Evaluation of the TNO Triskelion bv PCR method for the detection of ruminant DNA

Authors:

O. Fumière[1], G. van Duijn[2], A. Marien[1] and G. Berben[1]

[1] CRA-W, Knowledge and Valorisation of Agricultural Products department, Gembloux, Belgium

[2] TNO Triskelion bv, Zeist, The Netherlands

September 2021

European Union Reference Laboratory for Animal Proteins in feedingstuffs
Walloon Agricultural Research Centre
Department Knowledge and Valorisation of Agricultural Products, Henseval Building, Chaussée de Namur 24,
B-5030 GEMBLOUX (BELGIUM)
Phone: +32 (0) 81 87 52 28 - Fax : +32 (0) 81 87 40 19
secretary@eurl.craw.eu – website: eurl.craw.eu

ISBN 2-87286-119-X
Legal Deposit D/2021/1463/2

Editor :

Centre wallon de Recherches agronomiques
Service Communication
Rue de Liroux, 9
5030 Gembloux (Belgique)

TABLE OF CONTENTS

1. Introduction

In the TSE roadmap II[1], the Commission considers a possible lifting of the ban on the use of processed animal proteins (PAP) from non-ruminant in non-ruminant feed (except for herbivores) without the lifting of the existing prohibition on intra-species recycling. Such a measure would however be acceptable only if validated analytical techniques to determine the species origin of PAP are available. Polymerase Chain Reaction (PCR) would be helpful for that purpose.

In that framework, the detection of the ruminant PAP is of crucial importance. TNO Triskelion bv has developed a ruminant real-time PCR target that has already passed successfully an interlaboratory study conducted by the JRC-IRMM[2] and agreed to publicly disclose the protocol in case of a successful validation study[3]. Nevertheless, as for other PCR methods, the protocol was always used by their developers and only in-house validated. For this purpose, the EURL-AP implemented in its lab the assay, evaluated its performances and its fitness for a transferability to a network of labs before to decide about the opportunity of the organisation of a validation study. The evaluation was conducted according guidelines established in the framework of the European project GMOseek[4]. These guidelines are based on international texts such as the Codex Alimentarius[5] and ISO Guidelines[6,7,8,9,10] or other relevant publications[11,12,13] in the field. Most of them are mainly dedicated to PCR detection methods of GMOs but are also applicable to any other real-time PCR method and offer an objective frame to this study.

The report integrates Gert van Duijn as an author of TNO Triskelion b.v. because of his help on some points and his openness to questions of the EURL-AP but the assessment of the method itself was done completely independently by the EURL-AP.

2. Description of the target

The primers and probe as well as the cycling conditions for the ruminant PCR tests are provided in annex 1[14].

---

1 **The TSE Road map 2 - A Strategy paper on Transmissible Spongiform Encephalopathies for 2010-20.**
Communication from the Commission to the European parliament and the Council. Brussels, 16/07/2010, COM(2010)384 final.
https://www.eurl.craw.eu/legal-sources-and-sops/legal-sources/

2 **Detection of Ruminant Meat and Bone Meals in Animal Feed by Real-Time Polymerase Chain Reaction: Result of an Interlaboratory Study (2007).**
M. Prado, G. Berben, O. Fumière, G. Van Duijn, J. Mensinga-Kruize, S. Reaney, A. Boix, C. von Holst, Journal of Agricultural and Food Chemistry, 55, 7495-7501.

3 **Non disclosure agreement n° Q&S 10-0227/HaM.** TNO Triskelion bv.

4 **GMOseek: Development of screening methods for GMOs.** Deliverable D8/01.

5 **Guidelines On Performance Criteria And Validation Of Methods For Detection, Identification And Quantification Of Specific DNA Sequences And Specific Proteins In Foods (2010).**
Codex Committee On Methods Of Analysis And Sampling. Codex alimentarius commission - WHO Rome.

6 **ISO/IEC 17025 - General requirements for the competence of testing and calibration laboratories (2005).**
International Organization for Standardization, Geneva, Switzerland.

7 **ISO 24276:2006. Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - General requirements and definitions (2006).**
International Organization for Standardization, Geneva, Switzerland.

8 **ISO 21569:2005. Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods (2005).**
International Organization for Standardization, Geneva, Switzerland.

9 **ISO 21570:2005. Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Quantitative nucleic acid based methods (2005).**
International Organization for Standardization, Geneva, Switzerland.

10 **International Standard (ISO) 5725, Accuracy (trueness and precision) of measurement methods and results (1994).**
International Organization for Standardization, Geneva, Switzerland.

11 **Protocol for the design, conduct and interpretation of method-performance studies (1995).**
Horwitz W., Pure Applied Chemistry, 67, 331-343.

12 **Definition of minimum Performance requirements for analytical methods of GMO testing (2008).**
CRL-GMFF.
http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requir_Analyt_methods_131008.pdf. Accessed 21 Dec. 2010

13 **A protocol for the validation of qualitative methods of detection (2011).**
C. von Holst and R. MacArthur
http://www.iupac.org/web/ins/2005-024-2-600

14 The full description of the test is provided in an annex because this one remains confidential as long as the PCR test is not successfully validated through a collaborative test.

2.1. Information collected in sequence databanks

The information provided by TNO Triskelion about the target was that it had a length of 85 bp and originated from the bovine alpha subunit precursor of the acetylcholine receptor.

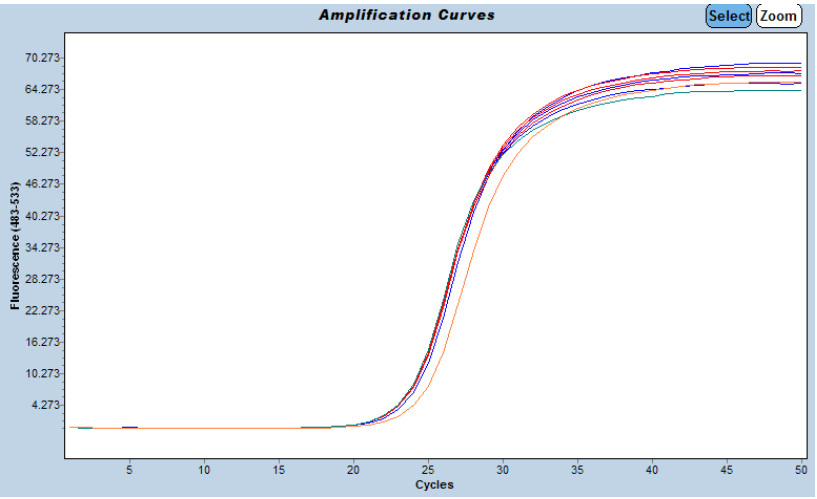
However a search in sequence databases shows it is an extremely abundant target in ruminants. It is rather difficult to estimate the quantity but by comparing the occurrence of the target in some sequenced pieces of bovine DNA compared with the size of the bovine genome we came to a rough estimate of at least 200 000 copies per cell. As will be seen further on, this feature has advantages (the method is sensitive) and drawbacks (the method may lead to false positive results if no appropriate cut-off is defined). Another point to stress is that when using only the primers for a search in the databases, the outcome is not a single sequence but a set of polymorphic sequences that are closely related.

2.2. Cloning and sequencing of PCR products

Pure DNAs extracted from blood samples of *Bos taurus*, *Ovis aries* and *Capra hircus* were amplified by PCR. The PCR products obtained were cloned into the pCR2 plasmid (Invitrogen, Carlsbad, CA, USA). Each of the transformed *Escherichia coli* was considered as an independent clone.

The PCR results obtained with plasmids isolated from each clone showed different efficiencies. Similar results were also observed with *Ovis aries* plasmids and *Capra hircus* plasmids.

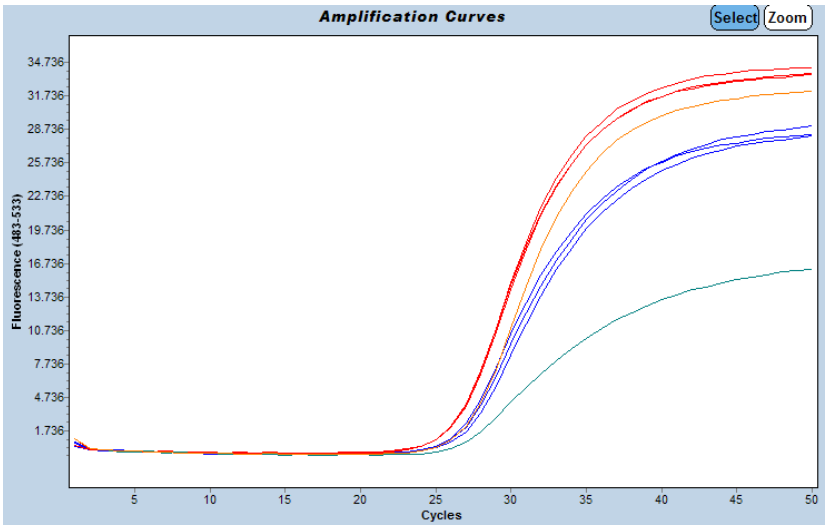
As it is shown in Figures 1 to 5, different clones of plasmids were analysed in the same conditions (same concentration or copy number in the reaction). When they were amplified using only primers and a mastermix containing SYBR® Green, the shape of the amplification curves was quite similar and the Ct values were very close (Figure 1).



Color of curve	Plasmid identification	Cp
	Bos Taurus A2C3	23.25
	Bos Taurus A1C8	23.23
	Bos Taurus A1C10	23.43
	Bos Taurus A2C8	24.22
	Bos Taurus A1C7	23.25
	Bos Taurus A1C3	23.60
	Bos Taurus A1C2	23.21
	Bos Taurus A2C5	23.11

***Figure 1**:* *Amplification signals obtained with plasmids containing various cloned Bos taurus amplicons. The PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) using SYBR® Green together with Universal Mastermix (Diagenode s.a.).*

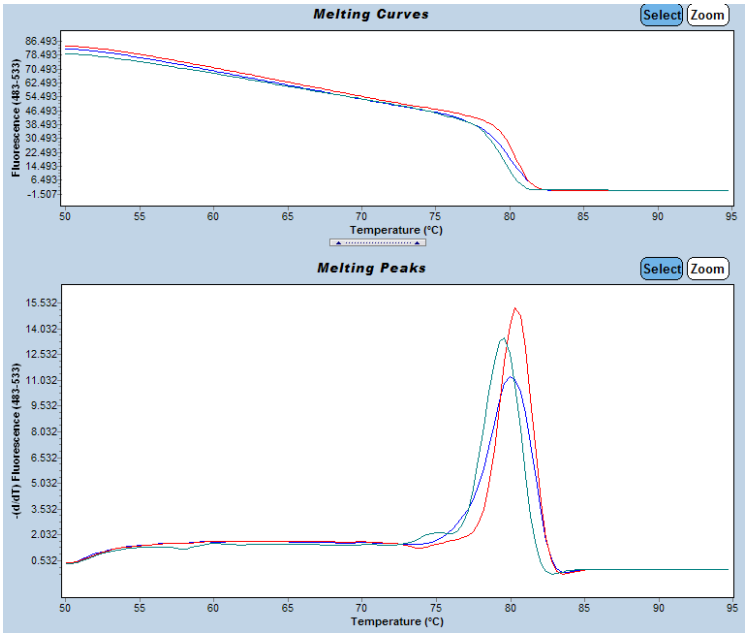
On the contrary, when amplifications were carried out with the appropriate TaqMan probe of the test, this resulted in clear differences of efficiency according to which clone was used and this whatever the species considered : *Bos taurus* (Figure 2), *Ovis aries* (Figure 4) or *Capra hircus* (Figure 5).



Color of curve	Plasmid identification	Cp
	Bos Taurus A2C3	26.12
	Bos Taurus A1C8	26.10
	Bos Taurus A1C10	26.02
	Bos Taurus A2C8	26.90
	Bos Taurus A1C7	26.61
	Bos Taurus A1C3	26.86
	Bos Taurus A1C2	26.34
	Bos Taurus A2C5	26.54

Figure 2: *Amplification signals obtained with plasmids containing various Bos taurus amplicons. The PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) using the TNO Triskelion ruminant TaqMan probe together with Universal Mastermix (Diagenode s.a.).*

Similarly, in SYBR® Green mode, different melting curves were observed according to the clone of plasmid considered (Figure 3). Only three clones (with a high, a medium and a low plateau level in Figure 2) were taken to avoid overloading of Figure 3.



Color of curve	Plasmid identification	Tm1
	Bos Taurus A1C8	80.41
	Bos Taurus A1C2	80.07
	Bos Taurus A2C5	79.51

Figure 3: *Melting curves obtained with plasmids containing various cloned Bos taurus amplicons. The PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) using SYBR® Green together with Universal Mastermix (Diagenode s.a.).*

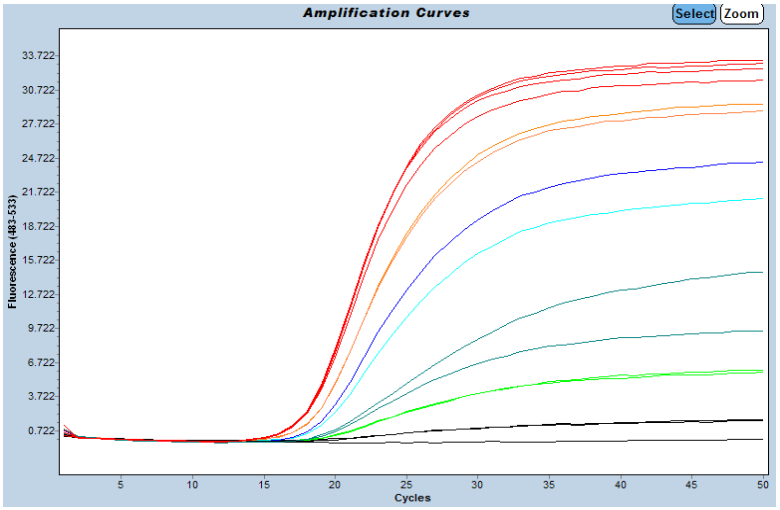
Several plasmid clones with inserts respectively from *Bos taurus*, *Ovis aries* and *Capra hircus* were sequenced with a PyroMark Q24 (Qiagen GmbH, Hilden, Germany) as pyrosequencing device. The resulting sequences showed differences at the level corresponding to the TaqMan probe (see Tables 1, 2 and 3 for *Bos taurus*, *Ovis aries* and *Capra hircus* plasmid clones respectively) and this explains the different efficiencies that were observed (Figures 4 and 5).

Table 1: *Sequences obtained on the PyroMark Q24 (Qiagen GmbH, Hilden, Germany) with "Bos taurus" plasmid clones.*

Species	Name of plasmid	Color of curve	Internal sequence (between primers)	Size of internal fragment	Size total (with primers)
Probe sequence			CAACTCTTCGCATGAGGTGGCCAAA		
Bos taurus	A2C3		GAGTCAACTCTTCGCATGAGGTGGCCAAAGTACTGGA	37	86
Bos taurus	A1C8		GAGTCAACTCTTCGCATGAGGTGGCCAAAGTACTGGA	37	86
Bos taurus	A1C10		GAGTCAACTCTTCGCATGAGGTGGCCAAAGTACTGGA	37	86
Bos taurus	A2C8		GCATCAACTCTTCGCATGAGGTGGCCAAAGTACTGGA	37	86
Bos taurus	A1C7		GAGTCAACTCTTCGCATAAGGTGGCCAAAGTCCTGGA	37	86
Bos taurus	A1C3		GAGTCAACTCTTCTCATGAGGTGGCCAAAGTACTGGA	37	86
Bos taurus	A1C2		GAGTCAAACTCTTCAATGAGGTGGCCAAAGTACTGGA	37	86
Bos taurus	A2C5		GAGTTAACTCTTCTCATGAGGTGGCCAAAGGACTGGA	37	86
Legend:			Sequence of the probe Difference with sequence of the probe Difference between the sequences of the plasmids		

Table 2: *Sequences obtained on the PyroMark Q24 (Qiagen GmbH, Hilden, Germany) with the "Ovis aries" plasmid clones.*

Species	Name of plasmid	Color of curve	Internal sequence (between primers)	Size of internal fragment	Size total (with primers)
Probe sequence			CAACTCTTCGCATGAGGTGGCCAAA		
Ovis aries	A1C22		GAGTCAACTCTTCGCATGAGGTGGCCAAAGTACTGGA	37	86
Ovis aries	A2C2		GAGTCAACTCTTCGCATGAGGTGGCCAAAGTACTGGA	37	86
Ovis aries	A1C17		AGTCAACTCTTCGCATGAGGTGGCCAAAGTACTGGA	36	85
Ovis aries	A1C14		GAGTCAACTCTTCGCATGAGGTGGCCAAAGTATTGGA	37	86
Ovis aries	A1C1		GAGTCAACTCTTCTCATGAGGTGGCCAAAGTACTGGA	37	86
Ovis aries	A1C15		GAGTCAACTCTTCTCATGAGGTGGCCAAAGTACTGGA	37	86
Ovis aries	A1C9		GAGTCAACTCTTTGCATGAGGTGGCCAAAGTACTGGA	37	86
Ovis aries	A1C7		AGTCAACTCTTTGCATGAGGTGGCCAAAGTACTGGA	36	85
Ovis aries	A1C11		GAGTCAGCTGTTCACATGAGGTGGCCAAAGTACTGGA	37	86
Ovis aries	A1C16		GAGTCAACTCTTCACATGATGTGGCCAAAGTACTGGA	37	86
Ovis aries	A1C4		GAGTCAACTCTTGGCATGAGGTGGCCAGAATACTGGA	37	86
Ovis aries	A1C8		GAGTCAACTCTTCACATGAGGTTGCCAAAGTACTGGA	37	86
Ovis aries	A1C5		GAGTTAACTCTTCACATGAAGTGGCCAAAGTACTGGA	37	86
Ovis aries	A1C10		GATTCAACTCTTTGCAAGAGGTGGCCAAAGTATTGGA	37	86
Ovis aries	A1C12		GAGTCAGTTCTTCATATCAGGTGGGCAAAGTATTGGA	37	86
Legend:			Sequence of the probe Difference with sequence of the probe Difference between the sequences of the plasmids		

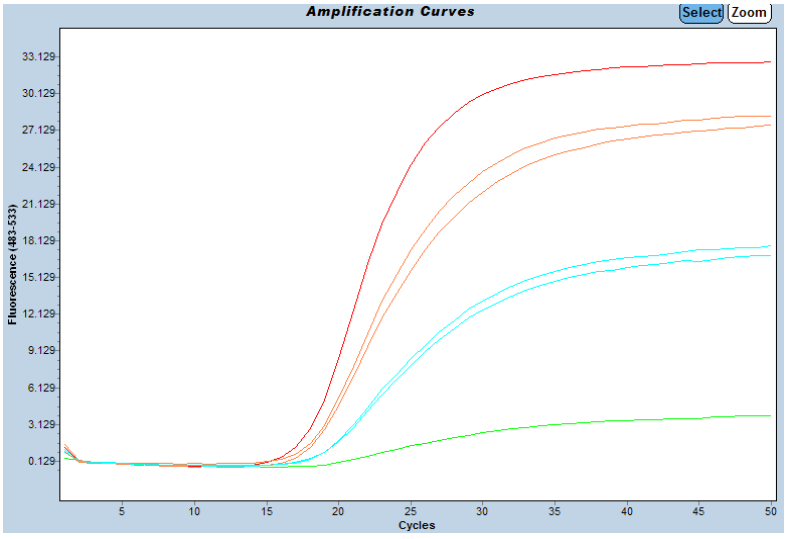


Color of curve	Plasmid identification	Cp
	Ovis Aries A1C22	17.89
	Ovis Aries A2C2	17.86
	Ovis Aries A1C17	17.77
	Ovis Aries A1C14	17.84
	Ovis Aries A1C1	18.29
	Ovis Aries A1C15	18.22
	Ovis Aries A1C9	18.62
	Ovis Aries A1C7	18.78
	Ovis Aries A1C11	19.12
	Ovis Aries A1C16	18.88
	Ovis Aries A1C4	19.29
	Ovis Aries A1C8	18.83
	Ovis Aries A1C5	18.83
	Ovis Aries A1C10	18.92
	Ovis Aries A1C12	50

Figure 4: *Amplification signals obtained with plasmids containing various cloned Ovis aries amplicons. The PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) using the TNO Triskelion ruminant TaqMan probe together with Universal Mastermix (Diagenode s.a.).*

Table 3: *Sequences obtained on the PyroMark Q24 (Qiagen GmbH, Hilden, Germany) with the "Capra hircus" plasmid clones.*

Species	Name of plasmid	Color of curve	Internal sequence (between primers)	Size of internal fragment	Size total (with primers)
Probe sequence			CAACTCTTCGCATGAGGTGGCCAAA		
Capra hircus	A1C2		GAGTCAACTCTTCGCATGAGGTGGCCAAAGTACTGGA	37	86
Capra hircus	A1C4		GAGTCAACTCTTCGCATGAAGTGGCCAAAGTACTGGA	37	86
Capra hircus	A2C1		GAGTCAACTCTTGGCATGAGGTGGCCAAAGTACTGGA	37	85
Capra hircus	A2C3		GAGTCAACTCTTCACATGAGCTGGCCAAAGTACTGGA	37	86
Capra hircus	A1C3		GAGTCAACTCTCTGCATGAGGTGGCCAAAGTACTGGA	37	86
Capra hircus	A1C1		GAGTCAACTCTTCTCATGAAGTGGCCAAAGTATTGGA	37	86
Legend:			Sequence of the probe Difference with the sequence of the probe Difference between the sequences of the plasmids		



Color of curve	Species/Samples	Cp
	Capra hircus A1C2	17.61
	Capra hircus A1C4	18.02
	Capra hircus A2C1	17.92
	Capra hircus A2C3	18.70
	Capra hircus A1C3	18.73
	Capra hircus A1C1	18.79

Figure 5: *Amplification signals obtained with plasmids containing various cloned Capra hircus amplicons. The PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) using the TNO Triskelion ruminant TaqMan probe together with Universal Mastermix (Diagenode s.a.).*

The comparison between the different sequences and the PCR efficiencies showed a direct link between the sequences of the amplicons and the sequence of the probe: the more similar the sequences, the higher the efficiency of the amplification.

From these different results, we can conclude that the target is not unique but is polymorphic. It was difficult to determine if the selected sequence of the test is among the most abundant targets or not. Empirically however it works on such a polymorphic system. It will nevertheless not be possible on the basis of the amplicon sequence to determine from which ruminant species precisely a target is coming.

