Guidelines for Developing Robust and Reliable PCR Assays

Leta Steffen, PhD
Applications Scientist
Outline

1) **PCR reaction components**
   - What is in the reaction?
   - How does it affect assay performance?
   - Where should you start?

2) **Analyzing qPCR performance**
   - Things to know
   - Performance criteria
   - Setting up the experiment

3) **qPCR products**

4) **Optimizing a qPCR assay**
PCR Reaction Components
What Is in a PCR Reaction?

**User-Defined Components**
- Primers
- Template
- Fluorescent Detection
- Cycling Conditions

**Master Mix Components**
- Amplification-grade water
- dNTPs
- MgCl₂
- Polymerase
- PCR Buffer
What Is in a PCR Reaction?

**User-Defined Components**
- Primers
- Template
- Fluorescent Detection
- Cycling Conditions

**Master Mix Components**
- Amplification-grade water
- dNTPs
- MgCl$_2$
- Polymerase
- PCR Buffer
Primers: Sequence Effects

Specificity
- Target
- DNA/RNA
- Primer interactions

Cycling conditions
- Annealing temperature
- Match primer Tms

Check your primers
- BLAST them!
- NCBI Primer-BLAST

Enter your primers
Choose your database
Choose your organism

NCBI Primer-BLAST: Finding primers specific to your PCR template (using Primer3 and BLAST).
Primers: Sequence Effects

**Primer Set #1**

- Specificity: ?
- Repeatability: ✓
- Linearity: 0.998 ✓
- Sensitivity: 10pg ✓
- Efficiency: 95% ✓
Primers: Sequence Effects

**Primers**

<table>
<thead>
<tr>
<th>Primer pair 1</th>
<th>Sequence 5'→3'</th>
<th>Length</th>
<th>Tm</th>
<th>GC%</th>
<th>Self complementarity</th>
<th>Self 3' complementarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>GTCCTAATGATCCACCC</td>
<td>20</td>
<td>53.69</td>
<td>45.00</td>
<td>4.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>CTCTGGAAGATGATGATGG</td>
<td>20</td>
<td>57.37</td>
<td>56.00</td>
<td>5.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Products on target templates**

>NC_000012.12 Homo sapiens chromosome 12, GRCh38 p2 Primary Assembly

- **Product length = 100**
- Features associated with this product:
  - glyceraldehyde-3-phosphate dehydrogenase isoform 1
  - glyceraldehyde-3-phosphate dehydrogenase isoform 1

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>GTCCTAATGATCCACCC</th>
<th>Template</th>
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<tbody>
<tr>
<td>6536692</td>
<td>6536711</td>
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<table>
<thead>
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<th>CTCTGGAAGATGATGATGG</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>6536791</td>
<td>6536772</td>
<td></td>
</tr>
</tbody>
</table>

**Product length = 2598**
- Features associated with this product:
  - ankyrin repeat and sterile alpha motif domain-containing...
  - ankyrin repeat and sterile alpha motif domain-containing...

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>GTCCTAATGATCCACCC</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>99017599</td>
<td>99017618</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reverse primer</th>
<th>CTCTGGAAGATGATGATGG</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>99020194</td>
<td>99020175</td>
<td></td>
</tr>
</tbody>
</table>

>NC_000001.11 Homo sapiens chromosome 1, GRCh38 p2 Primary Assembly

- **Product length = 82**
- Features flanking this product:
  - S5599 bp at 5' side: rho GTPase-activating protein 29 isoform X3
  - 115322 bp at 3' side: ATP-binding cassette sub-family D member 3 isoform X1

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>GTCCTAATGATCCACCC</th>
<th>Template</th>
</tr>
</thead>
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<tr>
<td>94302173</td>
<td>94302194</td>
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</table>

<table>
<thead>
<tr>
<th>Reverse primer</th>
<th>CTCTGGAAGATGATGATGG</th>
<th>Template</th>
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</thead>
<tbody>
<tr>
<td>94302236</td>
<td>94302237</td>
<td></td>
</tr>
</tbody>
</table>

**Product length = 101**
- Features associated with this product:
  - RHF/POZ domain-containing protein 8
Primers: Sequence Effects

**Primer Set #2**

- Specificity ✓
- Repeatability ✓
- Linearity 0.996 ✓
- Sensitivity 10pg ✓
- Efficiency 108% ✓
Primers: Concentration Effects

