A novel triplex real-time PCR method for the simultaneous authentication of meats and antlers from sika deer (*Cervus nippon*) and red deer (*Cervus elaphus*)

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Research Article

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Abstract

Deer products have been used as a traditional Chinese medicine for over 2000 years. Due to the high price, the meats and antlers are often adulterated with low-value materials. The present study developed a triplex real-time PCR assay to simultaneously identify the DNA from sika deer and red deer in deer products. The triplex real-time PCR approach showed high specificity for 16 animal species, and the method simultaneously amplified an endogenous control to eliminate false-negative results. The absolute limits of detection for raw meat, meat product, and antlers were 0.05–0.025 pg, 0.05–0.025 pg, and 0.1–0.025 pg, respectively, for sika deer and 0.05–0.025 pg, 0.5–0.25 pg, and 2.5–0.25 pg, respectively, for red deer. This approach could determine 0.1% (w/w) adulteration. In conclusion, the established triplex real-time PCR assay displayed high specificity and sensitivity and can be used for authentic animal products from sika deer and red deer.

Introduction

Deer products have been used in traditional Chinese medicines for over 2000 years as vitalizing, tonifying, hemopoietic, and strengthening agents for debilitation persons, mainly for treating Yang deficiency. The medicinal effects have been recorded in Li Shizhen's Compendium of Material Medica. In China, there are mainly two deer species: the sika deer (*Cervus nippon*) and the red deer (*Cervus elaphus*). Venison, the main game meat, is prone to adulteration due to its high price. Moreover, huge differences in the efficacy of phamacognosy and price between antlers from sika deer and red deer also have led to adulteration in the consumer market. Therefore, identification and authentication of deer products are essential for fair trade and consumer rights.

Due to high specificity and sensitivity and the high-throughput and low-cost traits, molecular biology techniques, such as DNA-sequencing and PCR, have been used for the authentication of anminal products (Guo et al. 2020; Hai et al. 2020; Liu et al. 2021; Scales et al. 2021; Guo et al. 2022; Vieira et al. 2022). In recent years, PCR-based approaches have been used to identify and authenticate deer meat (Druml et al. 2015; Kaltenbrunner et al. 2018) and antlers (Jiang et al. 2018; Yang et al. 2020), and multiplex PCR assay was developed to identify deer products including meats and antlers (Zha et al. 2011). However, no study has used the multiplex real-time PCR technique to identify and authenticate meat and antlers from different deer species (*Cervus nippon*, *Cervus elaphus*), and the endogenous control in the multiplex real-time PCR related to deer authentication has not yet been reported. Therefore, the present study aimed to develop a triplex real-time PCR technique with an endogenous control to simultaneously identify the DNA in meats and antlers from sika deer and red deer so as to authenticate deer products in practice.

Materials And Methods

Sample Preparation and DNA Extraction

Meat samples from deer were collected from the supermarket in Xilinhot, China, and the deer antlers were bought from the Jingdong online store. These samples were cut into small pieces and stored at −80°C to prevent DNA degradation. DNA was extracted from these samples using Takara MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa Bio, Dalian, China), according to manufacturer's protocol. The concentration and purity of the extracted DNA were determined based on the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) (ThermoFisher Scientific, Waltham, USA). The DNA samples with an A_{260}/A_{280} value of approximately 1.8 were used for real-time PCR, using a standard working concentration of 100 ng/μL.

Primer and Probe Design

Primers targeting a conserved region across species (species-conserved primers) were designed for the compatibility of three different probes for sika deer, red deer, and endogenous control, and species-specific probes were developed for the specificity of two probes for sika deer and red deer (Fig. 1). Species-conserved probe for endogenous control was designed to eliminate false negatives (Fig. 1). Primer and probe sequences, length, and final concentration used in this study are shown in Table 1. Different fluorescent reporters, fluorescent reporters 6-carboxyfluorescein (FAM), hexacholoro-6-corboxyfluorescein (HEX) and carboxy-X-rhodamine (ROX), were utilized to label sika deer, red deer, and the control probe for the triplex real-time PCR.

