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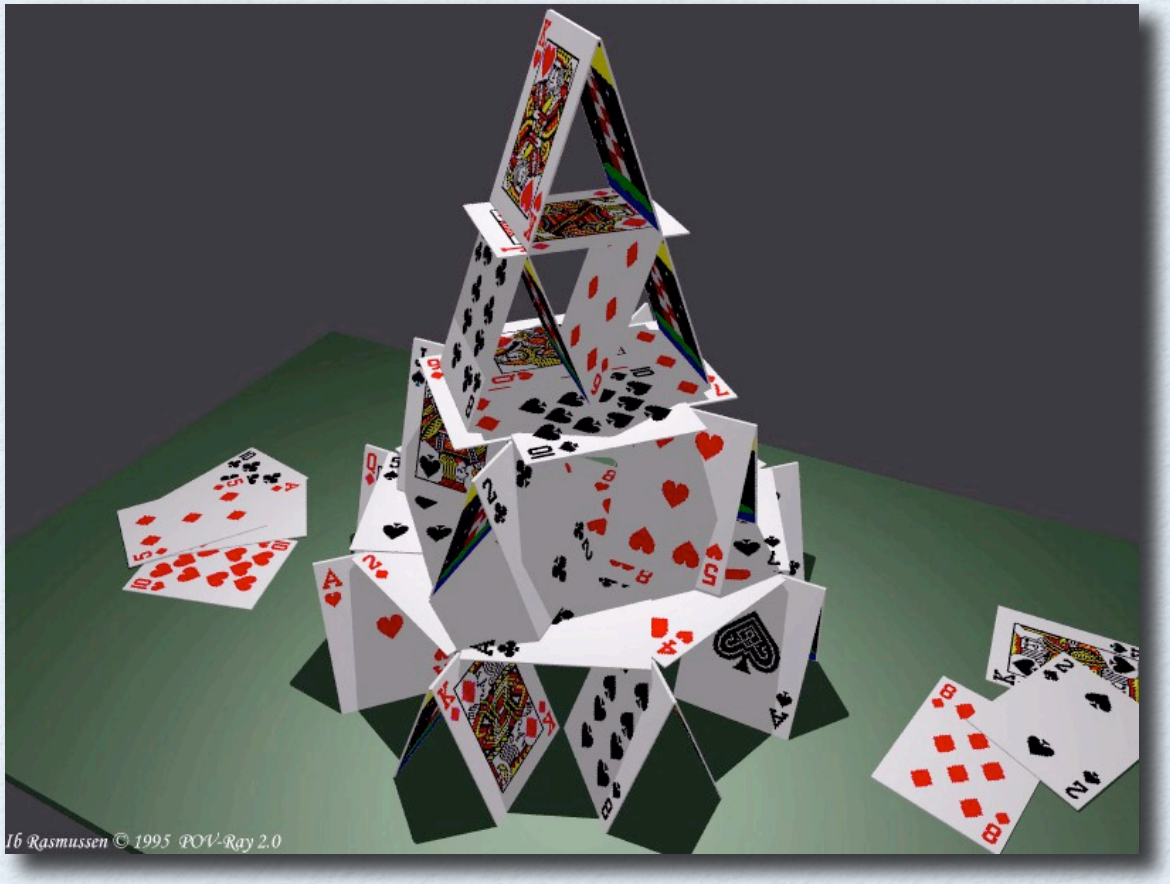
2nd qPCR meeting Freising, Germany 5th September 2005

mRNA quantification from archival cancer samples

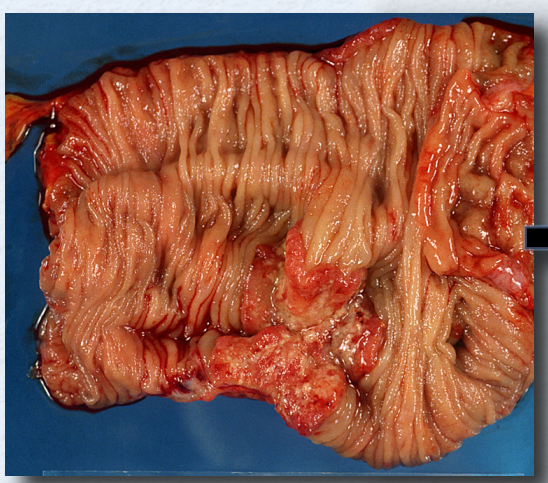
Professor Stephen A Bustin
s.a.bustin@qmul.ac.uk

Queen Mary
University of London

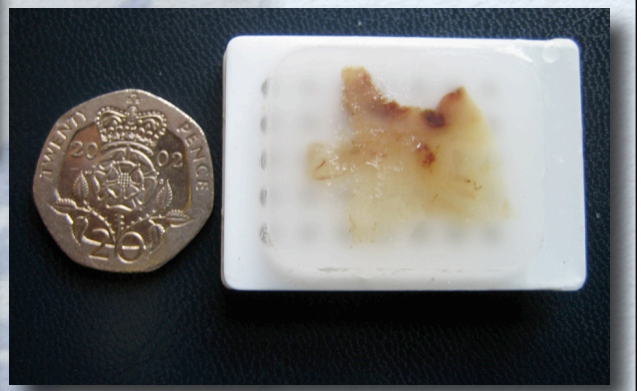
real-time RT-PCR

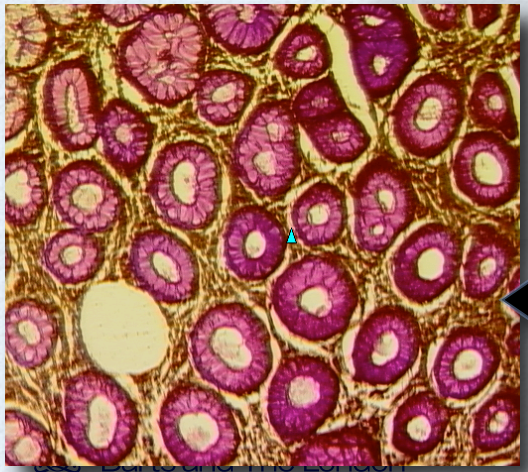


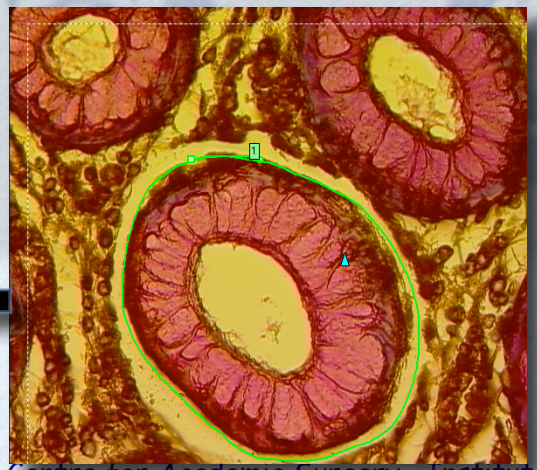
What is archival material ?