2.3. Specificity of the test

The specificity of the test was checked on a wide variety of animal and plant species. The DNAs tested came from the EURL-AP DNA sample bank. A maximum of precautions were taken to be sure of their purity: for animals, most of the DNAs were extracted from blood samples; when it was not possible, the DNAs were extracted from meat. In the case of fish species extraction was carried out on muscle using samples obtained from a German institute (Federal Research Centre for Nutrition and Food, Department of Fish Quality, Hamburg) with reference certificates; DNAs from sea mammals were also tested. They were extracted from muscle samples collected by Belgian and Italian specialised institutes.

The tests were performed with three real-time thermocyclers present in the EURL-AP facilities: one LightCycler LC480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland), one ABI7000 and one ABI7500 (Applied Biosystems, Foster City, CA, USA).

Two master mixes were used for the tests of specificity on the ABI7000 and the ABI7500: the Universal Mastermix (Diagenode s.a., Seraing, Belgium) and the TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA). A third master mix was added for these tests on the LightCycler LC480: the qPCR MasterMix for probe assay ROX (Eurogentec s.a., Seraing, Belgium).

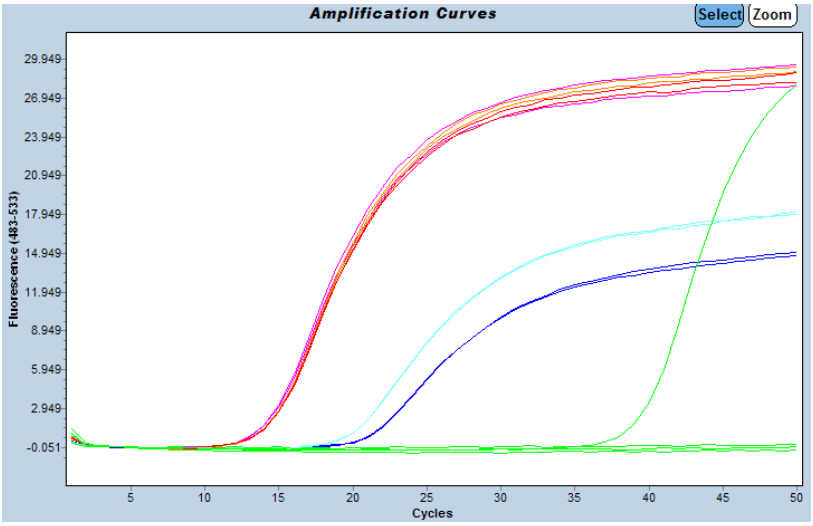
Finally, the specificity of the primers alone was also checked on a LightCycler LC480 using the Universal Mastermix (Diagenode s.a., Seraing, Belgium) containing SYBR® Green.

2.3.1. 1st PCR platform

The specificity of the test was firstly checked with DNA samples from ruminant samples and feed samples containing ruminant material. The species tested were major farmed ruminants (cow, sheep and goat) but also game species (stag or red deer - *Cervus elaphus* - and roe deer - *Capreolus capreolus*). Table 4 and Figure 6 summarize the results obtained on the LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.).

Table 4: *Specificity tests carried out on samples containing ruminant DNA. PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) with Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: Abs. quant/second derivative max and high confidence. The CRL samples tested are known to contain ruminant material.*

Species/samples	C_t	Mean C_t	Result	**Species/samples**	C_t	Mean C_t	Result
Bos taurus	14.01 14.02	14.02	**+**	**CRL 1302 extract 1**	22.78 22.78	22.78	**+**
Ovis aries	14.21 14.30	14.26	**+**	**CRL 1302 extract 2**	22.71 22.67	22.69	**+**
Capra hircus	14.21 14.28	14.25	**+**	**CRL 1356 extract 1**	23.91 23.91	23.91	**+**
Cervus elaphus	19.73 19.74	19.74	**+**	**CRL 1356 extract 2**	24.23 24.21	24.22	**+**
Capreolus capreolus	20.84 20.94	20.89	**+**	**CRL 1358 extract 1**	24.19 24.20	24.20	**+**
0.05 % ruminant PAP in feed (m/m)	30.49 30.39	30.44	**+**	**CRL 1358 extract 2**	24.06 24.13	24.10	**+**
0.0125 % ruminant PAP in feed (m/m)	32.53 32.57	32.55	**+**	**CRL 1373 extract 1**	22.79 23.14	22.97	**+**
				CRL 1373 extract 2	22.62 22.66	22.64	**+**

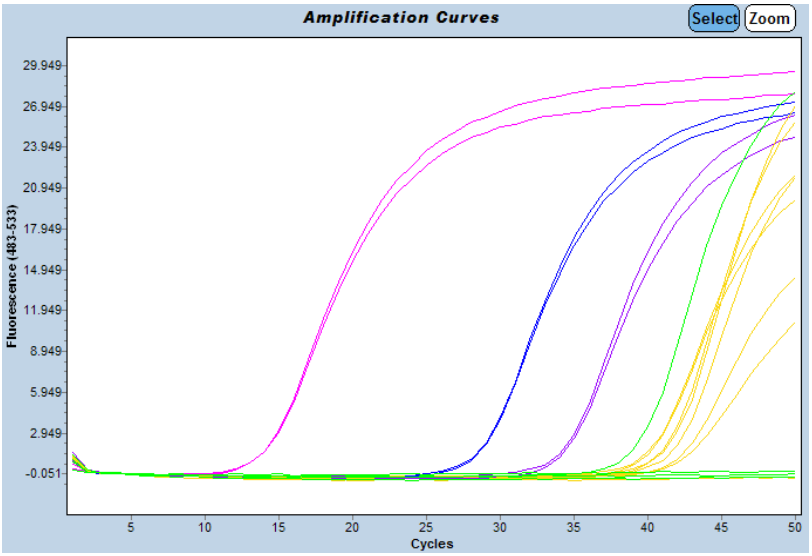


Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	14.01
	Bos Taurus 10ng	14.02
	Capra hircus 10ng	14.21
	Capra hircus 10ng	14.28
	Ovis aries 10ng	14.21
	Ovis aries 10ng	14.30
	Cervus Elaphus 10ng	19.73
	Cervus Elaphus 10ng	19.74
	Capreolus capreolus 10ng	20.84
	Capreolus capreolus 10ng	20.94
	No template control	39.31
	No template control	50
	No template control	50
	No template control	50

Figure 6: *Amplification signals obtained on DNAs extracted from ruminant blood samples and on no template control (H_2O) with the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Analysis mode: Abs. quant/second derivative max and high confidence*

All samples tested showed clear amplification signals. The earliest and highest signals are obtained with the ruminant farmed species. Compared to these signals, the Ct values obtained with the ruminant game species show a delay of 6 cycles and a final level of fluorescence reduced by half.

The tests of specificity continued with DNA samples from non-ruminant species. Different relevant categories were investigated: terrestrial mammalians, domestic birds, fish species and sea mammals. For the terrestrial mammalians, pig DNA was of course tested next to horse, donkey and rodents (rabbit, hare and rat). The possibility of a cross-reaction with human DNA was also considered. For the domestic birds, poultry species such as chicken, turkey, Muscovy duck and guinea fowl were tested. Different fish species potentially present in fishmeals and some sea mammals present in our sample bank were also tested. Figure 7 and Table 5 summarize the results obtained on a LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.).



Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	14.01
	Bos Taurus 10ng	14.02
	Tursiops truncatus 10ng	28.57
	Tursiops truncatus 10ng	28.63
	Ziphius cavirostris 10ng	34.16
	Ziphius cavirostris 10ng	34.23
	Grampus griseus 10ng	40.03
	Grampus griseus 10ng	41.72
	Stenella coeruleoalba 10ng	41.93
	Stenella coeruleoalba 10ng	42.09
	Phocoena phocoena 10ng	40.16
	Phocoena phocoena 10ng	41.62
	Phocidae 10ng	41.21
	Phocidae 10ng	50
	No template control	39.31
	No template control	50
	No template control	50
	No template control	50

Figure 7: *Amplification signals obtained with DNAs extracted from Bos taurus blood, sea mammal muscle and with no template control (H_2O) on the LC480 (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Analysis mode: Abs. quant/second derivative max and high confidence.*

Table 5: *Specificity tests performed on non-ruminant samples using as PCR platform the LC480 thermocycler (Roche Diagnostics Ltd.) combined with the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

Species	C_t	Mean C_t	Result	Species	C_t	Mean C_t	Result
Other terrestrial mammalians				**Fish species**			
Homo sapiens	50 50	50	-	***Gadus morhua***	50 50	50	-
Sus scrofa domesticus	41.21 50	45.61	-	***Pollachius virens***	50 50	50	-
Equus asinus	45 50	48	-	***Melanogrammus aeglefinus***	50 40.62	45.31	-
Equus caballus	43.70 50	46.85	-	***Micromesistius poutassou***	45 41.44	43.22	-
Oryctolagus cuniculus	39.34 40.30	39.82	-	***Sebastes spp.***	40.23 40.49	40.36	-
Lepus europaeus	50 50	50	-	***Mallotus villosus***	50 50	50	-
Rattus rattus	42.15 40.91	41.53	-	***Scomber scombrus***	41.92 43.52	42.52	-
Domestic birds				***Clupea harengus***	41.05 50	45.53	-
Gallus gallus	50 50	50	-	***Merluccius merluccius***	50 42.42	46.21	-
Numida meleagris	42.59 50	46.30	-	***Trachurus trachurus***	41.05 42.49	41.77	-
Meleagris gallopavo	41.63 50	45.82	-	***Trisopterus minutus***	41.25 41.00	41.13	-
Cairina moschata	45 43.46	44.23	-	***Sardina pilchardus***	50 39.95	44.98	-
Phasianus colchicus	50 40.89	45.45	-	***Engraulis encrasicolus***	39.58 41.81	40.71	-
Columba livia	50 42.16	46.08	-	***Gadus ogac***	42.67 50	46.34	-
Sea mammals				***Trisopterus esmarki***	40.56 50	46.34	-
Stenella coeruleoalba	42.09 41.93	42.01	-	***Ammodytes lancea***	41.14 44.09	42.62	-
Tursiops truncatus	28.63 28.57	28.60	+	***Ammodytes lancea***	42.68 42.33	42.51	-
Grampus griseus	40.03 41.72	40.88	-	***Sprattus sprattus***	43.98 50	46.99	-
Ziphius cavirostris	34.23 34.16	34.20	+	***Salmo salar***	50 42.40	46.20	-
Phocoena phocoena	40.16 41.62	40.89	-	***Raja* spp.**	45 50	47.5	-
Phocidae	50 41.21	45.61	-	***Paralithodes camtschaticus***	50 41.94	45.97	-

The results obtained for most of these non-ruminant samples showed late amplification curves resulting in Ct values that are not considered as significant[15]. Nevertheless, two of the sea mammals tested (the common Bottlenose Dolphin or *Tursiops truncatus* and the Cuvier's beaked whale or *Ziphius cavirostris* gave amplification signals with Ct values (of approximately 28 and 34 cycles respectively) that would be considered as positive results.

To complete the evaluation of the specificity of the assay, plant samples were also tested. Table 6 presents the results obtained on the LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.).

[15] At this step of the study, the decision criterion was based in first approximation on the cut-off value proposed by TNO Triskelion (35 cycles).

Table 6: *Specificity tests performed on plant species using as PCR platform the LC480 thermocycler (Roche Diagnostics Ltd.) combined with the Universal Mastermix (Diagenode s.a.). All plant extracts were at 2 ng/µl and 5 µl were tested. Analysis mode: Abs. quant/second derivative max and high confidence.*

Species	C_t	Mean C_t	Result	**Species**	C_t	Mean C_t	Result
Plant species							
Glycine max	40.72 42.26	41.49	-	***Oryza sativa***	50 50	50	-
Zea mays	50 43.83	46.92	-	***Lycopersicon esculentum***	43.73 42.72	43.23	-
Brassica napus	41.80 41.64	41.72	-	***Beta vulgaris***	50 43.40	46.70	-
Triticum aestivum	43.46 50	46.73	-				

In a wide majority of the reactions, a signal is observed but the Ct value has to be considered as a negative result according the decision criteria provided by TNO Triskelion[14].

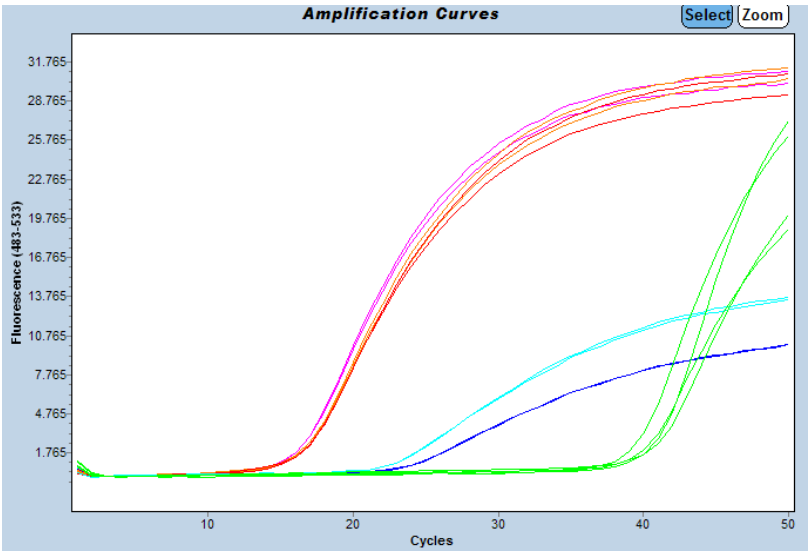
2.3.2. 2nd PCR platform

Tables 7 to 9 and Figures 8 and 9 summarise the same tests of specificity as in section 2.3.1 but performed on a platform combining the LC480 thermocycler (Roche Diagnostics Ltd.) with the TaqMan Universal PCR Mastermix (Applied Biosystems).

Table 7 and Figure 8 present the results obtained with ruminant samples and feed samples containing ruminant materials.

Table 7: *Specificity tests carried out on samples containing ruminant DNA. PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) with the TaqMan Universal PCR Mastermix (Applied Biosystems). Pure species DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: Abs. quant/second derivative max and high confidence. The CRL samples tested are known to contain ruminant material.*

Species/sample	C_t	Mean C_t	Result	**Sample**	C_t	Mean C_t	Result
Ruminant species and samples containing ruminant material							
Bos taurus	16.20 16.15	16.18	+	**CRL 1302 extract 1**	25.39 25.24	25.32	+
Ovis aries	16.58 16.77	16.68	+	**CRL 1302 extract 2**	25.06 25.17	25.13	+
Capra hircus	16.64 16.59	16.62	+	**CRL 1356 extract 1**	26.57 26.50	26.54	+
Cervus elaphus	22.72 22.61	22.67	+	**CRL 1356 extract 2**	26.89 26.80	26.85	+
Capreolus capreolus	23.67 23.80	23.74	+	**CRL 1358 extract 1**	27.19 26.88	27.04	+
0.05 % ruminant PAP in feed (m/m)	32.82 32.78	32.80	+	**CRL 1358 extract 2**	26.75 26.67	26.71	+
0.0125 % ruminant PAP in feed (m/m)	34.98 34.91	34.95	+	**CRL 1373 extract 1**	25.47 25.54	25.51	+
				CRL 1373 extract 2	25.27 25.16	25.22	+

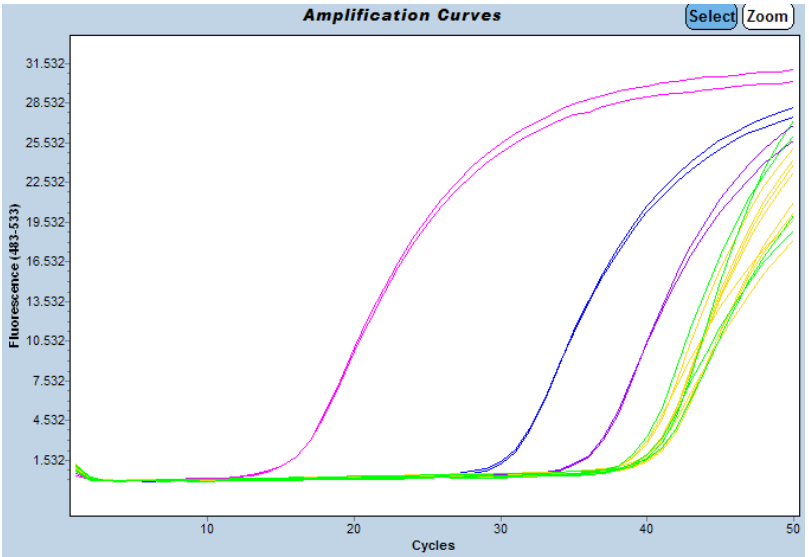


Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	16.15
	Bos Taurus 10ng	16.20
	Capra hircus 10ng	16.59
	Capra hircus 10ng	16.64
	Ovis aries 10ng	16.58
	Ovis aries 10ng	16.77
	Cervus Elaphus 10ng	22.61
	Cervus Elaphus 10ng	22.72
	Capreolus capreolus 10ng	23.67
	Capreolus capreolus 10ng	23.80
	No template control	39.35
	No template control	39.93
	No template control	40.85
	No template control	40.88

Figure 8: *Amplification signals obtained on DNAs extracted from ruminant blood samples and on no template control (H_2O) with the LC480 thermocycler (Roche Diagnostics Ltd.) using the TaqMan Universal PCR Mastermix (Applied Biosystems). Analysis mode: Abs. quant/second derivative max and high confidence.*

Overall, the tests performed with this set of samples are conclusive. The Ct values obtained show a delay of 2 to 2.5 cycles compared to the results obtained with the previous platform (LC480 - Universal Mastermix from Diagenode s.a.). The sample adulterated with 0.0125 % of bovine PAP is still positive but its Ct value is very close to the TNO Triskelion cut-off value whereas it was clearly positive (mean Ct = 32.55) with the first platform (LC480 - Universal Mastermix from Diagenode s.a.). The signals obtained with the game ruminant species samples show the same characteristics as with the previous platform (higher Ct and lower final fluorescence level). With this mastermix, the “no template controls” performed with water always gave a signal even though with Ct values after the cut-off (Ct values around 40 cycles). This is probably due to the presence of ruminant DNA traces introduced in the mastermix together with bovine serum albumin (BSA), that is meant as enzyme stabilizer.

Figure 9 and Table 8 present the results obtained on the LC480 (Roche Diagnostics Ltd.) with the TaqMan Universal PCR Mastermix (Applied Biosystems) for samples free of ruminant DNA.



Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	16.15
	Bos Taurus 10ng	16.20
	Tursiops truncatus 10ng	30.74
	Tursiops truncatus 10ng	30.78
	Ziphius cavirostris 10ng	36.17
	Ziphius cavirostris 10ng	36.47
	Grampus griseus 10ng	39.71
	Grampus griseus 10ng	40.75
	Stenella coeruleoalba 10ng	40.46
	Stenella coeruleoalba 10ng	40.97
	Phocoena phocoena 10ng	40.29
	Phocoena phocoena 10ng	40.35
	Phocidae 10ng	39.00
	Phocidae 10ng	40.23
	No template control	39.35
	No template control	39.93
	No template control	40.85
	No template control	40.88

Figure 9: *Amplification signals obtained with DNAs extracted from Bos taurus blood, sea mammal muscle and with no template control (H_2O) on the LC480 (Roche Diagnostics Ltd.) using the TaqMan Universal PCR Mastermix (Applied Biosystems). Analysis mode: Abs. quant/second derivative max and high confidence.*

Table 8: *Specificity tests performed on non-ruminant samples using as PCR platform the LC480 thermocycler (Roche Diagnostics Ltd.) combined with the TaqMan Universal PCR Mastermix (Applied Biosystems). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

Species	C_t	Mean C_t	Result	**Species**	C_t	Mean C_t	Result
Other terrestrial mammalians				**Fish species**			
Homo sapiens	39.67 40.76	40.22	-	***Gadus morhua***	40.32 39.82	40.07	-
Sus scrofa domesticus	40.98 39.79	40.39	-	***Pollachius virens***	40.01 39.92	39.97	-
Equus asinus	41.46 40.34	40.90	-	***Melanogrammus aeglefinus***	41.02 41.43	41.23	-
Equus caballus	40.78 40.99	40.89	-	***Micromesistius poutassou***	41.22 40.15	40.69	-
Oryctolagus cuniculus	40.52 40.56	40.54	-	***Sebastes spp.***	40.90 41.02	40.96	-
Lepus europaeus	40.24 40.71	40.48	-	***Mallotus villosus***	41.74 42.83	42.26	-
Rattus rattus	40.55 41.01	40.78	-	***Scomber scombrus***	39.62 39.92	39.77	-
Domestic birds				***Clupea harengus***	39.61 40.51	40.06	-
Gallus gallus	41.54 39.28	40.41	-	***Merluccius merluccius***	41.08 39.91	40.50	-
Numida meleagris	40.79 41.31	41.05	-	***Trachurus trachurus***	39.29 40.37	39.83	-
Meleagris gallopavo	39.84 41.06	40.45	-	***Trisopterus minutus***	41.13 40.70	40.92	-
Cairina moschata	40.53 40.38	40.46	-	***Sardina pilchardus***	41.08 41.28	41.18	-
Phasianus colchicus	40.33 39.67	40.00	-	***Engraulis encrasicolus***	40.09 40.68	40.39	-
Columba livia	41.58 40.21	40.90	-	***Gadus ogac***	41.71 40.68	41.27	-
Sea mammals				***Trisopterus esmarki***	41.00 41.79	41.40	-
Stenella coeruleoalba	40.46 40.97	40.72	-	***Ammodytes lancea***	40.21 40.56	40.39	-
Tursiops truncatus	30.78 30.74	30.76	+	***Ammodytes lancea***	41.96 40.23	41.10	-
Grampus griseus	40.75 39.71	40.23	-	***Sprattus sprattus***	41.48 40.85	41.17	-
Ziphius cavirostris	36.47 36.17	36.32	-	***Salmo salar***	39.30 41.48	40.39	-
Phocoena phocoena	40.35 40.29	40.32	-	***Raja* spp.**	40.63 42.02	41.33	-
Phocidae	40.23 39.00	39.62	-	***Paralithodes camtschaticus***	50 45	47.5	-

As already observed before, Figure 9 shows it is impossible to have no signal at all with the TaqMan Universal PCR Mastermix (Applied Biosystems), even with negative samples. This is probably due to the presence of ruminant DNA traces in the mastermix. However, based on the Ct values, these signals correspond to negative results. *Tursiops truncatus* (common Bottlenose Dolphin) leads to signals that have to be considered as positive while based on a cut-off value of 35 cycles, *Ziphius cavirostris* presents Ct values beyond the cut-off (36 cycles).

