BioFeedback

[Letter to the editor]

Combined FAM-labeled TaqMan probe detection and SYBR green I melting curve analysis in multiprobe qPCR genotyping assays

Since its first use, real-time quantitative PCR (qPCR) has evolved into a flexible, application-made method for the quantification and identification of nucleic acids (1-2). Depending on the application, most researchers choose between fluorescent nucleic acid binding dyes or labels that interact by fluorescence resonance energy transfer (FRET) as nucleic acid detection methods (2-3). Binding dyes are relatively cheap, easy to use, and generate high signals. The fact that they display sequence independent binding properties can be considered an advantage or disadvantage, depending on the application. Currently, SYBR green I is the most frequently used fluorescent nucleic acid binding dye (3-5). Among all available FRET-based nucleic acid detection methods (e.g. hybridization probes, molecular beacons, hydrolysis probes, dye-primer systems), TaqMan probes are currently the most popular (3,6-7). They are more expensive, more difficult to design, but sequence specific. Although both detection systems can generate melting curves in closed tube reactions without the need for post-qPCR gel electrophoresis, only those produced by binding dyes provide a total PCR amplicon readout, including nonspecific amplicons and oligo dimers, whereas those produced by probes only reflect the amount of PCR product detected by the probe (8-9).

So far, only two reports applied both types of detection in a single assay (10-11). Lind et al. described the combination of the nonspecific dsDNA binding dye BOXTTO with sequence-specific FAM-labeled TaqMan and LNA probes (10), and Cheah et al. described a two-tube combined TaqMan/SYBR green assay with a ROX- and a Cy5-labeled probe (11). For those applications, they needed to combine specific sequence detection by fluorescently labeled probes with the generation of the total PCR amplicon readout by binding dyes. However, both papers mention the incompatibility of combining the most popular intercalating dye SYBR green I with a TaqMan probe labeled with the most common fluorophore, FAM, due to their overlapping spectra (10-11; Figure 1).

Here, we performed qPCR assay 1 of the dual fluorescent multiprobe assay for prion protein genotyping in sheep, as described by Van Poucke et al. (12), containing FAM-, HEX-, Texas Red- and Cy5-labeled TaqMan probes (Sigma-Aldrich, St. Louis, MO, USA), with iQ SYBR green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), to show that FAM-labeled probe detection can be combined with SYBR green I melting curve analysis if the detection of the probe is performed at a temperature higher than the melting temperature of the amplicon. In addition, we formulated some compatibility guidelines for TaqMan probe detection and SYBR green I melting curve analysis in qPCR genotyping assays in general, which provide extra qPCR design flexibility for future assays.

As a first experiment, we performed the assay described by Van Poucke et al. (12) on the CFX96 Real-Time PCR Detection system (Bio-Rad). A 180 bp amplicon was generated using iQ Supermix (Bio-Rad) and two biallelic SNPs (at codons 136 (A/V) and 154 (R/H) of the PRNP gene) were simultaneously detected in a single tube with four allele-specific TaqMan probes, labeled with FAM, HEX, Texas Red and Cy5. Detection of fluorophores (released from the annealed probes due to the exonuclease activity of the polymerase during elongation) was performed at the end of the combined annealing-elongation step of 62°C (Supplementary Material 1). Homozygous positive (+/+), heterozygous (+/-) and homozygous negative (-/-) samples (previously genotyped by this assay and confirmed via PCR-RFLP and sequencing; 12), and a no template control (NTC) were analyzed for every probe/fluorophore. All four probes detected their specific alleles (Figure 2.A-D.1; Supplementary Material 2). In this application, allele calling is based on getting a Cq value or not, hence the quantification cycle (Cq) value itself is not important for genotyping purposes. However, the shape of the curve provides additional confirmation about homozygosity or heterozygosity, as amplification plots for homozygotes are steeper and higher than plots from heterozygotes (Figure 2.A-D.1; 12).

