**Letter to the Editor**

**Optimization of fluorescence measurement in duplex real-time PCR with TaqMan® probes labeled with VIC and quenched by TAMRA**

Real-time PCR with 5'-nuclease TaqMan® probes (1,2) is a relatively robust method for specific DNA sequence detection and quantification. The method is often used in duplex format, where the first set of primers and the probe target the specific DNA sequence of interest, and the second set is used as a reference. In the original duplex TaqMan system, the first probe is labeled with FAM (6-carboxyfluorescein) and quenched by TAMRA (6-carboxytetramethylrhodamine) and the second probe is labeled with VIC (4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein) and quenched by TAMRA (3). This system is widely used both in commercial kits and in original systems, mainly because of an affordable price. However, it produces fluorescence spectra with overlapping peaks that can be correctly measured only using advanced instruments equipped with a spectrograph on the emission side and supported with a powerful software to process the spectra. Real-time PCR cyclers equipped with filter-based optics are unable to correctly monitor fluorescence of the original duplex TaqMan system because of interference between optical channels. For example, amplification of the primers/probe system with a FAM label, which is reflected by an increase in the recorded fluorescence in the FAM channel, leads also to a certain increase in the recorded fluorescence in the VIC channel. The instruments offer an option to eliminate such interference between optical channels by data transformation using some undisclosed calculation. However, this option is imprecise, and an increase in the fluorescence in the FAM channel leads to a decrease in the calculated fluorescence in the VIC channel. Another source of problems is the emission of TAMRA, which is recorded in the same channel as VIC by several instruments. The reason for the described problems is that standard filters used in real-time PCR cyclers, with filter-based optics, are not optimal for work with a combination of dyes FAM, VIC, and TAMRA.

As a basis for optimization of optical channels, we measured a three-dimensional (3-D) fluorescence spectrum of the PCR system. The previously described real-time PCR for the detection of *Salmonella enterica* (4) with a FAM-labeled, TAMRA-quenched probe was used in duplex with the previously described real-time PCR for the detection of *Escherichia coli* (5) with a VIC-labeled, TAMRA-quenched probe. The ratio of the template DNA from *S. enterica* and *E. coli* was 1:1 (200:200 ng). Reactions were performed in TopYield™ flat-bottom 8-strips (Nunc, Roskilde, Denmark) in a GeneAmp® 9700

![Three-dimensional fluorescence spectrum of the products of duplex PCR with TaqMan probes labeled with FAM and VIC, respectively, and quenched by TAMRA.](image)

**Table 1. Parameters of Amplification Curves (Fluorescence vs. Cycle Number) at Different Measurement Settings**

<table>
<thead>
<tr>
<th>Setting</th>
<th>F&lt;sub&gt;max&lt;/sub&gt; (RFU)</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;</th>
<th>ΔF&lt;sub&gt;max&lt;/sub&gt; (RFU)</th>
<th>F&lt;sub&gt;0&lt;/sub&gt; (RFU)</th>
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<td>optimal</td>
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<td>7870</td>
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<tr>
<td>MiniOpticon</td>
<td>25987</td>
<td>20.4</td>
<td>9216</td>
<td>25277</td>
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</table>

F<sub>max</sub>, plateau fluorescence with subtracted baseline; C<sub>T</sub>, cycle threshold; ΔF<sub>max</sub>, difference between the plateau fluorescence of the complete duplex PCR system (including the FAM-labeled probe) and the plateau fluorescence of the PCR system without the FAM-labeled probe; F<sub>0</sub>, baseline fluorescence; RFU, relative fluorescent units.

*Excitation maximum 525 nm, emission maximum 555 nm.*
*Excitation maximum 530 nm, emission maximum 575 nm.*
*Excitation maximum 517 nm, emission maximum 570 nm.*
*Excitation maximum 500 nm, emission maximum 570 nm.*

0 RFU, relative fluorescent units.
thermal cycler (Applied Biosystems, Foster City, CA, USA) at usual reaction conditions (6). After PCR, the 3-D fluorescence spectrum was measured from the bottom orientation directly in the microtubes in a Safire2™ double-monochromator 96-well fluorimeter (Tecan, Grödig bei Salzburg, Austria).

Based on the 3-D spectrum obtained (Figure 1), an optimal fluorescence measurement setting was suggested for bandwidths of 20 nm, which are usual with narrow-band filters: excitation maximum of 525 nm, emission maximum of 555 nm. Fluorescence measurement at this setting was compared with settings used by three common real-time cyclers. A set of samples without the FAM-labeled probe was included in the experiment to facilitate identification of the interference between channels. PCR as described above was carried out in triplicate in 12 strips that were removed from the cycler after each 2–4 cycles. After finishing the entire reaction, strips were placed in a frame, and fluorescence was measured by Safire2 using integration time 40 μs and optimal gain setting. Because the maximum bandwidth of the fluorimeter was 20 nm, the measurements were done under more selective conditions than those used by real-time cyclers equipped with filters of wider bandwidths. Fluorescence data were processed, and amplification curves were constructed using Prism 4 software (GraphPad, San Diego, CA, USA).

The results (Table 1) demonstrated that, at optimized conditions, better fluorescence parameters were achieved; namely, higher detection sensitivity (documented by a higher plateau fluorescence, Fmax, and a lower cycle threshold, CT) and less interference between channels (lower difference between +FAM and -FAM plateau fluorescence, ΔFmax, and lower baseline fluorescence, F0). These data suggest that results of the duplex real-time PCR with TaqMan probes labeled with FAM and VIC, respectively, and quenched by TAMRA may be improved by using optimal excitation and emission narrow-band filters.

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REFERENCES