JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 7973−7978 **7973**

Evaluation of Post-Polymerase Chain Reaction Melting Temperature Analysis for Meat Species Identification in Mixed DNA Samples

MARÍA LÓPEZ-ANDREO, AMANDO GARRIDO-PERTIERRA, AND ANTONIO PUYET*

Departamento de Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain

Real-time uniplex and duplex polymerase chain reaction (PCR) assays with a SYBR Green I post-PCR melting curve analysis were evaluated for the identification and quantification of bovine, porcine, horse, and wallaroo DNA in food products. Quantitative values were derived from threshold-cycle (C_t) data obtained from serial dilutions of purified DNA. The limits of detection in uniplex reactions were 0.04 pg for porcine and wallaroo DNA and 0.4 pg for cattle and horse DNA. Species specificity of the PCR products was tested by the identification of peaks in DNA melting curves, measured as the decrease of SYBR Green I fluorescence at the dissociation temperature. The peaks could be distinguished above the background even at the lowest amount of template DNA detected by the C_t method. The system was also tested in duplex reactions, by use of either single-species DNA or DNA admixtures containing different shares of two species. The minimum proportions of each DNA species allowing the resolution of T_m peaks in the duplex reactions were 5% (cattle or wallaroo) in cattle/wallaroo mixtures, 5% porcine and 1% horse in porcine/horse mixtures, 60% porcine and 1% wallaroo in porcine/wallaroo mixtures, and 1% cattle and 5% horse in cattle/horse mixtures. A loss in the sensitivity of the method was observed for some DNA combinations in the duplex assay. In contrast, the results obtained from SYBR Green I uniplex and duplex reactions with single-species DNA were largely comparable to those obtained previously with species-specific TaqMan probes, showing the suitability of that simpler experimental approach for large-scale analytical applications.

KEYWORDS: Food authenticity; species identification; real-time PCR; cytochrome *b*; meat species

INTRODUCTION

The extensive development of technologies for identification and quantification of nucleic acids over the past decade reflects their importance in diagnostics, forensics, and food analysis. The application of fluorescent-based Polymerase Chain Reaction (PCR) assay formats greatly simplifies protocols for DNA and mRNA detection and quantification by using real-time analysis of PCR products during amplification (*1*). Currently, two formats for correlating the amount of DNA template with the fluorescent signal are available. The first one allows specific sequence detection and quantification by use of fluorescently labeled sequence-specific hybridization probes. Several types of probes [TaqMan (*2*), Molecular beacons (*3*), LightCycler (*4*), and Amplifluor (*5*)] can be used, all based on fluorescence resonance energy transfer. The second format (*6*) uses a DNA binding dye, like SYBR Green I, which binds to the minor groove of the double-stranded DNA in a sequence-independent way. Upon binding, its fluorescence increases over 100-fold. While probe-based methods have been shown to be useful for a large variety of analytical applications (*7*−*9*), SYBR Green I methods receive similar support due to its lower cost and simplicity (*10*−*12*). The main disadvantage of SYBR Green I methods is caused by the lack of sequence specificity of the fluorescent signal, which ultimately compels one to perform additional verifications of the PCR products, either by electrophoretic separation on agarose gels or by differentiation of the products by melting curve analysis (*13*), so that false positive signals due to unspecific amplification or the appearance of primer-dimers can be discarded.

The identification and quantification of animal species in food and feed products is one of the most rapidly developing fields of application for real-time PCR methods. Several methods have been proposed for the identification and, in some cases, quantification of several mammal, bird, and fish species, including beef, pork, lamb, chicken, turkey, mule duck, goose, ostrich, grouper, wreck fish, and Nile perch, either by use of fluorescently labelled probes (*8, 14*−*16*) or SYBR Green I methods (*10, 12, 17, 18*). While the majority of the SYBR Green I-based PCR methods for identification of species rely on the electrophoretic analysis of PCR products to confirm the specificity of the fluorescent signal, several authors propose the use of postrun melting-curve analysis, as a way to avoid the time-consuming and manual transfer operations associated with

* Corresponding author: fax +34 913943824; e-mail apuyet@vet.ucm.es.