Triplex real-time PCR

Triplex real-time PCR was performed in an optical 96-well reaction plate (200 μL, Axygen, Hangzhou, China) sealed with an optical adhesive film on an ABI 7300plus Real-time PCR System (Applied Biosystems). The PCR was performed in a total volume of 20 μL, comprising 10 μL of probe quantitative PCR supermix, 1 μL forward primer (FP; 10 μM) and 1 μL reverse primer (RP; 10 μM), 0.5 μL each of Sika-FAM (10 μM), Red-HEX (10 μM), and Control-ROX (10 μM), 2 μL of DNA (100 ng/μL), and 4.5 μL of nuclease-free water (Transgen, China). The reaction was performed using the following thermocycling program: an initial denaturation for 30 s at 94 °C, followed by amplification for 40 cycles of 5 s at 94 °C and 31 s at 60 °C.

Evaluation of Specificity

DNA extracts from sika deer, red deer, and other animal species (cattle, buffalo, yak, donkey, horse, sheep, goat and pig) were used as the templates to test the specificity of the triplex real-time PCR. PCR was carried out with 200–250 ng DNA per tube/well (100 ng/μL DNA). The Ct (cycle threshold) values were confirmed using four independent meat samples related to sika deer or red deer (3 replicates per run), and one meat sample from another species was used to verify the specificity (3 replicates per run).

Evaluation of Sensitivity and Authentication Ability

The limit of detection (LOD) of the triplex real-time PCR was determined by assaying dilutions of DNA in 20 replicates following the recommendations of the European Network of GMO Laboratories (Marchesi et al., 2015). The LOD values were calculated using 10-fold and 2-fold serial dilutions of DNA from deer

meat and antlers (100, 10, 1, 0.1, 0.01, 0.005, 0.0025, 0.001, 0.0005, 0.00025, 0.0001, 0.00005, 0.000025 and 0.00001 ng/μL). The LOD was defined as the lowest concentration that led to an increase in the fluorescence signal within 40 cycles (95% confidence limit) (Finney, 1971). The authentication ability of the triplex real-time PCR was evaluated by assaying the DNA mixture in 20 replicates in the artificial binary mixtures containing venison (ranging in concentration from 0.1% to 99.9%) mixed with chicken. Five independent DNA samples from the binary mixtures were used to confirm the results. First, binary meat mixtures containing sika deer and chicken meat, the percentages of sika deer meat in the mixtures were 0.1%, 1%, 10%, 30%, 70%, 90%, 99%, and 99.9% (w/w), and the corresponding percentage of chicken meat in the mixtures were 99.9%, 99%, 90%, 70%, 30%, 10%, 1%, and 0.1% (w/w). Second, binary meat mixtures containing red deer meat and chicken, the percentages of red deer meat in the mixtures were 0.1%, 1%, 10%, 30%, 70%, 90%, 99%, and 99.9% (w/w), and the corresponding percentage of chicken in the mixtures were 99.9%, 99%, 90%, 70%, 30%, 10%, 1%, and 0.1% (w/w). All samples were immediately stored at -20ºC until further analysis.

Results And Discussion

Specificity of the Triplex Real-time PCR

The specificity of the triplex real-time PCR was confirmed using DNA samples from deer and other animal species. The Sika-FAM amplification curves were specifically observed with sika deer meat (Fig. 2A), sika deer processed meat (Fig. 2B), and sika deer antlers (Fig. 2C), while the red-HEX amplification curves were specifically observed with red deer meat (Fig. 2D), red deer processed meat (Fig. 2E), and red deer antlers (Fig. 2F). The amplification curves of control-ROX, the endogenous control, were synchronously and steadily amplified to validate the triplex real-time PCR in the normal state of amplification and control false negatives effectively. As shown in Table 2, the Ct values (average ± SD) of the triplex real-time PCR were consistent with the amplification curves. These results indicated that the species-specific probes had high specificity for sika deer and red deer in meat and antlers, and the species-conserved probe was a competent endogenous control. The observations suggest that the developed triplex real-time PCR with the designed primers and probes can effectively identify the target DNA from meat and antlers.

