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Designing multiplex qPCR assays

Introduction

Quantitative, real-time PCR (qPCR) is used to confirm the presence of, and quantify the amount of, a nucleotide sequence within a complex sample. The accumulating PCR product can be detected in real time by measuring the accumulation of fluorescent signal that is directly proportionate to the amount of a specific nucleotide sequence or amplicon in the reaction mix and carrying out the reaction in a PCR cycler that can detect fluorescence. The accumulating PCR product is detected with either an intercalating dye that indiscriminately binds to double stranded DNA, or a fluorophore that is released from its conjugated probe.

The qPCR technique can be adapted to allow multiple targets to be detected simultaneously in a single reaction as a multiplex qPCR assay. When combined with PCR systems such as the Cielo Real-Time PCR System from Azure Biosystems that can process samples in 96-well plates, multiplex qPCR provides a high-throughput approach to assessing multiple targets across hundreds of samples in one experiment.

Depending on the design of the assay, multiplex qPCR can involve multiple primer pairs or primer-probe sets with multiple fluorophores to detect numerous targets within a single sample. Therefore, careful design and optimization of the assays is required to obtain the highest quality data. This technical note will describe preliminary steps and considerations when designing and optimizing multiplex qPCR assays that will help ensure a successful multiplex qPCR experiment.

Multiplex qPCR applications, advantages, and limitations

Multiplex qPCR can use multiple primer pairs or primer-probe sets to detect different nucleotide targets in a single PCR reaction. The probes for each target are labeled with different fluorophores with non-overlapping excitation and emission spectra so that each amplified product can be detected independently (Figure 1).

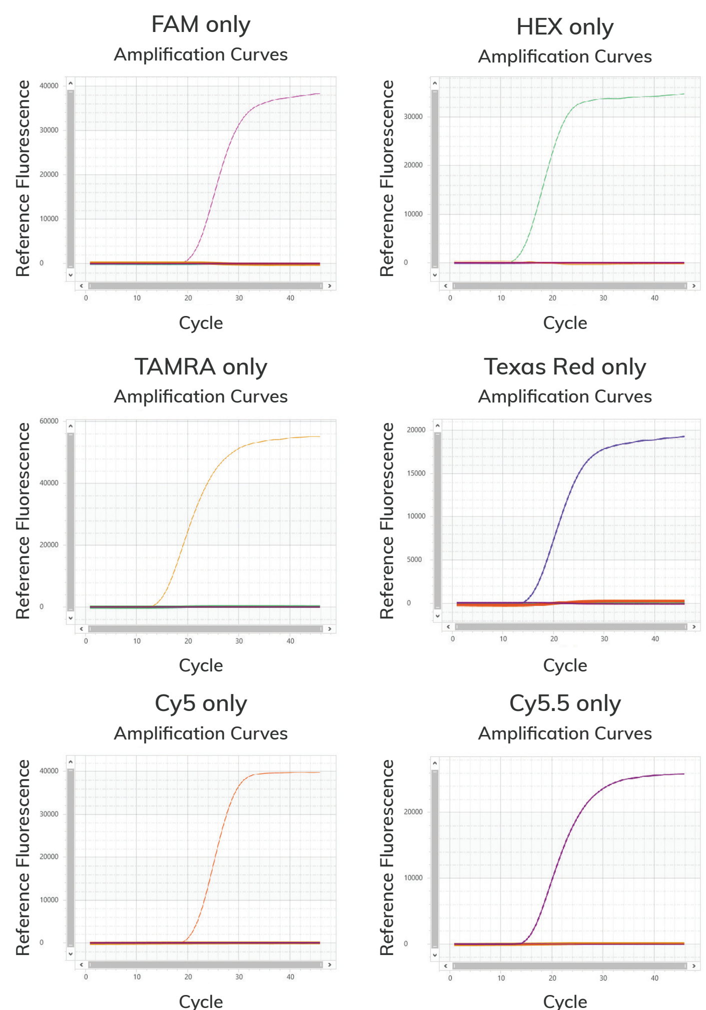


Figure 1. Detection of product accumulation in a six-plex qPCR reaction on the Azure Cielo 6. All six channels were scanned and no evidence of signal leakage or crosstalk was observed in the single channel amplification curves.