10.1021/jf0615045 CCC: $33.50
Published on Web 09/22/2006

Table 1. Oligonucleotide Primers and Probes

oligonucleotide	sequence (5′ → 3′)	target	ref
	Forward Primers		
FBOS3	CAAGAACACTAATGACTAACATTCGAAAG	*B. taurus*	*16*
FC1SUS	GAAAAATCATCGTTGTACTTCAACTACA	*S. scrofa*	*16*
FEQUS2	CATCGTTGTATTTCAACTATAAGAACACC	*E. caballus*	this work
FMAC4	AAAAACCAATAAAATAGAAAATAAAAAAACAACTA	*M. robustus*	this work
	Reverse Primers		
RBOS1	AAATGTTTGATGGGGCTGGA	*B. taurus*	*16*
RSUS2	GGTCAATGAATGCGTTGTTGAT	*S. scrofa*	*16*
REQUS3	GGATGAGAAGGCAGTTGTC	*E. caballus*	this work
RMAC3	CTGAAATGTTTGAGGGTGCAGG	*M. robustus*	this work
	MGB Probe		
S1 (FAM)	CCACCCACTAATAAA	consensus *B. taurus, S. scrofa*	*16*

Figure 1. Alignment of the 5′cytB region of *Bos taurus*, *Sus scrofa*, *Equus caballus*, and *Macropus robustus*. The positions of target sequences for the forward and reverse primers are indicated as shaded and empty boxes, respectively. Targets for Taqman MGB probes for bovine and porcine DNA are labeled as black boxes. S: position of the *cyt*B start codon.

agarose or polyacrylamide electrophoresis (*11*, *12*, *19*). In addition, some of the proposed methods made use of multiplex PCR reactions, allowing the detection of two or more species (or transgenic material) by use of a unique PCR reaction premix. Ultimately, if a correlation between the T_m peak height (or the area under the peak) and the number of copies of the template DNA in multiplex reactions is assumed, it might be possible to estimate the proportions for each species DNA in the mixture. However, the behavior of methods using the melting curve approach on mixed-species DNA samples and using multiplex reactions has not been fully evaluated yet.

In a previous work, we showed the feasibility of using a combination of species-specific primers and group-specific TaqMan probes for the detection and quantification of beef, pork, lamb, chicken, turkey, and ostrich DNA in complex mixtures (*16*). As a single fluorescent probe is shared by several species DNA, the method is significantly cheaper and simpler to run than species-specific probe methods. However, the extension of the method to other species may require the design of sequence-specific probes. The recurring health safety hazards associated to the consumption of meat in the last years have propelled the commercialization of less frequent species in European markets. Here, we have developed an alternative duplex SYBR Green I/melting curve approach for the identification of horse and kangaroo species, for which the use of the common beef/pork/lamb probe was not possible due to the presence of base mismatches at the target sequence. Foremost, the present work aims to evaluate and compare the results obtained with both real-time PCR formats for the detection and identification of meat species.

MATERIALS AND METHODS

DNA Extraction. Meat samples were obtained from commercial sources, and the DNA species were confirmed by sequencing of the *cytB* gene and its comparison with published sequences at NCBI GenBank. The samples of kangaroo meat matched the *cytB* sequence of *Macropus robustus* (wallaroo). DNA was purified from 30 mg of raw material by use of a Puregene DNA purification kit from Gentra Systems and following the manufacturer's protocol for solid tissue samples. Before extraction, muscle samples were ground to a fine powder in a hand-held macerator after having first been freeze-dried. Two independent extractions were performed from each sample and DNA was quantified after staining with Picogreen DSDNA quantification reagent (Molecular Probes), by use of a Perkin-Elmer LS-50B fluorescence spectrophotometer and comparison of the fluorescence with that from DNA standards of known concentration.

Primer and Probe Design. The primers were designed to amplify the mitochondrial region encompassing the 3′ end of ND6 and the 5′ end of *cytB* gene. The primer sequences were selected in such a way to include at least two species-specific base positions, one of them located at the 3′ end of the oligonucleotide. The sequences of primers and probes are summarized in **Table 1**. The amplicon lengths were as follows: cattle, FBOS3/RBOS1, 92 base pairs (bp); pork, FCSUS1/RSUS2, 100 bp; horse, FEQUS2/REQUS3, 250 bp; and wallaroo, FMAC4/RMAC3, 205 bp.

