Influence of RNA integrity on real-time RT-PCR quantification data

Tissue “matrix” interfere with real-time PCR efficiency and amplification fidelity
IGF-1 mRNA amplification in three cattle tissues
Noise in real-time PCR!

⇒ Effect of different types of background on an amplification history

A. No background
B. Constant background (Roche Biochemicals 1999)
C. Linear rising background (Tichopad et al., 2000 & 2003)
D. Decreasing non-linear background (Johnson et al., 2004)
E. Increasing non-linear background (SoFar, Wilhelm et al., 2003).

Y signal axis is in log. scale!
Bar, Tichopad, Pfaffl, 2007 (in preparation)
real-time PCR efficiency and amplification performance

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

- RNA / DNA degradation
- sampling and tissue degradation
- unspecific PCR products
- lab management
- DNA dyes
- cycle conditions
- DNA concentration
- PCR reaction components
- hardware: PCR platform & cups

Steps and variables of a successful mRNA quantification using real-time RT-PCR

Sampling method:
- Biopsy
- Fixed material
- Fresh blood
- Tissue storage
- Liquid Nitrogen
- RNA Later
- 1st extraction buffer
- RNA storage –80°C
  ⇒ native RNA

Extraction method:
- total RNA vs. mRNA
- liquid-liquid
- columns
- Automatic via robot
- RNA integrity:
  - Bioanalyzer 2100
  - Experion
  - Nano-Drop
  - mFold algorithm

Efficiency of RT:
- RT enzyme type
- RT temperature
- Primers:
  - poly-T Primer
  - Random-hexamers
  - Specific primer
  - Primer mixtures
- one-step qRT-PCR
- two-step RT-qPCR
**Determination the total RNA extraction efficiency**

- **tissue spike** with recombinant RNA
  - 100 – 100,000 pMS1 RNA molecules / tube
- **Bovine tissue samples:**
  - liver, spleen, kidney, caecum, colon, reticulum
- **total RNA extraction**
- **RNA OD260**
- **RT reverse transcription**
- **cDNA**
- **standard curve** with recombinant DNA
  - 10 – 100,000 pMS1 DNA molecules / capillary
- **qPCR**
- **CP data analysis**

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**Extraction Control Plasmid**

- artificial synthetic DNA and RNA standard sequence

- minimal homology to any DNA or RNA target gene
- any contamination can be excluded
- target compatible extraction efficiency
- exact known copy numbers (！)
- guaranteed DNA and RNA stability (！)

[Image of Roboscreen® logo]
Absolut quantification with a known and exact defined recRNA reference (RNA multi-standard pMS1)

Stahlberg et al., Clin Chem. 50(9) 2004

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 [mean±std.dev.]

mean daily recovery of recRNA MultiStandard (100 - 10,000 molecules)
overall mean = 61.3±17.4%

spiked molecules recRNA MultiStandard per reaction setup
overall mean = 61.3±17.4%
Table

Table 1. Absolute reverse transcription yields for RNA MultiStandard.

<table>
<thead>
<tr>
<th>RT enzyme and &quot;tissue background matrix&quot; affect the RT efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>External RNA molecules added⁶</td>
</tr>
<tr>
<td>MMLVH</td>
</tr>
<tr>
<td>Omniscript</td>
</tr>
<tr>
<td>AMV</td>
</tr>
<tr>
<td>MMLV</td>
</tr>
<tr>
<td>Imprrom-II</td>
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<tr>
<td>cAMV</td>
</tr>
<tr>
<td>ThermoScript</td>
</tr>
<tr>
<td>SuperScript III</td>
</tr>
<tr>
<td>Average</td>
</tr>
</tbody>
</table>

Stahlberg et al., Clin Chem. 50(9) 2004

Stahlberg et al., Clin Chem. 50(9) 2004
RT efficiency depends on enzyme and gene

Stahlberg et al., Clin Chem. 50(9) 2004

Fig. 1: qPCR Ct values reflecting the amounts of cDNA produced by the reverse transcription, with total RNA from spleen as input material. Color bars indicate (left) of samples treated with each enzyme. Relative quantification of total RT efficiency is indicated by the right y-axis. The reverse transcriptase are as follows: left to right MMLV, AMV, Improm, Improm II (Promega), and SuperScript III (Invitrogen).

