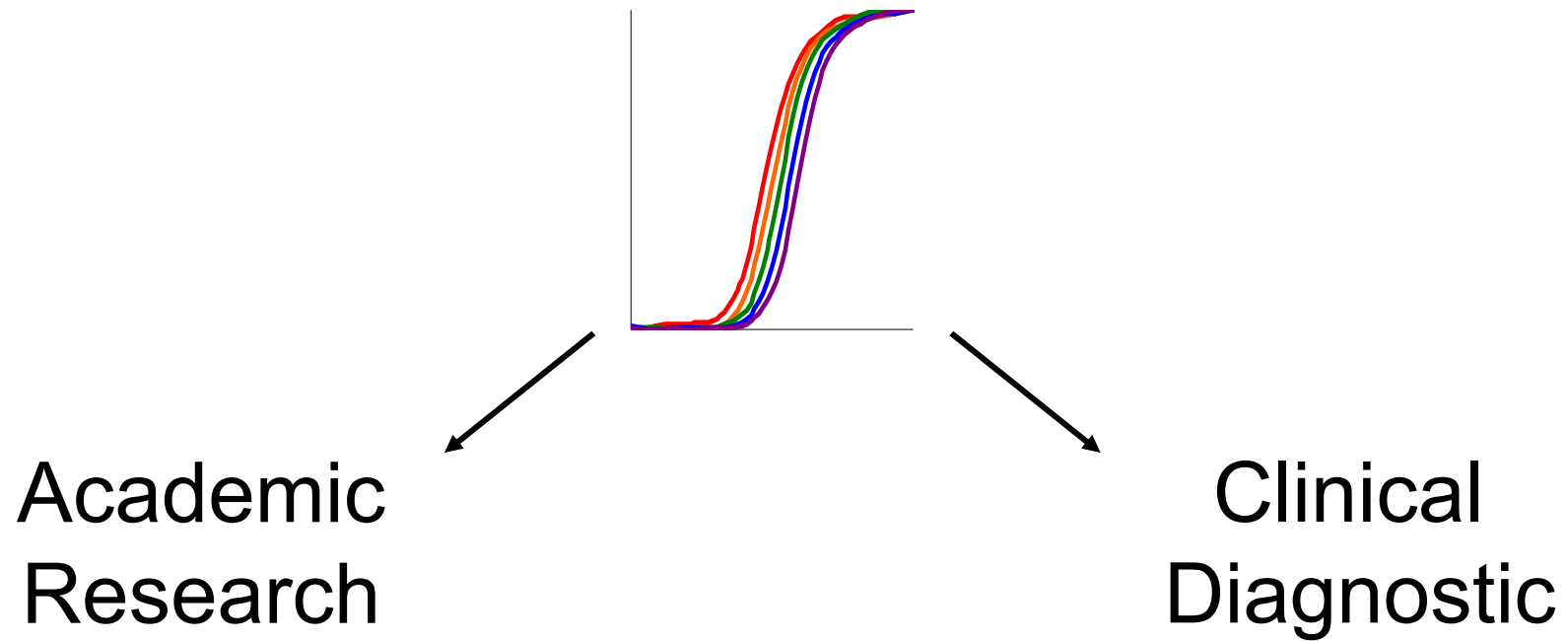
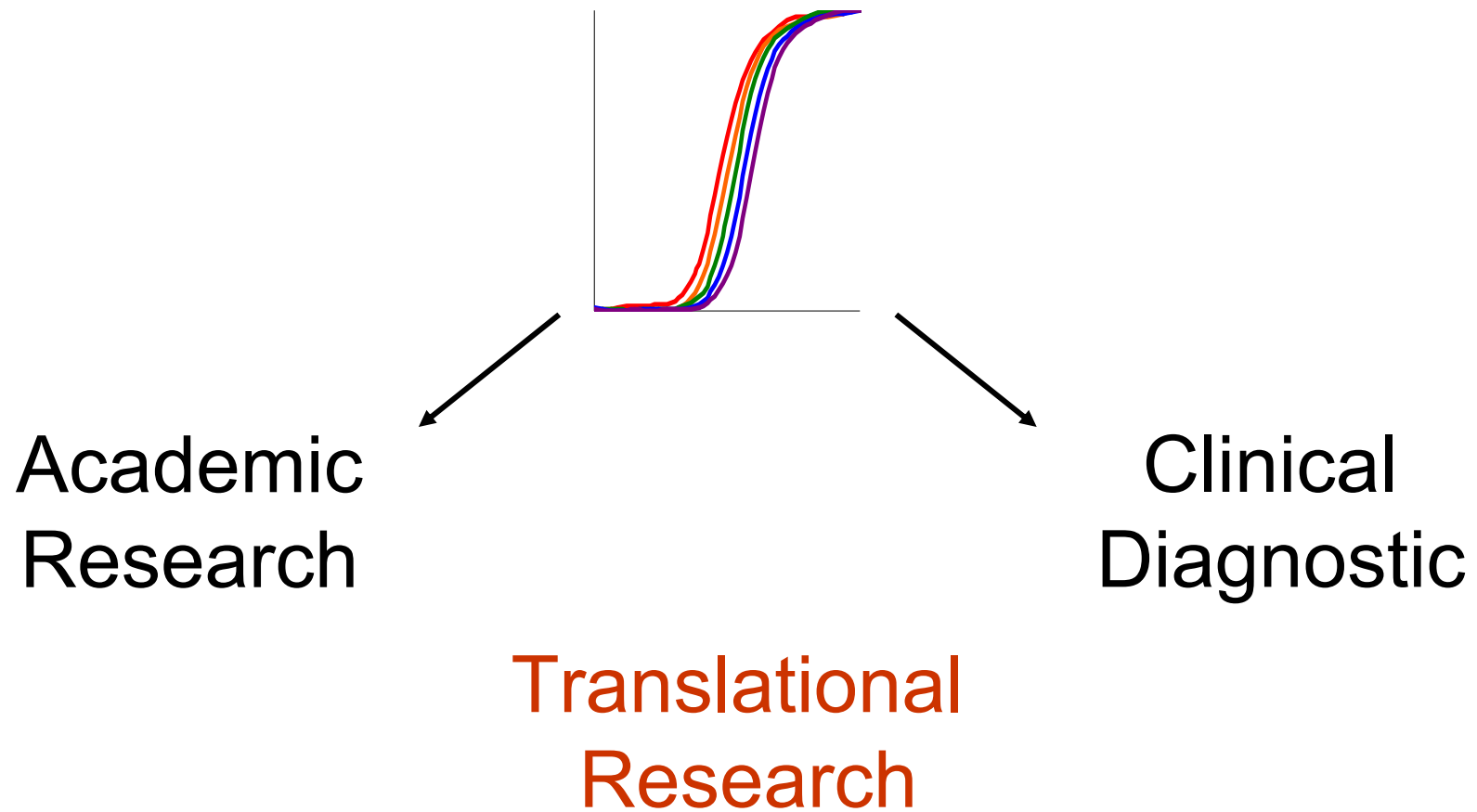


Relative Real Time PCR
for gene expression measurements
in breast cancer biopsies