The plant samples gave the results presented in Table 9.

Table 9: *Specificity tests performed on plant species using as PCR platform the LC480 thermocycler (Roche Diagnostics Ltd.) with the TaqMan Universal PCR Mastermix (Applied Biosystems). All plant extracts were at 2 ng/µl and 5 µl were tested. Analysis mode: Abs. quant/second derivative max and high confidence.*

Species	C_t	Mean C_t	Result	**Species**	C_t	Mean C_t	Result
Plant species							
Glycine max	41.18 40.37	40.78	-	***Oryza sativa***	39.94 41.05	40.50	-
Zea mays	41.60 40.82	41.21	-	***Lycopersicon esculentum***	41.02 40.52	40.77	-
Brassica napus	39.54 40.60	40.07	-	***Beta vulgaris***	40.47 41.61	41.04	-
Triticum aestivum	41.15 39.83	40.49	-				

As for the other negative samples, all replicates gave signals with Ct values comprised between 40 and 41 cycles.

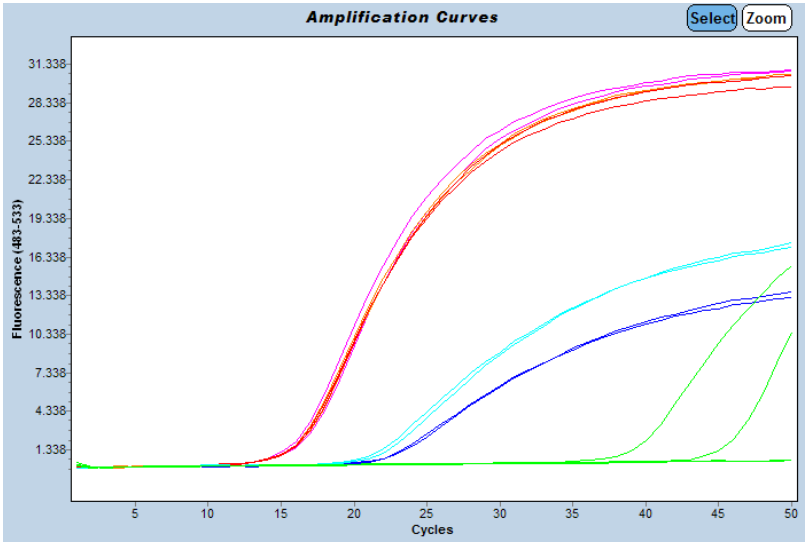
2.3.3. 3rd PCR platform

The third platform on which the specificity tests were done was the combination of the LC 480 thermocycler (Roche Diagnostics Ltd.) with the qPCR MasterMix for probe assay ROX (Eurogentec s.a.). The results are presented in Tables 10 to 12 and Figures 10 and 11.

Table 10 and Figure 10 present the results obtained with ruminant samples and feed samples containing ruminant materials.

Table 10: *Specificity tests carried out on samples containing ruminant DNA. PCR was performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the qPCR MasterMix for probe assay ROX (Eurogentec s.a.). Pure species DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: Abs. quant/second derivative max and high confidence. The CRL samples tested are known to contain ruminant material.*

Species/sample	C_t	Mean C_t	Result	**Sample**	C_t	Mean C_t	Result
Ruminant species and samples containing ruminant material							
Bos taurus	16.59 15.97	16.28	+	**CRL 1302 extract 1**	24.74 24.80	24.77	+
Ovis aries	16.10 16.25	16.18	+	**CRL 1302 extract 2**	24.75 24.73	24.74	+
Capra hircus	16.30 16.08	16.19	+	**CRL 1356 extract 1**	26.27 26.21	26.24	+
Cervus elaphus	21.72 21.19	21.46	+	**CRL 1356 extract 2**	26.52 26.39	26.46	+
Capreolus capreolus	22.09 22.53	22.31	+	**CRL 1358 extract 1**	26.47 26.37	26.42	+
0.05 % ruminant PAP in feed (m/m)	31.85 31.78	31.82	+	**CRL 1358 extract 2**	26.24 26.28	26.26	+
0.0125 % ruminant PAP in feed (m/m)	33.89 33.78	33.84	+	**CRL 1373 extract 1**	24.96 25.07	25.02	+
				CRL 1373 extract 2	24.75 24.73	24.74	+

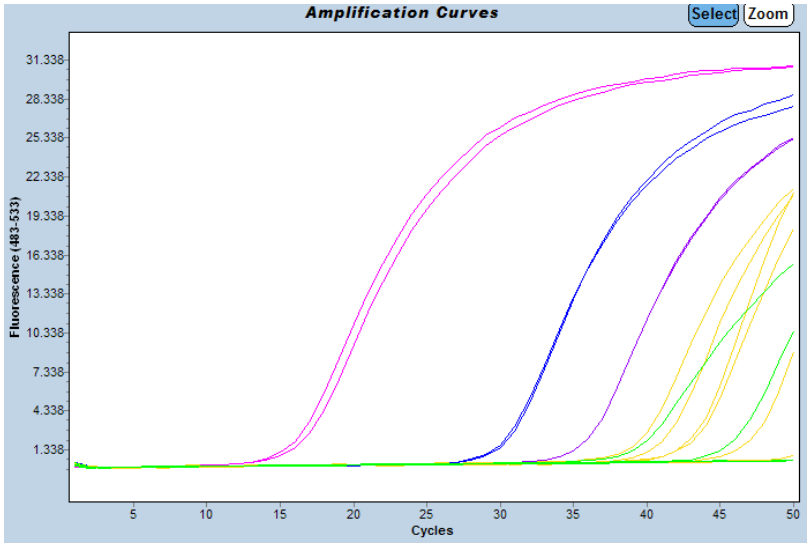


Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	15.97
	Bos Taurus 10ng	16.59
	Capra hircus 10ng	16.08
	Capra hircus 10ng	16.30
	Ovis aries 10ng	16.10
	Ovis aries 10ng	16.25
	Cervus Elaphus 10ng	21.19
	Cervus Elaphus 10ng	21.72
	Capreolus capreolus 10ng	22.09
	Capreolus capreolus 10ng	22.53
	No template control	39.39
	No template control	45
	No template control	50
	No template control	50

Figure 10: *Amplification signals obtained on DNAs extracted from ruminant blood samples and on no template control (H_2O) with the LC480 thermocycler (Roche Diagnostics Ltd.) using the qPCR MasterMix for probe assay ROX (Eurogentec s.a.). Analysis mode: Abs. quant/second derivative max and high confidence.*

As with the two first platforms, all the samples tested gave positive results. The occurrence of signals obtained with the replicates of the negative PCR controls is lower with this mastermix than with the one of Applied Biosystems.

The results of the analysis on DNA samples of non-target animal species using the LC480 (Roche Diagnostics Ltd.) with the qPCR MasterMix for probe assay ROX (Eurogentec s.a.) are presented in Figure 11 and Table 11.



Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	15.97
	Bos Taurus 10ng	16.59
	Tursiops truncatus 10ng	30.12
	Tursiops truncatus 10ng	30.31
	Ziphius cavirostris 10ng	35.73
	Ziphius cavirostris 10ng	35.77
	Grampus griseus 10ng	39.39
	Grampus griseus 10ng	41.04
	Stenella coeruleoalba 10ng	43.28
	Stenella coeruleoalba 10ng	45
	Phocoena phocoena 10ng	43.19
	Phocoena phocoena 10ng	45
	Phocidae 10ng	50
	Phocidae 10ng	50
	No template control	39.39
	No template control	45
	No template control	50
	No template control	50

Figure 11: *Amplification signals obtained with DNAs extracted from Bos taurus blood, sea mammal muscle and with no template control (H_2O) on the LC480 (Roche Diagnostics Ltd.) using the qPCR MasterMix for probe assay ROX (Eurogentec s.a.). Analysis mode: Abs. quant/second derivative max and high confidence.*

Table 11: *Specificity tests on non-ruminant samples using as PCR platform the LC480 thermocycler (Roche Diagnostics Ltd.) combined with the qPCR MasterMix for probe assay ROX (Eurogentec s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. Quant/second derivative max and high confidence.*

Species	C_t	Mean C_t	Result	Species	C_t	Mean C_t	Result
Other terrestrial mammalians				**Fish species**			
Homo sapiens	50 45	47.5	-	***Gadus morhua***	50 42.76	46.38	-
Sus scrofa domesticus	45 45	45	-	***Pollachius virens***	50 45	47.50	-
Equus asinus	45 43.72	46.86	-	***Melanogrammus aeglefinus***	40.96 41.32	41.14	-
Equus caballus	45 45	45	-	***Micromesistius poutassou***	41.24 45	43.12	-
Oryctolagus cuniculus	41.72 42.82	42.27	-	***Sebastes spp.***	45 50	47.50	-
Lepus europaeus	45 45	45	-	***Mallotus villosus***	40.80 44.02	42.41	-
Rattus rattus	41.19 40.03	40.61	-	***Scomber scombrus***	40.88 43.58	42.23	-
Domestic birds				***Clupea harengus***	41.03 45	43.02	-
Gallus gallus	50 45	47.5	-	***Merluccius merluccius***	45 44.25	44.63	-
Numida meleagris	45 45	45	-	***Trachurus trachurus***	45 50	47.50	-
Meleagris gallopavo	50 45	47.5	-	***Trisopterus minutus***	45 44.54	44.77	-
Cairina moschata	45 45	45	-	***Sardina pilchardus***	45 45	45	-
Phasianus colchicus	41.66 41.25	41.46	-	***Engraulis encrasicolus***	40.65 43.89	42.27	-
Columba livia	50 50	50	-	***Gadus ogac***	45 45	45	-
Sea mammals				***Trisopterus esmarki***	45 40.55	42.78	-
Stenella coeruleoalba	45 43.28	44.14	-	***Ammodytes lancea***	41.03 42.29	41.66	-
Tursiops truncatus	30.31 30.12	30.22	+	***Ammodytes lancea***	50 45	47.50	-
Grampus griseus	41.04 39.39	40.22	-	***Sprattus sprattus***	50 45	47.50	-
Ziphius cavirostris	35.73 35.77	35.75	-	***Salmo salar***	43.70 42.96	43.33	-
Phocoena phocoena	43.19 50	46.60	-	***Raja* spp.**	42.49 44.38	43.44	-
Phocidae	50 50	50	-	***Paralithodes camtschaticus***	45 45	45	-

A majority of the negative samples gave no signal or signals after 40 cycles. Once again, the aspecific signals appeared with two sea mammal species : *Tursiops truncatus* was clearly positive (Mean Ct = 30.22) while the two replicates of *Ziphius cavirostris* gave Ct values (35.73 and 35.77) close to the cut-off value defined by TNO Triskelion.

The plant samples tested gave the results presented in Table 12.

Table 12: *Specificity tests performed on plant species using as PCR platform the LC480 thermocycler (Roche Diagnostics Ltd.) with the qPCR MasterMix for probe assay ROX (Eurogentec s.a.). All plant extracts were at 2 ng/µl and 5 µl were tested. Analysis mode: Abs. quant/second derivative max and high confidence.*

Species	C_t	Mean C_t	Result	Species	C_t	Mean C_t	Result
Plant species							
Glycine max	40.78 50	45.39	-	***Oryza sativa***	45 45	45	-
Zea mays	45 41.93	43.47	-	***Lycopersicon esculentum***	45 40.71	42.86	-
Brassica napus	41.17 45	43.09	-	***Beta vulgaris***	50 45	47.50	-
Triticum aestivum	45 45	45	-				

Amplification curves were observed for all the replicates of the species tested but with Ct values higher than 40 cycles. So no false positive result can be picked out.

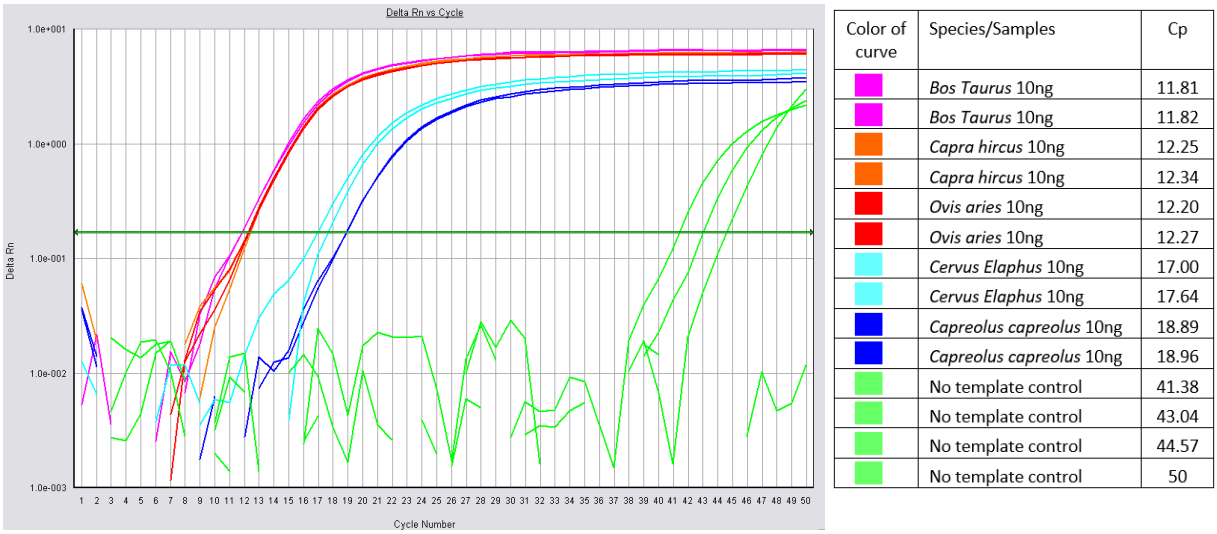
2.3.4. 4th PCR platform

Specificity tests were realised with an ABI7000 thermocycler (Applied Biosystems) combined with the Universal Mastermix (Diagenode s.a.).

Table 13 and Figure 12 present the results obtained with ruminant samples and feed samples containing ruminant material.

Table 13: *Specificity tests carried out on with samples containing ruminant DNA. PCR was performed on an ABI7000 thermocycler (Applied Biosystems) with the Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: automatic baseline and threshold at 0.17. The CRL samples tested are known to contain ruminant material.*

Species/sample	C_t	Mean C_t	Result	**Sample**	C_t	Mean C_t	Result
Ruminant species and samples containing ruminant material							
Bos taurus	11.82 11.81	11.82	+	**CRL 1302 extract 1**	20.52 21.18	20.85	+
Ovis aries	12.27 12.20	12.23	+	**CRL 1302 extract 2**	20.12 20.29	20.21	+
Capra hircus	12.34 12.25	12.29	+	**CRL 1356 extract 1**	21.72 21.11	21.42	+
Cervus elaphus	17.64 17.00	17.32	+	**CRL 1356 extract 2**	22.11 21.95	22.03	+
Capreolus capreolus	18.89 18.96	18.92	+	**CRL 1358 extract 1**	22.26 21.98	22.12	+
0.05 % ruminant PAP in feed (m/m)	28.45 28.60	28.53	+	**CRL 1358 extract 2**	21.85 21.56	21.70	+
0.0125 % ruminant PAP in feed (m/m)	30.68 30.56	30.62	+	**CRL 1373 extract 1**	19.69 20.79	20.24	+
				CRL 1373 extract 2	20.83 20.53	20.68	+



Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	11.81
	Bos Taurus 10ng	11.82
	Capra hircus 10ng	12.25
	Capra hircus 10ng	12.34
	Ovis aries 10ng	12.20
	Ovis aries 10ng	12.27
	Cervus Elaphus 10ng	17.00
	Cervus Elaphus 10ng	17.64
	Capreolus capreolus 10ng	18.89
	Capreolus capreolus 10ng	18.96
	No template control	41.38
	No template control	43.04
	No template control	44.57
	No template control	50

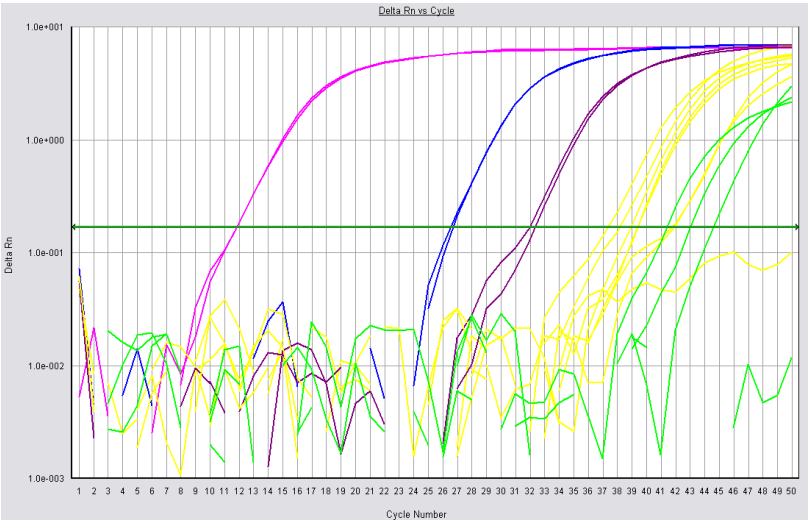
Figure 12: *Amplification signals obtained on DNAs extracted from ruminant blood samples and on no template control (H_2O) with the ABI7000 thermocycler (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.). Analysis mode: automatic baseline and threshold at 0.17.*

The Ct values obtained with this platform were the earliest compared to what was observed with the previous platforms and all the samples with the target gave positive results.

Table 14 and Figure 13 present the results obtained with samples from non-ruminant species.

Table 14: *Specificity tests performed on non-ruminant samples using as PCR platform the ABI7000 thermocycler (Applied Biosystems) with the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: automatic baseline and threshold at 0.17.*

Species	C_t	Mean C_t	Result	Species	C_t	Mean C_t	Result
Other terrestrial mammalians				**Fish species**			
Homo sapiens	39.64 39.06	39.35	-	***Gadus morhua***	38.47 40.15	39.31	-
Sus scrofa domesticus	39.09 50	44.54	-	***Pollachius virens***	50 40.32	45.16	-
Equus asinus	50 50	50	-	***Melanogrammus aeglefinus***	41.74 42.74	42.24	-
Equus caballus	47.90 40.03	43.97	-	***Micromesistius poutassou***	42.60 43.34	42.97	-
Oryctolagus cuniculus	37.49 39.34	38.42	-	***Sebastes spp.***	45.39 40.73	43.06	-
Lepus europaeus	50 50	50	-	***Mallotus villosus***	50 50	50	-
Rattus rattus	39.49 42.92	41.20	-	***Scomber scombrus***	50 41.46	45.73	-
Domestic birds				***Clupea harengus***	50 39.48	44.74	-
Gallus gallus	39.96 40.74	40.35	-	***Merluccius merluccius***	40.45 43.64	42.05	-
Numida meleagris	38.85 50	44.43	-	***Trachurus trachurus***	50 50	50	-
Meleagris gallopavo	39.18 41.03	40.10	-	***Trisopterus minutus***	42.06 40.87	41.47	-
Cairina moschata	50 50	50	-	***Sardina pilchardus***	50 38.76	44.38	-
Phasianus colchicus	50 42.15	46.07	-	***Engraulis encrasicolus***	50 50	50	-
Columba livia	50 50	50	-	***Gadus ogac***	44.12 43.10	43.61	-
Sea mammals				***Trisopterus esmarki***	50 41.00	45.50	-
Stenella coeruleoalba	39.32 39.33	39.32	-	***Ammodytes lancea***	39.16 38.70	38.93	-
Tursiops truncatus	26.67 26.49	26.58	+	***Ammodytes lancea***	41.21 42.66	41.93	-
Grampus griseus	37.35 38.15	37.75	-	***Sprattus sprattus***	50 50	50	-
Ziphius cavirostris	32.31 32.03	32.17	+	***Salmo salar***	45.05 42.62	43.83	-
Phocoena phocoena	50 38.94	44.47	-	***Raja* spp.**	39.58 50	44.79	-
Phocidae	41.78 41.99	41.88	-	***Paralithodes camtschaticus***	50 44.63	47.31	-



Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	11.81
	Bos Taurus 10ng	11.82
	Tursiops truncatus 10ng	26.49
	Tursiops truncatus 10ng	26.67
	Ziphius cavirostris 10ng	32.03
	Ziphius cavirostris 10ng	32.31
	Grampus griseus 10ng	37.35
	Grampus griseus 10ng	38.15
	Stenella coeruleoalba 10ng	39.32
	Stenella coeruleoalba 10ng	39.33
	Phocoena phocoena 10ng	38.94
	Phocoena phocoena 10ng	50
	Phocidae 10ng	41.78
	Phocidae 10ng	41.99
	No template control	41.38
	No template control	43.04
	No template control	44.57
	No template control	50

Figure 13: *Amplification signals obtained with DNAs extracted from Bos taurus blood, sea mammal muscle and with no template control (H_2O) on the ABI7000 (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.). Analysis mode: automatic baseline and threshold at 0.17.*

The only aspecific results appear with *Tursiops truncatus* and *Ziphius cavirostris* which respectively provide Ct values of 26 and 32 cycles.

The plant samples tested gave the results presented in Table 15.

Table 15: *Specificity tests performed on plant species using as PCR platform the ABI7000 thermocycler (Applied Biosystems) with the Universal Mastermix (Diagenode s.a.). All plant extracts were at 2 ng/µl and 5 µl were tested. Analysis mode: automatic baseline and threshold at 0.17.*

Species	C_t	Mean C_t	Result	**Species**	C_t	Mean C_t	Result
Plant species							
Glycine max	50 41.02	45.51	-	***Oryza sativa***	38.53 46.49	42.51	-
Zea mays	50 50	50	-	***Lycopersicon esculentum***	40.86 42.83	41.85	-
Brassica napus	41.45 38.50	39.97	-	***Beta vulgaris***	50 39.29	44.65	-
Triticum aestivum	41.33 42.04	41.69	-				

All the Ct values were determined after the cut-off when a signal was observed.

