Chapter 5

Design of Mismatch Primers to Identify and Differentiate Closely Related (Sub)Species: Application to the Authentication of Meat Products

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Abstract

Single-nucleotide polymorphisms (SNPs) are powerful molecular markers for the identification and differentiation of closely related organisms. A variety of methods can be used to determine the allele that is present at a specific locus in the genome, including real-time PCR by using an allele-specific primer. In order to increase the selectivity for the target allele, deliberate mismatch bases at the 3′ end of the allele-specific primer may be introduced. This strategy has already been used for the identification and differentiation of microorganisms and plants. We have recently developed real-time PCR assays involving mismatch primers for the identification and differentiation of closely related deer species (red deer, fallow deer, sika deer) or the discrimination of wild boar and domestic pig in game meat products. These methods are applicable to detect meat species adulteration in food products.

In this chapter, we offer a protocol for the design of PCR primer/probe systems suitable for meat species authentication in food. We address the retrieval and alignment of sequences, primer design by using a commercial software and the introduction of deliberate mismatch bases. In addition, we describe how the suitability of primer/probe systems can be tested in silico and in practice. We use the design of PCR primer/probe systems for wild boar and domestic pig as example.

Key words Primer design, SNP, Mismatch primer, Real-time PCR, Species identification, Subspecies, Meat, Food authentication

1 Introduction

Species and even subspecies can frequently be distinguished by morphological differences. In some situations, however, differentiation of organisms based on morphological characteristics is not possible, e.g., if only parts of the organisms are available. In these cases, molecular biology techniques targeting differences between the genome of organisms play an important role. For the identification and differentiation of closely related organisms, single-nucleotide polymorphisms (SNPs) are powerful molecular markers [1]. SNPs are variations in individual base pairs at specific positions

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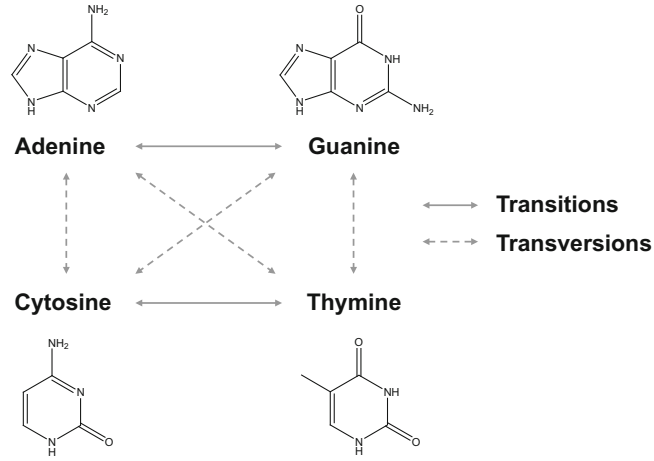


Fig. 1 Transitions and transversions

(=loci) in the genome. They are caused by mutations and/or recombination of DNA sequences and are evolutionarily stable and highly abundant. SNPs include transitions, transversions, and indels. The term transition is used for point mutations that result in the exchange of purine (adenine ↔ guanine, (A↔G)) or pyrimidine (cytosine ↔ thymine, (C↔T)) bases (Fig. 1). In case of transversions, a purine base is replaced by a pyrimidine base or vice versa (C↔G, A↔T, T↔G, C↔A). The term indel is used for the insertion or deletion of short sequences in the genome.

Various methodologies are suitable for SNP-based identification and differentiation of (sub)species. In addition to high-throughput technologies, e.g., next-generation sequencing (NGS) platforms [2], real-time polymerase chain reaction (PCR) plays an important role [3]. Real-time PCR allows efficient amplification of a target DNA sequence and monitoring formation of the PCR products in real-time, e.g., by using a sequence-specific fluorescent probe. To be applicable for SNP analysis, real-time PCR must yield the information which of the potential forms (=alleles) is present at the specific locus in the DNA sequence. So called allele-specificity may be achieved by using either an allele-specific primer (Fig. 2) or an allele-specific probe.

Under stringent real-time PCR conditions, a single-base mismatch may hamper primer extension, as shown in Fig. 2. In practice, however, alleles with one mismatch base are frequently amplified, although with lower efficiency than alleles without mismatch base. Various strategies have been developed aiming at enhancing selectivity for the target allele. Among them, the introduction of one or several deliberate mismatch bases at the 3′ end of the allele-specific primer turned out to be powerful [4]. The introduction of one deliberate mismatch base at the 3′ end of the allele-specific primer results in only one mismatch with the target allele,

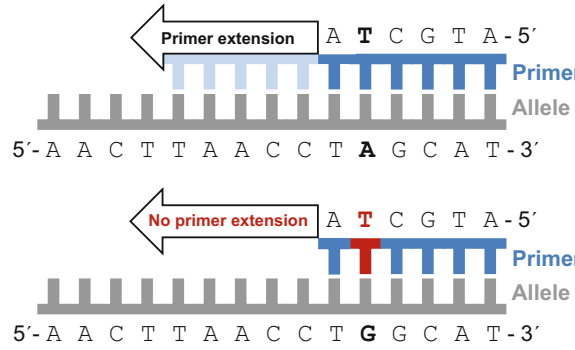


Fig. 2 Allele-specific PCR by using an allele-specific primer. Under stringent conditions, primer extension only occurs if the primer hybridizes perfectly, as it is the case with allele 1