Optimisation strategy depends on the task



Optimisation strategy depends on the task



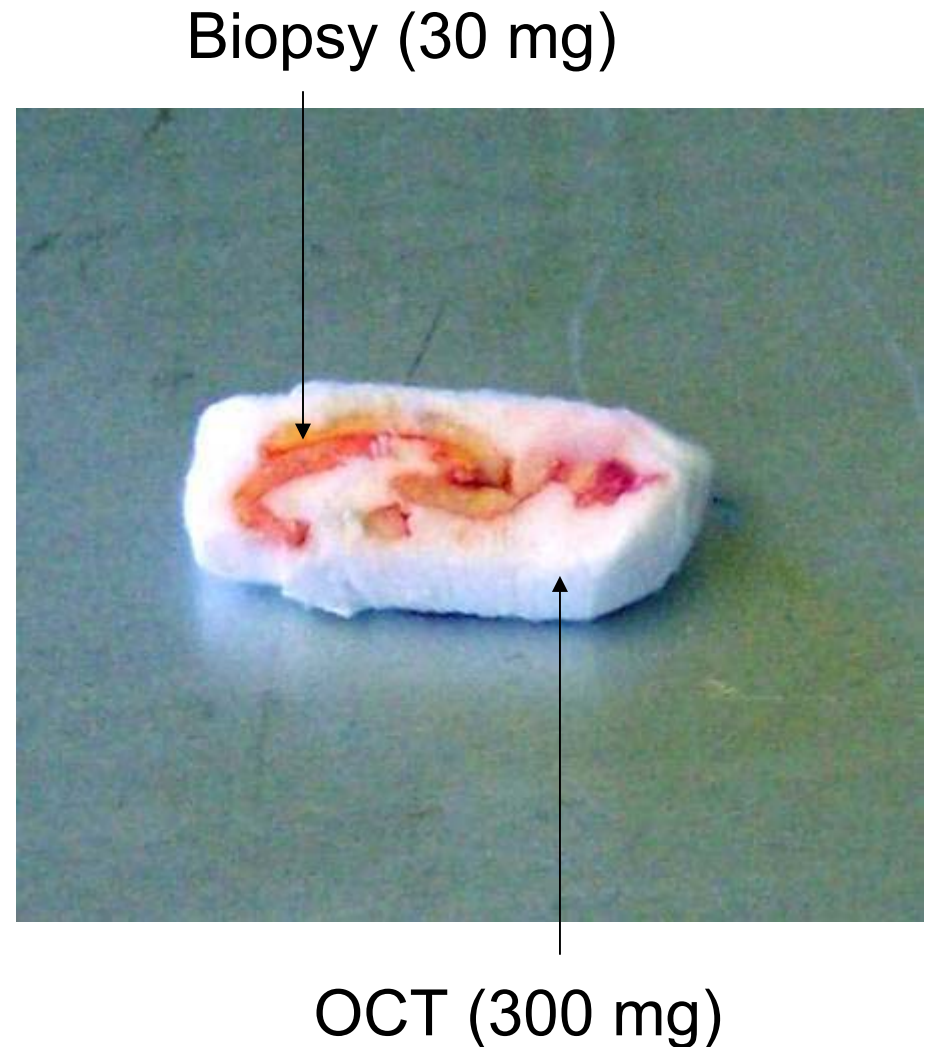
Main steps in Relative Real Time PCR

- Sample processing and RNA stabilization
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- Reverse Transcription
 - Enzyme
 - Primers
- PCR
 - Primer design
 - Reference gene(s) selection
 - Calibrator and standard curve
 - PCR chemistry and machine
 - PCR set up
- Data processing and interpretation

Breast biopsies – tissue processing

Complications

- Frozen section is required for histological verification
- RNA-later complicates tissue freezing and cutting for histology
- Excess of OCT complicates RNA extraction

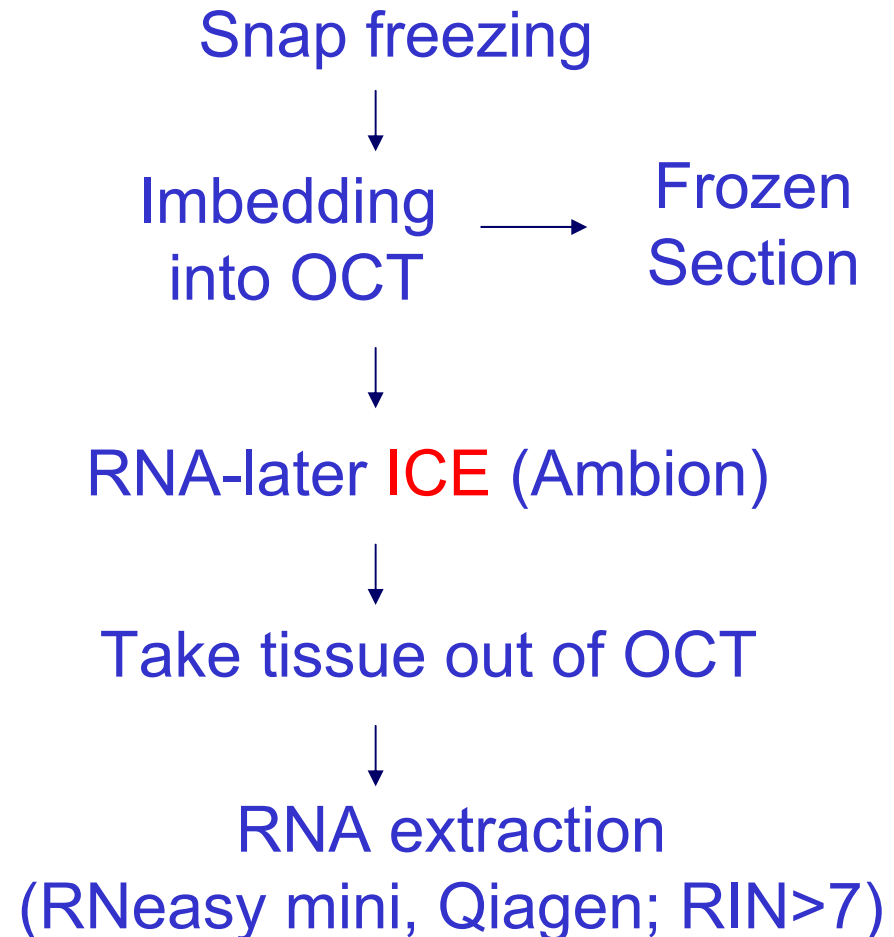


Breast biopsies – tissue processing

Complications

- Frozen section is required for histological verification
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Solution



Reverse transcription

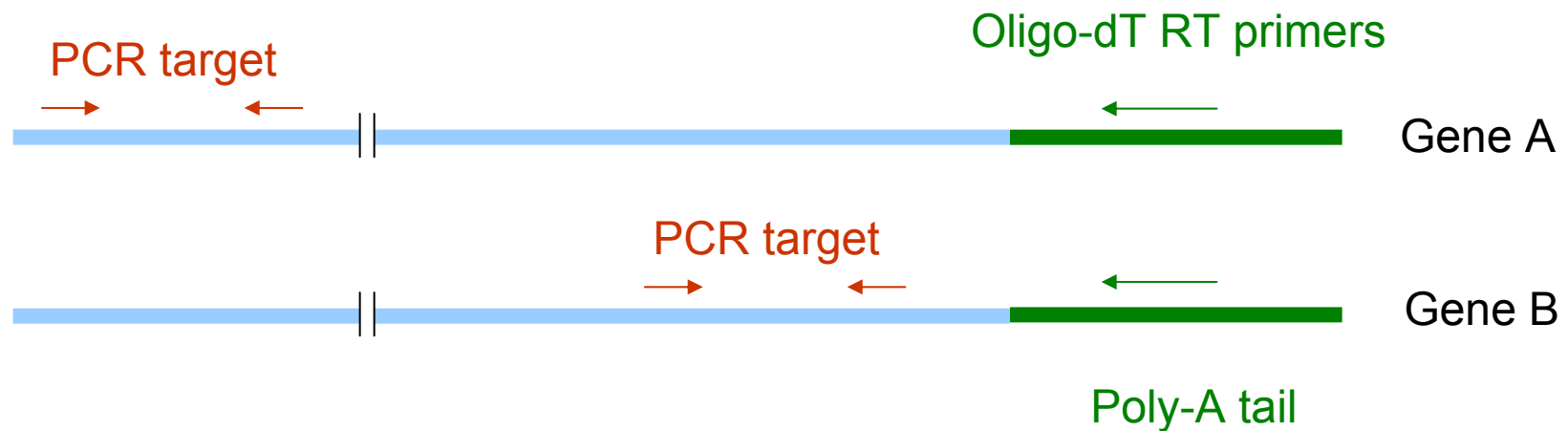
- Enzyme

- Superscript III, Invitrogen

- see Stahlberg A, Kubista M, Pfaffl M. - Comparison of reverse transcriptases in gene expression analysis. Clin Chem. 2004

- Primers

- Random Hexamers or Oligo-dT ?