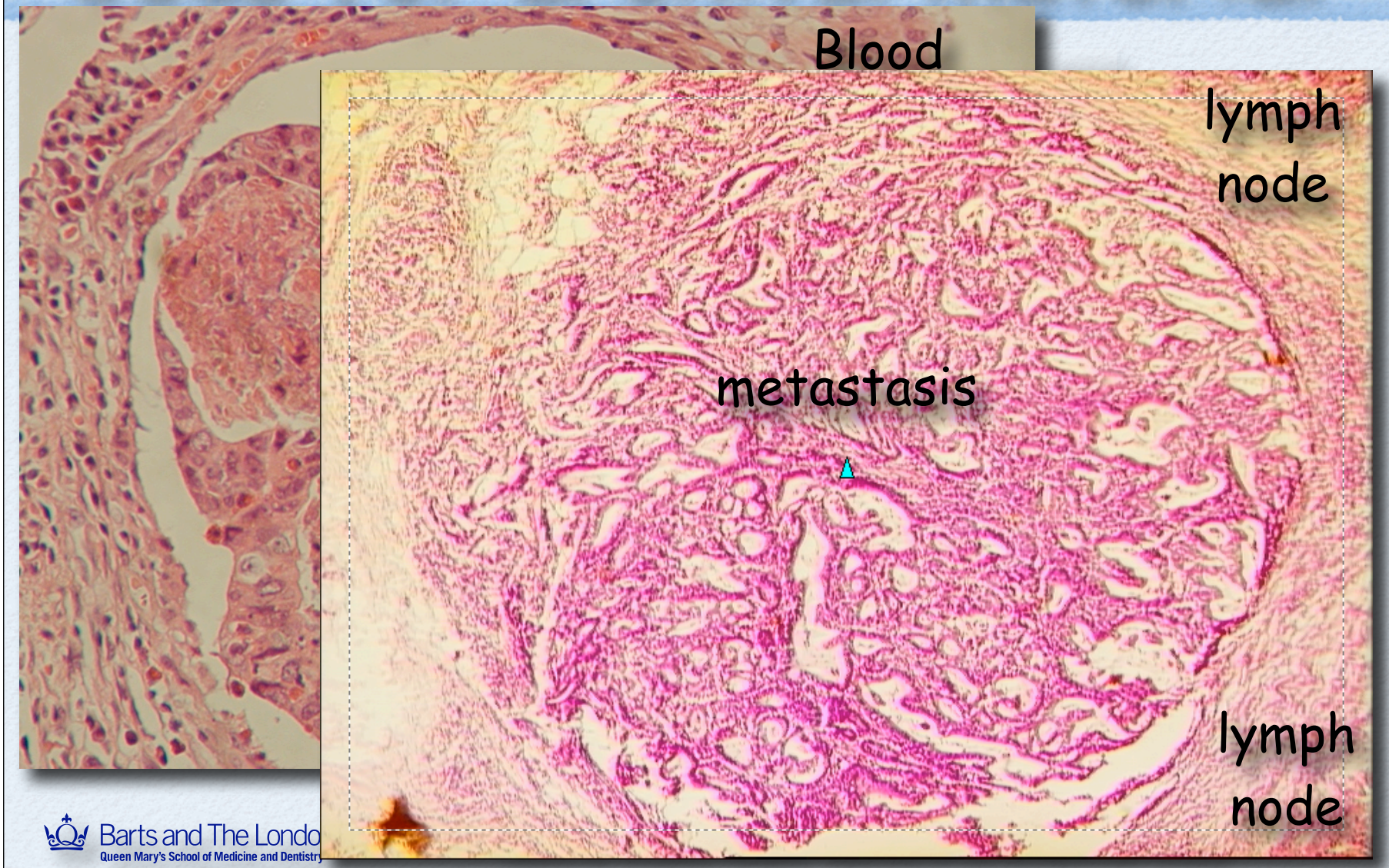




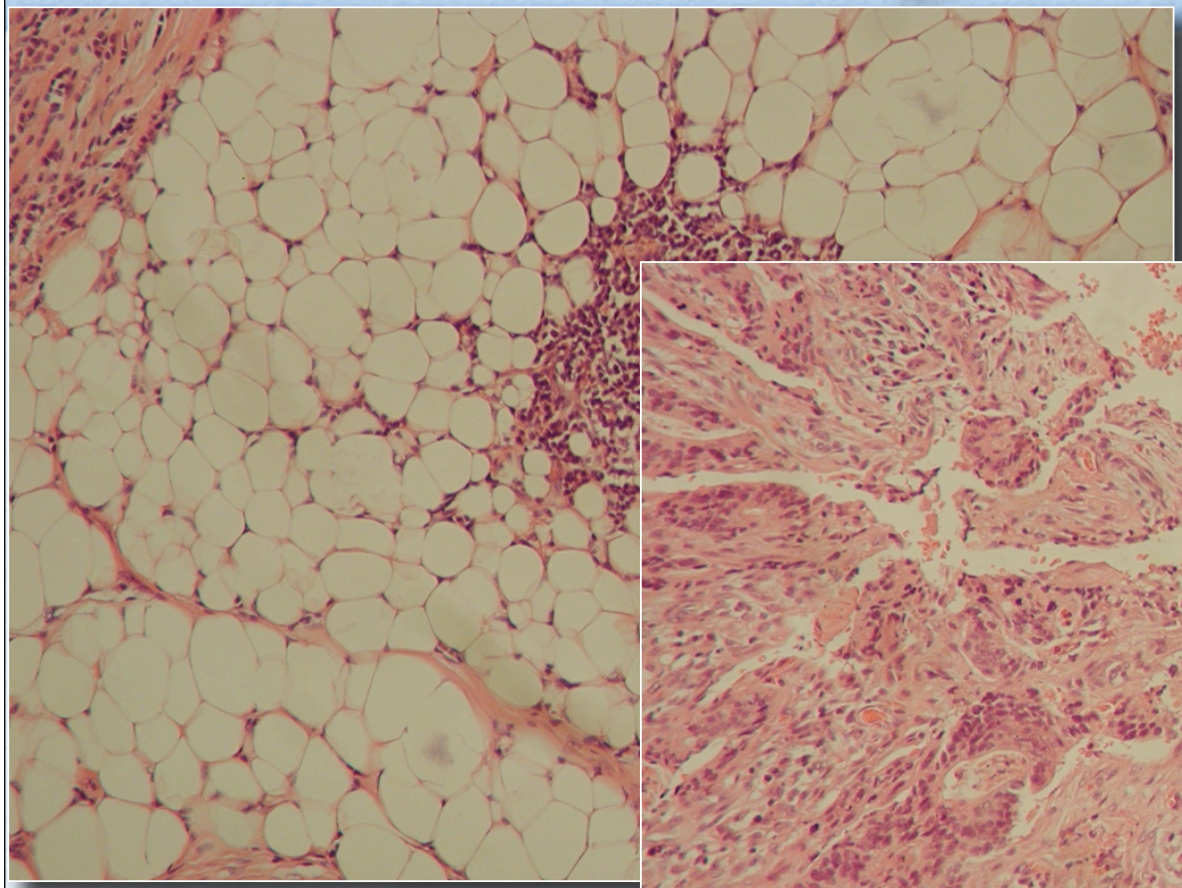
Why use archival material?

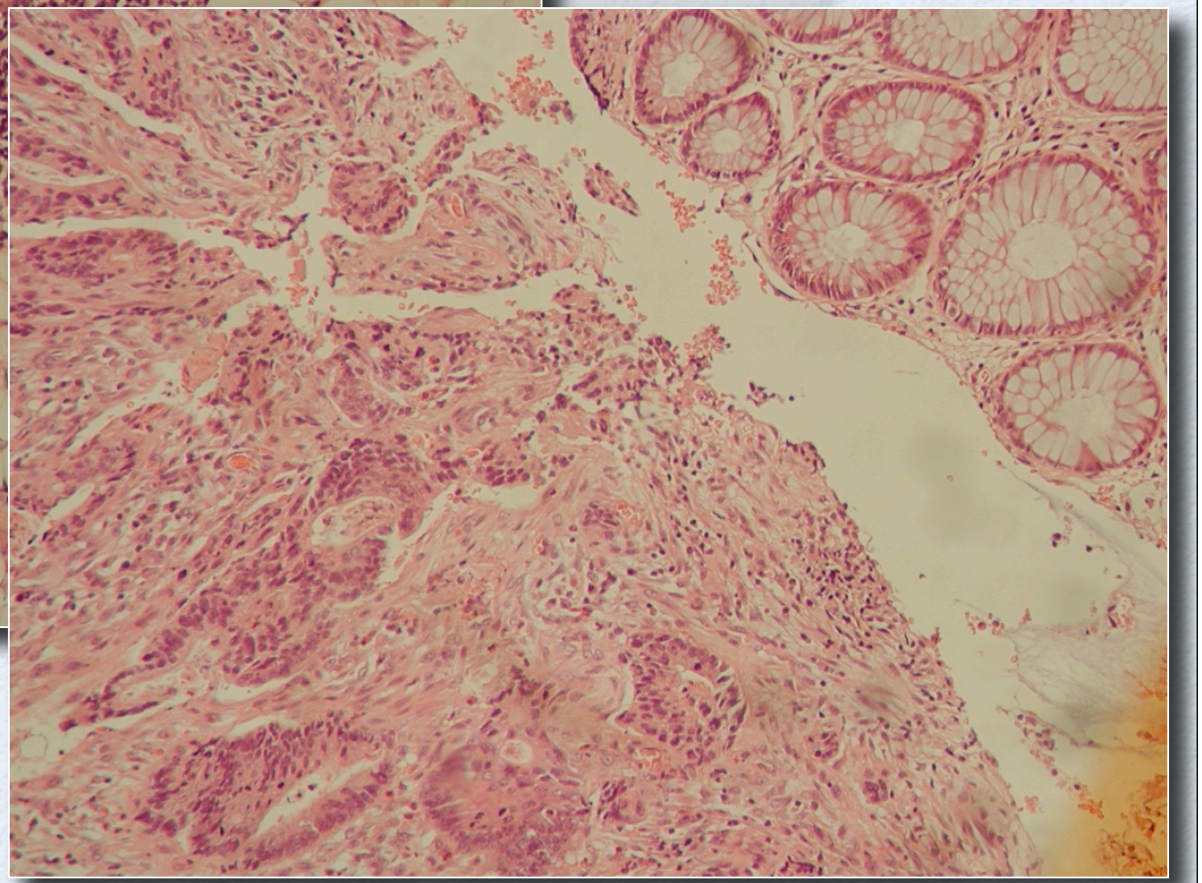


Uses for archival material



Heterogeneity





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BMC Clinical Pathology

BioMed Central

Research article

Open Access

In vitro mutation artifacts after formalin fixation and error prone translesion synthesis during PCR

Nancy Quach[1], Myron F Goodman[2] and Darryl Shibata*[1]

Address: [1]Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, CA, USA and [2]Hedco Molecular Biology Laboratories, Department of Biological Sciences and

Email: Nancy Quach - nquach@usc.edu; Myron F Goodman

* Corresponding author

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BMC Clinical Pathology 2004, **4**:1

This article is available from: http://www.biomedcentral.com/147

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Technical Advance

Evaluation of Non-Formalin Tissue Fixation for Molecular Profiling Studies

John W. Gillespie,*† Carolyn J.M. Best,†
Verena E. Bichsel,‡ Kristina A. Cole,†
Susan F. Greenhut,§ Stephen M. Hewitt,¶
Mamoun Ahram,† Yvonne B. Gathright,§
Maria J. Merino,¶ Robert L. Strausberg,§
Jonathan I. Epstein,‖ Stanley R. Hamilton,**
Gallya Gannot,†† Galina V. Baibakova,†
Valerie S. Calvert,‡ Michael J. Flaig,†
Rodrigo F. Chuaqui,† Judi C. Herring,‡‡
John Pfeifer,§§ Emmanuel F. Petricoin,‡
W. Marston Linehan,¶¶ Paul H. Duray,¶
G. Steven Bova,‖ and Michael R. Emmert-Buck†¶¶¶

From the Science Applications International Corporation, National Cancer Institute, Frederick, Maryland; the*

was superior to that obtained from formalin-fixed samples and sufficient to perform reverse transcription polymerase chain reactions. Recovery of DNA from ethanol-fixed specimens was superior to formalin-fixed samples as determined by one-dimensional gel electrophoresis and polymerase chain reaction. In conclusion, specimens fixed in 70% ethanol and embedded in paraffin produce good histology and permit recovery of DNA, mRNA, and proteins sufficient for several downstream molecular analyses. Complete protocols and additional discussion of relevant issues are available on an accompanying website (http://cgap-mf.nih.gov/). *(Am J Pathol 2002, 160:449–457)*

American Journal of Pathology, Vol. 161, No. 6, December 2002
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Review

Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids

Mythily Srinivasan, Daniel Sedmak, and Scott Jewell

From the Department and Pathology, The Ohio State University, Columbus, Ohio

Table 1. Fixation Parameters for Optimal Tissue Preservation

- Group I: Prefixation parameters
  - 1. Constant factors
    - a. Nature of the anesthetic
    - b. Duration of anesthesia
    - c. Anoxic injury *in situ*
  - 2. Variable factors
    - a. Prefixation time
- Group II: Intrafixation parameters
  - 1. Properties of the fixatives
    - a. Chemistry and mechanism of action
    - b. Tissue penetration
  - 2. Condition of fixation
    - a. Temperature
    - b. Duration
    - c. pH
    - d. Osmolarity
    - e. Concentration
    - f. Size of the specimen
    - g. Volume of the fixative
- Group III: Post-fixation parameters
  - 1. Storage parameters
    - a. Duration
    - b. Temperature
    - c. Condition (vacuum/nonvacuum packed)
  - 2. Nature of the biological factor to be analyzed
    - a. Proteins
    - b. Enzymes
    - c. Lipids
    - d. Nucleic acids
    - e. Mucopolysaccharides
    - f. Biogenic amines
    - g. Glycogen

Parameters

- Formalin fixation
- Paraffin-embedding
- Length of storage in block
- RNA extraction procedure
- RT-PCR protocol

RNA quality

- integrity
- yield
- inhibition
- accessibility
- Maintenance of expression profile

Formalin fixation

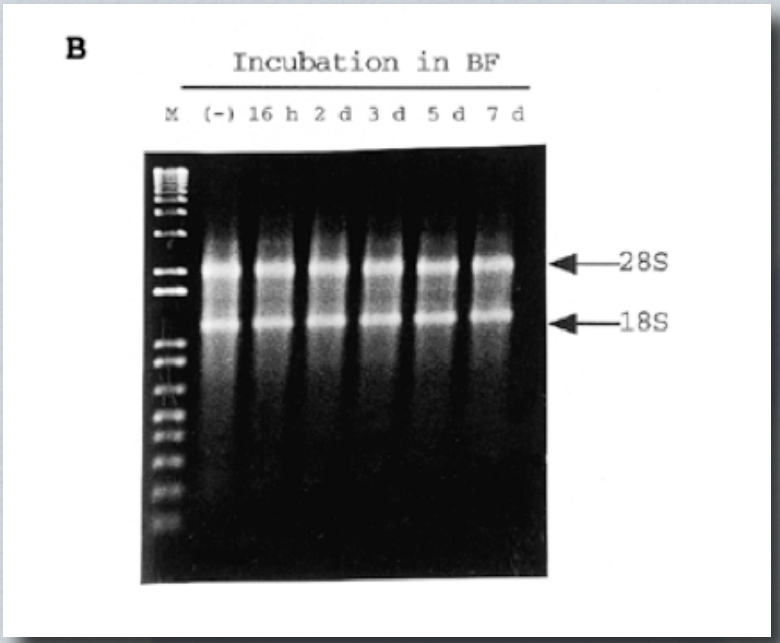
4436–4443 *Nucleic Acids Research, 1999, Vol. 27, No. 22* © 1999 Oxford University Press

Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples

Norikazu Masuda[1,2], Tadashi Ohnishi[1,2], Shoko Kawamoto[1], Morito Monden[2] and Kousaku Okubo[1,*]

- RNA to protein cross linking
- Addition of $-CH_2OH$ (40% A, 4% U)
- Adenine dimerisation through methylene bridges

Yield & integrity



Masuda *et al* Nucl Acids Res 27:4436-4443 (1999)

