QPCR Reagent Optimisation

2nd Nucleic Acid Quantification Meeting
An Independent workshop for real-time PCR and RT-PCR
Morris Lecture Theatre, Robin Brook Centre, St Bartholomew’s Hospital, West Smithfield, London
29th May 2003

Reinhold Mueller
Stratagene

QPCR Systems
Qualified QPCR Reagents: Maximum Optimisation and Convenience

Core Reagents for Optimisation

Master Mixes for Convenience

QPCR Reagents

QPCR Systems
Core reagents can be optimised for best results.

DNA Polymerase 10X Buffer dNTPs MgCl₂ Passive Reference Dye

Master Mixes are easy-to-use and convenient.

All components in 1 tube!
PCR Amplification

PCR: Correlation of amount of amplified DNA to amount of initial target DNA

\[ Y = X (1 + E)^n \]

- \( Y \) = PCR amplified quantity
- \( X \) = target DNA quantity prior to PCR
- \( E \) = amplification efficiency
- \( n \) = number of cycles
Reagent Optimisation: What Do You Mean?

- Optimisation (of the reagents) by the manufacturer
- Optimisation (of the assay) by the customer
Replicate Samples in Reagents, Which Have Not Been Optimized

The replicate samples do not have the same Ct value. A variability in the assay data is observed. However, operator and instrumentation may also affect the results.
Replicate Samples in a Robust Assay

Variable PCR Plateau

The replicate samples have the same Ct value. Tight data obtained when calculating the initial target concentration.
Amplification of RNA Template: Which Route to Take?

- Single-tube QRT-PCR
  
  Fast, homogenous assay with possibly lower sensitivity.

- Two-tube QRT-PCR
  
  Retain (archived) material; must open tube and maybe obtain higher sensitivity.
Performance of Optimized Single-Tube and 2-Tube QRT-PCR Reagents

Template: 10 ng of human total RNA (liver, brain and stomach)
Target: β-actin (FAM-MB) and GAPDH (JOE-MB) in multiplex

Using relative quantification, the difference in performance of a single-tube and a two-tube QRT-PCR assay might be small.
# Configuration of Qualified QPCR Core Reagents

<table>
<thead>
<tr>
<th></th>
<th>GATC + ROX</th>
<th>GATC - ROX</th>
<th>GAUC + ROX</th>
<th>GAUC - ROX</th>
<th>GAUC/UNG + ROX</th>
</tr>
</thead>
<tbody>
<tr>
<td>QPCR</td>
<td>Eurogentec/Stratagene</td>
<td>Eurogentec</td>
<td>Eurogentec</td>
<td>Eurogentec</td>
<td>ABI/Stratagene</td>
</tr>
<tr>
<td>1-Tube RT</td>
<td>Stratagene/Takara Mirus</td>
<td>Stratagene</td>
<td></td>
<td></td>
<td>ABI</td>
</tr>
<tr>
<td>2-Tube RT</td>
<td>Stratagene</td>
<td>Eurogentec</td>
<td>Eurogentec</td>
<td>ABI</td>
<td>Stratagene</td>
</tr>
<tr>
<td>SYBR Green I QPCR</td>
<td>Eurogentec/Stratagene</td>
<td>Eurogentec</td>
<td>Eurogentec</td>
<td>ABI</td>
<td></td>
</tr>
<tr>
<td>SYBR 1-Tube RT</td>
<td>Sigma</td>
<td>Ambion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYBR 2-Tube RT</td>
<td></td>
<td></td>
<td>Eurogentec</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Buffer Components

**Two-tube QRT-PCR**

- (SYBR® Green I)
- 10X Core RT Buffer
- Taq DNA Polymerase
- MgCl₂
- dGATC Mix (dGAUC Mix)
- UNG
- Glycerol
- DMSO
- Other Enhancing Agents
- Reference Dye

