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RealTimeDesign™ software

an advanced web-based program for real-time PCR sequence design

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Abstract: In a significant transformation of traditional PCR, real-time PCR reveals the target nucleic acid sequence through an accumulating fluorescent signal. Alongside this evolution, the design rules governing oligonucleotide sequence selection have also been refined with new insights and algorithms. We introduce a web-based software program, engineered to design TaqMan®[1] assays, that applies advanced computation toward the selection of primer and probe sequences. By fine-tuning a collection of parameters, the user can address primer-dimer formation, amplification efficiency, secondary structures, and mis-hybridizations. Interfacing with NCBI's databases facilitates sequence retrieval and specificity searches. Here, we demonstrate that this software program designs robust assays that efficiently amplify their targets from a panel of human genes, confirming its role as a valuable tool for qPCR applications.

Screenshots

Results

Expand All

Sequence — Select All Clear All — Assays

S31103720 — Targets: 1

Target	Status	Parameter Set	Assays
S31103720	Ok	Most Restrictive	1

Assay 0001 — 68.83

Oligo	Rank	Tm	GC%	Length	5' Pos	3' Pos	5'-Sequence-3'
Forward	48.92	59.9	59	17	284	300	GACGAGGAGGACATTGG
Reverse	58.18	59.1	47	17	372	356	TTGGTGGAGTTGAGGAT
Probe	72.22	69.5	58	24	302	325	TCCGAGACGAGAGCCATCTACTCC

Max Secondary Structure

Self Align	Dimer	3'Align	Hairpin	Total ΔG°	Pair Align	Dimer	3' Align	Bi-ΔG°
Forward	2	2	0	-10.85	Forward/Reverse	2	2	.00
Reverse	2	2	0	-10.83	Forward/Probe	4	3	.00
Probe	3	0	3	-11.92	Reverse/Probe	5	3	.00