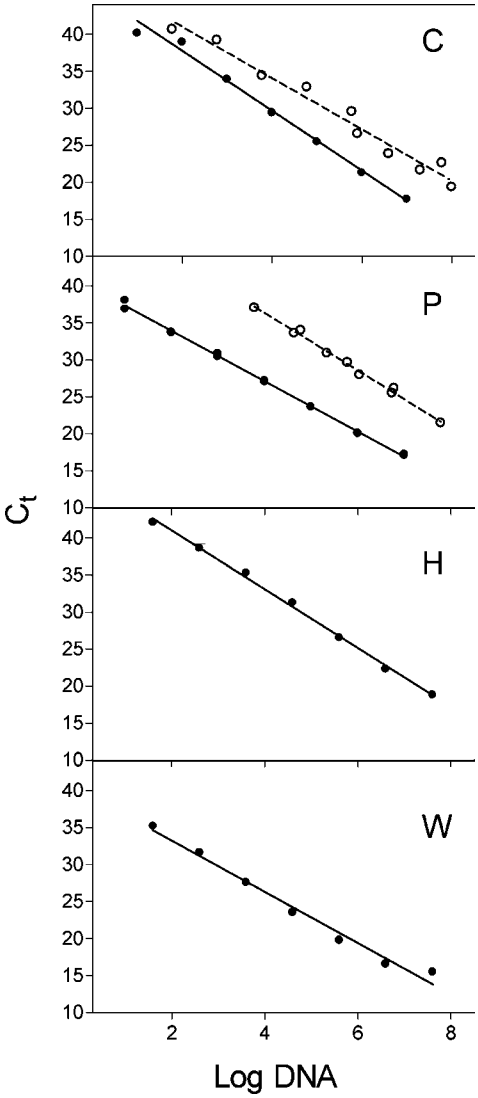

Figure 2. Quantitation ranges and linearity of the PCR assays. The effective ranges for cattle (C), pork (P), horse (H), and wallaroo (W) specific assays are shown for SYBR Green I (●) or S1 Taqman MGB probe (○). Ten-fold dilution series of DNA starting from 40 ng to 0.04 ng were used as standard in the reactions. The average C_t values obtained from three replicates (SD < 0.6) are plotted versus the logarithm of template DNA (in femtograms).

Real-Time PCR Analysis. DNA detection was performed by amplification by use of the real-time sequence-detection system 7000 from Applied Biosystems. Reactions were carried out by duplicate in 96-well plates. The 25 μL reaction mixtures contained 12.5 μL of SYBR green master mix (which includes the heat-activated AmpliTaq Gold enzyme), 2 μL of different dilutions of the DNA mixture, corresponding to 0.010 pg−50 ng of total DNA, and 300 nM of each primer oligonucleotide for uniplex reactions. In duplex reactions the concentrations (nanomolar) were optimized as follows: 150 (cattle)/900 (wallaroo), 150 (cattle)/900 (horse), 300 (pork)/300 (wallaroo), and 150 (pork)/900 (horse). The reaction conditions were 10 min at 95 °C for enzyme activation and 50 cycles of 15 s at 95 °C followed by 1 min at 55 °C. Readings were taken every cycle, and the logarithm of the increment in fluorescence was plotted versus the cycle number. The threshold level was fixed at the same midexponential position for all runs. The dissociation curves of the PCR products were monitored on the same instrument, and the derivatives of fluorescence values were plotted at 0.1 °C intervals from 60 to 95 °C. The signal-to-noise ratio R for each species-specific primer pair was calculated as