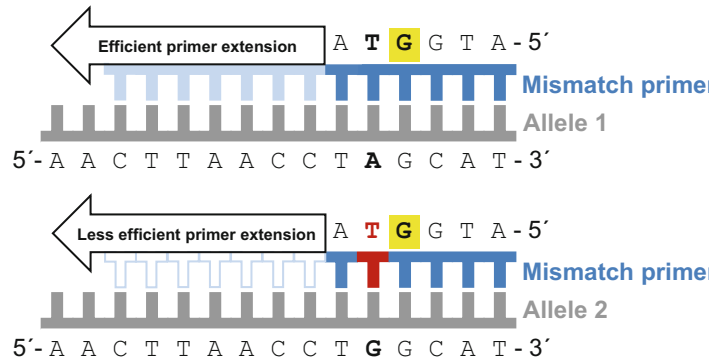


Fig. 3 Enhancing selectivity for the target allele by using a mismatch primer

but a double mismatch with the alternative allele(s) (Fig. 3). Thus, the target allele may be amplified with a higher efficiency than alternative alleles.

Real-time PCR based on so-called mismatch amplification mutation assays (MAMAs) has already been applied to various microorganisms [5–8] and plants [9, 10]. We demonstrated that real-time PCR assays involving mismatch primers enable the identification and differentiation of closely related deer species (red deer, fallow deer, sika deer) in game meat products [11–13]. In addition, we have designed mismatch primers for the discrimination of wild boar and domestic pig [14]. Substitution of more expensive meat species, e.g., game meat, by cheaper ones, e.g., meat from domesticated animals, is a common kind of food adulteration [15]. However, substitution is illegal because according to food legal regulations the species the meat originates from has to be declared correctly.

The design of appropriate primer/probe systems for (game) meat authentication in food is particularly challenging because of the following reasons:

1. Real-time PCR assays applied to food authentication, e.g., in official control laboratories, have to be selective to avoid false results.
2. Real-time PCR assays for food authentication must be applicable not only to raw but also to processed foodstuffs. Since food processing may affect DNA integrity, primers should be designed to result in PCR products <150 bp in length [16, 17]. In our studies on meat species identification and differentiation, we commonly aimed to obtain PCR products with a length from 60 to 100 bp [11–14, 18].
3. Genomes of common ancestry are highly homologous and thus the number of (sub)species-specific bases is very low.
4. For the quantification of meat species in food products, single-copy genes should be targeted (*see* **Note 1**). However, for some meat species, e.g., game species, the number of entries in the NCBI database are limited.

In the following, we offer a protocol for the design of PCR primer/probe systems suitable for meat species authentication in food. We address the introduction of mismatch bases and describe how primers and probes may be tested in silico and in practice for their suitability. We use the design of primer/probe systems for wild boar and domestic pig as example [14].

2 Materials

2.1 Sequence Retrieval and Design of Primer/Probe Systems

1. Ensemble database (www.ensembl.org/index.html).
2. NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore).
3. Sequence alignment program (e.g., CLC Genomics Workbench (Qiagen), MEGA7 (https://megasoftware.net/)).
4. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
5. PCR primer design software (e.g., Primer Express 3.0 (Applied Biosystems)).

2.2 In Silico Evaluation

1. PCR primer design software (e.g., Primer Express 3.0).
2. OligoAnalyzer.
3. RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

2.3 Evaluation by Real-Time PCR

1. Primers and probes: may be purchased from various manufacturers (e.g., Sigma Aldrich, Eurogentec).
2. DNA from the target (sub)species and closely realated (sub)species for performing cross-reactivity tests: DNA can be isolated from muscle meat with a variety of methods, including the CTAB (cetyltrimethylammonium bromide) method (*see* **Note 2**) or a commercial kit (e.g., Maxwell® Tissue DNA Purification Kit (Promega)).
3. UV/Vis spectrophotometer (e.g., Nanodrop instrument (Thermo Fisher)) or fluorometer (e.g., Qubit 4 including Qubit dsDNA BR Assay Kit (Thermo Fisher)).
4. Real-time PCR mix (e.g., QuantiTect Multiplex PCR Master Mix (Qiagen)).
5. Real-time thermocycler (e.g., Rotor Gene Q cycler (Qiagen), QuantStudio 5 (Thermo Fisher)).

3 Methods

3.1 SNP Search and Sequence Retrieval for Primer Design

1. Search in literature for a SNP potentially suitable for distinguishing the target (sub)species from closely related (sub)species. In our example on the design of primer/probe systems for wild boar and domestic pig, we selected the SNP g.118314929 A > G (rs81416363) on chromosome 9. The SNP had been reported to show high discrimination power for the two subspecies [19].
2. If you could not find a potentially suitable SNP in literature, continue with Subheading 3.2.
3. Enter the rs number of the SNP into the Ensemble database (https://www.ensembl.org) and press GO. In our example, we entered rs81416363 (Fig. 4a).
4. Click at the SNP in the search results (Fig. 4b) and select "Flanking sequence" (Fig. 4c).
5. Press "Configure this page" at the left side (Fig. 4d) and deselect "Show variants in flanking sequence" (Fig. 4e).
6. Close the window and press "Download sequence" (Fig. 4f). Select the file format "RTF (Word-compatible)" and download the sequence (Fig. 4g). The SNP is highlighted in red (Fig. 4h).
7. Continue with Subheading 3.3.