Sensitivity of the Triplex Real-time PCR

The LOD of the developed triplex real-time PCR assay was examined using 10-fold and 2-fold serial dilutions of DNA from meat and antlers. The LOD for sika deer meat, sika deer processed meat, and sika deer antlers were 0.05–0.025 pg (Fig. 3A), 0.05–0.025 pg (Fig. 3B), and 0.1–0.025 pg (Fig. 3C), respectively (confidence limit: 95%; Table 3). The LOD for red deer meat, red deer processed meat, and red deer antlers were 0.05–0.025 pg (Fig. 3D), 0.5–0.25 pg (Fig. 3E), and 2.5–0.25 pg (Fig. 3F), respectively (confidence limit: 95%; Table 3). These observations showed that the developed triplex real-time PCR is sensitive for the identification of DNA from meat and antlers from sika deer and red deer. The LOD of the method was significantly lower than in previous studies based on the developed multiple PCR assay, 0.05 ng for sika deer and 0.5 ng for red deer (Zha, et al., 2011). Although the economic cost of the real-time PCR base on probe is higher than conventional PCR, multiplex real-time PCR has demonstrated incomparable advantages in efficiency and sensitivity. Further, to determine the linearity of the triplex real-time PCR, DNA from meat and antlers were serially diluted (10-fold) and used as the templates. The obtained Ct values were plotted against the logarithmic DNA concentrations to construct the standard curve. The standard curve of sika deer meat (Fig. 4A), sika deer processed meat (Fig. 4B), sika deer antlers (Fig. 4C), red deer meat (Fig. 4D), red deer processed meat (Fig. 4E), and red deer antlers (Fig. 4F) are shown in Fig. 4. The slope of the calibration curve, efficiency, and coefficient of determination (R^2) for the above six samples are shown in Table 4. These results demonstrated high sensitivity of the real-time PCR, with good potential to identify deer DNA.

Authentication Ability of the Triplex Real-time PCR

The deer-based products are generally adulterated, and it is difficult to determine the ingredients based on visual inspection and conventional analysis. Therefore, the triplex real-time PCR should possess good authentication ability and detect the presence of other animal samples. We tested the authentication ability of the triplex real-time PCR using mixed samples (venison mixed with chicken in the eight different ratios). As shown in Table 5 and Table 6, the Ct values (mean ± SD) of the triplex real-time PCR assay increased with decreasing concentration of the corresponding component in the mixtures, and the Ct values were relatively stable in the five independent mixtures. The developed technique was able to detect as low as 0.1% target species (sika deer and red deer) in the mixed samples (Table 5 and Table 6), which is equal to or lower than the already reported methods (Druml et al., 2015; Kaltenbrunner et al., 2018). In short, the authentication ability of the triplex real-time PCR is adequate to identify the target species (sika deer and red deer) in the mixed samples.

Conclusions

The present study developed a triplex real-time PCR technique to authenticate venison and deer antlers. This method was based on species-conserved primers, species-specific probes, and the conserved probe for endogenous control, which target the conserved-specific-conserved region of the mitochondrial DNA through careful screening. The technique enables the simultaneous identification of DNA from sika deer and red deer in meat and antlers, using an endogenous control in one reaction. This approach saves time and cost, enhancing the detection throughput. This approach avoids false negatives through the use of an endogenous control. The study highlighted the assay's specificity, sensitivity, and authentication ability, which are adequate to authenticate venison and antlers in simple and complex products.

Declarations

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Data Availability Data will be made available on reasonable request.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

Informed consent is not applicable.

Conflict of Interest

The authors declare that they have no competing interests.

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Tables

Table 1. Triplex real-time PCR primers and probes

Primer and Probe	Length	Sequence (5′ to 3′)	Final concentration (mM)
Forward primer	22	CCTAGCAATAGCATGATTCCTC	10
Reverse primer	20	G(A/G)CCAAATTGGGCGGATTT	10
Sika-FAM	29	FAM-CATTAGGGGTATGTTGGAGTTGTTTGGTG-TAMRA	10
Red-HEX	30	HEX-CTATTAGGGGTATATTGGAGTCGTTTGGGT-TAMRA	10
Control-ROX	22	ROX-CCAGTTGCGGCTAGTGCAAGGC-BHQ-2	10

Table 2. The cycle threshold (Ct) values of the triplex real-time PCR in the specificity assay

