Application of High Resolution Melting to SNP genotyping of porcine Ryanodine Receptor Gene

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INTRODUCTION

High Resolution Melting Analysis (HRM) is a recently developed technique for fast and cost-effective SNP genotyping based on the analysis of the melting profile of PCR products. A single point mutation in the porcine ryanodine receptor (RYR1) gene was associated with malignant hyperthermia (MH) disease (Fujii et al., 1991). This disease causes important losses in porcine industry due to manifestation of MH post-mortem signals in susceptible animals that affect detrimentally meat quality. As RYR1 mutation plays a key role in animal production, it is of main importance to implement a fast and economical method of diagnosis.

An HRM protocol has been developed to genotype the C/T substitution at nucleotide 1843 of RYR1 gene. With the Eco[TM] Real-Time PCR System we have analysed DNA samples extracted from porcine blood with different methods in order to test the effects of genomic DNA quality on melting behaviour.

MATERIALS AND METHODS

DNA samples. Porcine genomic DNA was extracted from whole blood samples using three different methods. A standard phenol-chloroform method, a commercial kit (QIAamp DNA Blood Mini kit automated on the QIAcube platform of Qiagen) and a lysis with proteinase K without further purification (Roura et al, 1999) were used. Quantity and quality of genomic DNA retrieved was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDropTechnologies).

PCR and HRM conditions. Sequences of primers were: RYR-F 5'- TTGACCCCTAGGTGCTGGAT-3' and RYR-R 5'-TGGTGACATAGTTGATGAGGTTTGT-3' with an amplicon length of 144 bp. PCR cycling and HRM analysis were carried out on the Eco[TM] Real-Time PCR System (Illumina). Experiments were performed with Kapa[TM] HRM Fast PCR kit (Kapa biosystems) which contains the high sensitive "release-on-demand" dye EvaGreen®. PCR amplifications were performed in a total volume of 10 µl containing 2 µl of DNA, 1x of master mix, primers at 200 nM each and $MgCl_2$ at 2.5 mM. Each sample was analyzed by duplicate. Thermal cycle was: 3 min at 95° C and 40 cycles of 15s at 95° C and 30s at 60° C. After completion of the amplification process, the samples were heated to 95° C for 30s and for HRM step, the PCR products were heated from 60ºC to 95ºC.

RESULTS AND DISCUSSION

The RYR1 HRM assay developed has validated by screening 18 homozygous wild-type, 16 heterozygous and 9 homozygous mutant genomic DNA samples extracted with the three different methods described above. All tested samples were previously genotyped by pyrosequencing.

HRM analysis allows clear discrimination between the homozygous and heterozygous samples based on differences in melting curve shapes and independently of the extraction method.

