Evaluation of the PCR method for the detection of poultry DNA

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1. Introduction

In the TSE roadmap II[1], the Commission considers a possible lifting of the ban on the use of non-ruminant 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, the detection of the poultry PAP is of crucial importance. The Commission Regulation (EC) No 543/2008 of 16 June 2008[2] defines poultry carcasses as coming from *Gallus domesticus*, *Meleagris gallopavo*, duck (*Anas platyrhynchos* and *Cairina moschata*), *Anser anser* and *Numida meleagris*.

Different laboratories and publications proposed poultry, chicken or turkey real-time PCR methods. The EURL-AP implemented in its lab the assays and evaluated the performances of the different PCR methods.

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 targets.

The evaluation was conducted according to 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 detection methods for GMOs but are also applicable to any other real-time PCR method and offer an objective frame to this study.

2. Description of different poultry, chicken and turkey targets

2.1. Information collected in literature and sequence databanks

Two PCR methods for the detection of poultry DNA, 5 PCR methods for the detection of chicken DNA and 5 PCR methods for the detection of turkey DNA were at the disposal of the EURL-AP. The EURL-AP collected the information on these different PCR tests (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://intranet.crl.cra.wallonie.be/Document%20libraries/EC%20Directives/TSE%20Roadmap%20II.pdf

2 **The Commission Regulation (EC) No 543/2008 of 16 June 2008 laying down detailed rules for the application of Council Regulation (EC) No 1234/2007 as regards the marketing standards for poultrymeat.** Official journal of the European Union n° L 157 of 17/06/2008, 46-87.

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 method for the detection of poultry DNA, chicken DNA and turkey DNA put at the disposal of the EURL-AP.*

Targeted species*	Laboratories or publications of PCR test	Location of the target	Size of fragment	Type of probe
Poultry	Commercial kit producted by Institut für Produktqualität (Berlin, Germany)	mitochondrial DNA	227 bp	
	TNO Triskelion B.V., Zeist, The Netherlands	genomic DNA : Prolactin gene	75 bp	5'-FAM and 3'-TAMRA
Chicken	Krcmar P. and Rencova E. (2005) [13]	mitochondrial DNA : ATP8 and ATP6 gene	113 bp	5'-FAM and 3'-BH1
	Laube I. *at al.* [14]	genomic DNA : Interleukin-2 precursor gene	95 bp	5'-FAM and 3'-TAMRA
	Agroscope, Posieux, Switzerland	mitochondrial DNA : tRNA-Val and 16S ribosomal RNA sequences	100 bp	5'-FAM and 3'-TAMRA
	Departamento de Nutrición, Bromatología y Tecnología de los Alimentos – Facultad de Veterinaria – Universidad complutense de Madrid	mitochondrial DNA : D-loop	63 bp	5'-FAM and 3'-BBQ Contain 8 LNA
	CRA-W (Walloon Agircultural Research Centre – Valorisation of Agricultural Products Department), Gembloux, belgium	mitochondrial DNA : tRNA-Lys sequence and ATP8 gene	66 bp	5'-FAM and 3'-TAMRA
Turkey	AGES GmbH, Austria	mitochondrial DNA : tRNA-Lys sequence and ATP8 gene	98 bp	5'-FAM and 3'-MGB
	Lopez-Andreo M. *at al.* (2005) [15]	mitochondrial DNA : ND5 and CYTB gene	113 bp	5'-FAM and 3'-NFQ-MGB 2 probes possible
	Laube I. *at al.* (2007) [14]	Genomic DNA : Interleukin-2 precursor gene	86 bp	5'-FAM and 3'-TAMRA
	Departamento de Nutrición, Bromatología y Tecnología de los Alimentos – Facultad de Veterinaria – Universidad complutense de Madrid	mitochondrial DNA : D-loop	82 bp	5'-FAM and 3'-BBQ Contain 8 LNA

*defined according to those who developed the test

The EURL-AP analyzed the data of each test. The commercial kit proposed by the Hungarian NRL was discarded because this method is known to interfere with gull DNA and its target is rather large. The two tests developed by the Univesidad complutense de Madrid for the detection respectively of chicken DNA and turkey DNA, both require a probe containing 8 locked nucleic acids (LNA). This increases the cost of the assay. The EURL-AP favoured less expensive methods and therefore did not continue the study with these targets. For a similar reason the tests with a MGB probe were left aside; it is the case for the turkey PCR assays coming from AGES GmbH and from Lopez-Andreo *et al.* (2005).

The primers and probes of 6 other targets were synthesized by Eurogentec (Seraing, Belgium) to be evaluated experimentally.

[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] **Development and design of a 'ready-to-use' reaction plate for a PCR-based simultaneous detection of animal species used in foods (2007).**
Laube I. *at al.*
International Journal of food Science and Technology, 42, 9-17

[15] **Identification and quantization of species in complex DNA mixtures by real-time polymerase chain reaction (2005).**
Lopez-Andreo M. *and al.*
Analytical Biochemistry 339, 73-82

1) The “poultry” PCR method of TNO Triskelion BV (Figure 1).

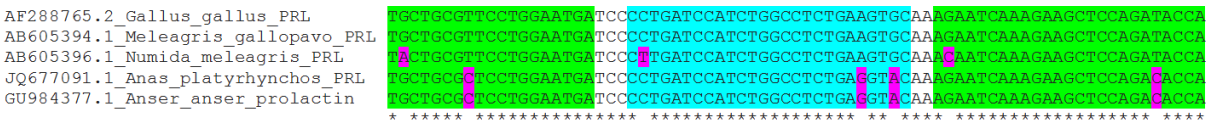

Figure 1: *Alignment of 5 poultry sequences of the prolactin gene targeted by the poultry PCR test of TNO Triskelion BV. Primer sequence location are in green and the probe in blue. The sequences originate from the NCBI database. Chicken is used as reference, different nucleotides in the other species are coloured in purple.*

2) The chicken PCR method published by Krcmar and Rencova (2005) (Figure 2).

Figure 2: *Alignment of 7 poultry mitochondrial DNA sequences targeted by the poultry PCR test published by Krcmar and Rencova (2005). Primer sequence locations are in green and the probe in blue. The sequences originate from the NCBI database. Chicken is used as reference, different nucleotides in the other species are coloured in purple.*

3 et 4) The chicken and turkey PCR methods published by Laube *at al.* (2007) (Figure 3 and 4).

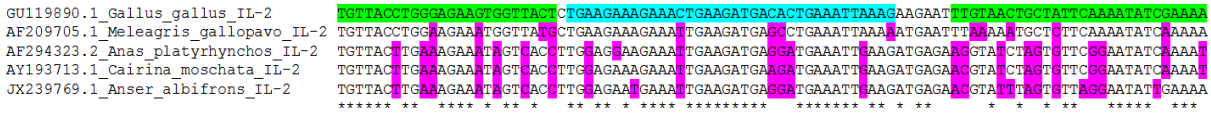

Figure 3: *Alignment of 5 poultry sequences of the interleukin-2 precursor gene targeted by the chicken PCR test published by Laube at al. (2007). Primer sequence locations are in green and the probe in blue. The sequences originate from the NCBI database. Chicken is used as reference, different nucleotides in the other species are coloured in purple.*

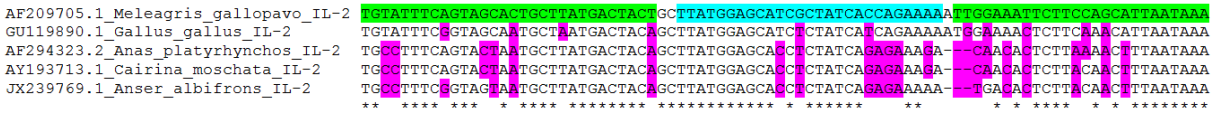

Figure 4: *Alignment of 5 poultry sequences of the interleukin-2 precursor gene targeted by the turkey PCR test published by Laube at al. (2007). Primer sequence locations are in green and the probe in blue. The sequences originate from the NCBI database. Turkey is used as reference, different nucleotides in the other species are coloured in purple.*

5) The PCR method for the chicken detection come from Switzerland (Figure 5).

Figure 5: *Alignment of 7 poultry mitochondrial DNA sequences targeted by the Swiss chicken PCR test. Primer sequence locations are in green and the probe in blue. The sequences originate from the NCBI database. Chicken is used as reference, different nucleotides in the other species are coloured in purple.*

6) The PCR method for the chicken detection of CRA-W (Figure 6).

