Accurate gene expression profiling –
Facing the issues of normalisation and efficiency

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Variations in RNA extraction

Cultured cells

Tissue section

Selected Cells

Total RNA extraction by column

Total RNA extraction by Guanidinium reagent

mRNA purification
Total RNA | mRNA
---|---
**One tube assay**
- target-specific primers
- *Tth* polymerase or RTase + *Taq* pol

**Two tube assay**
- oligo-dT or random primers
- random primers or oligo-dT
- target-specific primers

- **RT priming**
- **QPCR**
  - Reverse Transcriptase
  - Target-specific PCR primers
    - *Taq* polymerase
Favourite QRT-PCR Priming strategies

All onto total RNA

Data from participants of yahoo groups; qpcrlistserver
Bustin, Benes, Nolan, Pfaffl (2005) JME in press
Total RNA

Two tube assay

RT priming

oligo-dT or random primers

Reverse Transcriptase

Target-specific PCR primers and fluorescent probe

Tag polymerase

QPCR
Random primed RNA (2x) dilution series (QPCR NHE1)
Random primed RNA (2x) dilution series (QPCR NHE1)

Standard Curve

Log fit values
- SYBR Standards, R^2 = 0.939
- SYBR, Y = 2.101*LOG(X) + 26.04, Eff. = 199.2%
Random Priming RT and QPCR (100 fold dilutions, β-actin) is reproducibly none linear

RNA serial dilution 100ng to 1pg
Gene quantification is not reproducible between different RT reactions

**GAPDH**

**β-actin**

**NHE1**
Gene specific priming RT and QPCR (10 fold dilutions, GAPDH)
From RNA to cDNA to gene expression data

- Random priming of total RNA dilutions did not give a linear response.

✓ Random primed RT reactions appear to be reproducibly none linear from reaction to reaction when run together.

- Random primed RT reactions do not appear to be reproducible from reaction to reaction when run on different occasions.

✓ Specific priming of RNA dilutions appeared to give a linear response.
From RNA to cDNA
to gene expression data

Further Study:

• Investigate different RT priming strategies

• Investigate priming total RNA and mRNA

• Investigate the effects of these variables on data interpretation

• Investigate effect of template structure on RT-QPCR sensitivity and efficiency
Comparing RT Priming Strategies

GAPDH

Constant RNA input concentration

- Specific total RNA
- random total RNA
- oligo total
- r+o total
- Specific mRNA
- random mRNA
- oligo mRNA

Colorectal cancer sample

Ct
Sensitivity and efficiency using mRNA - GAPDH

GAPDH - mRNA serial dilution

specific y = -3.5067x + 49.745
R² = 0.9959

random+odT y = -3.5067x + 49.745
R² = 0.99897

random y = -3.602x + 51.251
R² = 0.9802

oligo-dT y = -4.0884x + 53.941
R² = 0.9897

% sensitivity rel to specific priming

% amplification efficiency

priming method

specific random oligo-dT ran+odT

50 60 70 80 90 100

0.001 0.01 0.1 1 10 100
GAPDH Comparisons:

• Using total or mRNA specific priming appears most sensitive
• When priming strategies are compared using mRNA serial dilutions specific priming appears most sensitive and results in highest amplification efficiency
Total RNA vs mRNA - IGF-I

- specific total
- specific mRNA

random total
random mRNA

specific total
specific mRNA

oligo-dT total
oligo-dT mRNA

Colorectal cancer sample
Sensitivity and efficiency using mRNA – IGF1

![Graph showing Ct vs Log input RNA concentration for different conditions: rand and dT, specific, random, oligo dT.]
Sensitivity and efficiency using mRNA – IGF1

\[
y = -3.2569x + 41.319 \quad R^2 = 0.996
\]

\[
y = -5.0145x + 50.925 \quad R^2 = 0.9788
\]

\[
y = -3.1575x + 40.274 \quad R^2 = 0.9979
\]

\[
y = -3.2569x + 41.319 \quad R^2 = 0.996
\]
IGF1 Comparisons:

• All priming strategies relatively insensitive
• Using total or mRNA Oligo dT priming appears most sensitive
• When priming strategies are compared using mRNA serial dilutions Oligo-dT or random priming appear most sensitive and results in highest amplification efficiency
• Specific priming is very poor (slight improvement on mRNA)
Data interpretation

GAPDH Total RNA

![Graph showing copy number of GAPDH Total RNA across different samples and conditions.]

GAPDH mRNA

![Graph showing copy number of GAPDH mRNA across different samples and conditions.]

Samples

<table>
<thead>
<tr>
<th>Specific</th>
<th>Random hexamers</th>
<th>Oligo (dT)15</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Graph showing copy number of GAPDH mRNA across different samples and conditions." /></td>
<td><img src="image2.png" alt="Graph showing copy number of GAPDH mRNA across different samples and conditions." /></td>
<td><img src="image3.png" alt="Graph showing copy number of GAPDH mRNA across different samples and conditions." /></td>
<td><img src="image4.png" alt="Graph showing copy number of GAPDH mRNA across different samples and conditions." /></td>
</tr>
</tbody>
</table>
IGF-I/GAPDH total RNA

IGF-I/GAPDH mRNA

Specific
Random hexamers
Oligo (dT)15
Mixed

Samples
Influence of priming strategy on data interpretation

Using constant input total or mRNA

• Interpretation of normalised data using random or oligo dT priming results in similar interpretation
• Specific priming results in great variation (due to IGF1 priming)
• mRNA appears to predict lower relative quantities than total RNA (by around 100 fold)
IGF1 structure

37°C

409-424

60°C
Comparing priming: IL-15

One tube assay (RNA dilutions) - Specific primers

Slope = -2.9

Two tube assay (cDNA dilutions) - Random primers

Slope = -3.7
IL-15
37°C

IL-15

60°C

Reverse primer

Reverse primer
Influence of target structure on priming efficiency

Using total RNA or cDNA dilution series

• Random priming appears to be more forgiving of secondary structure

• Specific primers need to be located within open regions (at 60°C or RT temperature)
37°C

GAPDH

60°C
GAPDH 5’

37°C

8 unpaired bases

60°C

12 unpaired bases
GAPDH Centre

1M NaCl

37°C

60°C

5 unpaired bases

10 unpaired bases
GAPDH 3’

37°C

18 unpaired bases

60°C

24 unpaired bases
Total RNA target
GAPDH specific primed dilution series

\[ y = -3.456x + 44.285 \]
\[ R^2 = 0.9992 \]

\[ y = -3.2243x + 43.256 \]
\[ R^2 = 0.9995 \]

\[ y = -3.308x + 42.935 \]
\[ R^2 = 0.9945 \]
QRT-PCR protocol considerations

- When possible quantify input RNA
- Include a constant RNA amount into each RT
- Correct for RT batch to batch variations (*Placenta* 26 (2005) 93-98)
QRT-PCR protocol considerations

For unknown amounts of RNA:

- Design specific reverse primers to open regions of transcript (at RT temperature) [www.designmyprobe.com]

- Use specific primers to increase sensitivity (especially when a large difference in transcript quantities is expected)

- Or use mRNA since all priming strategies appear to give limited linear response

- Whichever system is chosen check linearity and dynamic range of priming strategy for all genes to be measured.
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