Multiplex qPCR provides several logistical and technical advantages over carrying out multiple single plex assays when more than one target must be assessed in a sample. Multiplex qPCR conserves sample, particularly important when working with limited samples such as tissue or biopsy material. Multiplex qPCR also saves time by reducing the number of reactions that must be set up and the number of runs on the PCR machine, and it reduces costs by conserving reagents and consumables.

In addition to increased efficiency and cost savings, multiplex qPCR can simplify data analysis. When comparing targets within the same sample, assessing them in the same reaction removes the need to control for plate-to-plate or well-to-well variations that would be required if the targets were assessed in different reactions.

Multiplex qPCR is widely used across fields such as clinical diagnostics, forensics, and basic biomedical research. The high throughput and ability to assess multiple genes make multiplex qPCR a frequent choice for diagnosis of infectious diseases since it allows samples to be tested for multiple pathogens simultaneously and can also be used to characterize infections by assessing genes that predict response to various medications or antibiotics. Other applications of multiplex qPCR include confirming authenticity or detecting contamination of food and beverage products and estimating the quantity and quality of DNA in samples collected for forensic analysis.

The number of targets that can be detected in a multiplex qPCR reaction is limited by the channels and dye compatibility of the PCR instrument. Dyes must be selected with non-overlapping excitation and emission spectra, as will be described in more detail below. Another limitation of qPCR is that the likelihood of nonspecific products increases as more primers and probes are introduced. Avoiding primer dimers and other nonspecific products is another optimization step that will be described below.

Tips and strategies for designing and optimizing multiplex qPCR assays

Select the targets to be detected

The targets for a multiplex qPCR experiment will depend on the goal of the research. Once the specific genes or nucleotide sequences of interest have been selected, the specific sequence to be amplified by PCR must be chosen.

- Note that the number of products that can be assessed in a multiplex reaction is determined by the instrument that will be used and its detection channels.
- Assays to detect many genes are commercially available; search for assays and you may be able to avoid designing reactions from scratch.
- If no existing commercial assays are found, new assays may be designed using gene sequences available in public databases such as GenBank.

Design primers

Every target to be detected in a multiplex qPCR reaction must be efficiently amplified under the same PCR conditions. Therefore, the melting temperatures for primers and amplicons should be similar across all reactions and amplicon lengths should be consistent and able to be synthesized within the extension step time.

- Amplicon size should be short (less than 200 bp) for efficient amplification and reduced opportunity for secondary structure formation.[1]
- Target regions that are too rich in C and G bases should be avoided because they may be more difficult to amplify.[1]
- Free and commercially available software packages are available to assist with primer design, such as Primer-BLAST and Primer3. The software can help design primers with appropriate melting temperatures, with appropriate % G and C content, and less likely to form primer dimers.
- Assuming a PCR annealing temperature of 59 °C or 60 °C, melting temperatures for primers should be between about 63 °C to 64 °C; all primers should have identical melting temperatures within 1 °C.[2]
- Primers are generally 18 to 24 nucleotides long.[1,2]

- To prevent amplification of nonspecific products, it is important to ensure the primers cannot form hairpins or other secondary structures and that they cannot bind to each other; programs are available that can check for secondary structure such as mFold or OligoAnalyzer™.
- If using commercially available primer sets, it is still important to check that the various primers for the individual reactions are not able to interact with each other when all of the primers are assembled in one multiplex reaction.

Design probes

Several types of probes may be used to detect the amplicon.[3] If using TaqMan style qPCR reactions, oligos conjugated to fluorescent dyes are used as probes to detect the accumulating PCR products.

- Probe melting temperature should be 5 to 10 degrees higher than the melting temperatures of the primers; software programs are available to help in probe design.[1]
- The probe should bind the PCR product without blocking a primer binding site or otherwise interfering with amplification.
- Select fluorescent probes and quenchers that are compatible with the detection channels of the instrument that will be used.
- Select fluorophores with no or minimal spectral overlap. For the Cielo 6, Azure Biosystems recommends the following six dyes for 6-plex detection (Figure 2):
    - FAM™
    - HEX™
    - TAMRA™
    - Texas Red®
    - Cy5®
    - Cy5.5®