Figure 6: *Alignment of 7 poultry mitochondrial DNA sequences targeted by the CRA-W chicken PCR test. Primer sequence locations are in green and the probe in blue. The sequences originate from the NCBI database. Chicken is used as reference, different nucleotides in the other species are coloured in purple.*

Based on these alignments, 2 PCR methods retained the attention of EURL-AP.

The first one is the chicken PCR method of CRA-W that has a primer annealing perfectly with all poultry species. Nevertheless, further investigations on this target area did not allow to obtain a PCR method common to all poultry species. Indeed, there are too many differences in the target area to define a second common primer and a probe which would also be different enough from other animal species.

The second one is the chicken PCR method of Switzerland that has the forward primer fitting perfectly to the chicken and the turkey sequences if the primer is shortened by one base. Further investigations on this target did not allow to obtain a PCR method common to all poultry species either. However a test enabling simultaneous detection of chicken and turkey DNA could be developed (Figure 7). The EURL-AP searched for a reverse primer in the target area to allow the detection of *Gallus gallus* DNA but also *Meleagris gallopavo* DNA. The shortened forward primer from the Swiss test became the reverse primer of the new test for the detection of these 2 species (Figure 7). The specificity of the new test derives from the primers because sequences of the probe area show almost no differences with other bird species. Differences are more pronounced with other animals.

The primers and probe of this seventh method were also synthesized by the Eurogentec (Eurogentec s.a., Seraing, Belgium) to be evaluated experimentally.

Figure 7: *Alignment of 7 poultry mitochondrial DNA sequences of the target enabling simultaneous detection of chicken and turkey DNA. Primer sequence locations are in green and the probe in blue. The sequences originate from the NCBI database. Chicken is used as reference, different nucleotides in the other species are coloured in purple.*

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

The specificity of 7 PCR methods for the detection of "poultry" DNA was experimentally tested. The list consists of the six methods of Table 1 that were not discarded plus the chicken-turkey PCR test developed by the EURL-AP based on the received data. A wide variety of animal species were considered for the specificity. 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 DNAs were extracted from blood samples; when it was not possible, the DNAs were extracted from meat. In the case of fish species, extraction was carried out on muscle using samples obtained from a German institute (Federal Research Centre for Nutrition and Food, Department of Fish Quality, Hamburg) with reference certificates. DNAs from sea mammals were also tested. They were extracted from muscle samples collected by Belgian and Italian specialised institutes.

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

Each test was first checked against DNA samples from poultry species (Table 2) such as chicken – *Gallus gallus* –, turkey – *Meleagris gallopavo* –, duck – *Cairina moschata* –, guinea fowl – *Numida meleagris* – and goose – *Anser spp* –. Next to these poultry species, other species of birds were also analysed such as quail – *Coturnix japonica* –, pheasant – *Phasianus colchicus* –, pigeon – *Columba livia* –, black-backed gull – *Larus fuscus* – and the ostrich – *Struthio camelus* –. For almost all the species several animals were tested.

The pure species DNA extracts were at 2 ng/µl. Five µl of extract were tested per PCR. Each extract was tested in duplicate.

Table 2: *Specificity tests of 7 "poultry" targets carried out on DNA samples from different poultry species. PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) with Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng/µl. Five µl were tested per PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

	Poultry TNO Triskelion BV			**Chicken-Turkey EURL-AP**			**Chicken CRA-W**			**Chicken Krcmar and Rencova (2005)**			**Chicken Switzerland**			**Chicken Laube *et al.* (2007)**			**Turkey Laube *et al.* (2007)**		
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	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
Domestic birds																					
Gallus gallus	25.30 25.34	25.32	+	27.08 27.07	27.08	+	26.37 26.30	26.34	+	24.56 24.68	24.62	+	26.06 26.21	26.14	+	24.82 25.38	25.10*	+	50 50	50	-
***Meleagris gallopavo* (animal 1)**	25.79 25.76	25.78	+	26.94 26.91	26.93	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	29.19 29.19	29.19	+
***Meleagris gallopavo* (animal 2)**	25.69 25.73	25.71	+	26.28 26.39	26.34	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	28.46 28.67	28.57	+
***Numida meleagris* (animal 1)**	28.71 28.87	28.79	+	40.30 50	45.15*	?	50 50	50	-	37.65 50	43.83*	-**	39.70 50	44.85*	?	39.26 50	44.63*	?	50 50	50	-
***Numida meleagris* (animal 2)**	28.90 28.80	28.85	+	39.55 39.76	39.66	+	50 50	50	-	39.48 37.18	38.33*	-	38.14 38.34	38.24	+	50 50	50	-	50 50	50	-
***Cairina moschata* (animal 1)**	26.15 26.12	26.14	+	40.09 50	45.05*	?	50 50	50	-	50 50	50	-	39.94 50	44.97*	?	50 50	50	-	50 50	50	-
***Cairina moschata* (animal 2)**	26.10 26.10	26.10	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
***Cairina moschata* (animal 3)**	26.09 26.16	26.13	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
***Cairina moschata* (animal 4)**	25.97 25.99	25.98	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
***Cairina moschata* (animal 5)**	26.85 26.19	26.52*	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
***Anser spp.* (animal 1)**	25.79 25.74	25.77	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
***Anser spp.* (animal 2)**	25.79 25.64	25.72	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
***Anser spp.* (animal 3)**	25.88 25.84	25.86	+	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-

* > 0.5 cycle between replicates of the same extract
** ambiguous results also in second analysis, the final result is given negative
? ambiguous results
50 = no amplification signal

Table 3: *Specificity tests of 7 "poultry" targets carried out on DNA samples from different birds. PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) with Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng/µl. Five µl were tested per PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

	Poultry TNO Triskelion BV			**Chicken-Turkey EURL-AP**			**Chicken CRA-W**			**Chicken Krcmar and Rencova (2005)**			**Chicken Switzerland**			**Chicken Laube *et al.* (2007)**			**Turkey Laube *et al.* (2007)**		
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	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
Domestic birds																					
***Coturnix japonica* (animal 1)**	25.20 25.16	25.48	**+**	50 50	50	**-**	30.44 30.47	30.46	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
***Coturnix japonica* (animal 2)**	25.51 25.48	25.50	**+**	50 50	50	**-**	30.67 30.60	30.64	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
***Coturnix japonica* (animal 3)**	25.73 25.72	25.73	**+**	50 50	50	**-**	30.68 30.63	30.66	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
***Phasianus colchicus* (animal 1)**	25.25 25.24	25.25	**+**	36.88 36.77	36.83	**+**	50 50	50	**-**	50 50	50	**-**	39.68 50	44.84*	**?**	50 50	50	**-**	50 50	50	**-**
***Phasianus colchicus* (animal 2)**	25.15 25.47	25.31	**+**	37.18 37.02	37.10	**+**	50 50	50	**-**	38.98 50	44.49*	**-****	40.03 50	45.02*	**?**	39.34 50	44.67*	**?**	50 50	50	**-**
***Columba livia* (animal 1)**	31.97 31.97	31.97	**+**	36.62 36.54	36.58	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
***Columba livia* (animal 2)**	31.98 32.11	32.05	**+**	35.78 36.27	36.03	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
***Columba livia* (animal 3)**	32.16 32.07	32.12	**+**	35.75 35.93	35.84	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
Larus fuscus	25.98 26.20	26.09	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
***Struthio camelus* (animal 1)**	26.05 26.33	26.19	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
***Struthio camelus* (animal 2)**	26.26 26.20	26.23	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**
***Struthio camelus* (animal 3)**	26.23 26.23	26.23	**+**	50 50	50	**-**	50 50	50	**-**	50 50	50	**-**	38.36 50	44.18*	**?**	50 50	50	**-**	50 50	50	**-**

* > 0.5 cycle between replicates of the same extract
** ambiguous results also in second analysis, the final result is given negative
? ambiguous results
50 = no amplification signal

Table 3 shows the specificity results obtained on other bird species than poultry.

The "poultry" target of TNO detects not only poultry but all bird species tested. Figure 8 shows only one amplification curve per species tested, all animals of the same species give similar amplification signals.

