Influence of RNA matrix effect on qRT-PCR results
- an overview

Michael W. Pfaffl & Simone Fleige

qPCR 2005, Freising-Weihenstephan, 5 – 7th September 2005

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Fluorescence history

4 samples in quadruplicates
SYBR Green I
performed in LightCycler 1.0
PCR inhibitors:
- Hemoglobin
- Urea
- Heparin
- Organic or phenolic compounds
- Glycogen
- Fatty acids
- Ca²⁺
- 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 & performance

RNA / DNA degradation

Sampling and tissue degradation

Unspecific PCR products

Lab management

DNA dyes

DNA concentration

PCR reaction components

Hardware:
- PCR platform & cups

Cycle conditions

Pfaffl, qPCR 2004
Steps and variables of a successful mRNA quantification using real-time RT-PCR (1)

- **Sampling method:**
  - Biopsy
  - Fixed material
  - Fresh blood
  - Tissue storage
  - Liquid Nitrogen
  - RNA Later
  - 1st extraction buffer

- **RNA isolation:**
  - Extraction method:
    - total RNA vs. mRNA
    - liquid-liquid
    - columns
    - by Robot
  - **RNA integrity:**
    - Bioanalyzer, Experion
    - Nano Drop
    - various dyes
  - RNA storage –80°C

- **RT = reverse transcription:**
  - Efficiency of RT:
    - RT enzyme type
    - RT temperature
    - poly-T Primer
    - Random-hexamers
    - Specific primer
    - Primer mixtures
  - **One-step RT-PCR**
  - **Two-step RT-PCR**

- **PCR amplification:**
  - Efficiency & Specificity of real-time PCR:
    - Primer design
    - Primer specificity
    - multi-species Primer
    - mRNA abundance
    - cDNA input
    - Polymerase types & Mixtures
    - Robot vs. Technician
Steps and variables of a successful mRNA quantification using real-time RT-PCR

- **Detection method:**
  - SYBR Green I
  - Probes: Beacons, Scorpions etc.
  - Fit point method
  - TaqMan fitting (10x SD)
  - 2\textsuperscript{nd} derivative maximum
  - other models (Log. or Sig.)
  - raw data vs. background correction
  - other curve manipulations
  - 2-step, 3-step or 4-step PCR

- **Quantification strategy:**
  - “absolute” quantification
    - which standard curve
    - normalization with HKG
  - relative quantification
    - Total RNA, mass of tissue
    - normalization with HKG
    - normalization via an index of more (> 3) HKGs
    - geNorm, REST, BestKeeper, qBASE, Normfinder, etc.

- **BioInformatics:**
  - CP vs. molecules
  - Normality of data (???)
  - t-Test (?)
  - ANOVA (on the ranks ?)
  - SAS, SPSS, Excel, Sigma Stat
  - Permutation test
  - Randomization test (REST)
  - ..................???
Determination the extraction efficiency

**Spike** with rec. RNA
100 – 100,000 molecules per tube

**total RNA extraction**

**OD$_{260}$**

**RT reverse transcription**

**standard curve** using rec. DNA
10 – 100,000 molecules

**qPCR**

**CP data analysis**

**tissue sample:**
Liver, Spleen, Kidney, Caecum Colon, Reticulum
Tissue extraction efficiency [ mean±sem ]
(3 tissue repeats with 4 different spikes, n =12)
RT Efficiency
Qiagen SYBR Green I qRT-PCR Kit, performed in LightCycler

Mean daily recovery of recRNA MultiStandard (100 - 10,000 molecules)
Overall mean = 58.5±17.5%
RT Efficiency
Qiagen SYBR Green I qRT-PCR Kit, performed in LightCycler

overall mean = 61.3±17.4%
### Table

Table 1. Absolute reverse transcription yields for RNA MultiStandard.