Amplicon Detail

Amplicon	Rank	AT Run	GC Run	GC%	Length	Variation
Allele 1	75.80	2	3	57	89	

GACGAGGAGG ACATTGGCTC CGAGACGAGA GCCATCTACT CCATCGTGCT CAAGCTTCCG GGTCACAGCA CCATCCTCAA CTCCACCAA

ePCR Links

Forward-Reverse

Target Sequence

```
   1 TGAAAAGCGT ACCAAAGAGC TTGCTGGCCT GCAAGCCTCT GGGACAGAGG CAGAGACAGA
  61 AAACTTTGTC CACCCCACGG GCAGTTCTCG AAGCTGCAGC AGTTACGAAC TTCAACAGCA
 121 AAGCATGAAA CGCTCCAACA GGAGGAAGTA TGGCCGCTGC CACTTCTGGT TCACAACCAA
 181 GAGCTGGAAA CCCAGCTCCG AGCAGATGGA CCAAGACCAC AGCAGCAGTG ACAGTTGGAA
 241 CAACAATGAT GCTGCTGCCT CCCTGGAGAA CTCCGCCTCC TCCGACGAGG AGGACATTGG
 301 CTCCGAGACG AGAGCCATCT ACTCCATCGT GCTCAAGCTT CCGGGTCACA GCACCATCCT
 361 CAACTCCACC AAGTTACCCT CATCGGACAA CCTGCAGGTG CCTGAGGAGG AGCTGGGGAT
 421 GGTGGACTTG GAGAGGAAAG CCGACAAGCT GCAGGCCCAG AAGAGCGTGG ACGATGGAGG
 481 CAGTTTTCCA AAAAGCTTCT CCAAGCTTCC CATCCAGCTA GAGTCAGCCG TGGACACAGC
 541 TAAGACTTCT GACGTCAACT CCTCAGTGGG TAAGAGCACG GCCACTCTAC CTCTGTCCTT
 601 CAAGGAAGCC ACTCTGGCCA AGAGGTTTGC TCTGAAGACC AGAAGTCAGA TCACTAAGCG
 661 GAAAAGGATG TCCCTGGTCA AGGAGAAGAA AGCGGCCCAG ACCCTCAGTG CGATCTTGCT
 721 TGCCTTCATC ATCACTTGGA CCCCATACAA CATCATGGTT CTGGTGAACA CCTTTTGTGA
 781 CAGCTGCATA CCCAAAACCT TTTGGAATCT GGGCTACTGG CTGTGCTACA TCAACAGCAC
 841 CGTGAACCCC GTGTGCTATG CTCTGTGCAA CAAAACATTC AGAACCACTT TCAAGATGCT
 901 GCTGCTGTGC CAGTGTGACA AAAAAAAGAG GCGCAAGCAG CAGTACCAGC AGAGACAGTC
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Features

Splice Sites — Display

Design	Name	Position
☐	SJ1	93
☑	SJ2	298
☐	SJ3	679

Tandem Repeats — Display

Summary of Features

Available for free over the internet, RealTimeDesign can be run entirely through a web browser.

Interfacing with NCBI's databases enables sequence retrieval using accession numbers, as well as BLAST searches for SNP identification.[2]

The specificity of proposed assays can be confirmed by clicking the electronic PCR link, hosted by NCBI. "ePCR" identifies the desired target as well as any mis-hybridizations that could lead to false amplification.

Using Express Mode, the software will automate all steps of TaqMan® design, presenting the highest-ranked assay to the user for inspection.

Custom Mode allows the user to view the sequential nature of the program, offering input at every step of the process.

Custom Mode offers enhanced control over design:

- the selection of alternative highly-ranked assays
- the ability to target a splice site
- the choice of diverse fluors and quenchers
- the ability to designate or "anchor" an oligo's sequence
- the adjustment of parameter values to overcome difficult targets

A few of these parameters include:

- the distance between the probe and the upstream primer
- the magnitude of mis-alignments between the oligos
- the concentrations of the primers and the probe
- the G/C content within terminal 3' bases
- the stability of annealing across an oligo's length

Assays can be designed against 1-10 different targets simultaneously, the results of which are archived for inspection at a later time

Performance of TaqMan® Designs

Methods:

Eight human gene sequences were randomly retrieved from NCBI's databases and submitted to RealTimeDesign. The default assay proposed for each target using Express Mode was inspected for specificity using electronic PCR, but no further user insight went into assay design. Each assay's sequences were synthesized and tested for performance by amplifying from human genomic DNA. To gauge the lower limits of detection, a 1:4 dilution series was prepared for each target, encompassing 16,384 copies down to a single copy. To accurately determine the amplification efficiencies, the resulting PCR products were retrieved and purified using Qiagen MinElute™ columns. These were then provided as templates for amplification across a seven-log range of copy number, using a 1:10 dilution series.

PCR Reaction Components:	Volume	Final Concentration
• Nuclease-free H2O	7.48 µL	N/A
• Platinum Taq PCR Buffer (10X)	2.00 µL	1X
• SuperROX (15 µM)	0.13 µL	100 nM
• Magnesium Chloride (50 mM)	2.29 µL	5.75 mM
• dNTPs (2.5 mM each)	1.60 µL	200 µM each