Fluorophore	Dye-5′-T_{10}	
	EX	EM
Biosearch Blue™	352	447
FAM	495	520
TET	521	536
CAL Fluor® Gold 540	522	544
JOE	529	555
VIC	538	554
HEX	535	556
CAL Fluor Orange 560 (VIC/HEX/JOE replacement)	538	559
Quasar® 570 (Cy3 replacement)	548	566
Cy™3	549	566
TAMRA	557	583
CAL Fluor Red 590	569	591
Cy3.5	581	596
ROX	589	610
CAL Fluor Red 610	590	610
Texas Red®	597	616
CAL Fluor Red 635	618	637
Cy5	646	669
Quasar® 670 (Cy5 replacement)	647	670
Cy5.5	675	694
Quasar® 705	690	705

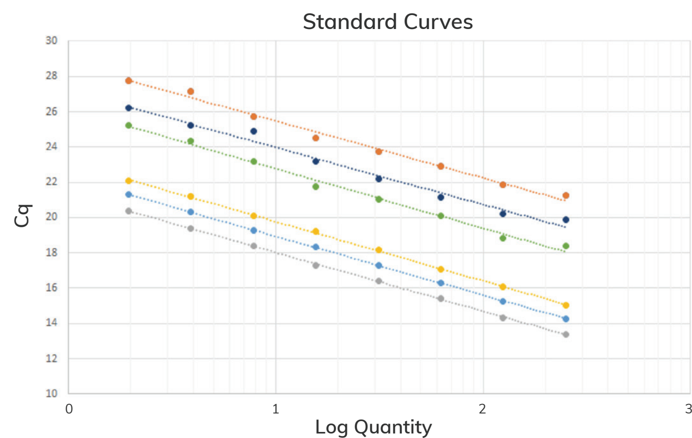
Figure 2. Dye compatibility with the Azure Cielo 6.

Validate primers and probes

Confirm the specificity and efficiency of each of the qPCR reactions and ensure that these are maintained when the reactions are run in a multiplex scenario.

- Before running any reactions, conduct validation “in silico” using a program such as Primer-BLAST to ensure that the primers and probes are specific to the intended target.
- Optimize PCR conditions and primer and probe concentrations for each reaction in monoplex reactions.

- Test a range of annealing temperatures around the predicted primer melting temperature to identify the best conditions.[1]
- Verify that only one product of the correct size is formed by carrying out a melting curve analysis and gel electrophoresis after PCR is complete.
- Carry out a standard curve analysis to demonstrate that the PCR efficiency of each reaction is between 90% and 110%.[1] An example of efficiency determination is shown in Figure 3.
- Once each individual assay is optimized, verify that the efficiency and specificity of the reactions are maintained when the reactions are carried out as multiplex reactions.



Report Dye	Gene name	Efficiency	R^2	Slope	Y-intercept
• FAM	RRP36	103.582	0.959	-3.239	28.684
• HEX	ACTB	100.456	0.995	-3.311	21.281
• TAMRA	RNASE-P	97.016	0.995	-3.396	22.157
• Texas Red	GAPDH	100.219	1.000	-3.317	22.307
• Cy5	TBP	96.811	0.993	-3.401	26.023
• Cy5.5	EF1a	97.224	0.993	-3.390	23.173

Figure 3. Efficiency and linearity determination for a six-plex qPCR experiment carried out on the Azure Cielo 6.

Best practices for successful multiplex qPCR

- Use the correct plates recommended for the machine being used, and the correct master mix.
- You may need to adjust relative primer concentrations to avoid depleting reaction components if one target is present in much higher amounts than other targets of the multiplex reaction.
- Adjust for spectral crosstalk; instructions for adding dyes and conducting color compensation on the Azure Cielo may be found in this Technical Note.

With the tips and resources described above, you are off to a great start creating successful multiplex qPCR assays.

References

1. Raymaekers M, Smets R, Maes B, Cartuyvels R. Checklist for optimization and validation of real-time PCR assays. *J Clin Lab Anal.* 2009;23:145–151.
2. Thornton B, Basu C. Real-time PCR (qPCR) primer design using free online software. *Biochem Mol Biol Educ.* 2011;39(2):145–154.
3. Juskowiak B. Nucleic acid-based fluorescent probes and their analytical potential. *Anal Bioanal Chem.* 2011;399:3157–3176.

Figure 4. The Azure Cielo 6 Real-Time PCR (PN AIQ060) supports up to 6 channel multiplex qPCR reactions.

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