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# RealTimeDesign™

## free web-based software for TaqMan® and Amplifluor® Design

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## Abstract:
In a significant transformation of traditional PCR, real-time PCR reveals the amplicon through an accumulating fluorescent signal. Emerging from this evolution is a diverse assortment of methods to probe DNA targets, all using fluorescent-labeled oligonucleotides. To assist with the technical task of selecting and crafting these primer and probe sequences, we present a software program titled RealTimeDesign™ for use over the internet. This program applies newly-developed algorithms toward designing TaqMan® and Amplifluor® assays, including a partition function to gauge mis-alignments within and between the primers. By submitting a panel of human genes to the software's scrutiny, we demonstrate that RealTimeDesign proposes robust assays without additional user expertise, amplifying across seven orders of magnitude of starting copy number. These results also provide an illuminating side-by-side comparison of the TaqMan® and Amplifluor® probing methodologies.

## Algorithms at Work

Melting temperatures of oligos are calculated according to the unified nearest-neighbor thermodynamics documented by John SantaLucia. The dangling ends of oligos are considered within the calculations, to improve the accuracy of prediction.

SantaLucia J Jr. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. Proc Natl Acad Sci USA. 1998. Feb 17;95(4):1460-5

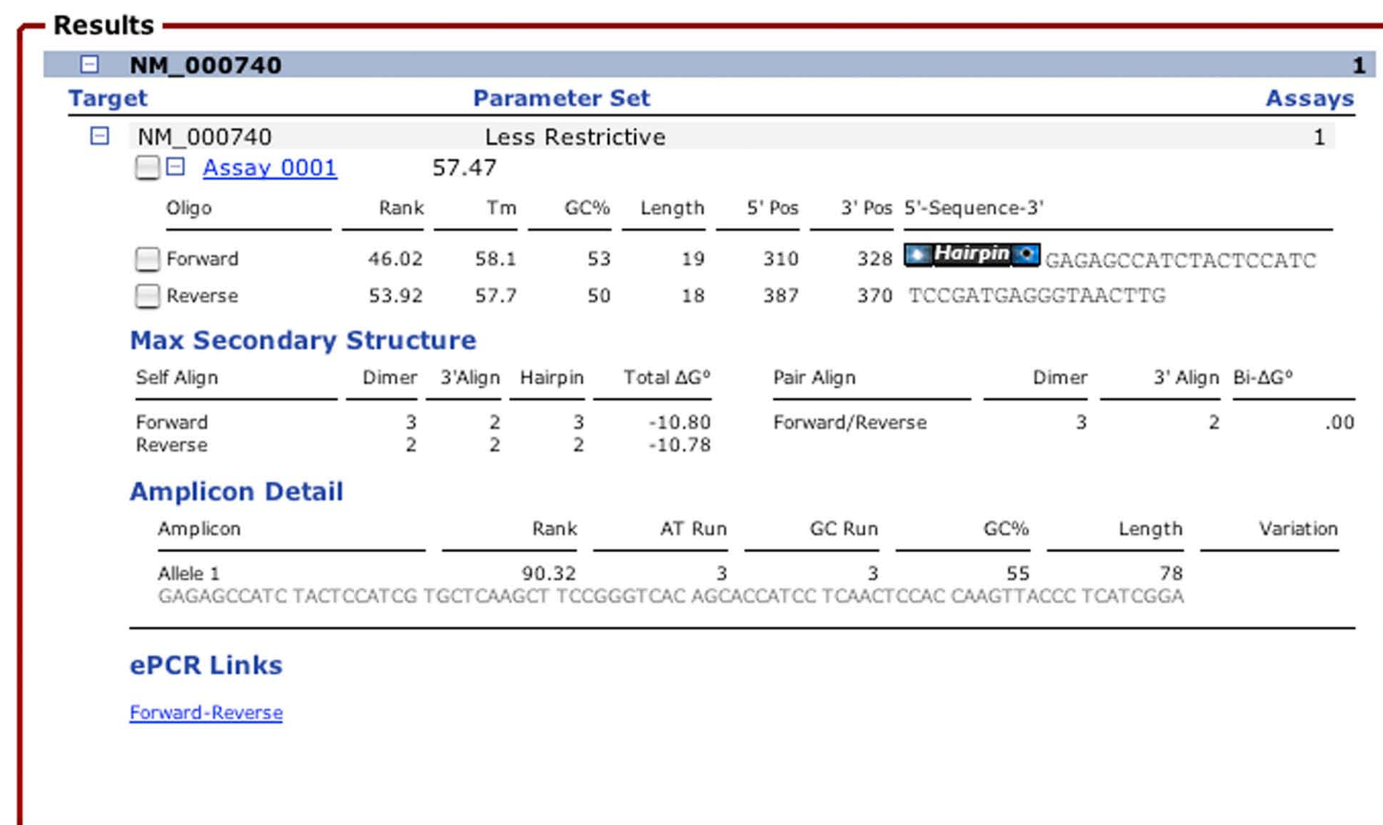



Figure 1: screenshot showing the sequences of an Amplifluor® assay proposed by RealTimeDesign

Numerous parameters govern every aspect of assay design and are user-accessible to overcome difficult targets. Parameters are traversed so that the most computationally-intense are processed last.

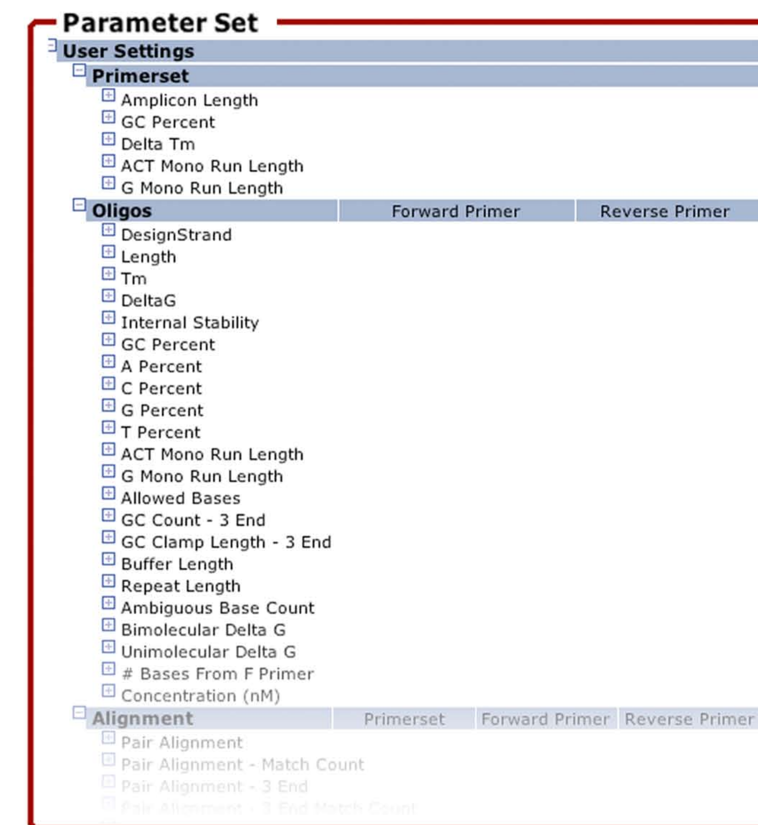



Figure 2: screenshot showing various software parameters that are accessible for adjustment

A Partition function predicts all possible hairpins and dimers within and between the primers. A composite free energy is then accumulated from this ensemble, with each alignment weighted by its probability of formation.

Publications describing partition functions:

Markham NR and Zucker M. DINAMelt web server for nucleic acid melting prediction. Nucleic Acids Res. 2005; 33: W577-81

Mathews DH. Revolutions in RNA Secondary Structure Prediction. J. Mol. Biol. 2006; February 6