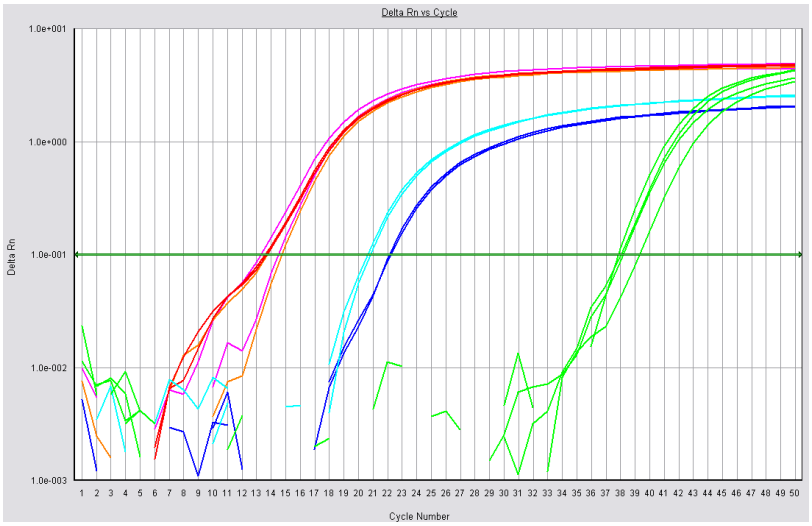
2.3.5. 5th PCR platform

The fifth platform on which the specificity tests were done was an ABI7000 thermocycler (Applied Biosystems) combined with the TaqMan Universal PCR Mastermix (Applied Biosystems).

Table 16 and Figure 14 present the results obtained with ruminant samples and feed samples containing ruminant material.

Table 16: *Specificity tests carried out on samples containing ruminant DNA. PCR was performed on a ABI7000 thermocycler (Applied Biosystems) with the TaqMan Universal PCR Mastermix (Applied Biosystems). Pure species DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: automatic baseline and threshold at 0.1. The CRL samples tested are known to contain ruminant material.*

Species/sample	C_t	Mean C_t	Result	**Sample**	C_t	Mean C_t	Result
Ruminant species and samples containing ruminant material							
Bos taurus	14.42 13.26	13.84	+	**CRL 1302 extract 1**	23.03 23.64	23.33	+
Ovis aries	13.69 13.56	13.62	+	**CRL 1302 extract 2**	22.96 22.48	22.72	+
Capra hircus	13.71 14.67	14.19	+	**CRL 1356 extract 1**	24.02 24.08	24.05	+
Cervus elaphus	20.77 20.57	20.67	+	**CRL 1356 extract 2**	24.50 24.48	24.49	+
Capreolus capreolus	22.12 22.21	22.17	+	**CRL 1358 extract 1**	24.45 24.45	24.45	+
0.05 % ruminant PAP in feed (m/m)	30.36 30.57	30.38	+	**CRL 1358 extract 2**	24.16 24.08	24.12	+
0.0125 % ruminant PAP in feed (m/m)	32.65 32.46	32.57	+	**CRL 1373 extract 1**	22.70 23.09	22.90	+
				CRL 1373 extract 2	22.74 22.60	22.67	+

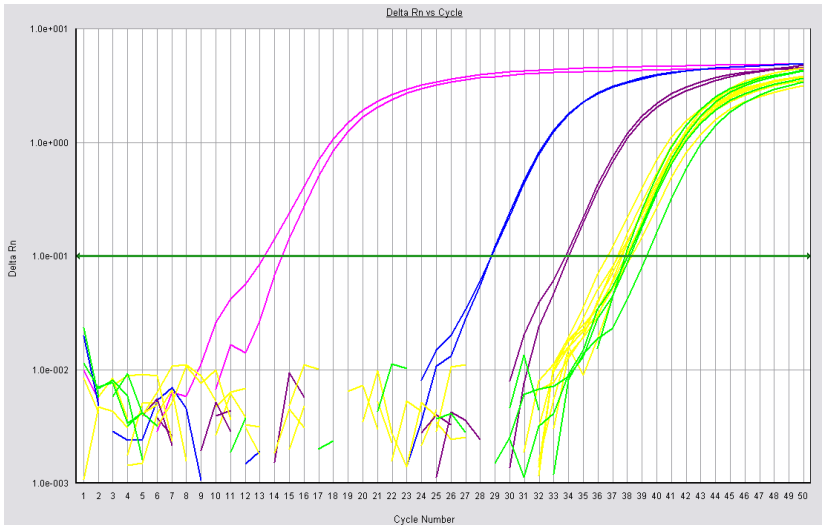


Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	13.26
	Bos Taurus 10ng	14.42
	Capra hircus 10ng	13.71
	Capra hircus 10ng	14.67
	Ovis aries 10ng	13.56
	Ovis aries 10ng	13.69
	Cervus Elaphus 10ng	20.77
	Cervus Elaphus 10ng	20.57
	Capreolus capreolus 10ng	22.12
	Capreolus capreolus 10ng	22.21
	No template control	37.83
	No template control	38.05
	No template control	38.15
	No template control	39.24

***Figure 14**: Amplification signals obtained on DNAs extracted from ruminant blood samples and on no template control (H_2O) with an ABI7000 thermocycler (Applied Biosystems) using the TaqMan Universal PCR Mastermix (Applied Biosystems). Analysis mode: automatic baseline and threshold at 0.1.*

With this second mastermix tested on the ABI7000 thermocycler, a delay of ~2 cycles is observed compared to the results obtained with Universal Mastermix (Diagenode s.a.) on the same thermocycler. A similar behaviour was also pointed out when this same mastermix was used with the LC 480 thermocycler. Nevertheless, all the samples gave clear positive results. The presence of ruminant DNA in the mastermix is confirmed by systematic amplifications with the PCR negative control (H_2O) but the Ct values obtained (~38 cycles) are after the cut-off value.

The results obtained with samples from non-ruminant species are presented in Table 17 and Figure 15.



Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	13.26
	Bos Taurus 10ng	14.42
	Tursiops truncatus 10ng	28.67
	Tursiops truncatus 10ng	28.71
	Ziphius cavirostris 10ng	33.75
	Ziphius cavirostris 10ng	34.02
	Grampus griseus 10ng	37.37
	Grampus griseus 10ng	37.50
	Stenella coeruleoalba 10ng	37.28
	Stenella coeruleoalba 10ng	38.26
	Phocoena phocoena 10ng	36.63
	Phocoena phocoena 10ng	37.59
	Phocidae 10ng	37.90
	Phocidae 10ng	38.01
	No template control	37.83
	No template control	38.05
	No template control	38.15
	No template control	39.24

***Figure 15**: Amplification signals obtained with DNAs extracted from Bos taurus blood, sea mammal muscle and with no template control (H_2O) on the ABI7000 (Applied Biosystems) using the TaqMan Universal PCR Mastermix (Applied Biosystems). Analysis mode: automatic baseline and threshold at 0.1.*

Table 17: *Specificity tests performed on non-ruminant samples using as PCR platform the ABI7000 thermocycler (Applied Biosystems) with the TaqMan Universal PCR Mastermix (Applied Biosystems). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: automatic baseline and threshold at 0.1.*

Species	C_t	Mean C_t	Result	Species	C_t	Mean C_t	Result
Other terrestrial mammalians				**Fish species**			
Homo sapiens	37.74 37.03	37.39	-	***Gadus morhua***	38.54 37.70	38.12	-
Sus scrofa domesticus	37.11 38.27	37.69	-	***Pollachius virens***	37.70 37.31	37.50	-
Equus asinus	37.33 37.84	37.59	-	***Melanogrammus aeglefinus***	38.01 38.15	38.08	-
Equus caballus	37.02 36.96	36.99	-	***Micromesistius poutassou***	38.94 38.63	38.79	-
Oryctolagus cuniculus	38.21 38.03	38.12	-	***Sebastes spp.***	38.56 37.40	37.98	-
Lepus europaeus	38.23 37.05	37.64	-	***Mallotus villosus***	38.03 38.62	38.33	-
Rattus rattus	38.31 37.56	37.94	-	***Scomber scombrus***	37.01 37.81	37.41	-
Domestic birds				***Clupea harengus***	38.08 40.03	39.05	-
Gallus gallus	38.12 37.59	37.86	-	***Merluccius merluccius***	38.23 39.39	38.81	-
Numida meleagris	37.04 37.57	37.30	-	***Trachurus trachurus***	38.95 37.56	38.26	-
Meleagris gallopavo	36.81 38.19	37.50	-	***Trisopterus minutus***	37.61 38.09	37.85	-
Cairina moschata	38.76 38.05	38.40	-	***Sardina pilchardus***	37.66 37.93	37.80	-
Phasianus colchicus	36.77 37.01	36.89	-	***Engraulis encrasicolus***	39.04 39.38	39.21	-
Columba livia	37.28 38.26	37.77	-	***Gadus ogac***	37.03 37.50	37.27	-
Sea mammals				***Trisopterus esmarki***	39.41 39.26	39.33	-
Stenella coeruleoalba	37.28 38.26	37.77	-	***Ammodytes lancea***	36.87 38.15	37.51	-
Tursiops truncatus	28.71 28.67	28.69	+	***Ammodytes lancea***	37.60 37.79	37.69	-
Grampus griseus	37.50 37.37	37.44	-	***Sprattus sprattus***	38.06 37.37	37.71	-
Ziphius cavirostris	34.02 33.75	33.89	+	***Salmo salar***	40.25 38.23	39.24	-
Phocoena phocoena	36.63 37.59	37.11	-	***Raja* spp.**	38.06 39.14	38.60	-
Phocidae	38.01 37.90	37.95	-	***Paralithodes camtschaticus***	43.99 42.95	43.47	-

With this platform, *Tursiops truncatus* and *Ziphius cavirostris* are positive (mean Ct values of 28.69 and 33.89 respectively). The shape of the curves are similar to these obtained with ruminant samples (slope and final fluorescence level).

The plant samples tested gave the results presented in Table 18.

Table 18: *Specificity tests performed on vegetal samples using as PCR platform the ABI7000 thermocycler (Applied Biosystems) with the TaqMan Universal PCR Mastermix (Applied Biosystems). All plant extracts were at 2 ng/µl and 5 µl were tested. Analysis mode: automatic baseline and threshold at 0.1.*

Species	C_t	Mean C_t	Result	Species	C_t	Mean C_t	Result
Plant species							
Glycine max	37.90 38.24	38.07	-	***Oryza sativa***	37.21 37.56	37.39	-
Zea mays	38.95 38.19	38.57	-	***Lycopersicon esculentum***	38.40 38.41	38.40	-
Brassica napus	37.77 36.99	37.38	-	***Beta vulgaris***	38.21 39.11	38.66	-
Triticum aestivum	36.69 37.02	36.85	-				

All the replicates of the plant samples tested gave amplification curves but with Ct values after the cut-off.

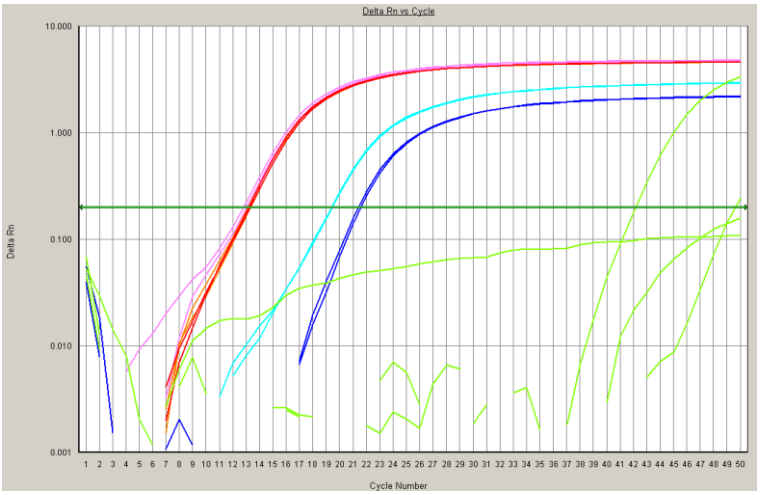
2.3.6. 6th PCR platform

The 6th platform used the third thermocycler tested, the ABI 7500 (Applied Biosystems), in combination with the Universal Mastermix (Diagenode s.a.).

Table 19 and Figure 16 present the results obtained with ruminant samples and feed samples containing ruminant materials.

Table 19: *Specificity tests carried out on samples containing ruminant DNA. PCR was performed on an ABI7500 thermocycler (Applied Biosystems) with the Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: automatic baseline and threshold at 0.2. The CRL samples tested are known to contain ruminant material.*

Species/sample	C_t	Mean C_t	Result	**Sample**	C_t	Mean C_t	Result
Ruminant species and samples containing ruminant material							
Bos taurus	13.04 12.77	12.90	+	**CRL 1302 extract 1**	21.83 21.92	21.87	+
Ovis aries	13.25 13.15	13.20	+	**CRL 1302 extract 2**	21.83 21.65	21.74	+
Capra hircus	13.12 13.27	13.19	+	**CRL 1356 extract 1**	22.91 22.95	22.93	+
Cervus elaphus	19.37 19.31	19.34	+	**CRL 1356 extract 2**	23.27 23.38	23.32	+
Capreolus capreolus	21.34 21.51	21.43	+	**CRL 1358 extract 1**	23.31 23.29	23.30	+
0.05 % ruminant PAP in feed (m/m)	29.61 29.51	29.56	+	**CRL 1358 extract 2**	23.04 23.09	23.07	+
0.0125 % ruminant PAP in feed (m/m)	31.67 31.81	31.74	+	**CRL 1373 extract 1**	21.75 21.84	21.80	+
				CRL 1373 extract 2	21.66 21.54	21.60	+

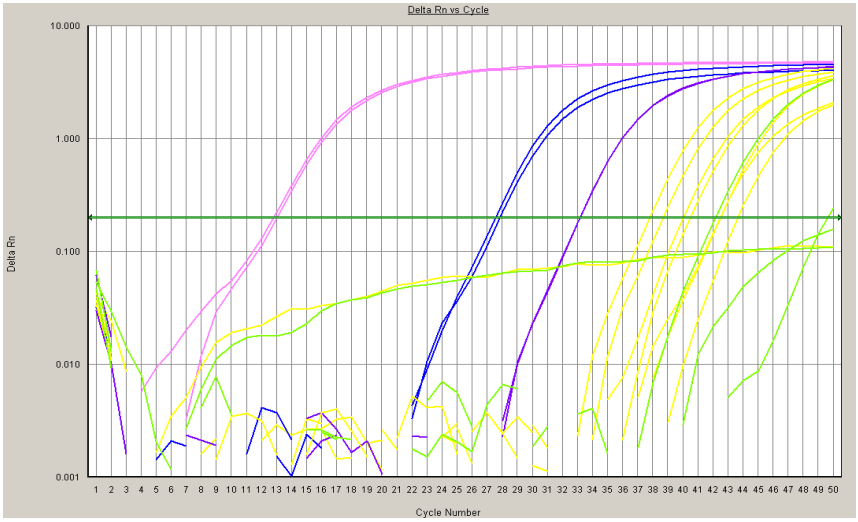


Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	12.7724
	Bos Taurus 10ng	13.0366
	Capra hircus 10ng	13.1159
	Capra hircus 10ng	13.2659
	Ovis aries 10ng	13.1543
	Ovis aries 10ng	13.2464
	Cervus Elaphus 10ng	19.3142
	Cervus Elaphus 10ng	19.3690
	Capreolus capreolus 10ng	21.3397
	Capreolus capreolus 10ng	21.5135
	No template control	42.1357
	No template control	49.5599
	No template control	50
	No template control	50

Figure 16: *Amplification signals obtained on DNAs extracted from ruminant blood samples and on no template control (H_2O) with an ABI7500 (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.). Analysis mode: automatic baseline and threshold at 0.2.*

As with the other platforms, the signals obtained with the farmed ruminants are earlier than with the other ruminant species which are nevertheless detected as positive.

The results obtained with samples from non-ruminant species are presented in Table 20 and Figure 17.



Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	12.7724
	Bos Taurus 10ng	13.0366
	Tursiops truncatus 10ng	27.4725
	Tursiops truncatus 10ng	27.7841
	Ziphius cavirostris 10ng	33.1318
	Ziphius cavirostris 10ng	33.1350
	Grampus griseus 10ng	37.7138
	Grampus griseus 10ng	38.6094
	Stenella coeruleoalba 10ng	40.0065
	Stenella coeruleoalba 10ng	42.4290
	Phocoena phocoena 10ng	40.4182
	Phocoena phocoena 10ng	42.4163
	Phocidae 10ng	43.5909
	Phocidae 10ng	50
	No template control	42.1357
	No template control	49.5599
	No template control	50
	No template control	50

Figure 17: *Amplification signals obtained with DNAs extracted from Bos taurus blood, sea mammal muscle and with no template control (H_2O) on the ABI7500 (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.). Analysis mode: automatic baseline and threshold at 0.2.*

Table 20: *Specificity tests performed on non-ruminant samples using as PCR platform the ABI7500 thermocycler (Applied Biosystems) with the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: automatic baseline and threshold at 0.2.*

Species	C_t	Mean C_t	Result	**Species**	C_t	Mean C_t	Result
Other terrestrial mammalians				**Fish species**			
Homo sapiens	50 39.70	44.85	-	***Gadus morhua***	50 41.30	45.65	-
Sus scrofa domesticus	42.61 41.40	42.00	-	***Pollachius virens***	42.06 40.90	41.48	-
Equus asinus	44.05 50	47.02	-	***Melanogrammus aeglefinus***	44.10 50	47.05	-
Equus caballus	46.80 42.06	44.43	-	***Micromesistius poutassou***	40.93 39.91	40.42	-
Oryctolagus cuniculus	38.19 41.47	39.83	-	***Sebastes spp.***	42.10 40.32	41.21	-
Lepus europaeus	50 40.52	45.26	-	***Mallotus villosus***	38.45 43.37	40.91	-
Rattus rattus	40.50 39.91	40.21	-	***Scomber scombrus***	49.40 42.07	45.74	-
Domestic birds				***Clupea harengus***	40.24 40.30	40.27	-
Gallus gallus	50 42.90	46.5	-	***Merluccius merluccius***	40.75 46.96	43.85	-
Numida meleagris	41.47 50	45.74	-	***Trachurus trachurus***	41.27 50	45.63	-
Meleagris gallopavo	50 40.35	45.18	-	***Trisopterus minutus***	41.24 44.54	42.89	-
Cairina moschata	44.74 50	47.37	-	***Sardina pilchardus***	41.78 41.13	41.46	-
Phasianus colchicus	39.45 40.45	39.95	-	***Engraulis encrasicolus***	43.25 45.94	44.60	-
Columba livia	50 43.16	46.58	-	***Gadus ogac***	50 50	50	-
Sea mammals				***Trisopterus esmarki***	43.26 38.38	40.82	-
Stenella coeruleoalba	42.43 40.01	41.22	-	***Ammodytes lancea***	39.48 42.60	41.04	-
Tursiops truncatus	27.78 27.47	27.63	+	***Ammodytes lancea***	41.08 42.39	41.74	-
Grampus griseus	38.61 37.71	38.16	-	***Sprattus sprattus***	50 50	50	-
Ziphius cavirostris	33.13 33.14	33.13	+	***Salmo salar***	43.02 50	46.51	-
Phocoena phocoena	42.42 40.42	41.42	-	***Raja* spp.**	38.46 44.21	41.33	-
Phocidae	50 43.59	46.80	-	***Paralithodes camtschaticus***	50 50	50	-

Tursiops truncatus and *Ziphius cavirostris* are both positive (mean Ct values of 27.63 and 33.13 respectively). On this platform, the shape of the sea mamal amplification curves is similar to what is obtained with ruminant samples (slope and final fluorescence level).

The plant samples tested gave the results presented in Table 21.

Table 21: *Specificity tests performed on plant species using as PCR platform the ABI7500 thermocycler (Applied Biosystems) with the Universal Mastermix (Diagenode s.a.). All plant extracts were at 2 ng/µl and 5 µl were tested. Analysis mode: automatic baseline and threshold at 0.2*

Species	C_t	Mean C_t	Result	**Species**	C_t	Mean C_t	Result
Plant species							
Glycine max	47.73 36.36	42.05	-	***Oryza sativa***	41.95 50	45.97	-
Zea mays	50 44.23	47.12	-	***Lycopersicon esculentum***	41.58 47.14	44.36	-
Brassica napus	40.27 39.57	39.92	-	***Beta vulgaris***	50 39.88	44.94	-
Triticum aestivum	50 49.04	49.52	-				

In general, amplification curves are observed but almost all with Ct values around or higher than 40 cycles. Only one of the duplicates on *Glycine max* gave an earlier Ct value of 36.36 cycles.

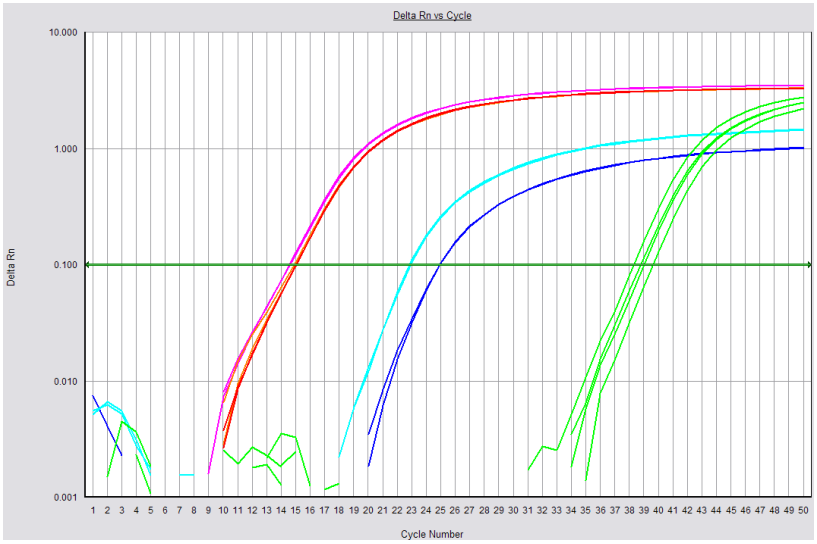
2.3.7. 7th PCR platform

This platform used the ABI 7500 (Applied Biosystems), in combination with the TaqMan Universal PCR Mastermix (Applied Biosystems).

Table 22 and Figure 18 present the results obtained with ruminant samples and feed samples containing ruminant material.