Figure 1. Melt curve profiles of DNA samples carrying different RYR1 genotypes.
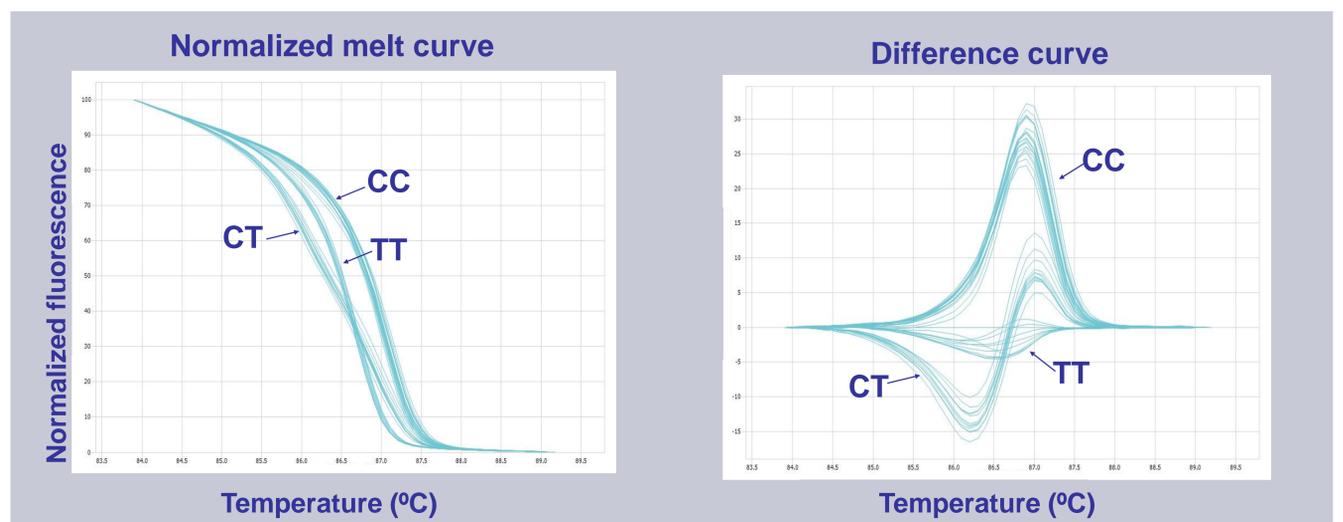
We have observed that using 10 ng of good quality DNA resulted always in a successful call. In lower quality samples, based on OD ratios, good results have been obtained decreasing DNA quantity to 2 ng. Expected problems associated with a proteinase k lysis protocol without further purification have been overcame with a dilution 1/10 of the sample with milli-Q water and incorporating 2 ul of the dilution to PCR reaction.
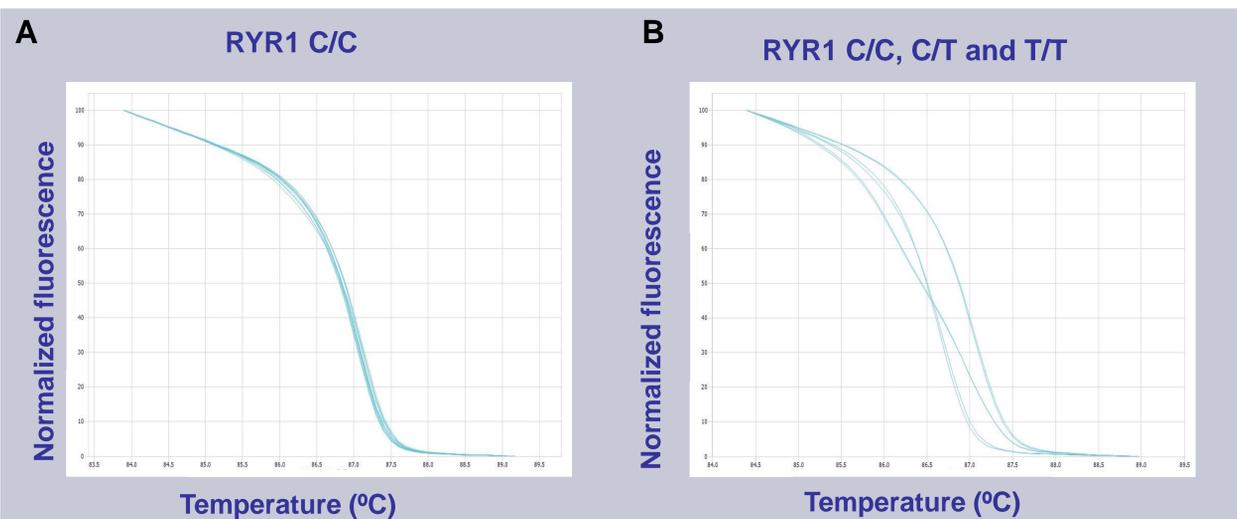
We have not found differences in the melting curve pattern between samples from the three different extraction protocols, as seen in figure 2.

In our study, quality of DNA has not been a limiting factor.

Figure 2. Normalized melt curve profiles from (A) five proteinase k lysed and six phenol-chloroform extracted blood samples of genotype C/C and from (B) one proteinase k lysed blood sample and one extracted with the QIAamp DNA Blood Mini kit of each genotype.

The HRM assay for RYR1 described here offers clear advantages over the PCR-RFLP methodology published by Fujii et al. (1991). Moreover, HRM requires less optimization than similar systems based on TaqMan and fluorescence resonance energy transfer (FRET) probes. Compared to these methods HRM is a simpler and more cost-effective way to characterize multiple samples. Identification of human RYR1 SNPs using HRM has been also described by Grievink and Stowell (2008).

In conclusion, the use of the HRM assay for porcine RYR1 genotyping considerably reduces hands-on-time and costs. In addition, it is a closed assay system requiring no post-PCR processing and results are comparable to conventional methods.

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