Methods for qPCR Analysis

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Fab. Absolute vs relative is a great idea, although you must bear in mind that as conference organiser if I do not agree with any speaker's opinion they will be bundled off to the Tower of London.

S.
Methods of Analysis

- Absolute quantitation
- Relative quantitation
- Comparative quantitation
Why absolute quantitation?

- Gives a measure of copy number
- Viral load determination
- FDA filing
- Inter-lab comparisons
Why is absolute quantitation not currently feasible?

- There is no reliable method for preparing, quantitating and storing RNA standards
- No NIST traceable standards
Next Best Alternatives?

• Synthetic templates known to come up at a certain Ct value—“semi quantitative PCR”
Why relative quantitation?

• Does not require that you know the copy numbers for the standard curve
• Can be used to determine fold increases and decreases in gene expression
• There is no need to “over optimize” the efficiencies
What is needed for relative quantitation?

• Any sample that can be used as a comparison for other samples—“calibrator”

• A serial dilution of the calibrator to give a standard curve in terms of 1x, 2x, 10x, etc
Relative qPCR Data

- GOI
  - E = 43%

- Normalizer
  - E = 68%
In both animals, the GOI is expressed twice as much as in the treated areas as the untreated areas. This data verifies the array data.
Why comparative quantitation?

• Mathematical determination of relative quantities
• No standard curve needed
• Higher throughput
• Best used when particular ratios are expected or are verifying a “trend”
What is needed for comparative quantitation?

- Calibrator sample used as a 1x standard
- Samples that are prepared identically
- Ideally, if normalizing the results, your GOI and the normalizer will have the same efficiency
Comparative Quantitation

\[ \text{Ct} \_\text{GOI} - \text{Ct} \_\text{norm} = \Delta \text{Ct} \]

\[ \Delta \text{Ct} \_\text{Sample} - \Delta \text{Ct} \_\text{Calibrator} = \Delta \Delta \text{Ct} \]

Relative quantity = \( 2^{-\Delta \Delta \text{Ct}} \)
## Genotyping Experimental Rationale

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Genome Equivalents GOI</th>
<th>Genome Equivalents norm</th>
<th>Normalized Equivalents</th>
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<tbody>
<tr>
<td>Homozygous</td>
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<tr>
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<td>2</td>
<td>0.5</td>
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<tr>
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<td>2</td>
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<tr>
<td>Well</td>
<td>Dye</td>
<td>Replicate</td>
<td>Ct</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>-----------</td>
<td>-----</td>
</tr>
<tr>
<td>E1</td>
<td>FAM</td>
<td>b</td>
<td>22.26</td>
</tr>
<tr>
<td>F1</td>
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<tr>
<td>E1</td>
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<td>F1</td>
<td>HEX</td>
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<tr>
<td>A3</td>
<td>FAM</td>
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<tr>
<td>A4</td>
<td>FAM</td>
<td>c</td>
<td>40</td>
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<tr>
<td>A3</td>
<td>HEX</td>
<td>c</td>
<td>24.84</td>
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<td>c</td>
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<tr>
<td>H12</td>
<td>HEX</td>
<td>zp</td>
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</tbody>
</table>

- **wt Calibrator**
  - ∆Ct = -3.77
  - ∆∆Ct = 0.00
  - 2^{-∆∆Ct} = 1.0
  - Genotype: wt

- **Sample MC305**
  - ∆Ct = 15.50
  - ∆∆Ct = 19.26
  - 2^{-∆∆Ct} = 0.0
  - Genotype: null

- **Sample AS103**
  - ∆Ct = -3.82
  - ∆∆Ct = -0.05
  - 2^{-∆∆Ct} = 1.0
  - Genotype: hm

- **Sample TH600**
  - ∆Ct = -2.33
  - ∆∆Ct = 1.44
  - 2^{-∆∆Ct} = 0.4
  - Genotype: ht
Distribution of Genotype Results

