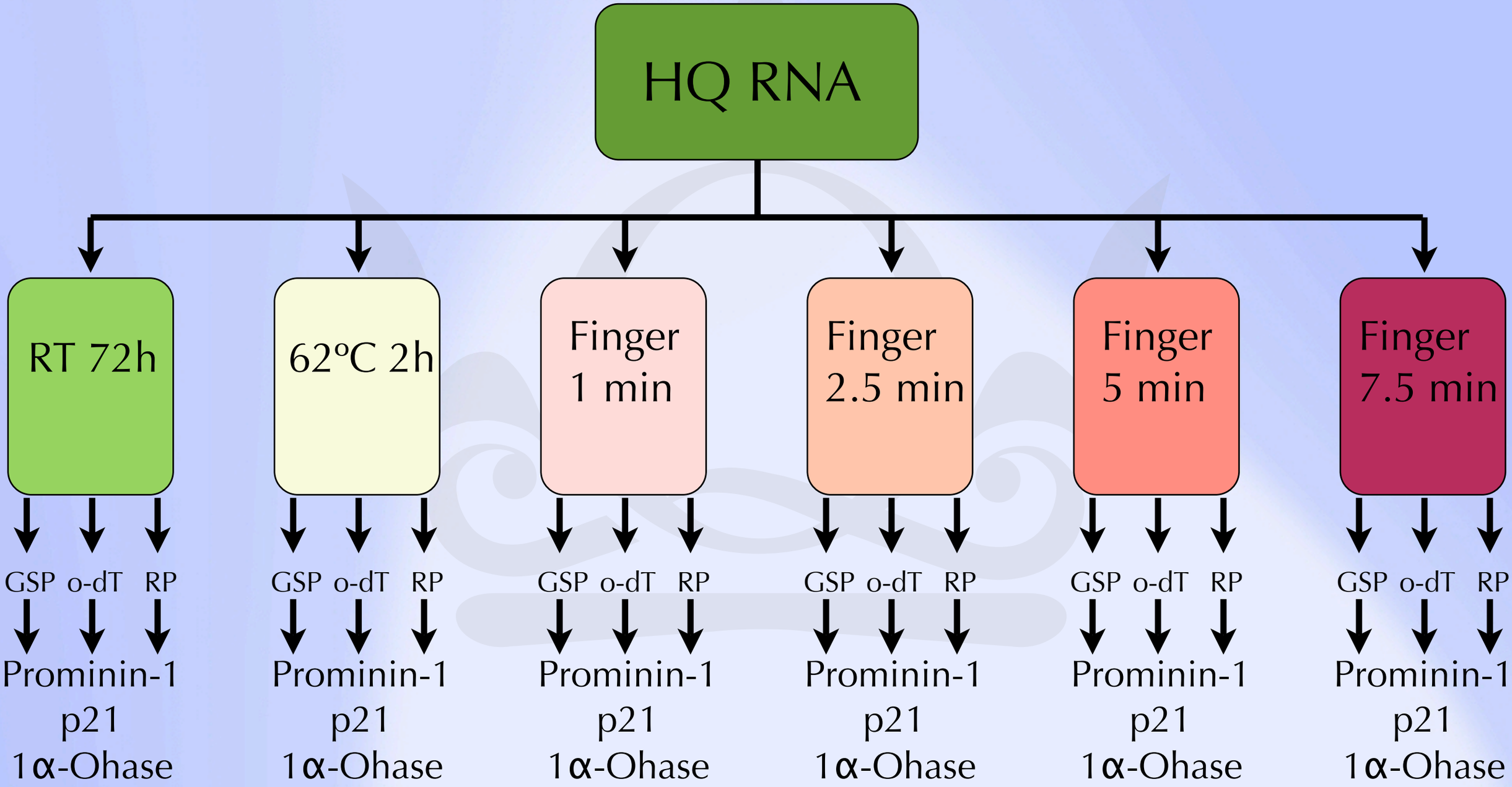
Gene-specific priming



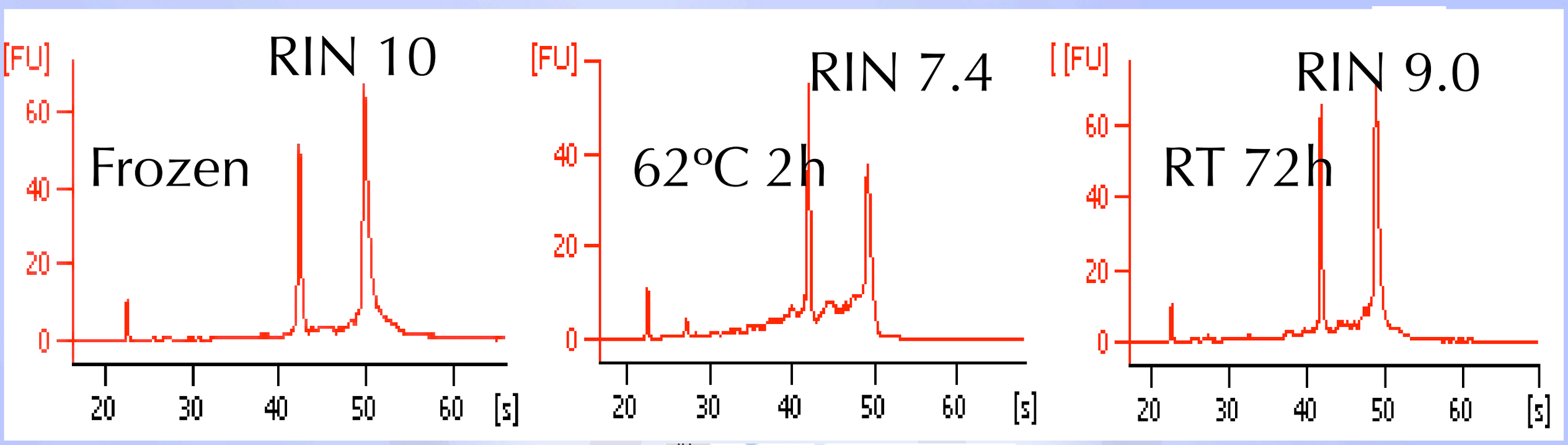
