Evaluation of the PCR method for the detection of pig DNA

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September 2021

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ISBN 2-87286-120-3
Legal Deposit D/2021/1463/3

Editor :

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TABLE OF CONTENTS

1. Introduction

In the TSE roadmap II[1], the Commission considers a possible lifting of the ban on the use of non-ruminant processed animal proteins (PAP) in non-ruminant feed 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, a PCR method for the detection of ruminant DNA was already validated and implemented in the EURL-AP network labs during 2012[2]. As a further step, the detection of the porcine PAP is also of importance. Different laboratories developed pig real time PCR targets. The EURL-AP assessed the performances of the different assays.

The choice of the target was made according to various criteria such as the cost of analysis, the specificity and the sensitivity of real time PCR tests.

The evaluation was conducted according guidelines established for validation of qualitative real-time PCR methods[3]. These guidelines are based on international texts such as the Codex Alimentarius[4] and ISO Guidelines[5,6,7,8,9] or other relevant publications[10,11,12] in the field. Most of them are mainly dedicated to PCR methods for GMO detection but are also applicable to any other real-time PCR method and offer an objective frame to this study.

2. Description of different PCR tests for pig detection

2.1. Information collected in literature and sequence databanks

The EURL-AP collected the information on the six PCR methods for the detection of pig DNA put at its disposal (Table 1).

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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 **EURL-AP PCR Implementation Test 2012 (2012).**
O. Fumière, A. Marien and G. Berben
https://www.eurl.craw.eu/wp-content/uploads/2019/10/eurl_ap_pcr_ils_2012_final_version.pdf

3 **Guidelines for validation of qualitative real-time PCR methods (2014).** Broeders *et al.* Trends in Food Science & Technology, 37(2), 115-126.

4 **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.

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

6 **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.

7 **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.

8 **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.

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

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

11 **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

12 **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

Table 1: *Information on the PCR methods for the detection of pig DNA put at the disposal of the EURL-AP.*

Country	Laboratories or references	Location of the target	Size of fragment	Type of probe
Poland	National Veterinary Research Institute, Partyzantów 57, PL-24-100 Pulawy, Poland	Mitochondrial DNA 12S rRNA	126 bp	No probe (classical PCR)
Spain	Departamento de Nutrición, Bromatología y Tecnología de los Alimentos – Facultad de Veterinaria – Universidad complutense de Madrid, 28040 Madrid, Spain	Mitochondrial DNA 12S rRNA	74 bp	5'-FAM and 3'-BBQ The probe contains 5 LNA
The Netherlands	TNO Triskelion B.V. Utrechtseweg 48, 3704 HE Zeist, The Netherlands	Genomic DNA Immunoglobulin heavy chain	70 bp	5'-FAM and 3'-TAMRA
Italy	Krcmar P. and Rencova E. (2005). Quantitative detection of species-specific DNA in feedstuffs and fish meals. J Food Prot: 68(6), 1217-21.	Mitochondrial DNA ATPase subunit 6	111 bp[13]	5'-FAM and 3'-BH1
Belgium	Walloon Agricultural Research Centre (CRA-W), Valorisation of Agricultural Products Department, Chaussée de Namur 24, 5030 Gembloux, Belgium	Mitochondrial DNA tRNA-Lys and ATP8 gene	68 bp[14]	5'-FAM and 3'-TAMRA
	EURL-AP (CRA-W)	Mitochondrial DNA ND4 gene	83 bp	5'-FAM and 3'-TAMRA

After the bibliographic analysis of each PCR assay, the ones of Poland and Spain were not considered as relevant. The pig PCR assay proposed by the Polish NRL is not a real-time PCR assay, no probe was indicated. As small targets have to be considered, end-point PCR is generally considered as less suitable. Moreover, the size of the amplicon (126 bp) is the largest of the list and close to the limit of what is acceptable for the purpose of the method. The pig PCR assay from the Spanish lab needs a probe containing 5 locked nucleic acids (LNA) thereby increasing the cost of the assay. The EURL-AP favours the less expensive assay presenting the requested performances.

The primers and probes of 4 other targets were synthesized by Eurogentec (Seraing, Belgium).

2.2. Specificity of different methods for the detection of pig DNA

The specificity of the 4 remaining PCR methods for the detection of pig DNA 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: most of the animal DNAs were extracted from blood samples; when it was not possible, the DNAs were extracted from meat. In the case of fish species, the DNA extractions were carried out on muscles using samples with reference certificates obtained from a German institute (Federal Research Centre for Nutrition and Food, Department of Fish Quality, Hamburg); DNAs from sea mammals were also tested. They were extracted from muscle samples collected by specialised institutes of Belgium and Italy.

The tests were performed with a real-time PCR platform combining a LightCycler 480 thermocycler (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with the Universal Mastermix (Diagenode s.a., Seraing, Belgium).

The specificity of the test was firstly checked with DNA samples from pig (*Sus scrofa domesticus*) blood, boar (wild species, *Sus scrofa scrofa*) blood and feed samples containing porcine material at different levels. These last samples used are of various origins: positive control for extraction and PCR used by EURL-AP, respectively 0.1 % and 0.025 % in mass fraction of PAP in plant matrix. The PAP

[13] **Quantitative detection of species-specific DNA in feedstuffs and fish meals (2005).**
Krcmar P., Rencova E.
J Food Prot, 68(6), 1217-1221

[14] **Effective PCR detection of animal species in highly processed animal byproducts and compound feeds (2006).**
Fumière O. *et al.*
Anal Bioanal Chem, 385, 1045-1054

used for these controls does not contain only pig, therefore the porcine PAP levels are lower than the nominal 0.1 and 0.025 % contents in PAP. Sample CRL 214 is a porcine PAP processed at 133 °C and CRL 558 is a porcine meal processed at 121 °C coming from Nutricontrol (Veghel, The Netherlands). It was purposedly treated below the legal requirement. Samples CRL 1370, 1374 and 1445 originate from PCR ring tests of APHA (Animal and Plant Health Agency-UK, formerly AHVLA) and sample CRL 1647 comes from a ring test of IAG (International Association for Feedingstuff Analysis).

The pure species DNA extracts were at 2 ng / µl. The meal samples were extracted with the kit Wizard® Magnetic DNA Purification System for Food (Promega, Madison, WI, USA). The DNAs were resuspended in 300 µl of PCR grade water (corresponding to what the report calls the 3-fold dilution). Five µl of extract were tested per PCR, generally at 3-fold and 30-fold dilutions. Each extract or dilution was tested in duplicate.

Table 2 summarises the results obtained on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Only the results obtained with the 3-fold dilution are presented, the tests with the 30-fold dilutions do not improve the results.

Table 2: *Specificity tests of 4 pig PCR tests carried out on samples containing porcine DNA. PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) with Universal Mastermix (Diagenode s.a.). DNA from fresh blood samples was extracted with the kit Qiagen Genomic DNA tip 20/G (Qiagen, Venlo, The Netherlands). Pure species DNA extracts were at a concentration of 2 ng / µl. Meal samples were extracted with the kit Wizard® Magnetic DNA Purification System for Food (Promega, Madison, WI, USA). The CRL samples tested are known to contain porcine material. Results were obtained with the so-called 3-fold diluted DNA extracts. Five µl of DNA extract were tested per PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

PCR method	**A**			**B**			**C**			**D**		
Species/sample	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
Pig, boar and samples containing porcine material												
Sus scrofa domesticus **(fresh blood sample)**	29.31 29.32	29.32	+	23.29 23.30	23.30	+	22.97 22.99	22.98	+	24.44 24.44	24.44	+
Sus scrofa scrofa **(animal 1) (fresh blood sample)**	29.70 29.58	29.64	+	20.88 22.15	21.52*	+	22.29 22.23	22.26	+	23.59 23.26	23.43	+
Sus scrofa scrofa **(animal 2) (fresh blood sample)**	28.27 28.24	28.26	+	21.17 21.19	21.18	+	21.20 21.17	21.19	+	22.71 22.67	22.69	+
Sus scrofa scrofa **(animal 3) (fresh blood sample)**	28.38 28.34	28.36	+	22.01 22.01	22.01	+	21.99 21.89	21.94	+	23.50 23.37	23.44	+
0.1 % (w/w) PAP extract 1	39.38 39.04	39.21	+	36.92 36.96	36.94	+	34.31 34.33	34.32	+	36.17 36.52	36.35	+
0.1 % (w/w) PAP extract 2	39.62 50	44.81*	?	35.51 35.01	35.26	+	33.53 33.59	33.56	+	35.22 35.22	35.22	+
0.025 % (w/w) PAP extract 1	50 50	50	-	36.69 37.36	37.03*	+	36.12 36.21	36.17	+	37.56 37.26	37.41	+
0.025 % (w/w) PAP extract 2	50 50	50	-	50 35.80	42.90*	?	35.12 34.60	34.86*	+	36.10 36.11	36.11	+
CRL 214 extract 1	39.64 50	44.82*	?	34.80 34.92	34.86	+	31.58 31.35	31.47	+	32.19 32.20	32.20	+
CRL 214 extract 2	41.62 41.18	41.40	+	36.31 37.10	36.71*	+	32.21 32.16	32.19	+	33.12 33.17	33.15	+
CRL 558 extract 1	26.93 26.99	26.96	+	19.33 19.30	19.32	+	17.94 17.97	17.96	+	19.76 19.76	19.76	+
CRL 558 extract 2	26.78 26.70	26.74	+	19.23 19.27	19.25	+	17.78 17.76	17.77	+	19.68 19.72	19.70	+
CRL 1370 extract 1	36.06 35.81	35.94	+	28.86 29.09	28.98	+	28.54 27.93	28.24	+	30.00 30.17	30.09	+
CRL 1370 extract 2	36.04 35.93	35.99	+	28.81 28.76	28.79	+	28.18 28.17	28.18	+	29.78 29.81	29.80	+
CRL 1374 extract 1	33.74 33.98	33.86	+	26.89 26.87	26.88	+	26.42 26.14	26.28	+	27.95 27.98	27.97	+
CRL 1374 extract 2	33.98 33.98	33.98	+	26.70 26.92	26.81	+	25.87 25.71	25.79	+	27.36 27.39	27.38	+
CRL 1445 extract 1	38.80 38.19	38.50*	+	36.27 37.24	36.76*	+	32.26 32.45	32.36	+	34.82 34.59	34.71	+
CRL 1445 extract 2	39.69 38.14	38.92*	+	37.35 36.95	37.15	+	33.06 34.49	33.78	+	35.31 35.20	35.26	+
CRL 1647 extract 1	33.10 33.52	33.31	+	27.16 27.22	27.19	+	26.80 26.99	26.90	+	28.61 28.72	28.67	+
CRL 1647 extract 2	33.54 33.57	33.56	+	27.52 27.54	27.53	+	26.84 26.91	26.88	+	28.91 28.71	28.81	+

* > 0.5 cycle between replicates of the same extract
? ambiguous results between PCR replicates

The 4 targets tested showed clear amplification signals with pure pig DNA (*Sus scrofa domesticus*) but also with pure wild boar DNA (*Sus scrofa scrofa*) but this lack of distinction of wild boar from pig is not at all a problem.

All samples tested showed clear amplification signals with assays C and D. The PCR assay B is generally close to the results of the D method even though somewhat more variable (4 results for which replicates differ by more than 0.5 cycle in between them). However, at lowest level the Ct is somewhat later with the B method than with the D method. The target A gives later signals than the other targets tested with even a loss of signal for the samples at lowest levels (0.1 % and 0.025 % w/w PAP as well as for one porcine PAP CRL 214 processed at 133 °C). The sensitivity will be developed in point 2.3.

The specificity test continued with DNA samples from non-target species. Different relevant categories were investigated: terrestrial mammalians, domestic birds, fish species and sea mammals. For the terrestrial mammalians, cattle – *Bos taurus* –, sheep – *Ovis aries* – and goat – *Capra hircus* –, were of course tested but also game species (deer – *Cervus elaphus* – and roe deer – *Capreolus capreolus*). Horse – *Equus caballus* – and donkey – *Equus asinus* – used in butcher's products as well as rodents such as hare – *Lepus europaeus* – and rat – *Rattus rattus* – were considered. The possibility of a cross-reaction with human – *Homo sapiens* – DNA was also considered. For the domestic birds, poultry species such as chicken – *Gallus gallus* –, turkey – *Meleagris gallopavo* –, Muscovy duck – *Cairina moschata* –, guinea fowl – *Numida meleagris* – and goose – *Anser spp.* – were tested. Next to this, other bird species of interest were analysed such as : quail – *Coturnix japonica* –, pheasant – *Phasianus colchicus* –, pigeon – *Columba livia* –, and ostrich – *Struthio camelus*. Different fish species potentially present in fish meals and some sea mammals present in the EURL-AP sample bank were also tested. Tables 3, 4 and 5 summarize the results obtained on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.).

