Design and preparation of filter sets for fourplex fluorescence PCR instruments

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Aiming at the crosstalk problem caused by small spectral intervals between fluorescent reagents in fourplex fluorescence quantitative polymerase chain reaction (PCR) detection analysis system, we calculate and analyse the effect of cut-off steepness, central wavelength positioning, and bandwidth of filters on crosstalk. Design and prepare four sets of fluorescence excitation and emission filters with proper shape cut-off steepness (optical density (OD) from OD 0.3 to OD 6 is less than 8 nm) and bandwidth (9–11 nm). In fourplex PCR instrument, crosstalk coefficient in all four channels are less than 0.3%.

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Multiplex real-time fluorescence quantitative polymerase chain reaction (PCR) detection analysis system has been added more spectrum detecting channels on the basis of real-time fluorescence quantitative PCR[1,2], and achieves the simultaneous detection of multiple fluorescent dyes or fluorescence probe[3−5], so more specimen information could be obtained within the same PCR amplification cycle time.

In multiplex real-time fluorescence quantitative PCR detection analysis system (multiplex PCR system), multiple fluorescent reagents coexist in one specimen, excitation and emission spectrum of multiple reagents will overlap and interact with each other, and it can lead to crosstalk between fluorescence detection signals, reducing the detection precision. Therefore how to optimise spectral characteristics of filters to reduce crosstalk between fluorescence detection signals of reagents are the key problems in multiplex PCR system.

In multiplex PCR system, multiple fluorescent reagents (focus on four types of fluorescent reagents) coexist in specimen. Four sets of light source and fluorescence filter system correspond to four types of fluorescent reagents respectively. The light source is using four different emission bands of light-emitting diode (LEDs). The fluorescence detection light path is shown in Fig. 1. Four types of LED light source spectral power distributions are shown in Fig. 2. Fluorescence excitation and emission spectra of four types of fluorescent reagents are overlapping to each other, as shown in Fig. 3.

In fourplex PCR system, four types of fluorescent reagents coexist in one specimen, and one reagent is corresponding to a set of filters (excitation filter and fluorescence emission filter). Due to the overlap of fluorescence excitation and emission spectrum of the four fluorescent reagents, when excitation light beam is irradiated onto the testing specimen, the corresponding fluorescent reagent is excited and emits fluorescence signal. Meanwhile, the other three fluorescent reagents are also excited in various degree and emit fluorescence crosstalk signal that influences the testing precision in the channel. The degree of crosstalk is influenced by the characteristics of emission source, fluorescent reagent, fluorescence filter spectrum, and fluorescence receiver. The target value of fourplex fluorescence PCR instruments on the crosstalk is less than 0.5% in the letter.

The degree of fluorescence signal crosstalk can be expressed by

$$C_n =$$

$$\frac{\int S_n(\lambda)*T_{\mathrm{EF}n}(\lambda)*(\sum_{n=1}^{4} M - M_n)*T_{\mathrm{FF}n}(\lambda)*R_n(\lambda)\mathrm{d}\lambda}{\int S_n(\lambda)*T_{\mathrm{EF}n}(\lambda)*M_n*T_{\mathrm{FF}n}(\lambda)*R_n(\lambda)\mathrm{d}\lambda},$$ (1)

$$M_n = \int Ex_n(\varphi)*Em_n(\lambda)\mathrm{d}\varphi,$$

where C_n is the crosstalk in the nth channel from other channels, $S_n(\lambda)$ is the relative spectral power distribution function of light source in the nth channel, $R_n(\lambda)$ is the relative spectral response function of photodetector in the nth channel, $T_{\mathrm{EF}n}(\lambda)$ is spectral transmittance function of excitation filter in the nth channel, $T_{\mathrm{FF}n}(\lambda)$ is spectral transmittance function of fluorescence emission filter in the nth channel, $Ex_n(\varphi)$ is excitation spectral function of fluorescent reagent in the nth channel, $Em_n(\lambda)$ is emission spectral function of fluorescent reagent in the nth channel, and φ and λ are variable quantities depending upon wavelength.

Equation (1) shows that the characteristics of transmission and cut-off spectra of fluorescence filter (passband position, bandwidth, cut-off steepness, and cut-off depth, etc.) will directly affect the crosstalk degree. In the fluorescence detection optical system with reagent, light source and photoelectric receiver are already established, and the spectral characteristic of filters is the key factor affecting the crosstalk.