• Platinum Taq Polymerase	0.10 µL	0.5 units total
• Forward Primer (10 µM)	0.60 µL	300 nM
• Reverse Primer (10 µM)	0.60 µL	300 nM
• Probe (10 µM)	0.20 µL	100 nM
• Template DNA	5.00 µL	varies
Total	**20.0 µL**	

Triplicate Reactions were prepared for all dilution points and NTCs.

PCR Thermal Cycling Conditions:
95°C for two minutes followed by 40 cycles of: 95°C for 20 seconds, 60°C for 60 seconds

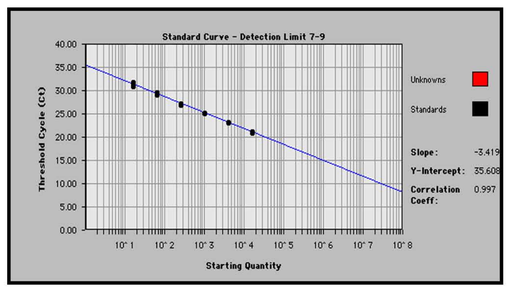
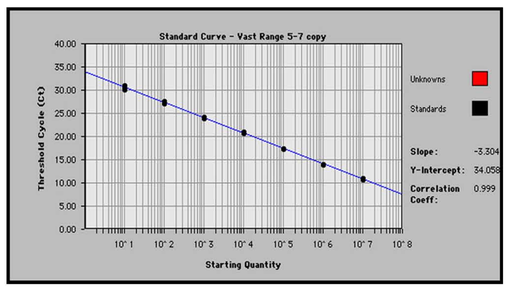
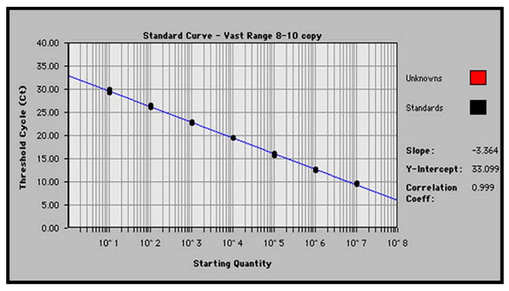
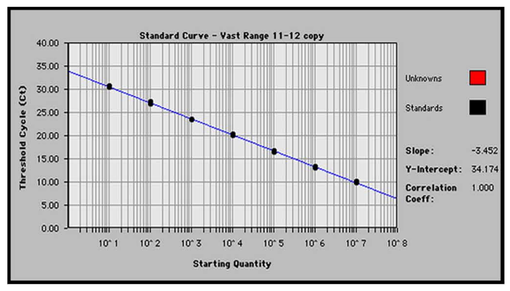
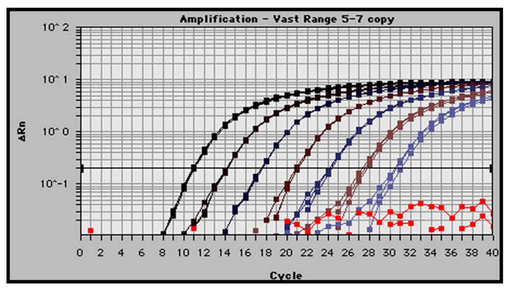
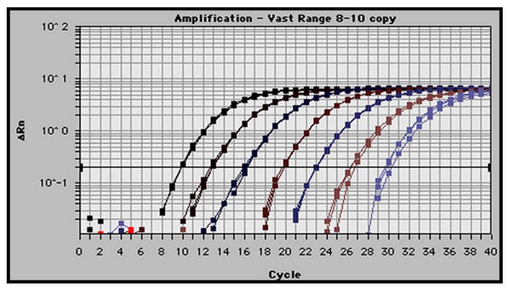
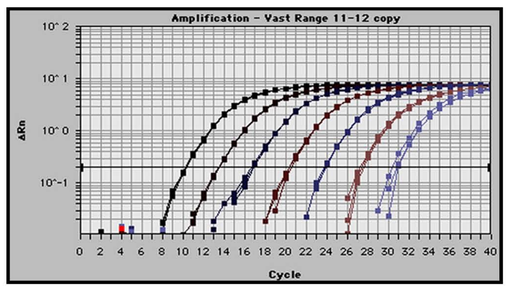
Qiagen Minelute™ PCR Purification Kit was used to purify the PCR products amplified from genomic DNA.

Serial Dilutions for the construction of standard curves were prepared in nuclease-free H2O containing 100 ng/µL of yeast tRNA, Roche Molecular systems,

Lower Limit of Detection is defined by the criteria when adding an additional dilution point drops the correlation coefficient of the standard curve below 0.997

Results:

Upon submitting the panel of gene sequences, RealTimeDesign proposes assays that are equally distributed across the available parameter sets. Each of these eight assays demonstrates robust performance on the ABI 7700 Sequence Detection System. Three of the assays that typify the group are presented below. When amplified from genomic DNA, nearly all have a limit of detection down to 64 copies. In fact, many successfully amplify the single-copy dilution (not shown), but the statistical effect of the Poisson distribution scatters the correlation of these points upon the standard curve. When amplifying from purified PCR product diluted across a 7-log range, these assays demonstrate an average amplification efficiency of 99% and correlation coefficients of 0.999.

	Cholinergic Receptor, Muscarinic 3 (CHRM3)	SMAD, mothers against DPP homolog 4 (SMAD4)	ATPase, V1 subunit C, isoform1 (ATP6V1C1)
Genomic DNA Standard Curve			
Purified PCR Product Standard Curve			
Semi-Log Plot of Purified PCR Product			

Parameter Set: Most Restrictive
Forward Primer: TTGGGTCATCTCCTTTG
Reverse Primer: GCACAGTTCTCTTTCCA
Probe: [FAM]-TCCTTTGGGCTCCTGCCATCT-[BHQ1]
Lower Limit of Detection: 64 Copies
Amplification Efficiency: 100.8%

Parameter Set: Less Restrictive
Forward Primer: GAGCATCAAAGAAACACC
Reverse Primer: GCAATCGGCATGGTA
Probe: [FAM]-TGAAATTCACTTACACCGGGCCCT-[BHQ1]
Lower Limit of Detection: 16 Copies
Amplification Efficiency: 98.3%

Parameter Set: Least Restrictive
Forward Primer: CTGTTTGAGAGTTAAATGATGTC
Reverse Primer: ACACCTCAGCATATAACAA
Probe: [FAM]-TCACTTGCTATGCTGCTTCCAGGAT-[BHQ1]
Lower Limit of Detection: 64 Copies
Amplification Efficiency: 94.8%

Conclusion:

RealTimeDesign proposes robust TaqMan® assays without additional user expertise. Demonstrating a vast dynamic range of detection and amplification efficiencies that average 99%, these designs are well-suited for most real-time PCR applications including multiplexed gene expression measurements. 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. This parameter versatility also provides RealTimeDesign users the potential to design probing methodologies beyond TaqMan®; a software module enabling the design of Amplifluor® Direct[3] primers will be available in the near future. Reflecting on its current performance, RealTimeDesign should provide significant utility to quantitative PCR investigations.

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1 "TaqMan" is a registered trademark of Roche Molecular Systems, Inc., Alameda, CA. PCR is a proprietary technology covered by several US patents including US Patent Nos. 4,683,195, 4,683,202 and 4,965,188, and by issued and pending counterparts outside the U.S. These patents are owned by Roche Molecular Systems, Inc., and 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

2 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

3 Amplifluor Direct is a product of Chemicon® International, a Serologicals® Company. www.chemicon.com