Recommended dye combinations for multiplex qPCR

Review these recommendations for selecting dyes for multiplex qPCR that minimize background and avoid overlap of fluorescent signals. Included is a table of compatible dyes for multiplexing on common qPCR instruments and a list of suggested quenchers.

Jan 14, 2014

Revised/updated Mar 2, 2016

The experimental design for multiplex qPCR is more complicated than for single reactions. The probes used to detect individual targets must contain unique reporter dyes with distinct spectra. The settings for excitation and emission filters of real-time detection systems vary from manufacturer to manufacturer; therefore, instruments must be calibrated for each dye as part of the experiment optimization process. Ensuring that instruments are appropriately calibrated will enhance dye specificity, and minimize background and overlap of fluorescent signals.

When selecting dyes:

- **Choose dyes that are compatible with your instrument.** The instrument must be capable of detecting the emission spectrum for each dye you are using. The manufacturer can provide instrument excitation and detectable emission wavelengths. Get more information on dye spectral emission and excitation values.
- **Choose dyes that can be calibrated on your instrument.** Your real-time PCR instrument must be calibrated for the set of dyes you choose (Table 1). If it is not, you must calibrate the instrument or select different dyes. IDT provides free, online dye calibration protocols for some common real-time PCR instruments.
- **Avoid overlap of emission spectra.** Choose dyes with appropriate excitation wavelengths and little to no overlap in their emission spectra (Table 1). Consider total fluorescence intensity as well. For example, FAM is a good dye choice to detect low copy transcripts because it has high fluorescent signal intensity. You can then use fluorophores with lower signal intensities for more abundant transcripts (e.g., housekeeping genes).

Instrument	Dye 1	Dye 2	Dye 3	Dye 4	Dye 5
Applied Biosystems 7300	FAM	HEX™ or JOE			
Applied Biosystems 7500	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665
Applied Biosystems 7900	FAM	HEX™ or JOE			
Applied Biosystems StepOne™	FAM	HEX™ or JOE			
Applied Biosystems StepOnePlus™	FAM	HEX™ or JOE	TAMRA		
Applied Biosystems ViiA™ 7	FAM	HEX™ or JOE	TAMRA		
Bio-Rad CFX384™	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665
Bio-Rad CFX96™	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665
Bio-Rad iCycler iQ™	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665
Bio-Rad Miniopticon™	FAM	HEX™ or JOE			
Bio-Rad MyiQ™2	FAM	HEX™ or JOE			
Bio-Rad MyiQ™5	FAM	HEX™ or JOE	TAMRA	TEX 615™	Cy5 or Tye™ 665
Roche Lightcycler® 480	FAM	HEX™ or JOE		LC Red 640	Cy5 or Tye™ 665
Stratagene Mx3000P™	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	
Stratagene Mx3005P™	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665

Table 1. Dye recommendations for multiplex qPCR. Shown are up to 5 pre-calibrated dye combinations for common real-time PCR instruments. Some machines (most of the Applied Biosystems instruments) will require calibration for HEX and JOE dyes. Also, additional dyes are available from IDT, but may require prior dye calibration. You can access free, online calibration protocols for some of the common real-time PCR instruments.

Minimize signal cross-talk by using probes that quench well. Highly efficient dark quenchers, especially those used in combination with a secondary quencher such as the ZEN™ Quencher (/pages/products/qpcr-and-pcr/gene-expression/primetime-qpcr-probes) (Table 2), considerably reduce background fluorescence leading to increased sensitivity and end-point signal, as well as earlier C_q values (see the data in the article, *Decrease qPCR Background, Improve qPCR Signal (/pages/education/decoded/article/zen)*). This is particularly useful for multiplex reactions because having several fluorophores in the same tube causes higher background fluorescence. We recommend using the same "quencher type" (all dark quenchers or all fluorescent quenchers) in assays that will be multiplexed.

Dye	Excitation Wavelength	Emission Wavelenth	Dark Quencher
FAM	495	520	Iowa Black® FQ/ZEN™
HEX™	538	555	Iowa Black® FQ/ZEN™
Cy3	550	564	Iowa Black® RQ
TEX 615™[2]	596	613	Iowa Black® RQ
LC Red 640[1]	620	635	Iowa Black® RQ
Tye™ 665	645	665	Iowa Black® RQ
Cy5	648	668	Iowa Black® RQ

Table 2. Suggested quenchers for probes used in multiplex qPCR. Note that you must verify that your qPCR instrument is calibrated for the dyes you choose, and if not, you must perform calibration or select different dyes. Read more about dye spectral emission and excitation values.

[1] NHS ester

[2] The TEX™ 615 spectrum overlaps with that of ROX; therefore, TEX 615 should not be used in a platform that requires a ROX passive reference or with master mixes containing high concentrations of ROX dye.

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