<table>
<thead>
<tr>
<th>External RNA molecules added&lt;sup&gt;a&lt;/sup&gt;</th>
<th>10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>10&lt;sup&gt;5&lt;/sup&gt;</th>
<th>10&lt;sup&gt;4&lt;/sup&gt;</th>
<th>10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Average&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMLVH</td>
<td>22</td>
<td>50</td>
<td>48</td>
<td>(125)</td>
<td>40 ± 16</td>
</tr>
<tr>
<td>Omniscrypt</td>
<td>7.2</td>
<td>3.1</td>
<td>11.5</td>
<td>(66)</td>
<td>7.3 ± 4.2</td>
</tr>
<tr>
<td>AMV</td>
<td>0.4</td>
<td>0.6</td>
<td>4.9</td>
<td>(44)</td>
<td>2.0 ± 2.5</td>
</tr>
<tr>
<td>MMLV</td>
<td>32</td>
<td>49</td>
<td>50</td>
<td>(110)</td>
<td>44 ± 10</td>
</tr>
<tr>
<td>Improm-II</td>
<td>32</td>
<td>22</td>
<td>12</td>
<td>(98)</td>
<td>22 ± 10</td>
</tr>
<tr>
<td>cAMV</td>
<td>6.3</td>
<td>17</td>
<td>35</td>
<td>(88)</td>
<td>19 ± 15</td>
</tr>
<tr>
<td>ThermoScript</td>
<td>1.1</td>
<td>9.0</td>
<td>14</td>
<td>(46)</td>
<td>8.0 ± 6.6</td>
</tr>
<tr>
<td>SuperScript III</td>
<td>87</td>
<td>72</td>
<td>90</td>
<td>(43)</td>
<td>83 ± 10</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>24±29</strong></td>
<td><strong>28±26</strong></td>
<td><strong>33±29</strong></td>
<td><strong>78±32</strong></td>
<td><strong>28 ± 27</strong></td>
</tr>
</tbody>
</table>
Influence of total RNA quality, quantity and purity on qRT-PCR results
total RNA extracted bovine WBC analysed in Bioanalyzer 2100

S. Fleige, V. Walf, S. Huch, MW. Pfaffl, 2005
Degradation scale

S. Fleige, V. Walf, S. Huch, MW. Pfaffl, 2005
Degradation of tissue extracted total RNA

The intensity of bands decreases with increasing degradation.
bovine ileum total RNA

28S

18S

β-Actin

IL-1β
### bovine ileum: CP = TOP

**Linear Regression Equations:**
- \( y = -0.2583x + 21.787 \)  
  \( R^2 = 0.8119 \)
- \( y = -0.1461x + 13.565 \)  
  \( R^2 = 0.4903 \)
- \( y = -0.2036x + 8.6557 \)  
  \( R^2 = 0.7612 \)

### Gene Expression Analysis

<table>
<thead>
<tr>
<th>gene</th>
<th>level of significance</th>
</tr>
</thead>
<tbody>
<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>
Relative Quantification in real time qRT-PCR

relative quantification

normalisation

<table>
<thead>
<tr>
<th>via one reference gene</th>
<th>via reference gene index &gt;3 HKG</th>
<th>external calibration curve without any reference gene</th>
</tr>
</thead>
</table>

ROX

without real-time PCR efficiency correction

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

with real-time PCR efficiency correction

REST, qBASE, qGene, LC software
bovine ileum: delta CP (compared to beta-actin expression)

\[
y = -0.057x - 4.909 \\
R^2 = 0.096 \\
n. s.
\]

\[
y = -0.112x + 8.222 \\
R^2 = 0.260 \\
P = 0.04
\]
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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulum</td>
<td>E</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>E</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>P</td>
</tr>
<tr>
<td>Colon</td>
<td>P</td>
</tr>
<tr>
<td>Lung</td>
<td>E</td>
</tr>
<tr>
<td>Corpus luteum</td>
<td>P</td>
</tr>
<tr>
<td>Caecum</td>
<td>P</td>
</tr>
<tr>
<td>Spleen</td>
<td>P</td>
</tr>
<tr>
<td>Abomasum</td>
<td>P</td>
</tr>
</tbody>
</table>
Influence of qRT-PCR product length on RIN
beta-actin procuts in various lengths

