

Collaborative ring trial for the detection of cow, sheep and goat milk in dairy samples by real-time PCR targeting mitochondrial DNA

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Online Resource 2, Supplementary Information

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1.1 Visualisation of binding sites of primers and probes

The binding sites of the primers and probes of the four tested real-time PCR assays targeting either cattle, sheep or goat mitochondrial DNA, or mammal mitochondrial DNA in total are shown (Fig. S1).

Cattle tRNA-Lys assay (Rentsch et al. 2013)

5'-RindAG1----->3'5'-RindAGFAM----->3'

AGTTAGAGATTGAGAGCCATATACTCTCC TGGTGACATGCCGCAACTAGACACG

Bos taurus : TTTTAAGTTAGAGATTGAGAGCCATATACTCTCCTTGGTGACATGCCGCAACTAGACACGTCACATGACTGACAATGATCTTATCAATATTC

Ovis aries :A...C.....ATAT...T.....A...T.....A.....G.....T.....A...TC.....T

Capra hircus :A...CC.....ATA.....A...T.....A.....G.....T.....CA...T.....T

GTTGTACTGACTGTTACTAGAAATAGTT

3'<-----RindAG2-5'

Sheep and goat NADH assays

5'-sheep-mt-F----->3'5'-sheep-mt-P----->3'

CCCTCACACTAGTCACCCCTAATCTTAC CCATACCCATCGCAGCAATCAATTTTAACA

5'-goat-mt-F----->3'5'-goat-mt-P----->3'

TGCACCTAACCCACCTAACCCCTATT CCGCACCCATCATAATAACCAACCTCAA

Ovis aries : TTCCCTCCCTCACACTAGTCACCCCTAATCTTACTAACCATACCCATCGCAGCAATCAATTTTAACACCCATAAATTCACCAATTATCCACTCTATGTAACCAACCACTTC

Capra hircus : ...T..T..TG.....AC.....CC...T...GC.....AT..AT..C...CC..C..T..T..C....C.....C...C.....T.....GGT..G....

Bos taurus : C.....A...T.....T..TT..C..CT.....T..C.....TAT..AT...A..GC.....T..C...CCTT...C..C.....C.....G..T...C...

GGTTAATAGGTGAGATACATTTTGTGTGG

TAGATGTTTAGGTGGTTGATGGGTG

3'<-----sheep-mt-R-5'

3'<-----goat-mt-R-5'

Universal mammal 16S rRNA gene assay

5'-mam-mt-F----->3'5'-mam-mt-P----->3'

GACCTCGATGTTGGATCAGGA ATGGTGCAACCGCTATCAAAGGTTTCG

Bos taurus : TTTACGACCTCGATGTTGGATCAGGACATCCTGATGGTGCAACCGCTATCAAAGGTTTCGTTTGTTCACGATTAAAGTCCTACGTGATCTG

Ovis aries :C..C.....

Capra hircus :ACAAGTTGCTAATTCAGGATGCAC

3'<-----mam-mt-R-5'

GenBank sequences used:	
Bos taurus:	NC_006853.1:8087-8179
Ovis aries:	NC_001941.1:7733-7825
Capra hircus:	NC_005044.2:7729-7821
Ovis aries:	NC_001941.1:11765-11875
Capra hircus:	NC_005044.2:11759-11869
Bos taurus:	NC_006853.1:12120-12230
Bos taurus:	NC_006853.1:2772-2862
Ovis aries:	NC_001941.1:2415-2505
Capra hircus:	NC_005044.2:2411-2501

Fig. S1 Binding sites of primers and probes with regard to the target genes of cattle, sheep and goat

2 Real-time PCR cycling conditions

The real-time PCR reaction mixtures for the singleplex assays (25 μ L total volume, each) were prepared with PerfeCTa qPCR ToughMix (Quantabio, Beverly, Massachusetts, USA) with either low ROX or without ROX, depending on the real-time PCR cycler, and 0.9 μ M forward and reverse primer (Biomers, Ulm, Germany), each as well as 0.1 μ M probe (Biomers) and 5 μ L DNA sample or standard in varying concentrations. The real-time PCR temperature-time programme was as follows: 10 min at 95 °C for initial denaturing and polymerase activation followed by 45 cycles of 15 s at 95 °C denaturing and 60 s at 60 °C annealing/amplification. The reaction conditions for the duplex real-time PCR assays were identical, but primer and probe concentrations had been optimised beforehand (data not shown), which are presented in Table S1.

Table S1 Primer and probe concentrations in duplex real-time PCR assays

Duplex real-time PCR assay	Primers and probes	Fluorophore/Quencher	Concentration in PCR (nM)
cattle + mammalian	RindAG1 / RindAG2		100
	RindAG-FAM	FAM/BMN-Q535	15
	mam-mt-F / mam-mt-R		100
	mam-mt-P	Cy5/2x BMN-Q650	100
sheep + mammalian	sheep-mt-F / sheep-mt-R		150
	sheep-mt-P	FAM/BMN-Q535	15
	mam-mt-F / mam-mt-R		150
	mam-mt-P	Cy5/BMN-Q650	50
goat + mammalian	goat-mt-F / goat-mt-R		200
	goat-mt-P	FAM/BMN-Q535	15
	mam-mt-F / mam-mt-R		200
	mam-mt-P	Cy5/BMN-Q650	50

All DNA samples, PCR reagent controls (no-template controls (NTCs) containing water instead of DNA sample) and DNA standards were measured in duplicate. DNA samples and NTCs were measured in all real-time PCR assays. Each species-specific assay was also applied for measurement of the six DNA standards prepared from the milk of the corresponding species. The mammalian assay was applied with the DNA standard series of all three species. The single-laboratory tests were performed on a CFX Opus real-time PCR cycler (Bio-Rad Laboratories, Feldkirchen, Germany). A variety of real-time PCR machines was used by the ring trial laboratories (see Table S2).