3.2 Sequence Retrieval in Case No SNP Has Been Found in Literature

1. Look for DNA sequences in the NCBI database that are available not only for the target (sub)species but also for (sub)species the target (sub)species shall be differentiated from (*see* **Note 3**). For sequence retrieval from the NCBI nucleotide database, *see* **Note 4**.
2. Select nuclear DNA sequences and BLAST them.

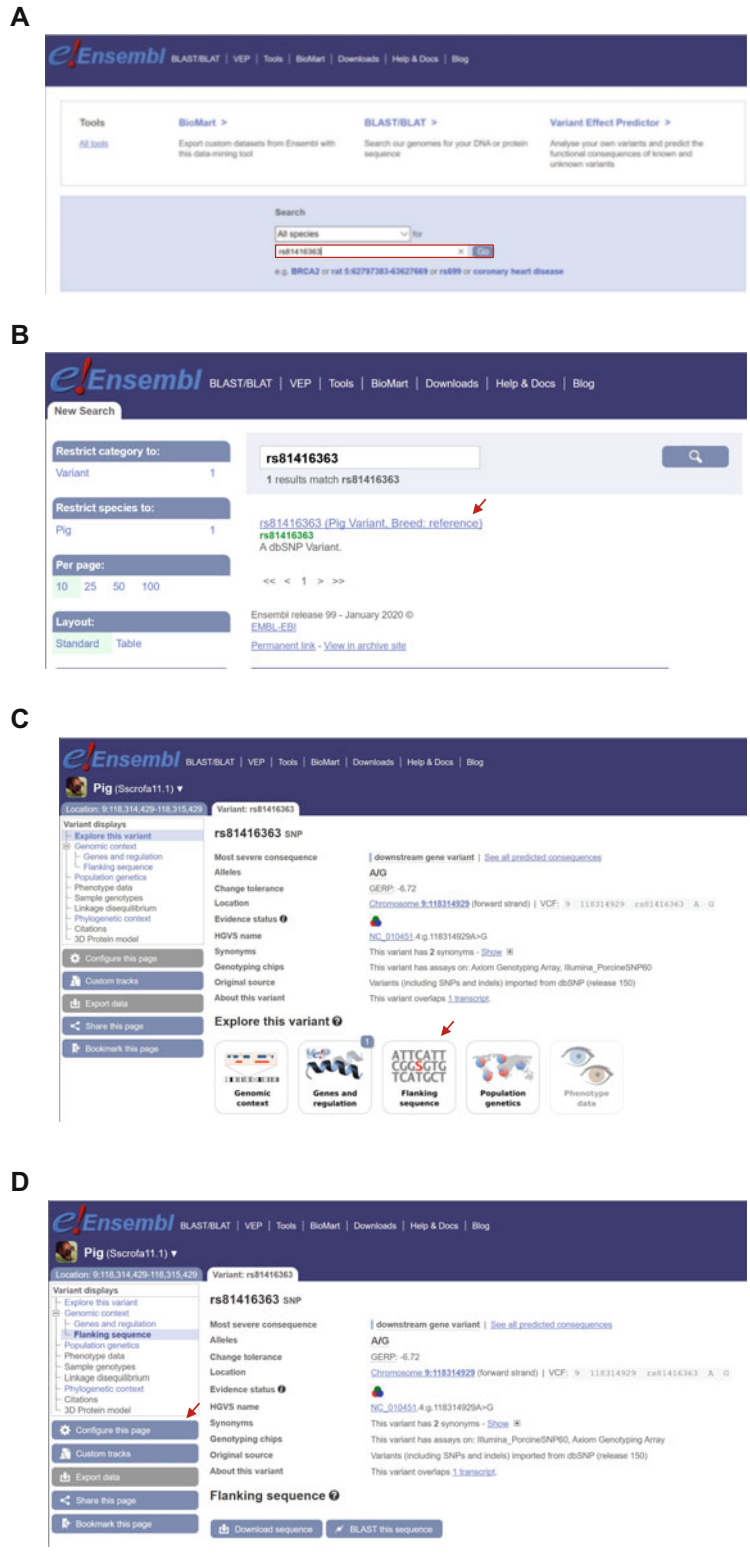
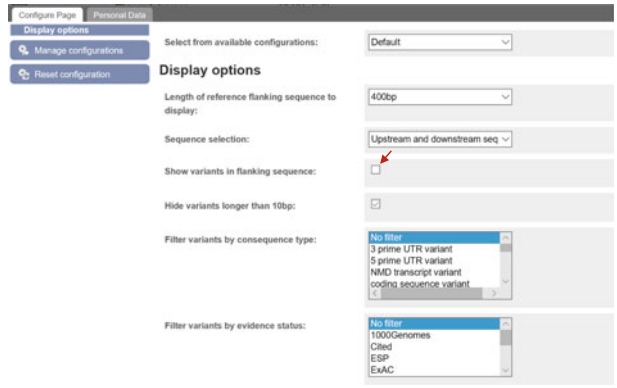
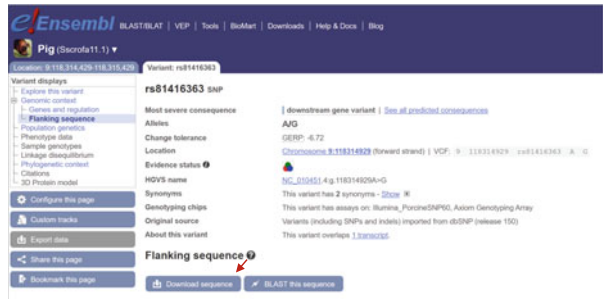