In order to analyse the effect of different cut-off steepnesses, aiming at the second channel reagent, we designed three sets of different cut-off steepness filter sets (as shown in Fig. 4) as contrast
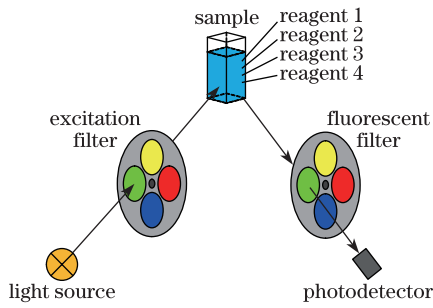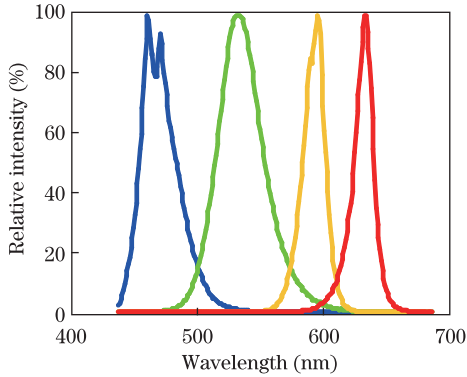
Fig. 1. Diagram of light path.

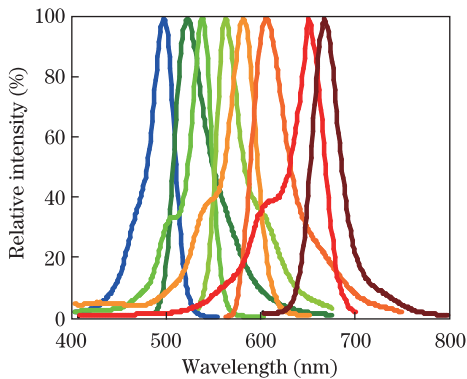Fig. 2. (Color online) Spectral power distribution of four types.

Fig. 3. (Color online) Overlapping between fluorescence excitation and emission spectra of four types of fluorescent reagents.

Table 1. Effect of Different Cut-off Steepness on Crosstalk Coefficient

Number	Cut-off Steepness of Filters (OD 0.3 to OD 6) (nm)	Crosstalk Coefficient
1	8	0.15%
2	11	0.23%
3	16	0.65%

specimen. The bandwidth of filter in these three sets is set to 10 nm. The cut-off background crosstalk point between excitation and emission filter is set to optical density (OD) 5[6]. As the cut-off steepness varies, the central wavelength position of excitation and emission filters are also different. The crosstalk coefficient of fluorescence signal from other fluorescent reagents to reagent in the second channel, when using these three filter sets with different steepness, is calculated by computer simulation. The results are shown in Table 1. We

can conclude that the higher cut-off steepness of filter, the smaller crosstalk.

Due to the overlap of the four sets fluorescent reagents's excitation and emission spectra, wavelength interval is only 20–30 nm. Therefore the effect of fluorescence filter wavelength position on crosstalk degree cannot be ignored. Taking the second channel filter set for example, on the premise of certain passband width, unchanged central wavelength interval between excitation and emission filter in the same set, wavelength position of filter sets movesat 1 nm pre step synchronously. Using Eq. (1) to calculate the crosstalk coefficient C_2, the variation of C_2 is shown in Fig. 5. We can conclude that if the wavelength positioning precision of fluorescence filter sets is controlled within the range of ± 2 nm, smaller crosstalk can be obtained.

The second channel filter set, for example, under the premise of the same wavelength position of excitation and emission filters, changes the passband width of the excitation and emission filters synchronously, and the variation is shown in Fig. 6.

We can conclude that the smaller the passband width, the smaller the crosstalk. However, small passband width will affect the intensity of the fluorescence signal in this channel. The relation of passband width and fluorescent signal intensity is shown in Fig. 7. The range of passband width 9–11 nm is the preferred selection by considering the influence trend of the passband width on both crosstalk and fluorescent signal intensity.

According to the calculation and analysis above, combined with the current technology capacity, the technical requirements of fluorescent filter spectral characteristics in multiplex PCR system are as follows: the cut-off of filteris designed as passband bilateral sharp cut-off, bilateral cut-off steepness (OD 0.3 to OD 6) is about 8 nm, central wavelength positioning accuracy is ± 2 nm, and passband width selection range is 9–11 nm (except for excitation filter in the first channel and emission filter in the fourth channel). Figure 8 is transmissivity and cut-off spectra of the four sets of excitation and emission filters designed. Simulated crosstalk coefficients in each channel are: $C_1=0.1\%$, $C_2=0.2\%$, $C_3=0.17\%$, and $C_4=0.19\%$.

The most critical part of the filter with the above spectral characteristics is the main film system design on passband and bilateral sharp cut-off, adopting TiO_2/SiO_2 as the materials,
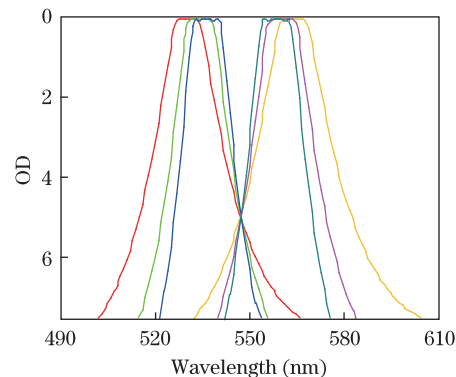

