Quantification strategies in real-time RT-PCR
with special focus on RNA quality,
relative expression and data normalisation

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www.gene-quantification.info
Genotype -> Phenotype -> Function

DNA => pre-mRNA => mRNA => Protein => Function

↓↓ ↓
Transcription
Splicing
Translation
post-translatorial modifications

Genom
Transcriptome & Splicome
Proteome
Metabolome
Transcriptome
RNA content of a cell
T.A. Brown, GENOMES 2nd Edition

- ribosomal RNA  
  rRNA  80-85%  (5S, 18S und 28S)
- transfer RNA  
  tRNA  10-15%
- messenger RNA  
  mRNA  2% (1-5%)  (Ø length 1930 bases)
  - high abundant  
    < 100 genes  > 10,000 copies/cell
  - intermediate abundant  
    ~ 500 - 1,000 genes  200 - 400 copies/cell
  - low abundant  
    ~ 27,000 genes  < 10 - 50 copies/cell

- RNA quantity & RNA quality
Quantification Strategies in real time qRT-PCR

M.W. Pfaffl, BioSpektrum 2004 (Sonderausgabe PCR)

**absolute quantification**
- external calibration curve
  - one color detection system
    - SYBR Green I
- external calibration curve
  - two color detection system
    - e.g. Probes, ROX

**relative quantification**
- normalisation
  - via one reference gene
  - via reference gene index
    - >3 HKG
  - external calibration curve without any reference gene

**without real-time PCR efficiency correction**
- 2 \((-\Delta\Delta CP)\)

**with real-time PCR efficiency correction**
- REST, qGene
- LC software

**external calibration curve**
- RT-PCR product
- plasmid DNA
- *in vitro* transcribed RNA
- synthetic DNA Oligos
- synthetic RNA Oligos
Quantification strategies in real-time RT-PCR

**Absolute quantification using calibration curves**

- recombinant DNA (recDNA) calibration curve  
  (Pfaffl & Hageleit, Biotechnol.Lett. 2001)
- recombinant RNA (recRNA) calibration curve  
  (Pfaffl & Hageleit, Biotechnol.Lett. 2001)
- calibration curve using a synthetic DNA oligo-nucleotide  
  (Bustin, JME 2000)
- calibration curve using a synthetic RNA oligo-nucleotide  
  (Bustin et al. 2000)
- calibration curve using a purified RT-PCR product  
  (Einspanier et al. 1999)
- „Copy & Paste“ of previously performed calibration curves  
  (LC software)
Absolute quantification using calibration curves

- **Calibration curve using a purified RT-PCR product or a synthetic ss/ds oligo-nucleotide**
  - **two-step RT-PCR**
  - advantages: quick, highly defined DNA content for the synthetic oligo
  - disadvantages: instable, “often problems with re-amplification”, „short“ templates

- **Calibration curve using a recombinant DNA (recDNA), e.g. plasmid DNA**
  - **two-step RT-PCR**
  - advantages: very stable, no problems with re-amplification, „mimic of mRNA“
  - disadvantages: cloning, linearization and purification of recDNA

- **Calibration curve using a recombinant RNA (recRNA)**
  - **one-step RT-PCR => recRNA and native mRNA undergoing RT and PCR in parallel**
  - advantages: mimics the natural mRNA situation best (recRNA = native mRNA)
  - disadvantages: very unstable recRNA, complicate cloning, linearization, purification of recRNA, storage problems, reproducibility (???)
  - => storage of recRNA !!

- **“Copy & Paste“ of previously performed calibration curves (e.g. LightCycler Software)**
  - advantages: very easy and very high reproducibility (at least for the calibration curve)
  - disadvantages: do not covers variations in real RT-PCR experiment: RNA quality, slope, qPCR efficiency ??? batch to batch variations, etc. => *truth ???*
ERα intra-assay & inter-assay variation

variation on the basis of detected molecules using a recombinant plasmid DNA calibration curve
intra-assay variation: within one run
inter-assay variation: between different runs

ER-alpha intra-assay variation CV = 18.7% (n = 3)

ER-alpha inter-assay variation CV = 28.6% (n = 7)
### Validation: absolute quantification of steroid receptors