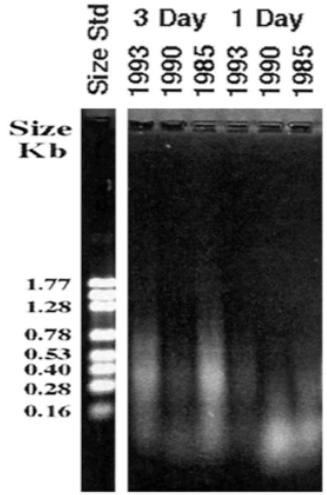
or use archival samples are as follows: (i) avoid prolonged fixation that results in irreversible modification; (ii) use a

Journal of Molecular Diagnostics, Vol. 2, No. 2, May 2000
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Quantitative mRNA Expression Analysis from Formalin-Fixed, Paraffin-Embedded Tissues Using 5′ Nuclease Quantitative Reverse Transcription-Polymerase Chain Reaction

Tony E. Godfrey,* Sun-Hun Kim,§ Marielena Chavira,* David W. Ruff,‡ Robert S. Warren,† Joe W. Gray,* and Ronald H. Jensen*

From the Departments of Laboratory Medicine and Surgery,† University of California San Francisco Cancer Center, San Francisco, California; P.E. Biosystems,‡ Foster City, California; and the School of Dentistry,§ Chonnam National University, Kwangju, South Korea*



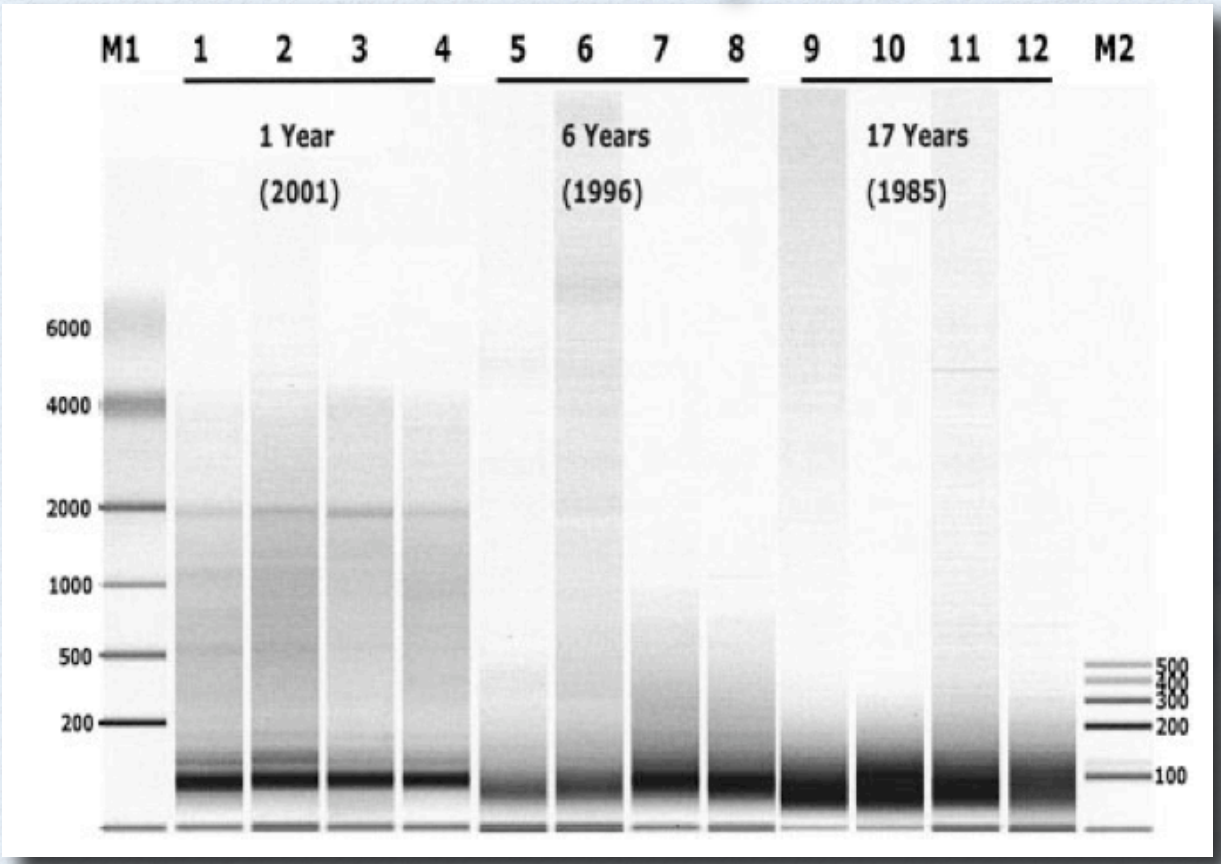
A

Year	RNA Yield per 20μM Section	
	1 Day Incubation	3 Day Incubation
1985	3.2μg	7.2μg
1990	3.0μg	4.6μg
1993	2.5μg	4.7μg

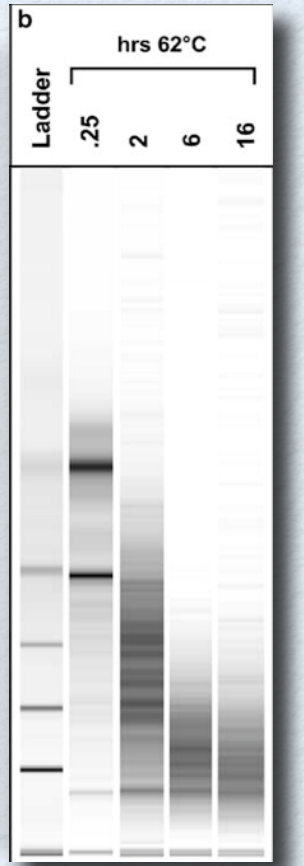
B

Figure 1. Denaturing agarose gel of RNA isolated from 3 archival prostate tissue blocks collected in 1985, 1990, and 1993, respectively. RNA isolated using the modified 3-day protocol is longer (**A**) and the yield is higher (**B**) than with the original protocol.

Effects of paraffin on RNA



Cronin et al, Am J Pathol (2004) 164, 35 - 42



Antonov et al, Lab Invest (2005) 85, 1040 - 1050

Archival material

- Delay between resection and fixation ?
  - min-max 12 hrs (Godfrey et al, J Mol Diagn 2:84-91 (2000)
- Length of time of formalin fixation ?
  - 2-7+ days
- Fixation protocol ?
  - dehydration? intra/inter-hospital variability
- Length of storage ?
  - years

RNA quality

- rRNA is degraded
- mRNA integrity: ?
- accessibility: ?
- inhibitors: ?

Impact of RNA quality on reference gene expression stability

Claudina Angela Pérez-Novo, Cindy Claeys, Frank Speleman, Paul Van Cauwenberge, Claus Bachert, and Jo Vandesompele

Ghent University Hospital, Ghent, Belgium

BioTechniques 39:52-56 (July 2005)

Our results show that the stability of reference genes not only is different in CRS and NP ethmoidal and maxillary sinus tissues (as can be expected due to differences in cellular origin), but also varies within the same tissue type according to the degradation status of the samples (Figure 1). The fact that in degraded samples, we propose performing RNA quality control prior to downstream quantification assays and discarding degraded samples, especially if one aims to accurately quantify small expression differences. Indeed, as it is of utmost importance to normalize samples using the same set of reference genes, our results suggest that it is inappropriate to compare degraded and intact samples.

1

2

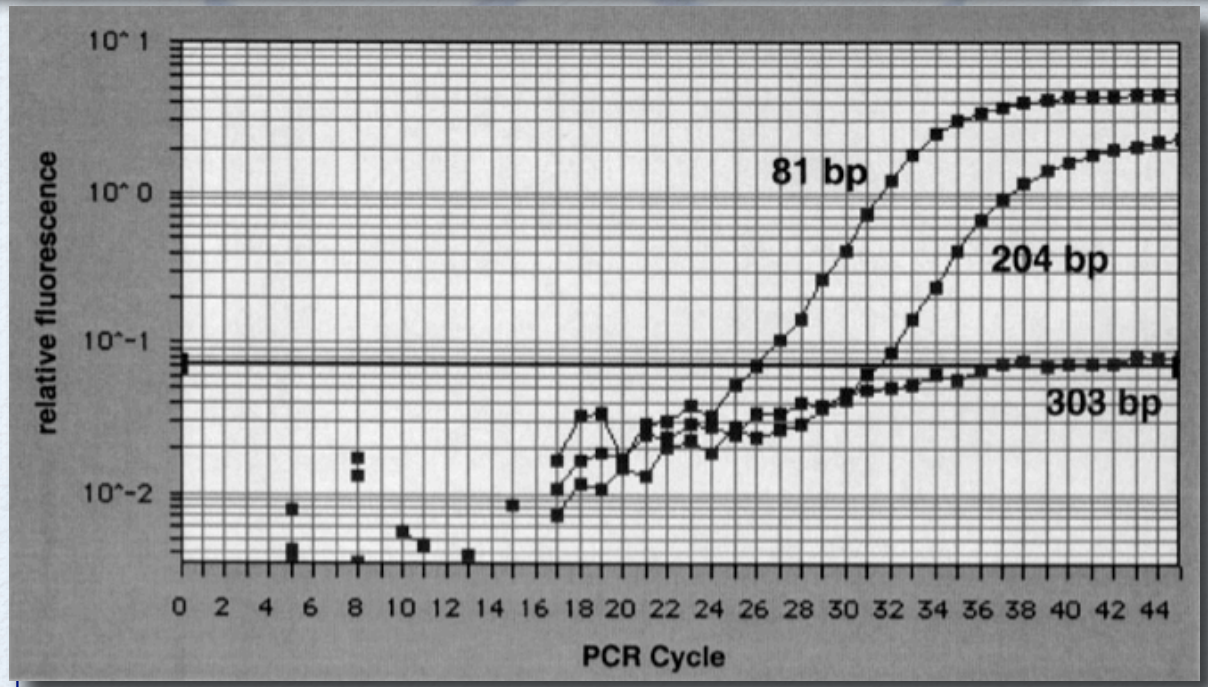
3

METHODS 25, 409–418 (2001)
doi:10.1006/meth.2001.1263, available online at http://www.idealibrary.com on IDEAL®

Real-Time PCR Analysis of DNA and RNA Extracted from Formalin-Fixed and Paraffin-Embedded Biopsies[1]

Ulrich Lehmann[2] and Hans Kreipe

Institute of Pathology, Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany



American Journal of Pathology, Vol. 158, No. 2, February 2001
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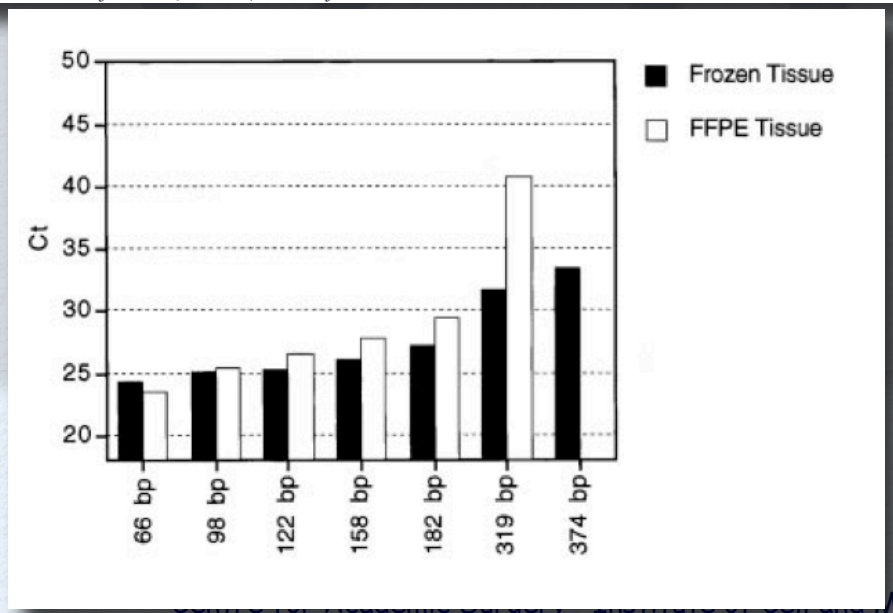
Technical Advance