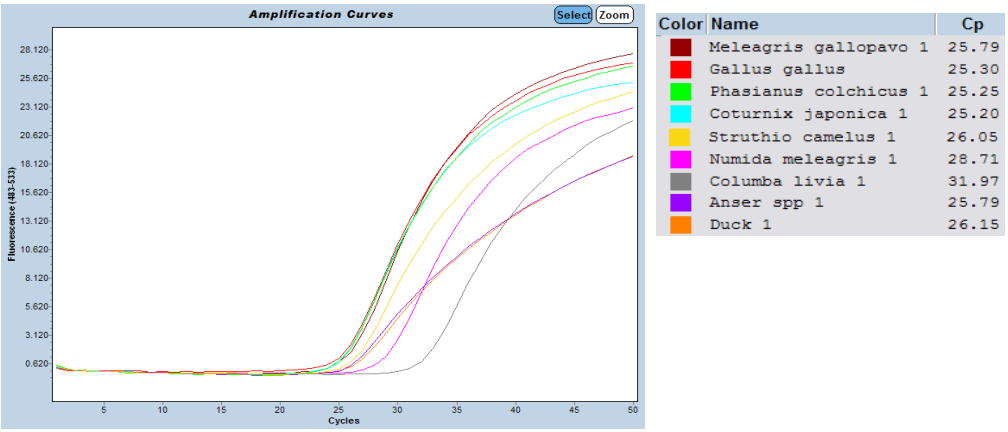

Figure 8: *Amplification signals obtained on DNAs extracted from 9 different bird species with the poultry PCR method of TNO. PCR was performed with a LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Only one amplification curve per species tested is presented, All animals of the same species and all replicates of the same animal give similar amplification signals. Analysis mode: Abs. quant/second derivative max and high confidence.*

The "chicken-turkey" PCR test of EURL-AP shows clear amplification signals with *Gallus gallus* DNA and *Meleagris gallopavo* DNA. Three other bird species give a late amplification signal. The two individuals of *Phasianus colchicus* provided an amplification curve with Ct values around 37 cycles. The three individuals of *Columba livia* species gave signals with Ct values close to 36 cycles but their amplification curve showed a poor efficiency (Figure 9). For *Numida meleagris*, amplification signals were late (Ct value close to 40 cycles) and not reproducible (Figure 10).

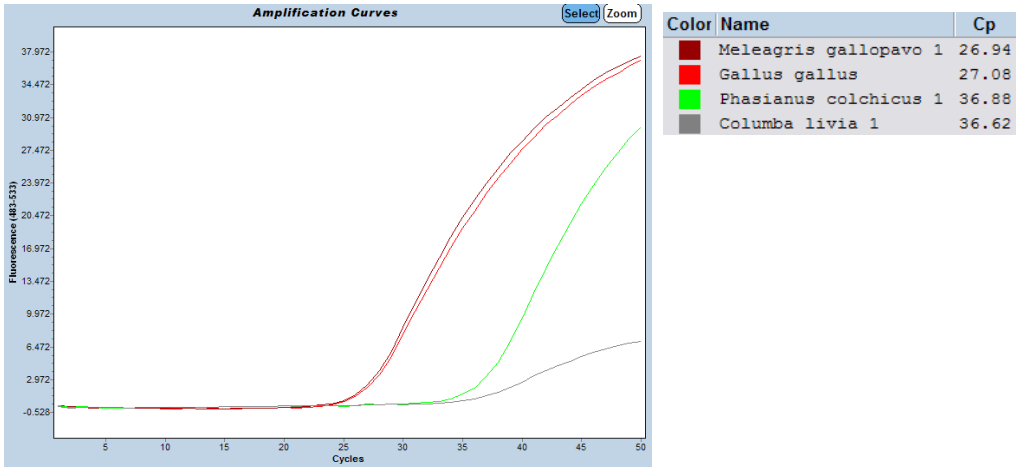

Figure 9: *Amplification signals obtained on DNAs extracted from 4 different birds with the "chicken-turkey" PCR method of EURL-AP. PCR was performed with a LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Only one amplification curve per species tested is presented, all animals of the same species and all replicates of the same animal give similar amplification signals. Analysis mode: Abs. quant/second derivative max and high confidence.*

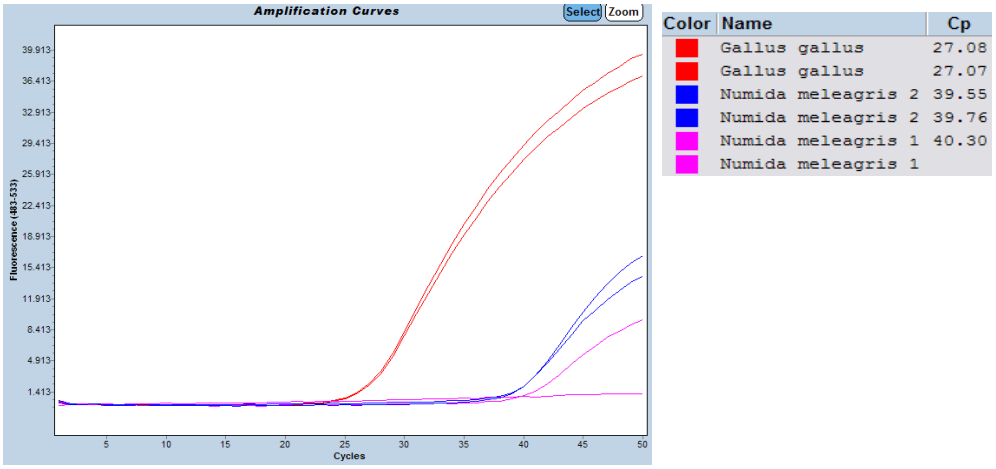

Figure 10: *Amplification signals obtained on DNAs extracted from Gallus gallus and Numida meleagris with the "chicken-turkey" PCR method of EURL-AP. PCR was performed with a LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

The chicken PCR test of CRA-W shows clear amplification signals with *Gallus gallus* DNA without any interference in the presence of other poultry species. However, it can show aspecificity in the presence of other species closely related to chicken. The three tested individuals of *Coturnix japonica* species gave amplification curves with Ct value between 30 and 31 cycles but the curves reached a much lower fluorescence level (Figure 11). A background signal was also observed with *Numida meleagris* DNA and *Phasianus colchicus* DNA (Figure 12).

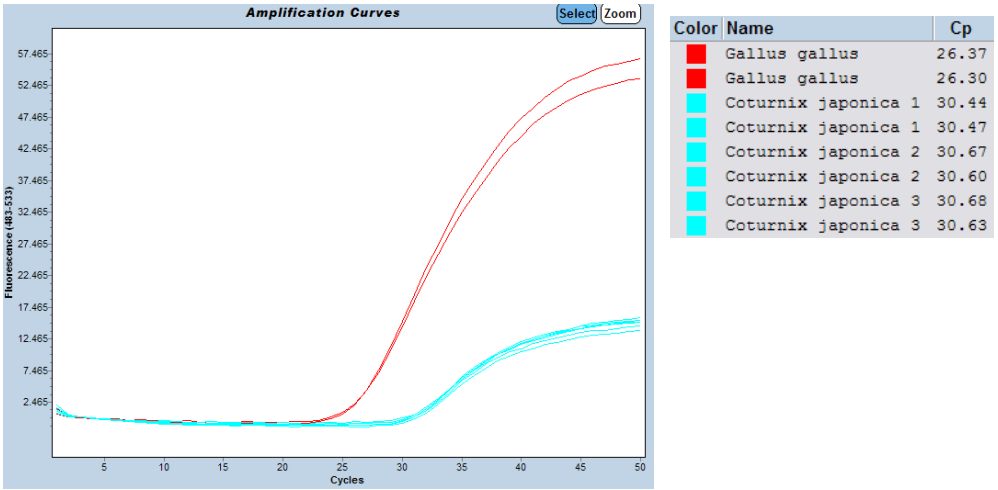

Figure 11: *Amplification signals obtained on DNAs extracted from Gallus gallus and Coturnix japonica with the chicken PCR method of CRA-W. PCR was performed with a LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

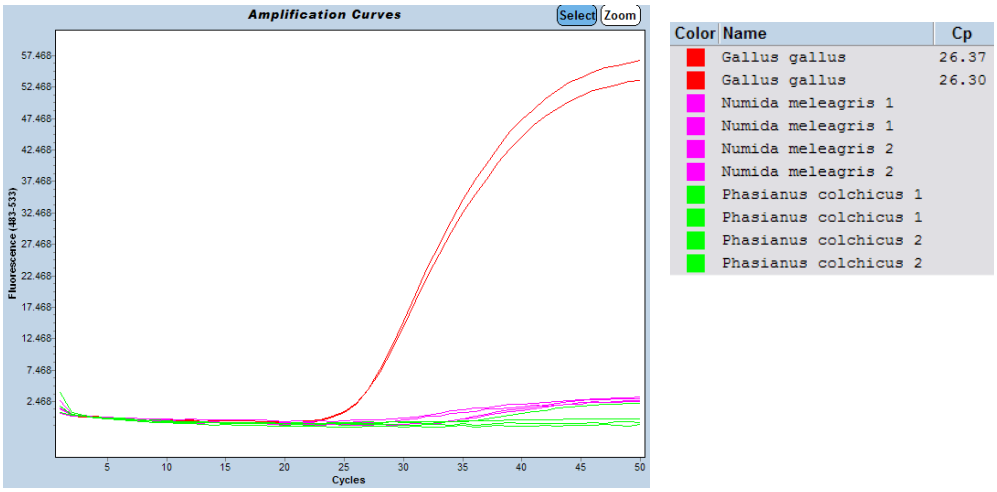

Figure 12: *Amplification signals obtained on DNAs extracted from Gallus gallus, Numida meleagris and Phasianus colchicus with the chicken PCR test of CRA-W. PCR was performed with a LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

The chicken PCR test proposed by Krcmar and Rencova (2005) gives amplification signals not only with *Gallus gallus* but also with *Numida meleagris* DNA (Figure 13). However, the amplification curves with *Numida meleagris* DNA were late and not always repeatable (1 signal on 2 replicates for animal 1). Moreover, the intensity of the fluorescence signal reached with chicken is rather low.