Table 22: *Specificity tests carried out on samples containing ruminant DNA. PCR was performed on an ABI7500 thermocycler (Applied Biosystems) with the TaqMan Universal PCR Mastermix (Applied Biosystems). Pure species DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: automatic baseline and threshold at 0.1. The CRL samples tested are known to contain ruminant material.*

Species/sample	C_t	Mean C_t	Result	**Sample**	C_t	Mean C_t	Result
Ruminant species and samples contain ruminants							
Bos taurus	14.51 14.57	14.54	+	**CRL 1302 extract 1**	23.96 23.84	23.90	+
Ovis aries	15.03 15.04	15.04	+	**CRL 1302 extract 2**	24.03 23.81	23.92	+
Capra hircus	14.88 15.03	14.95	+	**CRL 1356 extract 1**	25.20 25.24	25.22	+
Cervus elaphus	22.90 22.79	22.85	+	**CRL 1356 extract 2**	25.58 25.57	25.58	+
Capreolus capreolus	24.92 24.91	24.92	+	**CRL 1358 extract 1**	25.62 25.71	25.67	+
0.05 % ruminant PAP in feed (m/m)	31.56 31.42	31.49	+	**CRL 1358 extract 2**	25.29 25.23	25.26	+
0.0125 % ruminant PAP in feed (m/m)	33.88 34.01	33.95	+	**CRL 1373 extract 1**	23.88 23.87	23.88	+
				CRL 1373 extract 2	23.91 23.67	23.79	+

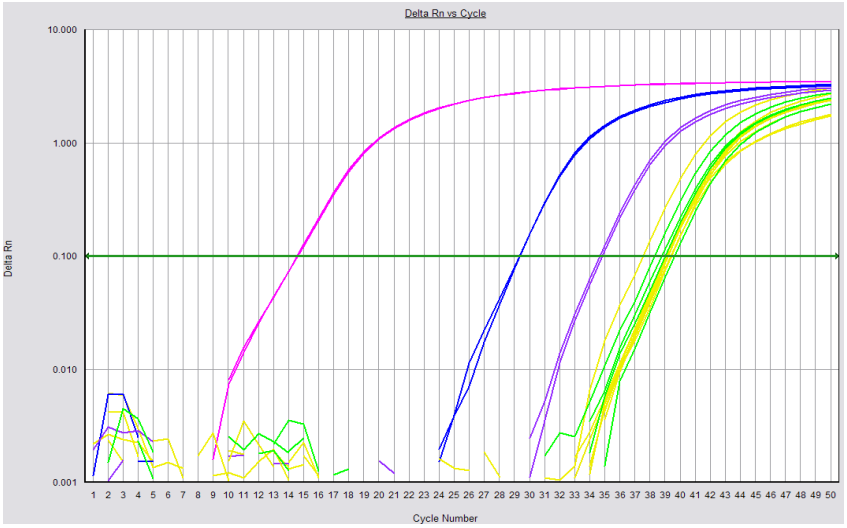


Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	14.5101
	Bos Taurus 10ng	14.5696
	Capra hircus 10ng	14.8752
	Capra hircus 10ng	15.0295
	Ovis aries 10ng	15.0306
	Ovis aries 10ng	15.0421
	Cervus Elaphus 10ng	22.7947
	Cervus Elaphus 10ng	22.8985
	Capreolus capreolus 10ng	24.9142
	Capreolus capreolus 10ng	24.9217
	No template control	38.2220
	No template control	38.7168
	No template control	38.9847
	No template control	39.5381

***Figure 18**: Amplification signals obtained on DNAs extracted from ruminant blood samples and on no template control (H_2O) with an ABI7500 thermocycler (Applied Biosystems) using the TaqMan Universal PCR Mastermix (Applied Biosystems). Analysis mode: automatic baseline and threshold at 0.1.*

As previously observed on other thermocyclers, the mastermix of Applied Biosystems results in a delay of 1.5 to 2 cycles when compared to the results obtained with Universal Mastermix (Diagenode s.a.) on the same thermocycler. All samples gave however clear positive results. Due to the presence of ruminant DNA in the mastermix, systematic amplifications are observed with the PCR negative control (H_2O) but the Ct values obtained (between 38 and 40 cycles) are after the cut-off value.

The results obtained with samples from non-ruminant species are presented in the Table 23 and the Figure 19.



Color of curve	Species/Samples	Cp
	Bos Taurus 10ng	14.5101
	Bos Taurus 10ng	14.5696
	Tursiops truncatus 10ng	29.2481
	Tursiops truncatus 10ng	29.2781
	Ziphius cavirostris 10ng	34.6180
	Ziphius cavirostris 10ng	34.8036
	Grampus griseus 10ng	37.4359
	Grampus griseus 10ng	39.2942
	Stenella coeruleoalba 10ng	39.0201
	Stenella coeruleoalba 10ng	39.3327
	Phocoena phocoena 10ng	39.0218
	Phocoena phocoena 10ng	39.1450
	Phocidae 10ng	39.1014
	Phocidae 10ng	39.1104
	No template control	38.2220
	No template control	38.7168
	No template control	38.9847
	No template control	39.5381

***Figure 19**: Amplification signals obtained with DNAs extracted from Bos taurus blood, sea mammal muscle and with no template control (H_2O) on the ABI7500 (Applied Biosystems) using the TaqMan Universal PCR Mastermix (Applied Biosystems). Analysis mode: automatic baseline and threshold at 0.1.*

Table 23: *Specificity tests performed on non-ruminant samples using as PCR platform the ABI7500 thermocycler (Applied Biosystems) with the TaqMan Universal PCR Mastermix (Applied Biosystems). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: automatic baseline and threshold at 0.1.*

Species	C_t	Mean C_t	Result	Species	C_t	Mean C_t	Result
Other terrestrial mammalians				**Fish species**			
Homo sapiens	39.08 37.96	38.52	-	***Gadus morhua***	38.38 38.09	38.24	-
Sus scrofa domesticus	39.70 37.59	38.65	-	***Pollachius virens***	39.88 39.39	39.63	-
Equus asinus	38.60 37.76	38.18	-	***Melanogrammus aeglefinus***	39.18 39.80	39.49	-
Equus caballus	40.14 38.95	39.54	-	***Micromesistius poutassou***	39.67 38.68	39.18	-
Oryctolagus cuniculus	38.96 39.32	39.14	-	***Sebastes spp.***	39.24 38.14	38.69	-
Lepus europaeus	38.79 39.61	39.20	-	***Mallotus villosus***	37.63 38.15	37.89	-
Rattus rattus	38.94 38.94	38.94	-	***Scomber scombrus***	39.09 38.12	38.61	-
Domestic birds				***Clupea harengus***	40.03 39.97	40.00	-
Gallus gallus	39.52 39.04	39.28	-	***Merluccius merluccius***	39.41 40.08	39.74	-
Numida meleagris	38.44 37.49	37.96	-	***Trachurus trachurus***	38.01 38.50	38.25	-
Meleagris gallopavo	38.91 40.62	39.76	-	***Trisopterus minutus***	39.71 37.37	38.54	-
Cairina moschata	39.05 39.27	39.16	-	***Sardina pilchardus***	39.63 37.76	38.69	-
Phasianus colchicus	39.11 40.21	39.66	-	***Engraulis encrasicolus***	39.61 39.96	39.79	-
Columba livia	39.18 37.92	38.55	-	***Gadus ogac***	40.08 39.23	39.65	-
Sea mammals				***Trisopterus esmarki***	39.64 38.98	39.31	-
Stenella coeruleoalba	39.33 39.02	39.18	-	***Ammodytes lancea***	38.25 40.01	39.13	-
Tursiops truncatus	29.25 29.28	29.26	+	***Ammodytes lancea***	39.24 39.89	39.56	-
Grampus griseus	39.29 37.44	38.37	-	***Sprattus sprattus***	38.06 40.15	39.10	-
Ziphius cavirostris	34.62 34.80	34.71	+	***Salmo salar***	39.09 39.41	39.25	-
Phocoena phocoena	39.02 39.15	39.08	-	***Raja* spp.**	40.19 39.08	39.64	-
Phocidae	39.11 39.10	39.11	-	***Paralithodes camtschaticus***	46.60 46.12	46.36	-

As with all the other platforms, *Tursiops truncatus* and *Ziphius cavirostris* gave earlier signals (mean Ct values of 29.26 and 34.71 respectively). All the replicates of the other non-ruminant samples tested gave amplification curves with Ct values ranging approximately from 38 to 40, except for the crab species that reached 46.

The plant samples tested gave the results presented in the Table 24.

Table 24: *Specificity tests performed on plant species using as PCR platform the ABI7500 thermocycler (Applied Biosystems) with the TaqMan Universal PCR Mastermix (Applied Biosystems). All plant extracts were at 2 ng/µl and 5 µl were tested. Analysis mode: automatic baseline and threshold at 0.1.*

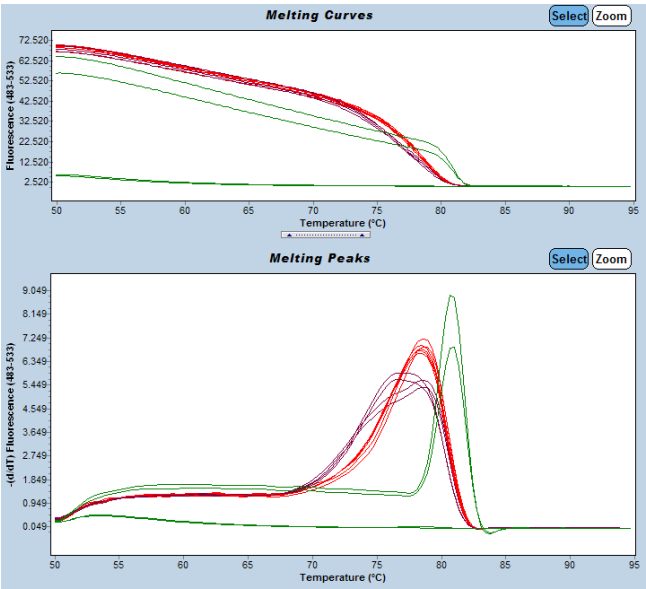
Species	C_t	Mean C_t	Result	**Species**	C_t	Mean C_t	Result
Plant species							
Soybean	38.10 38.93	38.52	-	***Oryza sativa***	38.45 39.13	38.79	-
Zea mays	38.63 39.32	38.97	-	***Lycopersicon esculentum***	37.73 38.92	38.33	-
Brassica napus	39.16 39.59	39.37	-	***Beta vulgaris***	38.58 40.38	39.48	-
Triticum aestivum	38.89 38.80	38.85	-				

No aspecific result was obtained but, as with most non-ruminant samples, the replicates gave amplification curves with Ct values in between 38 and 40 cycles.

2.3.8. Specificity of the primers

To evaluate the importance of the primers and of the probe in the specificity of the test, PCR were performed with the Universal Mastermix (Diagenode s.a.) containing SYBR® Green on the LC480 thermocycler (Roche Diagnostics Ltd.). Melting curves were also realised at the end of the PCR to characterize the amplicons produced.

Table 25 and Figure 20 present the results obtained with DNAs extracted from ruminant blood samples and from feed samples containing ruminant material.



Color of curve	Species/Samples	Tm1
	Bos Taurus 10ng	78.24
	Bos Taurus 10ng	78.32
	Capra hircus 10ng	77.95
	Capra hircus 10ng	77.99
	Ovis aries 10ng	78.02
	Ovis aries 10ng	78.02
	Cervus Elaphus 10ng	77.11
	Cervus Elaphus 10ng	77.17
	Capreolus capreolus 10ng	79.08
	Capreolus capreolus 10ng	79.08
	No template control	80.84
	No template control	80.88
	No template control	
	No template control	

Figure 20: *Melting curves obtained with DNAs extracted from ruminant blood samples and with no template control (H_2O) on the LC480 (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) containing SYBR® green. Melting curves are given with fluorescence levels in function of temperature (upper part of the figure) and also as the negative value of the first derivative of this fluorescence towards time and still in function of time in order to visualize more easily the Tm values (lower part of the Figure). Analysis mode: Tm calling.*

Table 25: *Specificity tests of primers performed with samples containing ruminant DNA on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) in SYBR® Green format. Pure species DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: Abs. quant/second derivative max, high confidence and Tm calling. The CRL samples tested are known to contain ruminant material.*

Species/sample	C_t	Mean C_t	Tm 1	Result	**Sample**	C_t	Mean C_t	Tm 1	Result
Ruminant species and samples containing ruminant material									
Bos taurus	13.29 13.46	13.38	78.32 78.24	**+**	**CRL 1302 extract 1**	21.96 21.94	21.95	78.45 78.51	**+**
Ovis aries	13.10 13.17	13.14	78.02 78.02	**+**	**CRL 1302 extract 2**	21.95 21.73	21.84	78.26 78.19	**+**
Capra hircus	13.11 13.12	13.12	77.99 77.95	**+**	**CRL 1356 extract 1**	23.26 23.17	23.22	78.12 78.16	**+**
Cervus elaphus	15.02 14.92	14.97	77.11 77.17	**+**	**CRL 1356 extract 2**	23.59 23.58	23.59	78.19 78.14	**+**
Capreolus capreolus	15.77 15.30	15.54	79.08 79.08	**+**	**CRL 1358 extract 1**	23.57 22.96	23.27	78.21 78.37	**+**
0.05 % ruminant PAP in feed (m/m)	29.59 29.74	29.67	77.95 77.92	**+**	**CRL 1358 extract 2**	23.27 23.22	23.25	78.35 78.34	**+**
0.0125 % ruminant PAP in feed (m/m)	31.89 31.85	31.87	78.48 77.90	**+**	**CRL 1373 extract 1**	22.02 22.15	22.09	78.37 78.38	**+**
					CRL 1373 extract 2	21.79 21.88	21.84	78.21 78.17	**+**
No template control									
H2O	45 50 50 45	47.5	80.84 / / 80.88	-					

Melting curves of the ruminant species tested show different characteristics: the ones obtained with the 3 farmed ruminant species (*Bos taurus*, *Ovis aries* and *Capra hircus*) present a well-defined peak with a maximum close to 78°C while the melting curves of *Capreolus capreolus* and *Cervus elaphus* show a more flattened peak associated with Tm figures around 79°C and 77°C respectively.

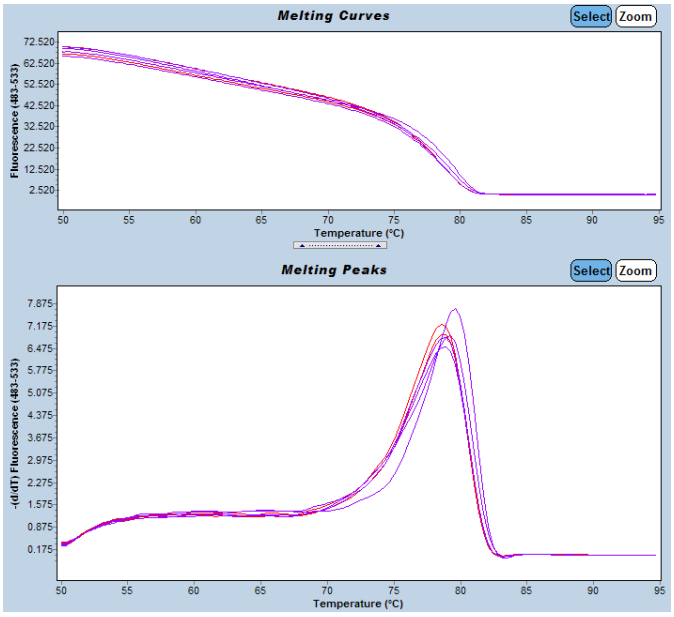
When an amplification occurs in the PCR negative control, the Tm calculated is after 80°C.

The same test was performed on non-ruminant samples. Table 26, Figures 21 and 22 summarise the results.

Table 26: *Specificity tests of primers performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) in SYBR® Green format. All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max, high confidence and Tm calling.*

Species	C_t	Mean C_t	Tm 1	Tm 2	Result	Species	C_t	Mean C_t	Tm 1	Tm 2	Result
Other terrestrial mammalians						**Fish species**					
Homo sapiens	40.87 40.97	40.92	76.69 68.05	82.81 78.58	-	***Gadus morhua***	40.06 45	42.53	77.39 78.46	/ /	-
Sus scrofa domesticus	37.95 38.62	38.29	77.13 77.06	86.19 /	-	***Pollachius virens***	45 42.78	43.89	78.49 79.18	/ /	-
Equus asinus	41.86 43.03	42.45	82.47 79.97	/ /	-	***Melanogrammus aeglefinus***	40.44 45	42.72	79.33 79.14	/ /	-
Equus caballus	40.88 39.25	40.07	80.12 79.24	/ /	-	***Micromesistius poutassou***	41.13 41.84	41.49	79.95 79.86	/ 83.17	-
Oryctolagus cuniculus	39.55 39.67	39.61	82.23 81.46	/ /	-	***Sebastes spp.***	39.23 40.51	39.87	78.05 75.90	/ 83.18	-
Lepus europaeus	38.70 41.74	40.22	81.07 79.82	/ 85.67	-	***Mallotus villosus***	39.98 42.83	41.41	79.60 79.90	/ /	-
Rattus rattus	38.61 40.04	39.33	80.05 79.66	/ /	-	***Scomber scombrus***	40.53 38.63	39.58	83.84 75.73	/ 83.67	-
Domestic birds						***Clupea harengus***	38.58 37.85	38.22	82.54 78.54	/ 82.64	-
Gallus gallus	37.92 38.50	38.21	76.07 82.05	81.60 /	-	***Merluccius merluccius***	41.12 38.90	40.01	74.22 75.00	81.23 79.23	-
Numida meleagris	38.13 38.15	38.14	76.18 75.25	79.91 80.16	-	***Trachurus trachurus***	41.49 42.36	41.93	75.18 74.53	/ /	-
Meleagris gallopavo	39.13 37.78	38.46	82.36 81.27	/ /	-	***Trisopterus minutus***	41.06 40.16	40.61	78.59 79.54	/ /	-
Cairina moschata	38.88 38.52	38.70	77.15 82.07	82.28 /	-	***Sardina pilchardus***	41.53 41.57	41.55	77.83 77.37	/ /	-
Phasianus colchicus	37.83 38.74	38.29	80.74 66.21	/ 78.71	-	***Engraulis encrasicolus***	41.35 44.38	42.87	86.33 74.45	/ 86.15	-
Columba livia	38.94 38.30	38.62	81.23 82.35	/ /	-	***Gadus ogac***	40.97 41.27	41.12	74.58 79.52	79.72 82.74	-
Sea mammals						***Trisopterus esmarki***	43.66 45	44.33	77.21 79.50	81.68 /	-
Stenella coeruleoalba	39.83 40.10	39.97	79.83 79.37	/ /	-	***Ammodytes lancea***	38.87 42.46	40.67	76.25 75.20	79.88 80.90	-
Tursiops truncatus	27.80 27.75	27.78	78.30 78.32	/ /	+	***Ammodytes lancea***	42.77 38.63	40.70	79.36 79.91	83.97 /	-
Grampus griseus	38.01 38.57	38.29	79.80 79.61	85.24 85.47	-	***Sprattus sprattus***	41.05 45	43.03	80.12 80.56	/ /	-
Ziphius cavirostris	33.29 33.29	33.29	79.22 78.62	/ /	+	***Salmo salar***	38.78 38.41	38.60	78.29 82.63	82.86 /	-
Phocoena phocoena	39.46 38.95	39.21	79.34 81.16	/ /	-	***Raja* spp.**	43.02 42.22	42.62	78.56 78.71	/ /	-
Phocidae	38.80 39.58	39.19	80.96 82.59	86.18 /	-	***Paralithodes camtschaticus***	45 45	45	/ 75.58	/ /	-

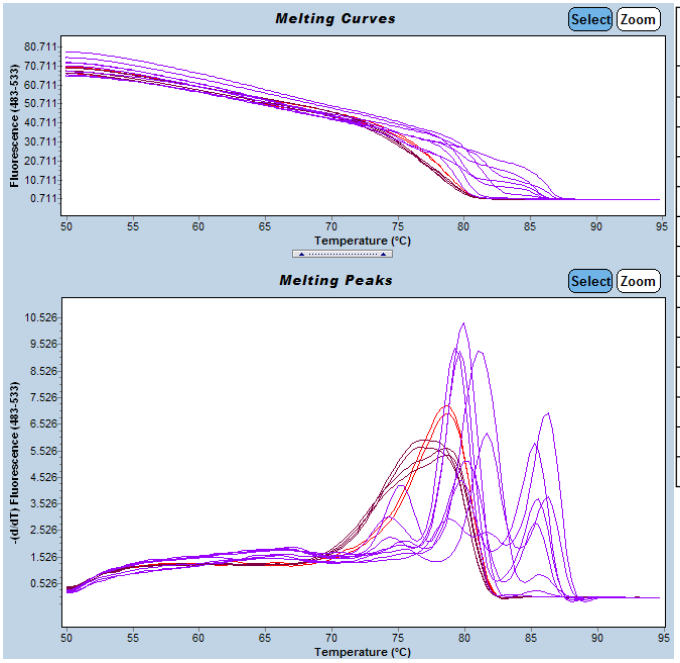
With several negative samples, a second peak is present in the melting curve. The Tm2 associated to this peak is close to or higher than 80°C. Looking at the Tm1, a wider variability is frequently observed with differences of more than 2°C between the replicates (e.g. *Homo sapiens*, *Phasianus colchicus*, ...). For *Tursiops truncatus* and *Ziphius cavirostris*, the signals are similar to these obtained with ruminants. Their melting curves show a single peak with a reproducible Tm (see also Figure 21).



Color of curve	Species/Samples	Tm1
	Bos Taurus 10ng	78.24
	Bos Taurus 10ng	78.32
	Tursiops truncatus 10ng	78.30
	Tursiops truncatus 10ng	78.32
	Ziphius cavirostris 10ng	78.62
	Ziphius cavirostris 10ng	79.22

Figure 21: *Melting curves obtained with DNAs extracted from a Bos taurus blood sample and from muscle coming from two species of sea mammals that give a positive result in TaqMan real-time PCR format with the TNO Triskelion PCR assay. The PCR was performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) in SYBR® Green format. Analysis mode: Tm calling.*

Figure 22 allows a comparison between melting curves of ruminants (*Bos taurus* and *Capreolus capreolus* or *Cervus elaphus* – red and maroon curves respectively) and the signals of sea mammals other than *Tursiops truncatus* and *Ziphius cavirostris* (purple curves).



