Normalization of real-time RT PCR data using an external RNA control

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Improved normalization of real-time reverse transcriptase polymerase chain reaction data using an external RNA control

Stian Ellefsen a,*, Kåre-Olav Stensløkken b, Guro K. Sandvik a, Tom A. Kristensen c, Göran E. Nilsson a

Anal Biochem (2008), 376, p 83-93
Introduction

A vertebrate with an extraordinary advantage:

*it produces ethanol!*

This enables survival without oxygen (anoxia)
Introduction
Introduction

What has anoxia tolerance to do with normalization of real-time RT PCR data?
a balanced ATP-budget is a prerequisite for anoxic survival

1) ATP production = ATP consumption

2) ATP production = ATP consumption

3) ATP production = ATP consumption
*Anoxia* is an extreme physiological challenge, and "everything" must be expected to change.
Aim of project: to develop a procedure for accurate assessment of gene expression in anoxic crucian carp
This is the first study to introduce an external RNA control gene prior to RNA extraction on a “per unit weight of tissue” basis, and to use it for normalization of real time RT PCR data.

**Material and Methods**

\[ \text{mw2060} \]
\[ \text{mw} = \text{Microcystis cf. wessenbergi} \]
\[ 2060 = \text{number of nucleotides} \]

External RNA control gene: an *in vitro* synthesized mRNA strand that does not have analogs in the experimental system of interest.
Standard curve

- using one batch of mw2060
- using one pool of homogenized brains

Fig. 1

Ellefsen et al. (2008)
Materials and Methods

**Anoxia exposures**

- Two anoxia experiments:
  1. 8 °C
  2. 13 °C

- Four oxygen regimes in each experiment:
  1. *Normoxia 7 days* (*N7*)
  2. *Anoxia 1 day* (*A1*)
  3. *Anoxia 7 days* (*A7*)
  4. *Anoxia 7 days/Normoxia 3 or 7 days* (*A7N3/A7N7*)

- Brain and heart were sampled for real-time RT PCR analyses
Results

**Internal RNA control genes in the crucian carp brain**

mw2060-normalized data:

**Fig. 2**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 ºC</td>
<td>β-actin, Cyclophilin A, GAPDH</td>
</tr>
<tr>
<td>13 ºC</td>
<td>β-actin, Cyclophilin A, GAPDH</td>
</tr>
</tbody>
</table>

**Ellefsen et al. (2008)**
Results

Internal RNA control genes in the crucian carp heart

mw2060-normalized data:

Fig. 3

8 ºC

NB!

N7 vs. A1

13 ºC

Ellenssen et al. (2008)
Results

Internal RNA control genes in the crucian carp heart

Non-normalized data:

Fig. 4

NB!
N7 vs. A1

Ellefsen et al. (2008)
Results

"Internal RNA control genes in the crucian carp heart"

Comparing mw2060-normalized and non-normalized data:

Ellefsen et al. (2008)
**Results**

Normalization of target gene expression in crucian carp heart: **HSP30**

**Fig. 5**

<table>
<thead>
<tr>
<th>Normalization:</th>
<th>mw2060</th>
<th>β-actin</th>
<th>geNorm</th>
</tr>
</thead>
</table>

**8 °C**

- N7 vs. A1

**13 °C**

- N7 vs. A1

**NB!**

Ellefsen et al. (2008)
## Results

Normalization of target gene expression in crucian carp heart: **HSC70**

Normalization:

<table>
<thead>
<tr>
<th></th>
<th>mw2060</th>
<th>β-actin</th>
<th>geNorm</th>
</tr>
</thead>
</table>

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSC70</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N7</td>
<td>A1</td>
<td>A7</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>A7N7</td>
</tr>
<tr>
<td></td>
<td>13 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N7</td>
<td>A1</td>
<td>A7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A7N3</td>
</tr>
</tbody>
</table>

**NB!**

N7 vs. A1

Fig. 6

*Ellefsen et al. (2008)*
Observed changes in internal RNA control gene expression had large consequences for target gene normalization.
## Results

### Evaluation

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>• standard curve</td>
<td>• batch-to-batch variation</td>
</tr>
<tr>
<td>• intra-experimental reproducibility (<em>low Coeff. of var.</em>)</td>
<td>• complete cell lysis?</td>
</tr>
<tr>
<td>• inter-experimental reproducibility (<em>similar expression profiles between experiments</em>)</td>
<td></td>
</tr>
<tr>
<td>• non-normalized data supports normalized data</td>
<td></td>
</tr>
<tr>
<td>• changes in mw2060-normalized gene expression could not be explained from changes in RNA yields</td>
<td></td>
</tr>
</tbody>
</table>

Our external RNA control gene approach seems to enable accurate normalization of real-time RT PCR data.
Expression of genes involved in excitatory neurotransmission in anoxic crucian carp (Carassius carassius) brain

Stian Ellefsen,1,2 Guro K. Sandvik,1 Helene K. Larsen,1 Kåre-Olav Stensløkken,3 Dag Are S. Hov,1 Tom A. Kristensen,4 and Göran E. Nilsson1

Physiol Genomics 35: 5–17, 2008

Expression of genes involved in GABAergic neurotransmission in anoxic crucian carp brain (Carassius carassius)

Stian Ellefsen,1,2 Kåre-Olav Stensløkken,3 Cathrine E. Fagernes,1 Tom A. Kristensen,4 and Göran E. Nilsson1

Physiol Genomics 36: 61-68, 2009

Differential regulation of AMP-activated kinase and AKT kinase in response to oxygen availability in crucian carp (Carassius carassius)

Kåre-Olav Stensløkken,1,2 Stian Ellefsen,3,4 Jonathan A. W. Stecyk,3 Mai Britt Dahl,2 Göran E. Nilsson,3 and Jarle Vaage1

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