Fig. 4. (Color online) Design curve of different cut-off steepness filter sets.
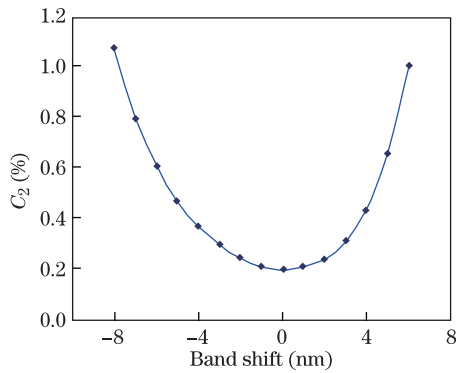
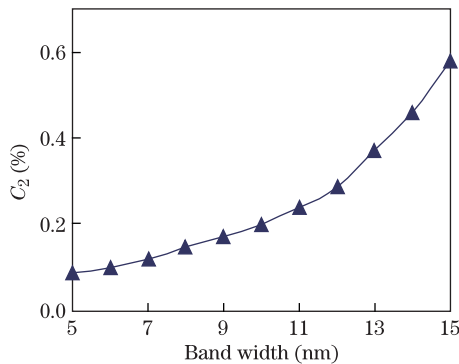Fig. 5. Effect of wavelength position of filter sets on crosstalk coefficient.

Fig. 6.   Effect of filter passband width on the crosstalk coefficient intensity.
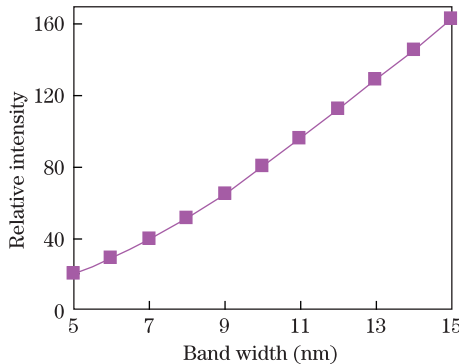

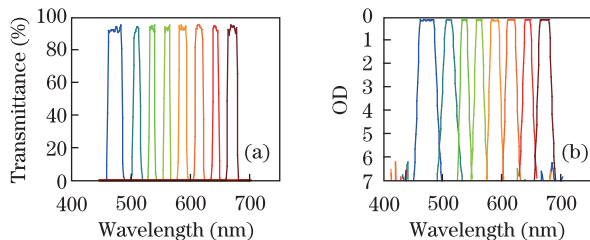Fig. 7. Effect of filter passband width on the fluorescent signal.

Fig. 8. (Color online) (a) Transmissivity and (b) OD of fluorescence filter sets in multiplex PCR system.
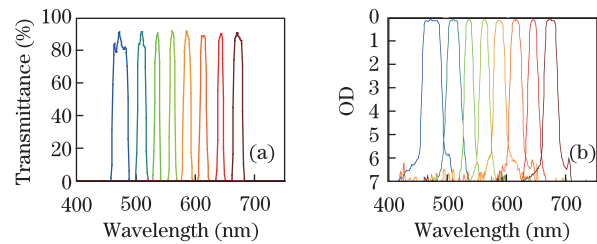

Fig. 9. (Color online) (a) Transmissivity and (b) OD of fluorescence filter sets in multiplex PCR system.

and the total number of layers is about 130. Cut-off range of the completed filters (together with the rest of cut-off auxiliary films) is ultraviolet (UV)~1 150 nm, cut-off depth is above OD 6, passband width is 9–11 nm, and cut-off steepness (OD 0.3 to OD 6) is about 8 nm.

The fluorescence filter sets of multiplex PCR system mentioned in this letter are prepared on Optorun coating equipment (OTFC1300) and Leybold coating equipment (SYRUS C). The fluorescence filter sets are produced by using radio frequency (RF) ion assisted deposition and plasma ion assisted deposition with coating material TiO_2, Ta_2O_5, and SiO_2.

Using Cary 6 000i from VARIAN to measure spectrum, the results are shown in Fig. 9.

Through application and verification by multiplex real-time fluorescence quantitative PCR detection analyzer, fluorescence filter sets show strong feasibility and reliability. Fluorescence signal to noise ratio (SNR) is high without standard specimen correction in the channel. The crosstalk coefficient in fluorescence channels is less than 0.3% without the design software correction.

In conclusion, to reduce the crosstalk of multiple fluorescence signals in multiplex fluorescence PCR system, the central wavelength positioning precision, cut-off steepness and passband width are optimally designed. The optimized parameters of filter spectrum are: the central wavelength positioning accuracy is ±2 nm, cut-off steepness (OD 0.3 to OD 6) is about 8 nm, passband width is 9–11 nm. The four sets excitation and emission filter with the above parameters are prepared by using RF ion assisted deposition and plasma ion assisted deposition. The measured fluorescence crosstalk coefficient between four reagents in fourplex fluorescence PCR analyzer is less than 0.3%.

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