A Rapid Bioinformatic Engine for Multiplexed qPCR Design

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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 multiplexed assays that target genes from the mouse. Each assay was designed using a free, online software program that carefully considers oligo interactions while simultaneously building 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 simultaneously but detected independently using distinct reporters. We select well resolved fluorophores with minimal spectral overlap. Emission Spectra of fluorophores for Multiplexing

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

Crossstalk is fluorescent bleed-through between adjacent channels. If unmitigated, crossstalk can produce false positive amplifications and impair quantification.

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.

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

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.

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