Fig. 4 Sequence retrieval from the Ensemble database; (**a**) Search for a specific SNP in the Ensemble database; (**b**) Selection of a SNP from the search results; (**c**–**e**) Selection of display options for the flanking sequence; (**f**) Download of the sequence; (**g**) Selection of the file format; (**h**) Data file, with the SNP highlighted in red

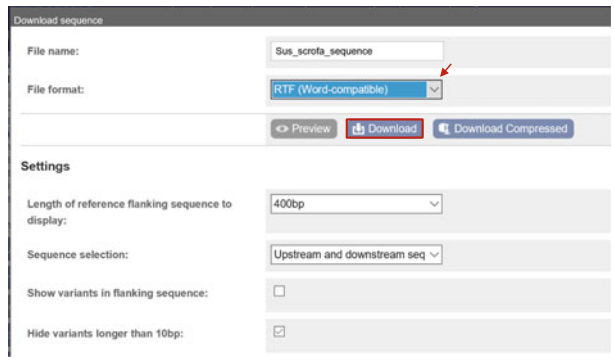
E



F



G



H

TCCTACCCTCGCTGCTTTGGGGGATGTTGGTCCACATCACCTTCACGTCCTCCAGAGCCA
CACACTGTTCTGGAGTTTCCTATGCCCACAGCCCAGGAGCTTCCTGTTGTGACTGTCGAT
GAACACTTGGTCATTGTTCTTGACTGACTCCGTGAGCACCGAGGATGGACCCGTCTTGAA
TCCTCCTCTGCTTCTGCAGCTCCTCCTGGCTGGCTCACTCCTCACTCAGGGGCTTCCTGA
GGAGGCTCTCCATGGCGGTGGCCATGCGCTCAGATCACATCTATCTCCTCTGTGTTGCTT
CTGCCTCTGAGATGCTAATTTAAATTGCTTTTTTCTAGCTAGCAACCAAGTAAGAAAATC
TTAACCTAGCAAATGGGTGTCTCACAGGTGATGTGACTGCRTGAAAGAACAAAAAACTCC
CTGGCCACATGAGAAACTGGTTTCTCACTAGGATGGGCCGTGAATTACCCGTGATTTCTC
GTCCATAGTAATCATTTCAGGGCCACATACTTCGGGGGATGATTATGTACTAAACCATGT
CTTGTTAATCCTTGTATCCAGGGAATAGTAAATTAATAATATATAATGGTTCAATTTAAT
TGATTAGAATTTCAGAATACGTGGCTAGTCCTGCCATGGTCCCAGAATAATAATGTAACC
TTTATGTATTCAGAATTCTGCCTCATATTTATTCTAAAGACTACCACACTCTGCAAGGCA
GTTTATCACACCCATTTTGAGAAAGTGGTTTTGAATTTGTCCTTGGGCCAGGAGATTCAC
AATTAAACAGGTCTTAGTTAG

Fig. 4 (continued)

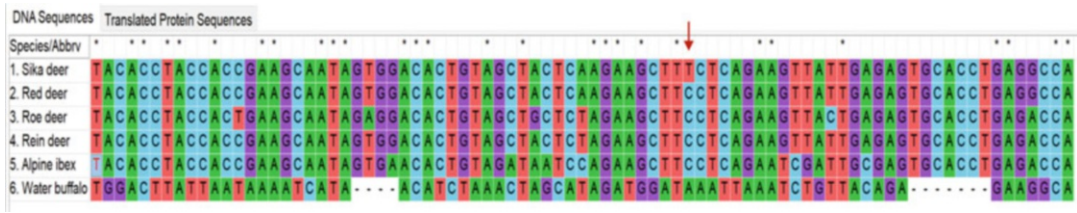


Fig. 5 Example of a multiple sequence alignment performed with MEGA7 for a fragment of the kappa-casein precursor gene. Our aim was to differentiate sika deer from closely-related deer species (e.g., red deer, roe deer, and reindeer) and other game species (e.g., alpine ibex and water buffalo) [13]. By sequence alignment, we could identify one sika deer-specific base, indicated by the red arrow

3. Exclude all sequences that are identical with sequences for (sub)species the target (sub)species shall be differentiated from.
4. Select one of the remaining DNA sequences. Download the DNA sequence for each of the (sub)species the target (sub)-species has to be differentiated from by clicking on FASTA. Copy the sequence into a word file.
5. Align the sequences according to the manual for the respective sequence alignment software (e.g., CLC Genomics Workbench or MEGA7). An example of a multiple sequence alignment is shown in Fig. 5.
6. If the sequence for the target (sub)species contains at least one (sub)species-specific base, continue with Subheading 3.3.
7. If the sequence for the target (sub)species does not contain a (sub)species-specific base, go back to **step 4**.

3.3 Design of a Primer/Probe System

1. Copy the sequence for the target (sub)species into the primer design software, e.g., Primer Express 3.0 (*see* **Note 5**).
2. Select "TaqMan MGB quantification" (*see* **Note 6**).
3. Use the default settings (*see* **Note 7**) and press "Find Primers/Probes".
4. Check if either the forward or the reverse primer of the primer/probe systems suggested by the software contains the (sub)species-specific base at position 2 from the 3′ end, as shown in Fig. 6. If this is the case, continue with Subheading 3.4.
5. If in none of the primers, the (sub)species-specific base is at position 2 from the 3′ end, open the "Primer/Probe Test Tool" and enter the sequences of the primers and the probe.
6. Manually modify the sequences of the primers by adding/deleting bases in order to shift the (sub)species-specific base to position 2 from the 3′ end. The Primer Express Software indicates if the melting temperature (T_m) and the GC content still meet (green) or do not meet (red) the presettings.