**Single-tube QRT-PCR**

- (SYBR® Green I)
- 10X Core RT Buffer
- Reverse Transcriptase
- RNase Inhibitor
- Taq DNA Polymerase
- MgCl₂
- dGATC Mix (dGAUC Mix)
- Glycerol
- DMSO
- Other Enhancing Agents
- Reference Dye

**QPCR Systems**
List of Core Reagent Components That Have a Known Effect on Ct and/or Fluorescence Intensity

- 10X Core RT-PCR Buffer
- 10X Core PCR Buffer
- SYBR Green I
- Reverse Transcriptase
- RNase Inhibitor
- Taq DNA Polymerase
- MgCl₂
- dGATC mix (dGAUC mix)
- UNG
- Glycerol
- DMSO
- Other enhancing agents
- Random Primers
- Oligo(dT) Primer
- Reference Dye

QPCR Systems
Different Strokes for Different Blokes?

• TaqMan® probes, Molecular Beacons, Hybridization probes, Scorpions® and Amplifluor® primers can be detected in the same Core Reagents.

• SYBR® Green I Core Reagents have their own set of ingredients.

• Core Reagents for samples to be run in plastic tubes have a different make-up than Core Reagents to be run in glass capillaries.
Requirements for Core Reagents

- Sensitivity
- Reproducibility
- Accuracy
- Versatility
Part I: Optimising the Reagents

Probe Detection

QPCR Systems
Optimisation of [Tris-HCl] for Single-Tube QRT-PCR Core Reagents

A matrix for [Tris] and [KCl] was set up. This plot represents one part of the matrix. Low Ct and high final fluorescence is desired.

Template: 10 ng of mouse total RNA
Target: β-actin (FAM-MB)
DNA Polymerase (Taq)

Hot Start vs. Non-Hot Start

The average of replicate QPCR reactions is shown. A FAM-labeled Molecular Beacon is used for detection.

Chemical and antibody hot start Taq DNA polymerase yields in higher final fluorescence, compared to reactions run with a non-hot start enzyme.
Polymerase (Taq) Concentration
(Chemically Modified Taq)

<table>
<thead>
<tr>
<th>Target Amount</th>
<th>1.25U</th>
<th>1.5U</th>
<th>1.75U</th>
<th>2.0U</th>
<th>2.5U</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ng</td>
<td>21.1</td>
<td>20.5</td>
<td>20.8</td>
<td>20.5</td>
<td>20.3</td>
</tr>
<tr>
<td>0.1ng</td>
<td>27.8</td>
<td>27.2</td>
<td>27.4</td>
<td>27.4</td>
<td>27.2</td>
</tr>
<tr>
<td>0.01ng</td>
<td>31.1</td>
<td>30.4</td>
<td>30.7</td>
<td>30.7</td>
<td>30.7</td>
</tr>
<tr>
<td>NTC</td>
<td>No Ct</td>
<td>No Ct</td>
<td>No Ct</td>
<td>No Ct</td>
<td>No Ct</td>
</tr>
</tbody>
</table>

Average Ct of duplicate 50 µl QPCR reactions using GAPDH TaqMan probe (PDAR) and cDNA from PMA-treated Raji cells.

The higher the [Taq], the lower the Ct.
Do We Need Contamination Control?

Security, but loss of sensitivity.
The GAPDH targets used are very likely different (the TaqMan PDAR site is not identified). The data indicate that [RT] may have a different effect on different targets.
### RNase H (+) vs. RNase H (-)