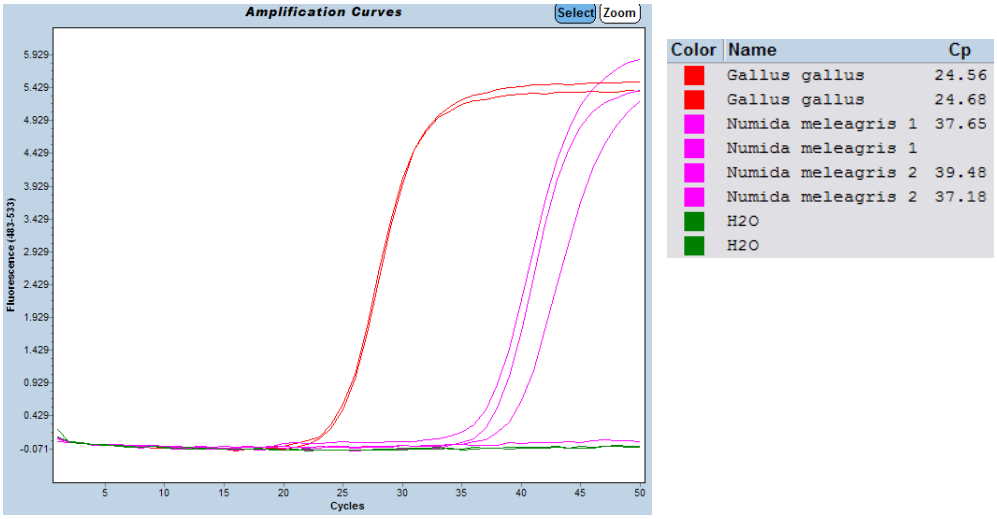

Figure 13: *Amplification signals obtained on DNAs extracted from Gallus gallus and Numida meleagris and no template control (H_2O) with the chicken PCR method of Krcmar and Rencova (2005). PCR was performed with a LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

The chicken PCR method coming from Switzerland shows clear amplification signals with *Gallus gallus* DNA. Late signals were obtained with *Numida meleagris* and *Phasianus colchicus* DNA. Amplification on these two species is not always repeatable (1 signal on 2 replicates for animal 1 of *Numida meleagris* and 1 signal on 2 replicates for both animal of *Phasianus colchicus*) (Figure 14).

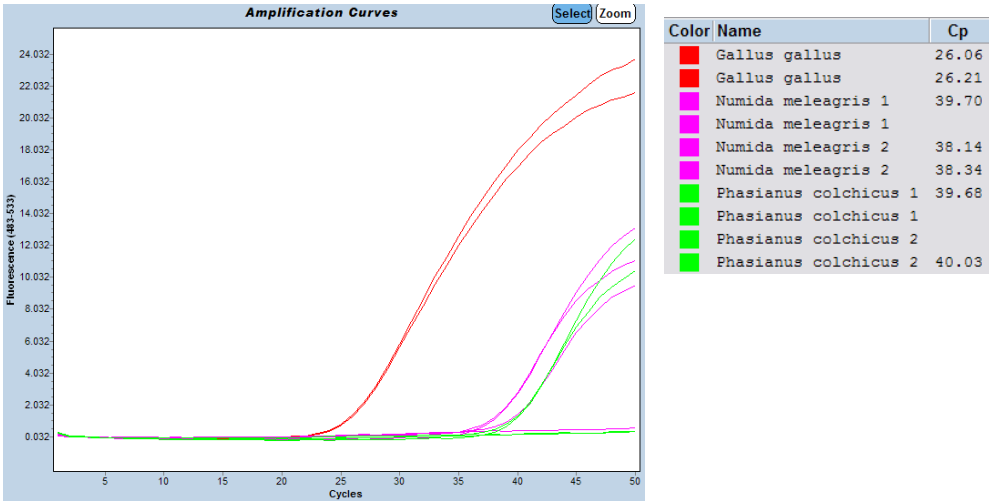

Figure 14: *Amplification signals obtained on DNAs extracted from Gallus gallus, Numida meleagris and Phasianus colchicus with the chicken PCR method coming from Switzerland. PCR was performed with a LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

The chicken PCR method published by Laube *et al.* (2007) gives amplification signals with *Gallus gallus* DNA. However, the fluorescence level reached is low and the shape of the amplification curves does not look fine (Figure 15).

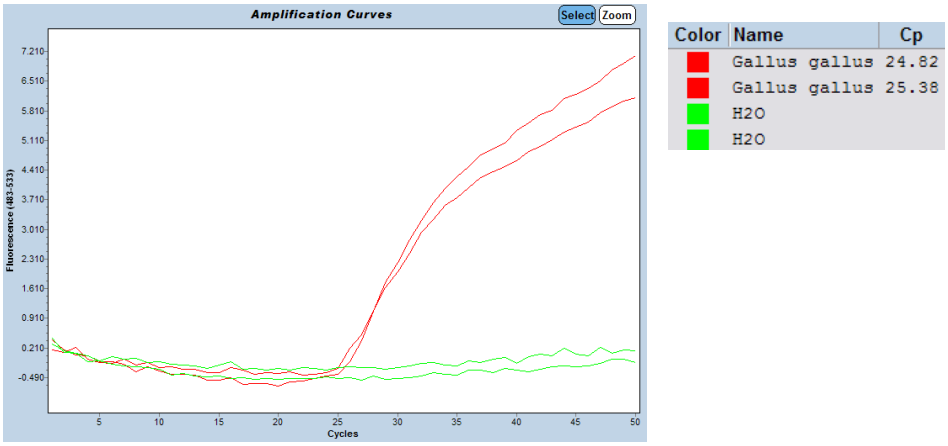

Figure 15: *Amplification signals obtained on DNAs extracted from Gallus gallus and no template control (H_2O) with the chicken PCR method of Laube et al. (2007). PCR was performed with a LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

The same remarks can be given about the turkey PCR method published by Laube *et al.* (2007). It detects *Meleagris gallopavo* DNA but the fluorescence level reached is low and the amplification curves do not show a nice shape (Figure 16).

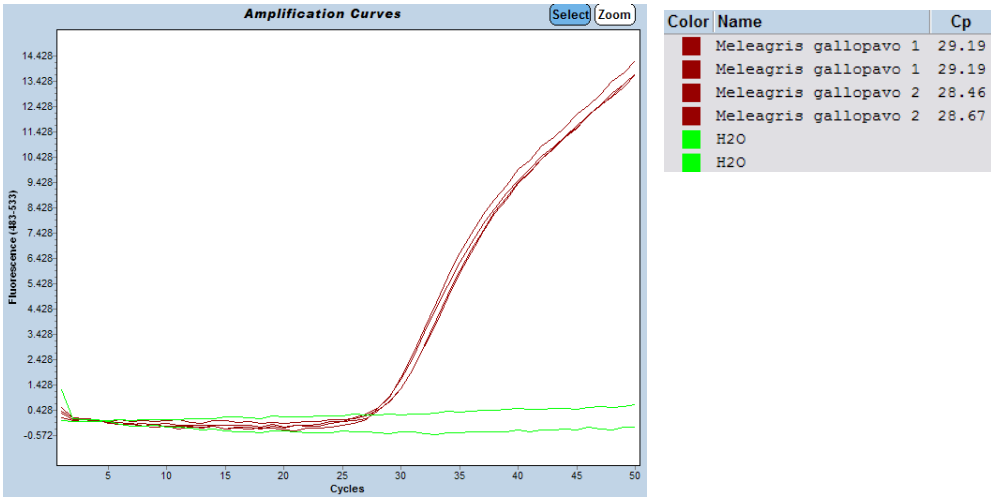

Figure 16: *Amplification signals obtained on DNAs extracted from Meleagris gallopavo and no template control (H_2O) with turkey PCR method of Laube et al. (2007). The PCR performed with the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

The tests of specificity continued with DNA samples from terrestrial and sea mammalian species. Cattle - *Bos Taurus* -, sheep - *Ovis aries* -, goat - *Capra hircus* -, pig - *Sus scrofa domesticus* -, were of course tested but also game species (stag - *Cervus elaphus* -, roe deer - *Capreolus capreolus* - and wild boar - *Sus scrofa scrofa*). 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 for the terrestrial mammals. The possibility of a cross-reaction with human - *Homo sapiens* - DNA was also envisaged. Some sea mammals present in our sample bank were tested too. Tables 4 and 5 summarize the results obtained on a LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.).