Quantitative Gene Expression Analysis in Microdissected Archival Formalin-Fixed and Paraffin-Embedded Tumor Tissue

Katja Specht,* Thomas Richter,† Ulrike Müller,* Axel Walch,† Martin Werner,† and Heinz Höfler*†

From the Institute of Pathology, GSF-National Research Center for Environment and Health, Neuherberg; and the Institute of Pathology,† Technical University Munich, Munich, Germany*

verse transcriptase-polymerase chain reaction opens new avenues for the investigation and clinical validation of gene expression changes in archival tissue specimens. ***(Am J Pathol 2001, 158:419–429)***



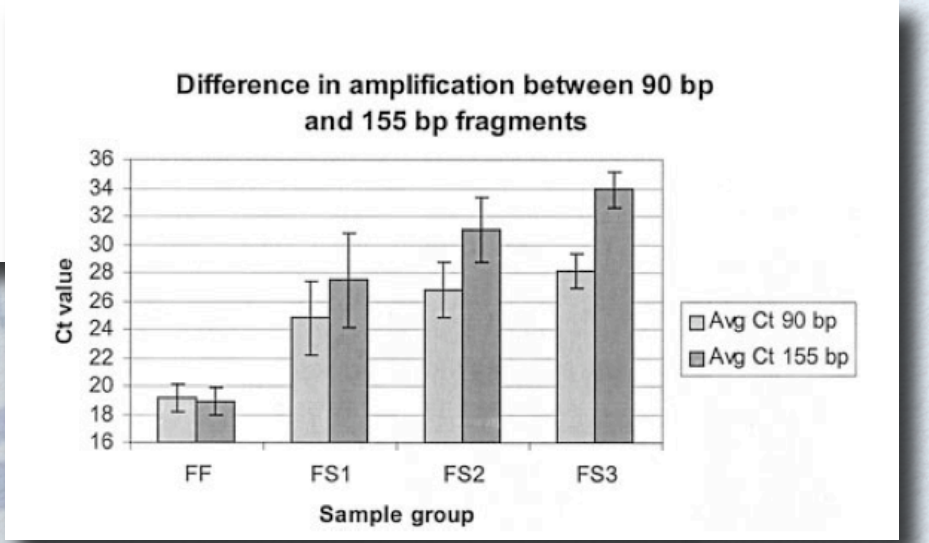
American Journal of Pathology, Vol. 165, No. 5, November 2004
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Tumorigenesis and Neoplastic Progression

Quantitative Gene Expression Profiling in Formalin-Fixed, Paraffin-Embedded Tissues Using Universal Bead Arrays

Marina Bibikova,* Dimitri Talantov,† Eugene Chudin,* Joanne M. Yeakley,* Jing Chen,* Dennis Doucet,* Eliza Wickham,* David Atkins,† David Barker,* Mark Chee,* Yixin Wang,† and Jian-Bing Fan*

From Illumina, Inc., San Diego; and Veridex, LLC,† a Johnson & Johnson Company, San Diego, California*



tissue- and cancer-specific markers derived from previous genome-wide expression profiling studies of fresh-frozen samples were validated in the formalin-fixed samples. The DASL assay system should prove useful for

Expression profile

- No "exact" correlation (Bibikova et al Am J Path 165: 1799-1807 2004)

	Differentially expressed genes (Normal vs Tumour)	
Tissue	Colon	Breast
Fresh	64	61
FFPE	38	33
in common	28 (74%)	20 (61%)

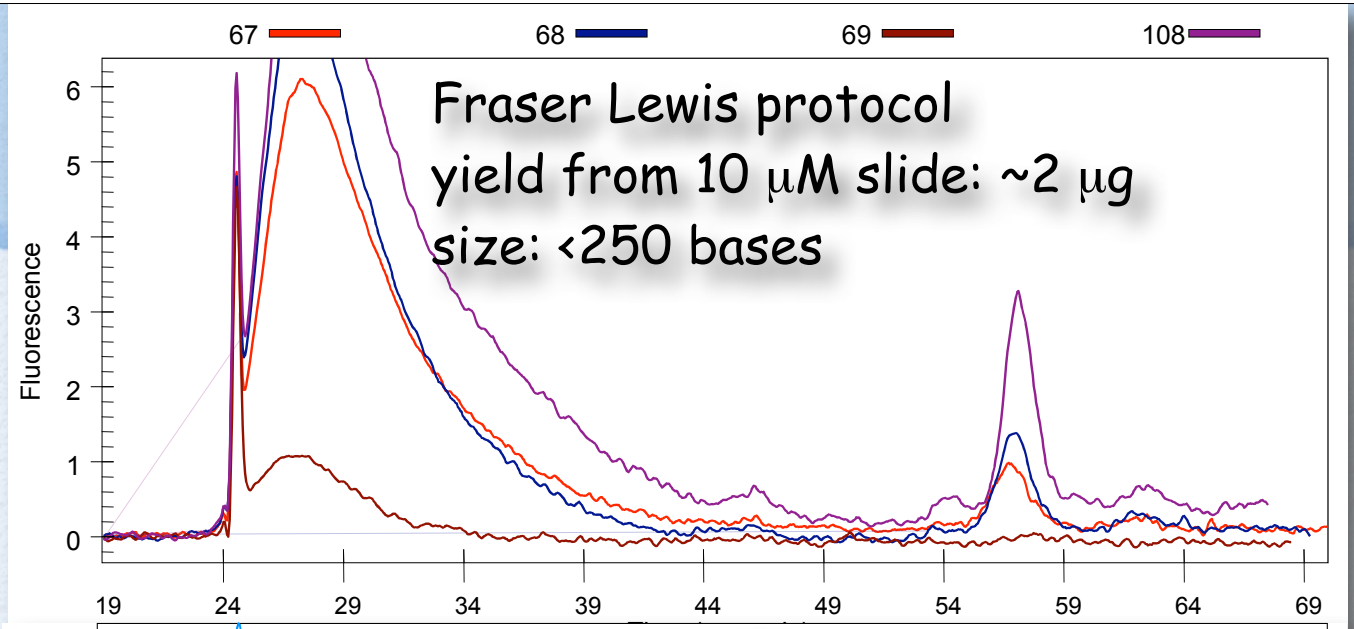
Results

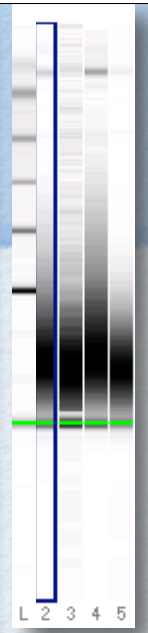
Methods

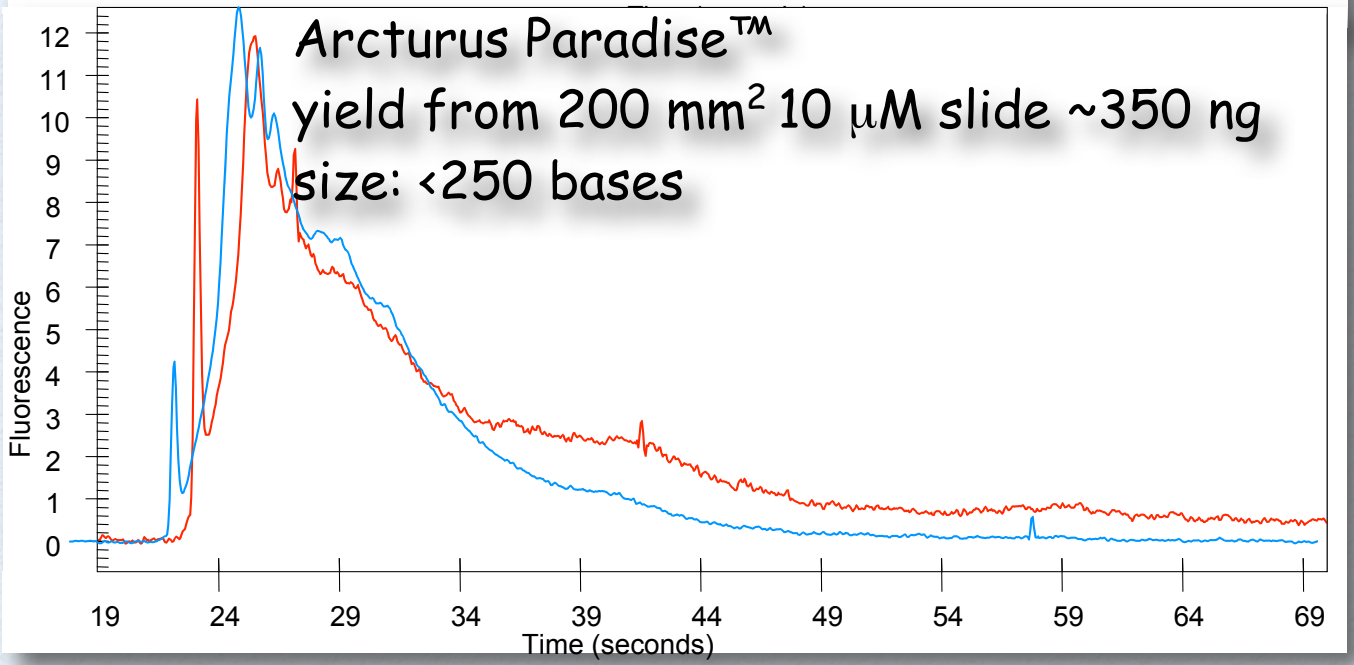
- Archival material from Pathology Dept, Royal London Hospital (median 5 (0.5-8 years old)
- PALM Laser microdissection (7-20µM)
- Fraser Lewis protocol (5 day proteinase K)
- Arcturus Paradise™ kit

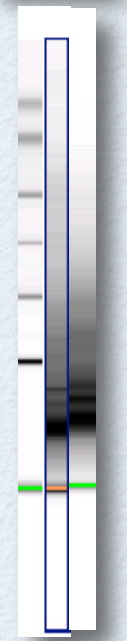
RNA extraction

- Removal of paraffin using organic solvents
- Digestion of protein matrix with proteases
- Column purification