Threshold RIN = 5.0
PCR efficiency in dependence of RIN

![Graph showing PCR efficiency vs RNA Integrity Number](image-url)
Comparison of Experion & Bioanalyzer 2100

Fluorescence

Area
Experion: 410.00
Bioanalyzer: 253.00
Comparison of 18S/28S rRNA ratio

Experion & Bioanalyzer 2100

**Bioanalyzer 2100**

\[ y = 0.177x + 0.2346 \]
\[ R^2 = 0.47 \]

**Experion**

\[ y = 0.107x + 0.475 \]
\[ R^2 = 0.32 \]

**Bioanalyzer 2100**

\[ y = 0.1201x + 0.092 \]
\[ R^2 = 0.53 \]

**Experion**

\[ y = 0.085x + 0.005 \]
\[ R^2 = 0.43 \]

200 ng total RNA analysed

50 ng
Run performance

Experion & Bioanalyzer 2100

**Experion:** 165.34 [71.47 ng/µl]
Ratio [28S/18S]: 0.93
Ladder Area: 370.14

**Bioanalyzer:** 63.3 [27.0 ng/µl]
Ratio [28S/18S]: 1.30
RIN: 7.4
Ladder Area: 354.1

**Experion:** 130.31 [45.07 ng/µl]
Ratio [28S/18S]: 1.36
Ladder Area: ----

**Bioanalyzer:** 44.8 [25.0 ng/µl]
Ratio [28S/18S]: 1.80
RIN: 5.2
Ladder Area: ----
A: Experion (50 ng/µl)
B: Bioanalyzer (50 ng/µl)
C: Experion (200 ng/µl)
D: Bioanalyzer (200 ng/µl)

mean [ng]

<table>
<thead>
<tr>
<th></th>
<th>Experion</th>
<th>Bioanalyzer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>54.2</td>
<td>43.4</td>
</tr>
<tr>
<td>B</td>
<td>211.1</td>
<td>235.8</td>
</tr>
</tbody>
</table>

CV [%]

<table>
<thead>
<tr>
<th></th>
<th>Experion</th>
<th>Bioanalyzer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>39.1</td>
<td>57.1</td>
</tr>
<tr>
<td>B</td>
<td>14.7</td>
<td>27.4</td>
</tr>
</tbody>
</table>

n = 207
n = 171
Sensitivity

Experion & Bioanalyzer 2100

Measured Concentration vs. Ratio

concentration [ng] vs. concentration [ng]

Ratio
Summary and Conclusions

- Total RNA Extraction efficiency is highly variable [ CV >50% ]
- Total RNA Extraction is very tissue dependent [ 20% to 70% extraction efficiency ]
- RT efficiency is highly enzyme dependent [ <10% for AMV, 50-85% for MMLV H- ]
- RT is very sensitive [ ~ 30% day-to-day variations ]
- RT is dependent of the abundancy [ 40% for low- and 75% for high abundant genes ]
- PCR performance is dependent on total RNA quantity and quality
- RNA quality (=RIN) is highly tissue dependent:
  - good RIN [8-10] for single cells like cell cultures and WBC
  - Lower RIN [5-8] for solid tissues, requiring more homogenisation
- Influence of RNA quality on qRT-PCR results:
  - Minor influence on classical qRT-PCR products under 200 bp
  - RIN threshold of RIN = 5 for longer qRT-PCR products over 400 bp
  - NO influence on amplification efficiency
  - Relative quantification can circumvents the RIN problematic
- Tools to measure RNA integrity:
  - Advantages of Bioanalyzer 2100: RIN algorithm and better 18S/28S ratio
  - Advantages of Experion: more sensitivity and less variability

=> Pre-analytical steps are HIGHLY variable and replicates should be done here and NOT later during PCR reaction.
Thank you team!
Thank you for your attention!