Common name	Ct value		
	Sika-FAM	Red-HEX	Control-ROX
Sika deer meat (n=4)	14.34±0.08[a]	-	13.86±0.00
	14.72±0.14	-	13.92±0.02
	14.70±0.15	-	13.91±0.03
	14.18±0.05	-	13.80±0.01
Sika deer processed meat (n=4)	13.74±0.04	-	13.71±0.02
	13.67±0.03	-	13.67±0.01
	13.75±0.01	-	13.68±0.01
	13.64±0.02	-	13.66±0.01
Sika deer antlers (n=4)	13.64±0.01	-	13.51±0.01
	13.64±0.01	-	13.50±0.03
	13.70±0.00	-	13.59±0.01
	13.72±0.01	-	13.62±0.02
Red deer meat (n=4)	-	15.33±0.09	13.78±0.01
	-	14.28±0.08	13.67±0.00
	-	14.68±0.17	13.68±0.08
	-	15.33±0.12	13.73±0.03
Red deer processed meat (n=4)	-	15.84±0.42	13.77±0.00
	-	17.63±0.54	13.81±0.01
	-	22.12±0.68	15.74±0.57
	-	22.00±0.32	15.25±0.11
Red deer antlers (n=4)	-	17.72±0.14	13.98±0.08
	-	17.78±0.33	13.91±0.05
	-	16.48±0.27	14.22±0.15
	-	17.19±0.03	14.41±0.03

[a]Average Ct value±SD shown from triplicate PCR reactions from each DNA extraction; "-" represents not detected.

Table 3. Sensitivity assay in deer meat and antlers (4 biological replicates)

sample	Limit of detection (ng)[a]
Skia deer meat	0.000025-0.00005
Skia deer processed meat	0.000025-0.00005
Skia deer antlers	0.000025-0.0001
Red deer meat	0.000025-0.00005
Red deer processed meat	0.00025-0.0005
Red deer antlers	0.00025-0.0025

[a]The limit of detection was estimated by Probit analysis using 20 replicates for each dilution

Table 4. The slope of the calibration curve, efficiency, and the coefficient of determination (R^2) for the deer meat and antlers (4 biological replicates)

Sample	R^2	slope	PCR efficiency (%)
Skia deer meat 1	0.9904	-3.691	86.61
Skia deer meat 2	0.9954	-3.746	84.91
Skia deer meat 3	0.9933	-3.804	83.18
Skia deer meat 4	0.9959	-3.735	85.24
Sika deer processed meat 1	0.9907	-3.534	91.85
Sika deer processed meat 2	0.9930	-3.618	88.97
Sika deer processed meat 3	0.9906	-3.791	83.56
Sika deer processed meat 4	0.9918	-3.722	85.64
Skia deer antlers 1	0.9948	-3.763	84.39
Skia deer antlers 2	0.9909	-3.862	81.52
Skia deer antlers 3	0.9947	-3.648	87.98
Skia deer antlers 4	0.9934	-3.700	86.32
Red deer meat 1	0.9886	-3.749	84.82
Red deer meat 2	0.9922	-3.830	82.43
Red deer meat 3	0.9938	-3.902	80.42
Red deer meat 4	0.9955	-3.946	79.23
Red deer processed meat 1	0.9892	-3.832	82.37
Red deer processed meat 2	0.9943	-3.902	80.42
Red deer processed meat 3	0.9939	-3.898	80.53
Red deer processed meat 4	0.9915	-3.851	81.83
Red deer antlers 1	0.9973	-3.756	84.06
Red deer antlers 2	0.9940	-3.780	83.89
Red deer antlers 3	0.9872	-3.689	86.67
Red deer antlers 4	0.9839	-3.793	83.50

Table 5. The cycle threshold (Ct) values (mean ± SD) of the triplex real-time PCR in the authentication assay of sika deer (5 biological replicates)

Mass (%)	Ct value[1]		Ct value[1]		Ct value[1]		Ct value[1]		Ct value[1]	
Skia deer	Sika-FAM	ROX	Sika-FAM	ROX	Sika-FAM	ROX	Sika-FAM	ROX	Sika-FAM	ROX
0.1	25.89±0.74	22.10±0.36	22.62±0.30	19.58±0.21	24.20±0.78	24.20±0.78	23.94±0.38	21.03±0.42	25.16±0.31	22.80±0.31
1	22.67±0.56	18.97±0.42	18.74±0.29	15.67±0.14	18.28±0.94	15.20±0.29	17.05±0.61	14.64±0.20	18.15±0.38	15.71±0.31
10	20.77±0.31	16.60±0.36	17.02±0.43	14.49±0.08	16.43±0.66	14.38±0.15	16.12±0.44	14.24±0.09	16.55±0.45	14.54±0.19
30	19.28±0.59	16.00±0.33	14.46±0.43	13.94±0.17	14.78±0.48	13.90±0.03	14.32±0.16	13.86±0.01	14.39±0.15	13.90±0.02
70	14.83±0.35	13.88±0.03	14.26±0.14	13.82±0.02	14.67±0.40	13.95±0.09	14.20±0.16	13.84±0.02	13.72±0.04	13.70±0.02
90	18.30±0.54	14.18±0.14	14.60±0.24	13.77±0.01	15.58±0.58	13.90±0.05	15.55±0.52	13.93±0.03	14.03±0.13	13.71±0.03
99	14.86±0.29	13.91±0.02	13.85±0.07	13.72±0.02	14.02±0.17	13.80±0.03	13.91±0.04	13.75±0.03	14.46±0.09	13.90±0.02
99.9	15.86±0.44	13.98±0.09	13.92±0.03	13.73±0.02	13.74±0.12	13.70±0.03	14.17±0.16	13.83±0.01	13.75±0.07	13.70±0.02