#### Reverse Transcriptases

**TaqMan Detection of Different Target genes**

<table>
<thead>
<tr>
<th>Target</th>
<th>Target Amnt.</th>
<th>RNase H (+)</th>
<th>RNase H (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-2 micro</td>
<td>1ng</td>
<td>27.4</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>0.01ng</td>
<td>34.3</td>
<td>34.5</td>
</tr>
<tr>
<td></td>
<td>NTC</td>
<td>no ct</td>
<td>no ct</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>1ng</td>
<td>26.7</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>0.01ng</td>
<td>33.7</td>
<td>33.4</td>
</tr>
<tr>
<td></td>
<td>NTC</td>
<td>no ct</td>
<td>no ct</td>
</tr>
<tr>
<td>GUS</td>
<td>10ng</td>
<td>26.1</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>0.1ng</td>
<td>32.7</td>
<td>32.9</td>
</tr>
<tr>
<td></td>
<td>NTC</td>
<td>no ct</td>
<td>no ct</td>
</tr>
<tr>
<td>IL-6</td>
<td>10ng</td>
<td>30.6</td>
<td>30.6</td>
</tr>
<tr>
<td></td>
<td>1ng</td>
<td>34.0</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td>NTC</td>
<td>no ct</td>
<td>no ct</td>
</tr>
</tbody>
</table>

The results indicate that in this single-tube QRT-PCR there is no significant difference in using RTs with or without RNase H activity.
## Effect of RNase Block on QRT-PCR

<table>
<thead>
<tr>
<th>Amt RNAse Block</th>
<th>Target conc (ng)</th>
<th>Avg Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>25.9</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>35</td>
</tr>
<tr>
<td>10U</td>
<td>100</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>34.8</td>
</tr>
<tr>
<td>20U</td>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>36.2</td>
</tr>
<tr>
<td>30U</td>
<td>100</td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>36</td>
</tr>
<tr>
<td>40U</td>
<td>100</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>36.1</td>
</tr>
<tr>
<td>50U</td>
<td>100</td>
<td>26.3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>36.6</td>
</tr>
</tbody>
</table>

**Target:** TATA Box Binding Protein/TaqMan Probe

**Template:** QPCR Human Reference RNA

Only at low template concentration is the effect of RNase inhibitor really apparent.
Optimising dNTP (GATC) Concentration

<table>
<thead>
<tr>
<th>Conc. dNTP each</th>
<th>Ct</th>
<th>FI</th>
</tr>
</thead>
<tbody>
<tr>
<td>50uM</td>
<td>23.6</td>
<td>2500</td>
</tr>
<tr>
<td>100uM</td>
<td>22.8</td>
<td>3800</td>
</tr>
<tr>
<td>200uM</td>
<td>21.9</td>
<td>4700</td>
</tr>
<tr>
<td>300uM</td>
<td>21.7</td>
<td>4700</td>
</tr>
</tbody>
</table>

Template: 10 ng mouse total RNA

Target: β-actin using single-tube QRT-PCR with Molecular Beacon detection (FAM)

By increasing the [dNTP] a decrease in Ct and an increase in final fluorescence is observed.
The Effect of Magnesium on QRT-PCR

Template: 10 ng of mouse total RNA
Target: β-actin (FAM-MB) in single-tube QRT-PCR
The Effect of Magnesium on QRT-PCR
Continued

4% agarose gel, stained with ethidium bromide.
Effect of DMSO on Ct

<table>
<thead>
<tr>
<th>DMSO Conc (%)</th>
<th>GAPDH</th>
<th>β-2 micro</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27.5</td>
<td>36.3</td>
</tr>
<tr>
<td>0.5</td>
<td>26.4</td>
<td>32.8</td>
</tr>
<tr>
<td>1</td>
<td>25.9</td>
<td>32.7</td>
</tr>
<tr>
<td>1.5</td>
<td>26.2</td>
<td>32.4</td>
</tr>
<tr>
<td>2</td>
<td>25.6</td>
<td>32.6</td>
</tr>
<tr>
<td>2.5</td>
<td>25.8</td>
<td>32.9</td>
</tr>
<tr>
<td>3</td>
<td>25.8</td>
<td>32.7</td>
</tr>
<tr>
<td>5</td>
<td>26.7</td>
<td>33.1</td>
</tr>
<tr>
<td>10</td>
<td>32.4</td>
<td>37.7</td>
</tr>
</tbody>
</table>

Single Tube QRT-PCR Core Reagents with TaqMan probes for GAPDH and β-2 microglobulin using 0.01ng of QPCR reference RNA.
Passive Reference Dye