# of Samples

Comparative Quantity

- Null
- Heterozygote
- Homozygote
Comparative quantitation

$Ct_{Sample} - Ct_{Calibrator} = \Delta Ct$

Relative quantity $= 2^{-\Delta Ct}$
Gene Expression Results

<table>
<thead>
<tr>
<th></th>
<th>Ave Ct</th>
<th>dct</th>
<th>$2^{\text{dct}}$</th>
<th>$1.8^{\text{dct}}$</th>
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<tbody>
<tr>
<td>WT</td>
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<tr>
<td>TG 1</td>
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<td>1.58</td>
<td>0.33</td>
<td>0.40</td>
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<tr>
<td>TG 2</td>
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<td>2.45</td>
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<tr>
<td>TG 3</td>
<td>27.25</td>
<td>0.38</td>
<td>0.77</td>
<td>0.80</td>
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<td>28.36</td>
<td>1.49</td>
<td>0.36</td>
<td>0.42</td>
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</tbody>
</table>

The expression of Gene X is repressed in the transgenic mouse lines relative to wild type mice.
Renee

attached is a slide that i use to describe how i analyze data the example has only 4 samples so it will fit on a slide, 2 controls and 2 experimentals the geometric Ct values are transformed to arithmetic emissions values by 1/2^CT this number for me is multiplied by 10^7 ... because then actin (Ct about 16) is equal to 100 then i transform the emission values to logs for logs, ratios are created by subtracting rather than division every value is transformed to a ratio with respect to the average of the 4 samples this removes the differences in the absolute emission from gene to gene the averages of the ratios for all the genes in each sample are determined and used as a "normalizer" alternatively, the averages of the ratios of selected genes can be used as a normalizer

rudy
• the geometric Ct values are transformed to arithmetic emissions values by $\frac{1}{2^{\text{CT}}}$ multiplied by $10^7$
• transform the emission values to logs for logs, ratios are created by subtracting rather than division
• every value is transformed to a ratio with respect to the average
• the averages of the ratios for all the genes in each sample are determined and used as a "normalizer"
• alternatively, the averages of the ratios of selected genes can be used as a normalizer
<table>
<thead>
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<th></th>
<th>g01</th>
<th>g02</th>
<th>g03</th>
<th>g04</th>
<th>g05</th>
<th>g06</th>
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</thead>
<tbody>
<tr>
<td>C1</td>
<td>g01</td>
<td>g02</td>
<td>g03</td>
<td>g04</td>
<td>g05</td>
<td>g06</td>
</tr>
<tr>
<td>C2</td>
<td>g01</td>
<td>g02</td>
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</tr>
<tr>
<td>S1</td>
<td>g01</td>
<td>g02</td>
<td>g03</td>
<td>g04</td>
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<td>g01</td>
<td>g02</td>
<td>g03</td>
<td>g04</td>
<td>g05</td>
<td>g06</td>
</tr>
</tbody>
</table>

Emission = \(2^{-Ct} \times 10^7\)

Log of Emission Value

<table>
<thead>
<tr>
<th></th>
<th>Avg g01</th>
<th>Avg g02</th>
<th>Avg g03</th>
<th>Avg g04</th>
<th>Avg g05</th>
<th>Avg g06</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>g01</td>
<td>g02</td>
<td>g03</td>
<td>g04</td>
<td>g05</td>
<td>g06</td>
</tr>
<tr>
<td>C2</td>
<td>Avg C1</td>
<td>Avg C2</td>
<td>Avg S1</td>
<td>Avg S2</td>
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<td>S2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Grand Avg</td>
</tr>
</tbody>
</table>

Log of Emission Value - gene Avg

(Log of Emission Value - gene Avg) - Sample Avg - Grand Avg
Conclusions

• Absolute quantitation
  • Standard curve
  • Standards must be accurately quantitated
  • Best used for viral load determination

• Relative quantitation
  • Standard curve
  • Standards are serial dilutions of a calibrator template
  • Best used for gene expression studies

• Comparative quantitation
  • Mathematical determination
  • Calibrator sample used as a 1x standard
  • Best used when particular ratios are expected or to verify trends
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