oligo-dT priming

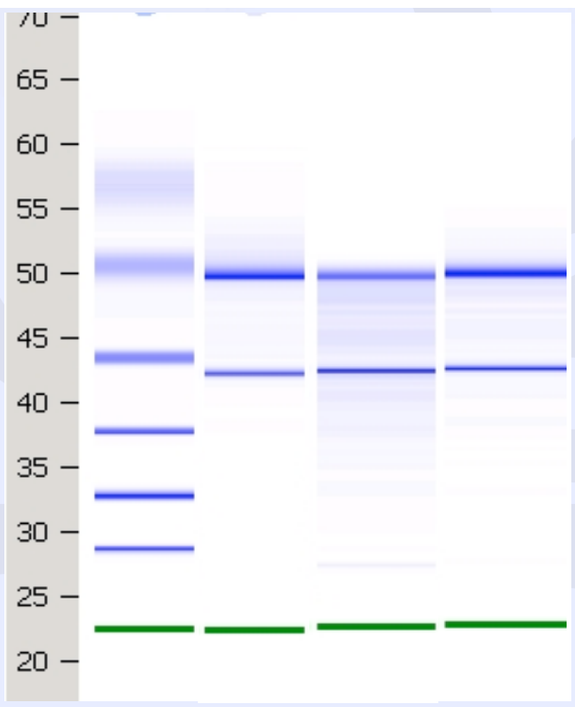


Random priming