Except a very late and not repeatable signal on *Ovis aries* DNA with the chicken test of Laube *et al.* (2007), all the PCR assays were specific on the several samples of tested mammalian DNA.

Table 4: *Specificity tests of 7 "poultry" targets carried out on sample DNA from terrestrials mammals. PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) with Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng/µl. Five µl were tested per PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

	Poultry TNO Triskelion BV			**Chicken-Turkey EURL-AP**			**Chicken CRA-W**			**Chicken Krcmar and Rencova (2005)**			**Chicken Switzerland**			**Chicken Laube *et al.* (2007)**			**Turkey Laube *et al.* (2007)**		
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	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
Terrestrial mammalians																					
Homo sapiens	50 50	50	-	50 50	50	-	50 50	50	-	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	-	50 50	50	-	50 50	50	-	50 50	50	-
Ovis aries	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	38.80 50	44.40*	**?**	50 50	50	-
Capra hircus	50 50	50	-	50 50	50	-	50 50	50	-	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	-	50 50	50	-	50 50	50	-	50 50	50	-
Capreolus capreolus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Sus scrofa scrofa	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-
Sus scrofa domesticus	50 50	50	-	50 50	50	-	50 50	50	-	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	-	50 50	50	-	50 50	50	-	50 50	50	-
Equus caballus	50 50	50	-	50 50	50	-	50 50	50	-	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	-	50 50	50	-	50 50	50	-	50 50	50	-
Rattus rattus	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-	50 50	50	-

* > 0.5 cycle between replicates of the same extract
? ambiguous results
50 = no amplification signal

Table 5: *Specificity tests of 7 "poultry" targets carried out on samples DNA from sea mammals. PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) with Universal Mastermix (Diagenode s.a.). Pure species DNA extracts were at 2 ng/µl. Five µl were tested per PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

	Poultry TNO Triskelion BV			**Chicken-Turkey EURL-AP**			**Chicken CRA-W**			**Chicken Krcmar and Rencova (2005)**			**Chicken Switzerland**			**Chicken Laube at al. (2007)**			**Turkey Laube at al. (2007)**		
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	C_t	Mean C_t	Result	C_t	Mean C_t	Result	C_t	Mean C_t	Result
Sea mammals																					
Stenella coeruleoalba	50 50	50	-	50 50	50	-	50 50	50	-	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	-	50 50	50	-	50 50	50	-	50 50	50	-
Grampus griseus	50 50	50	-	50 50	50	-	50 50	50	-	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	-	50 50	50	-	50 50	50	-	50 50	50	-
Phocoena phocoena	50 50	50	-	50 50	50	-	50 50	50	-	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	-	50 50	50	-	50 50	50	-	50 50	50	-

50 = no amplification signal

Considering that the PCR test should be able to detect poultry DNA, a first choice was made on assays detecting at least the two most abundant poultry species which are chicken and turkey. This limits the list to 2 PCR tests, the "poultry" test of TNO Triskelion BV and the "chicken-turkey" test of EURL-AP.

Sensitivity analysis started on these 2 PCR tests with samples containing avian material at different levels (Table 6).

Table 6: *Sensitivity analysis of the "poultry" PCR test from TNO Triskelion BV and the "chicken-turkey" PCR test from EURL-AP with samples containing avian material at different levels. PCR was performed on a LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Results obtained on extracts 3 fold diluted. Analysis mode: Abs. quant/second derivative max and high confidence.*

		Poultry TNO Triskelion BV			**Chicken-Turkey EURL-AP**		
Sample number and composition	N° extract	C_t	Mean C_t	Result	C_t	Mean C_t	Result
CRL 552 **Poultry meal standard (processed according to method 7)[16]**	1	24.96 24.86	24.91	+	17.72 17.73	17.73	+
	2	24.90 24.87	24.89	+	17.91 17.93	17.92	+
CRL 550 **Feather meal**	1	50 50	50	-	34.47 34.10	34.29	+
	2	50 50	50	-	34.27 34.39	34.33	+
CRL 1154 **Ring test VLA PCR – sample with chicken PAP**	1	35.63 36.37	36.00*	+	30.06 30.11	30.09	+
	2	36.58 36.02	36.30*	+	29.75 29.73	29.74	+
CRL 1448 **Ring test VLA PCR – sample with chicken PAP**	1	36.14 35.61	35.88*	+	31.06 31.10	31.08	+
	2	37.22 37.57	37.40	+	32.24 32.30	32.27	+
CRL 1394 **Ring test IAG microscopy – sample with chicken PAP**	1	35.40 35.68	35.54	+	30.98 30.95	30.97	+
	2	36.58 35.13	35.86*	+	30.95 30.95	30.95	+
CRL 1341 **1 % chicken PAP (treated at 133 °C) in soybean**	1	37.14 38.93	38.04*	+	34.69 34.71	34.70	+
	2	39.16 39.19	39.18	+	35.04 35.24	35.14	+
CRL 1340 **0.2 % chicken PAP (treated at 133 °C) in soybean**	1	37.47 50	43.74*	-**	35.64 35.85	35.75	+
	2	50 50	50	-	35.98 36.27	36.13	+
CRL 1365 **0.2 % chicken PAP (processed according to method 7) in soybean**	1	33.01 32.57	32.79	+	28.54 28.66	28.60	+
	2	33.85 33.32	33.59*	+	28.72 28.76	28.74	+

* > 0.5 cycle between replicates of the same extract
** ambiguous results between PCR replicates in first and second analysis, in final negative result
50 = no amplification signal

Results listed in Table 6 show that the "chicken-turkey" PCR test from EURL-AP is more sensitive than the "poultry" PCR test from TNO. Therefore the "chicken-turkey" PCR test is selected for further analysis as a "poultry" PCR assay.

The "chicken-turkey" PCR method was submitted to further specificity tests with DNA from Common blackbird (*Turdus merula*), from fish and from plant species. Table 7 shows the results obtained on a LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). No aspecificity was detected.

[16] **Method 7:** For these materials the temperature is minimum 90 °C for 30 minutes on cooking side. But with drying they get on top 60 minutes more with approximately 95 °C. There was no pressure on the product.

Table 7: *Further specificity tests of the "chicken-turkey" PCR method of EURL-AP carried out against non-target DNA samples. PCR was performed on a LC480 thermocycler (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). All DNAs were at 2 ng/µl and 5 µl were tested in each PCR. Analysis mode: Abs. quant/second derivative max and high confidence.*

Species	C_t	Mean C_t	Result	**Species**	C_t	Mean C_t	Result
Bird species							
Turdus merula	50 50	50	-				
Fish species							
Gadus morhua	50 50	50	-	***Trisopterus minutus***	50 50	50	-
Pollachius virens	50 50	50	-	***Sardina pilchardus***	50 50	50	-
Melanogrammus aeglefinus	50 50	50	-	***Engraulis encrasicolus***	50 50	50	-
Micromesistius poutassou	41.84 50	45.92 *	-	***Gadus ogac***	50 50	50	-
Sebastes spp.	50 50	50	-	***Trisopterus esmarki***	50 50	50	-
Mallotus villosus	50 50	50	-	***Ammodytes lancea***	50 50	50	-
Scomber scombrus	50 50	50	-	***Sprattus sprattus***	50 50	50	-
Clupea harengus	42.01 50	46.01 *	-	***Salmo salar***	50 50	50	-
Merluccius merluccius	50 50	50	-	***Raja spp.***	50 50	50	-
Trachurus trachurus	50 50	50	-				
Crustacean							
Paralithodes camtschaticus	50 50	50	-				
Plant species							
Glycine max	50 50	50	-	***Oryza sativa***	50 50	50	-
Zea mays	50 50	50	-	***Lycopersicon esculentum***	50 50	50	-
Brassica napus	50 50	50	-	***Beta vulgaris***	50 50	50	-
Triticum aestivum	50 50	50	-				

* > 0.5 cycle between replicates of the same extract
50 = no amplification signal

2.3. Specificity of the primers of the chicken-turkey PCR test with SYBR Green

Table 8 presents the results obtained with DNAs extracted from *Gallus gallus* and *Meleagris gallopavo* blood samples and with no template control. Melting curves of *Gallus gallus* and *Meleagris gallopavo* show a well-defined peak with a maximum close to 80 and 79.2 °C respectively. The PCR negative controls gave late amplification signals and a peak melting curve close to 86.5 °C.