$$R = E^{C^s} - C^{s,x} \quad (1)$$

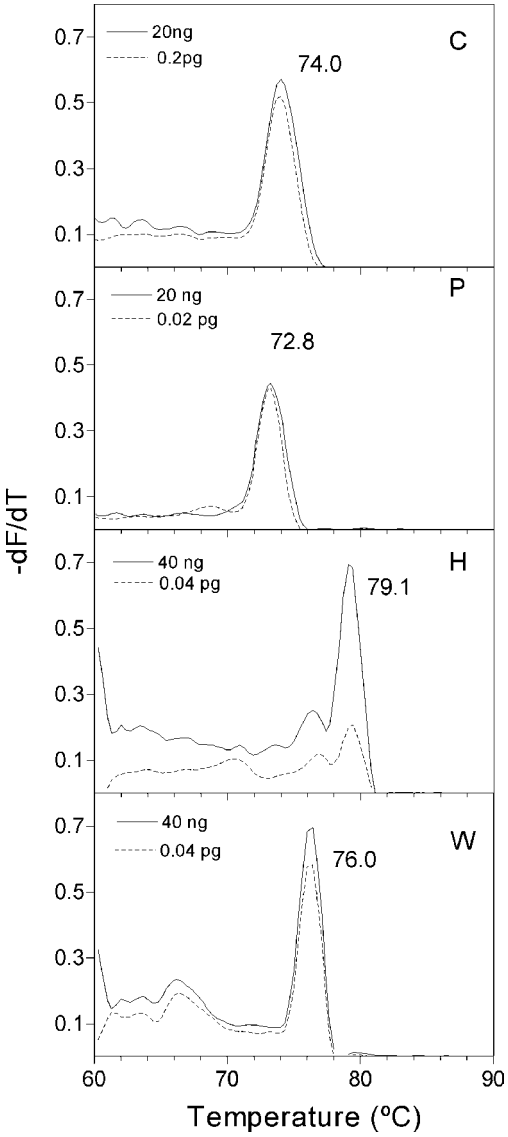

Figure 3. Melting temperature analysis of the PCR products in uniplex reactions. Melting curves for PCR products, obtained from purified DNA from wallaroo (W), horse (H), cattle (C), and pork (P), were transformed into first-derivative melting peaks. Representative results from duplicate experiments are shown for saturating (solid line) and limiting (dotted line) amounts of template DNA that allow the identification of the peaks. The average T_m values (degrees Celsius) are indicated at the peak positions.

where E is the efficiency of the PCR reaction for each primer pair, C^s is the C_t obtained with the target species DNA, and $C^{s,x}$ is the C_t obtained from a reaction containing the same amount of nontarget DNA.

RESULTS AND DISCUSSION

Initial Validation of the Method. The PCR assay was designed to test the resolution and sensitivity of a SYBR Green/melting curve analysis for the identification of mammal species DNA. Horse and wallaroo DNAs were selected as examples of non-commonly marketed species, for which few or no identification assays are available yet. The inclusion of beef and pork DNA in the assays allows the comparison of results with those reported for different real-time PCR based methods. A DNA alignment showing the positions of primers and probes for all four sequences is displayed in **Figure 1**. The sensitivity, specificity, and PCR amplification efficiency for each primer pair with the SYBR green format was calculated by plotting

Table 2. Efficiency and Specificity of Real-Time PCR Reactions

		std curve data			signal-to-noise ratio[a]			
primer pairs	label	slope	PCR efficiency	limit of detection (pg)	cattle	pork	wallaroo	horse
FBOS3/RBOS1	SYBR	–4.06	1.8	0.40	1	ND	10^{-4}	2×10^{-4}
	Taqman	–3.5	1.9	0.03	1	6×10^{-5}	ND[b]	ND
FC1SUS/RSUS2	SYBR	–3.40	2.0	0.04	N.D.	1	2×10^{-7}	7×10^{-6}
	Taqman	–3.88	1.8	0.07	7×10^{-5}	1	ND	ND
FMAC4/RMAC3	SYBR	–3.32	2.0	0.04	6×10^{-9}	3×10^{-9}	1	ND
FEQUS2/REQUS3	SYBR	–3.65	1.9	0.40	2×10^{-8}	10^{-8}	ND	1

[a] Ratio R of target to nontarget DNA amplification was detected by use of 40 ng of DNA as template. R values were calculated from eq 1. [b] ND, not determined.