Optimum varies between assays

Common range: 200nM – 1µM

Too low ⇒ Low amplification efficiency

Too high ⇒ Poor specificity, repeatability, or efficiency

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Linearity</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>100nM</td>
<td>0.991 ✓</td>
<td>68% ✗</td>
</tr>
<tr>
<td>500nM</td>
<td>0.988 ✓</td>
<td>102% ✓</td>
</tr>
<tr>
<td>10µM</td>
<td>0.812 ✗</td>
<td>156% ✗</td>
</tr>
</tbody>
</table>
Template

Concentration

*What range of sensitivity & linearity do I need?*

Fragmentation

*What size amplicon can I reproducibly amplify?*

Purity

*Does the sample contain contaminating RNA or DNA?*

*Does the sample contain inhibitors?*
Fluorescence Detection

**dsDNA binding dye**
- Signal/noise $\Rightarrow$ threshold

**Probes**
- Signal/noise $\Rightarrow$ threshold
- Sequence effects

*Analyze each assay separately!*

**Passive reference dye (ROX, CXR)**
- Use as required for your instrument

1X or 250nM
## Cycling Program

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp (°C)</th>
<th>Fast cycling time</th>
<th>Standard time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>95°C</td>
<td>2 min</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95°C</td>
<td>3 sec</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>30 sec</td>
<td>30 sec</td>
<td>40</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td></td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Melt Curve</td>
<td>65-95°C</td>
<td>Varies by instrument</td>
<td>30 sec</td>
<td>1</td>
</tr>
</tbody>
</table>

### Chart

- **55.1°C Efficiency**: 92% ✓
- **60.0°C Efficiency**: 81% ✓
- **66.2°C Efficiency**: 92% ✓
What Is in a PCR Reaction?

**User-Defined Components**
- Primers
- Template
- Fluorescent Detection
- Cycling Conditions

**Master Mix Components**
- Amplification-grade water
- dNTPs (200nM ea.)
- MgCl₂
- Polymerase
- Buffer

What is in a PCR Reaction?
**MgCl₂**

**Multiple functions**

- Divalent cationic cofactor for polymerase
- Sequestered with dNTPs 1:1
- Stabilizes nucleic acid hybridization

Lower efficiency

\[[\text{MgCl}_2]\]

Lower specificity

- [MgCl₂] concentrations: 1.5mM – 3mM

www.idtdna.com
Polymerase

Concentration

Minimum required for efficient amplification
Increase may improve plateau but may not affect exponential phase
May require increased Taq for high level multiplexing
PCR Buffer

Buffers vary in salts, additives, detergents, pH

Specificity ✗
Repeatability ✗
Linearity 0.942 ✗
Sensitivity ✗
Efficiency 127% ✗

Specificity ✓
Repeatability ✓
Linearity 0.992 ✓
Sensitivity ✓
Efficiency 100% ✓
Optimal for Each Component Depends on the Others

There may be many optimums, depending on where you start!
Analyzing PCR Performance
Analyzing qPCR Performance

The case against using Cq values

Thresholds are somewhat arbitrary

Thresholds can be drawn anywhere in the exponential phase
Lower thresholds yield earlier Cq values
A single threshold should NOT be applied across different assays
Use independent analyses and auto-threshold when comparing assays
Compare based on assay performance metrics, not Cq
# Analyzing qPCR Performance

<table>
<thead>
<tr>
<th>Specificity</th>
<th>NTC reactions should show no or late amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dye-based qPCR should show a single, sharp, symmetric melt peak</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repeatability</th>
<th>Standard deviations of technical replicate Cq values should be &lt; 0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assess qualitatively if only two replicates are performed</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Linearity</th>
<th>A linear regression fitted to the standard curve data should have a coefficient of determination ($r^2$) $\geq 0.98$ for qPCR</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>The lowest concentration at which a Cq can be reliably determined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific LOD/LOQ testing can be performed as follow-up if needed</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Efficiency</th>
<th>A linear regression fitted to the qPCR standard curve data should have a slope of -3.59 to -3.10, indicating 90-110% amplification efficiency</th>
</tr>
</thead>
</table>
How Do You Set Up the Experiment?