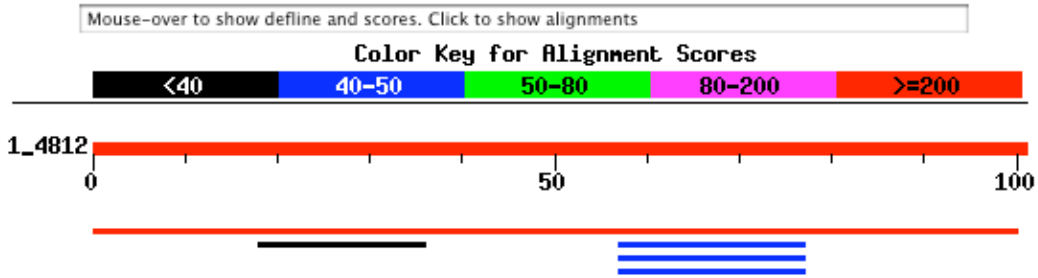




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Distribution of 5 Blast Hits on the Query Sequence



mplicon

vater, RNA

```
                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value

gi|2326871|emb|Y14572.1|STPHYB  Solanum tuberosum phyB gene         200   3e-49
gi|31560114|gb|AC145127.1|  Oryza sativa (japonica cultivar-...     42   0.17
gi|31429965|gb|AE017052.1|  Oryza sativa (japonica cultivar-...     42   0.17
gi|13384337|gb|AC079128.2|AC079128  Oryza sativa chromosome ...     42   0.17
gi|28604206|gb|AC123947.4|  Mus musculus BAC clone RP23-103O...     38   2.6

                                                Alignments


>gi|2326871|emb|Y14572.1|STPHYB   Solanum tuberosum phyB gene
          Length = 4907

 Score =  200 bits (101), Expect = 3e-49
 Identities = 101/101 (100%)
 Strand = Plus / Plus

