Validation study of a real-time PCR method developed for the detection of poultry DNA in feedingstuffs

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Table of contents

1. Summary

The development and validation of analytical methods for the detection and the species identification of processed animal proteins (PAPs) in animal feed has been indicated in the TSE Roadmap II as the main condition for a possible lifting of the extended feed ban. Alternative methods to the classical microscopy based on Polymerase Chain Reaction (PCR) are the privileged solution for this aim. A real-time PCR method able to detect simultaneously low levels of chicken and turkey DNA was developed to complete the panel of the assays available to detect and identify the PAPs in feed or feed ingredients. The fitness for purpose of this method was checked at the EURL-AP through an in-house validation study (Marien et al., 2023). A full validation through an interlaboratory study was conducted by the EURL-AP to provide evidence that the method is suitable for the detection of poultry PAP in a network of laboratories.

As for the ruminant and the pig methods, a transfer protocol based on plasmid calibrations combined with statistical considerations was used to set an accurate cut-off value specific of the PCR platforms (thermocycler + master mix). The setting of the cut-off value is crucial to distinguish accurately positive from negative results. In this case, the target of the method is present in multicopies per cell and therefore accurate determination of the cut-off is of importance to obtain reliable results.

The study took place from May 2017 (date of the call for participants) up to end of June 2017 (reporting of the results). Fifteen institutes agreed to participate to the study. A total of 15 thermocyclers dispatched between 5 major companies were tested. Taking into account the sensitivity of the method, a cut-off value calculated at 15 copies per reaction was tested (in fact cut-off value expressed in cycles corresponds to the upper limit of the interval confidence at 95 % for a Ct at 15 copies). Cut-off values calculated at 15 copies per reaction of the 15 platforms are in a range between 36.14 and 39.36 cycles, representing in terms of copy number a range of values from 8.63 to 11.52 copies. When using these cut-off values, the aimed 95 % level of correct assignments with the blind samples included in the trial was met. The rate of false positive results is 1.83 % and the rate of false negative results is 0.22 % considering the 3 levels of poultry PAP tested (0.2 %, 0.1 % and 0.04 % respectively). At the level of 0.1 % w/w of poultry PAP in feed, no false negative result was recorded. Based on the overall conclusions of the study, the method can therefore be considered as fit for purpose.

2. Introduction

Since the outbreak of bovine spongiform encephalopathy (BSE), the use of processed animal proteins (PAPs) as feed ingredients for farmed animals is drastically controlled within the European Union through several regulations (EU, 2001 ; EU, 2002 ; EU, 2003 and EU, 2013). Since 2013, classical light microscopy and PCR are the official methods for the detection of PAPs in compound feed in the European Union (EU, 2013). These two methods are complementary as PCR overcomes the limitations of light microscopy with respect to species discrimination.

PCR is currently the only technique able to determine the origin of animal by-products present in a feed at the species level. Thanks to the good stability of DNA to the high temperatures of the rendering processes, different PCR methods using small sized multi-copy targets already proved their efficiency for the detection of PAPs in animal feed at low level (Aarts *et al.*, 2006; Fumière *et al.*, 2006; Prado *et al.*, 2007; Cawthraw *et al.*, 2009).

The PCR method that is validated here through an interlaboratory study was developed by the EURL-AP and has been fully assessed through an in-house validation by the EURL-AP (Marien *et al.*, 2023). Conclusions of the study were that the method was fit for a validation in the sense that such a full validation with a collaborative trial would have a high chance to be successful. As with the ruminant and the pig methods, the EURL-AP completed the PCR assay for poultry with a protocol to define the cut-off value of any PCR platform (thermocycler and master mix).

A scientifically sound way to find out rapidly what is the cut-off value of any other PCR platform was defined by CRA-W based on a statistical approach (Planchon et al., 2010). By means of known amounts of plasmids carrying the PCR target, calibration curves were built. Through inverse regression (Draper and Smith, 1998) between the logarithm of the copy number and the Ct, a cut-off value is calculated. The way to define this value was adapted for the poultry PCR assay targeting a region located in the mitochondrial DNA which is less abundant than the previously validated ruminant target (Fumière *et al.*, 2021 ; Marien *et al.*, 2023).

This full validation of the EURL-AP poultry PCR assay will mainly check the transferability of this analytical assay in order to detect poultry PAP at 0.1% (w/w) in feedingstuffs. The assessment of the assay by in-house validation at the EURL-AP already pointed out the method was of interest by being able to meet acceptable performance criteria for a PCR method. Moreover the robustness of the assay was also confirmed. Transferability of the technique which mainly relies on the use of the cut-off will be tested here.

3. Organiser team

The study was conducted and coordinated by the EURL-AP. The EURL-AP was also responsible for the preparation of test materials and the overall compilation of the report.

4. Participants

The participants consisted of fifteen European institutes (named for the study "Lab #") using thermocyclers from 5 major companies:

- 1 LC480 and 1 LC96 (Roche Diagnostics)
- 1 ABI 7300, 1 ABI 7500, 2 StepOnePlus and 1 Quant Studio 6 Flex (Thermo Fisher Scientific)
- 3 CFX96, 1 iCycler and 1 C1000 Touch (Bio-Rad)
- 2 Mx3000P (Agilent)
- 1 RotorGene (QIAGEN)

The list of the participating institutes is given in the Annex I.