In experiment 2, the same assay was performed as in experiment 1, but with iQ SYBR green Supermix (Bio-Rad) instead of iQ Supermix (Bio-Rad), and with an additional melting curve analysis from 70°C to 95°C in 0.5°C incremental steps of 10 seconds (Supplementary Material 1). The presence of SYBR green didn’t have any effect on signal generation from HEX, Texas Red and Cy5-labeled probes as similar amplification plots (i.e. similar Cq value, RU value and shape) were generated as in experiment 1 (Figure 2.B-D.1-2; Supplementary Material 2). However, as expected, the FAM signal was not allele specific anymore, due to the simultaneous detection of the predominant SYBR green signal of the double-stranded amplicons at 62°C (Figure 2.A.1-2). So, in this experimental setup, FAM and SYBR green cannot be used together. A single melting peak at 81.5°C was generated for every sample (Figure 2.G.2; Supplementary Material 2). If nonspecific amplicons or oligo dimers had

Figure 1. Temperature-dependent fluorescence spectra of the fluorophores. Based on curves from the Fluorescence SpectraViewer (Life Technologies Europe BV, Ghent, Belgium) and data from Marras (13).
been produced, SYBR green, and possibly FAM signals, would produce an additional melting peak during melting curve analysis, necessitating assay optimization. The allele-specific FAM signals produced by the degraded probes during elongation will be incorporated in the melting curve as a constant signal, but will not be visible as a melting peak because a melting peak comes from a drop in fluorescence caused by higher temperatures melting a double-stranded amplicon (Figure 2.F-G.2). As a result, the peak height of a melting peak is generated only by SYBR green signals and reflects the total amount of amplicon produced by the primers, regardless of what part is detected by the allele-specific probes. In addition, the fluorescence detected in the melting curve at temperatures higher than the melting temperature of the amplicon is generated by the degraded FAM probes (during elongation) and reflect the amount of amplicon detected by the allele-specific probe (Figure 2.F-G.2).

Figure 2. Amplification plots of the fluorescent multiprobe assay for prion protein genotyping in sheep are shown with (A,E) FAM, (B) HEX, (C) Texas Red and (D) Cy5 detection, together with melting curves (F) and melting peaks (G). Plots 1 and 3 were generated with iQ Supermix (with all four probes) as a positive reference control. Plots 2 and 4 were generated with iQ SYBR green Supermix (with all four probes). Plots 5 and 6 were generated with iQ SYBR green Supermix (with all probes except FAM) to investigate the SYBR green interference with FAM detection. Plots 1, 2 and 5 were generated with fluorophore detection at the annealing temperature, while plots 3, 4 and 6 were generated with fluorophore detection at a temperature higher than the melting temperature of the amplicon. Assays were performed by different researchers and included technical and biological replicates. In order to give a clear overview of our findings, we only show 1 homozygous positive (+/+), 1 heterozygous (+/-), 1 homozygous negative (-/-) sample and a no template control (NTC) for each analyzed probe/fluorophore. Quantification cycle (Cq) and relative fluorescence unit (RFU) values of amplification plots, RFU values at 70°C and 95°C of the melting curves, and amplicon melting temperature (T) and peak height (-d(RFU)/dT) of melting peaks are listed in Supplementary Material 2.
Because the melting peak showed complete melting of the amplicon at 85°C (Figure 2.G.2), we investigated if it was indeed possible to specifically detect the FAM signal, in the presence of SYBR green, at a temperature higher than the melting temperature of the amplicon (i.e. 85°C). Marras (13) showed that the quantum yield of certain fluorophores (defined as the efficiency with which a fluorophore converts absorbed light into emitted light) decreases with increasing temperature. We designed experiment 3 to investigate the possibility of generating a sufficiently strong allele-specific signal by repeating experiment 1, but with detection of the fluorophores at an inserted step of 5 seconds at 85°C. (The fluorescent signals were still produced at the combined annealing/elongation step at 62°C; Supplementary Material 1). Although all Cq values were comparable to the ones from experiment 1 (except for Cy5), the RFU values dropped more than expected from the data of Marras (13; Figure 1; Figure 2.A-D.1-3; Supplementary Material 2). Due to a rise in the fluorophore detection temperature from 62°C to 85°C, FAM signals dropped by 50%, HEX signals by 40%, Texas Red signals by 50% and Cy5 signals even by 85%, but all signals were strong enough to allow allele-specific detection. We should note that the Cy5 signals were very low and the use of higher concentrations of the Cy5 probe did not improve the signal adequately. As a result, we do not recommend Cy5 labels for this experimental setup.