Stahlberg et al., Clin Chem. 50(9) 2004
RNA integrity $\Rightarrow$ RIN $\Rightarrow$ CP

Bioanalyzer 2100, Agilent Technologies

Bioanalyzer 2100

- Lab-on-chip technology
- Electrophoretic separation of total-RNA on mikrofabricated chips
- RNA samples are detected via laser induced fluorescence detection
Agilent 2100 Bioanalyzer
RNA chip

1. The sample is injected into the separation channel.
2. Sample components are electrophoretically separated.
3. Components are detected by their fluorescence and bounded into gel-like images (densit) and electropherograms (peaks).

Micro-channels are filled with a sizing polymer and fluorescence dyes.

Agilent Bioanalyzer 2100

<table>
<thead>
<tr>
<th>Time (seconds)</th>
<th>Fluorescence</th>
</tr>
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<tbody>
<tr>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0.5</td>
</tr>
<tr>
<td>29</td>
<td>1</td>
</tr>
<tr>
<td>34</td>
<td>1.5</td>
</tr>
<tr>
<td>39</td>
<td>2</td>
</tr>
<tr>
<td>44</td>
<td>2.5</td>
</tr>
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<td>49</td>
<td>3</td>
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<td>54</td>
<td>3.5</td>
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<tr>
<td>59</td>
<td>4</td>
</tr>
<tr>
<td>64</td>
<td>4.5</td>
</tr>
<tr>
<td>69</td>
<td>5</td>
</tr>
</tbody>
</table>

- Pre Region Marker
- 18S Fragment
- Inter Region
- 28S Fragment
- Fast Region
- 5S Region
- Precursor Region
- Post Region
Various total-RNA qualities analysed in the Bioanalyzer 2100

- Intact RNA
  - RIN: 9.5
  - ladder
  - marker
- degraded RNA
  - RIN: 2.8
  - marker


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Run standard experiment and use RIN to determine if sample integrity is sufficient:

- Cells/Culture
- Isolation of total RNA
  - RNA-QC via Agilent 2100 bioanalyzer
  - RIN
- RIN above threshold
  - Continue with downstream experiment (microarray, real-time PCR, etc.)
- RIN below threshold

Q: Impact of RNA integrity on the qRT-PCR performance?
Q: Impact on physiological result?
Influence of total RNA quality, quantity and purity on qRT-PCR results

total RNA extracted bovine WBC analysed in Bioanalyzer 2100

RIN: 9.5
ladder

RIN: 5.6

RIN: 2.8


RIN liver 1st and 2nd extraction

Leber 1.Extraktion

Leber 2.Extraktion

sample number
RIN in different tissues and cell lines

- Liver (n=22)
- Heart (n=17)
- Spleen (n=17)
- Lung (n=22)
- Rumen (n=23)
- Reticulum (n=26)
- omasum (n=17)
- Abomasum (n=17)
- Jejunum (n=20)
- Colon (n=19)
- Caecum (n=16)
- Lymph node (n=26)
- Kidney cell (n=3)
- Corpus luteum (n=5)
- Granulosa cell (n=5)
- Oviduct (n=5)
- WBC (n=5)

Degradation scale

- Total RNA extracted from bovine tissues
- Artificial degradation
- Degradation scale of mixtures between “good and bad” samples

Degradation of extracted total-RNA

The intensity of bands decreases with increasing degradation.

bovine ileum total-RNA

28S  18S  β-Actin  IL-1β
bovine WBC total-RNA

RNA integrity number [RIN]

crossing point [CP]

RNA integrity number [RIN]