Table 8: *Specificity tests of the "chicken-turkey" PCR method from EURL-AP carried out with target DNA of animal species and no template control. PCR assays was performed on a 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
Domestic birds				
Gallus gallus	25.93 25.89	25.91	80.24 80.08	/ /
Meleagris gallopavo **(animal 1)**	25.11 25.07	25.09	79.37 79.23	/ /
Meleagris gallopavo **(animal 2)**	28.40 28.13	28.27	79.24 79.26	/ /
No template control	37.53 38.20	37.87	86.55 86.74	/ /

The same test was performed on DNA from non-target species. Tables 9, 10 and 11 summarise the results. Melting curves of which fluorescence level did not exceed the level of the no template control were not taken into account.

Table 9: *Specificity tests of the "chicken-turkey" PCR method from EURL-AP carried out with non-target DNA of domestic bird species. PCR was performed on a 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
Domestic birds							
Numida meleagris **(animal 1)**	37.74 37.30	37.52	/ 80.64	***Coturnix japonica*** **(animal 1)**	37.54 36.93	37.24	77.77 /
Numida meleagris **(animal 2)**	37.76 37.61	37.69	/ 80.20	***Coturnix japonica*** **(animal 2)**	37.23 37.32	37.28	/ /
Cairina moschata **(animal 1)**	36.88 37.37	37.13	79.61 /	***Coturnix japonica*** **(animal 3)**	37.50 36.72	37.11	79.28 79.57
Cairina moschata **(animal 2)**	36.65 36.50	36.58	/ /	***Phasianus colchicus*** **(animal 1)**	35.91 35.55	35.73	79.24 /
Cairina moschata **(animal 3)**	36.74 36.52	36.63	/ /	***Phasianus colchicus*** **(animal 2)**	35.99 36.23	36.11	/ 83.47
Cairina moschata **(animal 4)**	37.39 36.39	36.89	79.17 /	***Columba livia*** **(animal 1)**	35.16 35.46	35.31	76.45 76.74
Cairina moschata **(animal 5)**	36.81 35.87	36.34	/ /	***Columba livia*** **(animal 2)**	36.70 35.85	36.28	85.19 76.78
Anser spp. **(animal 1)**	36.92 37.09	37.01	/ /	***Columba livia*** **(animal 3)**	35.53 35.34	35.44	76.78 76.78
Anser spp. **(animal 2)**	36.27 37.05	36.66	80.05 /	***Struthio camelus*** **(animal 1)**	35.86 35.80	35.83	/ /
Anser spp. **(animal 3)**	36.92 36.47	36.70	/ /	***Struthio camelus*** **(animal 2)**	35.61 35.64	35.63	/ 81.23
				Struthio camelus **(animal 3)**	35.85 36.25	36.05	81.26 81.15

Table 10: *Specificity tests of the "chicken-turkey" PCR method from EURL-AP carried out with non-target DNA of various animal species. PCR was performed on a 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
Terrestrial mammalians									
Homo sapiens	36.35 36.68	36.52	81.34 81.37	/ /	***Sus scrofa domesticus***	35.94 35.83	35.89	79.20 79.20	/ /
Bos taurus	37.52 37.96	37.74	/ /	/ /	***Sus scrofa scrofa***	35.85 35.65	35.75	79.24 79.24	/ /
Ovis aries	37.38 37.10	37.24	79.53 /	/ /	***Equus asinus***	36.32 36.36	36.34	82.07 82.03	/ /
Capra hircus	35.12 35.21	35.17	83.52 83.50	/ /	***Equus caballus***	35.51 35.59	35.55	81.52 81.70	/ /
Cervus elaphus	38.46 37.87	38.17	/ /	/ /	***Lepus europaeus***	36.13 36.04	36.09	81.74 /	/ /
Capreolus capreolus	37.17 37.39	37.28	78.95 79.24	/ /	***Rattus rattus***	36.31 36.50	36.41	78.91 /	/ /
Sea mammals									
Stenella coeruleoalba	37.31 37.85	37.58	80.23 80.57	/ /	***Ziphius cavirostris***	37.58 38.25	37.92	80.23 80.49	/ /
Tursiops truncatus	38.34 38.81	38.58	80.26 80.23	/ /	***Phocoena phocoena***	33.36 33.56	33.46	86.54 86.54	/ /
Grampus griseus	37.11 37.49	37.30	79.50 /	/ /	***Phocidae***	38.38 38.72	38.55	84.37 79.90	/ /
Fish species									
Gadus morhua	35.65 35.80	35.73	79.35 79.20	/ /	***Trisopterus minutus***	35.30 35.04	35.17	79.50 79.20	/ /
Pollachius virens	35.81 36.64	36.23	79.16 79.05	/ /	***Sardina pilchardus***	36.93 36.51	36.72	79.18 79.46	/ /
Melanogrammus aeglefinus	35.80 36.26	36.03	79.16 79.16	/ /	***Engraulis encrasicolus***	40.12 39.89	40.01	79.35 /	/ /
Micromesistius poutassou	35.53 35.48	35.51	79.16 79.39	/ /	***Gadus ogac***	36.63 36.01	36.32	79.27 79.39	/ /
Sebastes spp.	36.19 35.93	36.06	79.31 79.24	/ /	***Trisopterus esmarki***	35.04 34.96	35.00	80.42 80.15	/ /
Mallotus villosus	36.65 37.53	37.09	79.39 79.50	/ /	***Ammodytes lancea***	35.25 35.27	35.26	79.12 79.16	/ /
Scomber scombrus	36.94 37.67	37.31	79.50 79.50	/ /	***Sprattus sprattus***	38.58 38.92	38.75	79.43 80.45	/ /
Clupea harengus	36.73 36.26	36.50	79.46 79.50	/ /	***Salmo salar***	39.11 38.13	38.62	/ 82.66	/ /
Merluccius merluccius	35.63 35.57	35.60	79.16 79.12	/ /	***Raja spp.***	39.49 40.75	40.12	79.35 79.29	81.29 /
Trachurus trachurus	37.01 36.16	36.59	79.20 79.48	/ /					
Crustacean									
Paralithodes camtschaticus	44.36 41.44	42.90	/ 78.17	/ /					

Table 11: *Specificity test of the "chicken-turkey" PCR method of EURL-AP carried out against non-target DNA of various plant species. PCR was performed on a LC480 (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	**Species**	C_t	Mean C_t	Tm 1
Plant species							
Glycine max	35.93 36.80	36.37	/ /	***Oryza sativa***	37.36 37.08	37.22	/ 79.16
Zea mays	35.40 36.20	35.80	82.81 /	***Lycopersicon esculentum***	36.92 37.13	37.03	/ /
Brassica napus	34.58 34.57	34.58	84.07 83.72	***Beta vulgaris***	37.26 38.22	37.74	/ /
Triticum aestivum	34.72 34.90	34.81	/ 87.94				

The "chicken-turkey" PCR assay from EURL-AP in SYBR® Green format delivered a Ct above 35 cycles on most of the tested non-target DNA samples of animal or plant origin. The fish species gave melting curves with Tm values close to those obtained on *Gallus gallus* and *Meleagris gallopavo*. Nevertheless, given the Ct values obtained with these fish species, there is no specificity problem linked to the primers of the PCR assay.

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

To complete the evaluation of the method with performance parameters, the efficiency and the absolute LOD were measured with calibrants consisting of a dilution of a pUC18 plasmid in which the "chicken-turkey" PCR target had been cloned.

The cloned target sequence was checked by sequencing outsourced to Beckman Coulter Genomics firm (Bishop's Stortford, United Kingdom) (Table 12).

Table 12: *Comparison of the theoretical sequence of the NCBI databases and the sequence obtained by Beckman Coulter Genomics firm (Bishop's Stortford, United Kingdom) with plasmid. The size of fragment of "chicken-turkey" is of 84 bp. Primer sequence locations are in green and the probe in blue.*

	Fragment of "chicken-turkey EURL-AP" with primers sequence in green and probe sequence in blue
Theoretical sequence (NC_001323.1)	TAGACTACCAAGGCGTAGCTATAACTTCAAAGCATTCAGCTTACACCTGAAAGATACCCTCAACAGACAAGGTCGCCTTGACTT
Sequencing sequence	TAGACTACCAAGGCGTAGCTATAACTTCAAAGCATTCAGCTTACACCTGAAAGATACCCTCAACAGACAAGGTCGCCTTGACTT

This plasmid was used by JRC (Joint Research Centre, Reference Material unit, Geel, Belgium), to produce the three calibrants required for the cut-off value determination.