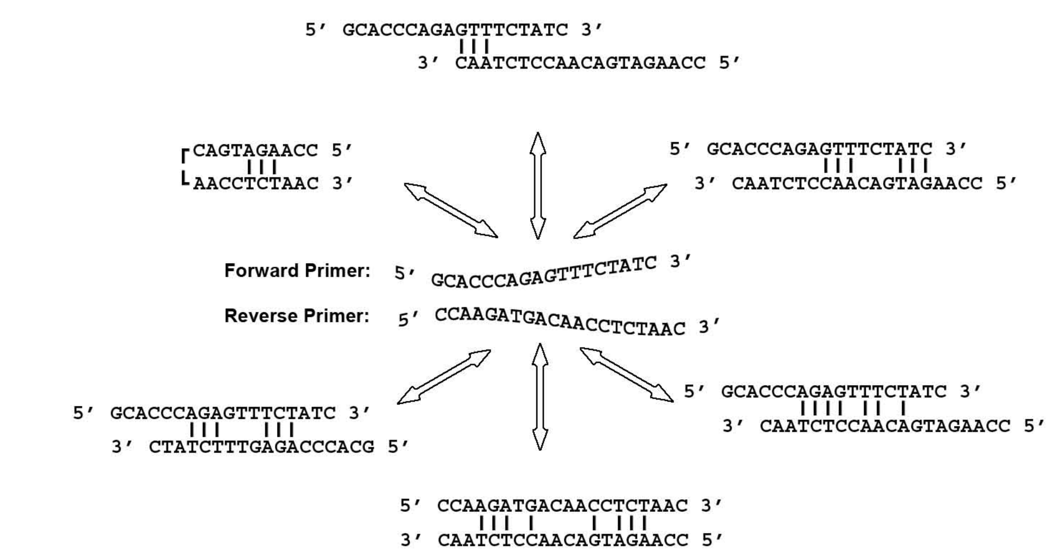
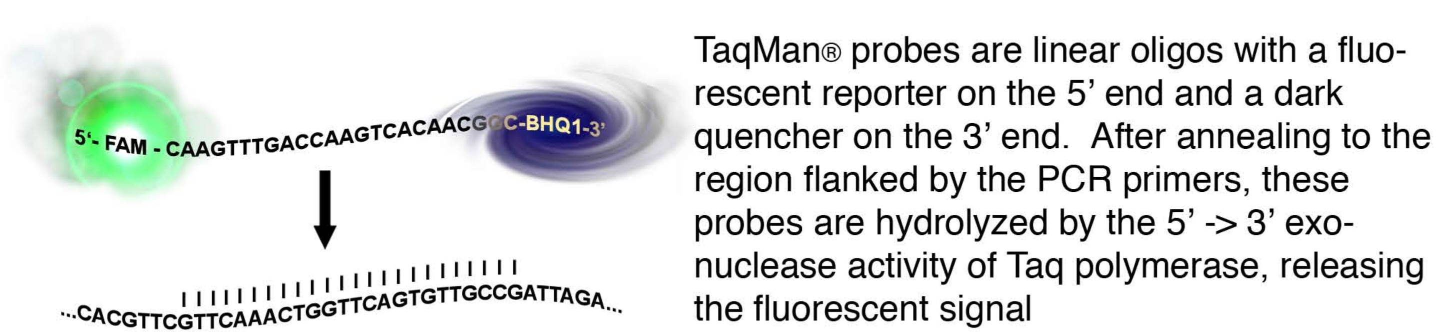



Figure 3: Illustration of several possible hairpins, homodimers, and heterodimers, each in equilibrium with the unhybridized primers.

## Results:
Eight human gene sequences were randomly retrieved from NCBI's databases and submitted to RealTimeDesign. The assay proposed for each target was inspected for specificity using electronic PCR, but no further user insight went into design. All sequences were synthesized and tested for performance by amplifying from purified PCR product and synthetic template. For both the TaqMan® and Amplifluor® modules, the proposed designs demonstrate good performance characteristics, amplifying across seven orders of magnitude starting copy number. Two of the gene targets that typify the group are shown below. A degree of template contamination is apparent in the Amplifluor® negative controls, obscuring the lower limit of detection, but this does not impact the efficiency calculations. For every target tested, the TaqMan® designs demonstrate a higher amplification efficiency than Amplifluor®. However, the Amplifluor® designs demonstrate higher signal/noise values, testifying to the efficient quenching provided by the hairpin sequence.

