A Rapid Bioinformatic Engine for Multiplexed qPCR Design

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REALTIMEDESIGN™

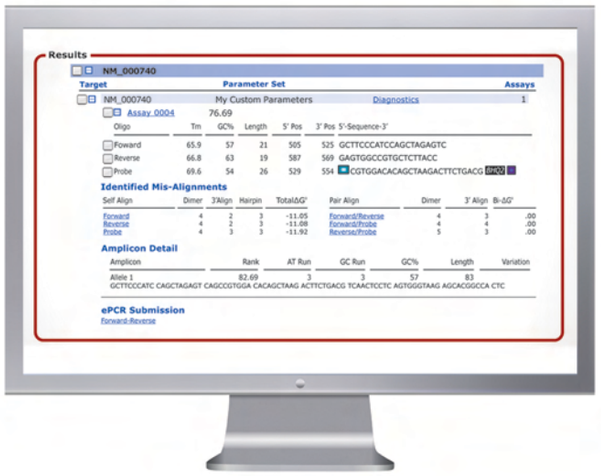


Figure 1: screenshot from RealTimeDesign™, showing oligo sequences and their properties. This software is available for free public use on the web at:

www.qPCRdesign.com

RealTimeDesign is a high-throughput web application for oligo sequence design. A new multiplexing module carefully considers interactions between the assays while combining them into sets. An instrument drop-down menu suggests the best reporter combination according to the degree of multiplexing. To validate the software module, we developed two pentaplexed assays targeting a panel of mouse genes. Both assays yield similar performance here we present the results from one.

Methods

Triplicate reactions were prepared for all dilution points. Mouse genomic DNA was amplified at a quantity of 3.75 ng per reaction to confirm singleplexing/multiplexing equivalence. PCR products from these reactions were then diluted in series to provide pentaplexed assays a disproportionate copy number. Serial dilutions were prepared in nuclease-free water containing 100 ng/µL of yeast tRNA. Real-Time PCR is performed on the Rotor-Gene 6000 using the following thermal cycling conditions: 95 °C for ten min. followed by 40 cycles of 95 °C for 20 s, 60 °C for 60 s.

Reaction Components	Volume	Final Conc.
• Sample Template	3.00 µL	N/A
• 2X Immomix (Bioline)	10.0 µL	1X
• Each Forward Primer (10 µM)	5x 0.60 µL	300 nM
• Each Reverse Primer (10 µM)	5x 0.60 µL	300 nM
• Each Probe (10 µM)	5x 0.20 µL	300 nM
Total Volume	20.0 µL	

Gene Target:	Oligo Sequences
Isg20	Forward: GGCACTGACATCCTTCATCTTCT
	Reverse: AGGCAGCTCGGAGGTAGAAAG
[FAM]-TGTCGGAATGGTCAGGATTGCTGATC-[BHQ1]	
Ppa2	Forward: GCCCAGTATGCTTGGGTATC
	Reverse: TGCTGACTCCCAGAACAGA
[CAL Fluor Red 610]-TGCAGGTGCTACAACGGCCAG-[BHQ2]	
Ube2o	Forward: TCTGCACTGCCACAGATAAGG
	Reverse: GAAGCCAGCTGTCACTAGACA
[Quasar 705]-TGGGCAGGCAGAATAGCCAGATTA-[BHQ2]	

Gene Target:	Oligo Sequences
F13a1	Forward: CCGGACCTCATTTCCCATGA
	Reverse: GTGGGCACCAGAGACTACAAG
[CAL Fluor Orange 560]-TGTCAGGACCCTCCTCCACAAA-[BHQ2]	
Camta1	Forward: TGTGCAGCTCTGAAGTCATTTC
	Reverse: CCCAGGAAAGTGTACGGAAAGAG
[Quasar 670]-AGGCAGGCAGACACTTCTTCCA-[BHQ2]	

We would like to acknowledge Dean Fiala, Raymond Peterson, and the rest of Celadon Laboratories, for developing the code that powers RealTimeDesign.

Multiplexed qPCR remains a challenging endeavor for reasons that include: 1) designing assays to combine without interference, 2) resolving fluorophores using the optics of each real-time instrument, and 3) optimizing and validating each assay's performance. Here, we address each of these issues when developing several pentaplexed assays that target genes from the mouse. Each assay was designed using a free, online, software program that carefully considers inter-oligo interactions while simultaneously buildd ing its multiplexed set. Situations of disproportionate copy number present a particular challenge upon multiplexed performance; additional effort is needed to validate a multiplexed set, as compared to individually amplified assays.

Fluorophores and Instrument Optics

Multiplexed qPCR amplifies several targets simull taneously but detected independently using diss tinct reporters. We select well resolved fluorophores with minimal spectral overlap

Emission Spectra of Fluorophores for Multiplexing

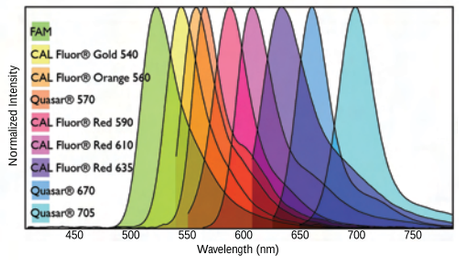


Figure 2: the normalized emission spectra for a series of fluorophores provide a reference to choose potential candidates for multiplexing.