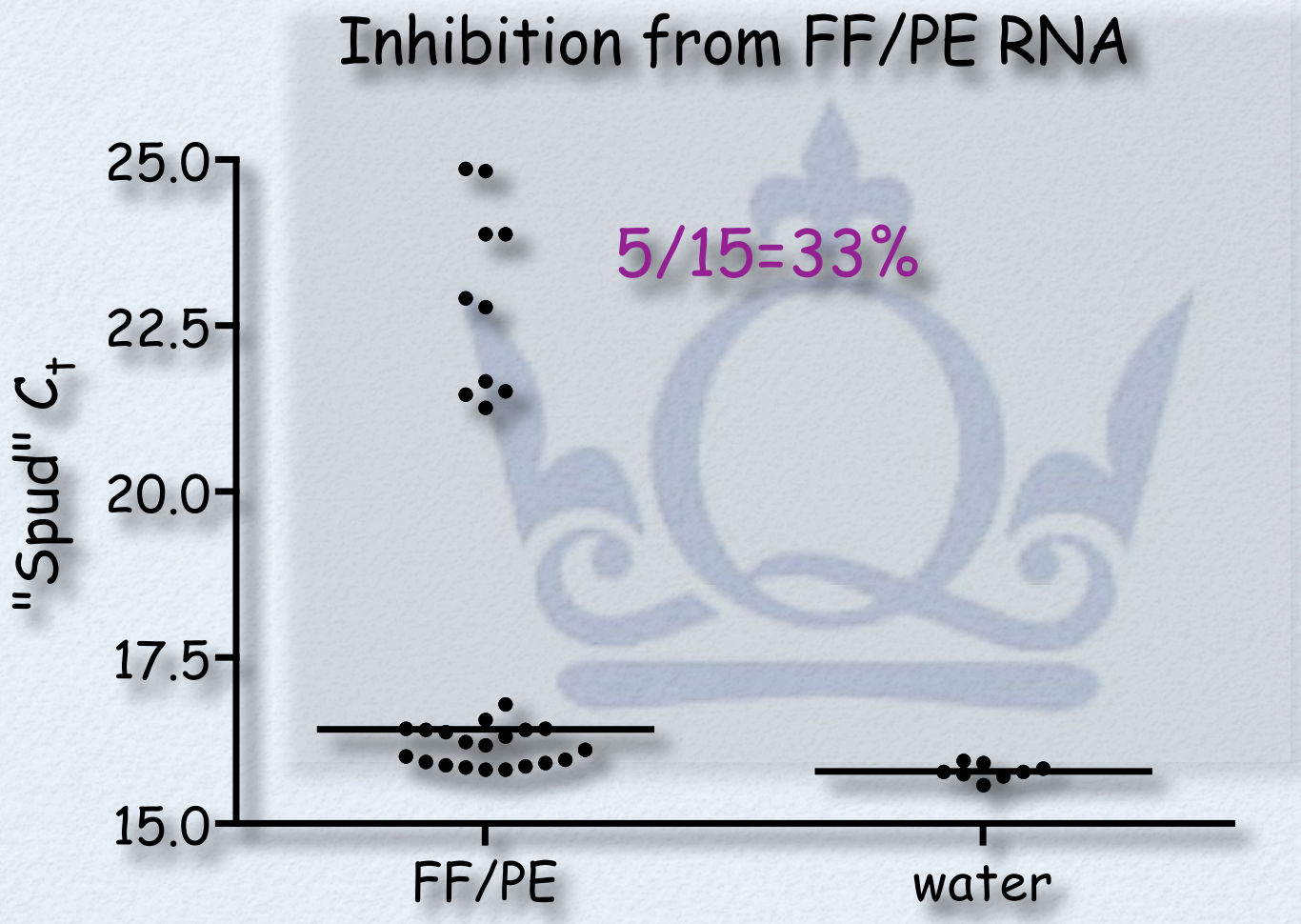

Query: 1   aacttggctttaatggacctccaattttgagtgtgcacaagctatggaacaccacgtaag 60
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Sbjct: 449 aacttggctttaatggacctccaattttgagtgtgcacaagctatggaacaccacgtaag 508


Query: 61  acataaaacggccacatatggtgccatgtaaggatgaatgt 101
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Sbjct: 509 acataaaacggccacatatggtgccatgtaaggatgaatgt 549
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cont

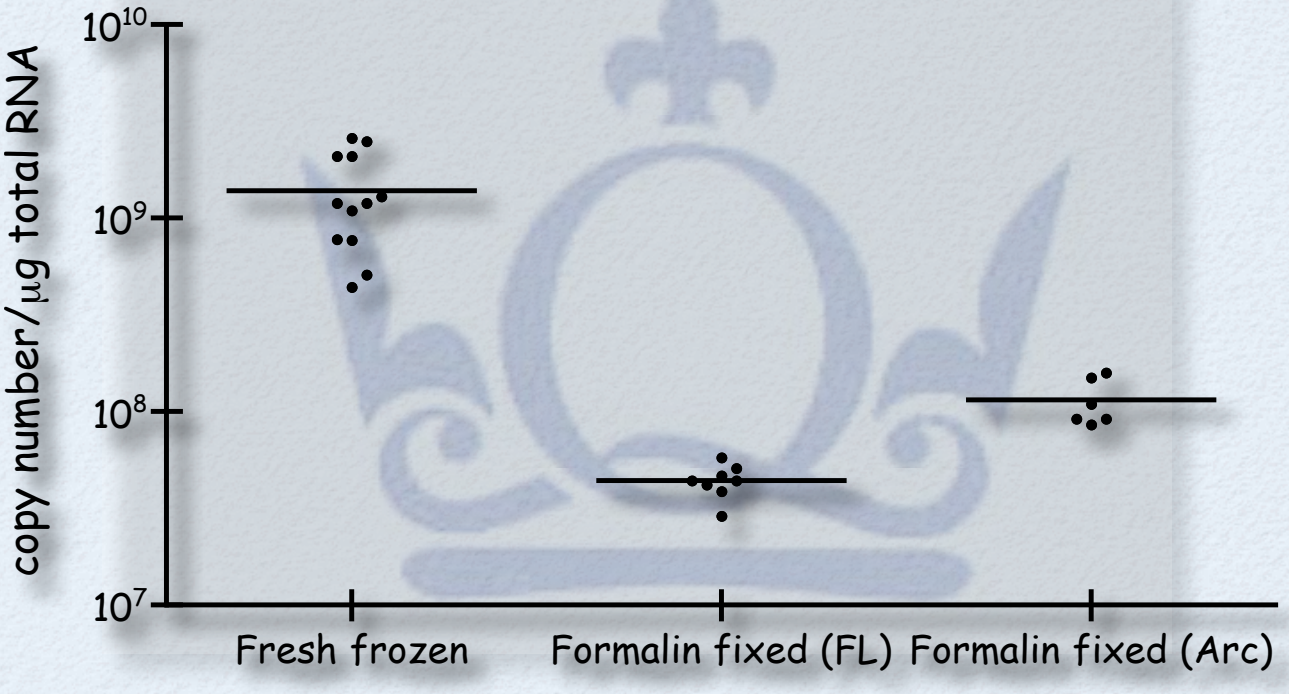
ds higher C_t

Inhibition

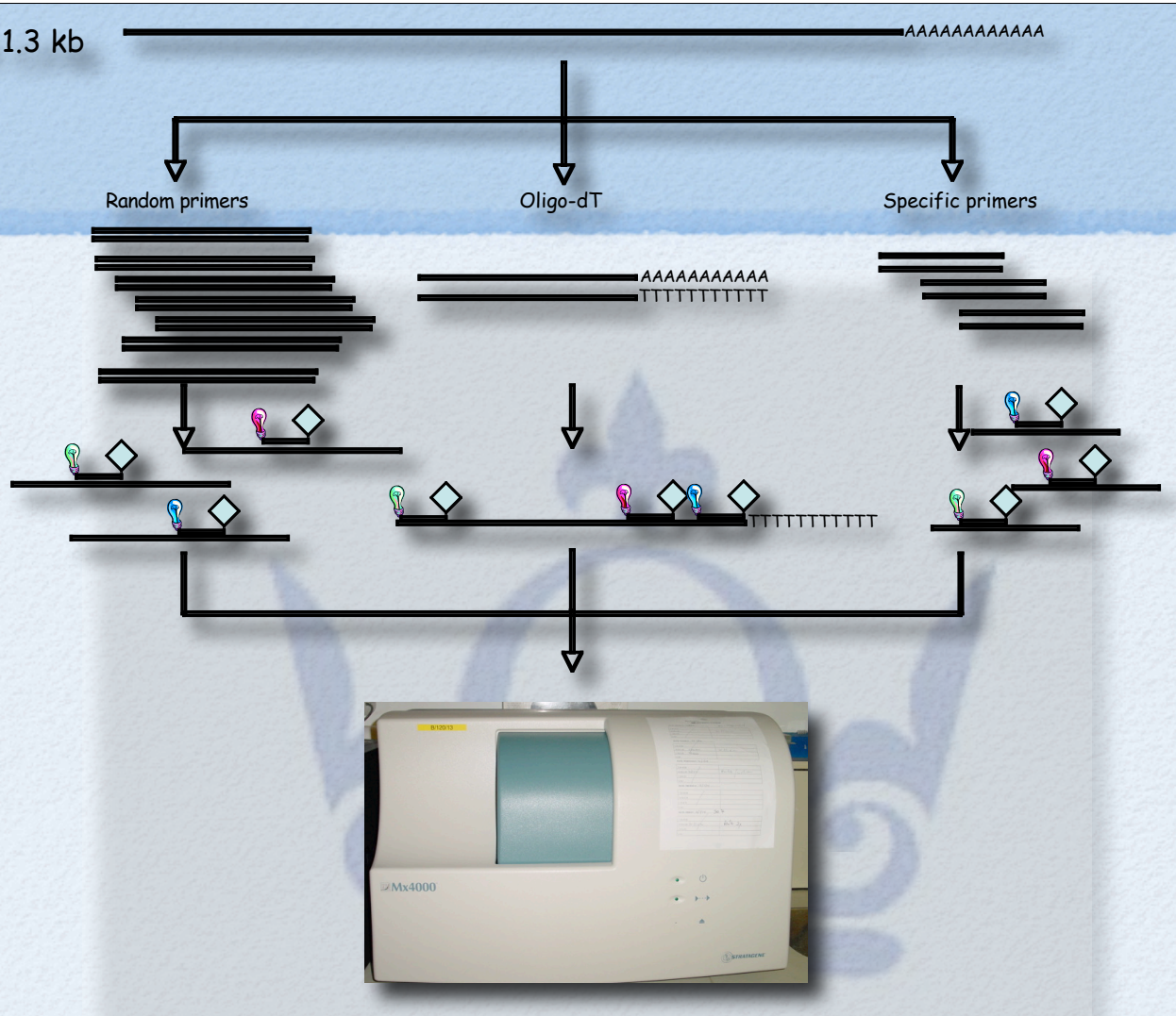

Inhibition from FF/PE RNA
"Spud" C_t
25.0
22.5
20.0
17.5
15.0
5/15=33%
FF/PE
water

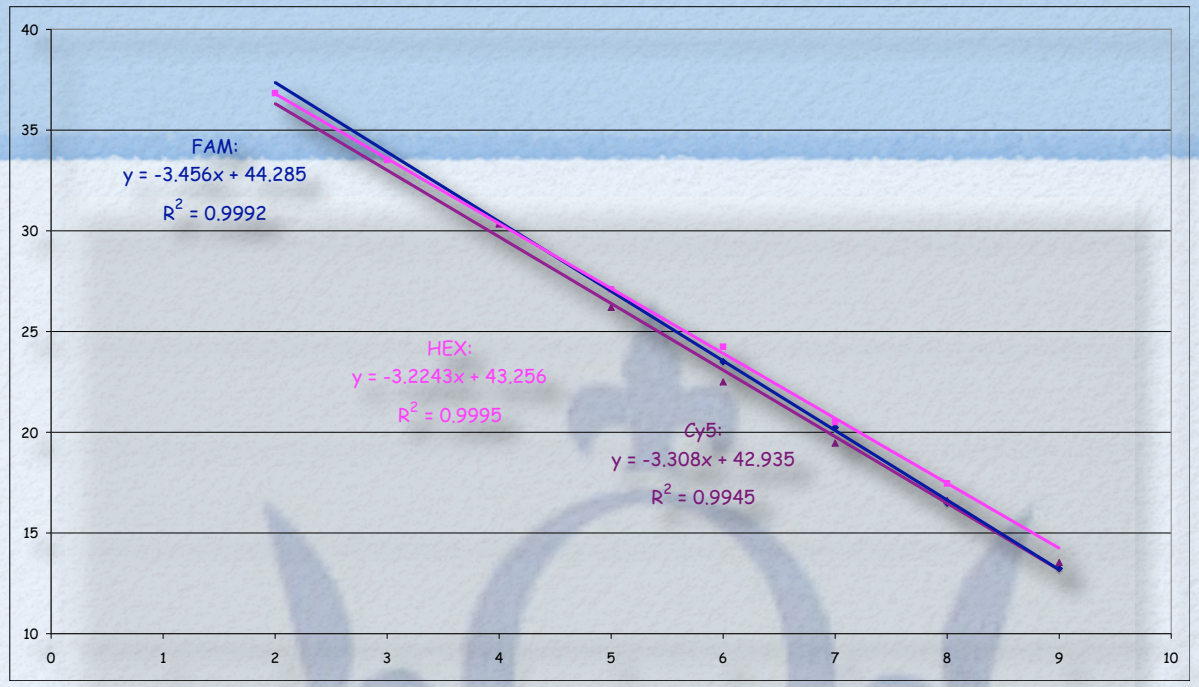
Accessibility

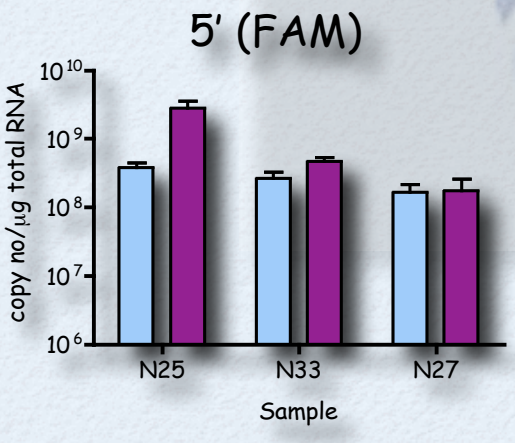
73bp amplicon, 5ng total RNA, GAPDH

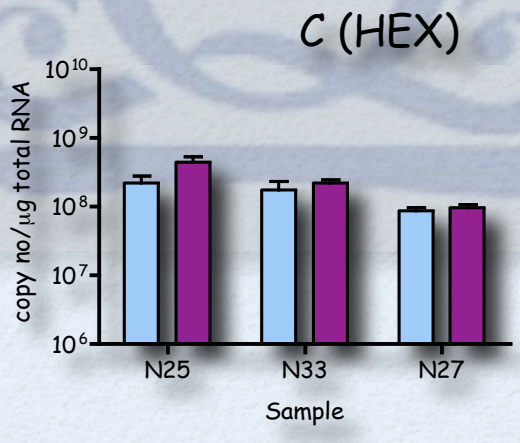


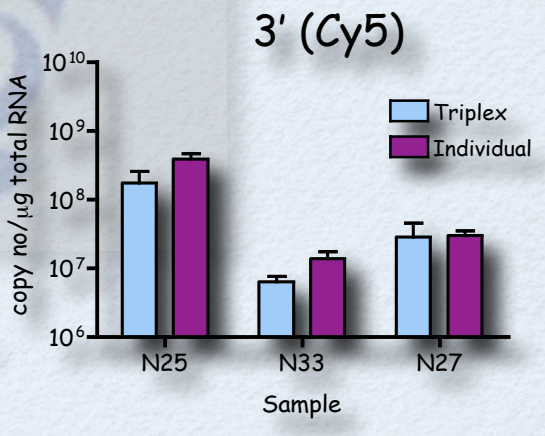
GAPDH mRNA: 1.3 kb