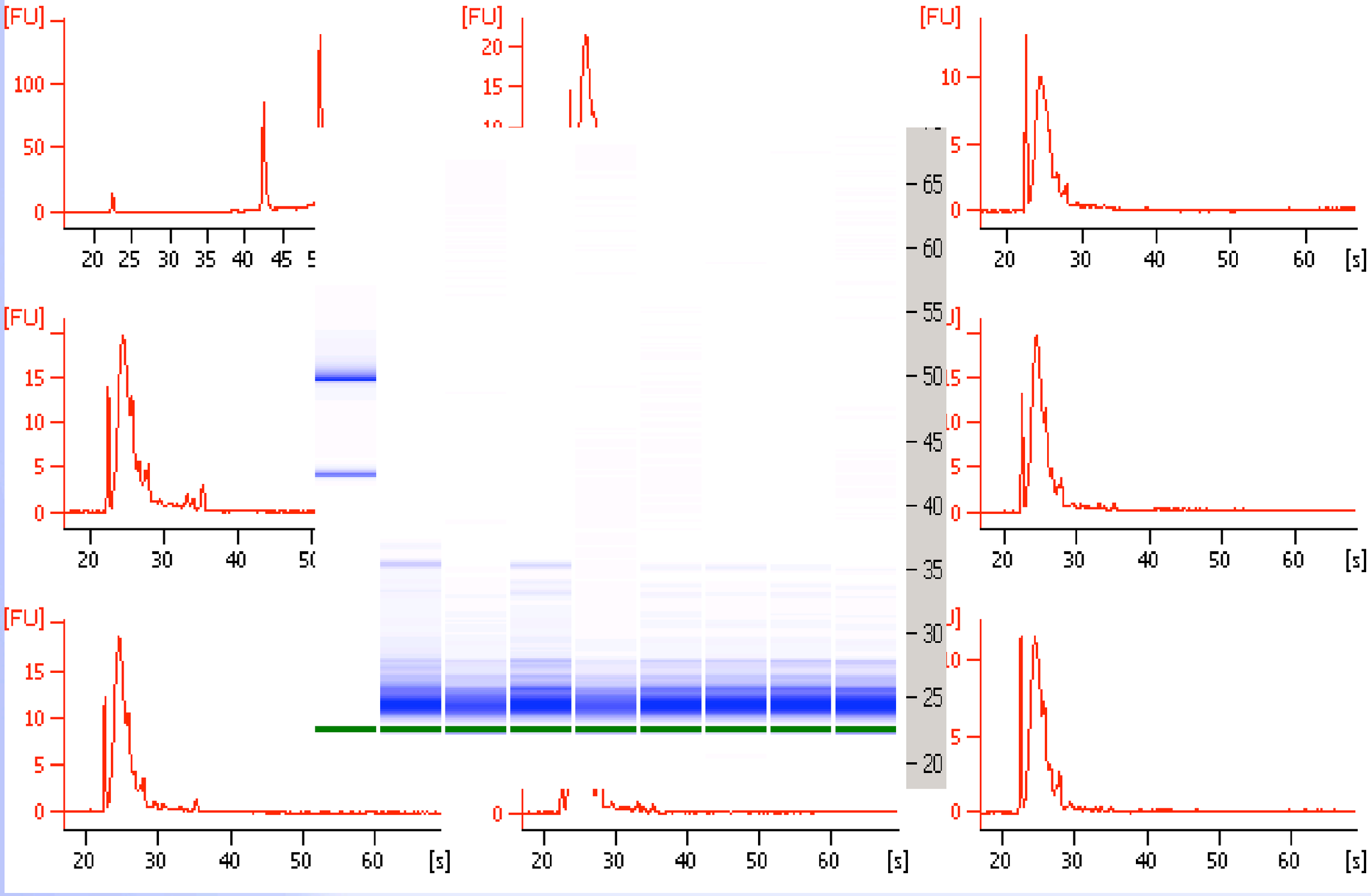


Degradation experiment





RNA integrity and cDNA synthesis