Table 3: *Specificity test of the 4 pig PCR assays on non-target material consisting of terrestrial mammals, sea mammals and domestic birds. The assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng / µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

PCR Method	**A**			**B**			**C**			**D**		
Species	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
Other terrestrial mammalians												
Homo sapiens	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Bos taurus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Ovis aries	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Capra hircus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Cervus elaphus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Capreolus capreolus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Equus asinus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Equus caballus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Lepus europaeus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Rattus rattus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Domestic birds												
Gallus gallus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Meleagris gallopavo	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Numida meleagris	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Cairina moschata	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Anser spp	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Coturnix japonica	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Phasianus colchicus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Columba livia	50 50	50	-	50 50	50	-	39.21 50	44.61*	-**	50 50	50	-
Struthio camelus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Sea mammals												
Stenella coeruleoalba	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Tursiops truncatus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Grampus griseus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Ziphius cavirostris	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Phocoena phocoena	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Phocidae	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-

* > 0.5 cycle between replicates of the same extract

** ambiguous results also during a second analysis, therefore the final result is reported as negative

The 4 pig PCR assays tested gave the same results. No aspecifity was detected with the different animal species tested considering the overall results. However, at individual results level, the method C interacted with pigeon DNA.

Table 4: *Specificity test of 4 pig PCR assays with non-target material consisting of fish and crustacean species. The assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng / µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

PCR Method	**A**			**B**			**C**			**D**		
Species	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
Fish												
Gadus morhua	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Pollachius virens	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Melanogrammus aeglefinus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Micromesistius poutassou	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Sebastes spp.	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Mallotus villosus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Scomber scombrus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Clupea harengus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Merluccius merluccius	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Trachurus trachurus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Trisopterus minutus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Sardina pilchardus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Engraulis encrasicolus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Gadus ogac	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Trisopterus esmarki	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Ammodytes lancea	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Sprattus sprattus	50 50	50	-	39.46 50	44.73*	-**	50 50	50	-	50 50	50	-
Salmo salar	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Raja spp	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Crustacean												
Paralithodes camtschaticus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-

* > 0.5 cycle between replicates of the same extract

** ambiguous results also during a second analysis, therefore the final result is reported as negative

The overall results obtained show that no aspecificity was detected with fish species and crab (*Paralithodes camtschaticus)*. However, at individual results level, the PCR method B interacted with *Sprattus sprattus* DNA.

To complete the evaluation of the specificity of the assay, plant samples were also tested.

Table 5: *Tests of specificity of 4 pig PCR assays with non-target material consisting of plant material. The assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng / µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

PCR Method	**A**			**B**			**C**			**D**		
Species	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
Plant species												
Glycine max	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Zea mays	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Brassica napus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Triticum aestivum	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Oryza sativa	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Lycopersicon esculentum	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Beta vulgaris	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-

The 4 pig PCR assays gave the same results. No aspecifity was detected with the different plant species tested.

2.3. Sensitivity of the 4 pig PCR methods

In order to be able to quickly make a choice between the 4 pig PCR assays, a first test of sensitivity was carried out. The sensitivity of the test was checked with feed samples containing porcine material at a known level (in mass fraction). The different porcine PAP used in mixes are identified with a number between brackets. Table 6 summarises the results obtained on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) on 3-fold diluted DNA extracts. The 30-fold dilutions were also tested but are not shown as they do not improve the results.

Table 6: *Sensitivity test of 4 pig PCR assays with samples containing porcine material at a known level (in mass fraction). The assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Five µl of 3-fold diluted DNA extract were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

PCR Method		**A**			**B**			**C**			**D**		
Sample number and composition	N° extract	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
CRL 1338 **1 % porcine PAP (1) + 99 % soya (w/w)**	1	50 50	50	-	38.83 39.11	38.97	+	38.41 36.05	37.23*	+	37.43 37.54	37.49	+
	2	38.83 50	44.42*	?	39.45 38.70	39.08*	+	37.01 37.32	37.17	+	37.85 38.12	37.99	+
CRL 1337 **0.2 % porcine PAP (1) + 99.8 % soya (w/w)**	1	50 50	50	-	50 50	50	-	39.86 39.72	39.79	+	40.04 39.86	39.95	+
	2	41.08 50	45.54*	?	50 50	50	-	40.73 38.15	39.44*	+	39.43 41.28	40.36*	+
CRL 1362 **1 % porcine PAP (2) + 99 % soya (w/w)**	1	50 44.31	47.16*	?	38.32 37.29	37.81*	+	34.50 34.99	34.75	+	35.63 36.09	35.86	+
	2	39.51 39.52	39.52	+	37.02 36.33	36.68*	+	34.95 34.67	34.81	+	35.72 35.42	35.57	+
CRL 1363 **0.2 % porcine PAP (2) + 99.8 % soya (w/w)**	1	50 50	50	-	39.54 50	44.77*	?	38.24 38.56	38.40	+	37.78 38.09	37.94	+
	2	50 50	50	-	39.44 50	44.72*	?	38.33 39.55	38.94*	+	38.74 39.29	39.02*	+
CRL 1349 **0.1 % porcine PAP (2) + 99.9 % blank feed (w/w)**	1	39.91 50	44.96*	?	36.54 35.62	36.08*	+	32.76 33.05	32.91	+	34.49 34.85	34.67	+
	2	50 50	50	-	39.33 38.91	39.12	+	34.69 34.93	34.81	+	36.62 36.96	36.79	+
CRL 1380 **0.2 % porcine haemoglobin powder + 99.8 % soya (w/w)**	1	39.68 37.29	38.49*	+	34.71 34.23	34.47	+	34.31 34.64	34.48	+	35.89 36.20	36.05	+
	2	36.68 36.91	36.80	+	34.48 32.17	33.33*	+	33.79 33.71	33.75	+	35.81 36.64	36.23*	+

* > 0.5 cycle between replicates of the same extract
? ambiguous results between PCR replicates

The gathered results in Table 2 and Table 6 show that target A is less sensitive. To confirm these results, reagents (primers and probe ordered by EURL-AP) and three extracts of DNA were sent to Lab A having developed the method (two extracts of porcine DNA at 2 ng / µl and at 20 pg / µl and one extract of bovine DNA at 2 ng / µl). It carried out a comparison with its reagents. The results are presented in Table 7.

Table 7: *Comparison between test results obtained by Lab A on its ABI7700 (Applied Biosystems) with either its own reagents (primers and probe) or those provided by the EURL-AP on a set of DNA samples.*

	Ct values obtained with the reagents ordered by	
Extracts	**EURL-AP**	**Lab A**
Porcine DNA at 2 ng/µl (EURL-AP)	27.6	29.2
Porcine DNA at 20 pg/µl (EURL-AP)	35.1	36.7
Bovine DNA at 2 ng/µl (EURL-AP)	> 40	> 40
0.1 % porcine (Lab A)	33.2	35.1
100 % porcine (Lab A)	22.1	23.3

The results show a slight Ct difference depending on which reagents were used. The Ct values obtained with the reagents ordered by EURL-AP were slightly earlier. This confirms that the results obtained by EURL-AP with the pig PCR method of Lab A are fine and are not underperformant due to the quality of the reagents (primer and probe). Consequently, this method was not further considered for evaluation.

Method B seems to be less sensitive than the methods C and D. In order to confirm that observation, some additional samples contaminated at low level with two other porcine PAPs were tested. Table 8 summarises the results obtained on the LC480 thermocycler (Roche Diagnostics Ltd.)

with the Universal Mastermix (Diagenode s.a.) on 3-fold diluted DNA extracts. The 30-fold dilutions were also tested but are not shown as it does not improve the results.

Table 8: *Sensitivity test of 3 pig targets with feed samples containing porcine material at low level (in mass fraction) performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Results obtained on extracts 3 fold diluted. Five µl of DNA extract were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

PCR Method		**B**			**C**			**D**		
Sample number and composition	N° extract	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
CRL 1625 **1 % porcine PAP (3)** **+ 99 % fish meal (w/w)**	1	34.64 34.82	34.73	+	33.21 33.20	33.21	+	34.80 34.64	34.72	+
	2	33.12 33.20	33.16	+	32.42 32.81	32.62	+	33.93 34.30	34.12	+
CRL 1627 **1 % porcine PAP (3)** **+ 99 % chicken PAP (w/w)**	1	33.85 33.89	33.87	+	33.08 33.12	33.10	+	34.21 34.15	34.18	+
	2	34.13 33.99	34.06	+	33.03 32.83	32.93	+	34.53 34.48	34.51	+
CRL 1637 **1 % porcine PAP (3)** **+ 99 % pig feed (w/w)**	1	30.07 30.22	30.15	+	28.54 28.43	28.49	+	30.12 30.29	30.21	+
	2	30.48 30.62	30.55	+	28.35 28.45	28.40	+	30.30 30.24	30.27	+
CRL 1730 **1 % porcine PAP (4)** **+ 99 % pig feed (w/w)**	1	50 50	50	-	36.37 35.91	36.14	+	37.80 37.82	37.81	+
	2	50 50	50	-	36.09 36.37	36.23	+	37.90 38.09	38.00	+

The additional analysis of samples containing 1 % (in mass fraction) of porcine PAP (4) confirms that the method B is less sensitive than the methods C and D.

The tests were continued with pig PCR assays C and D in order to choose one of them.

2.4. Specificity of the primers

An in-depth analysis of the specificity of the primers was carried out. To evaluate the importance of the primers and of the probe in the specificity of the test, two types of analysis were made. An *in silico* analysis based on sequences available from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) and a PCR analysis performed with the Universal Mastermix (Diagenode s.a.) containing SYBR® Green on the LC480 thermocycler (Roche Diagnostics Ltd.). At the end of the PCR with SYBR® Green the melting curves were performed to characterize the amplicons produced.

Item 2.4.1. presents the results obtained with the PCR assay C and the item 2.4.3. presents the results obtained with the PCR assay D.

2.4.1. Specificity of the primers of the PCR assay C

2.4.1.1. Analysis based on DNA sequences

Sequence alignments show that the specificity of the target C stems from the probe for a lot of species (Table 9).

Table 9: *In silico specificity test of the primers and probe from method C based on the alignment of mitochondrial DNA sequences. The sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank). On the Sus scrofa sequences, the primers are highlighted in green and the probe in blue. Mismatches within the so identified primer and probe regions are highlighted in pink. Fully conserved primers are highlighted in green while the legend below explains what characterizes regions highlighted in yellow or in grey.*