<table>
<thead>
<tr>
<th></th>
<th>AR</th>
<th>ERα</th>
<th>ERβ</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>product length</strong></td>
<td>172 bp</td>
<td>234 bp</td>
<td>262 bp</td>
<td>227 bp</td>
</tr>
<tr>
<td><strong>detection limit</strong></td>
<td>12 molecules</td>
<td>2 molecules</td>
<td>10 molecules</td>
<td>14 molecules</td>
</tr>
<tr>
<td><strong>quantification limit</strong></td>
<td>120 molecules</td>
<td>165 molecules</td>
<td>106 molecules</td>
<td>760 molecules</td>
</tr>
<tr>
<td><strong>quantification range</strong></td>
<td>120 - 1.20*10^{10} molecules (r = 0.998)</td>
<td>165 - 1.65*10^{9} molecules (r = 0.995)</td>
<td>106 - 1.06*10^{10} molecules (r = 0.996)</td>
<td>760 – 7.60*10^{9} molecules (r = 0.998)</td>
</tr>
<tr>
<td><strong>PCR efficiency</strong></td>
<td>90.7%</td>
<td>81.2%</td>
<td>81.3%</td>
<td>93.9%</td>
</tr>
<tr>
<td><strong>intra-assay variation</strong></td>
<td>31.2% (n = 3)</td>
<td>18.7% (n = 4)</td>
<td>17.6% (n = 4)</td>
<td>5.7% (n = 4)</td>
</tr>
<tr>
<td><strong>inter-assay variation</strong></td>
<td>24.3% (n = 7)</td>
<td>28.6% (n = 4)</td>
<td>29.7% (n = 4)</td>
<td>25.7% (n = 4)</td>
</tr>
</tbody>
</table>

**Species specific T_{melt} (°C)**

<table>
<thead>
<tr>
<th>Species</th>
<th>AR</th>
<th>ERα</th>
<th>ERβ</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Homo sapiens</em></td>
<td>85.4</td>
<td>86.0</td>
<td>[ 87.9 ]</td>
<td>83.5</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>84.4</td>
<td>85.0</td>
<td>89.0</td>
<td>[ 82.9 ]</td>
</tr>
<tr>
<td><em>Callithrix jacchus</em> (primate)</td>
<td>85.0</td>
<td>--</td>
<td>[ 89.9 ]</td>
<td>83.9</td>
</tr>
<tr>
<td><em>Bos taurus</em></td>
<td>85.5</td>
<td>85.3</td>
<td>90.1</td>
<td>83.8</td>
</tr>
<tr>
<td><em>Ovis aries</em></td>
<td>--</td>
<td>85.4</td>
<td>90.5</td>
<td>83.1</td>
</tr>
<tr>
<td><em>Sus scrofa</em></td>
<td>84.5</td>
<td>86.0</td>
<td>90.2</td>
<td>83.5</td>
</tr>
</tbody>
</table>
Estrogen receptors (ERα & ERβ) expression pattern in cattle tissues

Pfaffl et al., APMIS 2001
Relative Quantification

The mRNA expression is relative to WHAT

• relative to a non treated control
• relative to a time point zero
• relative to another gene of interest
• relative to the mean expression of a target gene
• relative to an universal calibration curve
• relative to the expression of one constant expressed HKG
  GAPDH, tubulins, various actins, albumins, cyclophilin, micro-globulins, histone subunits,
  ribosomal units (18S or 28S rRNA), ...........etc.
• relative to a HKG Index containing more HKGs (> 3)
  geNorm (Vandesompele et al., Genome Biology, 2002)
  BestKeeper (Pfaffl et al.; Biotechnology Letters 2004)
  Normfinder (Andersen et al., Cancer Research 2004)
  statistical modeling (Szabo et al., Genome Biology 2004)
• etc. ???
Normalisation strategies

According to known amounts of extracted RNA
(RIN quality check, molecules/ng RNA; ag transcript/ng RNA)

According to mass or volume of extracted tissue
(molecules/mg tissue; ag transcript/mg tissue; transcript/cells)

According to one known and NOT regulated HKG
GAPDH, tubulins, actins, albumins, cyclophilin, micro-globulins, histone subunits, ribosomal units (18S or 28S rRNA),

According to a HKG Index containing more HKGs (> 3)
gNorm (Vandesompele et al. 2002, Genome Biology)
Accurate normalization of real-time quantitative RT-PCR data by geometric veraging of multiple internal control genes.

