Quantitative, multiplexed amplification with the Plexor™ qPCR Systems

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Novel Base-Pairing

- IsoC & IsoG dNTPs
  - Recognized by DNA Polymerase
  - Do not base pair with ACGTU
  - Licensed from EraGen Biosciences

Use of IsoG and IsoC in Real-Time PCR

- Two primer method
  - One standard primer
  - One primer modified with iso-dC base and a fluorophore at 5´end

- Amplification master mix contains
  - Standard dNTPs
  - Dabcyl -labeled iso-dGTP

Dabcyl-iso-dGTP Contact Quenching

- Primers targeting different genes are labeled with iso-dC and different fluorophores
- Dabcyl-iso-dGTP incorporates opposite any iso-dC, resulting in contact quenching
<table>
<thead>
<tr>
<th>Dye</th>
<th>% Quenching</th>
<th>Emission Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>62%</td>
<td>518nm</td>
</tr>
<tr>
<td>TET</td>
<td>53%</td>
<td>536nm</td>
</tr>
<tr>
<td>HEX</td>
<td>71%</td>
<td>554nm</td>
</tr>
<tr>
<td>ROX</td>
<td>64%</td>
<td>606nm</td>
</tr>
<tr>
<td>LC™640</td>
<td>48%</td>
<td>640nm</td>
</tr>
<tr>
<td>Cy™5</td>
<td>35%</td>
<td>667nm</td>
</tr>
</tbody>
</table>

Typical Amplification Curves

- Gain of fluorescence

![Graph showing typical amplification curves with fluorescence on the y-axis, cycle on the x-axis, and a threshold line.](image)
Plexor™ Amplification Curves

- Quenching of fluorescence
Plexor™ Method…Same Data

- $C_t$ vs. concentration

![Graph showing $C_t$ vs. concentration with a linear regression line $y = -3.61x + 41.21$ and $R^2 = 0.999$.]
Plexor™ Method…Includes Melt Curves

• Confirmation of specificity
Multiplexing Applications

Gene Expression Analysis
Large Dynamic Range

- Detection of various concentrations of a synthetic transcript in a constant background of 10ng human total RNA using one-step qRT-PCR
  - Monoplex reaction
  - Duplex reaction (also amplifying GAPDH)

Dynamic Range: $10^1$ to $10^{10}$ copies
Quantitation in Multiplex

Experimental details
• 3 primer sets designed with Plexor™ Primer Design Software
• Biosearch Technologies dye sets
• All targets are human total RNA
• Plexor™ One-Step qRT-PCR System
• Thermal cycling performed on the Applied Biosystems 7500 Real Time PCR System
Consistent Quantitation in Multiplex

Fibroblast Growth Factor Receptor 1 (FGFR1) - FAM Channel

Monoplex $R^2 = 0.999$ 100% Eff.

Duplex $R^2 = 1.000$ 102% Eff.

Triplex $R^2 = 1.000$ 99.6% Eff.
Consistent Quantitation in Multiplex

Matrix Metalloproteinase 1 (MMP1) - CalFluor™ Red 610 Channel

Monoplex $R^2 = 0.999$ 100.8% Eff.

Duplex $R^2 = 0.999$ 100.6% Eff.

Triplex $R^2 = 0.995$ 102.3% Eff.
Consistent Quantitation in Multiplex

Glyceraldehyde-3-phosphotransferase (GAPDH) - JOE Channel

Monoplex $R^2 = 0.999$ 98.8% Eff.

Duplex $R^2 = 0.999$ 98.8% Eff.

Triplex $R^2 = 1.000$ 102% Eff.

- GAPDH
- GAPDH + FGFR1
- GAPDH + FGFR1 + MMP1
4-Color Multiplexing

Experimental Details

- 4 primer sets designed with Plexor™ Primer Design Software
- Biosearch Technologies dye sets
- All targets are human cDNA
- Plexor™ Two-Step qRT-PCR System
- Thermal cycling performed on the Applied Biosystems 7500 Real Time PCR System
Quantitate High- & Low-Copy Together

Red = Monoplex
Blue = 4-plex

Quasar™ 670
GAPDH
Δ = 0.1 cycles

Cal Fluor™
Red 610
Integrin
Δ = 0.4 cycles

Cal Fluor™
Orange 560
FGF receptor
Δ = 0.5 cycles

FAM
Sulfotransferase
Δ = 0.8 cycles

AB 7500
Plexor™ HY Assay

Autosomal

Titration series (3pg-50ng/µl)