## Performance of TaqMan® Designs




TaqMan® probes are linear oligos with a fluorescent reporter on the 5' end and a dark quencher on the 3' end. After annealing to the region flanked by the PCR primers, these probes are hydrolyzed by the 5' -> 3' exonuclease activity of Taq polymerase, releasing the fluorescent signal

### E74-like factor 5 (ELF5)

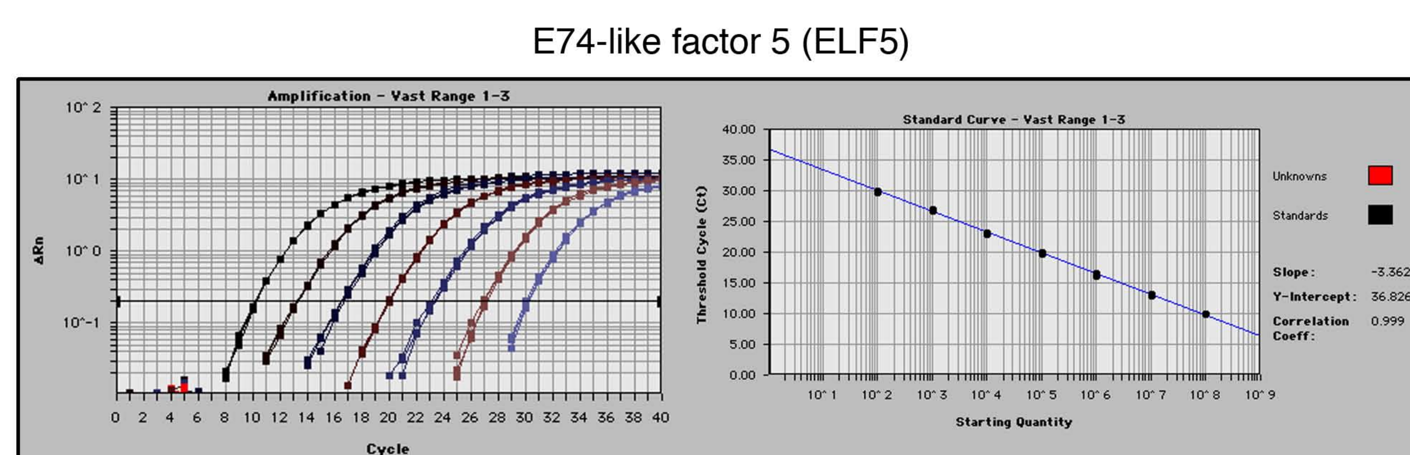



Figure 4: Semi-log amplification plot and its associated standard curve, illustrating the performance of a TaqMan® assay targeting E74-like factor 5. No template controls are shown in red.
Amplification Efficiency: 98.4% Correlation Coefficient: 0.999 Signal/Noise = 5.87
Forward Primer: CACTCCCTGAAAGAGGA
Reverse Primer: CCCAAGCTCCTTCTTCT
Probe: [FAM]-CAAGTTTGACCAAGTCACAACGGC-[BHQ1]