the C_t (cycle threshold) values versus the logarithm of target DNA amount (*1*), using serial dilutions of 40 ng of purified DNA from each species (**Figure 2**). The data derived from the plots are summarized in **Table 2**. Results obtained with Taqman MGB probes for bovine and porcine DNA are also included for comparison. Real-time PCR data obtained from different primer pairs are fully comparable only when the experimental efficiencies (E) for the different reactions are similar and approach the theoretical optimum of 2 (one doubling in the number of copies of the target DNA per PCR cycle). The efficiencies found were similar for all four primer pairs, with an average $E = 1.92 \pm 0.10$, which approaches the optimal of one duplication in the number of copies per cycle. The C_t registered showed a linear correlation with decreasing logarithm of template DNA (coefficient of correlation >0.99) in the range from 40 ng to 0.04 pg for porcine, horse, and wallaroo and 40 ng to 0.2 pg for bovine DNA. As some residual fluorescence could be detected in the negative template controls after the 40th PCR cycle, the limits for detection in uniplex reactions for these species were established as the amount of template DNA yielding a C_t significantly different ($p \leq 0.05$) from 40 in the linear range of the standard curve. These values were 0.4 pg for cattle and horse ($C_t = 37.06 \pm 0.61$ and 38.60 ± 0.63, respectively), and 0.04 pg for pork and wallaroo ($C_t = 36.25 \pm 0.68$ and 35.17 ± 0.21, respectively). Cross-reaction of the primers with unspecific species DNA was checked by running control real-time PCR reactions and calculation of the signal-to-noise ratio (R) of target to no-target DNA amplification. As shown in **Table 1**, the R values obtained with 40 ng of nonspecific DNA ranged from 10^{-4} to 10^{-9}. The absence of unspecific amplification products was further confirmed by electrophoresis in agarose gels (data not shown). While the Taqman approach allows the detection of smaller amounts of cattle DNA than the SYBR Green I protocol, the limit of detection for pork with FCSUS1/RSUS2 primers and SYBR Green I chemistry is similar to that achieved with TaqMan probes. Similar results showing comparable sensitivities for TaqMan and SYBR Green I detection methods have been reported for amplicons based in short interspersed elements in pork DNA (*18*). This suggests that, at least for 5′-FAM-labeled TaqMan probes, the inherent advantage in the specificity of the fluorescent signal may not necessarily be accompanied by an increase in the sensitivity of the method. The lower limit of detection for horse DNA reported here, 5 times lower than the value obtained with the nuclear growth hormone gene as target gene (*14*), is likely due to the higher copy number of mitochondrial DNA as template. A different copy number of target mitochondrial DNA among species also may be a possible explanation for the differences found in the cattle and pork limits of detection; however, these differences were not likewise observed when labeled Taqman probes were used. Other explanations, such as SYBR Green fluorescent signal variations depending on the amplicon size and sequence might also explain those differences. Thus, both DNA and detector characteristics and also the labeling approach appear to have an effect on the limit of detection reached.

The melting curve profiles obtained from the real-time PCR products are shown in **Figure 3**. Major peaks were observed for each uniplex reaction at 40 ng of template DNA at the following melting temperatures (average $T_m \pm$ standard deviation of four independent measurements): 74.0 ± 0.2 (cattle), 72.8 ± 0.2 (pork), 76.0 ± 0.08 (wallaroo), and 79.1 ± 0.2 (horse). When limiting amounts of DNA for each assay are used as template, the peaks were likewise distinguished above the background. Remarkably, DNA amounts below the limit of detection by C_t can be identified by the melting curve analysis for cattle, pork, and horse. As expected from a PCR final product, the size of the peaks was approximately constant throughout the standard curve.

To assess the specificity of the melting curve analysis when a mixture of DNA from different species is present in the PCR, a control experiment was run by using as template a four-species admixture containing 20 ng of each DNA. The melting curve results for each detector, shown in **Figure 4**, indicate that the position and specificity of the peaks are not affected by the presence of nontarget DNA. These results validate the use of post-PCR melting curve analysis as a control of the reaction specificity even for complex DNA samples.

Species Detection by Use of Duplex PCR Reactions. Assuming that T_m differences above 2 °C may allow discrimination of the PCR products, it was possible to arrange horse−pork, horse−cattle, wallaroo−pork, and wallaroo−cattle duplex reactions. As compared to uniplex reactions, no significant up- or downshifts (<0.2 °C) of T_m were observed when duplex reactions on a single species DNA were used, suggesting that the presence of the extra pair of primers in the PCR does not interfere with the amplification of the specific amplicon (not shown). The ability to detect and discriminate DNA from horse and wallaroo in mixed-template duplex reactions was tested by comparing the melting curves obtained from the PCR products. A series of DNA mixtures, containing (in percentage) 1/99, 5/95, 10/90, 40/60, 50/50, 60/40, 90/10, 95/5, and 99/1 ratios of cattle/horse, cattle/wallaroo, pork/horse, and pork/wallaroo was prepared in duplicate and amplified as before but by use of duplex PCR systems. The total amount of DNA in the mixtures was 40 ng. As shown in **Figure 5**, the melting curves obtained displayed peaks at the T_m expected for each species. The highest T_m deviation from the uniplex reaction was observed in wallaroo−pork mixtures, with an average T_m downshift of 0.65 ± 0.07 °C for wallaroo (average of all wallaroo−pork mixtures). **Figure 5** also shows the melting curves of DNA mixtures that allow discrimination of the peaks at the minimum proportions of each DNA species. Thus, the smaller percentage allowing identification of the peaks in double-species duplex reactions were established as follows: 5% (cattle or wallaroo) in cattle/wallaroo mixtures, 5% pork and 1% horse in porcine/horse