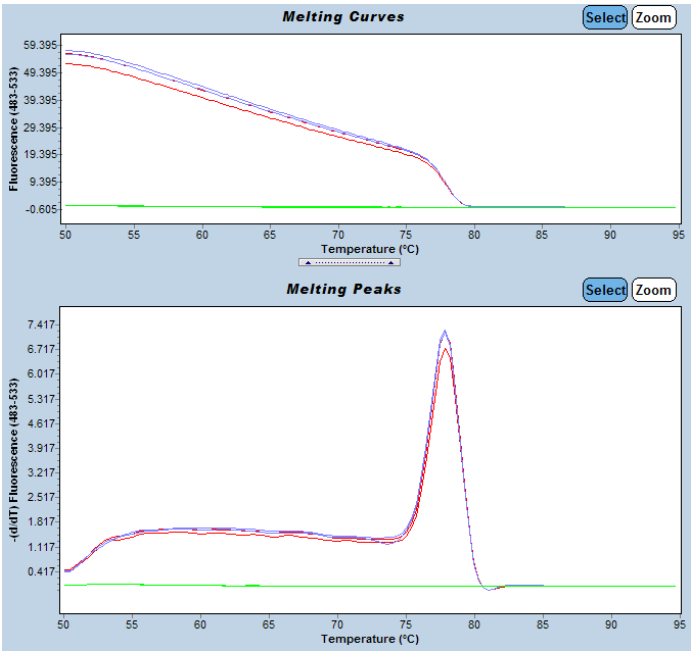
NCBI Accession number	Species	Mitochondrial DNA sequence from NCBI web site
Comparison between pig DNA sequence and terrestrial mammalian DNA sequences		
NC_012095.1	*Sus scrofa domesticus*	AGCACTAACCTTTTAAGTTAGAGATCGGGAGCCTAAATCT--CCCCT-CAATGGTATGCCACAACTAGATA
KP301137.1	*Sus scrofa scrofa*	AGCACTAACCTTTTAAGTTAGAGATCGGGAGCCTAAATCT--CCCCT-CAATGGTATGCCACAACTAGATA
NC_006853	*Bos taurus*	AGCACTAACCTTTTAAGTTAGAGATTGAGAGCCATATACT--CTCCT-TGGTGACATGCCGCAACTAGACA
NC_001941.1	*Ovis aries*	AGCGTTAACCTTTTAAGTTAAAGACTGAGAATATTATATT--CTCCT-TGATGATATGCCACAACTAGACA
NC_005044.1	*Capra hircus*	AGCGTTAACCTTTTAAGTTAAAGACCGAGAGCATAATACT--CTCCT-TGATGATATGCCACAACTAGACA
AB245427.2	*Cervus elaphus*	AGCGCTAGCCTTTTAAGCTAGAAATTGAGAGTATAATGCT--CTCCT-TAATGGAATGCCACAACTAGACA
JN632610.1	*Capreolus capreolus*	AGCACTAGCCTTTTAAGCTAGAGACTGAGAGCAATTAACT--CTCCT-TAATGATATGCCACAACTAGATA
NC_001788.1	*Equus asinus*	AGCGTTAACCTTTTAAGTTAAAGACTGAGGGTTCAATCTC--CCTCCCTAGTGATATGCCACAGTTGGATA
NC_001640.1	*Equus caballus*	AGCATTAACCTTTTAAGTTAAAGATTGAGGGTTCAACC-C--CCTCCCTAGTGATATGCCACAGTTGGATA
NC_004028.1	*Lepus europaeus*	AGCGCTAGCCTTTTAAGCTAGAGAATGAGAGCTAAGTAACACTCTCCATAATGGAATGCCACAACTTGACA
emb_AJ001588.1	*Oryctolagus cuniculus*	AGCGCTAGCCTTTTAAGCTAGAGAGTGAGAGTTAAATAGT-CTCTCCATAGTGAAATGCCACAACTTGACA
NC_012374.1	*Rattus rattus*	AGCGTTAACCTTTTAAGTTAAAGTTAGAGACCACAAATCT--CCAC--AATGGTATGCCACAACTAGACA
NC_001665.2	*Rattus norvegicus*	AGCGTTAACCTTTTAAGTTAAAGTTAGAGACAACAAATCT--CCAC--AATGACATGCCACAACTAGACA
Comparison between pig DNA sequence and domestic bird DNA sequences		
NC_012095.1	*Sus scrofa domesticus*	AGCACTAACCTTTTAAGTTAGAGATCGGGAGC-CTAAATCTCCCCTCAATGGTATGCCACAACTAGATA
NC_001323.1	*Gallus gallus*	AGCACTAGCCTTTTAAGCTAGAGAGAGGGGAC-ACCC-TC-CCCCTTAATGACATGCCCCAATTAAACC
NC_010195.1	*Meleagris gallopavo*	AGCACTAGCCTTTTAAGCTAGAGAAAGAGGAA-ACCT-TC-CTCCTTAATGATATGCCCCAACTAAACC
NC_006382.1	*Numida meleagris*	AGCACTAGCCTTTTAAGCTAGAGAAAGAGGAC-ACACTCCCTCCTTAATGACATGCCTCAACTAAACC
NC_009684.1	*Anas_platyrhynchos*	AGCACTAGCCTTTTAAGCTAGCTAAAGAGGAA-TTAT-CCCTCCTTAATGGCATGCCTCAACTCAACC
NC_010965.1	*Cairina moschata*	AGCACTAGCCTTTTAAGCTAGCTAAAGAGGAG-TCACTCC-CTCCTTAATGATATGCCCCAGCTCAACC
NC_004539.1	*Anser albifrons*	AGCACTAGCCTTTTAAGCTAGCCAAAGAGGGA-CTTC-CCCTCCTTAATGATATGCCACAGCTCAACC
NC_011196.1	*Anser anser*	AGCACTAGCCTTTTAAGCTAGCCAAAGAGGGA-CTTC-CCCTCCTTAATGATATGCCACAGCTTAACC
NC_003408	*Coturnix japonica*	AGCACTAGCCTTTTAAGCTAGAGAGAGGGGAT-GCCC-TC-CCCCTTAATGATATGCCACAACTAAACC
FJ752430.1	*Phasianus colchicus*	AGCACTAGCCTTTTAAGCTAGAGAAAGAGGAA-ATTA--CCCTCCTTAATGATATGCCCCAATTAAACC
NC_013978.1	*Columba livia*	AGCGCTAGCCTTTTAAGCTAGAGAAAGAGGATTGCCCGCCCCTCCTTAATGACATGCCCCAGCTCAATC
AF338715.1	*Struthio camelus*	AGCACTAGCCTTTTAAGCTAGAGAAAGAGGAA--TA-TCCCCTCCTTAATGACATGCCCCAGCTTAACC
Comparison between pig DNA sequence and sea mammal DNA sequences		
NC_012095.1	*Sus scrofa domesticus*	AGCACTAACCTTTTAAGTTAGAGATCGGGAGCCTA--AATCTCCCCTCAATGGTATGCCACAACTAGATA
NC_012053.1	*Stenella coeruleoalba*	AGCGTTAACCTTTTAAGTTAAAGATTGAGAGTAAT--AAACTCCCCTTAGTGATATGCCACAACTAGATA
NC_012059.1	*Tursiops truncatus*	AGCGTTAACCTTTTAAGTTAAAGATTGAGAGTTAT--AAACTCCCCTTAGTGATATGCCACAACTAGATA
NC_012062.1	*Grampus griseus*	AGCGTTAACCTTTTAAGTTAAAAATTGGGAATTAT--AAACTCCCCTTAGTGGCATGCCACAACTAGATA
KC776710.1	*Ziphius cavirostris*	AGCATTAACCTTTTAAGTTAAAGATTGAAAGCCCA--AA-CTTTCCTTAATGGTATGCCACAACTAGATA
NC_005280.1	*Phocoena phocoena*	AGCGTTAACCTTTTAAGTTAAAGATTGGGAGCTAC--ACATTCCCCTTAATGACATGCCACAACTAGACA
NC_008433.1	*Phoca hispida*	AGCATTAACCTTTTAAGTTAAAGACTGAGAGTACTCTAACCTCTCCTTAATGAAATGCCACAGTTAGATA
NC_001325.1	*Phoca_vitulina*	AGCATTAACCTTTTAAGTTAAAGACTGAGAGTCCTCTAATCTCTCCTTAATGAAATGCCACAGTTAGATA
NC_001602.1	*Halichoerus grypus*	AGCGTTAACCTTTTAAGTTAAAGACTGAGAGTACTCTAACCTCTCCTTAATGAAATGCCACAGTTAGATA
Comparison between pig DNA sequence and fish species DNA sequences		
NC_012095.1	*Sus scrofa domesticus*	AGCACTAACCTTTTAAGTTAGAGATCGGGAGCCTAAATCTCCCCTCAATGGTATGCCACAACTAGATA
NC_001960.1	*Salmo salar*	AGCGTTAGCCTTTTAAGCTAAAGATTGGTGGTCCCCAACCACCCCTTAGTGACATGCCCCAACTCAACC
NC_009581.1	*Engraulis encrasicolus*	AGCGTCAGCCTTTTAAGCTGAAGTTTGGTGACTCCGCCCCACCTCTGGTGACATGCCTCAATTAAACC
NC_009592.1	*Sardina pilchardus*	AGCGTCAGCCTTTTAAGCTGAAGATTGGTGACCCCCAACCACCTCTTAGTGACATGCCCCAATTGAATC
NC_012323.1	*Gadus ogac*	AGCACCAGCCTTTTAAGCTGGAAGCAGGTGACTCCCAACCACCCTTAATGAAATGCCCCAGTTAAACC
NC_007396.1	*Melanogrammus aeglefinus*	AGCACCAGCCTTTTAAGCTGGAAGTAGGTGATTCCCAACCACCCTTAATGAAATGCCCCAGTTAAACC
Legend :	In pink : different base compared to the sequences of primers and probe from method C In blue : same sequence as the one of the probe from method C In green : same sequence as one of the primers from method C In yellow : primer sequence highlighted because of a single sequence difference (highlighted in pink) compared to one of method C primers In grey : primer sequence highlighted because of only 2 sequence differences (highlighted in pink) compared to one of method C primers	

2.4.1.2. PCR analysis performed with SYBR® Green

Table 10 and Figure 1 present the results obtained with DNAs extracted from *Sus scrofa domesticus* and *Sus scrofa scrofa* blood samples and from feed samples containing porcine material (for more details on these samples see § 2.2.).

Table 10: *Specificity tests of the primers from method C carried out with samples containing porcine DNA. The PCR assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) in SYBR® Green format. DNA from fresh blood samples was extracted with the kit Qiagen Genomic DNA tip 20/G (Qiagen, Venlo, The Netherlands). Pure species DNA extracts were at 2 ng / µl. Meal samples were extracted with the kit Magnetic DNA Purification System for Food (Promega, Madison, WI, USA). The CRL samples tested are known to contain porcine material. Results were obtained with 3-fold diluted DNA extracts. Five µl of DNA extract were tested in each PCR. Analysis mode: Abs. quant/second derivative max, high confidence and Tm calling. Description of the feed samples is to be found in § 2.2.*

Species/sample	C_t	Mean C_t	Tm 1	Result	**Species/sample**	C_t	Mean C_t	Tm 1	Result
Pig, boar and samples containing porcine material									
Sus scrofa domesticus **(fresh blood sample)**	21.86 21.84	21.85	77.88 77.88	+	**CRL 558 extract 1**	16.77 16.83	16.80	77.73 77.73	+
Sus scrofa scrofa **(animal 1) (fresh blood sample)**	20.91 20.86	20.89	77.88 77.88	+	**CRL 558 extract 2**	16.40 16.34	16.37	77.73 77.73	+
Sus scrofa scrofa **(animal 2) (fresh blood sample)**	20.08 20.01	20.05	77.88 77.88	+	**CRL 1370 extract 1**	27.22 27.00	27.11	77.81 77.81	+
Sus scrofa scrofa **(animal 3) (fresh blood sample)**	20.71 20.69	20.70	77.81 77.88	+	**CRL 1370 extract 2**	26.80 26.83	26.82	77.75 77.75	+
0.1 % (w/w) PAP extract 1	32.78 32.17	32.48	77.63 77.32	+	**CRL 1374 extract 1**	25.09 25.04	25.07	77.75 77.75	+
0.1 % (w/w) PAP extract 2	32.29 32.41	32.35	77.12 77.40	+	**CRL 1374 extract 2**	24.54 24.56	24.55	77.75 77.75	+
0.025 % (w/w) PAP extract 1	35.75 36.20	35.98	77.79 77.79	+	**CRL 1445 extract 1**	31.15 31.15	31.15	77.96 77.81	+
0.025 % (w/w) PAP extract 2	33.08 33.15	33.12	77.40 77.66	+	**CRL 1445 extract 2**	31.76 31.67	31.72	77.79 77.79	+
CRL 214 extract 1	30.26 30.11	30.19	77.59 77.81	+	**CRL 1647 extract 1**	25.31 25.30	25.31	77.83 77.83	+
CRL 214 extract 2	30.71 30.78	30.75	77.42 77.46	+	**CRL 1647 extract 2**	25.56 25.56	25.56	77.84 77.84	+
H_2O (No template control)									
H2O	50 50 50 50	/	/ / / /	-					



Color	Name	Tm1
	Sus scrofa domesticus	77,88
	Sus scrofa domesticus	77,88
	Sus scrofa scrofa animal1	77,88
	Sus scrofa scrofa animal1	77,88
	H2O	
	H2O	
	H2O	
	H2O	

Figure 1: *Melting curves obtained with the primers from method C on DNAs extracted from Sus scrofa domesticus and Sus scrofa scrofa blood samples (5 µl of DNA extracts at 2 ng / µl) and with no template control (5 µl of H_2O). PCR assays were performed on the LC480 thermocycler (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.*

Melting curves of *Sus scrofa domesticus* and *Sus scrofa scrofa* show the same characteristics. The melting curves present a well-defined peak with a maximum close to 78 °C.

The PCR negative controls do not give any amplification.

The same test was performed on DNA from non-target animal species. Table 11 summarises the results.

Table 11: *Specificity tests of the primers from method C carried out against non-target DNA samples. The PCR assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) 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	**Species**	C_t	Mean C_t	Tm 1	Tm 2
Other terrestrial mammalians								
Homo sapiens	29.23 29.16	29.20	77.17 77.17	***Capreolus capreolus***	25.92 25.92	25.92	76.43 76.42	/ /
Bos taurus	22.69 22.49	22.59	77.11 77.11	***Equus asinus***	30.71 30.68	30.70	76.84 76.84	/ /
Ovis aries	28.88 28.91	28.90	73.62 73.62	***Equus caballus***	29.53 29.56	29.55	77.12 77.13	/ /
Capra hircus	28.39 28.57	28.48	76.03 76.11	***Lepus europaeus***	26.34 26.37	26.36	76.79 76.79	/ /
Cervus elaphus	26.15 26.19	26.17	76.41 76.41	***Rattus rattus***	24.23 24.23	24.23	75.77 75.98	/ /
Domestic birds								
Gallus gallus	37.71 37.09	37.40	73.03 73.62	***Coturnix japonica***	26.55 26.57	26.56	78.31 78.27	/ /
Meleagris gallopavo	30.90 30.81	30.86	74.00 74.00	***Phasianus colchicus***	34.11 34.83	34.47	73.23 73.35	/ /
Numida meleagris	33.23 33.69	33.46	78.28 76.09	***Columba livia***	32.71 32.61	32.66	79.90 79.90	/ /
Cairina moschata	33.88 34.09	33.99	77.54 77.54	***Struthio camelus***	36.74 36.62	36.68	73.61 73.62	/ 78.74
Anser spp.	30.09 30.08	30.09	78.20 78.20					
Sea mammals								
Stenella coeruleoalba	25.33 25.42	25.38	74.62 74.52	***Ziphius cavirostris***	24.91 25.07	24.99	75.08 75.13	/ /
Tursiops truncatus	23.95 23.89	23.92	75.00 74.98	***Phocoena phocoena***	23.10 23.08	23.09	76.58 76.58	/ /
Grampus griseus	25.05 24.86	24.96	75.90 75.81	***Phocidae***	29.78 29.79	29.79	76.47 76.47	/ /
Fish								
Gadus morhua	44.05 41.83	42.94	59.37 60.55	***Trisopterus minutus***	40.97 41.05	41.01	80.54 74.94	/ /
Pollachius virens	45 45	45	76.33 77.44	***Sardina pilchardus***	36.99 36.80	36.90	77.76 /	/ /
Melanogrammus aeglefinus	45 45	45	/ /	***Engraulis encrasicolus***	29.30 29.06	29.18	85.84 85.84	/ /
Micromesistius poutassou	41.68 40.27	40.98	78.36 60.18	***Gadus ogac***	42.29 42.14	42.22	/ 73.20	/ /
Sebastes spp.	39.17 37.85	38.51	60.78 74.65	***Trisopterus esmarki***	39.99 39.70	39.85	/ 76.37	/ /
Mallotus villosus	39.70 38.36	39.03	76.55 76.42	***Ammodytes lancea***	38.05 37.68	37.87	73.77 73.77	/ /
Scomber scombrus	39.84 41.49	40.67	57.62 79.26	***Sprattus sprattus***	41.77 37.54	39.66	/ 77.76	/ /
Clupea harengus	37.80 37.72	37.76	78.75 78.76	***Salmo salar***	35.77 35.82	35.80	70.09 70.09	/ /
Merluccius merluccius	39.56 41.06	40.31	57.01 58.15	***Raja spp.***	40.60 42.50	41.55	78.40 79.91	/ /
Trachurus trachurus	38.61 39.67	39.14	60.16 /					
Crustacean								
Paralithodes camtschaticus	50 50	50	/ /					

Various species tested gave an early amplification signal even though results of Table 10 show that the PCR assay does not yield primer dimers. It is especially true for the terrestrial mammalians with the earliest mean Ct value for the *Bos taurus* (22.59). Early signals also appear for some domestic birds (*Coturnix japonica*, *Anser spp.* and *Meleagris gallopavo*) for the sea mammals.