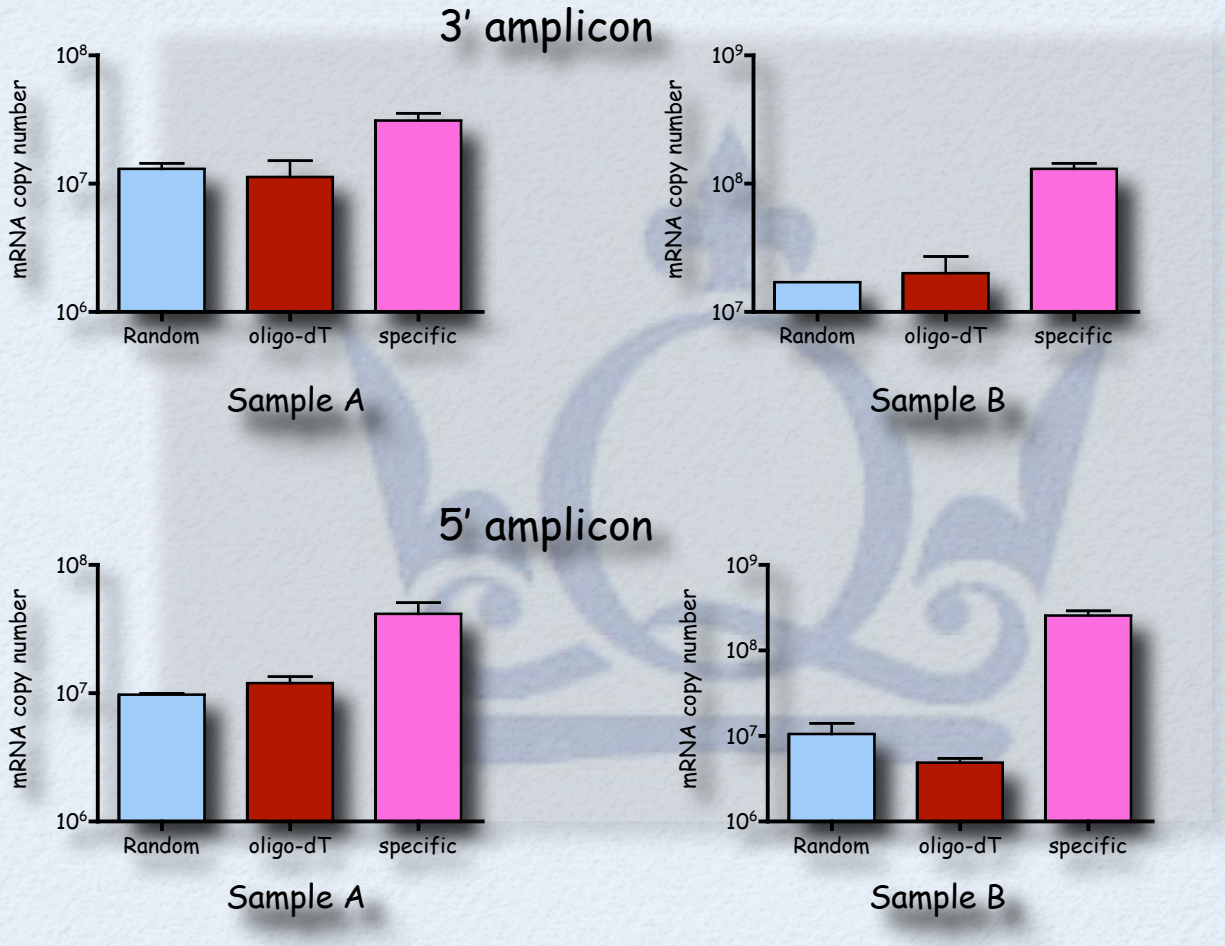




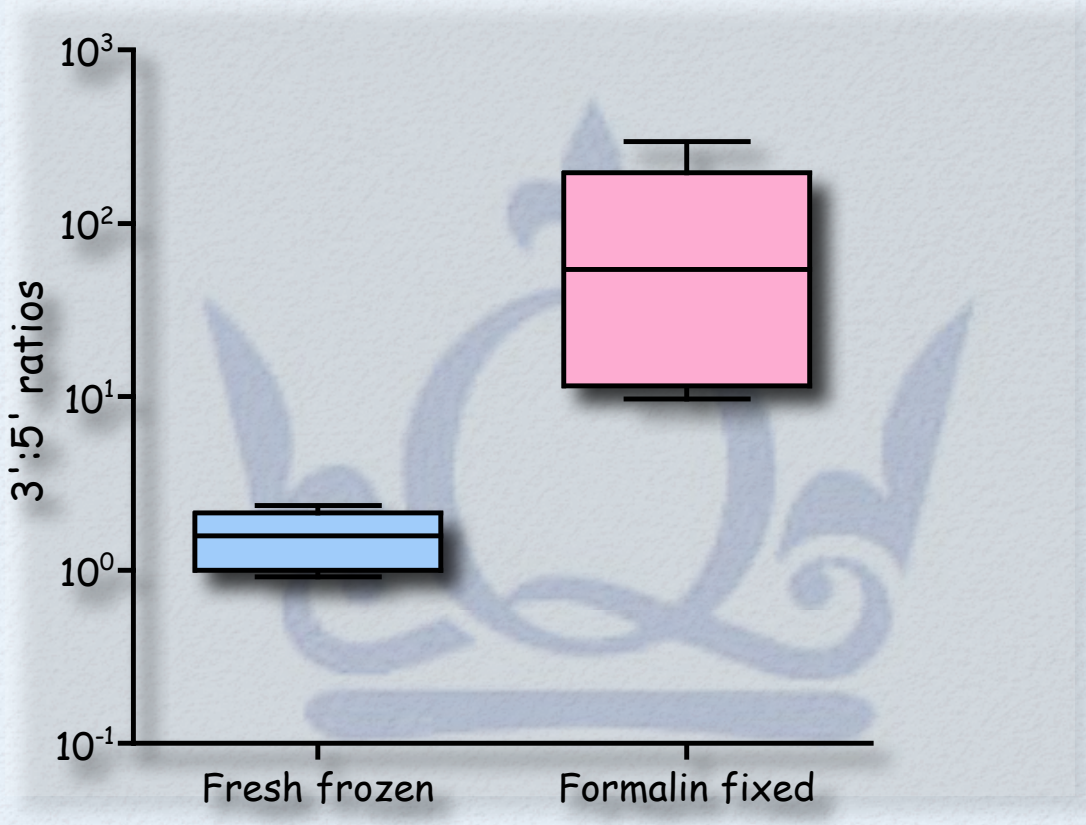
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FFPE: Integrity/accessibility



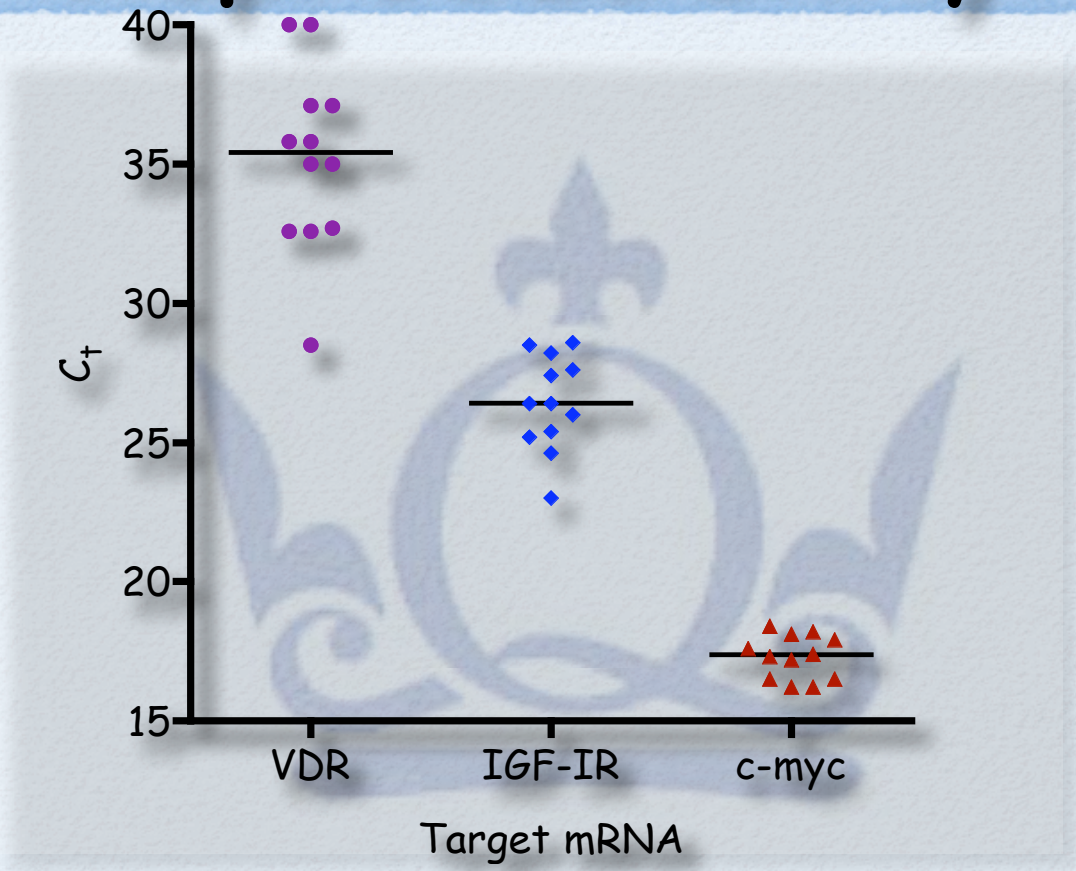
Integrity



Reproducibility

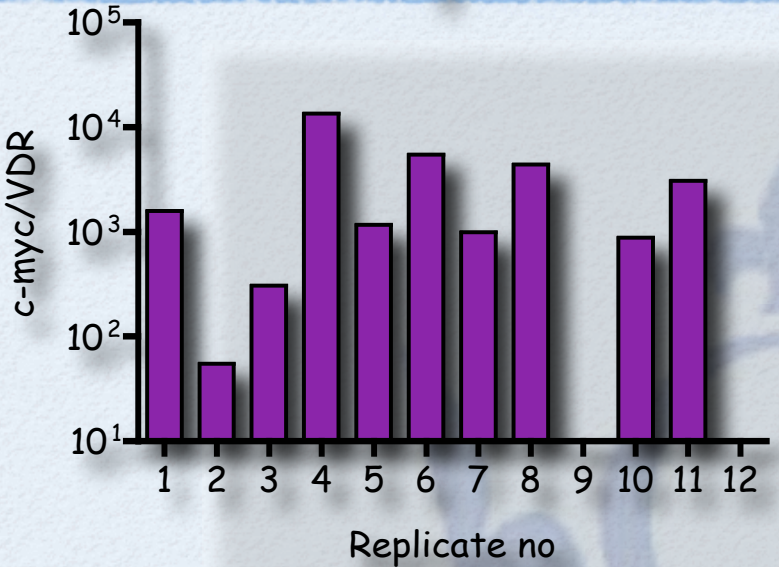
- Single paraffin block 5 yrs old
- Twelve 10µM sections
- RNA extracted from same area
- qRT-PCR: VDR, IGF-IR, c-myc

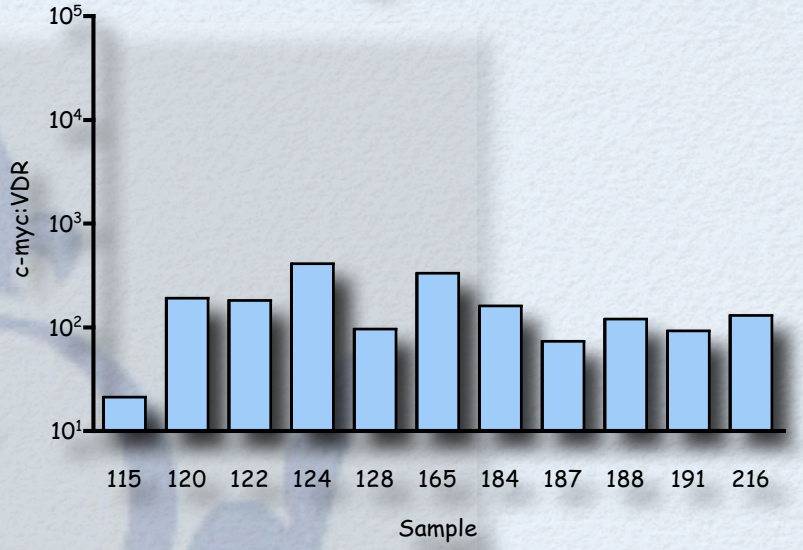
Reproducibility

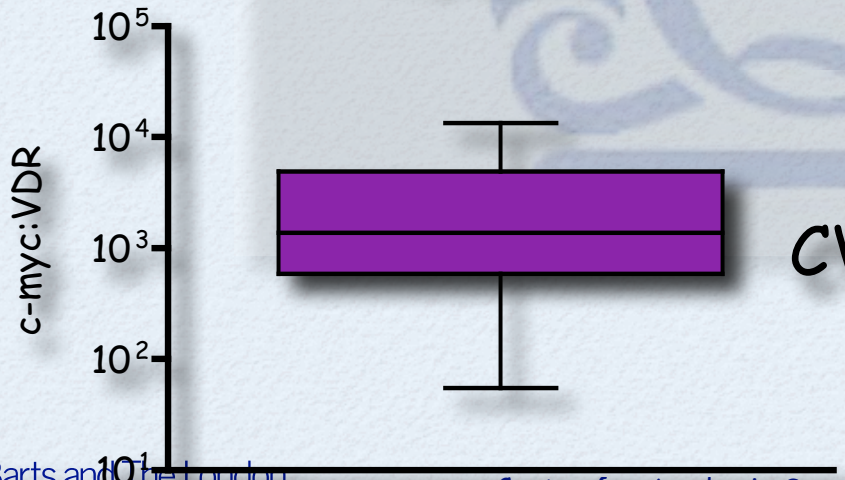


	VDR	IGF-IR	c-myc
Range:	11.5	5.6	2.2
CV:	9.34%	6.48%	4.56%

Reproducibility

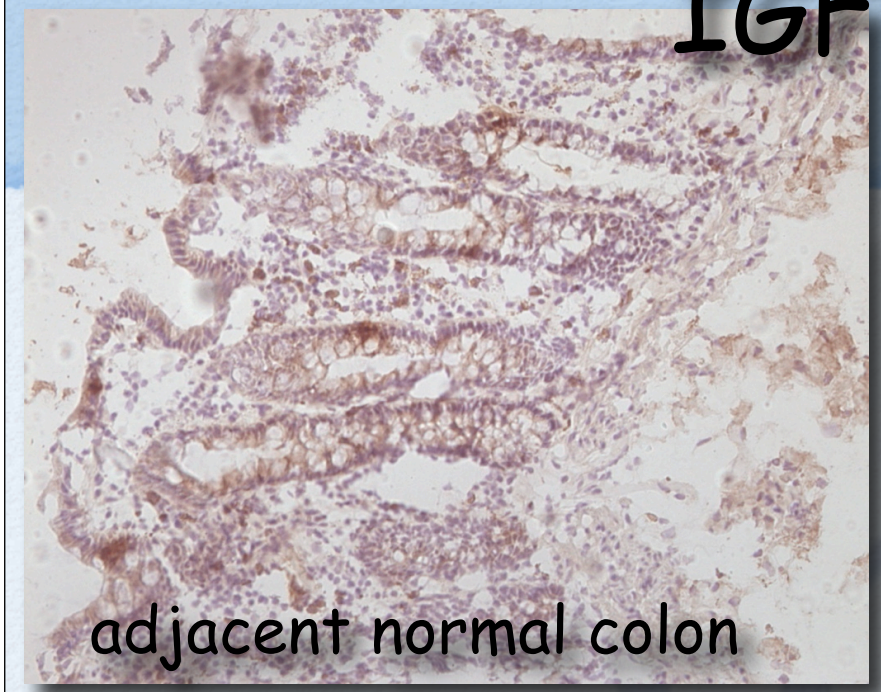


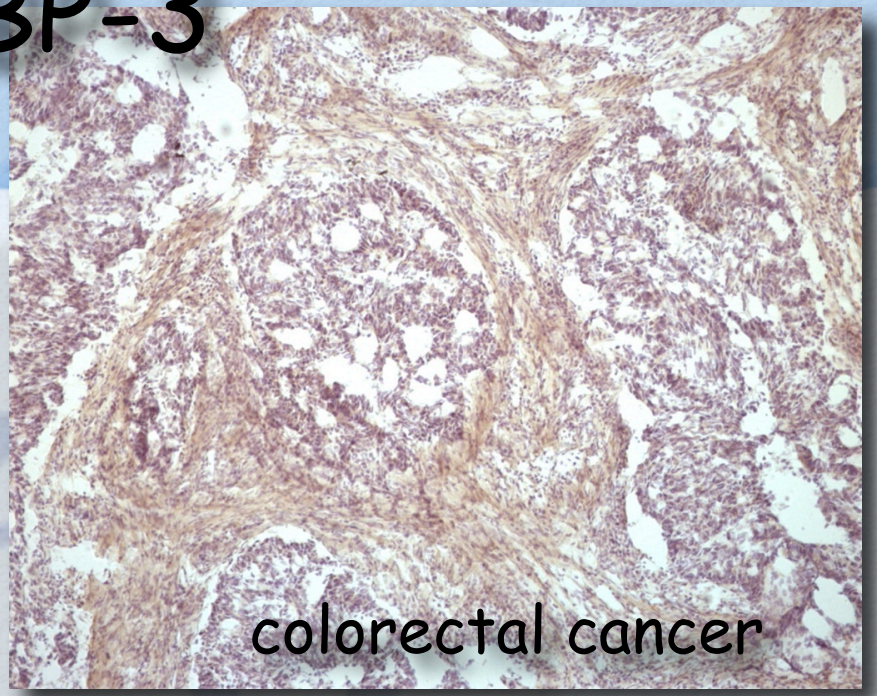


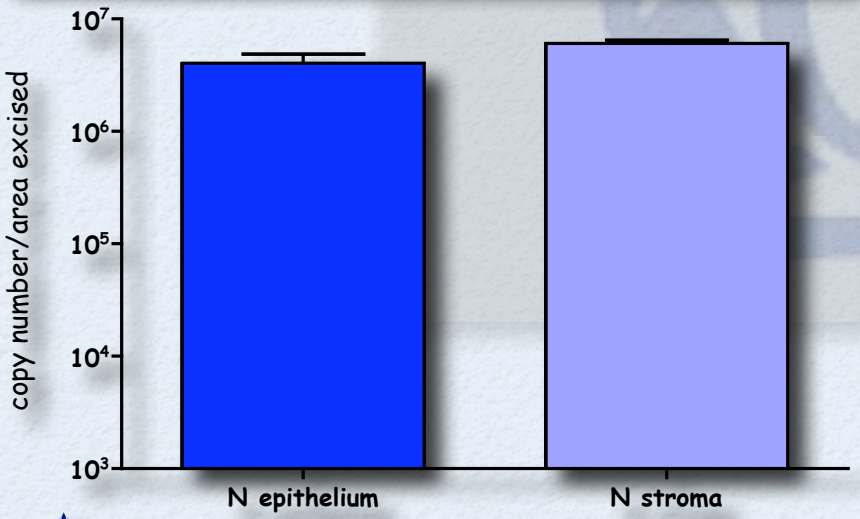


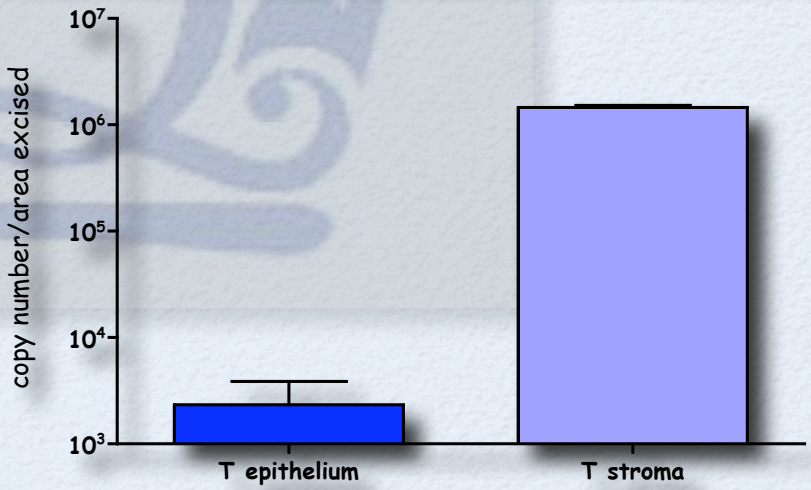
CV=129%

IGFBP-3









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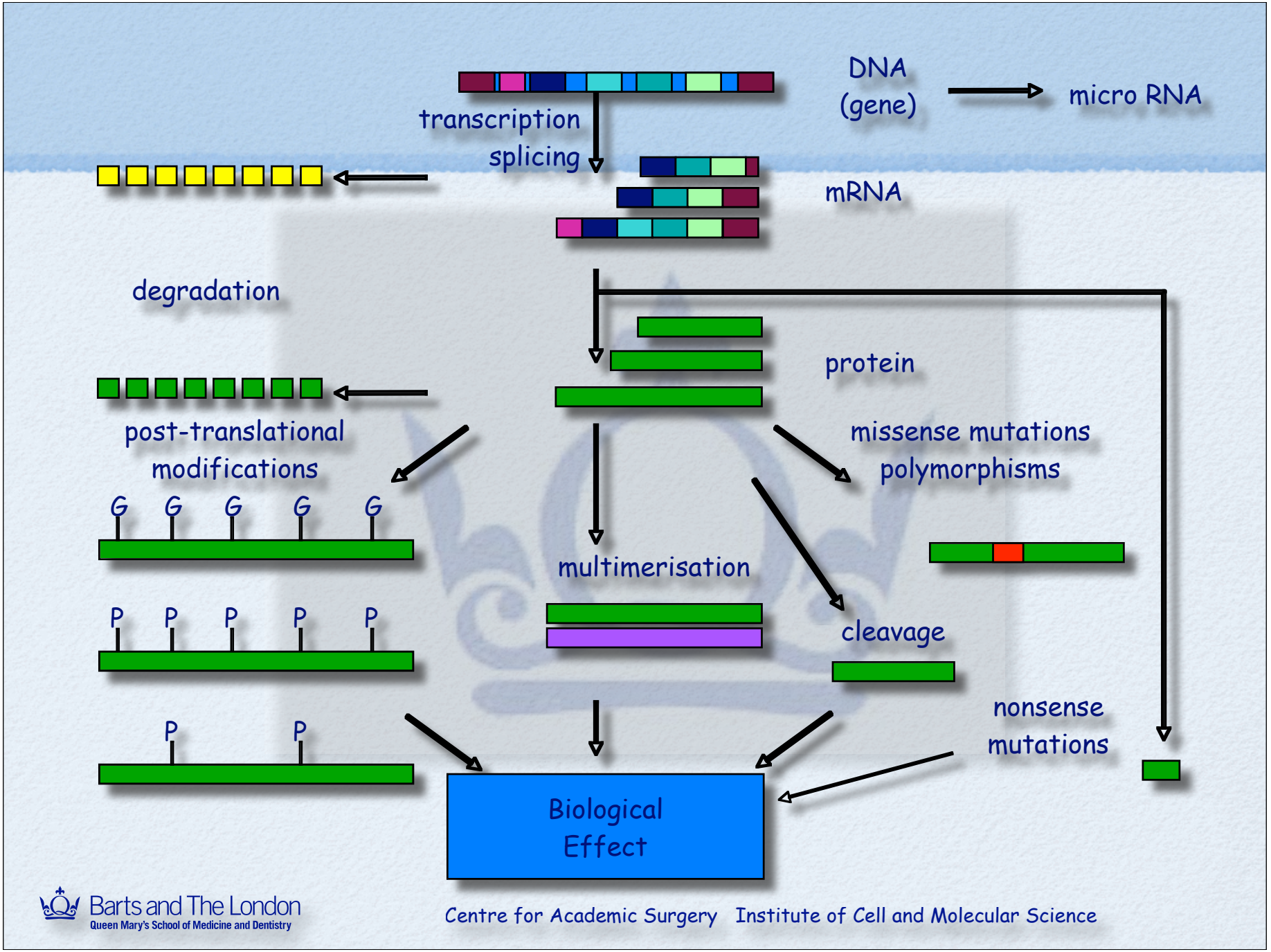