These calibrants produced by JRC were provided at 103, 24 and 8 copies/µl. The cut-off value of the platform combining a LightCycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and the Universal Mastermix (Diagenode s.a., Seraing, Belgium) was determined on 4 PCR plates with 12 replicates per concentration on each plate.

Analysis of results was carried out in "Abs. quant/second derivative max and high confidence" mode. Cut-off values (in cycles) at 1, 5, 10 and 15 copies were determined and are presented in Table 13.

Table 13: *Determination of cut-off values at different copy numbers for the PCR platform with LC480 thermocycler (Roche Diagnostics Ltd.) of the EURL-AP using the Universal Mastermix (Diagenode s.a.).*

Copies number used to determine the cut-off values	Cut-off value (in cycles)
1 copy	41.46
5 copies	38.96
10 copies	37.88
15 copies	37.25

All the runs performed on the same 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 concentrations at 5000, 2500, 1000, 500 and 100 copies / 5 µl respectively with 5 µl as volume of sample in the reaction. Six replicates per concentration were performed.

The tests were performed on a LightCycler 480 (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 setting of parameters.

The "chicken-turkey" PCR test efficiency is 94.22 %. The efficiency calculated on each plate is always higher than 90 %. Acceptance criterion for the efficiency is to get a value within the range 80-120 %.

4.2. LOD

The LOD of the PCR assay was calculated with dilutions of plasmid at low copy numbers (50 - 20 - 10 - 5 - 2 and 1 copy/5 µl respectively) in a solution with a background of autoclaved DNA from maize at 12 ng/µl. Two runs including 6 replicates per concentration were performed.

LOD_6 is determined starting from the results of these two runs. LOD_6 is the smallest copy number giving 6 signals out of 6 replicates of 2 runs.

The copy number corresponding to LOD_6 is then tested 60 times on the same plate. The LOD is validated if at least 95 % of signals are recorded out of the 60 replicates.

The tests were performed on a LightCycler 480 (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 platform were analysed using the same setting of parameters.

According to the acceptance criterion, the LOD must not be higher than 20 target copies. With a cut-off set at 1 copy or 5 copies, the LOD is less than 10 copies. With a cut-off set at 10 or 15 copies, the LOD is less than 20 copies for the platform tested. The acceptance criterion is reached.

Table 14: *Absolute LOD determined on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) with plasmid dilutions at 10 and 20 copies/5 µl performed. Analysis mode: Abs. quant/second derivative max and high confidence. Results calculated according cut-off set at 1, 5, 10 and 15 copies.*

	Positive results in % and number of positive reactions on 60 replicates (raw data between brackets) obtained with a cut-off set at			
Copy number tested for LOD	**1 copy [41.46 cycles]**	**5 copies [38.96 cycles]**	**10 copies [37.88 cycles]**	**15 copies [37.25 cycles]**
10	100 % (60/60)	100 % (60/60)		
20	100 % (60/60)	100 % (60/60)	100 % (60/60)	100 % (60/60)

4.3. Sensitivity

DNA from samples with known PAP contents (samples free of poultry material and samples containing 0.1 % of poultry PAPs) were analysed on a LightCycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) as real-time thermocycler using the Universal Mastermix (Diagenode s.a., Seraing, Belgium). The results were analysed in “Abs. quant/second derivative max and high confidence” mode.

Four tables summarise the results obtained with samples free of poultry material (Table 15), pure PAP samples (Table 16), samples containing 0.1 % in mass fraction of poultry PAPs (Tables 17 and 18). The DNAs were tested at the 3-fold and 30-fold dilutions. Only the results of the 3 fold dilutions are presented as the data at 30-fold dilution would not change the outcome. Except one late unrepeatable signal on a pure ruminant PAP, all samples free of poultry material delivered a negative result ; all samples containing poultry PAPs, even those at 0.1 % in mass fraction, gave rise to amplification curves that could be considered as positive at the various cut-off levels considered.

Table 15: *Sensitivity tests with samples free of poultry material performed on a LC480 (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Result interpretations according to cut-off values 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 nr and Description	**Extract**	**Dilution**	**C_t**	**Results interpretation with a cut-off set at**			
				1 copy (41.46)	**5 copies (38.96)**	**10 copies (37.88)**	**15 copies (37.25)**
CRL 2815 Broilers feed	1	3x	50 50 50	-	-	-	-
	2	3x	50 50 50	-	-	-	-
CRL 2821 Turkey feed	1	3x	50 50 50	-	-	-	-
	2	3x	50 50 50	-	-	-	-
CRL 2734 Hens feed	1	3x	50 50 50	-	-	-	-
	2	3x	50 50 50	-	-	-	-
CRL 2676 Pig feed	1	3x	50 50 50	-	-	-	-
	2	3x	50 50 50	-	-	-	-
CRL 2579 Fish feed	1	3x	50 50 50	-	-	-	-
	2	3x	50 50 50	-	-	-	-
CRL 2059 Pig PAP	1	3x	50 50 50	-	-	-	-
	2	3x	50 50 50	-	-	-	-
CRL 2706 Ruminant PAP	1	3x	39.75 50 50	?**	-	-	-
	2	3x	50 50 50	-	-	-	-

** ambiguous result for the first analysis and negative result for the second analysis, final conclusion negative result

Table 16: *Sensitivity tests with samples of poultry material performed on a LC480 (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Result interpretations according to cut-off values 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 nr and Description	Extract	Dilution	C_t	Mean C_t	Results interpretation with a cut-off set at			
					1 copy (41.46)	5 copies (38.96)	10 copies (37.88)	15 copies (37.25)
CRL 552 Poultry PAP (1)	1	3x	19.60 19.73 19.79	19.71	+	+	+	+
	2	3x	19.43 19.55 19.66	19.55	+	+	+	+
CRL 549 Poultry PAP (2)	1	3x	22.04 22.09 22.09	22.07	+	+	+	+
	2	3x	21.94 22.02 22.03	22.00	+	+	+	+
CRL 1563 Poultry PAP (3)	1	3x	19.40 19.49 19.52	19.47	+	+	+	+
	2	3x	19.52 19.54 19.62	19.56	+	+	+	+
CRL 545 Poultry PAP (4)	1	3x	24.79 24.94 24.98	24.90	+	+	+	+
	2	3x	24.97 25.08 25.13	25.06	+	+	+	+
CRL 1564 Poultry PAP (5)	1	3x	21.76 21.77 21.82	21.78	+	+	+	+
	2	3x	21.62 21.67 21.73	21.67	+	+	+	+

Table 17: *Sensitivity tests with samples containing 0.1 % (in mass fraction) of different poultry PAPs in a same background matrix. Tests were performed on a LC480 (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.). Result interpretations according to cut-off values 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 nr and Description	**Extract**	**Dilution**	**C_t**	**Mean C_t**	**Results interpretation with a cut-off set at**			
					1 copy (41.46)	**5 copies (38.96)**	**10 copies (37.88)**	**15 copies (37.25)**
CRL 2834 0.1 % poultry PAP (1) in broilers feed (CRL 2815)	1	3x	27.76 27.78 27.83	27.79	+	+	+	+
	2	3x	27.36 27.38 27.39	27.38	+	+	+	+
CRL 2835 0.1 % poultry PAP (2) in broilers feed (CRL 2815)	1	3x	28.75 28.85 28.87	28.82	+	+	+	+
	2	3x	28.43 28.50 28.57	28.50	+	+	+	+
CRL 2836 0.1 % poultry PAP (3) in broilers feed (CRL 2815)	1	3x	27.95 27.99 28.01	27.98	+	+	+	+
	2	3x	28.10 28.14 28.14	28.13	+	+	+	+
CRL 2837 0.1 % poultry PAP (4) in broilers feed (CRL 2815)	1	3x	30.73 30.92 30.96	30.87	+	+	+	+
	2	3x	30.31 30.31 30.44	30.35	+	+	+	+
CRL 2838 0.1 % poultry PAP (5) in broilers feed (CRL 2815)	1	3x	30.19 30.23 30.24	30.22	+	+	+	+
	2	3x	30.24 30.25 30.29	30.26	+	+	+	+