*Use a standard curve*

Start with a positive control

≥10-fold more concentrated than samples

Typically purified or purchased DNA, representative RNA, or pure target

Serially dilute in nuclease-free water (1/4 – 1/10 dilutions)

Pipetting accuracy is important

Use same day (no freeze/thaw)

Consider plastics

Amplify in triplicate

Include NTC reactions

Can include representative samples
**Data Analysis**

Analyze reactions for each assay separately

- In some software, this is done automatically if assays are labeled uniquely
- In others, you may need to set “well groups” or “omit” other reactions

**Use automatic baseline and threshold settings**

**Perform a quality control check on the data**

- Use linear view to verify baselines
- Use semi-log view to verify threshold

Flat and overlapping baselines

Crosses in exponential phase for all reactions
Assay Performance: Specificity

✓ NTC reactions

No or very late amplification (≥ 3 cycles later)
Or primer artifact only (low Tm peak)

✓ Melt curves (dye-based only)

Single sharp, symmetric peak

✓ Exclude reactions with non-specific amplification
Assay Performance: Repeatability

✓ Standard deviation <0.5 cycles for technical replicate reactions

Exclude points with standard deviation of the C_q ≥ 0.5
Assay Performance: Linearity

✓ Fit a line to the plot of Cq value vs log_{10}[DNA]
✓ $r^2$ should be $\geq 0.980$
✓ Exclude points that make $r^2 < 0.98$. 

![Linearity Graphs]

- Linearity: 0.998 ✓
- Linearity: 0.921 ✗
- Linearity: 0.978 ✗
Assay Performance: Sensitivity

✓ The lowest template concentration for reliable quantification

   Limit of quantification (LOQ) testing

✓ Inferred from lowest concentration included in the standard curve

   Standard deviation of Cq < 0.5
   Linear regression $r^2 \geq 0.980$
   No substantial deflection from the linear regression

Linearity 0.978 ✗
Linearity 0.997 ✓
Assay Performance: Efficiency

✓ Efficiency calculated from the slope of the standard curve

\[ Efficiency = 10^{\left(-\frac{1}{\text{slope}}\right)} - 1 \]

Ideal = 100%

Acceptable = 90-110%

Efficiency 102% ✓

Efficiency 68% ✗
### Tool for Comparing qPCR Reagents

<table>
<thead>
<tr>
<th>Specification</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Was there no or very late amplification in the NTC reaction? If you are using dye-based qPCR for amplification of a single target, did you observe a single peak in the melt analysis?</td>
</tr>
<tr>
<td>Repeatability</td>
<td>Which product has the fewest standard curve points excluded for high standard deviation ($\geq 0.5 C_q$)?</td>
</tr>
<tr>
<td>Linearity</td>
<td>Is the $r^2$ of the linear regression equation $\geq 0.98$?</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Which product has the best assay sensitivity? (The lowest DNA concentration included in the linear range should not negatively impact the $r^2$, and the standard deviation of the $C_q$ with this concentration of DNA should be $\leq 0.5$.</td>
</tr>
<tr>
<td>Efficiency</td>
<td>Is qPCR assay efficiency between 90–110%?</td>
</tr>
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qPCR Reagents
Promega’s qPCR Master Mix Offerings

Master Mix Components
- Water
- dNTPs
- MgCl₂
- Polymerase
- Buffer

Master Mix
- GoTaq® qPCR Master Mix
- GoTaq® Probe qPCR Master Mix

Custom Master Mix from individual components
- PCR Optimization Kit
  - 5X PCR Buffers A-H
  - MgCl₂ Solution, 25mM
  - GoTaq® MDx Hot Start Polymerase
- Made-to-order 2X PCR Master Mix
Optimizing a qPCR Assay
Step 1: Get to Know Your Assay

**User-Defined Components**
- Primers
- Template
- Fluorescent Detection
- Cycling Conditions

**BLAST your primers**
- Are they sequence specific?
- Are they RNA/DNA specific?
- Are they located properly on the template?
- Do they interact (self-prime)?
- What are the predicted Tms?

**Consider your template**
- Do you have a standard?
- What concentration range?
- Is the sample likely to be degraded?
- Are inhibitors a concern?