Using oligo-dT keep PCR primers close to poly-A tail

Main steps in Relative Real Time PCR

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PCR primers

Ready to use pre-designed primers

or

In-house design ?

Ready to use pre-designed PCR primers

- Available for different PCR designs
 - SYBR Green from Qiagen
 - TaqMan from Applied Biosystems
 - LUX primers from Invitrogen
 - *etc*
- Pre-validated in PCR
 - save time on design and validation
- **Primer sequences may be hidden!**
 - complicates reproducibility in academic research
 - not appropriate for development of clinical diagnostics

In-house PCR primers design

Principles

- Discriminate cDNA from genomic DNA
 - span exons' junctions or go into different exons
- Discriminate cDNA from pseudo-genes
 - pseudo-genes are not exact copies of mRNA
- Take into account
 - alternative splicing and known SNPs
- Keep close to Poly-A tail
 - if Oligo-dT was used in RT

In-house PCR primers design

Procedure

- Investigate gene structure
 - Be critical to databases!
- Select target areas (exons)
 - Different exons (exon junctions)
 - SNPs
- Use software for primer design
 - Primer3
- Use NCBI Electronic PCR to check primers
 - Pseudogenes
 - Change target areas and repeat design if needed
- Validate primers in real PCR
 - confirm annealing temperature by gradient PCR
 - confirm specificity by melting curve or electrophoresis



In-house PCR primers design

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e!Ensembl Human GeneSeqView

THIS STYLE: Location of other exons

THIS STYLE: Location of selected exons

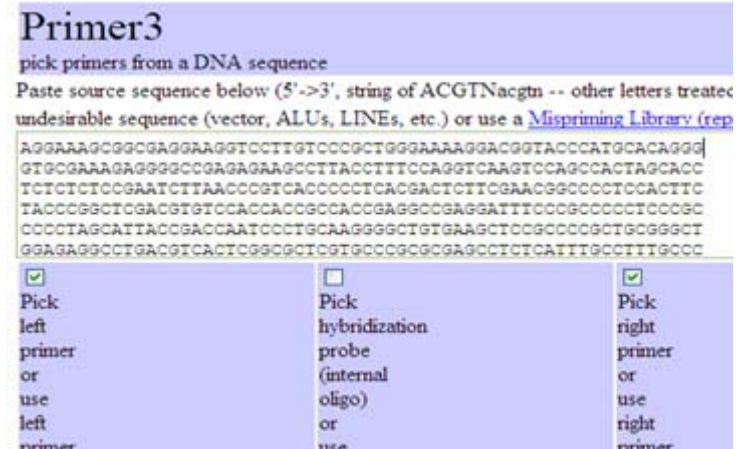
THIS STYLE: Location of SNPs

```
GGTCCAGGACATCCAGAGCCCTGAGGCTGGGAGGATTCCTTGGCCAGGAGTCCAGGAC
ACAGTGAGCTATGTCGTGTCATTGCACTCTAGCCTGGGTGACTGAGCGAGACCCCATCTC | (b
TAAAAAAAAAAAAAATTTTAAGTATACTGGTATAGGTATAATAGTATAGGAGTCATAATAG | (b
TAGTAGTAATATAATTTGTATAGGAGAGATGGTCCACCCCATAAATTTGAATTTGGTTTAA
GCAGGTTGAAGGCAGCCATAAATCCCTGTTTACTAGTTCAGTCTACATAGAGTTATGTGA
GGTTGGTTCATCACCTGCATGGAGAACACCTATGTTTAAAGTGACATCATACTTTGTTAT | (b
TATTCAGGCCAAGGTGACAGAGGAGTTAGCAGCGGCCACTGCACAGGTCTCTCATCTGCA | (b
GCTGAAAATGACTGCTCACCAAAAAAGGAAAACAGAGCTGCAGATGCAGGTGACAGAAA | (b
CCTGAAGGAGACAGATCTTCTCAGGGGCCAGCTCACCAAAGTGCAGGCAAGCTCTCAGG
TATGTGCTAGGTGGGGATGCTGGGATTTGTTGCCATTTAAACTCAAGGAATTAAGCTGTT
TTCCTCTCTCAATTTTTTTGGCATGATGCTCATGAAGTTTTTGAAGSACTACACCAAAA
ATTAAAGGAGACTAGTATAAAACCTACATGATTGTTTTACAGAGGAAACAGCAAAAGAAC
TGCATATTCTGCTGAAATGATCACTGCACTGCTTCCCTTTTGGTTGGGAGCTGTTGCT | (b
```

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NCBI >> SeqUtils >> e-PCR >> Reverse search

PubMed Protein Genome Structure PopSet Taxonomy MapViewer UniC

Search UniSTS Go Clear

Retrieve STS hits by RID: Go Clear

Reverse e-PCR

Dataset: Choose dataset

Text input Table input UniSTS input

Fill form with STSs: [example]

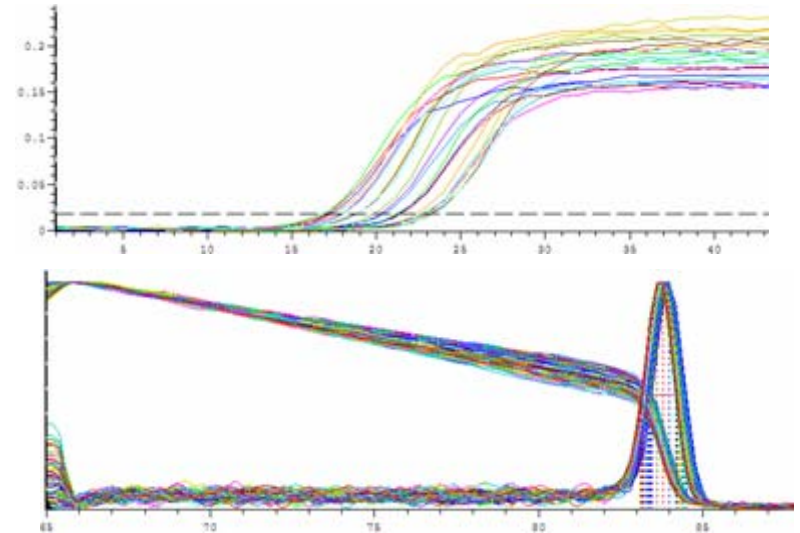
Label	First primer	Second primer	Size
MyTarget	ACTGTCAGTCATGACTG	TGTCAGTCATGACTGAT	100-200

Search is limited to 10 STSs per request

In-house PCR primers design

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Reference genes selection

Editorial

Novel Internal Controls For Real-Time PCR Assays

In the 19 years since the first descriptions of the PCR (1), nucleic acid amplification methods have made the trans-

sequences, which are expected to be present in all specimens, have also been used as IACs (12, 13). These endog-

ELSEVIER

Journal of Biotechnology 75 (1999) 291–295

www.elsevier.com/locate/jbiotec

Journal of Biotechnology

Short communication

Housekeeping genes as internal standards: use and limits

Method

Statistical modeling for selecting housekeeper genes

Aniko Szabo^a, Charles M Perou¹, Mehmet Karaca¹, Laurent Perreard², John F Quackenbush³ and Philip S Bernard^{2,5}

Open Access

Analytical Biochemistry 295, 17–21 (2001)
doi:10.1006/abio.2001.5171, available online at <http://www.idealibrary.com> on IDEAL[®]

Ribosomal 18S RNA Prevails over Glyceraldehyde-3-Phosphate Dehydrogenase and β -Actin Genes as Internal Standard for Quantitative Comparison of mRNA Levels in Invasive and Noninvasive Human Melanoma

RESEARCH REPORT

Validation of housekeeping genes for normalizing RNA expression in real-time PCR

Physiol Genomics 2: 143–147, 2000.

Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes

Available online at www.sciencedirect.com

SCIENCE @ DIRECT[®]

Biochemical and Biophysical Research Communications 313 (2004) 856–862

BBRC

Gene to reference gene selection for quantitative real-time PCR

JANET A. WARRINGTON, ARCHANA NAIR, MAMATHA DEVAPPA, AND MAYA TSYGANSKAYA
Affymetrix, Inc., Santa Clara, California

Warrington, Janet A., Archana Nair, Mamatha devappa, and Maya Tsyganskaya. Comparison of adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiol Genomics* 2: 143–147, 2000. Gene expression levels of about 7,000 genes were measured in 11 different human adult and fetal tissues using high-density oligonucleotide arrays to identify genes involved in maintenance. The tissues share a set of 535 transcripts that are turned on early in fetal development and stay on throughout adulthood. Because our goal was to identify genes

Research

Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes

Jo Vandesompele, Katleen De Preter, Filip Pattyn, Bruce Poppe, Nadine Van Roy, Anne De Paepe and Frank Speleman

Address: Center for Medical Genetics, Ghent University Hospital 1K5, De Pintelaan 185, B-9000 Ghent, Belgium.

Correspondence: Frank Speleman. E-mail: franki.speleman@rug.ac.be

<http://genomebiology.com/2002/3/7/research/0034.1>

in an accumulation of genes metastatic the under-

Identification of mRNA transcription. It is crucial to choose the appropriate reference gene for that purpose. However, it has

Reference genes selection



Breast cancer biopsies during hormonal treatment

- Literature: no validated genes for this system yet
- ~22k micro-arrays (215 specimens pre- & post- treatment)
- ~2000 genes were expressed in all specimens
- Ordered by coefficient of variation
- 10 candidates tested by PCR (38 specimens)
- 3 selected genes - KIAA0674, TBP and PUM1
calculated according to Vandesompele 2002 and Szabo 2004

Reference genes selection



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calculated according to Vandesompele 2002 and Szabo 2004

Excluded: despite stably expressed in micro-arrays

- Discrepancies between Affymetrix probes
- Sub-optimal gene structure
 - No long introns,
 - Complex alternative splicing
 - Pseudogenes, etc
- Inconsistent gene annotation
 - RefSeq, UniGene, Ensemble, Vega etc

Specific exclusion: protein biosynthesis

- No 18 and 28 S RNAs on micro-arrays
 - Difficulties in the genes annotation?
- Several ribosomal proteins
 - Often have pseudo-genes
 - Often have no introns
 - Often are very abundant
- Some other abundant genes related to protein bio-synthesis
 - E.g. translation elongation factors

Specific exclusion: protein biosynthesis

- No 18 and 28 S RNAs on micro-arrays

- Difficulties in the genes annotation?

- Several ribosomal proteins

- Often have pseudo-genes
- Often have no introns
- Often are very abundant

Protein
biosynthesis may
be associated
with proliferation !

- Some other abundant genes related to protein bio-synthesis

- E.g. translation elongation factors

Genes stably expressed in micro-arrays

Microsoft Excel - 200genes 100pc 10Mar05.xls

File Edit View Insert Format Tools Data Window Help

AK5

	A	B	C	D	E	F
1	Rank (by CV)	Name	Primers	Problems	Affy code	Annotation
2	1	SCLY	OK	AFFY target in intron	59705 at	Homo sapiens, Similar to putative selenocysteine lyase, c
3	2	KIAA0674	OK	NO	31826 at	KIAA0674 protein
4	3	PPP2R5D	NO	Pseudogenes by ePCR	211159 s at	Homo sapiens mRNA for protein phosphatase 2A delta (B) re
5	4	TAOK3	OK	AFFY target in intron	221508 at	Homo sapiens serinethreonine kinase (KDS) mRNA, complete
6	5	RPL37A	NO	Pseudogenes by ePCR	201429 s at	Homo sapiens ribosomal protein L37a (RPL37A), mRNA.
7	6	MYL4	OK	NO	216054 x at	Human MLC1emb gene for embryonic myosin alkaline light ch
8	7	TPT1	NO	Pseudogenes by ePCR	214327 x at	tumor protein, translationally-controlled 1
9	8	FKSG17	NO	No introns	211445 x at	Homo sapiens FKSG17 (FKSG17) mRNA, complete cds.
10	9	TPT1	NO	Pseudogenes by ePCR	212284 x at	tumor protein, translationally-controlled 1
11	10	TPT1	NO	Pseudogenes by ePCR	212869 x at	tumor protein, translationally-controlled 1
12	11	RPL41	NO	No introns, multiple ge	201492 s at	Homo sapiens ribosomal protein L41 (RPL41), mRNA.
13	12	SIRT3	OK	AFFY target in intron	49327 at	sirtuin (silent mating type information regulation 2 home
14	13	TNFRSF5	NO	No suitable exons	35150 at	Human CDw40 mRNA for nerve growth factor receptor-related
15	14	RRM1	OK	NO	201476 s at	ribonucleotide reductase M1 polypeptide
16	15	FLJ13848	NO	Pseudogenes? No suitabl	218734 at	Homo sapiens hypothetical protein FLJ13848 (FLJ13848), m
17	16	FLJ21865	NO	Two products from trans	65635 at	endo-beta-N-acetylglucosaminidase
18	17	FLJ10385	OK	NO	44563 at	hypothetical protein FLJ10385
19	18	CYB561	NO	Unclear gene structure	207986 x at	Homo sapiens cytochrome b-561 (CYB561), mRNA.
20	19	QRSL1	NO	Pseudogenes by ePCR	218949 s at	Homo sapiens hypothetical protein FLJ10989 (FLJ10989), m
21	20	RPS4X	NO	Pseudogenes by ePCR	213347 x at	ribosomal protein S4, X-linked
22	21	EEF1A1	NO	Pseudogenes	213583 x at	leukocyte receptor cluster (LRC) member 7
23	22	ASXL1	NO	Difficult gene structur	212238 at	Homo sapiens mRNA; cDNA DKFZp434N0535 (from clone DKFZp4
24	23	RPL39	NO	No introns	208695 s at	Homo sapiens, ribosomal protein L39, clone MGC:1636, mRNA
25	24	METTL4	OK	NO	219698 s at	Homo sapiens hypothetical protein FLJ23017 (FLJ23017), m
26	25	RPL23A	NO	Pseudogenes reported by	208834 x at	Homo sapiens, Similar to cadherin 1, type 1, E-cadherin