BestKeeper (Pfaffl et al. 2004; Biotechnology Letters 2004)
Determination of most stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper Excel based tool using pair-wise correlations.

Normfinder (Andersen et al., 2004 Genome Biology)

Statistical modeling for selecting housekeeper genes
(Szabo et al. 2004, Genome Biology)
Relative Quantification in real time qRT-PCR

relative quantification

normalisation

- via one reference gene
- via reference gene index >3 HKG
- external calibration curve without any reference gene

ROX

without real-time PCR efficiency correction

$2^{-\Delta\Delta CP}$
“Delta-delta method” for comparing relative expression results between treatments in real-time PCR

presented by PE Applied Biosystems (Perkin Elmer, Forster City, CA, USA)

ABI Prism 7700 Sequence detection System User Bulletin #2 (2001)

Relative quantification of gene expression.
http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf

\[
\begin{align*}
\text{expression ratio} &= 2 - [\Delta CP \text{ sample} - \Delta CP \text{ control}] \\
\text{expression ratio} &= 2 - \Delta \Delta CP
\end{align*}
\]

Livak KJ, Schmittgen TD. (2001)
Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 \(^{-\Delta \Delta C(T)}\) method.
“Delta-delta method” for comparing relative expression results between treatments in real-time PCR presented by ABI (Applied Biosystems Inc.)

assumptions:  \( E = 2 \)  &  \( \Delta CP \) is constant over a wide range

\( \Delta C_T \) is a constant value

When the PCR efficiency of both systems is the same the \( \Delta C_T \) remains constant

\( \Delta C_T \)s for 10 systems
FIG. 1. Validation of the $2^{-\Delta CT}$ method: Amplification of cDNA synthesized from different amounts of RNA. The efficiency of amplification of the target gene (c-myc) and internal control (GAPDH) was examined using real-time PCR and TaqMan detection. Using reverse transcriptase, cDNA was synthesized from 1 µg total RNA isolated from human Raji cells. Serial dilutions of cDNA were amplified by real-time PCR using gene-specific primers. The most concentrated sample contained cDNA derived from 1 ng of total RNA. The $\Delta CT$ ($CT_{c-myc} - CT_{GAPDH}$) was calculated for each cDNA dilution. The data were fit using least-squares linear regression analysis ($N = 3$).
expression relative to the time point zero & normalised by a HKG:

TNFα mRNA expression in cultured leukocytes after LPS stimulation:
- white blood cells [WBC] vs. somatic milk cells [SMC]
- isolated blood monocytes vs. isolated milk macrophages

PCR Efficiency = 2    n = 6    mean ± sem

Prgomet et al., 2004
Relative Quantification in real time qRT-PCR

- Relative quantification
  - Normalisation:
    - Via one reference gene
    - Via reference gene index >3 HKG
  - External calibration curve without any reference gene
  - ROX

2 (\(-\Delta\Delta\) CP)

Without real-time PCR efficiency correction

With real-time PCR efficiency correction

REST, qGene LC software
Tissue “background” interfere with real-time PCR efficiency and amplification fidelity

IGF-1 mRNA amplification in three cattle tissues

![Log Flo vs Cycle #](image1)

- liver

![Log Flo vs Cycle #](image2)

- m. splenius

![Log Flo vs Cycle #](image3)

- m. gastrocnemius
# Efficiency variation in real-time RT-PCR

<table>
<thead>
<tr>
<th>Detection Cycle (n)</th>
<th>PCR efficiency</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.97</td>
<td>16 %</td>
<td>25 %</td>
<td>35 %</td>
<td>46 %</td>
<td>57 %</td>
<td>70 %</td>
</tr>
<tr>
<td></td>
<td>1.95</td>
<td>29 %</td>
<td>46 %</td>
<td>66 %</td>
<td>88 %</td>
<td>113 %</td>
<td>142 %</td>
</tr>
<tr>
<td></td>
<td>1.90</td>
<td>67 %</td>
<td>116 %</td>
<td>179 %</td>
<td>260 %</td>
<td>365 %</td>
<td>500 %</td>
</tr>
<tr>
<td></td>
<td>1.80</td>
<td>187 %</td>
<td>385 %</td>
<td>722 %</td>
<td>1 290 %</td>
<td>2 260 %</td>
<td>3 900 %</td>
</tr>
<tr>
<td></td>
<td>1.70</td>
<td>408 %</td>
<td>1 045 %</td>
<td>2 480 %</td>
<td>5 710 %</td>
<td>13 000 %</td>
<td>29 500 %</td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>920 %</td>
<td>2 740 %</td>
<td>8 570 %</td>
<td>26 400 %</td>
<td>80 700 %</td>
<td>246 400 %</td>
</tr>
</tbody>
</table>