Fig. 6 Primer/probe system designed for wild boar. The red arrow indicates the subspecies-specific base at position 2 from the 3′ end of the reverse primer

Table 1
Characteristics of the mismatch primers designed for wild boar. The subspecies-specific base is highlighted in gray. Mismatch bases are highlighted in green (T), blue (C), yellow (G), and red (A). Transversions are underlined

Primer	Sequence (5′- 3′)	Length (bp)	Total number of mismatch bases	Number of transitions	Number of transversions
1	CAGGGAGTTTTTTGTTCTTTCACG	24	-	-	-
2	CAGGGAGTTTTTTGTTCTTTCTCG	24	1	-	1
3	GGGAGTTTTTTGTTCTTTCCCG	22	1	-	1
4	GGGAGTTTTTTGTTCTTTCGCG	22	1	1	-
5	CAGGGAGTTTTTTGTTCTATCACG	24	1	-	1
6	CAGGGAGTTTTTTGTTCTCTCACG	24	1	1	-
7	AGGGAGTTTTTTGTTCTGTCACG	23	1	-	1
8	GGGAGTTTTTTGTTCTTTGCCG	22	2	-	2
9	CAGGGAGTTTTTTGTTCTTTACCG	24	2	-	2
10	AGGGAGTTTTTTGTTCTTTTCCG	23	2	1	1
11	AGGGAGTTTTTTGTTCTTACCCG	23	2	-	2
12	GGGAGTTTTTTGTTCTTCCCCG	21	2	1	1
13	GGGAGTTTTTTGTTCTTGCCCG	21	2	-	2

3.4 Introduction of One Mismatch Base

Introduce mismatch bases to enhance the selectivity for the target (sub)species. Number, kind, and position of the mismatch bases may critically affect polymerase extension.

1. Start with introducing a single mismatch base in the primer containing the (sub)species-specific base. Locate the mismatch at position 3 from the 3′ end of the primer (in our example, *see* **primer 2–4,** Table 1 and **primer 2–4,** Table 2) (*see* **Note 8**).
2. Evaluate the primers in silico as described in Subheading 3.5.

Table 2
Characteristics of the mismatch primers designed for domestic pig. The subspecies-specific base is highlighted in gray. Mismatch bases are highlighted in green (T), blue (C), yellow (G), and red (A). Transversions are underlined

Primer	Sequence (5′- 3′)	Length (bp)	Total number of mismatch bases	Number of transitions	Number of transversions
1	CCAGGGAGTTTTTTGTTCTTTCATG	25	-	-	-
2	CCAGGGAGTTTTTTGTTCTTTCTTG	25	1	-	1
3	CAGGGAGTTTTTTGTTCTTTCCTG	24	1	-	1
4	CAGGGAGTTTTTTGTTCTTTCGTG	24	1	1	-
5	CCAGGGAGTTTTTTGTTCTTACTTG	25	2	-	2
6	CAGGGAGTTTTTTGTTCTTCCTTG	24	2	1	1
7	CAGGGAGTTTTTTGTTCTTGCTTG	24	2	-	2
8	CCAGGGAGTTTTTTGTTCTTACCTG	25	2	-	2
9	AGGGAGTTTTTTGTTCTTCCCTG	23	2	1	1
10	AGGGAGTTTTTTGTTCTTGCCTG	23	2	-	2
11	CAGGGAGTTTTTTGTTCTTACGTG	24	2	1	1
12	AGGGAGTTTTTTGTTCTTCCGTG	23	2	2	-
13	GGGAGTTTTTTGTTCTTGCGTG	22	2	1	1
14	CAGGGAGTTTTTTGTTCTTTGTTG	24	2	-	2
15	CCAGGGAGTTTTTTGTTCTTTATTG	25	2	-	2
16	CCAGGGAGTTTTTTGTTCTTTTTTG	25	2	1	1
17	CAGGGAGTTTTTTGTTCTTGGTTG	24	3	-	3
18	CCAGGGAGTTTTTTGTTCTTGATTG	25	3	-	3
19	CAGGGAGTTTTTTGTTCTTGTTTG	24	3	1	2
20	AGGGAGTTTTTTGTTCATGGTTG	23	4	-	4
21	CAGGGAGTTTTTTGTTCCTGATTG	24	4	1	3
22	AGGGAGTTTTTTGTTCGTGTTTG	23	4	1	3
23	CCAGGGAGTTTTTTGTTCTAGCTTG	25	3	-	3
24	AGGGAGTTTTTTGTTCTCGCTTG	23	3	1	2
25	AGGGAGTTTTTTGTTCTGGCTTG	23	3	-	3
26	AGGGAGTTTTTTGTTCATGCTTG	23	3	-	3
27	AGGGAGTTTTTTGTTCCTGCTTG	23	3	1	2
28	GGGAGTTTTTTGTTCGTGCTTG	22	3	-	3
29	CAGGGAGTTTTTTGTACATGCTTG	24	4	-	4
30	GGGAGTTTTTTGTCCCTGCTTG	22	4	2	2
31	GGAGTTTTTTGTGCGTGCTTG	21	4	-	4
32	CCAGGGAGTTTTTTATACATGCTTG	25	5	1	4
33	GGGAGTTTTTTCTCCCTGCTTG	22	5	2	3
34	GGAGTTTTTTTTGCGTGCTTG	21	5	0	5

3.5 In Silico Evaluation

Introduction of one or several mismatch bases may drastically influence the melting temperature of the primer. However, for efficient amplification, the melting temperature of forward primer, reverse primer, and probe has to be similar.