With regard to the melting curves, most of the curves obtained with DNAs extracted from species other than *Sus scrofa domesticus* and *Sus scrofa scrofa* show a profile that differs from that obtained with pig DNA.

Four terrestrial mammalians (*Homo sapiens, Bos taurus, Equus asinus* and *Equus caballus)* gave melting curves close to these obtained on *Sus scrofa domesticus* (Figure 2).

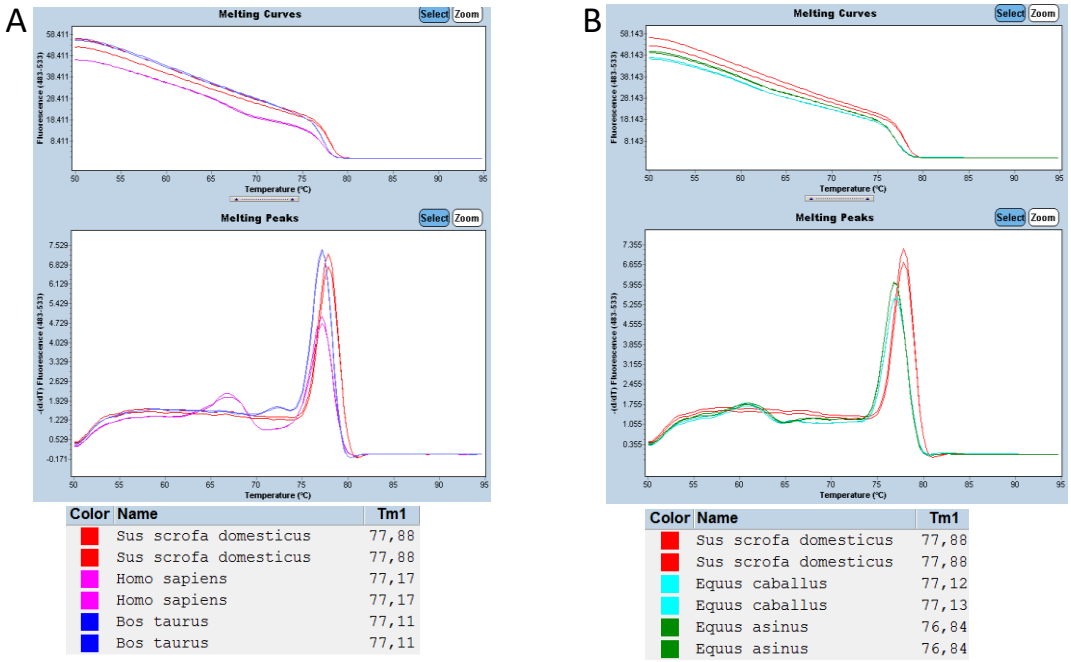


Figure 2: *A. Melting curves obtained with DNAs extracted from Sus scrofa domesticus blood (red curves) and melting curves obtained with DNAs extracted from Homo sapiens blood (pink curves) and Bos taurus (blue curves) blood samples that result in a Tm peak close to the one obtained with Sus scrofa domesticus.*
B. Melting curves obtained with DNAs extracted from Sus scrofa domesticus blood (red curves) and melting curves obtained with DNAs extracted from Equus asinus (green curves) and Equus caballus (light blue curves) blood samples that result in a Tm close to the one obtained with Sus scrofa domesticus.
A and B. The PCR was performed with primers from method C on the LC480 thermocycler (Roche Diagnostics Ltd.) using 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: Tm calling.

Three domestic birds (*Cairina moschata*, *Anser spp.* and *Coturnix japonica*) deliver melting curves with a Tm that is very similar to the one obtained on *Sus scrofa domesticus* (see also Figure 3).

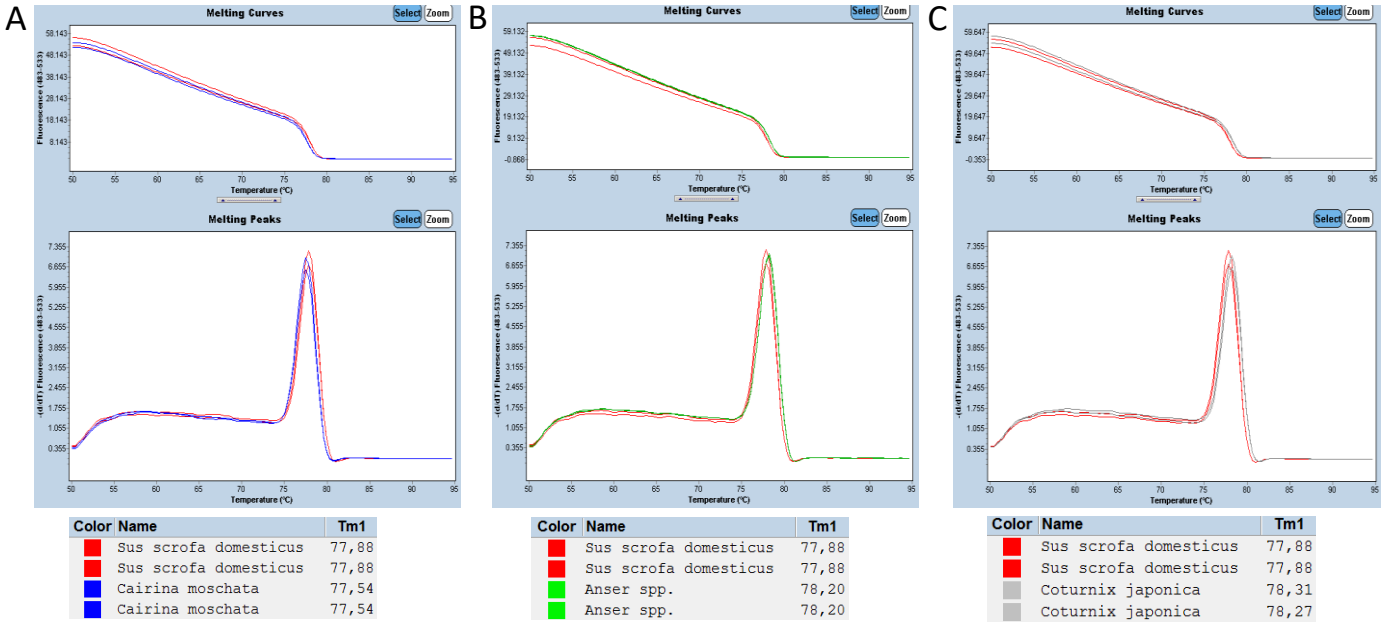


Figure 3: *A. Melting curves obtained with DNAs extracted from Sus scrofa domesticus blood sample (red curves) and with DNAs extracted from Cairina moschata blood sample (blue curves).*
B. Melting curves obtained with DNAs extracted from Sus scrofa domesticus blood sample (red curves) and with DNAs extracted from Anser spp. blood sample (green curves).
C. Melting curves obtained with DNAs extracted from Sus scrofa domesticus blood sample (red curves) and with DNAs extracted from Coturnix japonica blood sample (gray curves).
A, B and C. The PCR was performed in SYBR® Green format with the primers from method C on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng / µl and 5 µl were tested in each PCR. Analysis mode: Tm calling.

The results were completed with tests performed on plant samples. They are presented in Table 12.

Table 12: *Specificity tests of primers from method C performed with plant samples on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) containing SYBR green. 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	**Species**	C_t	Mean C_t	Tm 1
Plant species							
Glycine max	37.43 38.70	38.07	72.49 73.14	***Oryza sativa***	41.20 41.49	41.35	75.18 76.36
Zea mays	39.04 39.35	39.20	80.22 80.22	***Lycopersicon esculentum***	38.09 37.18	37.64	72.50 72.50
Brassica napus	37.00 36.98	36.99	71.43 71.43	***Beta vulgaris***	40.32 40.05	40.19	/ /
Triticum aestivum	34.72 34.92	34.82	71.86 71.86				

The melting curves obtained with DNAs extracted from plant species result in a Tm that is different from the one obtained with *Sus scrofa domesticus.*

2.4.2. Overall conclusion about the specificity of method C

The specificity of the PCR assay C comes from the probe. Therefore, competition problems with DNA from non-target species must be expected with this PCR assay.

2.4.3. Specificity of the primers of PCR assay D

2.4.3.1. Analysis based on DNA sequences

Sequences alignments show that the specificity of the PCR assay D springs from the primers even though the probe also contributes to provide it (Table 13).

Table 13: *In silico specificity tests of the primers and probe from method D based on the alignment of mitochondrial DNA sequences. The sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank). On the Sus scrofa sequences, the primers from method D are highlighted in green and probe in blue. Mismatches within the so identified primer and probe regions are highlighted in pink.*

