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INTRICACIES OF PCR MULTIPLEXING
AS REVEALED THROUGH A PATHOGEN DETECTION ASSAY

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ABSTRACT: The intensifying demand to detect pathogens in food products, agriculture, and the environment can be met with speed and confidence by multiplexing PCR assays. Spectrally-distinct fluorophores and quenchers provide the ability to detect multiple genetic signatures and distinguish closely related strains, all within the same reaction chamber. We present amplifications from a multiplexed TaqMan® assay engineered to detect the virulence factors of *Bacillus anthracis* (BA) and identify several common laboratory strains, distinguished from a closely related species. Multiplexing places increased demands on the performance of each amplification, and we outline our stepwise efforts to successfully quadruplex: sequence design, master mix formulation, and reaction optimization. With pathogen detection as one possible application, we show that multiplex qPCR is best-suited toward assays that will be run regularly, or when sample material is scarce.

SEQUENCE DESIGN

RealTimeDesign is a free software program hosted by Biosearch Technologies (www.qpcrdesign.com) and was used to rapidly design all TaqMan® assays. This program applies advanced oligonucleotide modeling to automate primer and probe selection. Several parameters that influence design include the melting temperatures of the primers and probe, the distance between the probe and the upstream primer, the magnitude of misalignments between the oligos, and the stability of annealing across an oligo's length.

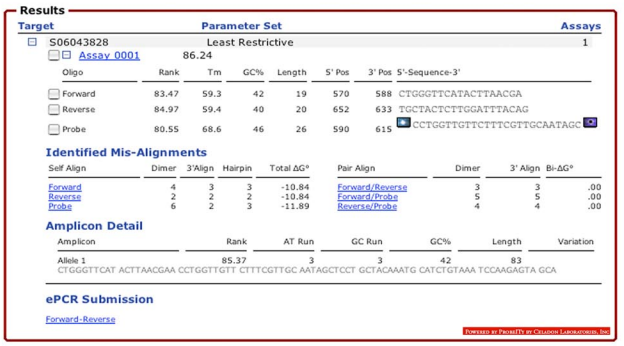


FIGURE 1. Screenshot taken from RealTimeDesign, showing the proposed sequences for the assay targeting the gene coding the capsule biosynthesis protein A, on the pXO2 plasmid.

To develop TaqMan® assays for the detection of *Bacillus anthracis*, we targeted our designs to each of the pXO1 and pXO2 plasmids, as well as the genome. The virulence factors housed within the two plasmids provide a specific genetic signature to identify the organism, but one or both of these plasmids can be absent from certain laboratory strains and in natural Anthrax isolates. For this reason, we designed a third assay to a chromosomal region unique to Anthrax, and a fourth to a region common to organisms within the Cereus-group. TaqMan® probes were synthesized with spectrally-distinct fluorophores and paired with Black Hole Quenchers® (BHQ). These oligos should distinguish laboratory strains of Anthrax from nearest neighbor organisms, all within a single four-color qPCR reaction.

pXO2-SPECIFIC: targets the capsule biosynthesis protein A on the pXO2 plasmid (NCBI accession #: NC_002146)
Forward Primer: 5'-CTGGGTTCATACTTAACGA-3' Reverse Primer: 5'-TGCTACTCTTGGATTTACAG-3'
TaqMan® Probe: 5'-[Quasar® 670]-CCTGGTTGTTCTTTCGTTGCAATAGC-[BHQ2]-3' Detects only BA Ames

pXO1-SPECIFIC: targets the lethal factor precursor (NCBI accession #: M29081) on the pXO1 plasmid
Forward Primer: 5'-GAGGTACAAGAAGTATTTGC-3' Reverse Primer: 5'-GCTTCCGGTGCATAAAG-3'
TaqMan® Probe: 5'-[CAL Fluor® Red 610]-TGCATATTATATCGAGCCACAGCATCG-[BHQ2]-3' Detects BA Ames and Sterne

CHROMOSOMAL, BA-SPECIFIC: targets the putative bacitracin synthetase gene (TIGR locus #: BA_2725)
Forward Primer: 5'-GTCGTATTGCCTAATCTAGTATC-3' Reverse Primer: 5'-GTGAACAGACCGAACATA-3'
TaqMan® Probe: 5'-[CAL Fluor® Orange 560]-TCAGACCTCCCGAAGTAGAAGGA-[BHQ1]-3' Detects BA Ames and Sterne