Degraded RNA

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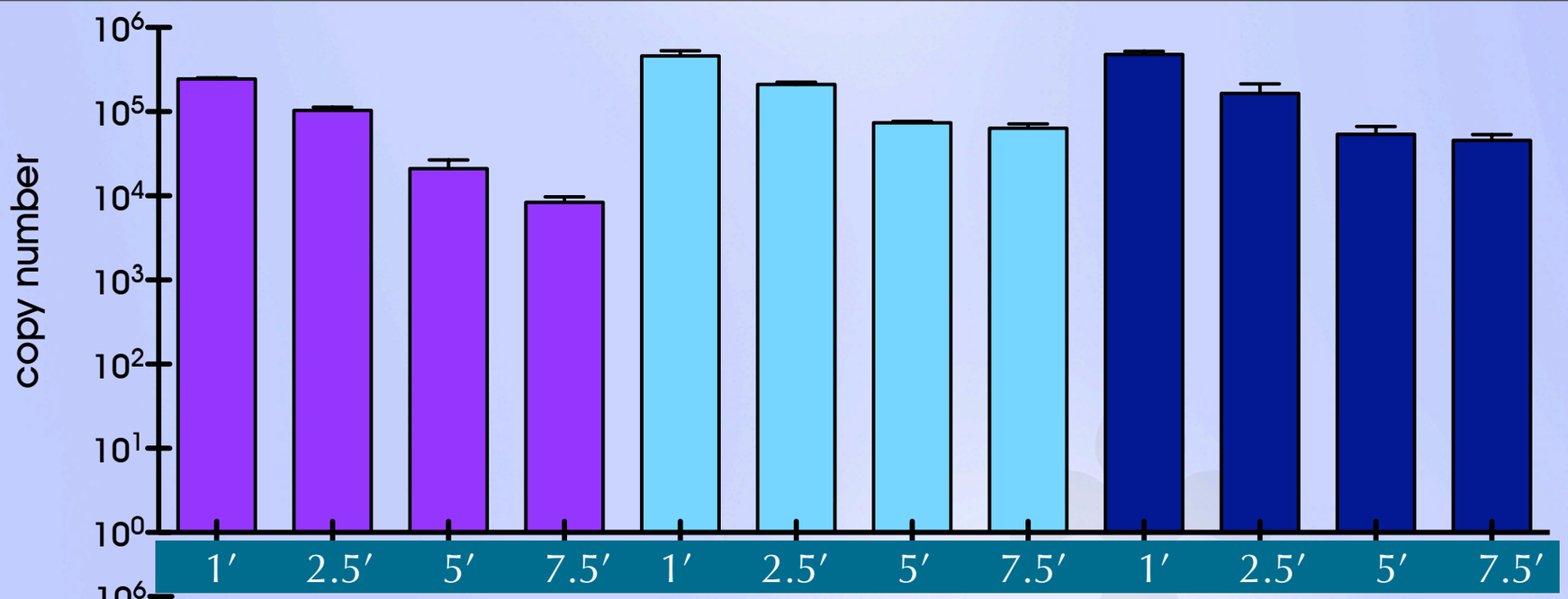