[1] The result was estimated by Probit analysis using 20 replicates for each mixture.

Table 6. The cycle threshold (Ct) values (mean ± SD) of the triplex real-time PCR in the authentication assay of red deer (5 biological replicates)

Mass (%)	Ct value[1]		Ct value[1]		Ct value[1]		Ct value[1]		Ct value[1]	
Red deer	Red-HEX	ROX	Red-HEX	ROX	Red-HEX	ROX	Red-HEX	ROX	Red-HEX	ROX
0.1	26.30±0.44	19.31±0.22	24.63±0.42	17.75±0.13	24.75±0.68	18.02±0.52	23.94±0.38	19.28±0.20	27.61±0.51	20.62±0.24
1	22.04±0.38	16.25±0.35	20.87±0.44	15.79±0.30	20.05±0.67	15.12±0.33	17.05±0.61	15.00±0.24	20.11±0.32	15.33±0.26
10	16.53±0.34	13.94±0.04	16.33±0.30	13.91±0.04	15.60±0.49	13.87±0.04	16.12±0.44	13.87±0.03	15.59±0.29	13.88±0.02
30	16.63±0.92	13.91±0.08	15.73±0.33	13.79±0.01	16.14±0.52	13.88±0.03	14.32±0.16	13.73±0.04	16.73±0.36	13.90±0.02
70	14.73±0.35	13.79±0.02	14.84±0.18	13.77±0.01	14.42±0.24	13.76±0.02	14.20±0.16	13.76±0.01	15.05±0.17	13.82±0.01
90	14.38±0.30	13.73±0.04	14.76±0.16	13.74±0.01	14.18±0.20	13.68±0.02	15.55±0.52	13.70±0.01	14.06±0.09	13.66±0.02
99	14.73±0.34	13.67±0.05	14.30±0.15	13.55±0.03	14.74±0.44	13.73±0.04	13.91±0.04	13.58±0.02	14.19±0.12	13.63±0.03
99.9	15.46±0.50	13.83±0.04	14.74±0.15	13.77±0.01	16.61±0.50	13.99±0.06	14.17±0.16	13.65±0.03	14.21±0.10	13.69±0.03