1. Open the "Primer Probe Test Tool" of Primer Design 3.0 and enter the sequences of the primers and probes (*see* Fig. 7).
2. Check the melting temperatures, GC content, and length displayed in the "Primer Probe Test Tool" by selecting the primers or the probe.
3. If necessary, delete or add one or several base(s) at the 5′ end of the primer to adjust the melting temperature (as shown in Fig. 7).

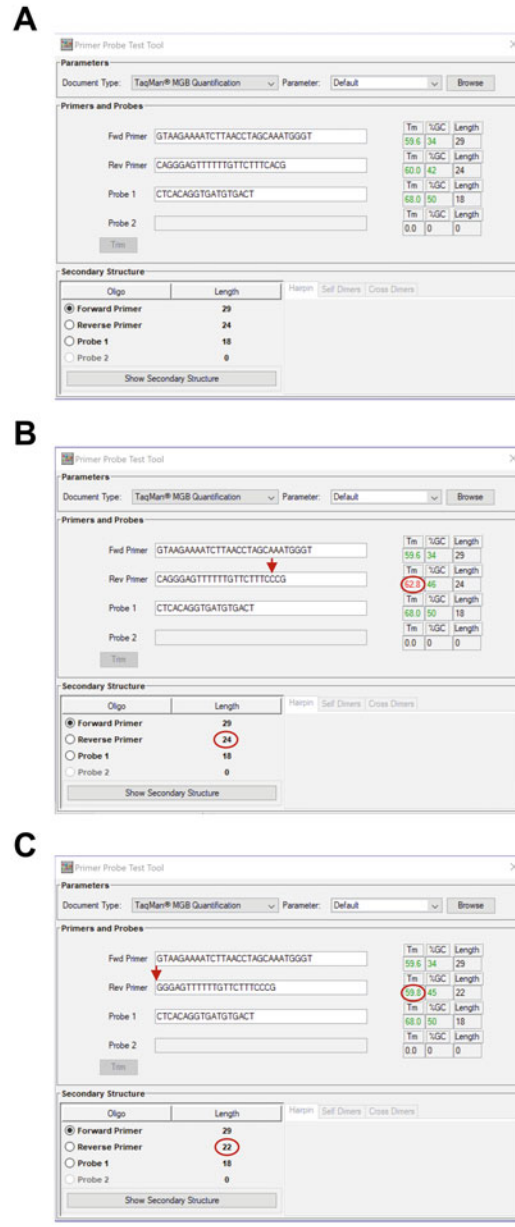


Fig. 7 Modification of the primer length to adjust the melting temperature (T_m). (**a**) Reverse primer without any mismatch base (primer 1, Table 1). (**b**) Reverse primer with one mismatch base at position 3 from the 3′ end. (**c**) Reverse primer with the mismatch base, meeting the settings after deleting two bases at the 5′ end (primer 3, Table 1)

4. In addition, test the primers and the probe for the formation of secondary structures, homodimers, and heterodimers (*see* **Note 9**).
5. If the primers meet the criteria in silico, order the primer and test them by running real-time PCR (*see* Subheading 3.6).

3.6 Evaluation by Real-Time PCR

1. Subject the primer/probe systems (including the mismatch primer) to cross-reactivity tests with DNA isolates (*see* **Notes 10–12**) from the target (sub)species and (sub)species the target (sub)species shall be differentiated from. Use a commercial real-time PCR mix (*see* **Note 13**).
2. Determine C_t values for all (sub)species (*see* **Note 14**).
3. For each mismatch primer, plot the C_t values as shown in Figs. 8 and 9 and calculate the ΔC_t values between the cross-reacting species and the target species.
4. If any of the primer/probe systems results in a low C_t value (~20.0–25.0) for the target species and a high ΔC_t value (>11.0) between the target (sub)species and the (sub)species the target (sub)species shall be differentiated from, *see* **Note 15**.
5. If a primer/probe system results in a low C_t value (~20.0–25.0) for the target (sub)species, and the ΔC_t value is between 8.0 and 11.0, try to enhance selectivity by varying the real-time PCR conditions (*see* Subheading 3.9).

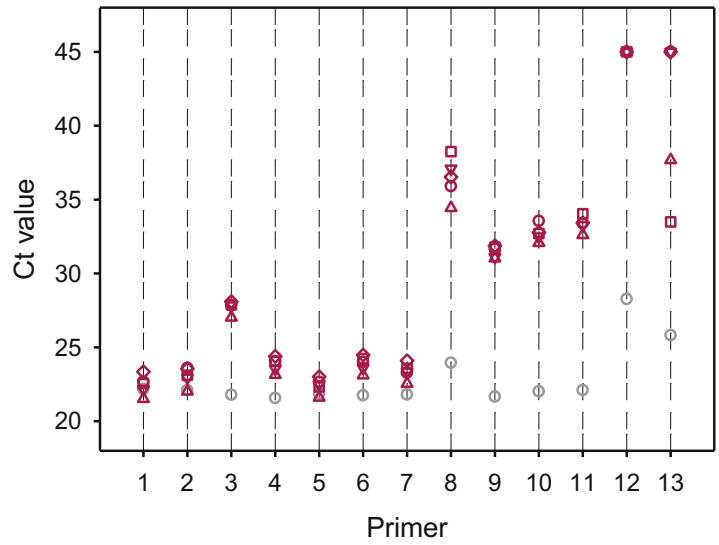


Fig. 8 C_t values obtained with the primers designed for wild boar. Primer numbers refer to Table 1. Gray: wild boar, red: domestic pig breeds. A C_t value of 45 indicates that we did not observe an increase in the fluorescence signal within 40 cycles