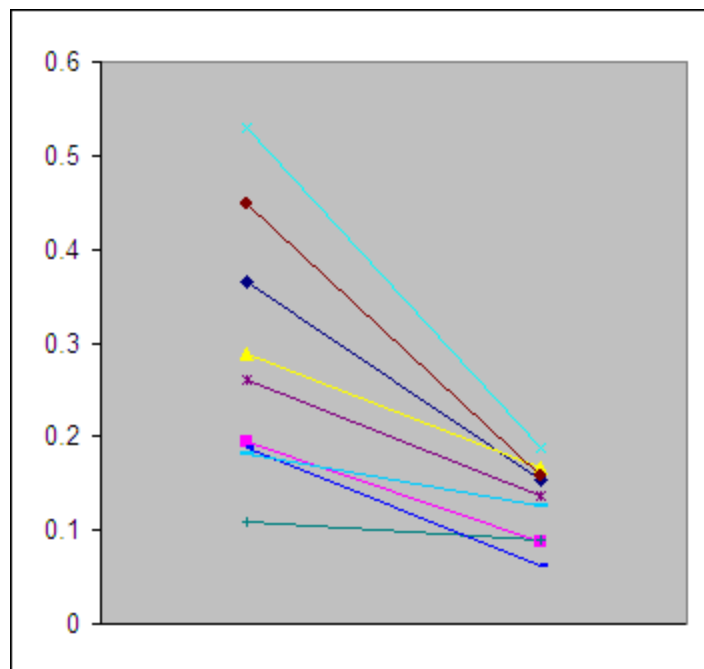
Housekeeper Gene List

Ready NUM

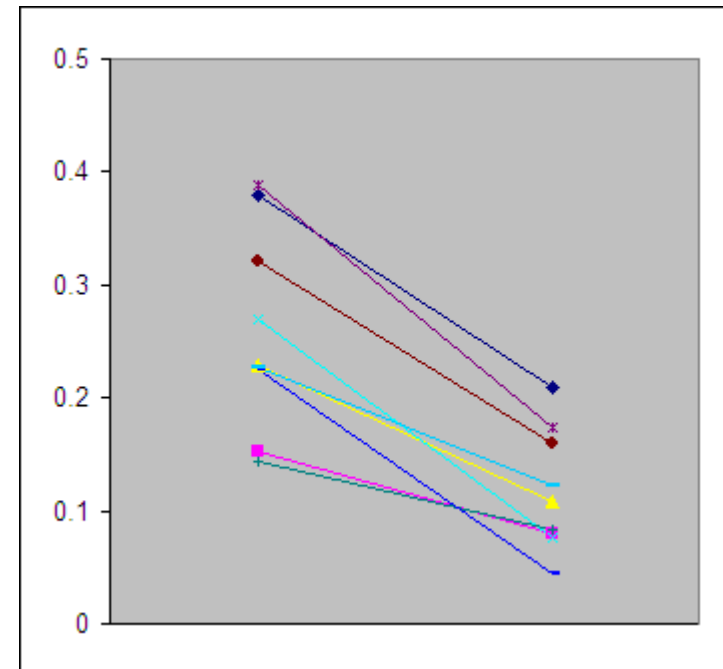
Are results based on ACTB & GAPD wrong ?

For measurement of estrogen-dependent gene expression in breast cancer biopsies ACTB and GAPD may be not optimal but they are not grossly misleading either

Effect of treatment on CKS2 expression



ACTB & GAPD



KIAA0674 & TBP

Non-stably expressed reference genes

$$\text{Result} = \frac{\text{Target 1}}{\text{Target 2}}$$

CKS2 (*cell cycle progression*)

MGB1 (*associated with differentiation*)

Do we need ACTB (cytoskeleton) or GAPD (glucose metabolism)?

Main steps in Relative Real Time PCR

- Sample processing and RNA stabilization
- RNA extraction and evaluation
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 - Enzyme
 - Primers
- PCR
 - Primer design
 - Reference gene(s) selection
 - **Calibrator and standard curve**
 - **PCR chemistry and machine**
 - **PCR set up**
- Data processing and interpretation

PCR machine, chemistry, design and set-up

- **Calibrator**

Pool of cDNA aliquots
from all specimens

- **Standard curve**

7 points of 3-fold dilutions

- **Machine**

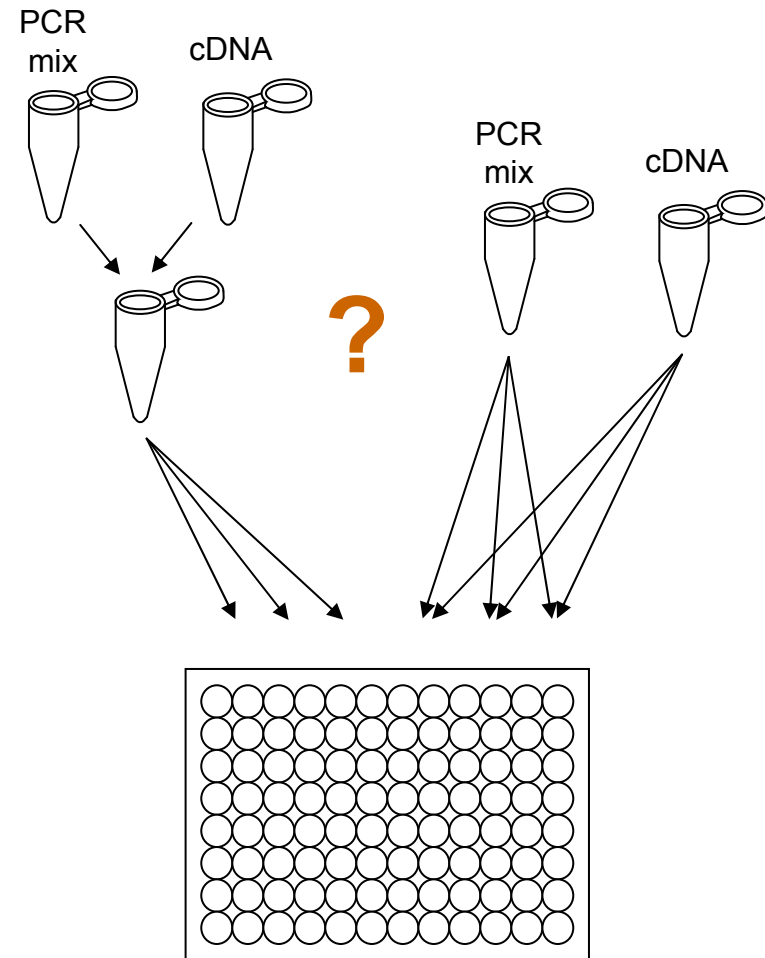
Opticon Monitor 2,
MJR – Biorad

- **Chemistry**

Sybr green, Hot Start
Quanti-Tect, Qiagen

- **Set-up**

When to split
intra-assay replicas?