CHROMOSOMAL, PAN-CEREUS: targets a conserved region within the hemolysin III gene (TIGR locus #: BA_2241)
Forward Primer: 5'-GGATGGCTCATAATCGTTG-3' Reverse Primer: 5'-TTCCACCTGCTAAAAGTAG-3'
TaqMan® Probe: 5'-[FAM]-TTAAACCACTTTATGAAAATC-[BHQplus]-3' Detects all Cereus-group organisms

AMPLIFICATION PERFORMANCE

Before multiplexing, we confirmed that each assay detects the appropriate organisms by amplifying them independently on the iQ®5 real-time thermal cycler, kindly provided by Bio-Rad Laboratories. Templates for amplification include purified nucleic acid extracted from BA Ames (contains both plasmids), BA Sterne (only pXO1), and the closely related species, *Bacillus cereus* (BC) strain ATCC 10987.

Independent Amplifications of Each Assay Upon the Template DNA

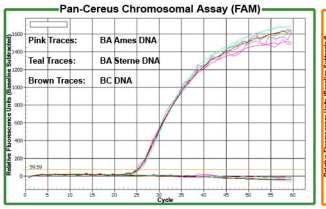


FIGURE 2. Independent amplifications of the FAM assay. This pan-Cereus assay appropriately detects all organisms.

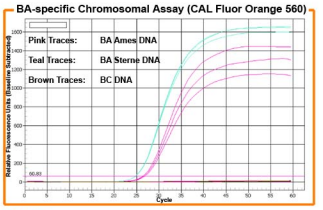


FIGURE 3. Independent amplifications of the CAL Fluor Orange 560 assay. This BA-specific assay only detects BA Ames and Sterne.

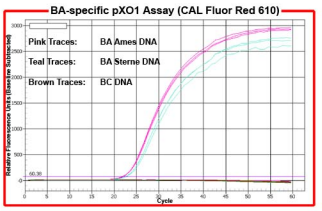


FIGURE 4. Independent amplifications of the CAL Fluor Red 610 assay. This pXO1 assay only detects BA Ames and Sterne.

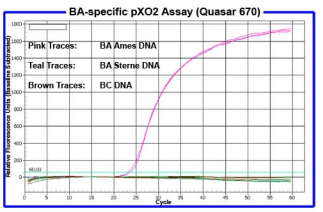


FIGURE 5. Independent amplifications of the Quasar 670 assay. This pXO2 assay only detects BA Ames.

When multiplexing, it is important the individual assays share similar amplification efficiencies so that one target does not out-compete the others for reaction components and cause a discrepancy in C_T values. Each assay's efficiency and lower limit of detection was tested by constructing a 6-order dilution series of BA Ames DNA: 1.00 ng, 100 pg, 10.0 pg, 1.00 pg, 100 fg, and 10.0 fg. DNA in these dilutions was amplified by our quadraplexed assay to generate the traces below.

Quadruplexed Amplifications from a Dilution Series of Template DNA

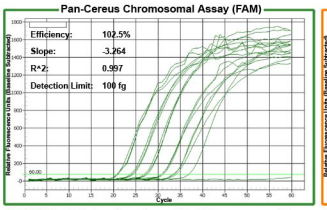


FIGURE 6. Multiplexed reactions of the FAM assay, amplifying from a ten-fold dilution series of BA Ames DNA

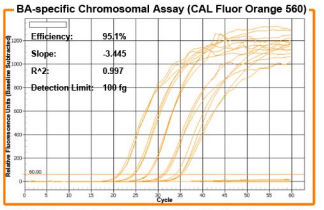


FIGURE 7. Multiplexed reactions of the CAL Fluor Orange 560 assay, amplifying from a ten-fold dilution series of BA Ames DNA

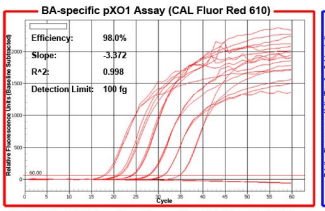


FIGURE 8. Multiplexed reactions of the CAL Fluor Red 610 assay, amplifying from a ten-fold dilution series of BA Ames DNA

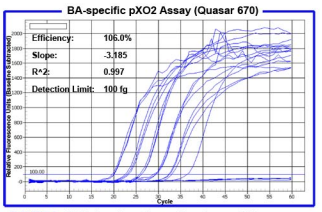
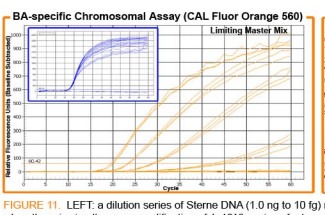