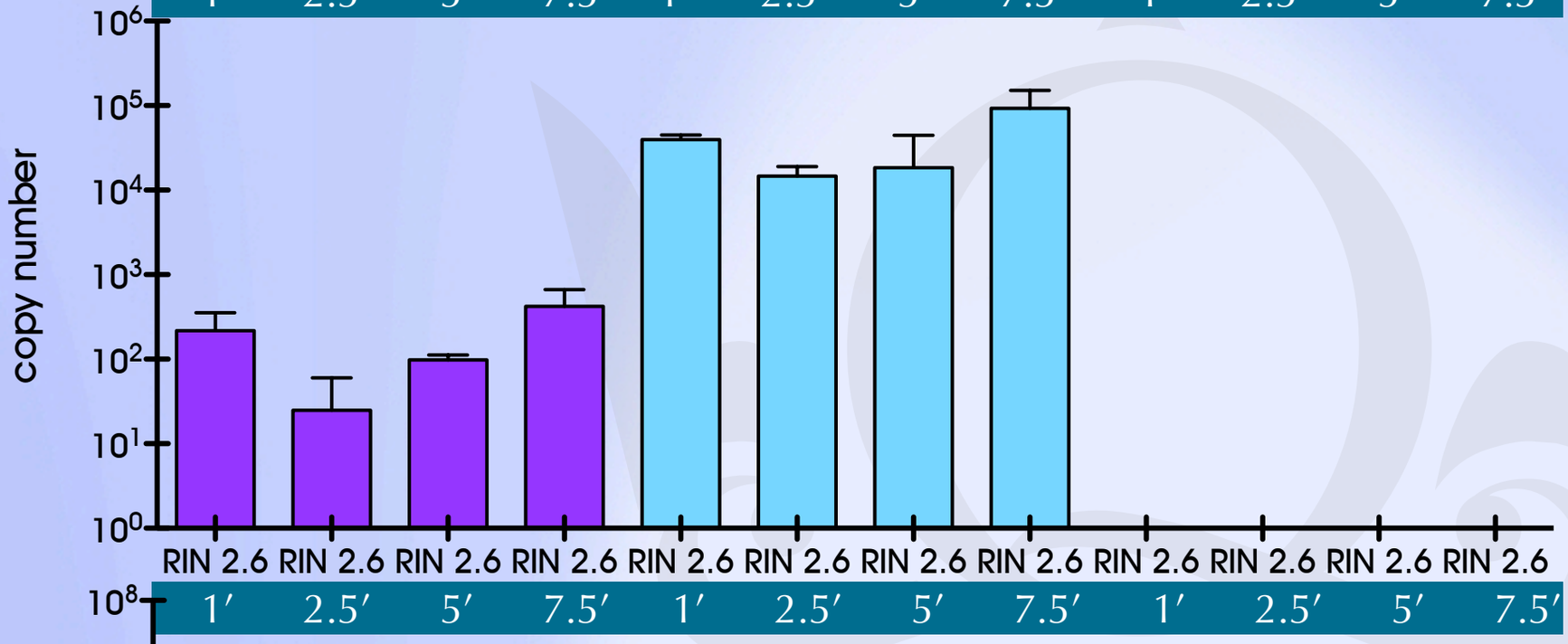
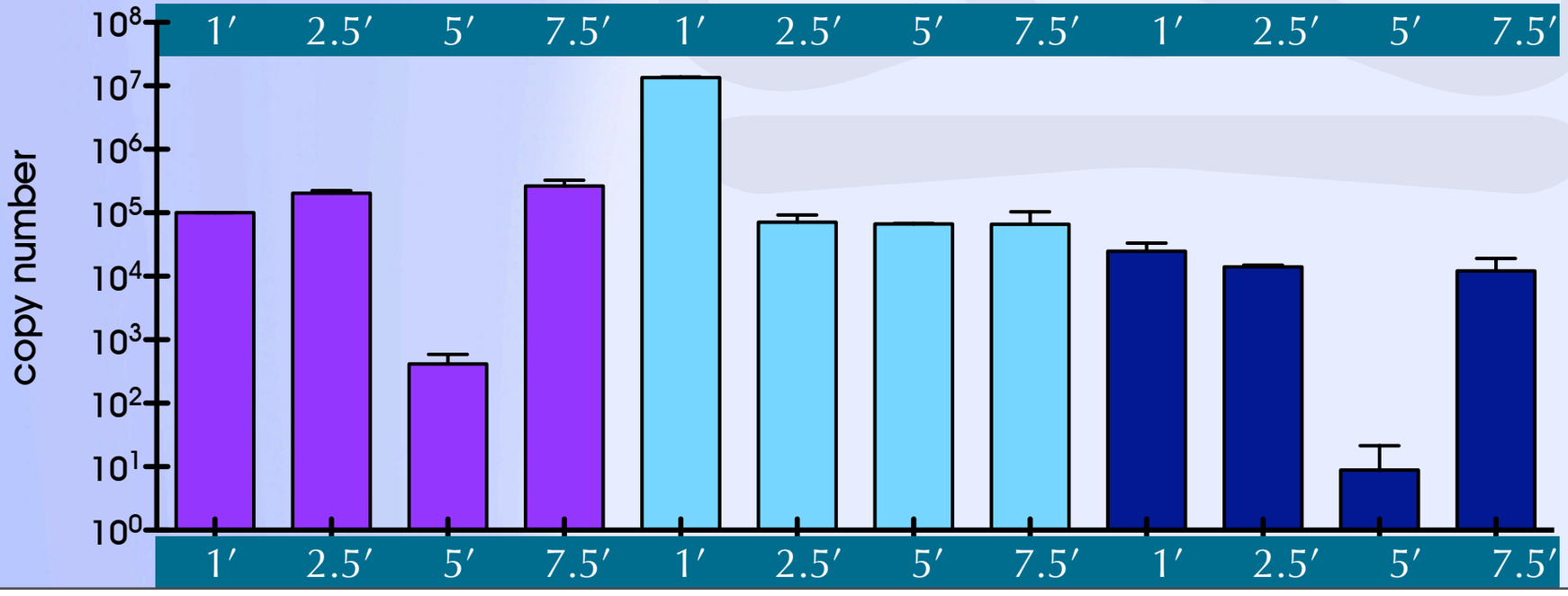


