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® assays, that applies a proprietary algorithm 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 structure, 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.

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.
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 fluorophores 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 GC 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 databases and submitted to RealTimeDesign. The default set of parameters proposed for each target using Express Mode was inspected for specificity using electronic PCR, but no further user input went into assay design. Each assay’s sequence was retrieved 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 purified and analyzed using Melt Curve Analysis columns. The concentration of PCR products was determined using a broad range of copy numbers, using a 1:10 dilution series.

PCR Thermal Cycling Conditions: 95°C for two minutes followed by 40 cycles of 95°C for 20 seconds, 60°C for 30 seconds, 72°C for 1 minute

QX200™ 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 HO-DE detergent containing 100 µg/ml of yeast RNA. Real-time PCR was performed.

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.

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 toward splice junctions, designed from difficult AT-rich sequences, or composed 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 AmpliFam Direct® primers will be available in the near future. Reflecting on its current performance, RealTimeDesign should provide significant utility to quantitative PCR investigations.

Acknowledgements: I would like to acknowledge Raymond Peterson, Dean Fiala, and the rest of the Celldion team for their ongoing support of this program, and especially their patience accommodating our many requests.

Greg Shipley, Ph.D., University of Texas Health Science Center, has provided critical insight and suggestions regarding the features of this program, and toward refining its ease-of-use. 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.