FIGURE 9. Multiplexed reactions of the Quasar 670 assay, amplifying from a ten-fold dilution series of BA Ames DNA

MULTIPLEXING CONSIDERATIONS

In certain applications such as gene expression measurement, one or more targets may be present in vast excess, creating a challenge for the multiplexed measurement of the other targets. It has been reported that supplementing the master mix with additional reagents, particularly the polymerase, can overcome a loss in sensitivity caused by competing amplifications.[1]



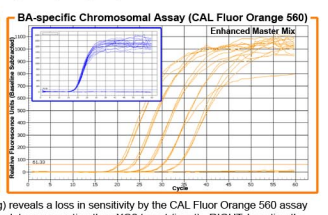


FIGURE 11. LEFT: a dilution series of Sterne DNA (1.0 ng to 10 fg) reveals a loss in sensitivity by the CAL Fluor Orange 560 assay when there is simultaneous amplification of $1x10^{\wedge}6$ copies of a template representing the pXO2 target (inset). RIGHT: boosting the reagents in the master mix rescues the amplification of the lower dilutions in competition with this dominant pXO2 amplification.

[1] K. Persson, K. Hamby, L.A. Ugozzoli, Analytical Biochemistry 344 (2005) 33-42.

Multiplexing requires pairing distinct fluorescent reporters that can be resolved from one another. Any crosstalk between channels might cause false positives or compromise quantitation.

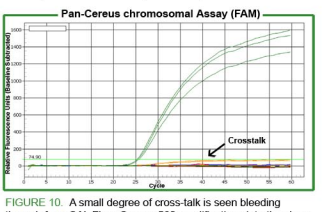


FIGURE 10. A small degree of cross-talk is seen bleeding through from CAL Fluor Orange 560 amplifications into the channel oriented to detect FAM.

METHODS

Triplicate reactions were prepared for all dilution points and NTCs. Serial dilutions for the construction of standard curves were prepared in nuclease-free water containing 100 ng/µL of yeast tRNA, Roche Molecular systems. *Bacillus anthracis* Ames and *Bacillus anthracis* Sterne DNA was obtained through the Critical Reagents Program within the Department of Defense. *Bacillus cereus* strain ATCC 10987 was obtained from ATCC. PCR thermal cycling conditions: 95°C for two min. followed by 60 cycles of: 95°C for 20 s, 60°C for 60 s. All independent reactions were constructed using a master mix with limited reagent concentrations. All multiplexed reactions were constructed using a master mix with more generous reagent concentrations, except when testing for the loss of sensitivity in the presence of a dominant amplification. Both of these master mix formulations are listed below.

Reaction Components:	Limiting Master Mix		Enhanced Master Mix	
• Nuclease-free water	4.40 µl	N/A	2.40 µl	N/A
• Platinum Taq PCR Buffer (10X)	2.00 µl	1X	2.00 µl	1X
• Magnesium Chloride (50 mM)	2.30 µl	5.75 mM	2.40 µl	6 mM
• dNTPs (2.5 mM each)	1.60 µl	200 µM	3.20 µl	400 µM
• Platinum Taq Polymerase	0.10 µl	0.5 units	0.40 µl	2.0 units
• Template DNA (variable)	4.00 µl	variable	4.00 µl	variable
• Each Forward Primer (10 µM)	4x 0.60 µl	300 nM	4x 0.60 µl	300 nM
• Each Reverse Primer (10 µM)	4x 0.60 µl	300 nM	4x 0.60 µl	300 nM
• Each Probe (10 µM)	4x 0.20 µl	100 nM	4x 0.20 µl	100 nM
Total	20.0 µl		20.0 µl	

CONCLUSIONS

By carefully selecting the target sequences, fluorescent reporters, and the real-time PCR instrument for detection, we have developed a high-performing quadruplexed assay that distinguishes several strains of *Bacillus anthracis* from a closely-related organism, *Bacillus cereus*. The software program RealTimeDesign was used to rapidly generate TaqMan® assays to the species-specific sequences. To successfully multiplex these assays together, we characterized dye crosstalk, confirmed that each assay has a high amplification efficiency, and identified their detection limit, particularly in the context of disproportionate targets. For situations where one sequence may be in vast excess over the others, we show the benefit of supplementing master mix components so that depleted reagents don't limit detection sensitivity. Multiplexing demands increased effort to characterize amplification performance, but is ideally suited to interrogate multiple genetic signatures from small quantities of sample DNA.

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