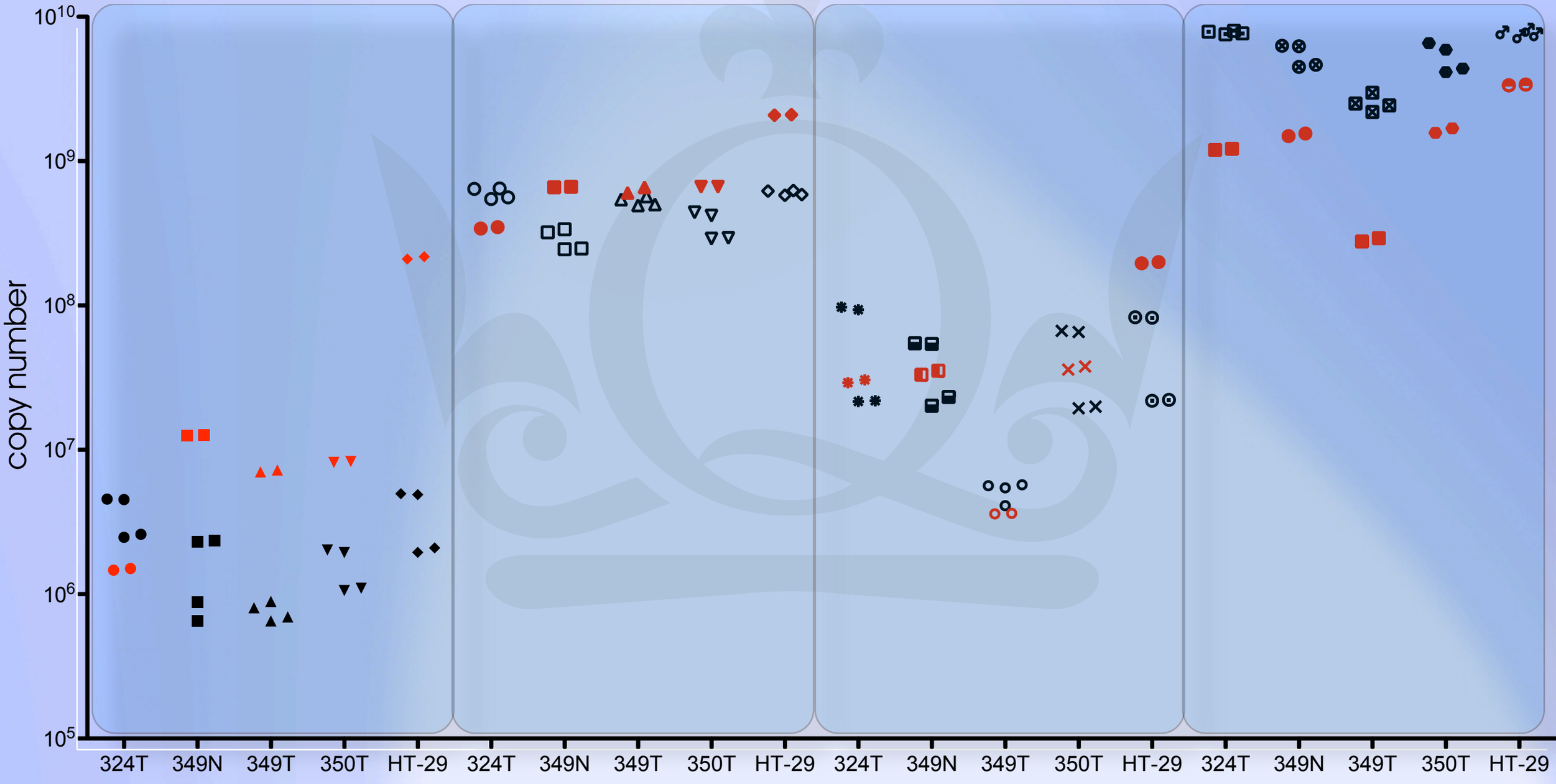
Gene-specific priming



oligo-dT priming



Random priming



Influence of RT

- accurate selection of starting material
- quantification & quality assessment of mRNA
- consistent priming strategies
- quality assessment of reagents and operators
- appropriate data analysis