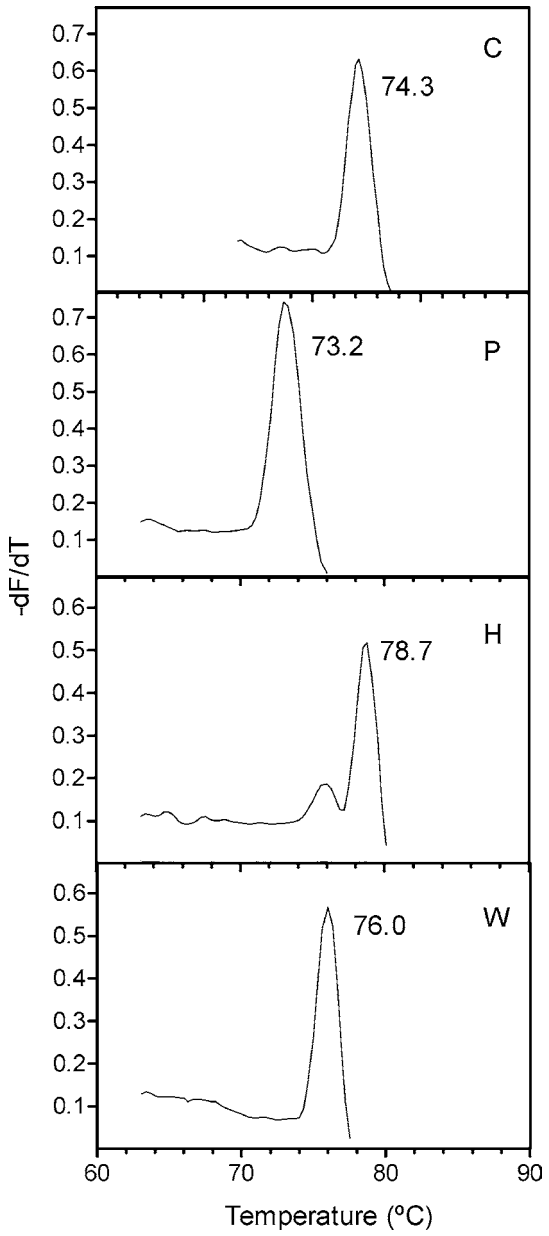

Figure 4. Melting temperature analysis of PCR products in uniplex reactions run on DNA admixtures. First-derivative melting peaks are shown for PCR products obtained from wallaroo (W), horse (H), cattle (C), and pork (P) primer pairs on a DNA mixture containing 20 ng each beef, pork, wallaroo, and horse DNA. The average T_m values (degrees Celsius) are indicated at the peak positions.

mixtures, 60% pork and 1% wallaroo in porcine/wallaroo mixtures, and 1% cattle and 5% horse in cattle/horse mixtures. In all cases, 1% corresponds to 0.4 ng of DNA. The criterion for positive identification of the peaks was to observe at least a 1% $-dF/dT$ increment above the baseline at the expected T_m in all duplicates.

The curve profiles also showed that, depending on the species mixture, the height of the peaks did not reflect necessarily the actual proportions of template DNA. Cattle/wallaroo 95/5 mixtures produce comparable peaks, while the signal for porcine DNA is underestimated in pork/wallaroo mixtures, in such a way that the peak is not detected in mixtures containing less than 50% pork, which corresponds to 20 ng of porcine DNA. In contrast, such amount of DNA template was correctly detected in FCSUS1/RSUS2 uniplex reactions. Similarly, a fair