**Identify your detection method**
- Screening, or specificity information? DYE
- Repeated assay or multiplexing? PROBE
- Do you have the appropriate filter sets?
### Step 2: Determine Your Initial Cycling Program

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<tr>
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<td>3 sec</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C*</td>
<td>30 sec</td>
<td>30 sec</td>
<td>40</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec</td>
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<td>1</td>
</tr>
</tbody>
</table>

#### Fast or standard cycling?
- Fast for ≤ 20µL reactions, small amplicons

#### 2-step or 3-step cycling?
- Consider 2-step if annealing is ≥ 60°C

#### Annealing temp?
- Existing: Start with current annealing temp
- New: Start at Tm - 5°C, usually ~60°C
Step 3: Perform the Experiment and Analyze Data

Specificity
Was there no or very late amplification in the NTC reaction? If you are using dye-based qPCR for amplification of a single target, did you observe a single peak in the melt analysis?

Repeatability
Which product has the fewest standard curve points excluded for high standard deviation (≥0.5C₀)?

Linearity
Is the r² of the linear regression equation ≥0.98?

Sensitivity
Which product has the best assay sensitivity? (The lowest DNA concentration included in the linear range should not negatively impact the r², and the standard deviation of the Cₜ with this concentration of DNA should be ≤0.5.)

Efficiency
Is qPCR assay efficiency between 90–110%?
Step 4: Optimize

Check that primers, probe(s), and passive reference dye are within the indicated concentration ranges

Optimize cycling conditions

Annealing temperature gradient

Non-specific amplification? Increase annealing temperature

Poor linearity or efficiency? Test ± 2-5°C annealing temperature, 3-step cycling

Optimize primer concentrations

Test with low (200nM), medium (500nM), and high (900nM) primer concentrations

Screen different primers to the same target
Optimize Cycling Conditions

*Human GAPDH DNA, dye-based qPCR*

Primer Tms: 61°C, 65°C

- **55.2°C**
  - Specificity: ❌
  - Repeatability: ✓
  - Linearity: 0.984 ✓
  - Sensitivity: ✓
  - Efficiency: 92% ✓

- **60.0°C**
  - Specificity: ✓
  - Repeatability: ✓
  - Linearity: 0.988 ✓
  - Sensitivity: ✓
  - Efficiency: 92% ✓

- **66.2°C**
  - Specificity: ✓
  - Repeatability: ✓
  - Linearity: 0.989 ✓
  - Sensitivity: ✓
  - Efficiency: 92% ✓
Optimize Primer Concentration

Human APP DNA, dye-based qPCR

100nM primers

- Specificity ✅
- Reproducibility ✅
- Linearity 0.991 ✅
- Sensitivity ✅
- Efficiency 68% ☠

500nM primers

- Specificity ✅
- Reproducibility ✅
- Linearity 0.988 ✅
- Sensitivity ✅
- Efficiency 102% ☑
What if Basic Optimization Doesn’t Work?

Or what if you need long term reproducibility?

Customize your master mix

Increased flexibility: Buffer, MgCl₂, Polymerase

Increased reproducibility: cGMP process control and QC

*PCR Optimization Kit*

**STEP 1**
Survey to identify candidate buffer formulations specific to application.

**STEP 2**
Perform assay optimization with candidate buffers.

**FINAL**
Optimized PCR formulation

<table>
<thead>
<tr>
<th>Buffer A</th>
<th>Buffer D</th>
<th>Buffer G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer A</td>
<td>Buffer D</td>
<td>Buffer G</td>
</tr>
</tbody>
</table>

Vary annealing temperature

-6°C -4°C -2°C Calculated \(T_m\) +2°C +4°C +6°C
titrate MgCl₂ (mM) titrate enzyme (units)

Buffer D
2mM MgCl₂
2.5 units Taq
Beyond Basic Optimization
78% GC-rich human RB gene, GoTaq® qPCR Master Mix

Initial gradient testing of annealing/extension temperatures

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq® activation</td>
<td>1</td>
<td>95°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>40</td>
<td>95°C</td>
<td>10 sec</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60</td>
<td>60°C-72°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Dissociation/Melt</td>
<td>1</td>
<td>Instrument default</td>
<td></td>
</tr>
</tbody>
</table>

Ta = 72.0°C

- Specificity: ✓
- Repeatability: ❌
- Linearity: 0.921 ❌
- Sensitivity: 1 ng ❌
- Efficiency: 69% ❌
Step 1: Survey PCR Buffers