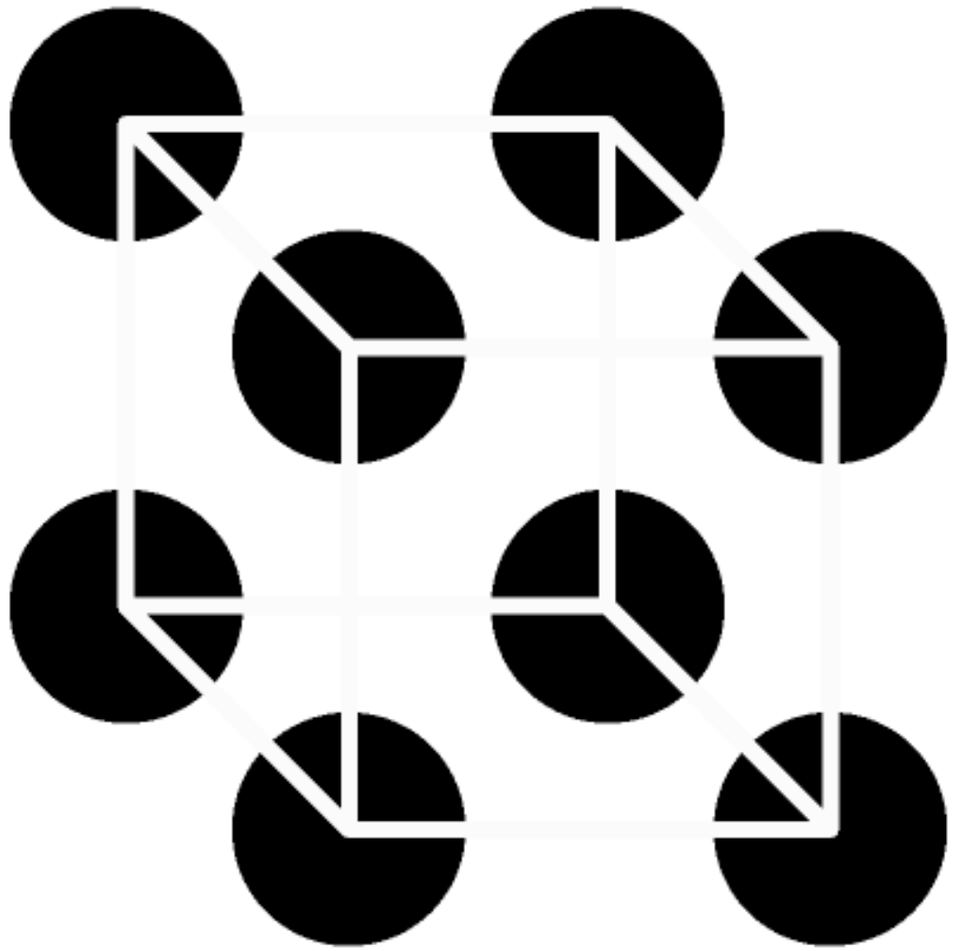
Summary: mRNA quantification

- Analyse biological replicates
- Two targets/mRNA
- Inhibition analysis
- Integrity assay
- Appropriate analysis

Suggested workflow

- RT-qPCR is not a robust assay
- Pre-assay steps critical for data quality

Conclusions



What shape do you see?

Data analysis