Optical specifications are different for each therr mal-cycler. When choosing fluors, we consider the excitation source, whether it is LASER, lamp, or LED, as well as the filters for detection.

Emission Filters Overlaid onto Reporter Spectra

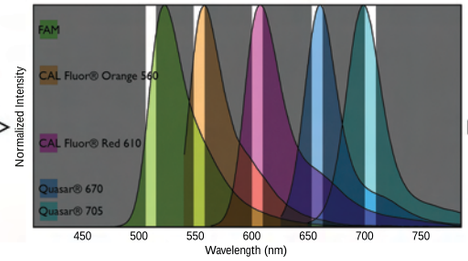


Figure 3: optimal reporters for a pentaplexed assay on the Rotor-Gene Q are identified by comparing spectra to the instrument's filter specifications.

Crosstalk is fluorescent bleed-through between adjacent channels. If unanticipated, crosstalk can produce false positive amplifications and impair quantification.

Crosstalk Amplifications

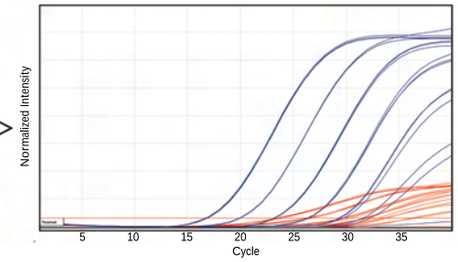
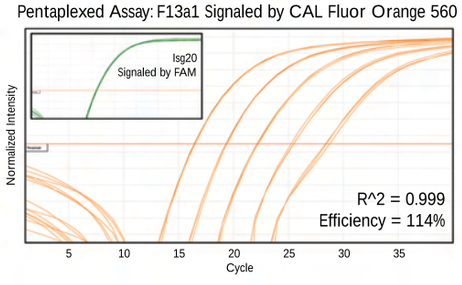


Figure 4: signal bleeds through from CAL Fluor Red 610 (red traces) into the channel detecting Quasar 670 (blue traces). Crosstalk is subsequently removed using software settings.

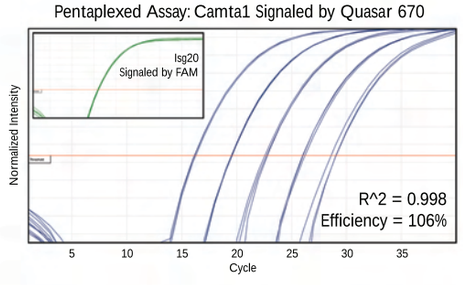
Validating Amplification Performance

Beyond the bioinformatics, further effort is needed to validate a multiplexed assay. In certain applications such as gene expression analysis, one or more targets may be in vast excess over others. We determine assay efficiency and sensitivity when amplifying from genes present at disproportionate quantities.

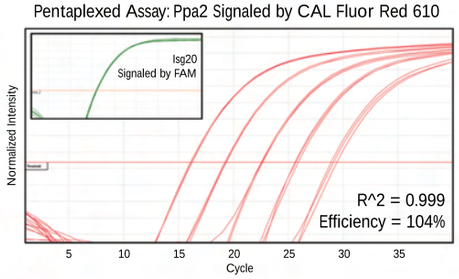
Pentaplexed Assay: F13a1 Signaled by CAL Fluor Orange 560



Pentaplexed Assay: Camta1 Signaled by Quasar 670



Pentaplexed Assay: Ppa2 Signaled by CAL Fluor Red 610



Pentaplexed Assay: Ube2o Signaled by Quasar 705

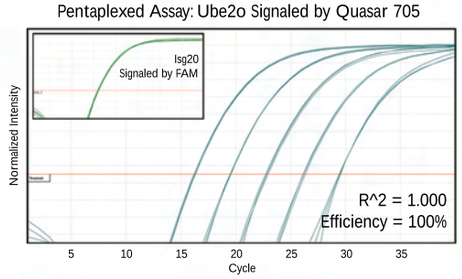


Figure 5: four assays signaled by different reporters are amplified across a dilution series of their gene targets. The fifth assay signaled by FAM is amplified from a fixed, high, copy number in all multiplexed reactions (inset).

To confirm that performance remains uncompromised upon combining the assays, the C_T values for individual reactions should overlay those multiplexed.

Isg20 Signaled by FAM

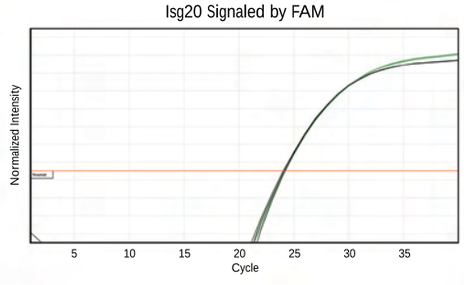


Figure 6: multiplexed reactions (black) overlay those amplified individually (green) at a given target quantity.

Conclusion

RealTimeDesign now enables multiplexed qPCR with rapid oligo design. We have shown that these oligos may be combined into pentaplexed assays with excellent performance characteristics.