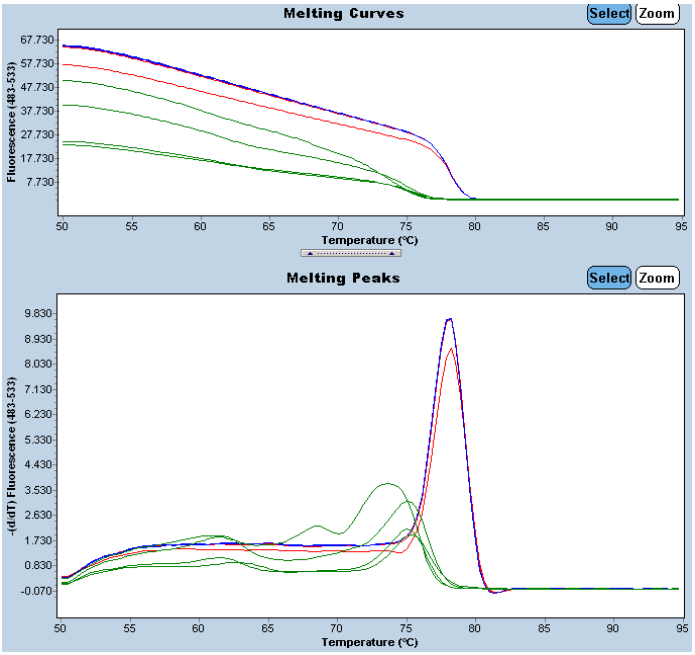
NCBI Accession number	Species	Mitochondrial DNA sequence from NCBI web site
Comparison between pig DNA sequence and terrestrial mammalian DNA sequences		
NC_012095.1	*Sus scrofa domesticus*	ACAACATAATCTGAATCAATGCAACAGTACATAGTCTCCTCATTAGCCTGATCAGTCTATCCCTACTAAACCAACTAGGCGAA
KP301137.1	*Sus scrofa scrofa*	ACAACATAATCTGAATCAATGCAACAGTACATAGTCTCCTCATTAGCCTGATCAGTCTATCCCTACTAAACCAACTAGGCGAA
NC_006853	*Bos taurus*	ATAATATAATTTGGGTTAACTCCACAGCACACAGCCTTCTAATTAGCTTTACAAGCCTCCTCCTCATAAACCAGTTTGGCGAC
NC_001941.1	*Ovis aries*	ATAGCATAATCTGAATCAACACCACACTTCACAGCTTGCTAATCAGCCTCACAAGCCTTCTCCTCCTAAATCAATTCGGTGAT
NC_005044.1	*Capra hircus*	ATAACATAATCTGAATTAACTCCACACTTCATAGCCTACTAATTAGCTTCACAAGCCTACTCCTTATAAACCAATTCGGCGAT
AB245427.2	*Cervus elaphus*	GCAATATAATTTGAATTAATTCTACAACCCATAGCCTATTAATTAGCCTCACAAGCCTTCTCCTTATAAATCAATTCAATGAC
JN632610.1	*Capreolus capreolus*	GTACCATAATCTGAATTAACTCCACAATTCACAGCCTAGTGATTAGTCTCACAAGCCTTCTTCTTATAAATCAGTTCAATGAC
NC_001788.1	*Equus asinus*	AGAACATAATCTGAATTAACACCACAACCTACAGCCTATTAATCAGCCTTATCAGCTTATCCCTCCTAAACCAGCCTAACAAC
NC_001640.1	*Equus caballus*	AGAATATAATCTGAATCAACACTACAACCTATAGTCTATTAATCAGCCTTATCAGCCTATCCCTCCTAAACCAACCTAGCAAC
NC_004028.1	*Lepus europaeus*	GTCGCATAATCTGAATCAACGCAACAGTTTACAGCCTACTAATTAGCCTAACTACTTTTCTTCTACTTTAATCAACCAAACGAC
AJ001588.1	*Oryctolagus cuniculus*	CCCATATAATTTGAATTAACGCAACAGTTTACAGCCTATTAATTAGCCTAACTACCCTCTCTCTACTTTAATCAGCCAAGTGAC
NC_012374.1	*Rattus rattus*	ACAAGAAAACCTGAACTAACGTCACCTCCTACAGCTTCCTAGTAAGCCTAGCAAGCTTATCGTTACTTTGACAAAACGACGAA
NC_001665.2	*Rattus norvegicus*	ACAAAAAAATCTGAACCAATGTCACCTCCTACAGCTTTCTAGTGAGCCTATTAAGCTTATCACTCCTATGACAAAATGACGAA
Comparison between pig DNA sequence and domestic bird DNA sequences		
NC_012095.1	*Sus scrofa domesticus*	ACAACATAATCTGAATCAATGCAACAGTACATAGTCTCCTCATTAGCCTGATCAGTCTATCCCTACTAAACCAACTAGGCGAA
NC_001323.1	*Gallus gallus*	CAAAATCCATATGAACTAACACCACACATATACAGCCTTCTAATCGCCTCGATCAGCCTACACTGACTAACCCCATCATACTAC
NC_010195.1	*Meleagris gallopavo*	CAAAATTCATATGAACTAACACCACAGCACATAGCCTTATAATTGCCCTAATCAGCTTACACTGATTAACCCCATCATACTAC
NC_006382.1	*Numida meleagris*	CCAAATTTTATGGACCAACACCACAGCATACAGCCTCCTAATTGCTTTAATCAGCCTACAATGACTTACCCCATCATACTAC
NC_009684.1	*Anas platyrhynchos*	CAAAATTCCTGTGAACTAATACTACCATGTATAGCCTGCTAATTGCCGCCCTCAGCCTCCAATGACTAATCCCGACCTACTAC
NC_010965.1	*Cairina moschata*	CAAAATTCCTGTGAACCAGCACCACCACATACAGCCTACTGATCGCCACCCTTAGCCTCCAATGACTGATCCCAACCTACTAC
NC_004539.1	*Anser albifrons*	CAAAATTTCTATGAACTAACACCACCCTATACAGCCTCCTAATCGCTGCCCTAAGCCTACAGTGATTAATCCCAACCTACTAC
NC_011196.1	*Anser anser*	CAAAATTTCTATGGACTAACACCACCTTATACAGCCTCCTAATCGCTGCCCTAAGCCTACAGTGATTAATCCCAACCTACTAC
NC_003408	*Coturnix japonica*	CAAAATCCATATGAACTAACACCACAACCATAGTCTCCTAATCGCCATAATCAGCCTACACTGACTAGCCCCATCCTACTAC
FJ752430.1	*Phasianus colchicus*	CAAAATCTATATGAACTAACACCACAGCCCACAGTCTTCTAATCGCTCTAATTAGCCTACACTGACTAACCCCATCATATTAC
NC_013978.1	*Columba livia*	AAAAATTCTTATGAACTAACACCACCACGTACAGCCTACTAATCGCCACCCTAAGCCTCCAGTGACTACTCCCCTCGCACTAC
AF338715.1	*Struthio camelus*	AAGCCTCACTATGGACAAATACCACCACCTACAGCCTCTTGATTGCCACCATCAGCCTTCAATGACTTAACCCAACATACTTC
Comparison between pig DNA sequence and sea mammal DNA sequences		
NC_012095.1	*Sus scrofa domesticus*	ACAACATAATCTGAATCAATGCAACAGTACATAGTCTCCTCATTAGCCTGATCAGTCTATCCCTACTAAACCAACTAGGCGAA
NC_012053.1	*Stenella coeruleoalba*	GCAACTTTATCTGAATCAATACTACAACCCATAGCTTATTAATTAGCTTTACAAGTTTACTCTTACTTAATCAATTCAACGAT
NC_012059.1	*Tursiops truncatus*	GCAACTTTATCTGAATCAATACTACAACTCATAGCTTATTAATTAGCTTTACAAGTTTACTCTTACTTAATCAATTTAACGAT
NC_012062.1	*Grampus griseus*	GTAATTATATCTGAATCAATACTACAACCCACAGTCTATTAATTAGTTTTACAAGTTTACTTCTACTTAACCAATTTAACGAT
KC776710.1	*Ziphius cavirostris*	GCAACTATATCTGAATTAACTCCACAGCCCACAGCCTACTAATTAGCTTTACAAGTCTCCTCCTCCTGAATCAATTCAACGAC
NC_005280.1	*Phocoena phocoena*	GCAGTTTTATTTGAATCAACACCACAGCTCACAGCCTATTAATTAGCTTCACAAGCCTACTTCTACTGAACCAATTTAACGAC
NC_008433.1	*Phoca hispida*	CTAACATAATCTGAATCAACACGACAGCCTATAGCCTACTAATGAGCCTTGTCAGCCTGTCCTTCTCTAAATCAACTCGGTGAC
NC_001325.1	*Phoca vitulina*	CTAACATGATCTGAATCAACACGACAGCCTATAGCCTACTAATGAGCCTTATCAGCCTATCCTTCTCTAAATCAACTCGGTGAC
NC_001602.1	*Halichoerus grypus*	CTAACATGATCTGAATCAACACAACAGCCTATAGCCTACTAATGAGCCTTATCAGTCTGTCCTTCTCTAAATCAACTCGGTGAC
Comparison between pig DNA sequence and fish species DNA sequences		
NC_012095.1	*Sus scrofa domesticus*	ACAACATAATCTGAATCAATGCAACAGTACATAGTCTCCTCATTAGCCTGATCAGTCTATCCCTACTAAACCAACTAGGCGAA
NC_001960.1	*Salmo salar*	CGAAATGAGCTCTGAACAACATCAATCGCACAAAGTTTAGTCATTGCCCTAGCAAGTTTATCCTGACTTAAATGA-TCGTCAGA
NC_009581.1	*Engraulis encrasicolus*	AGAAGTGACTATGAACTGCTACCACCGCACACAGCTTAATCATTGCGCTTCTGAGCTTTAGCTGATTAAACTGACCGTCTGAG
NC_009592.1	*Sardina pilchardus*	AGAAGTGGGTTTGGGCCTCAGTTGTCTCCCACAGCCTGATCATCGCCTCTCTGAGCCTAACCTGACTTAATTGGTCGGGGAA
NC_012323.1	*Gadus ogac*	GTAAATGACTATGACCTACTGCTCTAGCGCAAAGCATGTTGATTGCCTTGGGCAGTATTACCTGACTAAATAACACCACCGAT
NC_007396.1	*Melanogrammus aeglefinus*	GCAAGTGACTATGACCCACTGCTCTAACGCAAAGCATACTAATTGCCTTGGGCAGTATCACTTGATTGAATAATACTACCGAT
Legend :	In pink : different base compared to the sequences of primers and probe from method D In blue : same sequence that the pig for the probe from method D In green : same sequence that the pig for the primers from method D	

2.4.3.2. PCR analysis performed with SYBR® Green

Table 14 and Figure 4 present the results obtained with DNAs extracted from *Sus scrofa domesticus* and *Sus scrofa scrofa* blood samples and from feed samples containing porcine material (for more details on these samples see § 2.2.).

Table 14: *Specificity tests of the primers from method D carried out with samples containing porcine DNA. The PCR assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) in SYBR® Green format. DNA from fresh blood samples was extracted with the kit Qiagen Genomic DNA tip 20/G (Qiagen, Venlo, The Netherlands). Pure species DNA extracts were at 2 ng / µl. Meal samples were extracted with the kit, Magnetic DNA Purification System for Food (Promega, Madison, WI, USA). The CRL samples tested are known to contain porcine material. Results were obtained with 3-fold diluted DNA extracts. Five µl were tested per PCR. Analysis mode: Abs. quant/second derivative max, high confidence and Tm calling. Description of the feed samples is to be found in § 2.2.*

Species/sample	C_t	Mean C_t	Tm 1	**Species/sample**	C_t	Mean C_t	Tm 1
Pig, boar and samples containing pig material							
Sus scrofa domesticus **(fresh blood sample)**	21.19 21.40	21.30	78.25 78.15	**CRL 558 extract 1**	16.90 16.91	16.91	78.10 78.10
Sus scrofa scrofa **(animal 1) (fresh blood sample)**	20.61 20.66	20.64	78.11 78.11	**CRL 558 extract 2**	16.62 16.57	16.60	78.00 78.00
Sus scrofa scrofa **(animal 2) (fresh blood sample)**	19.63 19.64	19.64	78.12 78.21	**CRL 1370 extract 1**	27.13 27.06	27.10	78.10 78.04
Sus scrofa scrofa **(animal 3) (fresh blood sample)**	20.32 20.21	20.27	78.03 78.10	**CRL 1370 extract 2**	26.75 26.77	26.76	77.91 78.01
0.1 % (w/w) porcine extract 1	32.60 32.43	32.52	78.10 77.86	**CRL 1374 extract 1**	25.05 25.03	25.04	78.10 78.10
0.1 % (w/w) porcine extract 2	32.10 32.10	32.10	78.14 78.14	**CRL 1374 extract 2**	24.51 24.55	24.53	78.10 78.10
0.025 % (w/w) porcine extract 1	34.86 34.11	34.49	78.19 78.19	**CRL 1445 extract 1**	31.34 31.28	31.31	78.20 78.20
0.025 % (w/w) porcine extract 2	33.02 33.09	33.06	78.14 78.14	**CRL 1445 extract 2**	31.98 32.08	32.03	78.20 78.20
CRL 214 extract 1	29.06 29.05	29.06	78.16 78.16	**CRL 1647 extract 1**	25.76 25.75	25.76	78.14 78.10
CRL 214 extract 2	29.57 29.95	29.76	78.16 78.16	**CRL 1647 extract 2**	25.88 25.84	25.86	78.14 78.17
H_2O (No template control)							
H_2O	42.74 38.77 45 45	/	75.07 73.65 / /				



Color	Name	Tm1
	Sus scrofa domesticus	78,25
	Sus scrofa domesticus	78,15
	Sus scrofa scrofa animal1	78,11
	Sus scrofa scrofa animal1	78,11
	H2O	75,07
	H2O	73,65
	H2O	
	H2O	

Figure 4: *Melting curves obtained with the primers from method D on DNAs extracted from Sus scrofa domesticus and Sus scrofa scrofa blood samples (5 µl of DNA extracts at 2 ng / µl) and with no template control (5 µl of H_2O). PCR assays were performed on the LC480 thermocycler (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.*

Melting curves of *Sus scrofa domesticus* and *Sus scrofa scrofa* show the same characteristics. The melting curves present a well-defined peak with a maximum close to 78 °C.

The PCR negative controls gave late amplification signals and a Tm between 73 and 75 °C when present.

The same test was performed on DNA from non-target animal species. Table 15 summarises the results.