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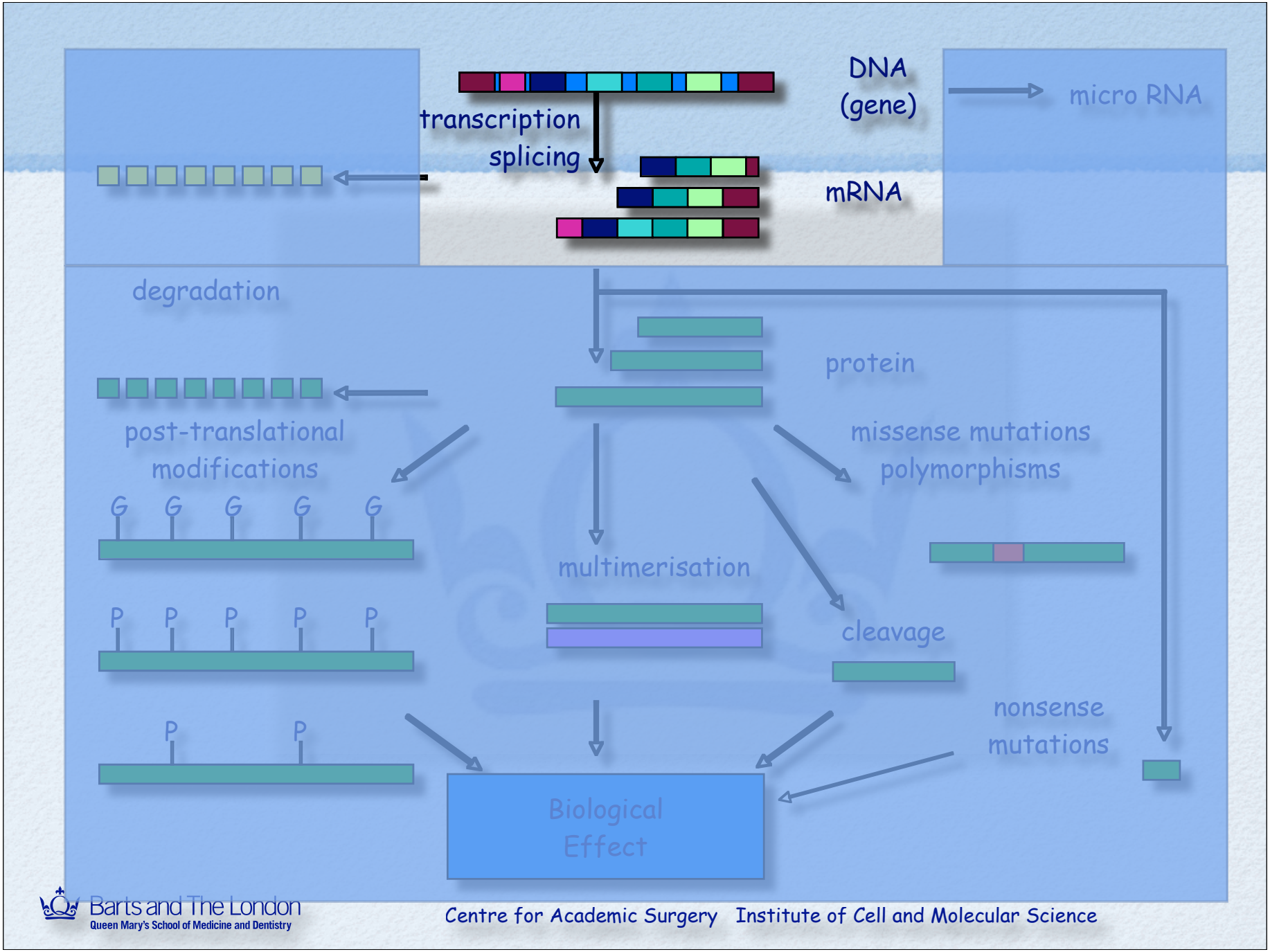
Summary

- Amplifiable RNA can be extracted from "real-life" FFPE tissue
- Yield is sufficient for qRT-PCR
- Integrity of mRNA is compromised
- Variability is significantly greater than with fresh/frozen samples

Conclusions

- Archival material may provide a useful resource
- Significant variability between biological replicates
- Results must be treated with caution

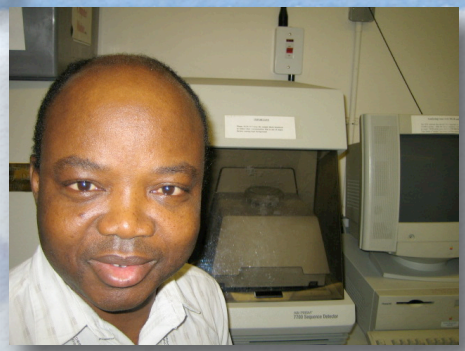

DNA
(gene)
micro RNA
transcription
splicing
mRNA
degradation
protein
post-translational
modifications
G G G G G
missense mutations
polymorphisms
multimerisation
P P P P P
cleavage
P P
nonsense
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Biological
Effect
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bowel & cancer research

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