Figures

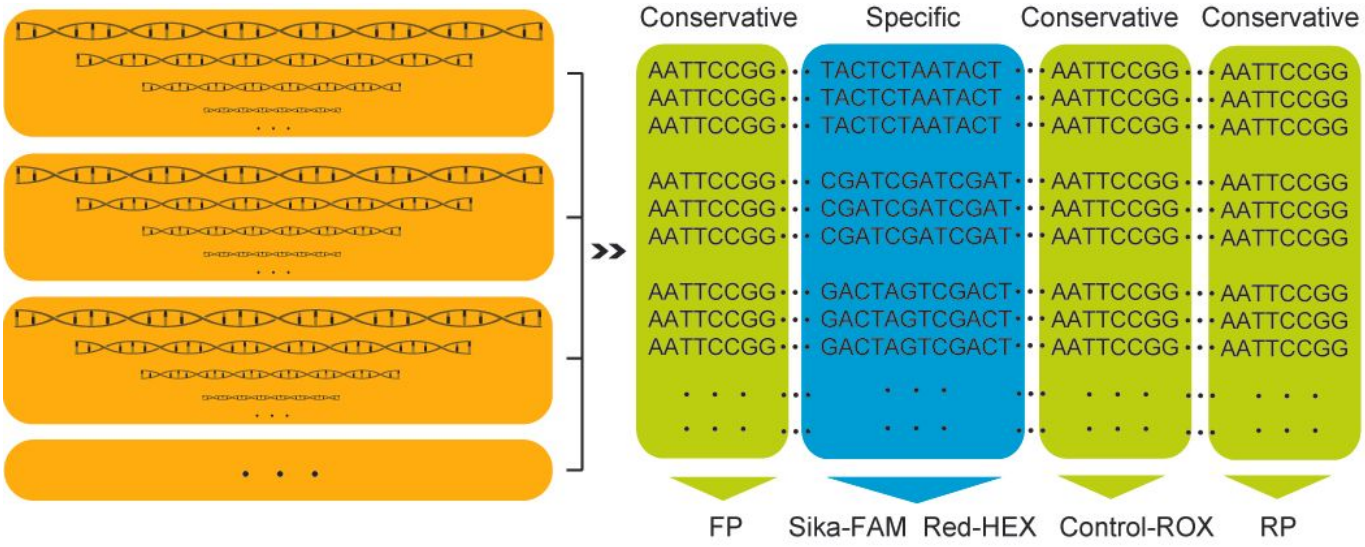

Figure 1

Development of species-conserved primers (FP and RP), species-specific probes (Sika-FAM and Red-HEX), and the species-conserved probe (Control-ROX) for the triplex real-time PCR.