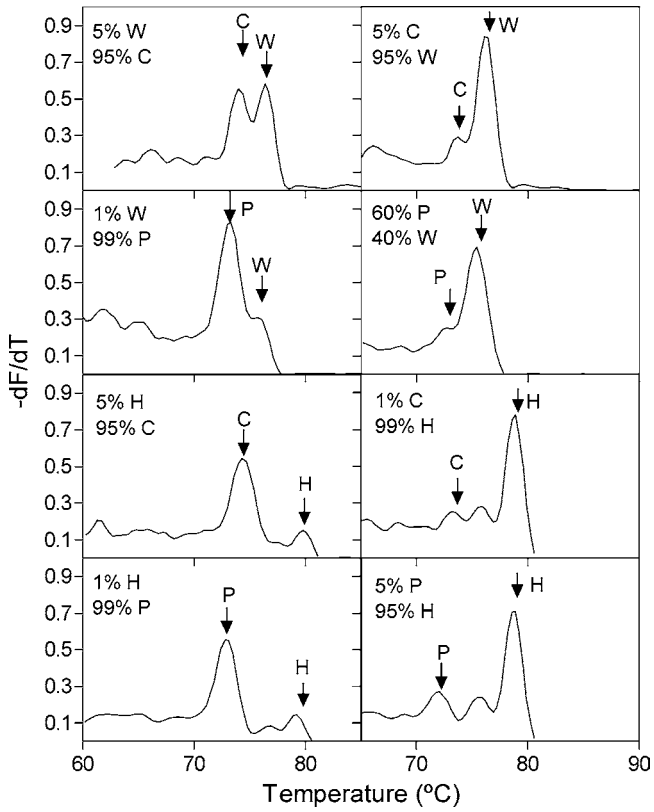

Figure 5. Melting curve analysis of PCR products obtained from duplex reactions on DNA admixtures. The panels show representative results obtained from duplicate independent experiments containing DNA admixtures of wallaroo (W), horse (H), cattle (C), and pork (P) at the percentages indicated. Only the curves corresponding to assays allowing the differentiation of peaks at the lowest percentage for each species DNA are displayed. The position of the T_m values obtained in the uniplex reactions for each species are indicated by arrows.

correlation between the relative height of the T_m peaks and the actual proportions of mixed-DNA templates has been reported by others for uniplex reactions detecting two different PCR products (*19*). These results suggest that the real-time SYBR Green I format can yield accurate results when either mixed-DNA templates in uniplex reactions or duplex PCR assays on single-DNA templates are used, but the combination of multiplex reactions with mixed-species DNA may produce a sensitivity loss for some DNA combinations. Although duplex reactions on mixed samples require larger amounts of the less abundant species DNA for detection, a 1−5% sensitivity may be sufficient for most practical applications. Identification of genetically modified material by means of duplex real-time PCR followed by melting curve analysis has been shown to be feasible with a sensitivity of 0.1% (*11*). The results reported here indicate that the applicability of this multiplex approach to meat species may largely be dependent on the choice of species to be identified. Such a drawback may restrict the widespread use of mutiplex SYBR Green I analysis for the identification and/or quantification of species in complex samples. In contrast, the performance (in terms of PCR efficiency, detection limits and resolution of species-specific amplicons) of the SYBR Green I format for uniplex reactions (or duplex reactions on single-species DNA) shown here appears to be comparable to that obtained by utilization of TaqMan-based amplicons. Thus, the lack of correlation between the peak size and the amount of DNA in the sample do not invalidate the use of the SYBR Green-based detectors for quantitative analysis, it just advises

against its use in a multiplex format for identification in complex samples. Quantitative data can be derived in any case from C_t values obtained from uniplex reactions. This may promote the use of the SYBR Green I format for uniplex reactions in detriment of fluorescently labeled probes, due to its lower cost and higher simplicity.

LITERATURE CITED

(1) Higuchi, R.; Fockler, C.; Dollinger, G.; Watson, R. Kinetic PCR analysis: real-time monitoring DNA amplification reactions. *Biotechnology* **1993**, *11*, 1026−30.

(2) Holland, P. M.; Abramson, R. D.; Watson, R.; Gelfand, D. H. Detection of specific polymerase chain reaction product by utilizing the 5′−3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci.* **1991**, *88*, 7276−7280.

(3) Tyagi, S.; Kramer, F. R. Molecular beacons probes that fluoresce upon hybridization. *Nat. Biotechnol.* **1996**, *14*, 303−308.