**Table 1:** Errors of quantification relative to a theoretical PCR efficiency of 2.00

Error calculation: \( (2^n/E^n-1) \times 100 \)

**Example:**

When the difference in PCR efficiency is 0.2 between two samples in the same run and both samples are detected in cycle 25 a more than 10-fold difference in the amount of the samples will be calculated.
PCR inhibitors:
- Hemoglobin, Urea, Heparin
- Organic or phenolic compounds
- Glycogen, Fats, Ca^{2+}
- Tissue matrix effects
- Laboratory items, powder, etc.

PCR enhancers:
- DMSO, Glycerol, BSA
- Formamide, PEG, TMANO, TMAC etc.
- Special commercial enhancers:
  - Gene 32 protein, Perfect Match, Taq Extender, AccuPrime, E. Coli ss DNA binding

real-time PCR efficiency

NA degradation = RIN

tissue degradation

unspecific PCR products

lab management

DNA dyes

cycle conditions

DNA concentration

PCR reaction components

hardware: PCR platform & cups
Relative quantification of a target gene versus a reference gene (housekeeping gene)

Single data ($n = 1$) e.g. array results:

$$\text{relative expression} = \frac{E_{\text{target}}^{\Delta CP_{\text{target}} \ (\text{control - sample})}}{E_{\text{reference}}^{\Delta CP_{\text{ref}} \ (\text{control - sample})}}$$

Roche Diagnostics, LC relative Quantification software, March 2001
Relative quantification of a **target gene** versus a **reference gene** (*housekeeping gene*)

**single data** \( (n = 1) \) e.g. array results:

\[
\text{relative expression} = \frac{E_{target} \Delta C_{P_{target}} \text{(control - sample)}}{E_{ref} \Delta C_{P_{ref}} \text{(control - sample)}}
\]

*Pfaffl, Nucleic Acids Research 2001*

**multiple data** \( (1 < n < 100) \) e.g. experimental groups via REST-XL©:

\[
\text{relative expression} = \frac{E_{target} \Delta C_{P_{target}} \text{(MEAN control – MEAN sample)}}{E_{ref} \Delta C_{P_{ref}} \text{(MEAN control – MEAN sample)}}
\]

*Pfaffl et al., Nucleic Acids Research 2002*
Relative Expression Software Tool - 384 = REST-384 © - version 1
Calculation Software for the Relative Expression in real-time PCR
using Pair Wise Fixed Reallocation Randomisation Test ©

Publication: Nucleic Acids Research 2002 Vol 29 (9) e45

Direct support: http://rest.gene-quantification.info

© 2001 & 2004 M.V. Pfaff & G.Y. Morgan
© 2005 M.V. Pfaff & G.Y. Morgan & Y.Y. Yairi

Reference gene

Target gene 1
Target gene 2
Target gene 3
Target gene 4
Target gene 5
Target gene 6
Target gene 7
Target gene 8
Target gene 9
Target gene 10
Target gene 11
Target gene 12
Target gene 13
Target gene 14
Target gene 15

Directional Ratio

Genes

Histon
MMP-2
MMP-14
MMP-19
TMP-1
TMP-2
IPA
uPA
uPAR
Angiopoitin-1
Caspase 3
Caspase 6
Caspase 7
Fas
Fas Lig.
Influence of total RNA quality, quantity and purity on qRT-PCR results

total RNA extracted bovine WBC analyzed in Bioanalyzer 2100

V. Walf, S. Huch, MW. Pfaffl, 2005
RIN in different bovine tissues (Box Plot)

- Liver (n = 22)
- Heart (n = 17)
- Spleen (n = 17)
- Lung (n = 22)
- Rumen (n = 23)
- Reticulum (n = 26)
- Omasum (n = 17)
- Abomasum (n = 17)
- Ileum (n = 17)
- Jejunum (n = 20)
- Colon (n = 19)
- Caecum (n = 16)
- Mes. lymphnode (n = 26)