Serial dilution of template DNA in water

Choose a single MgCl\(_2\) and Taq concentration

Example: PCR Buffer A

- Specificity: ✓
- Repeatability: ✓
- Linearity: 0.999 ✓
- Sensitivity: 100pg ✗
- Efficiency: 84% ✗

PCR Buffers A, E, G
Step 2: Optimize Annealing Temperature

Example: PCR Buffer A

- Specificity: Ta sensitive
- Repeatability: ✔
- Linearity: > 0.99 ✔
- Sensitivity: 100pg ✗
- Efficiency: <87% ✗

Serial dilution of template DNA in water

PCR Buffers A, E, G

1ng template Various Ta
Step 2: Optimize Annealing Temperature

Example: PCR Buffer G

- **Specificity**: ✓ all temps
- **Repeatability**: ✓ 67.6-71.1°C
- **Linearity**: ≥0.99 ✓ 67.6-71.1°C
- **Sensitivity**: 10pg ✓ 67.6-70.0°C
- **Efficiency**: ≥89% ✓ 67.6-70.0°C

Serial dilution of template DNA in water

PCR Buffers A, E, G

- PCR Buffer G, 68.6°C
Step 3: Optimize MgCl$_2$ and GoTaq® Concentrations

Example: PCR Buffer G

- **Specificity**: All MgCl$_2$ x Taq
- **Repeatability**: All MgCl$_2$ x Taq
- **Linearity**: ≥0.99 All MgCl$_2$ x Taq
- **Sensitivity**: 10pg All MgCl$_2$ x Taq
- **Efficiency**: ≥90% 3-4mM MgCl$_2$ x 3.5U Taq

Made-to-order 2X PCR Master Mix
PCR Buffer G, 3.5U Taq, 3-4mM MgCl$_2$
Real-Time PCR Resources On-line

qPCR Introduction:
Webinar: Introduction to Real-Time PCR: Basic Principles and Chemistries

qPCR Assay Performance:

Primer design software & Information:
NCBI - Primer-BLAST
How to: Design PCR primers and check them for specificity
IDT - PrimerQuest
Decoded: PCR and qPCR
Real-Time PCR Product Resources

Catalog master mixes

qPCR and RT-qPCR

PCR and RT-PCR

Flexible components and custom master mixes

PCR Optimization Kit

Made-to-Order 2X PCR Master Mixes
Technical Services Scientists Ready to Help

Customer & Technical Support

<table>
<thead>
<tr>
<th>Customer Support</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact customer service to check price, availability, place an order, check delivery or check on an order status, or get a copy of your invoice. We are here to help you!</td>
</tr>
</tbody>
</table>

- **Chat with Customer Service**

<table>
<thead>
<tr>
<th>Technical Support</th>
</tr>
</thead>
<tbody>
<tr>
<td>To answer technical questions about our products, request technical seminars and for information about our new products, our team of scientists are here to support you!</td>
</tr>
</tbody>
</table>

- **Chat with a Scientist**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Phone:</strong></td>
<td>(608) 274-4330</td>
</tr>
<tr>
<td><strong>Toll-Free Phone:</strong></td>
<td>(800) 356-9526</td>
</tr>
<tr>
<td><strong>Fax:</strong></td>
<td>(608) 277-2516</td>
</tr>
<tr>
<td><strong>Toll-Free Fax:</strong></td>
<td>(800) 356-1970</td>
</tr>
<tr>
<td><strong>Email Address:</strong></td>
<td><a href="mailto:custserv@promega.com">custserv@promega.com</a></td>
</tr>
<tr>
<td><strong>Hours:</strong></td>
<td>7am - 6pm CST, Monday-Friday</td>
</tr>
</tbody>
</table>

- **Contact Us Form**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tr>
<td><strong>Phone:</strong></td>
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<tr>
<td><strong>Fax:</strong></td>
<td>(608) 277-2516</td>
</tr>
<tr>
<td><strong>Toll-Free Fax:</strong></td>
<td>(800) 356-1970</td>
</tr>
<tr>
<td><strong>Email Address:</strong></td>
<td><a href="mailto:techserv@promega.com">techserv@promega.com</a></td>
</tr>
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- **Technical Request Form**
Questions Welcome