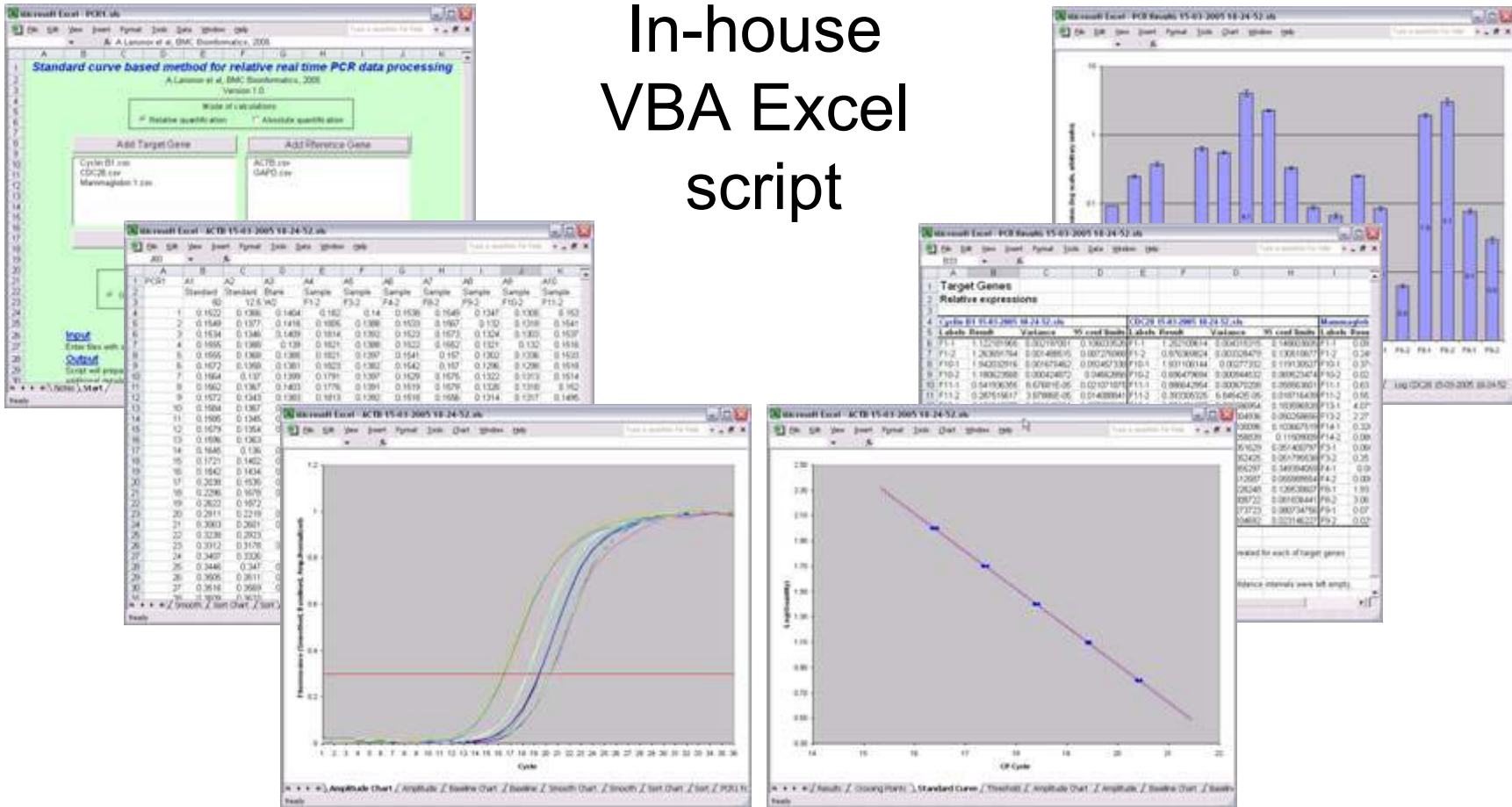


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PCR data processing

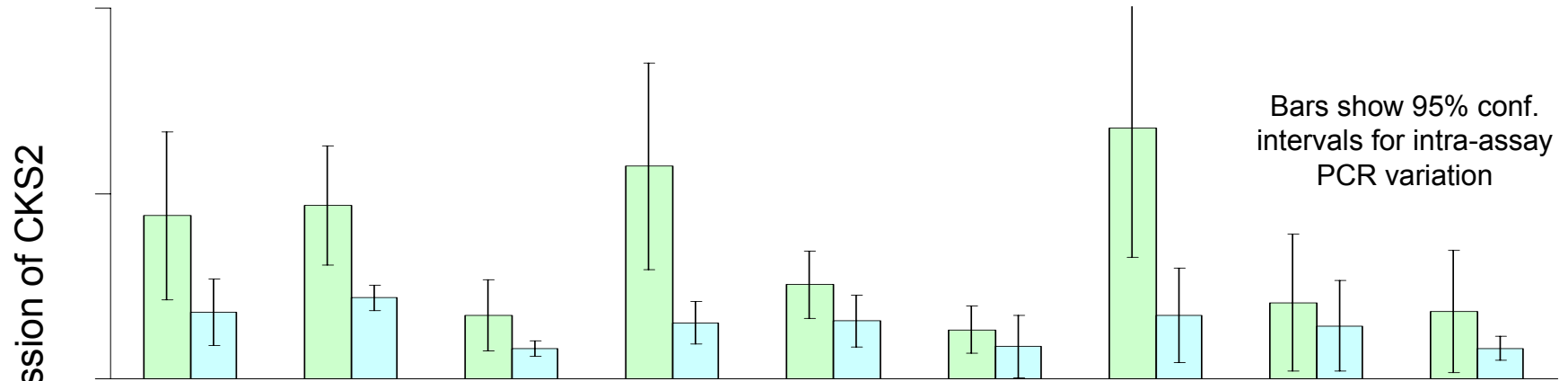
In-house
VBA Excel
script



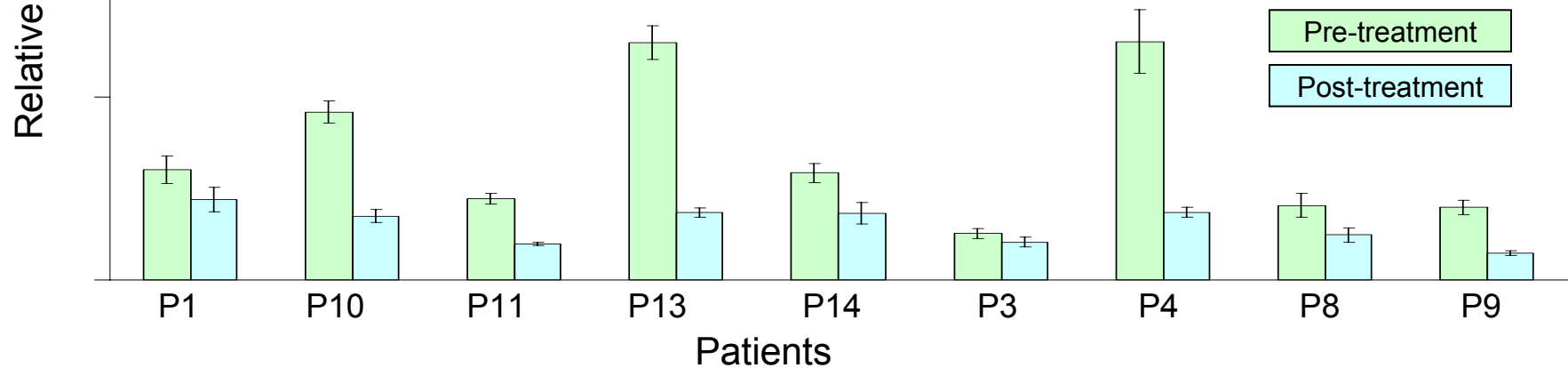
Larionov A, Krause A, Miller W, **2005**, BMC Bioinformatic