S. Fleige & MW. Pfaffl, 2005
RIN  liver 1st and 2nd extraction

- Leber 1. Extraktion
- Leber 2. Extraktion

Sample number vs. RIN
## Enzymatic or UV degradation of tissue extracted total RNA

<table>
<thead>
<tr>
<th>RIN</th>
<th>10.0</th>
<th>9.2</th>
<th>9.2</th>
<th>8.6</th>
<th>8.1</th>
<th>7.8</th>
<th>7.2</th>
<th>6.7</th>
<th>6.0</th>
<th>5.0</th>
<th>4.4</th>
<th>4.0</th>
</tr>
</thead>
</table>

- **nt**: 4,000, 2,000, 1,000, 500, 200, 25

### Notes
- RIN values range from 10.0 to 4.0, indicating varying degrees of RNA integrity.
- The graph shows the degradation pattern across different samples.
bovine WBC total RNA

RIN 9.5

RIN 2.8

Δ CP
Influence of total RNA quality on qRT-PCR results

\[ y = -0.206x + 7.6396 \]
\[ R^2 = 0.5363 \]

\[ y = -0.887x + 21.398 \]
\[ R^2 = 0.7165 \]

\[ y = -0.7211x + 27.077 \]
\[ R^2 = 0.7682 \]

**RNA Integrity Number**

- IL-1
- beta actin
- 28S

**CP = Take Off Point**

- **y = -0.7211x + 27.077**
  - \( R^2 = 0.7682 \)
- **y = -0.887x + 21.398**
  - \( R^2 = 0.7165 \)
- **y = -0.206x + 7.6396**
  - \( R^2 = 0.5363 \)
bovine ileum total RNA

28S

18S

β-Actin

IL-1β
\[ y = -0.2036x + 8.6557 \quad R^2 = 0.7612 \]

\[ y = -2E-15x + 6.00 \quad R^2 = 1.0 \]

\[ y = -0.1461x + 13.565 \quad R^2 = 0.4903 \]

\[ y = -0.2036x + 8.6557 \quad R^2 = 0.7612 \]

\[ y = -2E-15x + 6.00 \quad R^2 = 1.0 \]

\[ \Delta CP = \text{constant in relative quantification} \]

<table>
<thead>
<tr>
<th>Gene</th>
<th>( CP )</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>n.s.</td>
</tr>
<tr>
<td>Interleukin-1beta</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>beta-Actin</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>18S</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>
Influence of total RNA quality on qRT-PCR CP (Ct)

IL-1: Crossing Point

- Reticulum (E)
- Lymph nodes (E)
- Lymph nodes (P)
- Colon (P)
- Lung (E)
- Corpus luteum (P)
- Caecum (P)
- Spleen (P)
- Abomasum (P)
Influence of total RNA quality on qRT-PCR efficiency

28S: Amplification

- Reticulum (E)
- Lymph nodes (E)
- Lymph nodes (P)
- Colon (P)
- Lung (E)
- Corpus luteum (P)
- Caecum (P)
- Spleen (P)
- Abomasum (P)
Relative quantification using efficiency corrected calculation

Advantages:

- each sample derived from identical total RNA (one-step quantification) or cDNA source (two-step quantification) = identical inhibitors and enhancers
- tissue specific matrix effects are given, but not existent in relative quantification
- total RNA quantity, RT efficiency and cDNA quantity are minor relevant
- effect of total RNA quality and influence on qRT-PCR have to be tested in detail
- optimized assays are highly reproducibility (variations only given by researcher, kit, robot and real-time platform)
- each sample will be normalised with HKG or better by a HKG INDEX
- no production of a calibration curve: e.g. cloning, linearization or purification of calibration curve material
- no data conversion: molecules, concentrations or molarities
- => direct procedure: measured data were inserted in mathematical model
- => relative quantification by software applications, e.g. qGene, REST, LightCycler
- => automatic calculation => bio-informatics session

Disadvantages:

- No information about the effective molecule concentration present in the tissues (molecules/mg tissue; molecules/ng RNA; molecules /cell, etc.)
Thank you team!
Thank you for your attention!