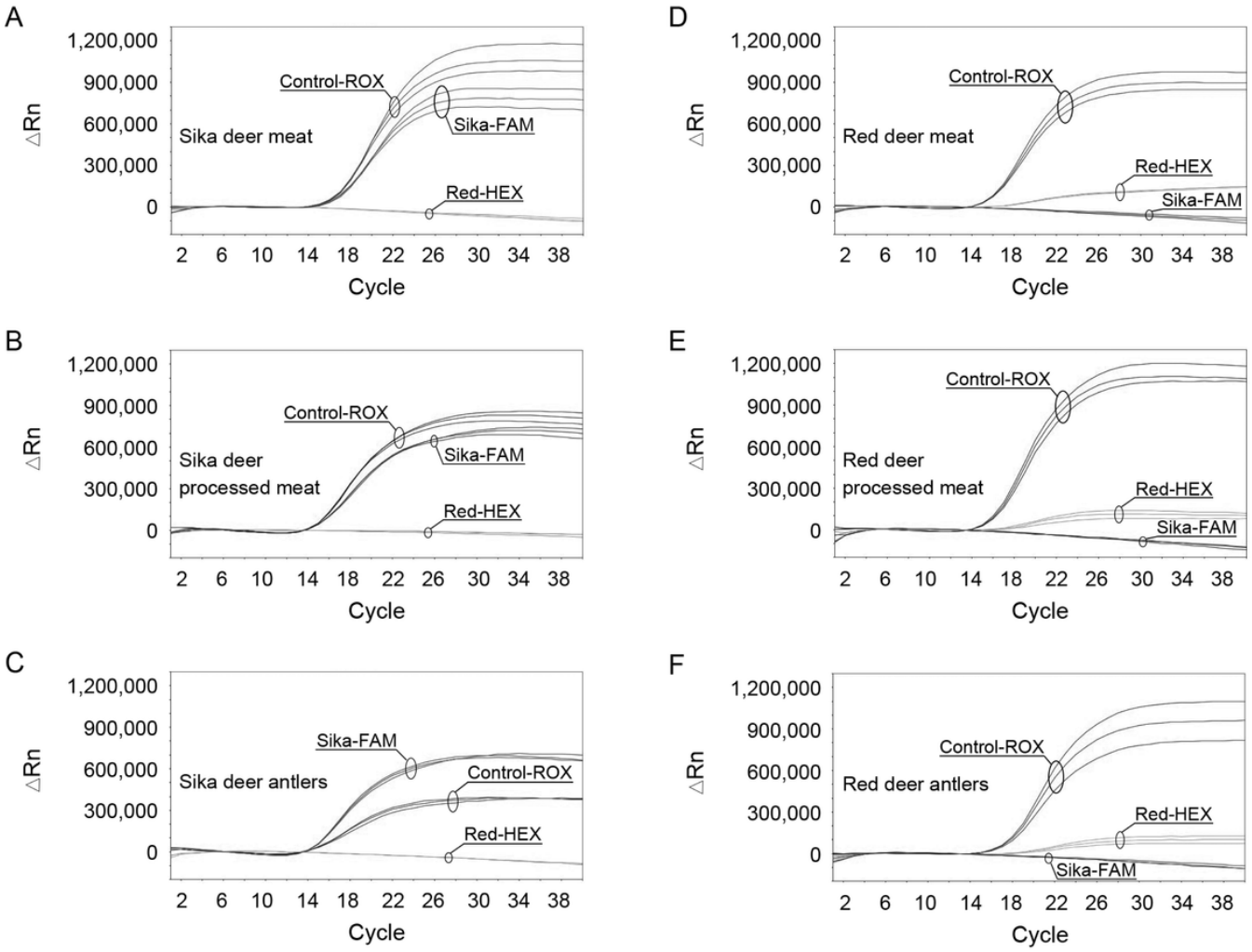

Figure 2

Amplification curves of the triplex real-time PCR in the specificity assay: Sika deer meat (A), sika deer processed meat (B), sika deer antlers (C), red deer meat (D), red deer processed meat (E), red deer antlers (F). The results were confirmed by 3 replicates. ΔRn = change in normalized reported values.