Color of curve	Species/Samples	Tm1	Tm2
	Bos Taurus 10ng	78.24	
	Bos Taurus 10ng	78.32	
	Capreolus capreolus 10ng	79.08	
	Capreolus capreolus 10ng	79.08	
	Cervus Elaphus 10ng	77.11	
	Cervus Elaphus 10ng	77.17	
	Stenella coeruleoalba 10ng	79.37	
	Stenella coeruleoalba 10ng	79.83	
	Grampus griseus 10ng	79.61	85.47
	Grampus griseus 10ng	79.80	85.24
	Phocoena phocoena 10ng	79.34	
	Phocoena phocoena 10ng	81.16	
	Phocidae 10ng	80.96	86.18
	Phocidae 10ng	82.59	

Figure 22: *Melting curves obtained with DNAs extracted from a ruminant blood sample and from muscle of several sea mammal species that don't provide a positive result in TaqMan real-time PCR format with the TNO Triskelion ruminant PCR assay. The PCR was performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) in SYBR® Green format. Analysis mode: Tm calling.*

The signals of *Stenella coeruleoalba*, *Grampus griseus* and *Phocoena phocoena* are clearly different from the ones for *Bos taurus*, *Capreolus capreolus* and *Cervus elaphus*.

The results were completed with tests performed on plant samples (Table 27).

Table 27: *Specificity tests of primers performed with plant samples on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) in SYBR® Green format. All plant DNA extracts were at 2 ng/µl and 5 µl were tested per PCR. Analysis mode: Abs. quant/second derivative max, high confidence and Tm calling.*

Species	C_t	Mean C_t	Tm 1	Tm 2	Result	**Species**	C_t	Mean C_t	Tm 1	Tm 2	Result
Plant species											
Soybean	38.90 37.62	38.26	**79.50** **68.13**	***I*** **78.42**	-	***Oryza sativa***	41.92 38.62	40.27	**80.08** **78.50**	***I*** ***I***	-
Zea mays	38.85 41.81	40.33	**79.83** **82.98**	***I*** ***I***	-	***Lycopersicon esculentum***	41.02 40.58	40.80	**79.47** **80.83**	***I*** ***I***	-
Brassica napus	39.10 39.06	39.08	**82.84** **66.26**	***I*** **79.32**	-	***Beta vulgaris***	43.79 43.49	43.64	**78.45** **74.50**	***I*** **80.21**	-
Triticum aestivum	41.69 42.82	42.26	**74.53** **79.89**	**80.50** ***I***	-						

On this set of negative samples, the results can be commented in the same way: a second peak is sometimes present in the melting curve and the Tm of the first peak (Tm1) is quite variable between replicates of a sample.

2.3.9. Overall conclusion about the specificity

In conclusion, the specificity of the test can be considered as satisfactory whatever the platform used. Among the various species tested, aspecific signals only occur in presence of some sea mammals and to be more precise in fact only in presence of some cetaceans (the common Bottlenose Dolphin or *Tursiops truncatus* and the Cuvier's beaked whale or *Ziphius cavirostris*). This aspecificity is not completely abnormal because among sea mammals, cetaceans are taxonomically rather close to ruminants. However the signals are late because the delay in Ct compared to the farmed ruminants ranges in-between 14 and 20 cycles. The amplification products of some of the sea mammals were sequenced (Table 28).

Table 28: *Sequences obtained on the PyroMark Q24 (Qiagen GmbH, Hilden, Germany) with the DNA from amplified from 4 cetacean species.*

Species	Biotinylated primers	Number of PCR products	Internal sequence (between primers)
Probe sequence :			C AACTCTTCG CATGAGGTGG CCAAA
Tursiops truncatus	F	3	GAGTC AACTCTTC CATGAGGTGG CCAAAGTAC T GG A
	R	2	GAGTC AACTCTTC CATGAGGTGGACCGAAGTACGT GGTA
Ziphius cavirostris	F	2	GAGTC AACTCTTCG CATGAGGTGG CCAAAGTAC T GG A
	F	2	GAGTC AACTCTTC CATGAGGTGG CCAAAGTAC T GG A
	R	1	GAGTC AACTCTTC CATGAGGTGGACG AAGTAC GTGGTA
	R	2	GAGTC AACTCTTC CATGAGGTGGACC AAGTAC GTGGTA
Stenella coeruleoalba	F	2	CTAGAGTCTAACTCTTCGTCATGAGGTGG CCAAAGTAC T GG A
	F	2	GAGTC AACTCTTCG CAT AGGTGG CCAAAGTA T?GG A
	R	2	GAGTC AACTCTTC CATGAGGTGG CC AAGTAC T GG A
Grampus griseus	F	1	AGAGTC AACTCTTC CATGAGGTGG CCAAAGTA? T GG A
Legend:	Sequence of the probe Difference with the sequence of the probe Difference with the sequences of the cloned *Bos taurus* target		

The internal target sequence may sometimes completely fit with what is expected for ruminants even if it cannot be excluded that this very precise sequence is not coming from the master mix and would then rather be of bovine origin. Is this lack of specificity a problem with respect to the purpose of the assay? The presence of material from cetaceans at very low level content cannot be totally excluded in fishmeals. Taking into account the kinetics of the PCR obtained with pure DNAs from the two species of cetaceans and the production process of the fishmeals, the presence of cetacean DNA should not lead to strong signals, neither to misinterpretations by well-informed PCR users. Indeed even if we consider a level of 0.1% of cetacean material in fishmeal which is already more than a trace level this would increase the Ct by ten units (a 1000-fold dilution implies a delay of about ten cycles because 2^{10} = 1024) and the outcome of the PCR should then be beyond the cut-off. Nevertheless it should be

stressed that this lack of specificity of the test is **one of the potential limitations of the assay**. In most of the cases it will not at all be a source of problem but it is not totally excluded that in very special circumstances this might lead to false positive results, the more as not all species of cetaceans were considered in this study.

It has to be stressed here that the specificity was considered here with the provisional cut-off of 35 in terms of Ct as set by TNO Triskelion. In the further sections, we will determine what is the exact cut-off for each platform and the obtained results of the specificity will have to be checked again in the light of the newly selected cut-off. In the further sections only 4 PCR platforms will be taken into consideration. We will abandon the use of TaqMan Universal PCR Mastermix (Applied Biosystems) because it is a source of ruminant DNA. Attention will be focussed on two types of thermocycler : the lightcycler LC480 (Roche Diagnostics Ltd) and the ABI7500 thermocycler (Applied Biosystem). Moreover, we will keep only two mastemixes: the Universal Mastermix of Diagenode and the qPCR MasterMix for probe assay ROX of Eurogentec which had been used in the third platform but not yet on an ABI thermocycler.

3. Production, validation of calibrants and determination of cut-off values at different levels

3.1. Production and validation of calibrants

To complete the evaluation of the method, parameters like the cut-off, the sensitivity, the efficiency and the absolute LOD were estimated with calibrants that are dilutions of a plasmid with an insert having exactly the sequences of the primers and probe of the ruminant PCR test under evaluation in this dossier (for details on the test see annex 1). The insert was obtained by cloning amplification products starting from *Bos taurus* blood DNA as template. To that purpose we used the bovine target of clone A1C8 (Table 1) that was transferred into pUC18 and the inserted target was checked again by sequencing. It is this version of clone A1C8 in pUC18 that is used as plasmid material for the calibrations.

The plasmid was produced in *Escherichia coli* and purified from the bacterial culture. The so-produced plasmid was linearized by the restriction endonuclease *Hin*dIII and afterwards submitted to a phenol-chloroform extraction to eliminate the endonuclease. The resulting amount of linearized plasmid in the stock solution was determined based on spectrophotometrical measurements of the DNA solution at 260 nm and taking into account the size of the plasmid. Having no access at the time of the assessment to a digital PCR facility, the solutions produced at low levels of copy number are merely based on the dilution pattern that was followed without any possibility to check directly the real amount in the final solutions, the more as maize DNA is used as background material. However a quality check was done through the verification of the frequency of positive PCR results on a dilution at 0.1 copy/5 µl. At this concentration, the frequency of positive PCR results when using 5 µl of the solution in a PCR should not be more than 1 signal out of 6 replicates. The results obtained at 0.1 copy/5 µl are in Table 29. The cut-off used here was set at 40 because the aim is to detect the presence of at least one plasmid. Signals appearing later are supposed as unlinked to the plasmid and are therefore not considered. As will be seen in the next section the precise cut-off for 1 copy is not 40 but lays in a range between 38.693 and 39.874 according to the considered platform but this has no impact on the observed frequency of positive results.

Run 2 of the platform combining ABI7500 with Diagenode mastermix is the only one giving more than 1 positive result on 6 replicates at 0.1 copy/5µl. A third run with 6 replicates at 0.1 copy/5µl was performed on the ABI7500 with both mastermixes resulting in no additional positive results. These results validate the dilutions used for the calibrants.

Table 29: *C_t (or C_p) values obtained with the plasmid at 0.1 copy/5µl and with the no template control (H_2O) on the four PCR platforms.*

	Device →	**LightCycler LC480**		**ABI7500**	
Run ↓	**Master Mix →**	**Diagenode**	**Eurogentec**	**Diagenode**	**Eurogentec**
1	C_t with 0.1 copy	41.80	39.60	39.34	37.82
		45	40.54	39.91	39.73
		50	42.83	40.19	42.46
		50	45	40.79	44.13
		50	45	40.98	47.30
		50	50	50	50
	C_t with H_2O mQ	50	45	40.41	40.77
		50	50	42.16	47.21
		50	50	47.92	47.90
		50	50	50	49.65
		50	50	50	50
		50	50	50	50
2	C_t for 0.1 copy	50	45	37.98	42.88
		50	50	38.34	43.53
		50	50	41.26	50
		50	50	50	50
		50	50	50	50
		50	50	50	50
	C_t for H_2O mQ	38.74	44.57	50	45.38
		41.51	45	50	48.35
		50	50	50	50
		50	50	50	50
		50	50	50	50
		50	50	50	50
3	C_t for 0.1 copy	/	/	45.78	44.38
				50	45.39
				50	50
				50	50
				50	50
				50	50
	C_t for H_2O mQ	/	/	40.36	38.01
				40.60	43.53
				40.91	45.00
				49.90	49.98
				50	50
				50	50

3.2. Determination of cut-off values for different copy numbers

The definition of the cut-off used here was an extension of the definition used at CRA-W for the bovine target where the protocol to define it was validated during an inter-laboratory study led by the EURL-AP. For the bovine target the cut-off was defined as the Ct corresponding to the upper limit of the confidence interval at level of 95% for the detection of one copy of the target. The extension applied here is that we consider the cut-off value (i.e. a Ct for a upper confidence limit at 95%) for several copy numbers and not only for a single copy.

The cut-off values[16] of 4 platforms were determined with 20 calibrations. A calibration is composed of 3 levels in number of copies (640 – 160 – 40 copies/5 µl) and 3 replicates per concentration level. The 20 calibrations were spread over 7 runs. The repetitions of calibrations allow to define an upper confidence level at 95% of the Ct of a given copy number.

The calibrations were performed with two real-time thermocyclers present in the EURL-AP facilities: one LightCycler LC480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and one ABI7500 (Applied Biosystems, Foster City, CA, USA).

Two mastermixes were also used for these cut-off settings: the Universal Mastermix (Diagenode s.a., Seraing, Belgium) and the qPCR MasterMix for probe assay ROX (Eurogentec s.a., Ougrée, Belgium).

[16] **EURL-AP Standard Operating Procedure. 2014. Detection of ruminant DNA in feed using real-time PCR.** https://www.eurl.craw.eu/legal-sources-and-sops/method-of-reference-and-sops/

Analysis of results with the LC480 thermocycler used « Abs. quant/second derivative max and high confidence » mode whatever the Mastermix used. On the ABI 7500, automatic baseline parameter was used while the threshold was set by the operator. The amount of ROX being different in the 2 mastermixes, the threshold was set differently. With the mastermix from Diagenode, the threshold was set at 0.2 while with the mastermix from Eurogentec the threshold was set at 0.015. However, all the runs performed on the same platform were analysed using the same criteria (baseline and threshold).

The results of these tests are presented in Table 30.

Table 30: *C_t (or C_p) values obtained for the cut-off at different copies number on 4 PCR platforms.*

Device	LightCycler 480		ABI7500	
Master Mix	Diagenode	Eurogentec	Diagenode	Eurogentec
Cut-off at 1 copy	39.874	39.302	38.693	38.853
Cut-off at 5 copies	37.444	36.878	36.284	36.411
Cut-off at 10 copies	36.399	35.836	35.248	35.361
Cut-off at 15 copies	35.787	35.226	34.642	34.747
Cut-off at 20 copies	35.354	34.794	34.213	34.312

4. Performances of the test

4.1. PCR Efficiency

The efficiency of the test was checked with a calibration involving the concentrations at 5000, 2500, 1000, 500 and 100 copies/5 µl respectively with 5 µl as volume of sample in the reaction. Four runs were done per platform and at each run six replicates per concentration were used.

The tests were carried out with two real-time thermocyclers present in the EURL-AP facilities (one LightCycler LC480 - Roche Diagnostics Ltd., Rotkreuz, Switzerland and one ABI7500 - Applied Biosystems, Foster City, CA, USA) and two mastermixes (the Universal Mastermix - Diagenode s.a., Seraing, Belgium and the qPCR MasterMix for probe assay ROX - Eurogentec s.a., Ougrée, Belgium).

Analysis of results with the LC480 used « Abs. quant/second derivative max and high confidence » mode whatever the Mastermix used. On the ABI 7500, automatic base line parameter was used while the threshold was set by the operator. The amount of ROX being different in the 2 mastermixes, the threshold was set differently. With the mastermix from Diagenode, the threshold was set at 0.2 while with the mastermix from Eurogentec the threshold was set at 0.015. However, all the runs performed on the same platform were analysed using the same parameters once they were set.

The results of these tests are presented in Table 31.

Table 31: *Efficiency determined on 4 PCR platforms.*

Device	LightCycler 480		ABI7500	
Master Mix	Diagenode	Eurogentec	Diagenode	Eurogentec
Efficiency	95.49 %	97.21 %	94.78 %	97.67 %

Acceptance criterion for the efficiency value must be in a range of 90 to 110 %. The 4 PCR platforms tested meet the set criterion.

4.2. The absolute limit of detection (LOD_{abs})

The LOD_{abs} of the test was calculated with dilutions of the same plasmid as the one used for the determination of the cut-off and the efficiency.

The LOD_{abs} was first calculated according to the LOD_6 approach.

Table 32: *Determination of the absolute LOD with the LOD_6 approach on 4 PCR platforms (Ĉt = mean Ct).*

	LC480 - Master mix Diagenode Cut-off (1 copy) : 39.874					LC480 - Master mix Eurogentec Cut-off (1 copy) : 39.302					ABI7500 - Master mix Diagenode Cut-off (1 copy) : 38.693					ABI7500 - Master mix Eurogentec Cut-off (1 copy) : 38.853			
	Run 1		Run 2			Run 1		Run 2			Run 1		Run 2			Run 1		Run 2	
copies/5µl	**Ct**	**Ĉt**	**Ct**	**Ĉt**		**Ct**	**Ĉt**	**Ct**	**Ĉt**		**Ct**	**Ĉt**	**Ct**	**Ĉt**		**Ct**	**Ĉt**	**Ct**	**Ĉt**
50	33,74	33,83	33,79	33,77		33,49	33,20	32,77	32,96		32,70	32,61	32,57	32,57		32,17	32,40	32,19	32,54
50	33,70		33,61			33,21		33,31			32,53		32,33			32,34		32,30	
50	34,13		33,69			33,36		33,44			32,45		32,90			32,78		32,41	
50	34,12		33,96			33,02		32,88			32,47		32,52			32,43		32,87	
50	33,64		33,86			33,04		32,78			33,06		32,46			32,29		32,54	
50	33,63		33,73			33,09		32,59			32,46		32,65			32,39		32,90	
20	35,22	35,49	35,72	35,40		34,13	34,16	34,24	34,31		34,10	33,98	34,20	33,92		34,02	34,04	33,78	34,10
20	36,11		35,60			34,81		34,20			33,66		33,85			34,25		34,02	
20	34,65		35,29			33,84		34,65			34,01		34,21			33,86		34,38	
20	35,72		35,24			34,30		33,95			34,07		33,90			34,13		33,82	
20	35,78		34,99			33,96		33,86			33,59		33,58			34,19		34,23	
20	35,45		35,56			33,91		34,94			34,46		33,78			33,79		34,37	
10	35,89	36,31	35,71	35,87		35,66	35,49	34,95	35,46		34,38	34,88	35,01	35,13		35,27	35,21	34,90	35,06
10	36,29		35,82			35,69		34,74			34,85		35,46			35,53		35,32	
10	36,29		36,17			34,91		35,80			35,33		34,82			34,72		35,80	
10	36,25		35,79			35,46		35,79			34,45		35,72			35,09		34,83	
10	36,58		35,73			35,36		35,54			36,03		34,85			35,48		35,38	
10	36,56		36,02			35,85		35,95			34,22		34,92			35,16		34,11	
5	36,51	37,05	38,16	39,52		36,03	36,78	37,69	36,57		34,75	35,66	36,47	36,38		36,24	35,69	35,38	35,98
5	37,31		36,99			36,03		36,46			35,69		36,13			36,17		35,35	
5	36,85		37,46			36,04		36,47			35,19		36,56			35,34		35,76	
5	36,79		36,95			37,58		36,52			36,52		36,95			35,82		38,06	
5	37,95		50			38,70		36,02			35,11		36,10			35,10		35,01	
5	36,86		37,54			36,29		36,23			36,67		36,06			35,45		36,31	
2	39,67	38,45	37,57	38,00		37,94	37,41	50	40,11		36,24	40,18	37,14	37,05		37,01	37,57	37,43	39,46
2	38,97		37,32			37,08		36,96			36,99		36,78			36,95		37,22	
2	37,81		38,77			37,75		36,89			50		38,00			37,24		50	
2	39,31		38,85			36,80		39,61			44,50		36,54			39,18		35,95	
2	37,44		37,05			37,59		38,62			36,19		36,76			36,02		39,24	
2	37,49		38,46			37,30		38,55			37,15		37,05			39,02		36,91	
1	40,46	42,99	38,50	41,10		36,98	38,35	38,96	40,46		50	44,74	37,26	40,77		50	40,90	37,86	40,13
1	39,18		40,80			37,85		37,76			50		41,28			36,54		37,79	
1	50		38,37			42,46		43,73			38,38		36,89			42,05		39,31	
1	50		38,73			36,36		38,66			50		41,13			37,93		36,80	
1	38,75		50			38,69		38,67			38,05		50			41,35		39,00	
1	39,52		40,20			37,77		45			42,03		38,05			37,51		50	
LOD_6	**10**					**5**					**5**					**5**			

Two runs including dilutions at low copy numbers (50 – 20 – 10 – 5 – 2 and 1 copy/5 µl respectively) were performed on each platform. Six replicates were tested per concentration.

The LOD_6 corresponds to the lowest copy number of the target tested for which 6 signals out of 6 replicates are positive in the 2 runs.

Results of the LOD_6 approach are given in Table 32. Use of mastermix of ABI was avoided here as it would have been difficult to differentiate a positive result coming from the low copy number of the sample and one due to contaminating ruminant DNA originating from the mastermix. As can be concluded from the results, the absolute LOD varies in a range from 5-10 copies according to the platform and therefore meets the performance parameter of not being higher than 20 copies.

In a second approach the absolute LOD was determined on the basis 60 replicates on the same plate. The LOD is validated if at least 95 % of signals are recorded as positive out of the 60 replicates.

Four platforms were tested combining two real-time thermocyclers (one LightCycler LC480-Roche Diagnostics Ltd., Rotkreuz, Switzerland-and one ABI7500-Applied Biosystems, Foster City, CA, USA) and two master mixes (the Universal Mastermix-Diagenode s.a., Seraing, Belgium-and the qPCR MasterMix for probe assay ROX-Eurogentec s.a., Ougrée, Belgium). The result analysis with the LC480 used « Abs. quant/second derivative max and high confidence » mode whatever the mastermix used. On the ABI 7500, automatic base line parameter was used while the threshold was set by the operator. The amount of ROX being different in the 2 mastermixes, the threshold was set differently. With the mastermix from Diagenode, the threshold was set at 0.2 while with the mastermix from Eurogentec the threshold was set at 0.015. However, all the runs performed on the same platform were analysed using the same parameters once they were set.

The results of LOD are presented in Tables 33 to 36 according to the cut-off set at different copy numbers (1, 5, 10, 15, 20 copies respectively). On each platform 3 plasmid levels were tested : the first one corresponding to the LOD_{abs} that resulted from the LOD_6 approach for that platform and subsequently two additional levels at 15 and 20 copies were considered.

Table 33: *Absolute LOD determined on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) with plasmid dilutions at 10, 15 and 20 copies/5 µl performed. Analysis mode: Abs. quant/second derivative max and high confidence. Results calculated according cut-off set at 1, 5, 10, 15 and 20 copies*

	Positive results in % and number of positive reactions on 60 replicates (between brackets) obtained with a cut-off set at				
Copy number tested for LOD	**1 copy**	**5 copies**	**10 copies**	**15 copies**	**20 copies**
10	100 % (60/60)	100 % (60/60)	80.0 % (48/60)	26.7 % (16/60)	1.7 % (1/60)
15	100 % (60/60)	100 % (60/60)	96.7 % (58/60)	68.3 % (41/60)	21.7 % (13/60)
20	100 % (60/60)	100 % (60/60)	100 % (60/60)	95.0 % (57/60)	85.0 % (51/60)

.