Table 15: *Specificity tests of the PCR assay D carried out against non-target DNA of animal species. The PCR assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) 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	Species	C_t	Mean C_t	Tm 1	Tm 2
Other terrestrial mammalians									
Homo sapiens	36.03 35.97	36.00	/ /	/ /	***Capreolus capreolus***	36.49 37.08	36.79	64.55 63.55	/ /
Bos taurus	35.94 35.58	35.76	84.36 84.20	/ /	***Equus asinus***	36.97 36.74	36.86	65.20 62.34	/ /
Ovis aries	35.00 35.25	35.13	76.95 77.07	/ /	***Equus caballus***	33.80 33.75	33.78	77.66 77.75	/ /
Capra hircus	35.64 35.09	35.37	76.79 77.11	/ /	***Lepus europaeus***	30.93 31.01	30.97	75.21 75.25	/ /
Cervus elaphus	33.44 33.84	33.64	78.99 79.01	/ /	***Rattus rattus***	36.54 36.51	36.53	61.19 60.78	/ /
Domestic birds									
Gallus gallus	37.05 37.35	37.20	/ /	/ /	***Coturnix japonica***	35.05 35.08	35.07	74.26 /	/ /
Meleagris gallopavo	36.08 35.28	35.68	/ /	/ /	***Phasianus colchicus***	34.85 35.25	35.05	81.53 81.51	/ /
Numida meleagris	35.20 35.11	35.16	77.69 77.73	/ /	***Columba livia***	36.46 36.44	36.45	72.92 72.58	/ /
Cairina moschata	35.09 34.99	35.04	/ /	/ /	***Struthio camelus***	35.21 35.61	35.41	76.82 77.03	/ /
Anser spp.	36.76 36.36	36.56	/ /	/ /					
Sea mammals									
Stenella coeruleoalba	34.42 34.58	34.50	81.35 81.35	/ /	***Ziphius cavirostris***	34.21 34.33	34.27	81.19 81.29	/ /
Tursiops truncatus	34.04 34.04	34.04	81.33 81.33	/ /	***Phocoena phocoena***	35.64 35.71	35.68	81.22 81.08	/ /
Grampus griseus	33.65 33.18	33.42	81.31 81.11	/ /	***Phocidae***	36.56 37.87	37.22	/ /	/ /
Fish									
Gadus morhua	39.79 39.54	39.67	73.74 72.88	/ /	***Trisopterus minutus***	33.10 33.39	33.25	73.90 73.90	/ /
Pollachius virens	36.24 35.97	36.11	73.84 73.91	/ /	***Sardina pilchardus***	31.93 31.91	31.92	72.12 72.12	/ /
Melanogrammus aeglefinus	40.20 40.61	40.41	/ /	/ /	***Engraulis encrasicolus***	28.90 28.92	28.91	85.91 85.91	/ /
Micromesistius poutassou	36.50 36.22	36.36	71.93 72.45	/ /	***Gadus ogac***	37.26 36.87	37.07	72.88 /	/ /
Sebastes spp.	33.57 33.56	33.57	72.20 72.20	/ /	***Trisopterus esmarki***	33.09 33.14	33.12	73.93 73.93	/ /
Mallotus villosus	41.70 40.88	41.29	72.21 72.21	/ /	***Ammodytes lancea***	38.19 39.36	38.78	/ /	/ /
Scomber scombrus	37.25 36.94	37.10	72.98 72.95	/ /	***Sprattus sprattus***	38.78 37.28	38.03	72.16 71.88	/ 78.37
Clupea harengus	38.78 38.10	38.44	/ /	/ /	***Salmo salar***	34.57 34.69	34.63	80.18 80.18	/ /
Merluccius merluccius	36.06 36.23	36.15	72.98 73.17	/ /	***Raja spp.***	38.22 38.77	38.50	/ /	/ /
Trachurus trachurus	40.70 39.63	40.17	72.55 /	/ /					
Crustacean									
Paralithodes camtschaticus	45 45	45	/ /	/ /					

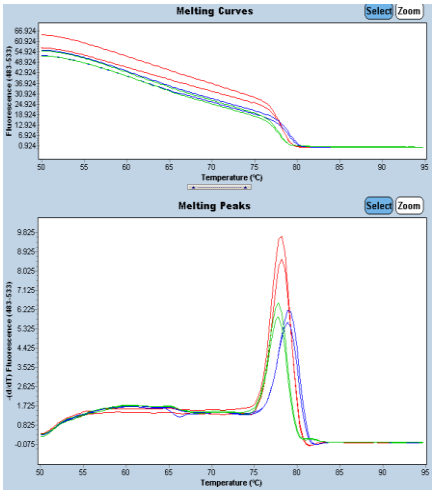
Table 16: *Specificity test of the PCR assay D carried out against non-target DNA of plant species. The PCR assays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.) containing SYBR® Green format. All DNAs were at 2 ng / µl and 5 µl were tested 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	Species	C_t	Mean C_t	Tm 1	Tm 2
Plant species									
Glycine max	32.26 32.00	32.13	77.63 77.54	/ /	***Oryza sativa***	37.50 36.70	37.10	/ /	/ /
Zea mays	35.72 35.69	35.71	72.87 /	81.34 /	***Lycopersicon esculentum***	32.61 32.57	32.59	72.15 72.21	/ /
Brassica napus	35.47 35.22	35.35	/ /	/ /	***Beta vulgaris***	35.74 35.69	35.72	78.14 78.21	/ /
Triticum aestivum	33.85 33.43	33.64	78.96 79.02	/ /					

The PCR assay D in SYBR® Green format delivered a Ct above 32 on most of the tested non-target DNA samples of animal or plant origin. Only 3 out of the 52 species tested gave an earlier amplification signal: *Lepus europaeus* with a mean Ct value at 30.97, *Engraulis encrasicolus* with a mean Ct value at 28.91 and *Sardina pilchardus* with a mean Ct value at 31.92. However, the melting curves obtained for these three species show a different pattern from that of *Sus scrofa domesticus* and *Sus scrofa scrofa*.

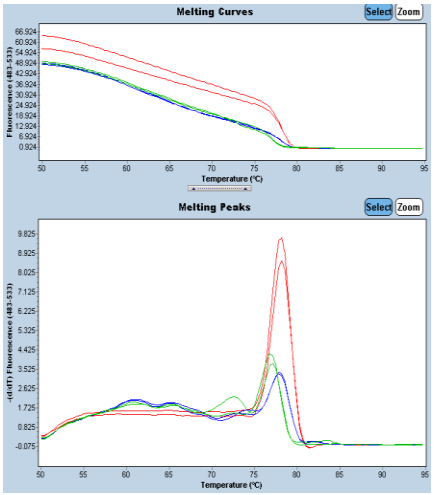
Two terrestrial mammalians (*Cervus elaphus* and *Equus caballus*) out of the 10 tested, two domestic birds (*Numida meleagris* and *Struthio* camelus) out of the 9 tested and three plant species (*Glycine max*, *Triticum aestivum* and *Beta vulgaris*) out of the 7 tested gave melting curves with Tm values close to the one obtained on *Sus scrofa domesticus* (Figure 5). Nevertheless, given the Ct values obtained with these seven species, there is no specificity problem linked to the primers of the PCR assay.

A



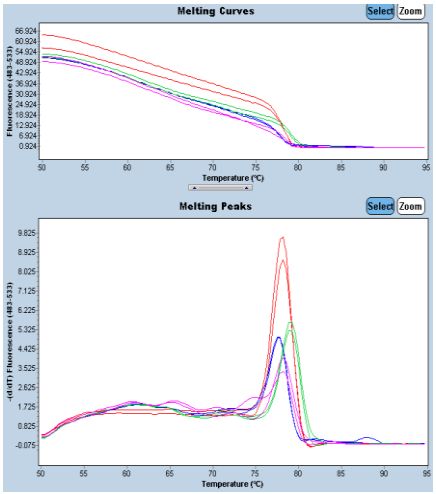
Color	Name	Tm1
	Sus scrofa domesticus	78,25
	Sus scrofa domesticus	78,15
	Cervus elaphus	78,99
	Cervus elaphus	79,01
	Equus caballus	77,66
	Equus caballus	77,75

B



Color	Name	Tm1
	Sus scrofa domesticus	78,25
	Sus scrofa domesticus	78,15
	Numida meleagris	77,69
	Numida meleagris	77,73
	Struthio camelus	76,82
	Struthio camelus	77,03

C



Color	Name	Tm1
	Sus scrofa domesticus	78,25
	Sus scrofa domesticus	78,15
	Glycine max	77,63
	Glycine max	77,54
	Triticum aestivum	78,96
	Triticum aestivum	79,02
	Beta vulgaris	78,14
	Beta vulgaris	78,21

Figure 5: *A. Melting curves obtained with DNAs extracted from Sus scrofa domesticus blood sample (red curves) and with DNAs extracted from Cervus elaphus (blue curves) and Equus caballus (green curves) blood samples.*
B. Melting curves obtained with DNAs extracted from Sus scrofa domesticus blood sample (red curves) and with DNAs extracted from Numida meleagris (blue curves) and Struthio camelus (green curves) blood samples.
C. Melting curves obtained with DNAs extracted from Sus scrofa domesticus blood sample (red curves) and with DNAs extracted from plant samples: Glycine max (blue curves), Triticum aestivum (green curves) and Beta vulgaris (pink curves).
A, B and C. The PCR was performed in SYBR® Green format with the primers from method D on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng / µl and 5 µl were tested in each PCR. Analysis mode: Tm calling.

2.4.4. Overall conclusion about the specificity of the PCR assay (method D)

In conclusion, the specificity of the PCR assay D in TaqMan format is better than that of the PCR assay C in the same format as its specificity is primarily brought by the primers rather than by the probe.

2.5. Competition

The results of the specificity tests showed that on some non-target species the specificity of the PCR assay C was due to the probe. Such a situation might be a drawback in case of competition effects.

The experiment to test this competition effect was performed with mixes containing 0.1 % (in mass fraction) of porcine PAP in the presence of material with competitive effect (cattle PAP or milk powder). The pig PCR assays C and D were compared. The cattle PAP was also tested with pig PCR assays and gives no signal.

Table 17: *Sensitivity test of the pig PCR assays C and D in the presence of potential competition effects. Samples under analysis contained porcine material at 0.1 % and 5 % (in mass fraction) and the competitive effect was achieved by cattle PAP or milk powder. PCR asssays were performed on the LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Results were obtained on 3-fold diluted DNA extracts and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

PCR Method		**C**			**D**		
Sample number and Composition	N° extract	C_t	Mean C_t	Result	C_t	Mean C_t	Result
CRL 1348 **0.1 % porcine PAP (2) + 99.9 % cattle PAP (1) (w/w)**	1	50 50	50	-	39.40 39.66	39.53	+
	2	50 50	50	-	38.61 38.97	38.79	+
CRL 1655 **0.1 % porcine PAP (3) + 5 % milk powder + 94.9 % pig feed (w/w)**	1	32.96 32.83	32.90	+	34.75 34.37	34.56	+
	2	32.31 32.17	32.24	+	33.80 34.38	34.09	+
CRL 1654 **0.1 % porcine PAP (4) + 5 % milk powder + 94.9 % pig feed (w/w)**	1	50 50	50	-	40.19 39.22	39.71*	+
	2	50 50	50	-	39.44 39.93	39.69	+

* > 0.5 cycle between replicates of the same extract

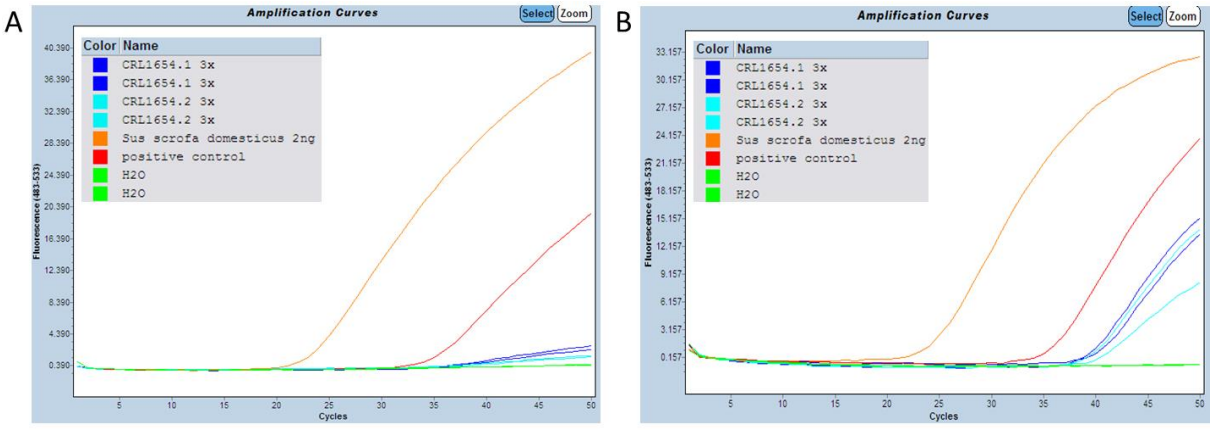


Figure 6: *Amplification signals obtained with PCR replicates performed on 2 DNA extracts from sample CRL 1654 consisting of a pig feed with 0.1 % porcine PAP and 5 % milk powder. Sus scrofa domesticus DNA at 2 ng / µl and positive control mix at 0.1 % (in mass fraction) in PAP (containing porcine PAP) and on no template control (H_2O). The PCR assays were performed with the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Analysis mode: Abs. quant/second derivative max and high confidence. A) Results obtained with the Pig PCR assay C. B) Results obtained with the Pig PCR assay D.*

The results show a competition effect between pig and cattle DNA when the PCR assay C is used (Table 17 and Figure 6). For sample CRL 1654, the LC480 software gives a Ct (or Cp) value but there is no clear amplification signal. This sample is therefore considered as negative.

On this same sample, the PCR assay D allows the detection of 0.1 % (in mass fraction) porcine PAP in presence of cattle. The target does not show a competition effect.

2.6. Choice of the pig PCR target

The PCR assay D is selected for the pig DNA detection. The primers and probe as well as the cycling conditions for the porcine PCR test are provided in annex 1.

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, performance parameters like the efficiency and the absolute LOD were measured with calibrants consisting of a dilution of a pUC18 plasmid in which the PCR target D had been cloned. This plasmid is named *pUC18-pig-AM*.