PCR data processing

Data processing based on PCR machine software



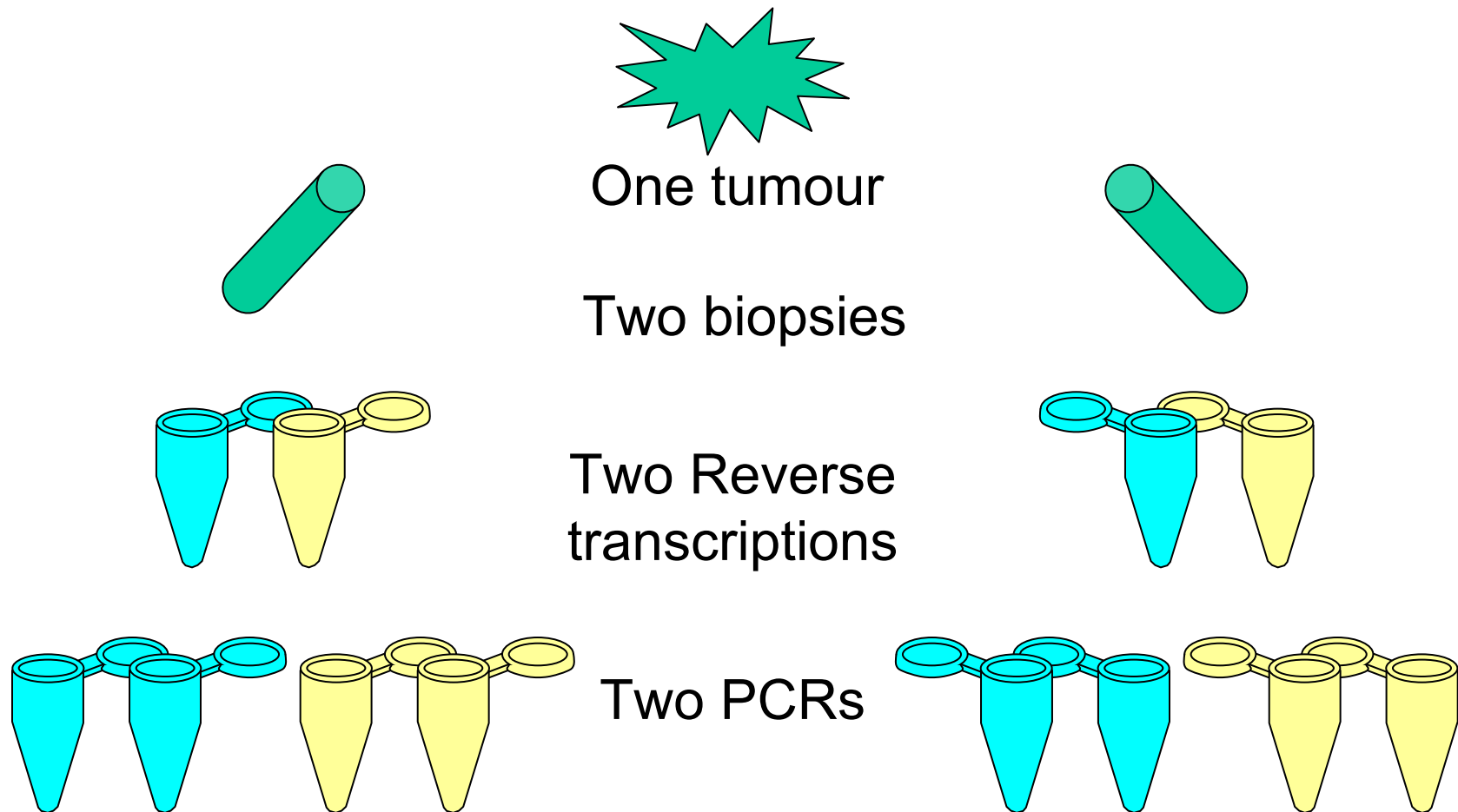
Optimized data processing



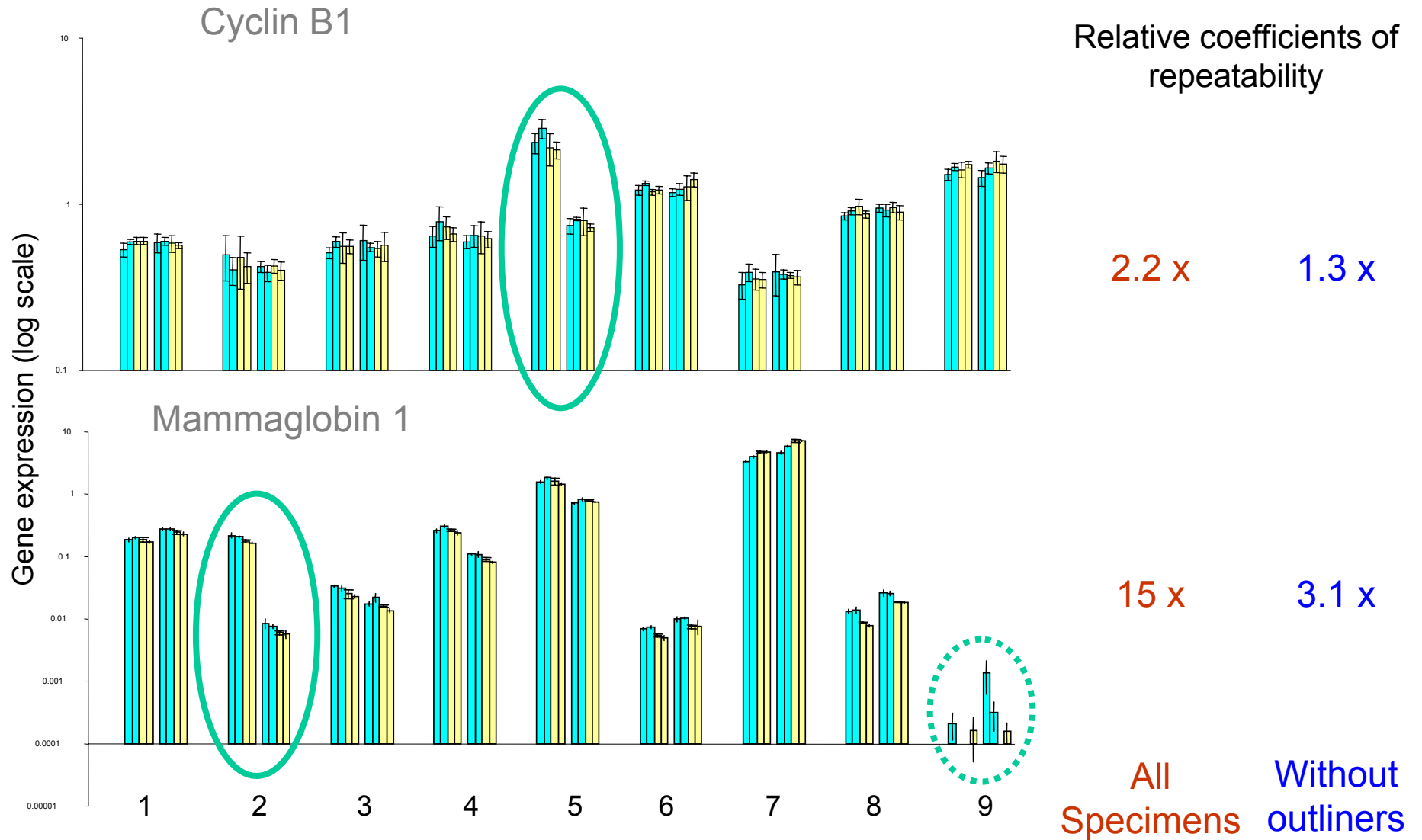
PCR results interpretation

- **Intra**-assay PCR variation
 - Can be used for PCR quality control
 - Can **not** be used for statistical inferences comparing different specimens
- **Inter**-assay variation
 - Academic Research
 - **Whole experiment** replicas
 - Verifications by other (non-PCR) methods
 - Clinical diagnostics
 - Repeatability analysis using training set of specimens