Table 34: *Absolute LOD determined on the LC480 thermocycler (Roche Diagnostics Ltd.) using the qPCR Master-Mix for probe assay ROX (Eurogentec s.a.) with plasmid dilutions at 5, 15 and 20 copies/5 µl. Analysis mode: Abs. quant/second derivative max and high confidence. Results calculated according cut-off set at 1, 5, 10, 15 and 20 copies.*

	Positive results in % and number of positive reactions on 60 replicates (between brackets) obtained with a cut-off set at				
Copy number tested for LOD	**1 copy**	**5 copies**	**10 copies**	**15 copies**	**20 copies**
5	98.3 % (59/60)	71.7 % (43/60)	16.7 % (10/60)	1.7 % (1/60)	0 % (0/60)
15	100 % (60/60)	100 % (60/60)	98.3 % (59/60)	85.0 % (51/60)	38.3 % (23/60)
20	100 % (60/60)	100 % (60/60)	100 % (60/60)	100 % (60/60)	93.3 % (56/60)

Table 35: *Absolute LOD determined on the ABI7500 thermocycler (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.) with plasmid dilutions at 5, 15 and 20 copies/5 µl. Analysis mode: automatic base line and threshold at 0.2. Results calculated according cut-off set at 1, 5, 10, 15 and 20 copies.*

	Positive results in % and number of positive reactions on 60 replicates (between brackets) obtained with a cut-off set at				
Copy number tested for LOD	**1 copy**	**5 copies**	**10 copies**	**15 copies**	**20 copies**
5	100 % (60/60)	75.0 % (45/60)	18.3 % (11/60)	1.7 % (1/60)	0 % (0/60)
15	100 % (60/60)	100 % (60/60)	95.0 % (57/60)	71.7 % (43/60)	33.3 % (20/60)
20	100 % (60/60)	100 % (60/60)	100 % (60/60)	95.0 % (57/60)	61.7 % (37/60)

Table 36: *Absolute LOD determined on the ABI7500 thermocycler (Applied Biosystems) using the qPCR Master-Mix for probe assay ROX (Eurogentec s.a.) with plasmid dilutions at 5, 15 and 20 copies/5 µl performed. Analysis mode: automatic base line and threshold at 0.015. Results calculated according cut-off at 1, 5, 10, 15 and 20 copies.*

	Positive results in % and number of positive reactions on 60 replicates (between brackets) obtained with a cut-off set at				
Copy number tested for LOD	**1**	**5**	**10**	**15**	**20**
5	96.7 % (58/60)	71.7 % (43/60)	25.0 % (15/60)	0 % (0/60)	0 % (0/60)
15	100 % (60/60)	100 % (60/60)	100 % (60/60)	83.3 % (50/60)	46.7 % (28/60)
20	100 % (60/60)	100 % (60/60)	100 % (60/60)	96.7 % (58/60)	90.0 % (54/60)

According to the acceptance criterion, the LOD must not be higher than 20 copies of the target. As could be seen from the results gathered in Table 32, this criterion was met for the 4 PCR platforms considered. However, this statement has in fact to be revised when cut-off levels at more than one copy of the target are considered. With a cut-off calculated based on 1 copy, the LOD is at 10 copies for the platform LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) and at 5 copies for the 3 other platforms.

For a cut-off calculated based on 5 copies of the target, the LOD of the 4 PCR platforms is at 10 copies of the target when combining the data of Tables 30 and 32. This is confirmed in with the absolute LOD in Table 33 for the platform combining the lightcycler LC480 (Roche Diagnostics Ltd) and the Universal Mastermix (Diagenode s.a.) while for the other three platforms the absolute LOD does not exceed 15 copies of the target (Tables 34 to 36).

For the 4 platforms, the absolute LOD does not exceed 15 copies of the target when the cut-off is calculated at 10 copies. When the cut-off is calculated at 15 copies, the LOD_6 and the absolute LOD of the 4 PCR platforms don't exceed 20 copies of the target. While if the cut-off is calculated at 20 copies, then the LOD_6 and the absolute LOD of the 4 PCR platforms exceed a copy number of 20 targets and the acceptance criterion for this performance parameter is no longer met.

4.3. Sensitivity

DNAs from samples with known contents (samples free of ruminant material and samples containing 0.1 % of bovine or ovine PAPs) were analysed with the 4 platforms combining two real-time thermocyclers (one LightCycler LC480-Roche Diagnostics Ltd., Rotkreuz, Switzerland and one ABI7500-Applied Biosystems, Foster City, CA, USA) and two master mixes (the Universal Mastermix-Diagenode s.a., Seraing, Belgium and the qPCR MasterMix for probe assay ROX-Eurogentec s.a., Ougrée, Belgium). Analysis of results with the LC480 used « Abs. quant/second derivative max and high confidence » mode whatever the mastermix used. On the ABI 7500, automatic base line parameter was used while the threshold was set by the operator. The amount of ROX being different in the 2 mastermixes, the threshold was set differently. With the mastermix from Diagenode, the threshold was set at 0.2 while with the mastermix from Eurogentec the threshold was set at 0.015. However, all the runs performed on the same platform were analysed using the same parameters once they were set.

Results are presented in Tables 37 to 48. For each platform, three tables summarise the results obtained with i) feed samples free of ruminant material, ii) feed samples containing 0.1% (mass/mass)

of ruminant PAPs in another PAP or in feedingstuffs of plant origin eventually in presence of some other PAPs but which is then explicitly stated and iii) various controls. The DNAs were tested at the 3-fold and 30-fold dilutions.

It may appear as strange to involve samples free of ruminant material at this stage of the assessment as this would be more in line with the specificity. However, in the specifity section we worked with a cut-off set at 35 cycles as given by TNO Triskelion. In the mean time, potential cut-off values in terms of Ct could be calculated for each platform (at different copy numbers) and due to the large natural abundance of the target (over 200 000 copies per cell), it is not abnormal that a blank feed sample may contain low contaminating quantities of the ruminant target. The study on sensitivity therefore also aims to look what seems to be the optimal cut-off that has to be chosen to keep a good sensitivity together with a good specificity. Of course, the choice of a well-defined cut-off might interfere with the specificity but as the Ct-values for the cut-off are generally above 35 (unless sometimes when the cut-off is calculated on the basis of 15 or 20 copies) there is no interference with the specificity in terms of ruminant-species that have to be detected. Once a cut-off will have been defined in this sensitivity section we will however have to reconsider the specificity results towards the non-target species.

PCR inhibition was detected in the DNA extracted from sample 11, a blank feed containing 0.1 % in weight of ruminant PAP and 5 % in weight of fishmeal particularly when it was tested on platforms using the mastermix provided by Eurogentec. Additional dilutions (6-, 15- and 60-fold) were prepared to remove the PCR inhibition. In the Tables, only the results obtained with the 15-fold dilution are presented.

Table 37: *Sensitivity tests with samples free of ruminant material performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: Abs. quant/second derivative max and high confidence.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (39.874)	5 copies (37.444)	10 copies (36.399)	15 copies (35.787)	20 copies (35.354)
1	Soybean	1	3x	50 50 50	50	-	-	-	-	-
			30x	50 42.21 41.31	44.51*	-	-	-	-	-
		2	3x	45 50 50	48.33*	-	-	-	-	-
			30x	42.23 50 50	47.41*	-	-	-	-	-
2	Pig meal 1 (133 °C)	1	3x	38.72 37.83 38.93	38.49*	+	-	-	-	-
			30x	41.70 42.14 45	42.95*	-	-	-	-	-
		2	3x	39.90 39.78 39.33	39.67*	Ambiguous (2+/3)	-	-	-	-
			30x	42.67 39.96 50	44.21*	-	-	-	-	-
3	Pig meal 2 (133 °C)	1	3x	37.65 37.10 38.26	37.67*	+	Ambiguous (1+/3)	-	-	-
			30x	40.48 39.64 38.95	39.69*	Ambiguous (2+/3)	-	-	-	-
		2	3x	40.09 41.04 41.72	40.95*	-	-	-	-	-
			30x	50 45 43.02	46.01*	-	-	-	-	-
4	Fish meal	1	3x	50 50 50	50	-	-	-	-	-
			30x	50 45 42.25	45.75*	-	-	-	-	-
		2	3x	50 50 50	50	-	-	-	-	-
			30x	43.10 50 50	47.70*	-	-	-	-	-

* > 0.5 cycle between replicates of the same extract

Table 38: *Sensitivity tests with samples containing 0.1 % (w/w) of ruminant material performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: Abs. quant/second derivative max and high confidence.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (39.874)	5 copies (37.444)	10 copies (36.399)	15 copies (35.787)	20 copies (35.354)
5	0.1 % bovine PAP (processed at 133 °C) in pig meal 2 (sample # 3)	1	3x	26.66 26.60 26.64	26.63	+	+	+	+	+
		2	3x	29.03 29.00 28.94	28.99	+	+	+	+	+
6	0.1 % ovine PAP (processed at 133 °C) in pig meal 2 (sample # 3)	1	3x	29.45 29.47 29.68	29.53	+	+	+	+	+
		2	3x	28.93 29.02 29.10	29.02	+	+	+	+	+
7	0.1 % bovine PAP (processed at 133 °C) in fish meal (sample # 4)	1	3x	29.98 29.93 29.95	29.95	+	+	+	+	+
		2	3x	30.61 30.60 30.61	30.61	+	+	+	+	+
8	0.1 % ovine PAP (processed at 133 °C in fish meal (sample # 4)	1	3x	32.97 32.95 32.89	32.94	+	+	+	+	+
		2	3x	32.66 32.69 32.68	32.68	+	+	+	+	+
9	0.1 % ovine PAP (processed at 133 °C in blank feed	1	3x	50 50 50	50	-	-	-	-	-
			30x	32.67 32.65 32.68	32.67	+	+	+	+	+
		2	3x	50 50 39.60	46.53*	Ambiguous (1+/3)	-	-	-	-
			30x	31.99 31.93 32.08	32.00	+	+	+	+	+
10	0.1 % ruminant PAP in blank feed	1	3x	34.21 34.47 34.01	34.23	+	+	+	+	+
			30x	32.24 32.28 32.25	32.26	+	+	+	+	+
		2	3x	34.78 34.64 34.36	34.59	+	+	+	+	+
			30x	31.93 31.87 31.83	31.88	+	+	+	+	+
11	0.1 % ruminant PAP and 5 % fish meal in blank feed	1	3x	35.65 35.59 35.24	35.49	+	+	+	+	Ambiguous (1+/3)
			30x	34.09 34.20 34.03	34.11	+	+	+	+	+
		2	3x	35.05 35.25 35.06	35.12	+	+	+	+	+
			30x	34.52 34.51 34.34	34.46	+	+	+	+	+

* > 0.5 cycle between replicates of the same extract

Table 39: *Sensitivity tests with positive and negative controls performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: Abs. quant/second derivative max and high confidence.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (39.874)	5 copies (37.444)	10 copies (36.399)	15 copies (35.787)	20 copies (35.354)
12	Extraction positive control (0.05 % ruminant PAP in blank feed)	1	3x	30.70 30.62 30.67	30.66	+	+	+	+	+
		2	3x	30.72 30.69 30.73	30.71	+	+	+	+	+
13	Extraction negative control	1	3x	45 41.82 50	45.61	-	-	-	-	-
		2	3x	50 50 50	50	-	-	-	-	-
14	PCR negative control (H_2O in PCR)	/	/	50 50 50 50 50 50	50	-	-	-	-	-
		/	/	50 50 42.20 50 50 50	48.70	-	-	-	-	-

Table 40: *Sensitivity tests with samples free of ruminant material performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the qPCR Master-Mix for probe assay ROX (Eurogentec s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: Abs. quant/second derivative max and high confidence.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (39.302)	5 copies (36.878)	10 copies (35.836)	15 copies (35.226)	20 copies (34.794)
1	Soybean	1	3x	50 50 50	50	-	-	-	-	-
			30x	50 50 50	50	-	-	-	-	-
		2	3x	50 50 50	50	-	-	-	-	-
			30x	50 50 50	50	-	-	-	-	-
2	Pig meal 1 (133 °C)	1	3x	38.85 41.68 42.69	41.07*	Ambiguous (1+/3)	-	-	-	-
			30x	45 41.30 43.21	43.17*	-	-	-	-	-
		2	3x	41.52 45 43.51	43.34*	-	-	-	-	-
			30x	41.00 50 42.87	44.62*	-	-	-	-	-
3	Pig meal 2 (133 °C)	1	3x	39.02 40.18 39.69	39.63*	Ambiguous (1+/3)	-	-	-	-
			30x	41.65 45 44.37	43.67*	-	-	-	-	-
		2	3x	41.52 42.64 40.56	41.57*	-	-	-	-	-
			30x	50 45 42.09	45.70*	-	-	-	-	-
4	Fish meal	1	3x	50 50 50	50	-	-	-	-	-
			30x	50 50 50	50	-	-	-	-	-
		2	3x	50 50 50	50	-	-	-	-	-
			30x	50 50 50	50	-	-	-	-	-

* > 0.5 cycle between replicates of the same extract

Table 41: *Sensitivity tests with samples containing 0.1 % in (w/w) of ruminant material performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the qPCR Master-Mix for probe assay ROX (Eurogentec s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: Abs. quant/second derivative max and high confidence.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (39.302)	5 copies (36.878)	10 copies (35.836)	15 copies (35.226)	20 copies (34.794)
5	0.1 % bovine PAP (processed at 133 °C) in pig meal 2 (sample # 3)	1	3x	27.50 27.34 27.45	27.43	+	+	+	+	+
		2	3x	29.83 29.85 29.80	29.83	+	+	+	+	+
6	0.1 % ovine PAP (processed at 133 °C) in pig meal 2 (sample # 3)	1	3x	30.30 30.24 30.15	30.23	+	+	+	+	+
		2	3x	30.16 30.24 30.30	30.23	+	+	+	+	+
7	0.1 % bovine PAP (processed at 133 °C) in fish meal (sample # 4)	1	3x	31.29 31.20 31.22	31.24	+	+	+	+	+
		2	3x	31.83 31.94 31.86	31.88	+	+	+	+	+
8	0.1 % ovine PAP (processed at 133 °C) in fish meal (sample # 4)	1	3x	34.03 34.03 33.73	33.93	+	+	+	+	+
		2	3x	33.72 33.72 33.69	33.71	+	+	+	+	+
9	0.1 % ovine PAP (processed at 133 °C) in blank feed	1	3x	50 50 50	50	-	-	-	-	-
			30x	33.94 34.21 34.13	34.09	+	+	+	+	+
		2	3x	37.61 38.22 37.56	37.80*	+	-	-	-	-
			30x	33.14 33.58 33.52	33.41	+	+	+	+	+
10	0.1 % ruminant PAP in blank feed	1	3x	33.19 33.22 33.19	33.20	+	+	+	+	+
			30x	33.23 33.13 33.48	33.28	+	+	+	+	+
		2	3x	33.22 33.09 33.28	33.20	+	+	+	+	+
			30x	32.89 32.79 32.79	32.82	+	+	+	+	+
11	0.1 % ruminant PAP and 5 % fish meal in blank feed	1	3x	35.48 35.64 35.72	35.61	+	+	+	-	-
			15x	35.08 35.38 35.12	35.19	+	+	+	Ambiguous (2+/3)	-
			30x	35.34 35.38 35.61	35.44	+	+	+	-	-
		2	3x	35.83 36.10 36.02	35.98	+	+	Ambiguous (1+/3)	-	-
			15x	35.33 35.59 35.60	35.51	+	+	+	-	-
			30x	36.10 35.87 35.97	35.98	+	+	-	-	-

* > 0.5 cycle between replicates of the same extract

Table 42: *Sensitivity tests with positive and negative controls performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the qPCR Master-Mix for probe assay ROX (Eurogentec s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: Abs. quant/second derivative max and high confidence.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (39.302)	5 copies (36.878)	10 copies (35.836)	15 copies (35.226)	20 copies (34.794)
12	Extraction positive control (0.05 % ruminant PAP in blank feed)	1	3x	31.65 31.58 31.64	31.62	+	+	+	+	+
		2	3x	31.76 31.66 31.72	31.71	+	+	+	+	+
13	Extraction negative control	1	3x	50 41.12 50	47.04*	-	-	-	-	-
		2	3x	50 44.36 45	46.45*	-	-	-	-	-
14	PCR negative control (H_2O in PCR)	/	/	50 50 50 50 50 50	50	-	-	-	-	-
		/	/	50 45 45 43.74 50 50	47.29	-	-	-	-	-

* > 0.5 cycle between replicates of the same extract

Table 43: *Sensitivity tests with samples free of ruminant material performed on the ABI7500 therrmocycler (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: auto baseline and threshold at 0.2.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (38.693)	5 copies (36.284)	10 copies (35.248)	15 copies (34.642)	20 copies (34.213)
1	Soybean	1	3x	40.53 38.57 50	43.03*	Ambiguous (1+/3)	-	-	-	-
			30x	45.26 50 50	48.42*	-	-	-	-	-
		2	3x	50 50 50	50	-	-	-	-	-
			30x	50 50 40.46	46.82*	-	-	-	-	-
2	Pig meal 1 (133 °C)	1	3x	38.22 37.40 35.63	37.08*	+	Ambiguous (1+/3)	-	-	-
			30x	38.08 40.08 40.78	39.65*	Ambiguous (1+/3)	-	-	-	-
		2	3x	38.05 39.52 39.45	39.01*	Ambiguous (1+/3)	-	-	-	-
			30x	50 46.24 40.21	45.48*	-	-	-	-	-
3	Pig meal 2 (133 °C)	1	3x	36.38 36.40 36.24	36.34	+	Ambiguous (1+/3)	-	-	-
			30x	40.48 39.80 39.84	40.04*	-	-	-	-	-
		2	3x	37.09 38.69 39.15	38.31*	Ambiguous (2+/3)	-	-	-	-
			30x	44.90 50 50	48.30	-	-	-	-	-
4	Fish meal	1	3x	50 50 50	50	-	-	-	-	-
			30x	47.85 50 50	49.28*	-	-	-	-	-
		2	3x	50 50 50	50	-	-	-	-	-
			30x	50 41.05 50	47.02*	-	-	-	-	-

* > 0.5 cycle between replicates of the same extract

Table 44: *Sensitivity tests with samples containing 0.1 % (w/w) of ruminant material performed on the ABI7500 thermocycler (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: auto baseline and threshold at 0.2.*

Sample	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (38.693)	5 copies (36.284)	10 copies (35.248)	15 copies (34.642)	20 copies (34.213)
5	0.1 % bovine PAP (processed at 133 °C) in pig meal 2 (sample # 3)	1	3x	25.76 25.65 25.58	25.66	+	+	+	+	+
		2	3x	28.04 28.08 28.17	28.10	+	+	+	+	+
6	0.1 % ovine PAP (processed at 133 °C) in pig meal 2 (sample # 3)	1	3x	28.40 28.42 28.34	28.39	+	+	+	+	+
		2	3x	27.81 27.97 28.12	27.97	+	+	+	+	+
7	0.1 % bovine PAP (processed at 133 °C) in fish meal (sample # 4)	1	3x	28.70 28.62 28.52	28.61	+	+	+	+	+
		2	3x	29.14 29.24 29.34	29.24	+	+	+	+	+
8	0.1 % ovine PAP (processed at 133 °C) in fish meal (sample # 4)	1	3x	32.12 31.82 31.60	31.85*	+	+	+	+	+
		2	3x	31.34 31.32 31.52	31.39	+	+	+	+	+
9	0.1 % ovine PAP (processed at 133 °C) in blank feed	1	3x	50 50 50	50	-	-	-	-	-
			30x	31.88 31.81 31.73	31.81	+	+	+	+	+
		2	3x	42.30 43.00 41.48	42.26*	-	-	-	-	-
			30x	31.05 31.19 31.14	31.13	+	+	+	+	+
10	0.1 % ruminant PAP in blank feed	1	3x	34.54 33.72 33.64	33.97*	+	+	+	+	Ambiguous (2+/3)
			30x	31.45 31.47 31.32	31.41	+	+	+	+	+
		2	3x	34.95 35.66 35.11	35.24*	+	+	Ambiguous (2+/3)	-	-
			30x	30.94 30.87 30.90	30.90	+	+	+	+	+
11	0.1 % ruminant PAP and 5 % fish meal in blank feed	1	3x	34.34 34.39 34.79	34.51	+	+	+	Ambiguous (2+/3)	-
			30x	32.92 33.18 33.26	33.12	+	+	+	+	+
		2	3x	34.27 34.19 34.15	34.20	+	+	+	+	Ambiguous (2+/3)
			30x	33.53 33.57 33.52	33.54	+	+	+	+	+

Table 45: *Sensitivity tests with positive and negative controls performed on the ABI7500 thermocycler (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: auto baseline and threshold at 0.2.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (38.693)	5 copies (36.284)	10 copies (35.248)	15 copies (34.642)	20 copies (34.213)
12	Extraction positive control (0.05 % ruminant PAP in blank feed)	1	3x	30.12 29.75 29.95	29.94	+	+	+	+	+
		2	3x	29.89 29.75 29.79	29.81	+	+	+	+	+
13	Extraction negative control	1	3x	42.24 50 44.31	45.52*	-	-	-	-	-
		2	3x	50 39.30 39.29	42.86*	-	-	-	-	-
14	PCR negative control (H_2O in PCR)	/	/	50 50 50 50 50 50	50	-	-	-	-	-
		/	/	50 50 42.45 50 50 41.99	47.41	-	-	-	-	-

* > 0.5 cycle between replicates of the same extract

Table 46: *Sensitivity tests with samples free of ruminant material performed on the ABI7500 thermocycler (Applied Biosystems) using the qPCR Master-Mix for probe assay ROX (Eurogentec s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: auto baseline and threshold at 0.015.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (38.853)	5 copies (36.411)	10 copies (35.361)	15 copies (34.747)	20 copies (34.312)
1	Soybean	1	3x	50 46.33 50	48.78*	-	-	-	-	-
			30x	42.81 50 50	47.60*	-	-	-	-	-
		2	3x	47.44 37.43 50	44.96*	Ambiguous (1+/3)	-	-	-	-
			30x	50 50 50	50	-	-	-	-	-
2	Pig meal 1 (133 °C)	1	3x	41.77 40.76 39.05	40.53*	-	-	-	-	-
			30x	45.25 43.10 50	46.12*	-	-	-	-	-
		2	3x	41.96 39.86 44.34	42.05*	-	-	-	-	-
			30x	46.83 50 50	48.94*	-	-	-	-	-
3	Pig meal 2 (133 °C)	1	3x	39.10 38.09 38.01	38.40*	Ambiguous (2+/3)	-	-	-	-
			30x	45.54 39.47 41.51	42.17*	-	-	-	-	-
		2	3x	40.75 45 41.17	42.30*	-	-	-	-	-
			30x	40.24 50 45.26	45.16*	-	-	-	-	-
4	Fish meal	1	3x	50 47.40 46.09	47.83*	-	-	-	-	-
			30x	50 50 50	50	-	-	-	-	-
		2	3x	50 50 50	50	-	-	-	-	-
			30x	42.02 42.77 42.48	42.42*	-	-	-	-	-