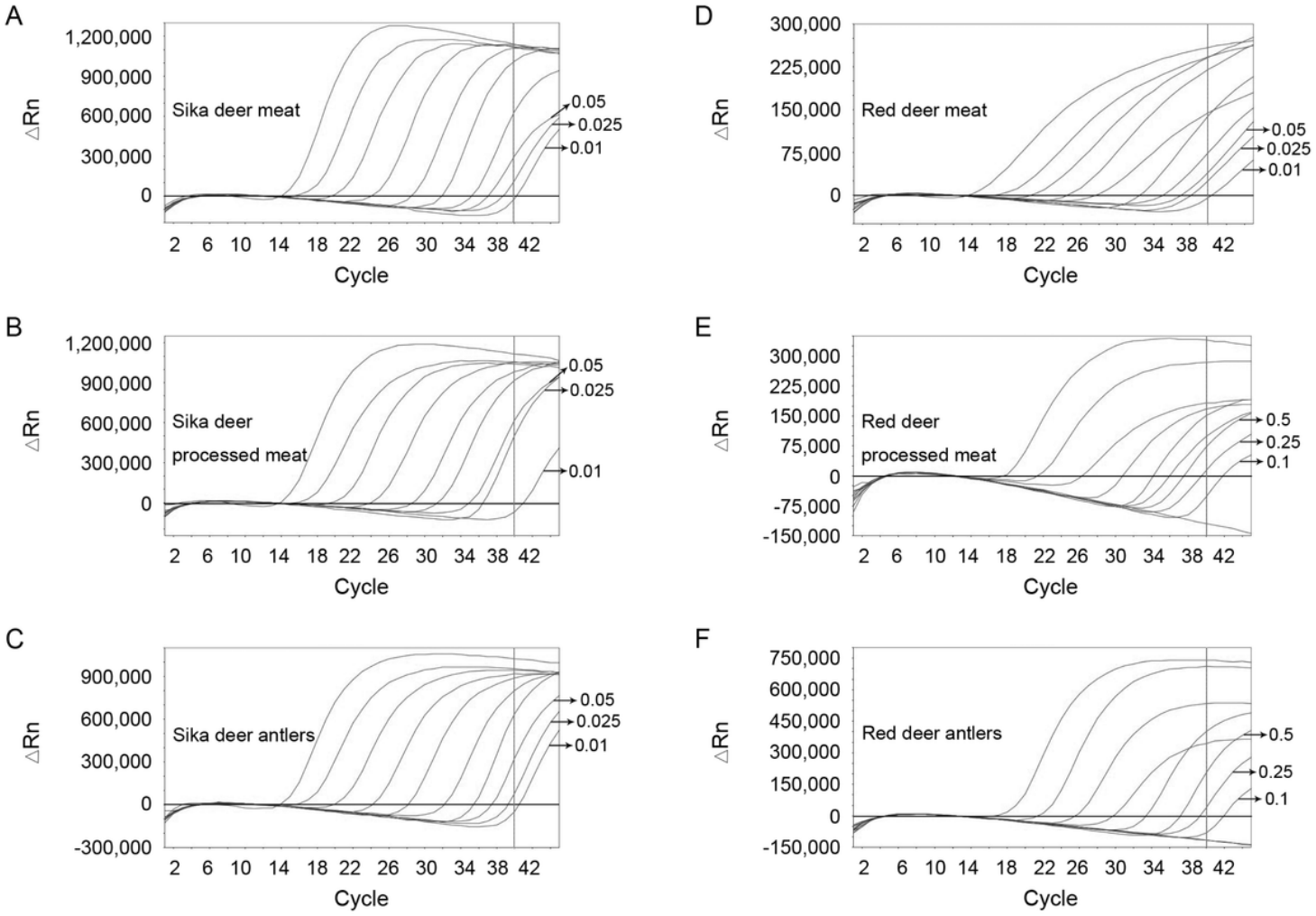

Figure 3

Amplification curves of the triplex real-time PCR assay in the sensitivity assay: Sika deer meat (A), sika deer processed meat (B), sika deer antlers (C), red deer meat (D), red deer processed meat (E), red deer antlers (F). ΔRn = change in normalized reported values.

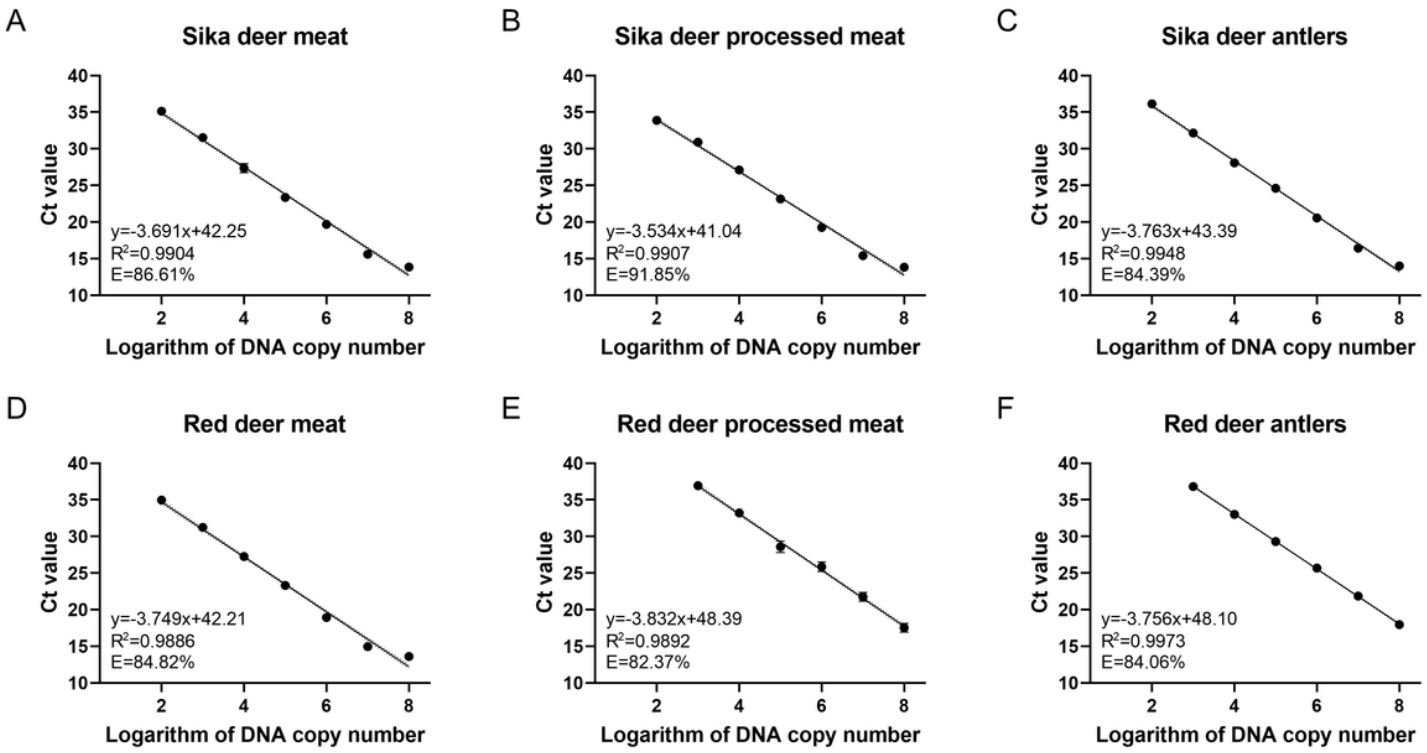

Figure 4

Standard curves for the triplex real-time PCR in the sensitivity assay: Sika deer meat (A), sika deer processed meat (B), sika deer antlers (C), red deer meat (D), red deer processed meat (E), red deer antlers (F).