Table S2 Real-time PCR cyclers used by the ring trial participants

Laboratory	Real-time PCR cycler
A	Rotor Gene Q (Qiagen, Hilden, Germany)
B	Applied Biosystems 7500 (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
C	Applied Biosystems 7500 Fast (Thermo Fisher Scientific)
D	CFX96 (Bio-Rad Laboratories, Feldkirchen, Germany)
E	CFX96 (Bio-Rad Laboratories)
F	Applied Biosystems 7500 (Thermo Fisher Scientific)
G	Applied Biosystems 7500 (Thermo Fisher Scientific)
H	LightCycler 96/LightCycler 480 (Roche, Basel, Switzerland)
I	CFX96 (Bio-Rad Laboratories)
J	QuantStudio 5/Applied Biosystems 7500 (Thermo Fisher Scientific)
K	CFX96 DeepWell (Bio-Rad Laboratories)
L	QuantStudio 6 Flex (Thermo Fisher Scientific)
M	AriaMx (Agilent Technologies, Santa Clara, California, USA)
N	LightCycler 480 II (Roche)
O	AriaMx (Agilent Technologies)

3 In-silico specificity (exclusivity and inclusivity)

3.1 PrimerBLAST matches of primers and probes

For checking the primer pair and probe specificities of the real-time PCR assays, Primer-BLAST (Ye et al. 2013) was used. First, an automatic search using the sequences of the forward and reverse primers, and second, the respective probes and the reverse primers was performed in April 2023 with the following parameters: primer must have at least 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 bps at the 3' end; ignore targets that have 4 or more mismatches to the primer; Max target amplicons size = 1000. All primers and probe combinations with a maximum of 2 mismatches in the respective primer or probe binding sites were recorded (Table S3).

Table S3 Primers and probe combinations that exhibited a maximum of 2 mismatches in the respective primer and probe binding sites in the Primer-BLAST analysis

Species	Forward primer	Reverse primer	Probe
Cow assay			
<i>Bos primigenius</i>	0	0	0
<i>Bos indicus</i>	0	0	0
<i>Bos frontalis</i>	0	1	
<i>Bos javanicus</i>		0	0
<i>Bos grunniens</i>		1	1
<i>Bison priscus</i>		1	1
Sheep assay			
<i>Ovis orientalis</i>	0	0	0
<i>Ovis canadensis</i>	1	0	
<i>Ovis ammon</i>	0	1	2
<i>Ovis dalli</i>	2	0	
<i>Ovis nivicola</i>	2	0	
<i>Ovis vignei</i>	1	1	
Goat assay			
<i>Capra falconeri</i>	0	0	2
<i>Capra aegagrus</i>	0	0	0
<i>Capra ibex</i>	0	0	0
<i>Capra</i>	0	0	1
<i>cylindricornes</i>			
<i>Capra pyrenaica</i>	0	0	0
<i>Capra sibirica</i>	1	0	0
<i>Capra walie</i>	1	0	2
<i>Capra nubiana</i>	1	0	2
<i>Capra caucasica</i>	1	1	

References (Supplement)

Ye J, Coulouris, G, Zaretskaya, I, Cutcutache, I, Rozen, S, Madden, TL (2012) Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13(1):134.
<https://doi.org/10.1186/1471-2105-13-134>

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Bison_priscus_MN549280.1 : TTTTAAGTTAGAGATTGAGAGCAATATACTCTCCTGGTGACATGCCCAACTAGACACCGCAACATGACTGACAATAATCTTATCAATATTC : 93
Bison_priscus_NC_027233.1 : TTTTAAGTTAGAGATTGAGAGCAATATACTCTCCTGGTGACATGCCCAACTAGACACCGCAACATGACTGACAATAATCTTATCAATATTC : 93
Bison_priscus_OL741537.1 : TTTTAAGTTAGAGATTGAGAGCAATATACTCTCCTGGTGACATGCCCAACTAGACACCGCAACATGACTGACAATAATCTTATCAATATTC : 93
Bison_priscus_OL741538.1 : TTTTAAGTTAGAGATTGAGAGCAATATACTCTCCTGGTGACATGCCCAACTAGACACCGCAACATGACTGACAATAATCTTATCAATATTC : 93

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Fig. S2 Binding sites of primers and probe in *Bos* and *Bison* sequences

3.2.2 Sheep primers and probes; DNA sequence sections annotated as *Ovis* species

In May 2023, a BLAST search in GenBank was conducted with a *Ovis aries* ND5 sequence (NC_001941.1) segment spanning the binding regions from forward to reverse primer including 5 additional bases before and after the first and last primer bases, respectively. The search was restricted to DNA sequences annotated with the genus *Ovis*. 521 sequences were retrieved and upon filtering for hits with 100 % query coverage, 501 sequences remained. The binding site of the forward primer is marked in red, of the probe in yellow, and of the reverse primer in green. Non matching bases in the binding sites are depicted without red, yellow or green colour (Fig. S3).

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In May 2023, a BLAST search in GenBank was conducted with a *Capra hircus* ND5 sequence (AB735749.1) segment spanning the binding regions from forward to reverse primer including 5 additional bases before and after the first and last primer bases, respectively. The search was restricted to DNA sequences annotated with the genus *Capra*. 384 sequences were retrieved and upon filtering for hits with 100 % query coverage, 366 sequences remained. The binding site of the forward primer is marked in red, of the probe in yellow, and of the reverse primer in green. Non matching bases in the binding sites are depicted without red, yellow or green colour (Fig. S4).

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Fig. S4 Binding sites of primers and probe in *Capra* sequences