* > 0.5 cycle between replicates of the same extract

Table 47 : *Sensitivity tests with samples containing 0.1 % (w/w) of ruminant material performed on the ABI7500 thermocycler (Applied Biosystems) using the qPCR Master-Mix for probe assay ROX (Eurogentec s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: auto baseline and threshold at 0.015.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (38.853)	5 copies (36.411)	10 copies (35.361)	15 copies (34.747)	20 copies (34.312)
5	0.1 % bovine PAP (processed at 133 °C) in pig meal 2 (sample # 3)	1	3x	27.10 26.96 27.02	27.03	+	+	+	+	+
		2	3x	29.33 29.43 29.49	29.42	+	+	+	+	+
6	0.1 % ovine PAP (processed at 133 °C) in pig meal 2 (sample 3)	1	3x	29.96 30.02 29.99	29.99	+	+	+	+	+
		2	3x	29.87 29.54 29.97	29.79	+	+	+	+	+
7	0.1 % bovine PAP (processed at 133 °C) in fish meal (sample # 4)	1	3x	31.05 30.96 30.84	30.95	+	+	+	+	+
		2	3x	31.46 31.74 31.72	31.64	+	+	+	+	+
8	0.1 % ovine PAP (processed at 133 °C) in fish meal (sample # 4)	1	3x	34.19 33.69 33.49	33.79*	+	+	+	+	+
		2	3x	33.33 33.09 33.35	33.26	+	+	+	+	+
9	0.1 % ovine PAP (processed at 133 °C) in blank feed	1	3x	50 50 50	50	-	-	-	-	-
			30x	33.57 33.48 33.48	33.51	+	+	+	+	+
		2	3x	45.11 48.05 46.43	46.53*	-	-	-	-	-
			30x	32.84 32.78 32.91	32.84	+	+	+	+	+
10	0.1 % ruminant PAP in blank feed	1	3x	33.75 33.72 33.45	33.64	+	+	+	+	+
			30x	32.84 32.60 32.75	32.73	+	+	+	+	+
		2	3x	34.48 34.41 34.56	34.48	+	+	+	+	-
			30x	32.28 32.42 32.38	32.36	+	+	+	+	+
11	0.1 % ruminant PAP and 5 % fish meal in blank feed	1	3x	35.71 36.16 36.11	36.00	+	+	-	-	-
			15x	35.06 34.24 34.78	34.69	+	+	+	Ambiguous (1+/3)	Ambiguous (1+/3)
			30x	34.58 34.75 34.92	34.75	+	+	+	Ambiguous (1+/3)	-
		2	3x	35.43 35.22 35.31	35.32	+	+	Ambiguous (2+/3)	-	-
			15x	35.06 34.33 34.85	34.75*	+	+	+	Ambiguous (1+/3)	-
			30x	35.15 35.25 35.54	35.32	+	+	Ambiguous (2+/3)	-	-

* > 0.5 cycle between replicates of the same extract

Table 48: *Sensitivity tests with positive and negative controls performed on the ABI7500 thermocycler (Applied Biosystems) using the qPCR Master-Mix for probe assay ROX (Eurogentec s.a.) - Interpretation of results is based on a cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding cut-off values in terms of Ct are given between brackets). Analysis mode: auto baseline and threshold at 0.015.*

Sample #	Description	Extract	Dilution	C_t	Mean C_t	Interpretation of results with a cut-off set at				
						1 copy (38.853)	5 copies (36.411)	10 copies (35.361)	15 copies (34.747)	20 copies (34.312)
12	Extraction positive control (0.05 % ruminant PAP in blank feed)	1	3x	31.47 31.29 31.24	31.33	+	+	+	+	+
		2	3x	31.50 31.48 31.28	31.42	+	+	+	+	+
13	Extraction negative control	1	3x	50 47.45 50	49.15*	-	-	-	-	-
		2	3x	42.19 46.90 42.41	43.83*	-	-	-	-	-
14	PCR negative control (H_2O in PCR)	/	/	50 41.04 42.35 46.74 50 50	46.69*	-	-	-	-	-
		/	/	50 50 46.66 50 50 44.96	48.60*	-	-	-	-	-

* > 0.5 cycle between replicates of the same extract

As can be seen from the results of Tables 37 to 48, the choice of a cut-off level has a direct impact on the sensitivity and it also influences the specificity. In order to make a choice of a cut-off level, sensitivity and specificity results on different platforms as obtained at different cut-off levels (calculated for 1, 5, 10, 15 and 20 copies) are summarized in Tables 49 to 52 with the rates of false positive or false negative results.

Table 49: *Number false negative and false positive results with the samples used for specificity and sensitivity on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) according to cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding number of cycles between brackets).*

Sample types	Number of samples tested	Cut-off for platform LC480 (Roche Diagnostics Ltd.) - Universal Mastermix (Diagenode s.a.)									
		1 copy (39.874)		5 copies (37.444)		10 copies (36.399)		15 copies (35.787)		20 copies (35.354)	
		Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results
Ruminant species and samples containing ruminant material	11	0	0	0	0	0	0	0	0	0	0
Terrestrial mammalian species other than ruminants	7	0	0 but 1 ambiguous sample	0	0	0	0	0	0	0	0
Domestic bird species	6	0	0	0	0	0	0	0	0	0	0
Sea mammal species	6	0	2	0	2	0	2	0	2	0	2
Fish and crab species	21	0	0 but 1 ambiguous sample	0	0	0	0	0	0	0	0
Plant species	7	0	0	0	0	0	0	0	0	0	0
Pure PAP without ruminant material	3	0	0 and 2 ambiguous samples	0	0 but 1 ambiguous sample	0	0	0	0	0	0
Samples containing 0.1 % (m/m) ruminant PAP	7	0	0	0	0	0	0	0	0	0 but 1 ambiguous sample	0
Extraction positive control	2	0	0	0	0	0	0	0	0	0	0
Extraction negative control	2	0	0	0	0	0	0	0	0	0	0
% of false results calculated on 72 samples		0	2.8 and 5.6 % ambiguous samples	0	2.8 and 1.4 % ambiguous samples	0	2.8	0	2.8	0 but 1.4 % ambiguous samples	2.8

Table 50: *Number false negative and false positive results with the samples used for specificity and sensitivity on the LC480 thermocycler (Roche Diagnostics Ltd.) using the qPCR MasterMix for probe assay ROX (Eurogentec s.a.) according to cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding number of cycles between brackets).*

		Cut-off for platform LC480 (Roche Diagnostics Ltd.) and qPCR MasterMix for probe assay ROX (Eurogentec s.a.)									
Sample types	**Number of samples tested**	**1 copy (39.302)**		**5 copies (36.878)**		**10 copies (35.836)**		**15 copies (35.226)**		**20 copies (34.794)**	
		Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results
Ruminant species and samples containing ruminant material	11	0	0	0	0	0	0	0	0	0	0
Terrestrial mammalian species other than ruminants	7	0	0	0	0	0	0	0	0	0	0
Domestic bird species	6	0	0	0	0	0	0	0	0	0	0
Sea mammal species	6	0	2	0	2	0	2	0	1	0	1
Fish and crab species	21	0	0	0	0	0	0	0	0	0	0
Plant species	7	0	0	0	0	0	0	0	0	0	0
Pure PAP without ruminant material	3	0	0 but 2 ambiguous samples	0	0	0	0	0	0	0	0
Samples containing 0.1 % (m/m) ruminant PAP	7	0	0	0	0	0	0	0 but 1 ambiguous sample	0	1	0
Extraction positive control	2	0	0	0	0	0	0	0	0	0	0
Extraction negative control	2	0	0	0	0	0	0	0	0	0	0
% of false results calculated on 72 samples		0	2.8 and 2.8 % ambiguous samples	0	2.8	0	2.8	0 and 1.4 % ambiguous samples	1.4	1.4	1.4

Table 51: *Number false negative and false positive results with the samples used for specificity and sensitivity on the thermocycler ABI7500 (Applied Biosystems) using the Universal Mastermix (Diagenode s.a.) according to cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding number of cycles between brackets).*

Sample types	Number of samples tested	Cut-off for platform ABI7500 (Applied Biosystems) and Universal Mastermix (Diagenode s.a.)									
		1 copy (38.693)		5 copies (36.284)		10 copies (35.248)		15 copies (34.642)		20 copies (34.213)	
		Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results
Ruminant species and samples containing ruminant material	11	0	0	0	0	0	0	0	0	0	0
Terrestrial mammalian species other than ruminants	7	0	0 but 1 ambiguous sample	0	0	0	0	0	0	0	0
Domestic bird species	6	0	0	0	0	0	0	0	0	0	0
Sea mammal species	6	0	3	0	2	0	2	0	2	0	2
Fish and crab species	21	0	0 but 2 ambiguous samples	0	0	0	0	0	0	0	0
Plant species	7	0	0 but 1 ambiguous sample	0	0	0	0	0	0	0	0
Pure PAP without ruminant material	3	0	0 but 2 ambiguous samples	0	0 but 2 ambiguous samples	0	0	0	0	0	0
Samples containing 0.1 % (m/m) ruminant PAP	7	0	0	0	0	0	0	0	0	0	0
Extraction positive control	2	0	0	0	0	0	0	0	0	0	0
Extraction negative control	2	0	0	0	0	0	0	0	0	0	0
% of false results calculated on 72 samples		0	4.2 and 8.3 % ambiguous samples	0	2.8 and 2.8 % ambiguous samples	0	2.8	0	2.8	0	2.8

Table 52: *Number false negative and false positive results with the samples used for specificity and sensitivity on the thermocycler ABI7500 (Applied Biosystems) using the qPCR MasterMix for probe assay ROX (Eurogentec s.a.) according to cut-off set at 1, 5, 10, 15 and 20 copies respectively (corresponding number of cycles between brackets).*

Sample types	Number of samples tested	Cut-off for platform ABI7500 (Applied Biosystems) and qPCR MasterMix for probe assay ROX (Eurogentec s.a.)									
		1 copy (38.853)		5 copies (36.411)		10 copies (35.361)		15 copies (34.747)		20 copies (34.312)	
		Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results	Number false negative results	Number false positive results
Pure PAP without ruminant material	3	0	0 but 1 ambiguous sample	0	0	0	0	0	0	0	0
Samples containing 0.1 % (m/m) ruminant PAP	7	0	0	0	0	0	0	0 but 1 ambiguous sample	0	0 but 1 ambiguous sample	0
Extraction positive control	2	0	0	0	0	0	0	0	0	0	0
Extraction negative control	2	0	0	0	0	0	0	0	0	0	0
% of false results calculated on 14 samples		0	0 and 7.1 % ambiguous samples	0	0	0	0	0 and 7.1 % ambiguous samples	0	0 and 7.1 % ambiguous samples	0

Looking as a whole at these results, it is clear that the cut-off should at least be calculated at 10 copies because when calculated at 5 copies, some blank samples (especially the pig meal tested here) could sometimes be interpreted as positive. At the other side, a cut-off calculated on 20 copies is clearly too high as some samples at 0.1% (w/w) of ruminant material in feed might no longer be detected. This means that in practice the cut-off should be calculated at 10 or 15 copies to reach a good compromise between sensitivity and specificity. If only considering the results of the assessment, a cut-off calculated at 10 copies appears as the best option but when calculated at 15 copies it is still very good. Such cut-off levels have no impact on the specificity study made towards non-ruminant target (see section 2.3.). Results would be strictly identical, except in some cases like detection of the cetacean *Ziphius cavirostris*. A last point to stress about sensitivity is that the level of 0.1% (w/w) of ruminant material can be detected as well in plant material (because what is called blank feed in tables 37 to 48 is based only on feedingstuffs of plant origin) as in another processed animal protein.

4.4. Robustness

The robustness of the method was tested by introducing some deviations in the standard experimental conditions on two thermocyclers (LC480 and ABI7500) and with two mastermixes (Diagenode versus Eurogentec): the annealing t° (60°C +/- 1°C), the primer concentrations (standard or reduced by 30%), the probe concentration (standard or reduced by 30%) and the PCR volume (25 µl +/- 1µl). Six replicates of plasmid at 20 copies/5 µl were performed in the conditions described in Table 53.

Table 53: *Experimental conditions tested to evaluate the robustness of method.*

PCR machine	LC480 and ABI7500				
PCR reagent kit	Universal Mastermix (Diagenode s.a.) and qPCR MasterMix for probe assay ROX (Eurogentec s.a.)				
Annealing temperature	59, 60 and 61 °C				
Primer concentration	Minus 30 %	Standard	Standard	Standard	Standard
Probe concentration	Standard	Minus 30 %	Standard	Standard	Standard
PCR volume	Standard	Standard	Standard (20 µl mix + 5 µl DNA)	Standard + 1 µl Mastermix (21 µl mix + 5 µl DNA)	Standard – 1 µl Mastermix (19 µl mix + 5 µl DNA)

The tested parameters taken over here are the ones considered by the German experts for GMO detection in a collection of methods called “chapter 64”. Normally the acceptance criterion is that the method must give positive results in all the cases considered during an orthogonal experiment where not all possible combinations of parameters are tested but only some.

Here we performed a somewhat more detailed experiment than what the orthogonal experiment would have foreseen. The numbers of positive results on 6 replicates in each condition are presented for each platform in Tables 54 to 57. The results were analyzed with cut-off values set at 10 copies and 15 copies. The percentages calculated do not include the 6 replicates realized in standard conditions (standard conditions with annealing temperature at 60 °C).

Table 54: *Robustness test performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Analysis mode: Abs. quant/second derivative max and high confidence.*

		Standard conditions		**Primers concentration minus 30 %**		**Probe concentration minus 30 %**		**+ 1 µl Mastermix (21 µl mix + 5 µl DNA)**		**- 1 µl Mastermix (19 µl mix + 5 µl DNA)**	
		Cut-off at 10 copies	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**
Annealing temperature	**59 °C**	6/6	6/6	6/6	4/6	6/6	6/6	6/6	5/6	6/6	6/6
	60 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	61 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6

With the platform LC480 with Universal Mastermix provided by Diagenode (Table 54), **100 %** of results are considered as positive with a cut-off set at 10 copies. With a cut-off set at 15 copies, the rate of positive results falls to **96.4 %.** The few false negative results observed appear when the concentration of the primers is reduced by 30 % (2 false negative results / 6 replicates) or when the total volume of the PCR mix is raised up to 21 µl.

Table 55: *Robustness test performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the qPCR MasterMix for probe assay ROX (Eurogentec s.a.). Analysis mode: Abs. quant/second derivative max and high confidence.*

		Standard conditions		**Primers concentration minus 30 %**		**Probe concentration minus 30 %**		**+ 1 µl Mastermix (21 µl mix + 5 µl DNA)**		**- 1 µl Mastermix (19 µl mix + 5 µl DNA)**	
		Cut-off at 10 copies	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**
Annealing temperature	**59 °C**	6/6	6/6	6/6	5/6	6/6	6/6	5/6	5/6	6/6	5/6
	60 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	61 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6

With the platform combining a LC480 thermocycler with qPCR MasterMix provided by Eurogentec (Table 55), **98.8 %** of results are considered as positive with a cut-off set at 10 copies and **96,4 %** of the replicates give the expected positive result with a cut-off at 15 copies.

Table 56: *Robustness test performed on the ABI7500 thermocycler (Applied Biosystems) with the Universal Mastermix (Diagenode s.a.). Analysis mode: automatic base line and threshold at 0.2*

		Standard conditions		Primers concentration minus 30 %		Probe concentration minus 30 %		+ 1 µl Mastermix (21 µl mix + 5 µl DNA)		- 1 µl Mastermix (19 µl mix + 5 µl DNA)	
		Cut-off at 10 copies	Cut-off at 15 copies	Cut-off at 10 copies	Cut-off at 15 copies	Cut-off at 10 copies	Cut-off at 15 copies	Cut-off at 10 copies	Cut-off at 15 copies	Cut-off at 10 copies	Cut-off at 15 copies
Annealing temperature	**59 °C**	6/6	6/6	6/6	4/6	5/6	3/6	6/6	6/6	6/6	6/6
	60 °C	6/6	5/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6	6/6
	61 °C	6/6	5/6	6/6	5/6	6/6	6/6	6/6	5/6	6/6	6/6

With the platform combining an ABI7500 thermocycler and Universal Mastermix by Diagenode (Table 56), **98.8 %** of results are considered as positive with a cut-off set at 10 copies while only **89.3 %** of results are considered as positive with a cut-off at 15 copies. However, in this assay, even the standard conditions resulted in only 83.3 % of positive results with a cut-off at 15 copies.

Table 57: *Robustness test performed on the ABI7500 thermocycler (Applied Biosystems) with the qPCR MasterMix for probe assay ROX (Eurogentec s.a.). Analysis mode: automatic base line and threshold at 0.015.*

		Standard conditions		Primers concentration minus 30 %		Probe concentration minus 30 %		+ 1 µl Mastermix (21 µl mix + 5 µl DNA)		- 1 µl Mastermix (19 µl mix + 5 µl DNA)	
		Cut-off at 10 copies	Cut-off at 15 copies	Cut-off at 10 copies	Cut-off at 15 copies	Cut-off at 10 copies	Cut-off at 15 copies	Cut-off at 10 copies	Cut-off at 15 copies	Cut-off at 10 copies	Cut-off at 15 copies
Annealing temperature	**59 °C**	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	60 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	61 °C	6/6	6/6	6/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6

With the last platform combining an ABI7500 thermocycler with qPCR MasterMix by Eurogentec (Table 57), **100 %** of results are considered as positive whatever the deviation with a cut-off set at 10 copies. In the case of a cut-off set at 15 copies, **98.8 %** of results are positive.

In conclusion of these robustness tests, the rate of positive results was always higher than 95 % with a cut-off set at 10 copies and was once below 95% (89.3 % - platform combining ABI7500 and Universal Mastermix by Diagenode) with a cut-off set at 15 copies. However, this is linked to the fact that the test was performed on a rather limited number of copies of the target (20 copies) for the cut-off considered. Moreover this less suitable result also appears because both thermocyclers were tested with both mastermixes, which in a merely orthogonal design would not necessarily have been seen because then we would only have either the combinations of Tables 54 and 57 (the best combination) or that of Tables 55 and 56 (the worst combination).

The test has been done on a copy number close to 40 which in practice is still far below what is the amount of copies of the targets that would be tested on a sample at 0.1% (w/w) of ruminant material in feed. This test was performed on 2 platforms (Tables 58 and 59) and the cut-off at 10 and 15 copies were applied to interpret the results.

Table 58: *Robustness test performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the qPCR MasterMix for probe assay ROX (Eurogentec s.a.) on the calibrant at 40 copies/5 µl. Analysis mode: Abs. quant/second derivative max and high confidence*

		Standard conditions		**Primers concentration minus 30 %**		**Probe concentration minus 30 %**		**+ 1 µl Mastermix (21 µl mix + 5 µl DNA)**		**- 1 µl Mastermix (19 µl mix + 5 µl DNA)**	
		Cut-off at 10 copies	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**
Annealing temperature	**59 °C**	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	60 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	61 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6

With the platform combining a LC480 thermocycler with qPCR MasterMix provided by Eurogentec, **100 %** of results are considered as positive with a cut-off set at 10 copies and at 15 copies.

Table 59: *Tests of robustness performed on the ABI7500 (Applied Biosystems) with the Universal Mastermix (Diagenode s.a.) on the calibrant at 40 copies/5 µl. Analysis mode: automatic base line and threshold at 0.2*

		Standard conditions		**Primers concentration minus 30 %**		**Probe concentration minus 30 %**		**+ 1 µl Mastermix (21 µl mix + 5 µl DNA)**		**- 1 µl Mastermix (19 µl mix + 5 µl DNA)**	
		Cut-off at 10 copies	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**	**Cut-off at 10 copies**	**Cut-off at 15 copies**
Annealing temperature	**59 °C**	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	60 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	61 °C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6

With the last platform combining ABI7500 with Universal Mastermix provided by Diagenode, **100 %** of results are considered as positive whatever the deviation with a cut-off set at 10 copies and at 15 copies.

Thus, the complete proof is provided here for the robustness with a threshold calculated at 15 copies.

5. Final conclusions

The TNO Triskelion ruminant PCR assay was assessed in this study for its specificity, its sensitivity and its robustness in order to check if it was fit for a validation in an interlaboratory study.

The method focuses a highly multicopy and polymorphic target that is however **specific for ruminants**, especially the farmed ones which are also the most important ones in processed animal proteins. Some game species of ruminants were tested too. They were still detected but in a somewhat less efficient way. However this is not a problem for the test. A more tricky point to stress is the **possible interference with cetacean species** that are taxonomically rather close to ruminants. Here too it was considered that taking into account the purpose of the assay, this drawback does not impair the use of the test. However users of the test should be aware of this possible limitation.

The test is able to achieve what is the minimum requirement expected of a PCR assay, i.e. to detect **0.1% (w/w) of ruminant material in feed**. It is able to reach that goal as well in presence of feed composed only of plant material as in presence of another species of processed animal proteins.

For implementation of the transferability, the study arrives to the conclusion that **cut-off levels calculated at 10 or 15 copies** have both to be considered in the validation study (a level in between could also be suitable).

The very final conclusion of this study is that the TNO Triskelion ruminant PCR assay used with an appropriate cut-off may be considered as **fit for validation** in the sense that the risk of failure of such a validation organized as interlaboratory study appears as very low.

Acknowledgments

The EURL-AP wishes to thank Dr Philippe Corbisier and Mr Stéphane Mazoua of JRC (Joint Research Centre, Geel, Belgium) for their assistance.

Annex 1 : Detection of ruminant DNA by real-time PCR

1. Primers and probe sequences

Forward primer : 5'-CCA GCA TCA GAG TCT TTT CCA AAT-3'
Reverse primer : 5'-GAA GGA ATG ATG CTA AAG CTG AAA C-3'
Probe : 5'-CAA CTC TTC GCA TGA GGT GGC CAA A-3'

Reporter dye : FAM (position 5' of the probe)
Quencher dye : TAMRA (position 3' of the probe)

2. Real-time PCR mix

After complete thawing of the reagents, in a DNAse free microfuge tube, the reagents are mixed in the following order for a final volume of 25 µl :

PCR grade water, 11 picomoles of forward primer and reverse primer, 3.65 picomoles of probe, mastermix with $MgCl_2$ at the final concentration of 3.86 mmole/l.

The examples of mixes are given in Table 1:

Table 1: Examples of mixes

	1 réaction	96 réactions	105 réactions (1 plate)*
PCR grade water	5.00 µl	480.00 µl	525.00 µl
Forward primer (10 µmole/l)	1.10 µl	105.60 µl	115.50 µl
Reverse primer (10 µmole/l)	1.10 µl	105.60 µl	115.50 µl
Probe (5 µmole/l)	0.73 µl	70.08 µl	76.65 µl
Master mix 2x	12.07 µl	1158.72 µl	1267.35 µl
Total PCR mix volume/reaction	20 µl		
DNA to be added in each PCR	5 µl		
Total reaction volume	25 µl / well		

* A larger volume than the one required to fill the wells has to be prepared

3. Thermal program

The thermal program to follow is outlined in Table 2:

Table 2: Thermal program of the ruminant PCR assay

Process		**Time (min:s)**	**Temperature (°C)**
Pre-PCR: decontamination (optional)		02:00	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA (mandatory)		10:00	95
PCR (50 cycles)			
Step 1	Denaturation	00:15	95
Step 2	Annealing and elongation	01:00	60