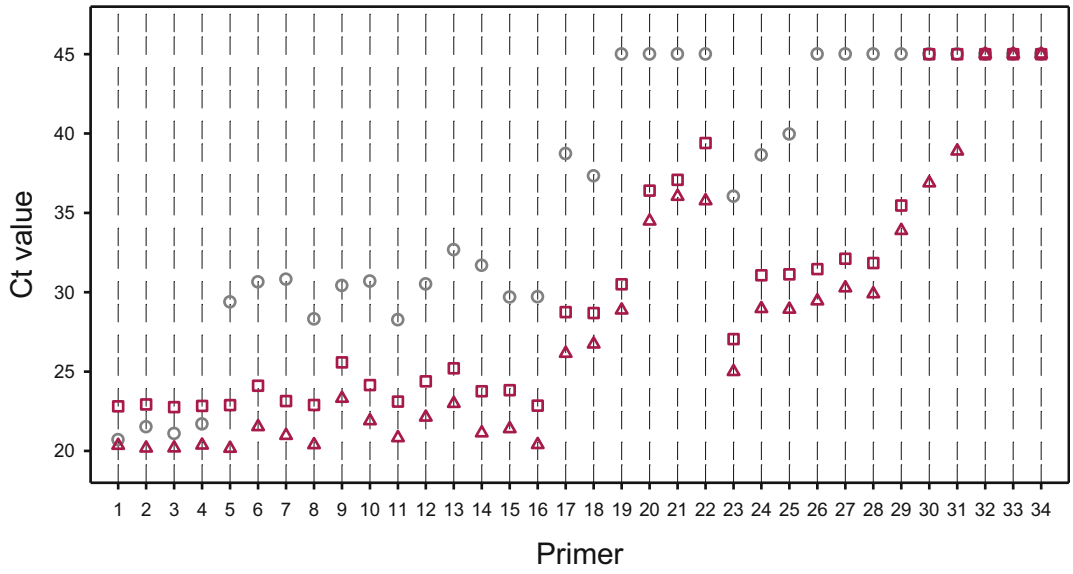


Fig. 9 C_t values obtained with the primers designed for domestic pig. Primer numbers refer to Table 2. A C_t value of 45 indicates that we did not observe an increase in the fluorescence signal within 40 cycles

6. If the ΔC_t value is still too low, introduce a further mismatch base, as described in Subheading 3.7.

3.7 Introduction of Two Mismatch Bases

1. Select the most selective primer containing one mismatch base.
2. Introduce a second mismatch base either at position 4 (in our example, *see* **primer 8–10**, Table 1 and **primer 14–16**, Table 2) or position 5 (*see* **primer 11–13**, Table 1 and **primer 5–13**, Table 2) from the 3′ end of the primer.
3. Evaluate the primers in silico (*see* Subheading 3.5).
4. If the primers meet the criteria in silico, order the primers and test them by running real-time PCR (*see* Subheading 3.6).
5. If any of the primer/probe systems results in a low C_t value (~20.0–25.0) for the target species and a high ΔC_t value (>11.0) between the target (sub)species and the (sub) species the target (sub)species shall be differentiated from, *see* **Note 15**.
6. If a primer/probe system results in a low C_t value (~20.0–25.0) for the target (sub)species, and the ΔC_t value is between 8.0 and 11.0, try to enhance selectivity by varying the real-time PCR conditions (*see* Subheading 3.9).
7. If the ΔC_t value is still too low, introduce a third mismatch base, as described in Subheading 3.8.

3.8 Introduction of Three Mismatch Bases

In case, the introduction of one or two mismatch bases was not sufficient, introduce a third mismatch base and vary kind and position.

1. Select the most selective primer containing two mismatch bases.
2. Introduce the third mismatch base either at position 5, 6 or 7 from the 3′ end of the primer. (In our example, primers with three mismatch bases are **primer 17–19**, **primer 23–25**, and **primer 26–28**, Table 2).
3. Evaluate the primers in silico (*see* Subheading 3.5).
4. If the primers meet the criteria in silico, order the primers and test them by running real-time PCR (*see* Subheading 3.6).
5. If any of the primer/probe systems results in a low C_t value (~20.0–25.0) for the target species and a high ΔC_t value (>11.0) between the target (sub)species and the (sub)species the target (sub)species shall be differentiated from, *see* **Note 15**.
6. If a primer/probe system results in a low C_t value (~20.0–25.0) for the target (sub)species, and the ΔC_t value is between 8.0 and 11.0, try to enhance selectivity by varying the real-time PCR conditions (*see* Subheading 3.9).
7. If the ΔC_t value is still too low, we do not suggest introducing further mismatch bases (*see* **Note 16**). We suggest looking for a SNP at another locus in the genome.

3.9 Optimization of Real-Time PCR Conditions

A number of parameters affect amplification efficiency, including the primer concentration, and the concentration of magnesium ions.