This plasmid *pUC18-pig-AM* was produced in *Escherichia coli* and purified from the bacterial culture. The cloned target sequence was checked by sequencing outsourced to Beckman Coulter Genomics (Bishop's Stortford, United Kingdom) (Table 18).

Table 18: *Comparison of the theoretical sequence of the NCBI databases and sequence obtained by Beckman Coulter Genomics firm (Bishop's Stortford, United Kingdom) with pUC18-pig-AM. The size of fragment is 83 bp.*

	Fragment of PCR method D with primers sequence in green and probe sequence in blue
Theoretical sequence (NC_012095.1)	ACAACATAATCTGAATCAATGCAACAGTACATAGTCTCCTCATTAGCCTGATCAGTCTATCCCTACTAAACCAACTAGGCGAA
pUC18-pig-AM sequence	ACAACATAATCTGAATCAATGCAACAGTACATAGTCTCCTCATTAGCCTGATCAGTCTATCCCTACTAAACCAACTAGGCGAA

The so-produced plasmid was linearized by the restriction endonuclease *Hind*III. Afterwards it was 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. To limit the loss of plasmid DNA through sticking to the plastic vials when the plasmid is in solution at low levels, it is diluted in a solution of background DNA consisting of salmon sperm DNA (Sigma-Aldrich, Saint-Louis, Missouri, U.S.A) at a concentration of 50 ng / µl.

This concentration at 50 ng / µl was verified by spectrophotometrical measurements with the nanodrop (Thermo Fisher Scientific, Wilmington, North Carolina, U.S.A.). A test by real-time PCR was carried out to check the absence of the pig target in the salmon sperm DNA of commercial origin. This DNA solution was autoclaved 3 times, filtered with Millex Express® PES Membrane 0.22 µm (EMD Millipore Corporation, Billerica, Massachusetts, U.S.A.) and potassium azide (azide has biocidal properties without interfering with the PCR) was added to obtain a final concentration at 0.1 % (w/v).

The calibrant solutions produced were aliquoted. Their content in pig target was measured by digital PCR (Fluidigm, South San Francisco, California, U.S.A.) at JRC (Geel, Belgium). The vials containing the aliquots to analyse were taken randomly from the whole batch. One analysis was made per vial. Ten measurements (thus 10 vials) for the levels at 640 and 160 copies / 5 µl, distributed on 3 plates (digital Array™ IFC's Fluidigm), and 19 measurements (thus 19 vials) for the level at 40 copies / 5 µl, distributed on 4 plates, were carried out by digital PCR. Table 19 gathers the main results in terms of mean copy number, standard deviation and relative standard deviation (RSD). The RSD for the levels at 640 and 160 copies / 5 µl are below 16 % and therefore the mean figure is reliable (Martin and Gendron, 2004). For the level at 40 copies / 5 µl, variation is somewhat more important but remains below 33 %, threshold above which the mean is no longer reliable.

Table 19: *Mean copy number, standard deviation and coefficient of variation calculated on 10 measurements by digital PCR of levels at 640 and 160 copies / 5 µl and 19 measurements by digital PCR of level at 40 copies / 5µl*

Number of copies expected	Mean number of copies obtained by digital PCR analysis	Standard deviation	Coefficient of variation
640 copies / 5 µl	647 copies / 5 µl	7.78	6.0 %
160 copies / 5 µl	147 copies / 5 µl	3.92	13.4 %
40 copies / 5 µl	38 copies / 5 µl	1.66	21.7 %

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

The cut-off value of one PCR platform was determined with 16 calibrations including three copy numbers (647 – 147 – 38 copies / 5 µl) and 3 replicates per concentration. The 16 calibrations were spread over 4 runs.

The calibrations were performed on a LightCycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and the Universal Mastermix (Diagenode s.a., Seraing, Belgium).

Analysis of results was carried out in "Abs. quant/second derivative max and high confidence" mode. The cut-off value at 1, 5, 10 and 15 copies were determined respectively at 42.75, 40.36, 39.81 and 38.73 cycles.

All the runs performed on the same PCR platform must be analysed using the same setting of parameters (baseline and threshold).

4. Performances of the test

4.1. Efficiency

The efficiency of the test was checked within four runs involving a calibration made with 5000, 2500, 1000, 500 and 100 copies respectively in the reaction. Six replicates per concentration were performed.

The tests were performed on a LightCycler 480 thermocycler (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with the Universal Mastermix (Diagenode s.a., Seraing, Belgium). Results were analysed in the "Abs. quant/second derivative max and high confidence" mode. All the runs performed were analysed using the same parameters setting.

The overall pig target efficiency is 92.34 %. The efficiency calculated on each plate is always higher than 90 %. This meets the acceptance criterion for the efficiency which should be within the range of 90 to 110 %[15].

4.2. LOD

The LOD of the PCR assay was calculated with dilutions of plasmid *pUC18-pig-AM* at low copy numbers (50 – 20 – 10 – 5 – 2 and 1 copy / 5 µl respectively) in a solution with a background DNA of salmon sperm at 50 ng / µl. Two runs including 6 replicates per concentration were performed.

LOD_6 is the smallest copy number giving 6 signals out of 6 replicates of 2 runs.

Results of the LOD_6 approach are given in Table 20. The absolute LOD varies in a range from 5-20 copies according to cut-off values applied and therefore meets the performance parameter of not being higher than 20 copies when the cut-off is set at 10 copies or lower.

[15] **Guidelines for validation of qualitative real-time PCR methods (2014).** Broeders et al. Trends in Food Science & Technology, 37(2), 115-126.

Table 20: *Absolute LOD_6 determined on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) with plasmid dilutions at 50, 20, 10, 5, 2 and 1 copies/5 µl performed. Analysis mode: Abs. quant/second derivative max and high confidence. Results calculated without cut-off applied and according cut-off set at 1, 5, 10 and 15 copies.*

Copy number tested for LOD_6	Number of positive reactions on 6 replicates obtained with a cut-off set at									
	Without cut-off value applied		1 copy (42.75 cycles)		5 copies (40.36 cycles)		10 copies (39.81 cycles)		15 copies (38.73 cycles)	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
50	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
20	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6
10	6/6	6/6	6/6	6/6	5/6	5/6	4/6	5/6	2/6	4/6
5	6/6	6/6	6/6	6/6	0/6	2/6	0/6	1/6	0/6	0/6
2	5/6	4/6	4/6	4/6	0/6	1/6	0/6	1/6	0/6	0/6
1	2/6	2/6	2/6	2/6	0/6	0/6	0/6	0/6	0/6	0/6

Given that the cut-off level was not yet fixed, the copy number corresponding to LOD_6 without cut-off value applied was then tested 60 times on the same plate but also the upper copy levels (5-10-20 copies respectively - Table 21). The LOD is validated if at least 95 % of signals are recorded positive out of the 60 replicates.

The tests were performed on a LightCycler 480 thermocycler (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with the Universal Mastermix (Diagenode s.a., Seraing, Belgium). Results were analysed in “Abs. quant/second derivative max and high confidence” mode. All the runs performed on a same PCR platform were analysed using the same parameters settings. The results are presented in Table 21.

According to the acceptance criterion, the LOD must not be higher than 20 target copies. Without cut-off value applied and with a cut-off set at 1 copy, the LOD is at 10 copies for the PCR platform tested. The acceptance criterion is still reached when the cut-off is placed at 5 and 10 copies. In this case, the LOD is at 20 copies for the PCR platform tested. With a cut-off set at 15 copies, it is quite normal to no longer meet the criterion.

Table 21: *Absolute LOD determined on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) with plasmid dilutions at 5, 10 and 20 copies / 5 µl. Analysis mode: Abs. quant/second derivative max and high confidence. Results calculated without cut-off applied and according cut-off set at 1, 5, 10 and 15 copies.*

Copy number tested for LOD	Positive results in % and number of positive reactions on 60 replicates (between brackets) obtained with a cut-off set at				
	Without cut-off value applied	1 copy (42.75 cycles)	5 copies (40.36 cycles)	10 copies (39.81 cycles)	15 copies (38.73 cycles)
5	93.3% (56/60)	90 % (54/60)	45 % (27/60)	20 % (12/60)	0 % (12/60)
10	98.3 % (59/60)	98.3 % (59/60)	90 % (54/60)	80 % (48/60)	23.3 % (14/60)
20	100 % (60/60)	100 % (60/60)	100 % (60/60)	96.7 % (58/60)	83.3 % (50/60)

4.3. Additional specificity tests

DNAs from samples free of pig material and samples of porcine PAPs were analysed on the real-time thermocycler LightCycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with Universal Mastermix (Diagenode s.a., Seraing, Belgium). Results were analysed in “Abs. quant/second derivative max and high confidence” mode.

Three tables summarise the results obtained with: Table 22- samples free of porcine material; Table 23- pure PAP samples and Table 24- various controls. The DNAs were tested at the 3 fold and 30 fold dilutions.

Table 22: *Specificity tests with samples free of porcine material performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Result interpretations according to cut-off set at 1, 5, 10 and 15 copies respectively (corresponding number of cycles between brackets).*
Analysis mode: Abs. quant/second derivative max and high confidence.

Sample number and Description	Extract	Dilution	C_t	Mean C_t	Result interpretation with a cut-off set at			
					1 copy (42.75 cycles)	5 copies (40.36 cycles)	10 copies (39.81 cycles)	15 copies (38.73 cycles)
CRL 1328 Maize	1	3x	39.71 50 50	46.57*	-**	-**	-**	-
		30x	50 50 50	50				
	2	3x	50 50 50	50	-	-	-	-
		30x	50 50 50	50				
CRL 1282 Fish meal	1	3x	50 50 50	50	-	-	-	-
		30x	50 50 50	50				
	2	3x	50 50 50	50	-	-	-	-
		30x	50 50 50	50				
CRL 552 Chicken meal	1	3x	40.08 40.14 50	43.41*	-**	-**	-	-
		30x	50 50 50	50				
	2	3x	50 50 50	50	-	-	-	-
		30x	50 50 50	50				
CRL 2055 Feed for pig	1	3x	50 50 50	50	-	-	-	-
		30x	50 50 50	50				
	2	3x	50 50 50	50	-	-	-	-
		30x	50 50 50	50				
CRL 1753*** Aquafeed	1	3x	50	50	-	-	-	-
		30x	50	50				
	2	3x	50	50	-	-	-	-
		30x	50	50				

* > 0.5 cycle between replicates of the same extract
** ambiguous results for the first and second analysis, final conclusion negative sample
*** routine analysis

Table 23: *Specificity tests with samples of porcine material performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Result interpretations according to cut-off set at 1, 5, 10 and 15 copies respectively (corresponding number of cycles between brackets).*
Analysis mode: Abs. quant/second derivative max and high confidence.

Sample number and Description	Extract	Dilution	C_t	Mean C_t	Result interpretation with a cut-off set at			
					1 copy (42.75 cycles)	5 copies (40.36 cycles)	10 copies (39.81 cycles)	15 copies (38.73 cycles)
CRL 2059 Porcine PAP (5)	1	3x	25.73 25.69 25.67	25.70	+	+	+	+
	2	3x	25.68 25.69 25.69	25.69	+	+	+	+
CRL 2011 Porcine PAP (6)	1	3x	25.20 25.23 25.09	25.17	+	+	+	+
	2	3x	25.49 25.42 25.33	25.41	+	+	+	+
CRL 2012 Porcine PAP (7)	1	3x	24.69 24.67 24.76	24.71	+	+	+	+
	2	3x	24.23 24.30 24.36	24.30	+	+	+	+
CRL 214 Porcine PAP (4)	1	3x	32.69 32.83 32.76	32.76	+	+	+	+
	2	3x	32.93 32.99 32.85	32.92	+	+	+	+

Table 24: *Specificity tests with positive and negative controls performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Result interpretations according to cut-off set at 1, 5, 10 and 15 copies respectively (corresponding number of cycles between brackets).*
Analysis mode: Abs. quant/second derivative max and high confidence

Sample number and Description	Extract	Dilution	C_t	Mean C_t	Result interpretation with a cut-off set at			
					1 copy (42.75 cycles)	5 copies (40.36 cycles)	10 copies (39.81 cycles)	15 copies (38.73 cycles)
CRL 291 PCR positive control (0.025 % PAP in blank feed (w/w))	1 140121	3x	38.65 37.59 37.60	37.95*	+	+	+	+
	2 140120	3x	36.74 36.86 37.25	36.95*	+	+	+	+
CRL 297 Extraction positive control (0.1 % PAP in blank feed (w/w))	1 120328	3x	36.77 37.17 36.48	36.81*	+	+	+	+
	2 120201	3x	35.66 36.55 36.09	36.10*	+	+	+	+
Extraction negative control	1 - 140121	3x	50	50	-	-	-	-
	2 - 140120	3x	50	50	-	-	-	-
PCR negative control (H_2O in PCR)	PCR 1 480-14-008	/	50 50	50	-	-	-	-
	PCR 2 480-14-014	/	50 50	50	-	-	-	-

* > 0.5 cycle between replicates of the same extract

4.4. Sensitivity

DNAs from samples with known PAP contents (1, 0.2 and 0.1 % of porcine PAPs (in mass fraction)) were analysed on the real-time thermocycler LightCycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with Universal Mastermix (Diagenode s.a., Seraing, Belgium). Results were analysed in “Abs. quant/second derivative max and high confidence” mode.