Analysis without Passive Reference - 110698

Analysis With Passive Reference - 110698

Total Human RNA
- 10 ng
- 1 ng
- 0.1 ng
- 0.01 ng
- NTC

Human Total RNA
- 10 ng
- 1 ng
- 0.1 ng
- 0.01 ng

QPCR Systems
dNTP Concentration in Multiplex Reactions

Template: QPCR human reference total RNA

Target: Cystic fibrosis transmembrane conductance regulator (CFTR);
(Detection: TaqMan ROX-BHQ2)
Assay Reproducibility in 96 Wells, Very Tight Run.
Reproducibility of “Optimized” QPCR Core Reagents From Two Vendors

Target: β-actin detection, linear probe, human genomic DNA as template.

Platform: ABI 7700

The plot shows the reproducibility and sensitivity when 8 replicates at two target concentrations (100ng, 1ng) were run (0.3Ct spread for Vendor 1 vs 0.8 Ct spread for Vendor 2).
2-Fold Discrimination

β-actin Molecular Beacon assay

10,000 copies

5,000 copies
Intra-Assay Variability Using the Single-Tube RT-PCR Core Reagent Kit

<table>
<thead>
<tr>
<th>pg Total RNA</th>
<th>Ct for Exp 1</th>
<th>Ct for Exp 2</th>
<th>Ct for Exp 3</th>
<th>Ct for Exp 4</th>
<th>Avg. Ct</th>
<th>Std. Dev</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35.1</td>
<td>35.9</td>
<td>35.6</td>
<td>32.5</td>
<td>34.8</td>
<td>1.3</td>
<td>3.9</td>
</tr>
<tr>
<td>10</td>
<td>32.1</td>
<td>31.8</td>
<td>31.9</td>
<td>31.1</td>
<td>31.7</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>100</td>
<td>28.2</td>
<td>28.7</td>
<td>28.7</td>
<td>29.0</td>
<td>28.7</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>1000</td>
<td>24.7</td>
<td>25.5</td>
<td>25.8</td>
<td>25.2</td>
<td>25.3</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>10000</td>
<td>20.8</td>
<td>21.3</td>
<td>22.1</td>
<td>21.6</td>
<td>21.5</td>
<td>0.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Optimized QRT-PCR Core Reagents show good reproducibility.
Part I: Optimising the Reagents:

SYBR Green I
SYBR Green I Titration With 900bp α-1 Anti Trypsin Target

SYBR Green I has an inhibitory effect on amplification/detection of target.
Effects of SYBR Green I Concentration On Amplicon Length

The SYBR Green I inhibitory effect is related to amplicon length.
Effects of SYBR Green I Concentration
Continued

Dissociation profile

Agarose gel stained with EtBr

QPCR Systems
SYBR Green I: Effects of DMSO and Glycerol (Amplification Plot)

<table>
<thead>
<tr>
<th>GLYCEROL (%)</th>
<th>DMSO (%)</th>
<th>AVG. Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>25.6</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>24.7</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>21.8</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>20.4</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>20.2</td>
</tr>
</tbody>
</table>

Template: Plasmid DNA
Target: Mouse muscle nicotinic acetylcholin receptor $\gamma$ (FAM-MB)
As expected, glycerol and DMSO change the melting of specific amplicon, in this case by 2°C.
Part II: Optimising the Reactions
Optimizing Primer Sets for SYBR Green

**Amplification Plot**
- Blue = Primer set 1 (247bp)
- Red = Primer set 2 (151bp)

**Dissociation Curve**
- Target = SERCA1 (ATP2A1)
- Template = cDNA from Human QPCR Reference Total RNA

**Legend**
- NTC
- Unknown
Acknowledgements

Gothami Padmabandu
Jeanette Quinn
Andrew Firmin
For Optimisation of Reactions Using Core Reagents One Should Consider Varying First:

- Primers
- Template
- MgCl\textsubscript{2}
- Enzymes
- dNTPs
- DMSO, Glycerol
- (SYBR Green I)