(4) Wittwer, C. T.; Ririe, K. M.; Andrew, R. V.; David, D. A.; Goudry, R. A.; Balis, U. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **1997**, *22*, 176−181.

(5) Uehara, H.; Nardone, G.; Nazarenko, I.; Omán, R. J. Detection of Telomerase Activity Utilizing Energy Transfer Primers: Comparison with Gel- and ELISA-Based Detection. *Biotechniques.* **1999**, *26*, 552−558.

(6) Wittwer, C. T.; Herrmann, M. G.; Moss, A. A.; Rasmussen, R. P. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* **1997**, *22*, 134−138.

(7) Rott, M. E.; Lawrence, T. S.; Wall, E. M.; Green, M. J. Detection and quantification of roundup ready Soy in foods by conventional and real-time polymerase chain reaction. *J. Agric. Food Chem.* **2004**, *52*, 5223−5232.

(8) Rodríguez, M. A.; García, T.; González, I.; Asensio, L.; Hernández, P. E.; Martín, R. Quantitation of mule duck in goose foie gras using TaqMan real-time polymerase chain reaction. J. *Agric. Food Chem.* **2004**, *52*, 1478−1483.

(9) Ballester, M.; Castelló, A.; Ibáñez, E.; Sánchez, A.; Folch, J. M. Real-time quantitative PCR-based system for determining transgene copy number in transgenic animals. *Biotechniques* **2004**, *37*, 610−613.

(10) Walker, J. A.; Hughes, D. A.; Hedjes, D. J.; Anders, B. A.; Laborde, M. E.; Shewale, J.; Sinha, S. K.; Batzer, M. A. Quantitative PCR for DNA identification based on genome-specific interspersed repetitive elements. *Genomics* **2004**, *83*, 518−527.

(11) Hernández, M.; Rodríguez-Lázaro, D.; Esteve, T.; Prat, S.; Pla, M. Development of melting temperature-based SYBR Green I polymerase chain reaction methods for multiplex genetically modified organism detection. *Anal. Biochem.* **2003**, *323*, 164−170.

(12) Trotta, M.; Schönhuth, S.; Pepe, T.; Cortesi, M. L.; Puyet, A.; Bautista, J. M. Multiplex PCR method for use in real-time PCR for identification of fish fillets from grouper (*Epinephelus* and *Mycteroperca* sp.) and common substitute species. *J. Agric. Food Chem.* **2005**, *53*, 2039−2045.

(13) Ririe, K. M.; Rasmunssen, R. P.; Wittwer, C. T. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.* **1997**, *245*, 154−160.

(14) Brodmann, P. D.; Moor, D. Sensitive and semi-quantitative TaqMan real-time polymerase chain reaction systems for the detection of beef (*Bos taurus*) and the detection of the family *Mammalia* in food and feed. *Meat Sci.* **2003**, *65*, 599−607.

(15) Laube, I.; Spiegelberg, A.; Butschke, A.; Zagon, J.; Shauzu, M.; Kroh, L.; Broll, H. Methods for the detection of beef and pork in foods using real-time polymerase reaction. *Int. J. Food Sci. Technol.* **2003**, *38*, 111−118.

(16) López-Andreo, M.; Lugo, L.; Garrido-Pertierra, A.; Prieto, M. I.; Puyet, A. Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. *Anal. Biochem.* **2005**, *339*, 73−82.

(17) Sawyer, J.; Wood, C.; Shanahan, D.; Gout, S.; McDowell, D. Real-time PCR for quantitative meat species testing. *Food Control* **2003**, *14*, 579−583.

(18) Walker, J. A.; Hughes, D. A.; Anders, B. A.; Shewale, J.; Sinha, S. K.; Batzer, M. A. Quantitative intra-short interspersed element PCR for species-specific DNA identification. *Anal. Biochem.* **2003**, *316*, 259−269.

(19) Freitas, J. M.; Lages-Silva, E.; Crema, E.; Pena, S. D. J.; Macedo, A. M. Real time PCR strategy for the identification of major lineages of *Trypanosomoa cruzi* directly in chronically infected human tissues. *Int. J. Parasitol.* **2005**, *35*, 411−417.

Received for review May 29, 2006. Revised manuscript received August 17, 2006. Accepted August 24, 2006. This work was supported by Grant AGL2001-1423 from the Ministerio de Ciencia y Tecnología of Spain.

JF0615045