Repeatability analysis

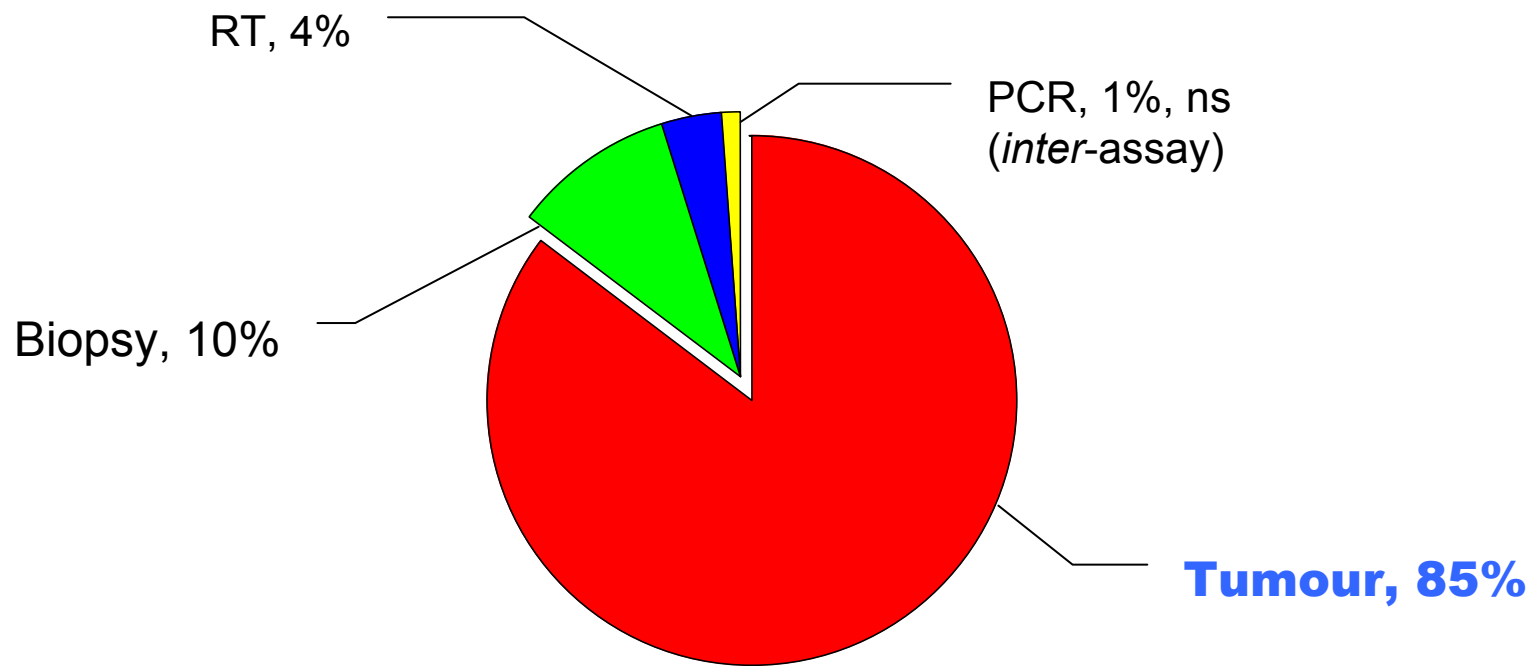


Repeatability analysis



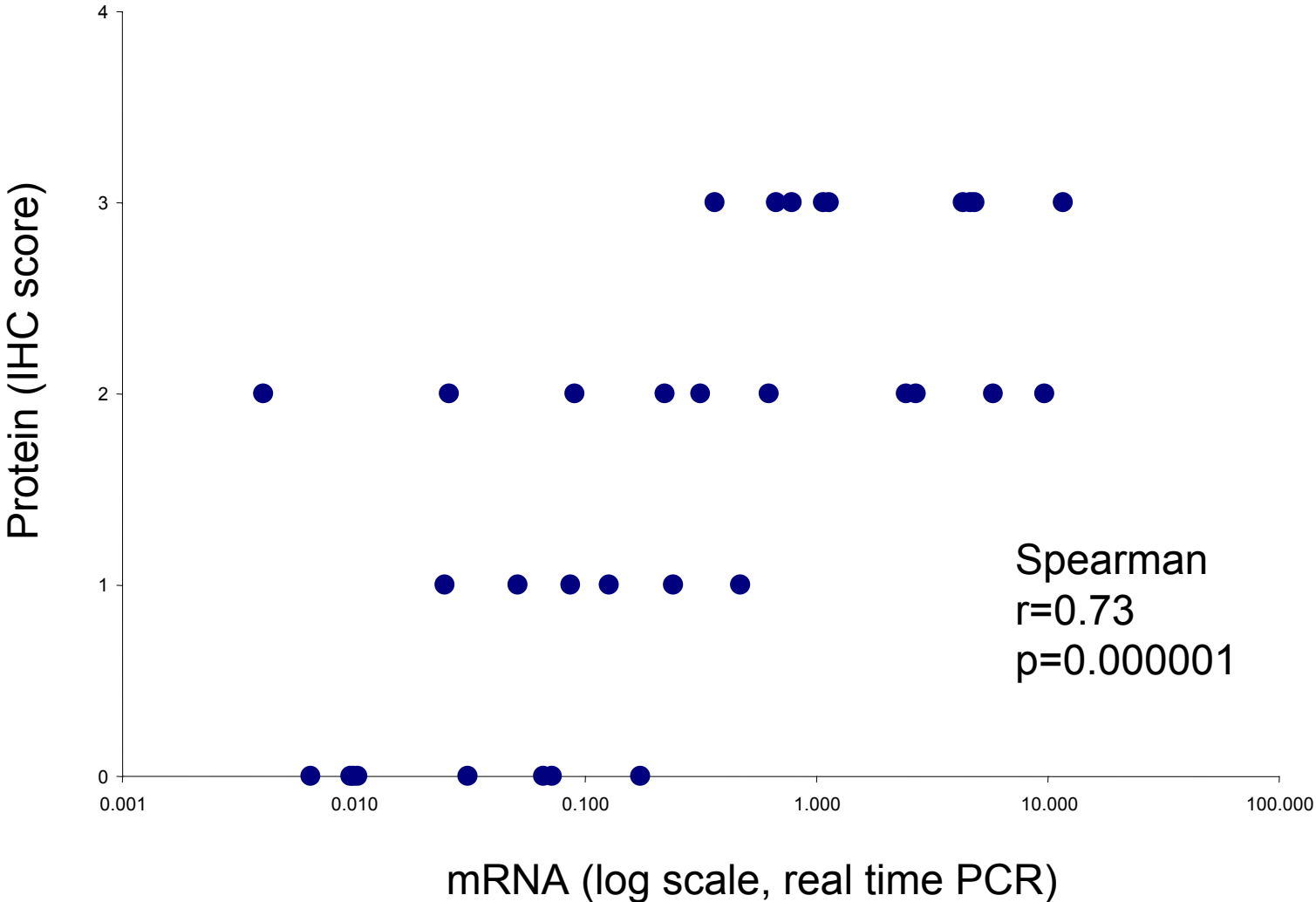
Sources of uncertainty

Components of Variance



Correlation of qPCR to IHC

Expression of MGB1 in breast cancer biopsies



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Strategy to Successful Real Time PCR

Attention to **EACH** step

Acknowledgements

Breast Unit Research Group Edinburgh Western General Hospital

- Prof. W Miller
- Prof. T Anderson
- Sharon White
- Alexey Larionov
- Mr. JM Dixon
- Juliette Murray
- Oliver Young
- Lorna Renshaw

Novartis Institutes for BioMedical Research

- Dean Evans
- Andreas Krause

Sir Alastair Currie laboratories, CRUK