Two tables summarise the results obtained with: Table 25- samples containing 0.1 % (in mass fraction) of porcine PAPs; Table 26- samples analysed for the choice of the pig PCR target. The DNAs were tested at the 3-fold and 30-fold dilutions.

Table 25: *Sensitivity tests with samples containing 0.1 % in mass fraction of porcine material performed on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Result interpretations according to cut-off set at 1, 5, 10 and 15 copies respectively (corresponding number of cycles between brackets). Analysis mode: Abs. quant/second derivative max and high confidence.*

Sample number and Description	Extract	Dilution	C_t	Mean C_t	Result interpretation with a cut-off set at			
					1 copy (42.75 cycles)	5 copies (40.36 cycles)	10 copies (39.81 cycles)	15 copies (38.73 cycles)
CRL 2222 0.1 % porcine PAP (5) in maize (CRL 1328) (w/w)	1	3x	33.59 33.71 33.52	33.61	+	+	+	+
	2	3x	32.11 32.21 32.24	32.19	+	+	+	+
CRL 2223 0.1 % porcine PAP (5) in fish meal (CRL 1282) (w/w)	1	3x	37.81 37.61 37.47	37.63	+	+	+	+
	2	3x	35.79 36.14 36.28	36.07	+	+	+	+
CRL 2224 0.1 % porcine PAP (5) in chicken meal (CRL 552) (w/w)	1	3x	37.96 37.90 37.82	37.89	+	+	+	+
	2	3x	38.11 37.91 37.73	37.92	+	+	+	+
CRL 2225 0.1 % porcine PAP (5) in aquafeed (CRL1753) (w/w)	1	3x	33.16 32.91 32.86	32.98	+	+	+	+
	2	3x	32.22 32.34 32.37	32.31	+	+	+	+
CRL 2232 0.1 % porcine PAP (5) in feed for pig (CRL 2055) (w/w)	1	3x	32.95 33.11 33.03	33.03	+	+	+	+
	2	3x	32.99 33.01 33.01	33.00	+	+	+	+
CRL 2218 0.1 % porcine PAP (4) in maize (CRL 1328) (w/w)	1	3x	38.41 40.80 40.05	39.75*	+	?	?	?
	2	3x	40.78 39.82 40.93	40.51*	+	?	-	-
CRL 2236 0.1 % porcine PAP (6) in feed for pig (CRL 2055) (w/w)	1	3x	29.69 29.78 29.72	29.73	+	+	+	+
	2	3x	30.30 30.22 30.43	30.32	+	+	+	+
CRL 2237 0.1 % porcine PAP (6) in fish meal (CRL 1282) (w/w)	1	3x	34.14 34.02 34.28	34.15	+	+	+	+
	2	3x	34.03 33.95 34.03	34.00	+	+	+	+
CRL 2238 0.1 % porcine PAP (7) in aquafeed (CRL 1753) (w/w)	1	3x	30.67 30.62 30.75	30.68	+	+	+	+
	2	3x	29.27 29.26 29.28	29.27	+	+	+	+
CRL 2239 0.1 % porcine PAP (7) in chicken meal (CRL 552) (w/w)	1	3x	35.59 35.58 35.91	35.69	+	+	+	+
	2	3x	36.65 36.36 36.71	36.57	+	+	+	+

* > 0.5 cycle between replicates of the same extract

? ambiguous results

Table 26: *Result interpretations according to a cut-off set at 1, 5, 10 and 15 copies respectively (corresponding number of cycles between brackets) for samples already analysed in Table 6 and 17.*
Analysis mode: Abs. quant/second derivative max and high confidence

Sample number and Description	Extract	Dilution	C_t	Mean C_t	Results interpretation with cut-off at			
					1 copy (42.75 cycles)	5 copies (40.36 cycles)	10 copies (39.81 cycles)	15 copies (38.73 cycles)
CRL 1338 1 % porcine PAP (1) + 99 % soya (w/w)	1	3x	37.43 37.54	37.49	+	+	+	+
	2	3x	37.85 38.12	37.99	+	+	+	+
CRL 1349 0.1 % porcine PAP (2) + 99.9 % blank feed (w/w)	1	3x	34.49 34.85	34.67	+	+	+	+
	2	3x	36.62 36.96	36.79	+	+	+	+
CRL 1348 0.1 % porcine PAP (2) + 99.9 % cattle PAP (w/w)	1	3x	39.40 39.66	39.53	+	+	+	-
	2	3x	38.61 38.97	38.79	+	+	+	?
CRL 1380 0.2 % porcine haemoglobin powder + 99.8 % soya (w/w)	1	3x	35.89 36.20	36.05	+	+	+	+
	2	3x	35.81 36.64	36.23*	+	+	+	+
CRL 1654 0.1 % porcine PAP (4) + 5 % powder milk + 94.9 % pig feed (w/w)	1	3x	40.19 39.22	39.71*	+	+	?	-
	2	3x	39.44 39.93	39.69	+	+	?	-

* > 0.5 cycle between replicates of the same extract
? ambiguous results

4.5. Choice of a cut-off level

The results obtained with samples tested for checking the specificity and the sensitivity of the method were compared to the Ct values corresponding to different cut-off set respectively at 1, 5, 10 and 15 copies of PCR platform. These results are summarized in Table 27 with the rates of false positive and false negative results.

Table 27: *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 and 15 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 (42.75 cycles)		5 copies (40.36 cycles)		10 copies (39.81 cycles)		15 copies (38.73 cycles)	
		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
Samples containing porcine material	22	0	0	0 but 1 ambiguous sample	0	0 but 1 ambiguous sample	0	1 and 1 ambiguous sample	0
Terrestrial mammalian species other than pig	10	0	0	0	0	0	0	0	0
Domestic bird species	9	0	0	0	0	0	0	0	0
Sea mammal species	6	0	0	0	0	0	0	0	0
Fish and crab species	20	0	0	0	0	0	0	0	0
Plant species	7	0	0	0	0	0	0	0	0
Pure PAP without porcine material	2	0	0	0	0	0	0	0	0
Samples without porcine material	3	0	0	0	0	0	0	0	0
Samples containing 0.1 % (w/w) porcine PAP	14	0	0	0 but 1 ambiguous sample	0	0 but 2 ambiguous samples	0	1 and 2 ambiguous samples	0
Extraction positive control	4	0	0	0	0	0	0	0	0
Extraction negative control	2	0	0	0	0	0	0	0	0
% of false results calculated on 99 samples		0	0	0 and 2.0 % ambiguous samples	0	0 and 3.0 % ambiguous samples	0	2.0 and 3.0 % ambiguous samples	0

The cut-off set at 5 copies is the best compromise to minimize the rate of false results but also to avoid false positives results in future analyzes.

With a cut-off value at 5 copies, sample CRL 2218 gives an ambiguous result (Table 25) while sample CRL 1654 containing also 0.1 % in mass fraction of the same porcine PAP is positive. This porcine PAP was collected by EURL-AP in 2007 but the 2 analysed mixes at 0.1 % were prepared at a two-years interval (2012 and 2014). During this period, the DNA of the PAP in one of the mixes must have been degraded explaining the late signal on CRL 2218.

4.6. Robustness

The robustness of the method was tested by deviations from the experimental conditions on two thermocyclers (LC480 and ABI7500): the annealing temperature (50 °C +/- 1 °C), the primer concentrations (standard or reduced of 30 %), the probe concentration (standard or reduced of 30 %) and the PCR volume (35 µl +/- 1 µl). Six replicates of plasmid at 20 copies / 5 µl were performed in the conditions described in Table 28.

Table 28: *Experimental conditions tested to evaluate the robustness of method*

PCR machine	LC480 (Roche Diagnostics Ltd.) and ABI7500 (Applied Biosystems) thermocyclers				
PCR reagent kit	Universal Mastermix (Diagenode s.a.) and TaqMan 2x Universal PCR Master mix No AmpErase UNG (Applied Biosystems)				
Annealing temperature	49 and 51 °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 acceptance criterion is that the method must give the expected results in 95 % of the cases. The numbers of positive results on 6 replicates in each condition are presented for each PCR platform in Tables 29 and 30. The results were analyzed with a cut-off set at 5 copies.

Table 29: *Tests of robustness 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.

		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)**
Annealing temperature	**49 °C**	6/6	6/6	6/6	6/6
	51 °C	6/6	6/6	6/6	6/6

With this PCR platform (LC480 thermocycler combined with Universal Mastermix provided by Diagenode), **100 %** of results are considered as positive with a cut-off set at 5 copies.

Table 30: *Tests of robustness performed on the ABI7500 thermocycler (Applied Biosystems) with the TaqMan 2x Universal PCR Master mix No AmpErase UNG (Applied Biosystems).*
Analysis mode: automatic base line and threshold at 0.14.

		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)**
Annealing temperature	**49 °C**	6/6	6/6	6/6	6/6
	51 °C	6/6	6/6	6/6	6/6

With the second PCR platform combining an ABI7500 thermocycler with TaqMan 2x Universal PCR Master Mix no AmpErase by Applied Biosystems, the cut-off at 5 copies is 39.79 cycles. With this PCR platform, **100 %** of results are considered as positive whatever the deviation with a cut-off set at 5 copies.

In conclusion, with a rate of positive results was always higher than 95 %, the PCR test is considered as robust.

5. Calibrants of JRC

Three calibrants (dilution of *pUC18-pig-AM)* for the determination of cut-off value are now produced by JRC (Joint Research Center, Geel, Belgium). The first batch of these calibrants are provided at 635, 170 and 45 copies / 5 µl.

The cut-off value of PCR platform LightCycler 480 thermocycler (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and the Universal Mastermix (Diagenode s.a., Seraing, Belgium) was also determinate with these calibrants. Analysis of results was carried out in "Abs. quant/second derivative max and high confidence" mode for all the runs. The cut-off value at 5 copies was determined at 40.08 cycles.

The cut-off value calculated with the JRC calibrants is very close to the cut-off value calculated with calibrants produced by EURL-AP, respectively 40.08 and 40.36. This change of value has no impact on the specificity and sensitivity.

The comparison of calibrants of EURL-AP and JRC showed that the solution at 20 copies / 5 µl used for the LOD and robustness tests is in fact somewhat lower than 20 copies / 5 µl. The tests were thus carried out in more challenging conditions and therefore all conclusions made (LOD, robustness) remain valid.

6. Analysis mode on the LightCycler 480

During the preparation of the inter-laboratory study, it was observed that the use of the automatic algorithm (Abs Quant – 2nd Derivative max) of the LightCycler 480 software (Roche Diagnostics) could give rise to erroneous values on late signals showing low amplification efficiency. There is no problem for the signals with good amplification efficiency. For that reason, it is advised to analyze the results in manual mode "Abs Quant/Fit Points" was made (with the analysis parameters: Background at 2-15, Noiseband (Fluoresc) at 2.5 and Threshold at 3.2). This change does not imply any modification of the PCR protocol and does not modify the final result for the samples tested. Except for sample CRL 1654 (former results in Table 27) which becomes ambiguous (a second analysis should be made in this case to obtain the final conclusion). However, as already pointed out, this result is probably due to the fact that the sample contains a poorly preserved porcine PAP (point 4.5).

7. Final conclusions

Out of the 6 pig PCR methods assessed by the EURL-AP, the specificity and sensitivity tests performed were decisive for the final choice of the pig PCR target to validate. The pig target designed in method D is specific and is the most sensitive.

The different performance criteria considered have been reached. The pig PCR method D has an efficiency at 92.34 %. The limit of detection was determined at 20 copies with a cut-off value at 5 copies. The test is able to achieve the minimum requirement expected of a PCR assay, i.e. to detect 0.1 % (w/w)) of porcine PAP in feed. This goal is achieved in presence of feed composed only of plant material as well as in presence of another species of processed animal proteins. The PCR method is also robust.

For the transferability of the method, the study of different cut-off levels comes to the conclusion that a cut-off calculated at 5 copies is the best one.

The final conclusion of this study is that the pig PCR assay D may be considered as fit for purpose for an interlaboratory assay.

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 pig DNA by real-time PCR

1. Primers and probe sequences

Forward primer: 5'-ACA ACA TAA TCT GAA TCA ATG C-3'

Reverse primer: 5'-TTC GCC TAG TTG GTT TAG TAG-3'

Probe: 5'-AGT ACA TAG TCT CCT CAT TAG CCT GAT C-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 35 µl :

PCR grade water, 8.75 picomoles of primer A and primer B, 8.75 picomoles of probe, mastermix with $MgCl_2$ at the final concentration of 5 mmole/l.

The examples of mixes are given in Table 1:

Table 1: Examples of mixes

	1 réaction	96 réactions	102 réactions (1 plate)*
PCR grade water	8.75 µl	840 µl	892.5 µl
Forward primer (7 µmole/l)	1.25 µl	120 µl	127.5 µl
Reverse primer (7 µmole/l)	1.25 µl	120 µl	127.5 µl
Probe (7 µmole/l)	1.25 µl	120 µl	127.5 µl
Master mix 2x	17.50 µl	1680 µl	1785 µl
Total PCR mix volume/reaction	30 µl		
DNA to be added in each PCR	5 µl		
Total reaction volume	35 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 pig 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	50