Normalisation of mRNA against gDNA

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Normalisation

- Tissue Mass
- Cell number
- Alien spike area (LCM)

Reference genes

- Whole error
- Specific internal reference
- Alu repeats in transcriptome
  Vandesompele (2005)
- Multiple internal references

Internal reference

RNA

External reference

Total RNA
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

- Tissue Mass
- Cell number
- Alien spike area (LCM)

- External reference

- Internal reference

- RNA
- DNA

- Total RNA
- Reference genes
- Genomic DNA

- Whole error

- Multiple internal references

- Specific internal reference

- Alu repeats in transcriptome
  - Vandesompele (2005)

- GHR, IGF-IR
- K-ras, hMLH1
  - Bustin (2005)

Reference genes

Barts and The London
Queen Mary’s School of Medicine and Dentistry
Centre for Academic Surgery
Institute of Cell and Molecular Science
Genomic DNA standards for gene expression profiling in *Mycobacterium tuberculosis*

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

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.
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

Multiplex assay:
- qPCR - gDNA target(s)
- qRT-PCR - mRNA targets

DNA, RNA Extraction

mRNA copy number calculation:
- x copies/genome equivalent
gDNA markers

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no</th>
<th>Location</th>
<th>sense (targets intron)</th>
<th>antisense (targets exon)</th>
<th>amplicon size</th>
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<tbody>
<tr>
<td>hMLH-1</td>
<td>NM_00249</td>
<td>3p21.3</td>
<td><strong>FAM</strong>-isoC-(\text{GCCGTTAAGTCGTAGCCCTT})</td>
<td><strong>GCCAGCTAATGCTATCAAAG AGATGAT</strong></td>
<td>86 bp</td>
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<tr>
<td>GHR</td>
<td>NM_000163</td>
<td>5p13-p12</td>
<td><strong>Quasar 670</strong>-isoC-(\text{TGCTATGAAAACTTGCATCTTITTAATA AAATCATCCAAA})</td>
<td><strong>CAGGATCAAGTGATGCCTTT TCTGGA</strong></td>
<td>147 bp</td>
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<tr>
<td>IGF-IR</td>
<td>NM_000875</td>
<td>15q26.3</td>
<td><strong>CAL Fluor Red 610</strong>-isoC-(\text{CTCGGGTTTCGCAAACAGGG})</td>
<td><strong>TCTGCGCAGGAGGGA</strong></td>
<td>99 bp</td>
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<tr>
<td>K-Ras</td>
<td>NM_004985</td>
<td>12p12.1</td>
<td><strong>CAL Fluor Orange 560</strong>-isoC-(\text{GGGTGGTCCGCTCCGTAC})</td>
<td><strong>CATTTCCGACTGGGAGCGAG</strong></td>
<td>104 bp</td>
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</table>
Chemistry
Plexor (1)

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

d-iG
d-iC
A
G
C
T
Plexor (2)
Primer evaluation

IGF-IR

K-Ras

hMLH-1

GHR
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
c-myc expression in CRC

Normalised against Total RNA

up: 8/11 = 70%
down: 1/11 = 10%
same: 2/11 = 20%
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

1 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

Normalised against SIR (VDR)

Sample c-myc T:N

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c-myc expression in CRC

Normalised against GAPDH 5'

Normalised against GAPDH C

Normalised against GAPDH 3'

Sample

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c-myc expression in CRC

Normalised against K-Ras/GHR S

Normalised against K-Ras/GHR M
Microdissection
Sample

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

Methodology article

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

Cristina Hartshorn *1, Aleksandra Anshelevich1,2 and Lawrence J Wangh1

Address: 1Division of Medical Cen...

Published: 13 March 2009

BMC Biotechn...
No need for extraction
c-myc expression N vs T

Samples

copies/µg total RNA

10^5 10^6 10^7 10^8 10^9

A B C D E

normal colon  tumour

normal colon  tumour

10^0 10^1 10^2 10^3

A B C D E

normal colon  tumour
c-myc expression N vs T

Graphs showing c-myc expression in normal colon and tumour samples. The x-axis represents different samples (A to E), and the y-axis represents expression levels on a logarithmic scale. Each sample is represented by two bars: one for normal colon (purple) and one for tumour (blue).
c-myc expression N vs T

Samples

c-myc:DNA

normal colon

tumour

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Reproducibility

CV = 47.6%

CV = 6.2%
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

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Dr Ethan Strauss

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