18 S
28 S
ß-Actin
IL-1ß
Normalisation according to an internal reference gene
“delta-delta Ct method” for comparing relative expression results between treatments in real-time PCR
ABI Prism Sequence detection System User Bulletin #2 (2001)
Relative quantification of gene expression

\[ \Delta CP = CP_{target \text{ gene}} - CP_{reference \text{ gene}} \]

Expression ratio = \[2^{\Delta CP \text{ treatment} - \Delta CP \text{ control}}\]

Expression ratio = \[2^{-\Delta \Delta CP}\]

Livak KJ, Schmittgen TD. (2001)
Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2^−ΔΔCt Method.
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)

**RNA integrity number [RIN]**

**delta CP**

- 18 S - β-actin
- 28 S - β-actin
- IL-1β - β-actin
Influence of total RNA quality on qRT-PCR efficiency
Relative Quantification in real time qRT-PCR using an internal control for normalisation

- 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

With real-time PCR efficiency correction

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

REST, qBASE, qGene, LC software

Influence of qRT-PCR product length on RIN

Beta-actin products in various lengths

Threshold RIN = 5.0

RNA Integrity Number

Crossing Point

66 bp, 99 bp, 201 bp, 480 bp, 795 bp, 975 bp
PCR efficiency in dependence of RIN

Comparison of Experion & Bioanalyzer 2100

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

Bioanalyzer 2100
\[ y = 0.1201x + 0.092 \]
\[ r^2 = 0.53 \]

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

Bioanalyzer 2100
\[ y = 0.177x + 0.2346 \]
\[ r^2 = 0.47 \]

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

200 ng
n = 171
total RNA analysed

50 ng
n = 207

Run performance

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

Experion:
- 165.34 [71.47 ng/µl]
- Ratio [28S/18S]: 0.93
- RIN: n.a.
- Ladder Area: 370.14

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)

**Variability**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean [ng]</td>
<td>54.2</td>
<td>43.4</td>
<td>211.1</td>
<td>235.8</td>
</tr>
<tr>
<td>CV [%]</td>
<td>39.1</td>
<td>57.1</td>
<td>14.7</td>
<td>27.4</td>
</tr>
</tbody>
</table>

n = 207 (50 ng)  n = 171 (200 ng)

**Sensitivity**

![Graph showing sensitivity of Experion and Bioanalyzer 2100]
Part 1 - Summary and Conclusion

- 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 mRNA abundancy [ 40% for low- and 75% for high abundant genes ]

Part 2 - Summary and Conclusion

- qRT-PCR performance is dependent on total-RNA quantity and quality !
- RNA quality (= RIN value) 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 homogenization during extraction
- Total RNA classification using the RIN:
  - RIN > 8:         perfect total-RNA
  - 5 < RIN < 8:   good RNA
  - RIN < 5:         RNA quality is highly questionable

  => RIN threshold = 5

- Effects 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
  - minor influence on amplification efficiency
  - relative quantification using an internal control gene, performing the ΔCP approach, can partly circumvent the RIN problematic

- Tools to measure RNA integrity:
  - Bioanalyzer 2100 => Advantages in RIN algorithm & “better” 18S/28S ratio
  - Experion => Advantages in more sensitivity and less variability
  - mFOLD software => future studies !
CONCLUSION:

Pre-PCR analytical steps (sampling, extraction and reverse transcription) are HIGHLY VARIABLE and replicates should be done at the pre-PCR analytical level and not during later PCR reaction!

References:

• Lightfood, S. (2002) Quantitation comparison of total RNA using the Agilent 2100 bioanalyzer, ribogreen analysis, and UV spectrometry. Agilent Application Note, Publication Number 5988-7650EN.

Web resources:

• http://www.gene-quantification.info/
• http://RNA-integrity.gene-quantification.info/
• http://relative.gene-quantification.info/
• http://REST.gene-quantification.info/
Thank you team!
Thank you for your attention!