1. Vary the primer concentration and the ratio of forward and reverse primer (*see* **Notes 17** and **18**).
2. Test another real-time PCR kit (*see* **Note 13**).

4 Notes

1. In meat species authentication, one is frequently interested not only in qualitative but also quantitative determination of meat species. It is, for example, necessary to verify if in a sausage declared as “game sausage”, at least 38% (w/w) of the total meat content derives from game species. If the game species content in a “game sausage” is <38% (w/w), it does not comply with the guidelines of the Codex Alimentarius Austriacus [20]. Due to the constant copy number, single-copy genes are more suitable for obtaining accurate quantitative results than mitochondrial DNA sequences. For more information on relative quantification of meat species in food, *see* [21].

2. A CTAB protocol for DNA extraction from muscle meat can be found in [22]. With this protocol, extracts with a DNA concentration from 100 to 300 ng/μL were obtained for most meat species [14]. (Note, that at the end of the lysis step, the tissue must be dissolved completely).
3. If available, select genes that are associated with traits like coat color, body composition, reproduction or behavior. In our study on the differentiation of wild boar and domestic pig, we targeted genes that have been selected in the process of wild boar domestication, e.g., *melanocortin 1 receptor* (*MC1R*), *v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog* (*KIT*), *insulin like growth factor-2* (*IGF2*), *ryanodine receptor 1* (*RYR1*), and *nuclear receptor subfamily 6 group A member 1* (*NR6A1*).
4. Enter the name of the target (sub)species, preferentially the Latin name. Subsequently, all sequences available for this (sub)species are listed.
5. We recommend starting with a ~150 bp long fragment flanking the (sub)species-specific base. By varying the number of bases upstream and downstream of the (sub)species-specific base, one can control if the (sub)species-specific base will be located in the forward or in the reverse primer. If the number of bases upstream of the (sub)species-specific base is higher than downstream, the (sub)species-specific base will be located in the reverse primer.
6. We recommend the use of probes conjugated with (nonfluorescent) minor groove binding (MGB) quenchers because they form very stable duplexes with single-stranded DNA sequences. Thus, MGB probes are more sensitive to single-base mismatches and can be shorter than probes labeled with alternative quenchers.
7. A trend in routine analysis in official food control laboratories is method standardization. In the Austrian Agency for Health and Food Safety (AGES), real-time PCR assays should run at a standardized annealing temperature of 60 °C. Thus, in our studies we used the following settings for the melting temperature (T_m): forward and reverse primer: 58–60 °C, probe: 68–70 °C [11–14].
8. Introduction of a single mismatch base at position 3 from the 3′ end of the primer commonly enhances selectivity more than introducing the mismatch base at other positions, e.g., position 6. Most commonly, transversions are more efficient in enhancing selectivity than transitions.
9. With RNAfold, primers and probes can be tested for secondary structures. If the sequence is displayed as red ring, the formation of secondary structures is not likely. Avoid primers/probes

for which secondary structures involving more than five bases are predicted. The probability of forming homodimers or heterodimers can be tested with the OligoAnalyzer tool. Avoid primers or probes for which dimers with more than seven consecutive base pairs are predicted.

10. The concentration of the DNA isolates can be determined either by UV or fluorescent measurement. Although fluorescent measurement is more specific and sensitive, UV measurement is frequently used in routine analysis because it is faster and cheaper. For comparison reasons, use one and the same method for all DNA extracts.
11. We recommend using a DNA concentration of 10 ng/μL (determined photometrically).
12. Use identical primer and probe concentrations for all primer/probe systems, e.g., 200 nM for primers and 100 nM for probes. After initial testing, the primer and probe concentrations for the most selective primer/probe system may be further optimized.
13. In combination with mismatch primers, we found the QuantiTect Multiplex PCR Master Mix (Qiagen) to be very suitable. Alternatively, the Takyon™ No Rox Probe MasterMix dTTP Blue (Eurogentec), the GoTaq® Probe qPCR Master Mix (Promega), or the PerfeCTa® qPCR ToughMix™ UNG (Biosciences) may be used. Our results and those from Livak et al. indicate that the TaqMan® Universal PCR Master Mix (Applied Biosystems) is too sensitive to single-base mismatches and therefore not applicable for real-time PCR involving mismatch primers [11, 12, 14, 23].
14. For comparison reasons, the same threshold line should be used for all primer/probe systems (unless different probe concentrations are used).
15. The applicability of the primer/probe system should be further investigated. In our example on the differentiation of wild boar and domestic pig, we analyzed DNA extracts from muscle meat of 30 wild boar individuals and 60 domestic pig individuals, belonging to different pig breeds [14]. In addition, in our studies we commonly investigated cross-reactivity with other (sub)species potentially occuring in commercial meat products, e.g., plant species including spices. The real-time PCR assay should also be validated by analyzing meat extract mixtures and DNA extracts from model food, as described in detail in our papers [11–14].
16. For the discrimination of domestic pig from wild boar, we designed primers containing four (*see* **primer 20–22**, **primer 29–31**, Table 2) or five (**primer 32–34**, Table 2) mismatch

bases. Figure 9 shows that the introduction of more than three mismatch bases drastically lowered amplification of the target sequence, resulting in C_t values > 35.

17. We suggest varying the primer and probe concentration in the range from 50 nM to 1000 nM and 50 nM to 300 nM, respectively.
18. Investigate whether the selectivity for the target species can be further enhanced by using a higher concentration of the primer containing the species-specific base and the mismatch base (s) compared to the other primer.

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