### Glycogen synthase 2 (GYS2)

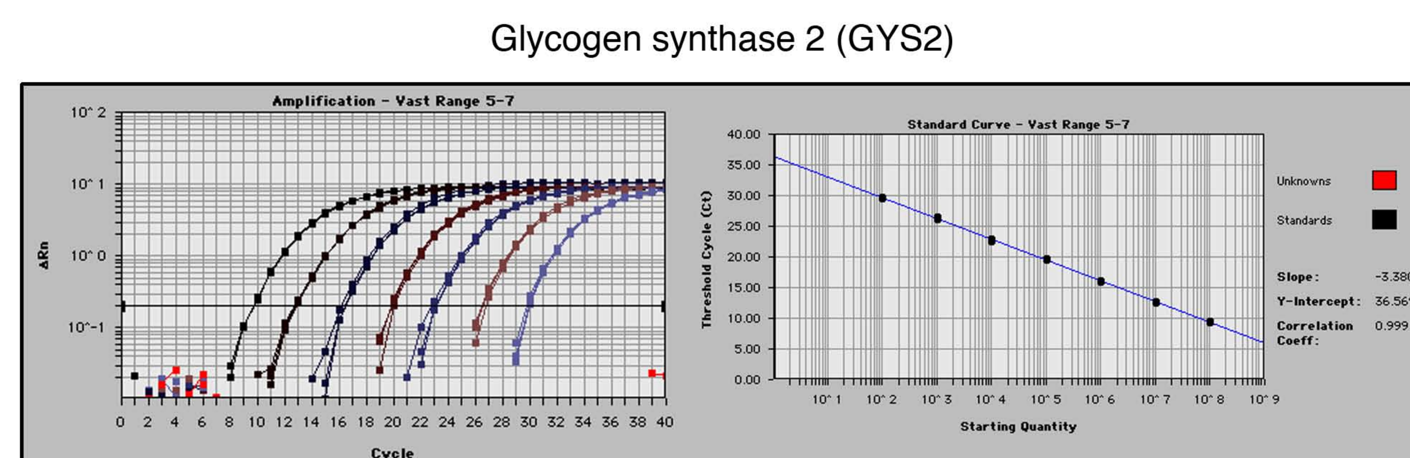
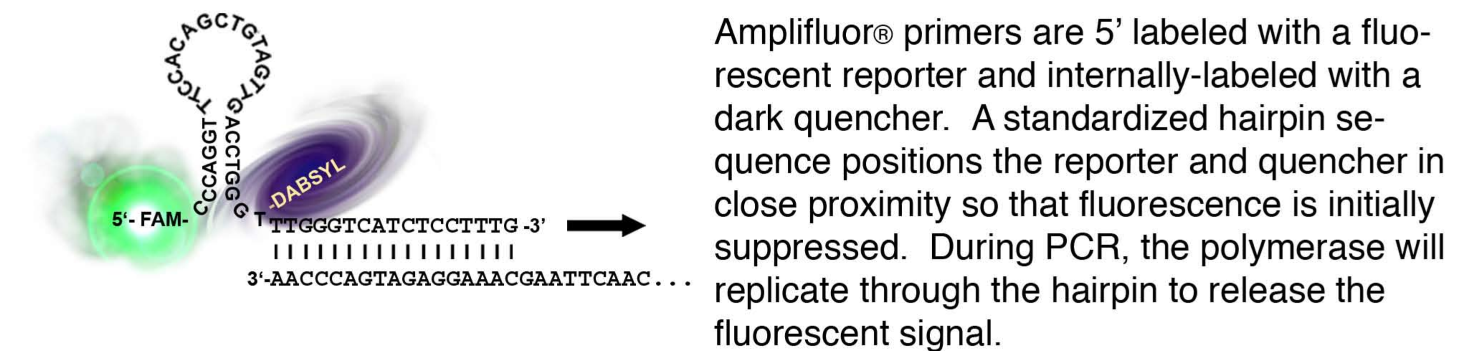



Figure 5: Semi-log amplification plot and its associated standard curve, illustrating the performance of a TaqMan® assay targeting glycogen synthase 2. No template controls are shown in red.
Amplification Efficiency: 97.6% Correlation Coefficient: 0.999 Signal/Noise = 3.21
Forward Primer: GCACCCAGAGTTTCTATC
Reverse Primer: GACAACCTCTAACAAACTCT
Probe: [FAM]-TCCACCAGTCCCTTACTACCCATG-[BHQ1]

## Performance of Amplifluor® Designs




Amplifluor® primers are 5' labeled with a fluorescent reporter and internally-labeled with a dark quencher. A standardized hairpin sequence positions the reporter and quencher in close proximity so that fluorescence is initially suppressed. During PCR, the polymerase will replicate through the hairpin to release the fluorescent signal.