Data provided by J. Butler (NIST)
Summary

- Two primer method allows easy multiplexing
- High correlation of $C_t$ values between monoplex and multiplex (less than 1 $C_t$ change)
- Accurate quantitation of lower copy messages in multiplex with high copy messages
Assay Design and Analysis

Plexor™ Primer Design System and Analysis Software
Plexor™ Technology in Research

- Plexor™ Assay Design
- Plexor™ Reaction
- Plexor™ Data Analysis
Plexor™ Primer Design Website

- Free access (registration required)
Plexor™ Primer Design Web Site

- Sequence #1
- Sequence #2
- Sequence #3
- Sequence #4

Designed amplimers & primers checked for interactions
Suggested primers with BLAST links

Powered by DNA Software
BLAST Parser

Plexor Primer Design BLAST analysis results
Wednesday, March 14, 2007

You may select a single species or genus to view

NM_006617 - analyzed results from BLAST performed against the RefSeq RNA database.

<table>
<thead>
<tr>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCTGTAGGCCCCCTTTTTCTCTCG</td>
<td>AGGCGTTGGAAACGAGGTGGA</td>
</tr>
</tbody>
</table>

A BLAST search was performed against the RefSeq database. The primer appears likely to anneal to a single gene from each of the species shown below in green. It is unlikely to misprime other genes from these species. If you are using RNA samples from one of these species and are examining all forms of the gene listed, the primer is unlikely to show non-specific amplification.

- Boe baurus (cattle):
  - LOC595236 (similar to nestin)
- Canis familiaris (dog):
  - LOC485490 (similar to nestin)
- Homo sapiens (human):
  - NES (nestin)
- Macaque mulatta (macaque monkey):
  - LOC21856 (similar to nestin)
- Pan troglodytes (chimpanzee):
  - NES (nestin)
- Tetrahymena thermophila 59210:
  - THERM_00532885 (hypothetical protein)
- Trypanosoma cruzi strain CL Brener:
  - T009_004705300851_154 (hypothetical protein)

The species shown in red have hits from more than one gene in the RefSeq RNA Database. If you wish to use this primer for one of these species, you should be cautious.

There were no species in the RefSeq database for which this primer looks like it will prime.

- Oryza sativa (sativa cultivar-group) (Japanese rice):

Promega
Plexor™ Technology in Research

- Plexor™ Assay Design
- Plexor™ Reaction
- Plexor™ Data Analysis
Plexor™ Systems

- **qPCR System**
  - Quantitation from genomic DNA, SNP genotyping
  - For 2-step qRT-PCR methods with your cDNA

- **Two-step qRT-PCR System**
  - ImProm-II™ Reverse Transcriptase reagents for cDNA synthesis
  - Plexor™ Master Mix for qPCR from cDNA template

- **One-step qRT-PCR System**
  - Combines ImProm-II™ Reverse Transcriptase with Plexor™ Master Mix for qRT-PCR directly from RNA template
Plexor™ Reactions

- 25µl standard reaction
- 2X Plexor™ Master Mix contains:
  - Enzyme (devoid of 5´ exonuclease activity)
  - High performance buffer
  - Proprietary primer-dimer inhibitor
  - dATP, dCTP, dGTP, dTTP and Dabcyl-iso-dGTP
  - For incorporation opposite the iso-dC in the Plexor™ primers
  - For amplification of the DNA sequence of interest
Plexor™ Technology in Research

Plexor™ Assay Design

Plexor™ Reaction

Plexor™ Data Analysis
Raw Data to Analyzed Data

- ABI Instruments
- Roche Instruments
- Other Instruments

Import Raw Data

Plexor Software

Promega
Plexor™ reagents and Data Analysis Software for:

- ABI PRISM® 7000
- ABI PRISM® 7700
- AB 7300, 7500, 7900HT
- Corbett Rotor-Gene™
- Roche LightCycler® 1 & 2
- Roche LightCycler® 480
- BioRad iCycler™ (2 & 4 color)
- MJ Opticon® Instruments
- Cepheid Instruments
- Stratagene Instruments
- *et al.*
Plexor™ Analysis Software Desktop
Summary

• **Specificity** from novel base-pairing
• **Simple** assay design
• **Easy** multiplex reactions
  – Increased productivity
  – Accurate quantitation
• **Tools and support to employ** Plexor™ technology freely available
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