Table 18: *Sensitivity tests with samples containing 0.1 % (mass fraction) of a same poultry PAP in different feed matrices. The poultry PAP used is the one giving the latest Ct values in Table 16. Tests were performed on a LC480 (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 nr and Description	**Extract**	**Dilution**	**C_t**	**Mean C_t**	**Results interpretation with a cut-off set at**			
					1 copy (41.46)	**5 copies (38.96)**	**10 copies (37.88)**	**15 copies (37.25)**
CRL 2839 0.1 % poultry PAP (4) in turkey feed (CRL 2821)	1	3x	35.23 35.25 35.31	35.26	+	+	+	+
	2	3x	33.88 33.98 34.12	33.99	+	+	+	+
CRL 2840 0.1 % poultry PAP (4) in hen feed (CRL 2734)	1	3x	30.58 30.71 30.77	30.69	+	+	+	+
	2	3x	30.72 30.76 30.80	30.76	+	+	+	+
CRL 2841 0.1 % poultry PAP (4) in pig feed (CRL 2676)	1	3x	29.10 29.12 29.18	29.13	+	+	+	+
	2	3x	29.69 29.74 29.75	29.73	+	+	+	+
CRL 2842 0.1 % poultry PAP (4) in fish feed (CRL 2579)	1	3x	30.05 30.12 30.22	30.13	+	+	+	+
	2	3x	30.06 30.19 30.20	30.15	+	+	+	+
CRL 2843 0.1 % poultry PAP (4) in pig PAP (CRL 2059)	1	3x	33.96 34.12 34.72	34.27*	+	+	+	+
	2	3x	34.34 34.40 34.49	34.41	+	+	+	+
CRL 2844 0.1 % poultry PAP (4) in ruminant PAP (CRL 2706)	1	3x	35.03 35.18 35.33	35.18	+	+	+	+
	2	3x	35.15 35.71 36.02	35.63*	+	+	+	+

* > 0.5 cycle between replicates of the same extract

4.4. 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 values set at 1, 5, 10 and 15 copies respectively. These results are summarized in Table 19 with the rates of false positive and false negative results.

With the cut-off set at 5 copies, there is an aspecificity with *Numida meleagris* and *Cairina moschata*.

A cut-off set at 15 copies was chosen as the best compromise to avoid false positive results in future analyses.

Table 19: *Number of false negative and false positive results with the samples used for specificity and sensitivity tests on the LC480 thermocycler (Roche Diagnostics Ltd.) using the Universal Mastermix (Diagenode s.a.) according to cut-off values 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 (41.46 cycles)		5 copies (38.96 cycles)		10 copies (37.88 cycles)		15 copies (37.25 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 poultry material	15	0	/	0	/	0	/	0	/
Samples containing 0.1 % (w/w) porcine PAP	11	/	0	/	0	/	0	/	0
Domestic bird species other than chicken and turkey*	9	/	2 and 2 ambiguous samples	/	2	/	2	/	2
Terrestrial mammals	12	/	0	/	0	/	0	/	0
Sea mammals	6	/	0	/	0	/	0	/	0
Fish and crab species	20	/	0	/	0	/	0	/	0
Plant species	7	/	0	/	0	/	0	/	0
Pure PAP without poultry material	2	/	0 and 1 ambiguous sample	/	0	/	0	/	0
Feed samples without poultry material	5	/	0	/	0	/	0	/	0
% of false results (calculated on 15 samples for the false negative results and on 72 samples for the false positive results)		0	2.8	0	2.8	0	2.8	0	2.8

*Species are listed in Tables 2 and 3 with in addition Black bird

4.5. Robustness

The robustness of the method was tested by deviations from the experimental conditions on two thermocyclers (one LC480 and one QuantStudio6): the annealing temperature (50 °C +/- 1 °C), the primer concentrations (standard or reduced by 30 %), the probe concentration (standard or reduced by 30 %) and the PCR volume (25 µl +/- 1 µl). Six replicates of plasmid at 20 copies/5 µl (thus using normally 20 copies per PCR) were performed in the conditions described in Table 20.

To determine the positive or negative character of amplification on a second platform, the cut-off value was determined for the platform combining the real-time thermocycler QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with Brilliant II QPCR Low ROX Master Mix (Agilent technologies, Santa Clara, CA, USA).

Table 20: *Experimental conditions tested to evaluate the robustness of method*

PCR machine	LC480 (Roche Diagnostics Ltd.) and QuantStudio6 (Applied Biosystems)				
PCR reagent kit	Universal Mastermix (Diagenode s.a.) and Brilliant II QPCR Low ROX Master Mix (Agilent technologies)				
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 % cases. The numbers of positive results on 6 replicates in each condition are presented for each platform in Tables 21 to 22. The results were analyzed with a cut-off value set at 15 copies.

Table 21: *Tests of robustness performed on a LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). The cut-off value at 15 copies for this platform is 37.25.*
Analysis mode: Abs. quant/second derivative max and high confidence.

		Primers concentration minus 30 %	**Probe concentration minus 30 %**	**Standard**	**+ 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	6/6
	51 °C	6/6	6/6	6/6	6/6	6/6

With this platform (LC480 with Universal Mastermix provided by Diagenode), **100 %** of results are considered as positive with a cut-off set at 15 copies.

Table 22: *Tests of robustness performed on the QuantStudio6 (Applied Biosystems) with Brilliant II QPCR Low ROX Master Mix (Agilent technologies). The cut-off value at 15 copies for this platform is 36.92.*
Analysis mode: automatic base line and automatic threshold.

		Primers concentration minus 30 %	**Probe concentration minus 30 %**	**Standard**	**+ 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	6/6
	51 °C	6/6	6/6	6/6	5/6	6/6

With the second platform combining QuantStudio™ 6 with Brilliant II QPCR Low ROX Master Mix by Agilent technologies, the cut-off at 15 copies is 36.92 cycles. With this platform, **98.3 %** of results are considered as positive with a cut-off set at 15 copies whatever the deviation.

In conclusion of these robustness tests, the rate of positive results was always higher than 95 %.

5. Final conclusions

The study of 6 “poultry” PCR methods made available to EURL-AP, led to the development of a combined chicken-turkey PCR test. This assay allows the detection of 2 poultry species (chicken - *Gallus gallus* - and turkey - *Meleagris gallopavo*) without aspecificity related to other bird species and is the most sensitive assay.

The different performance criteria were considered and have been reached. The EURL-AP “chicken-turkey” PCR method has an efficiency at 94.22 %. The limit of detection (LOD) was determined at less than 20 copies with a cut-off value set at 15 copies. This test is able to

achieve what is the minimum requirement expected of a PCR assay, i.e. to detect 0.1 % (w/w) of poultry material in feed composed only of plant material as well as in presence of processed animal proteins from other species. The PCR method is robust.

For the transferability, the study of different cut-off levels arrives to the conclusion that a cut-off set at 15 copies is best level.

This combined chicken-turkey test will be considered as a poultry test even though some poultry species are not detected by it. Developing a PCR poultry test that detects only those species legally considered as poultry is a quite impossible task because the so-defined group is not taxonomically homogeneous. However, chicken and turkey are by far the most abundant species present in poultry PAP and the developed test is able to detect them. That is why this test has to be considered as fit for purpose as a poultry test. Moreover we learned from EFPRA that if it is indeed not impossible to have a pure single species PAP made out of poultry species other than chicken or poultry, its value is much higher for pet food and will therefore not enter the food chain.

Acknowledgment

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Annex 1 : Protocol of poultry DNA detection by real-time PCR

1. Primers and probe sequences

Forward primer : 5′-TAG ACT ACC AAG GCG TAG CT-3′
Reverse primer : 5′-AAG TCA AGG CGA CCT TG-3′
Probe : 5′-AAA GCA TTC AGC TTA CAC CTG AAA-3′
Reporter dye : FAM (position 5' of the probe)
Quencher dye : TAMRA (position 3' of the probe)

2. Real-time PCR mix

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

PCR grade water, 7.65 picomoles of forward primer and reverse primer, 9 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 reaction	96 reactions	105 reactions (1 plate)*
PCR grade water	4.8 µl	460.8 µl	504 µl
Forward primer (8.5 µmole/l)	0.9 µl	86.4 µl	94.5 µl
Reverse primer (8.5 µmole/l)	0.9 µl	86.4 µl	94.5 µl
Probe (10 µmole/l)	0.9 µl	86.4 µl	94.5 µl
Master mix 2x	12.5 µl	1200 µl	1312.5 µl
Total PCR mix volume/reaction	20 µl		
DNA to be added in each PCR	5 µl		
Total reaction volume	25 µl / well		

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

3. Thermal program

The thermal program to follow is outlined in Table 2:
Table 2: Thermal program of the chicken and turkey 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