### E74-like factor 5 (ELF5)

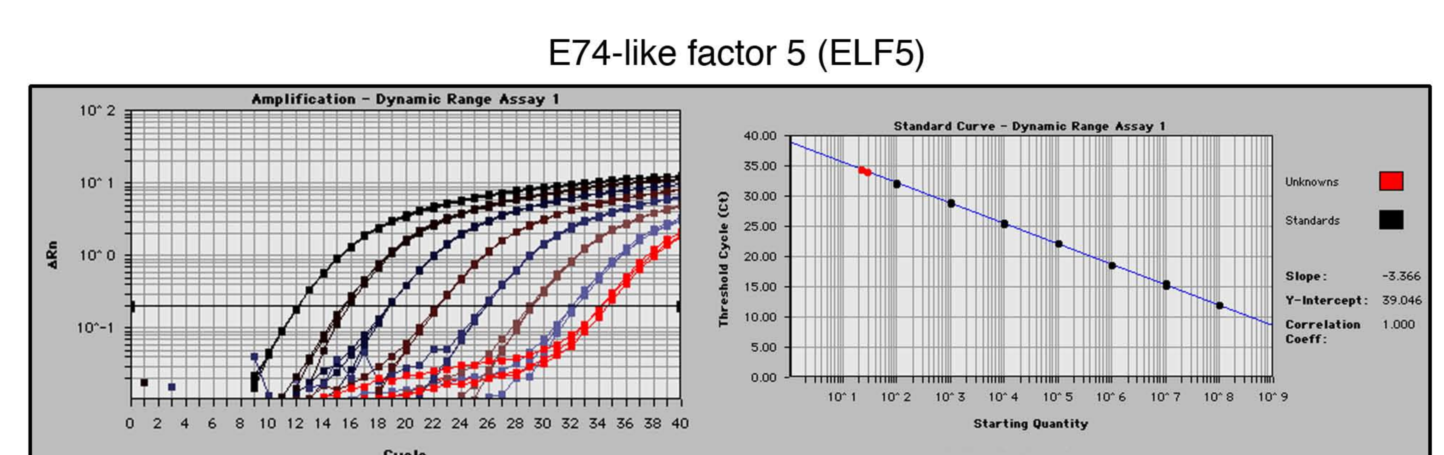



Figure 6: Semi-log amplification plot and its associated standard curve, illustrating the performance of an Amplifluor® assay targeting E74-like factor 5. No template controls are shown in red.
Amplification Efficiency: 98.2% Correlation Coefficient: 1.000 Signal/Noise = 25.05
Forward Primer: [FAM]-AGCGATGCGTTCGAGCATCGC-[T-DABSYL]-CAGAAGATACAGGAGATAGG
Reverse Primer: GCTGTCATATCCTCCTCTTC

### Glycogen synthase 2 (GYS2)

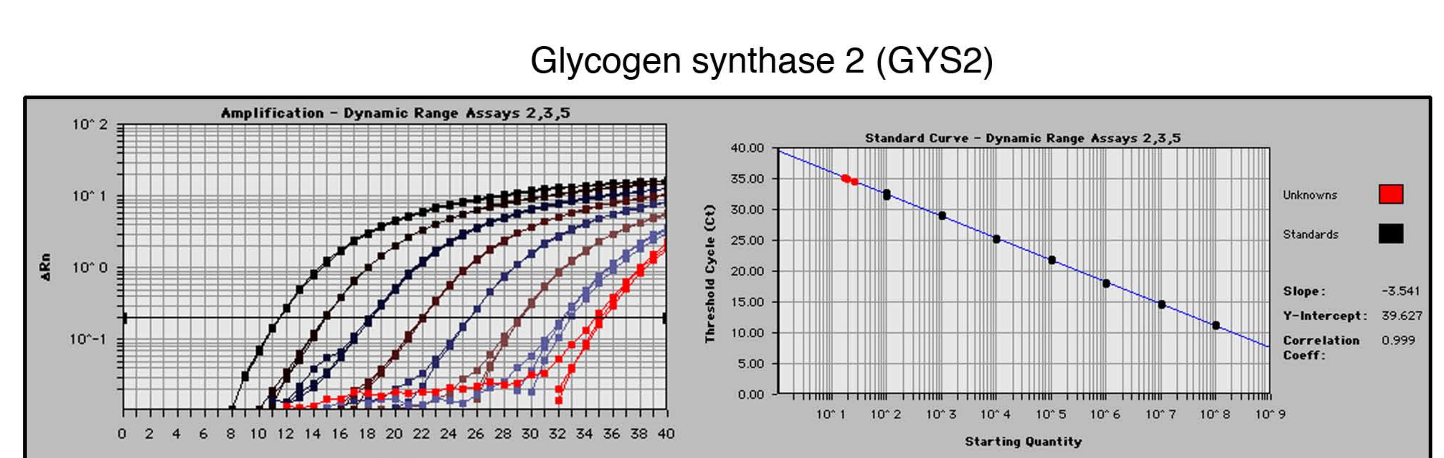



Figure 7: Semi-log amplification plot and its associated standard curve, illustrating the performance of an Amplifluor® assay targeting glycogen synthase 2. No template controls are shown in red.
Amplification Efficiency: 91.6% Correlation Coefficient: 0.999 Signal/Noise = 23.66
Forward Primer: [FAM]-AGCGATGCGTTCGAGCATCGC-[T-DABSYL]-CACCCAGAGTTTCTATCC
Reverse Primer: GACAACCTCTAACAAACTC