In experiment 4 we repeated experiment 2, but with the detection of fluorophores at a 5 seconds at 85°C step as in experiment 3 (Supplementary Material 1). The amplification plots in Figure 2.A.4 and the melting peaks in Figure 2.G.4 show that this experimental setup enables a differentiation between FAM and SYBR green signals in a single tube. In contrast to experiment 2, FAM signals were allele specific due to fluorophore detection at 85°C, which prevents SYBR green signal interference when measuring the allele-specific FAM signal because the amplicon is melted at this temperature. As in experiment 2, clear melting peaks were produced. As explained above, the FAM signals produced by the degraded probes during elongation will not interfere with melting peak formation because their fluorescence remains constant during melting curve analysis. In addition, amplification plots (i.e. RFU values, Cq values and shapes) generated for all other tested probes (Figure 2.B-D.3-4) were similar to those seen in experiment 3.

To confirm that both FAM and SYBR green signals in experiment 4 were specific, we repeated experiment 2 and 4 without adding the FAM probe (viz. experiment 5 and 6; Supplementary Material 1). With fluorophore detection at 62°C (experiment 5), the interference of the SYBR green signal in the amplification plot is clear (Figure 2.E.5; Supplementary Material 2). However, with fluorophore detection at 85°C (experiment 6), no signal is detected (Figure 2.E.6; Supplementary Material 2), proving that no SYBR green signal is generated at this temperature and that the signal observed in experiment 4 (Figure 2.A.4) is only produced by the FAM probe. The melting curves produced in experiment 5 show the drop of SYBR green fluorescence due to the melting of the double-stranded amplicon until a background level is reached, without the additional FAM signals produced by the degraded probes as in experiment 2. The melting peaks produced in experiment 5 and 6 are similar to the ones produced in experiments 2 and 4 (Figure 2.G; Supplementary Material 2).

In qPCR genotyping applications, combining specific sequence detection by fluorescently labeled probes with the generation of binding dye based melting peaks is useful for a number of reasons. First, nonspecific amplicons and oligo dimers are unwanted because they might influence the specificity of the assay and production of the amplicon of interest. They are easily detected by melting curve analysis and demand further optimization of the assay. Second, when testing different primer/probe concentrations during optimization, low probe signals can be probe related (suboptimal detection of the allele) or primer related (suboptimal production of the amplicon). A comparison of the RFU value of the amplification plot (reflecting the amount of amplicon detected by the allele-specific probe) with the height of the amplicon melting peak (reflecting the total amount of amplicon generated by the primers and detected by the binding dye) will indicate that a low probe signal is probe related (in case of a high melting peak) or primer related (in case of a low melting peak), and will demand probe or primer related optimization. Third, unknown variation in the probe binding site (i.e. in case of triallelic or additional SNPs) can lead to genotyping errors due to the inability of the probes to bind (14). Samples homozygous for these null alleles will be detected because they would not generate a signal. However, samples with one null allele will be genotyped incorrectly as homozygous for the detected allele. In comparison with control samples in an optimized assay, the comparison of the probe signal (reflecting the amount of amplicon detected by the allele-specific probe) with the height of the amplicon melting peak (reflecting the total amount of amplicon generated by the primers and detected by the binding dye) will provide an indication of the presence of a null allele if only one allele is detected and the probe signal is too small to account for the height of the melting peak. Such samples should be sequenced for verification.

Here, we showed that it is possible to differentiate between FAM and SYBR green signals in a single tube genotyping assay, despite the fact that their spectra are overlapping. This will provide extra flexibility when designing multi-probe genotyping assays. For optimal allele-specific detection, a few considerations should be taken into account: 1) if using a combination of fluorophores with spectra that do not overlap with the spectrum of SYBR green (e.g. HEX, Texas Red, Cy5), the fluorescence detection should be performed at the elongation temperature to assure high quantum yields (i.e. high signals) of the fluorophores; 2) if using a combination of fluorophores with spectra that do overlap with the spectrum of SYBR green (e.g. FAM), the fluorescence detection should be performed at a temperature higher than the melting temperature of the generated amplicon in order to circumvent the interference of SYBR green. In this case, we do not recommend fluorophores with quantum yields that drop substantially at higher temperatures (e.g. Cy5), since they may compromise allelic-specific detection. Instead, thermostable alternatives (e.g. ATTO647N for Cy5) can be used. Existing assays may be adapted by switching to qPCR buffers containing SYBR green (which are the same price as qPCR buffers without SYBR green), and in the case of FAM probes by adjusting the fluorescence detection temperature to perform an additional melting curve analysis. Depending on the detection system, both the probe detection and the melting curve analysis can be performed in a single run or in two consecutive runs. Similar guidelines can be applied for other fluorescent nucleic acid binding dyes.

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Competing interests
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References


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