## Conclusion:
RealTimeDesign proposes robust TaqMan® and Amplifluor® assays without additional user expertise. Demonstrating a vast dynamic range of detection and high amplification efficiencies, these designs are well-suited for most real-time PCR applications including multiplexed gene expression measurements. This software program incorporates the latest algorithms to model nucleic acid hybridization, including a partition function to gauge the severity of mis-alignments. With the capability to fine-tune many parameters, assays can be targeted to span splice junctions, designed from difficult A/T-rich sequences, or completed around a pre-defined oligo sequence. TaqMan® and Amplifluor® represent two very different probing methodologies, and the software designs have revealed interesting performance differences between the two: TaqMan® assays yield higher amplification efficiencies while Amplifluors® yield higher signal/noise values. Regardless of the choice in probing method, RealTimeDesign should provide significant utility to quantitative PCR investigations.

RealTimeDesign is available for use free-of-charge after registration at: www.qpcrdesign.com

## Methods:
Triplicate Reactions were prepared for all dilution points and NTCs. The standard curves capture a range of 100 - 100,000,000 starting copies of the template. Serial dilutions for the construction of standard curves were prepared in nuclease-free H2O containing 100 ng/μL of yeast tRNA, Roche Molecular systems. PCR Thermal Cycling Conditions: 95˚C for two minutes followed by 40 cycles of: 95˚C for 20 seconds, 60˚C for 60 seconds.

| Reaction Components: | TaqMan® Volumes | Final Concentration | Amplifluor® Volumes | Final Concentration |
|---|---|---|---|---|
| • Nuclease-free H2O | 7.47 μL | N/A | 2.27 μL | N/A |
| • Platinum Taq PCR Buffer (10X) | 2.00 μL | 1X | 2.00 μL | 1X |
| • SuperROX (15 μM) | 0.13 μL | 100 nM | 0.13 μL | 100 nM |
| • Magnesium Chloride (50 mM) | 2.30 μL | 5.75 mM | 2.30 μL | 5.75 mM |
| • dNTPs (2.5 mM each) | 1.60 μL | 200 μM each | 1.60 μL | 200 μM |
| • Platinum Taq Polymerase | 0.10 μL | 0.5 units total | 0.10 μL | 0.5 units total |
| • Forward Primer (10 μM) | 0.60 μL | 300 nM | 0.80 μL | 400 nM |
| • Reverse Primer (10 μM) | 0.60 μL | 300 nM | 0.80 μL | 400 nM |
| • Probe (10 μM) | 0.20 μL | 100 nM | N/A | N/A |
| • Template DNA | 5.00 μL | varies | 10.0 μL | varies |
| Total | 20.0 μL | | 20.0 μL | |

I would like to acknowledge Raymond Peterson, Dean Fiala, and the rest of the team at Celadon Laboratories for their sophisticated expertise in developing this program. Critical insight and suggestions toward the features of this program have been provided by Greg Shipley, Ph.D. and other members of the Nucleic Acids Research Group--Association of Biomolecular Resource Facilities. For this I am grateful. Finally, I would like to acknowledge A-Z of Quantitative PCR by Stephen Bustin, Ph.D. The information contained within was essential during the fine-tuning of parameter values.

"TaqMan" is a registered trademark of Roche Molecular Systems, Inc., Alameda, CA. PCR is a proprietary technology covered by several US patents owned by Roche Molecular Systems, Inc., which have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, Inc. 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc., and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

The use of "ePCR" and "BLAST" involves accessing algorithms hosted by the National Center for Biotechnology Information (NCBI), and public domain information within the web pages of the National Library of Medicine (NLM). More information regarding the use of this work can be obtained at: http://www.ncbi.nlm.nih.gov/About/disclaimer.html

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