5. Time schedule of the study

The 19th of May 2017, an invitation letter (Annex II) was sent to 30 potential participants with already some experience in PCR to announce officially the interlaboratory study and to know whether they were interested in participating in the study. The document described the following points:

- ✓ objective of the study,
- ✓ organiser team,
- ✓ material provided,
- ✓ material and equipment required but not provided,
- ✓ general outline of the exercise,
- ✓ time schedule of the study.

The laboratories had to confirm their participation by the 29th of May 2017 by sending back a reply form indicating all important information about the laboratories and the models of thermocyclers participating to the study. Fourteen labs answered positively at the deadline. A fifteenth lab sent its reply only the 31th of May. Its participation was accepted under the condition that the other participants received their study material in good conditions.

The 30th of May 2017, the instructions and the protocol of the study were sent to the participants.

The 6th of June 2017, the experimental material was sent to all the participating laboratories which received the material within two days (between the 7th and the 8th of June

2017). A last shipment was sent the 12th of June 2017 to the last participant. In this case, the reception of the material was recorded the day after, 13th of June 2017.

Results were collected between the 14th and the 29th of June 2017, deadline for the reporting.

6. Purpose of the study

The objective of this study was to evaluate the fitness of a protocol used to detect the presence of poultry DNA in feedingstuffs. A cut-off value able to delimit signals due to the presence of the target from unspecific or late signals was determined according the protocol developed in 2009 and already successfully tested through the validation studies of the ruminant PCR method in 2012 and of the pig PCR method in 2015 (Planchon *et al.*, 2010 ; Fumière *et al.*, 2010 ; Fumière *et al.*, 2016 ; Fumière *et al.*, 2023). As for the ruminant PCR assay, the determination of the cut-off value corresponds to the upper limit of the confidence interval of a Ct value for 15 copies of the target in the reaction. This adapted cut-off value was determined during the evaluation of the method to keep a good sensitivity and to minimize the rate of false positive results (Marien *et al.*, 2023).

Ten blind samples containing 0, 0.04, 0.1 and 0.2 % of poultry PAP in mass fraction were tested by the participants. Each sample was analysed 20 times by performing 10 reactions (replicates) on 2 runs.

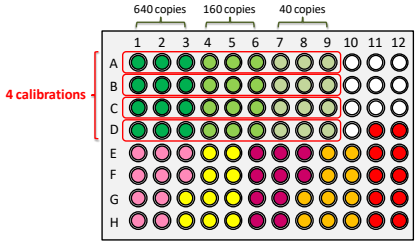
The present method will be considered as fit for the purpose if > 95 % of the reactions with DNA extracted from samples at least 0.1 % in mass fraction of poultry PAPs are positive and if the rate of false positive results does not exceed 5 %. As the use of the cut-off in that perspective is absolutely crucial, it was decided to focus the validation on the sole PCR step and thus to send the same DNA extracts to all participants. This avoids interference due to the DNA extraction technique.

7. Design of the study

The participation of the laboratories consisted in 4 PCR runs to perform within 2 or 3 consecutive days. On each plate, 4 calibrations were performed and 5 samples containing or not a low level of poultry PAP content or not were tested in blind (10 replicates / sample) on two consecutive runs. The position of the samples on the plates was inverted between the two runs in order to avoid any bias in the results due to an edge effect of the thermal block.

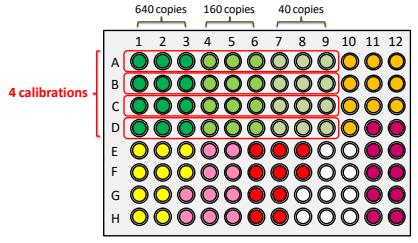
The design of the 2 first plates is presented in Figure 1.[1]

Run 1



		Wells
Calibrators		
	640 copies	A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3
	160 copies	A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6
	40 copies	A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9
Control		
	Negative PCR control	A10, A11, A12, B10, B11, B12, C10, C11, C12, D10
Samples		
	# 1	D11, D12, E11, E12, F11, F12, G11, G12, H11, H12
	# 2	E9, E10, F9, F10, G8, G9, G10, H8, H9, H10
	# 3	E6, E7, E8, F6, F7, F8, G6, G7, H6, H7
	# 4	E4, E5, F4, F5, G3, G4, G5, H3, H4, H5
	# 5	E1, E2, E3, F1, F2, F3, G1, G2, H1, H2

Run 2



		Wells
Calibrators		
	640 copies	A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3
	160 copies	A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6
	40 copies	A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9
Control		
	Negative PCR control	E9, E10, F9, F10, G8, G9, G10, H8, H9, H10
Samples		
	# 1	E6, E7, E8, F6, F7, F8, G6, G7, H6, H7
	# 2	A10, A11, A12, B10, B11, B12, C10, C11, C12, D10
	# 3	D11, D12, E11, E12, F11, F12, G11, G12, H11, H12
	# 4	E1, E2, E3, F1, F2, F3, G1, G2, H1, H2
	# 5	E4, E5, F4, F5, G3, G4, G5, H3, H4, H5

Figures 1 : Design of the 2 first PCR plates to perform by the participants

The participants had to report their results by filling in a dedicated Excel file with the Ct values obtained as well on the calibrants as on the blind samples (Figure 2).

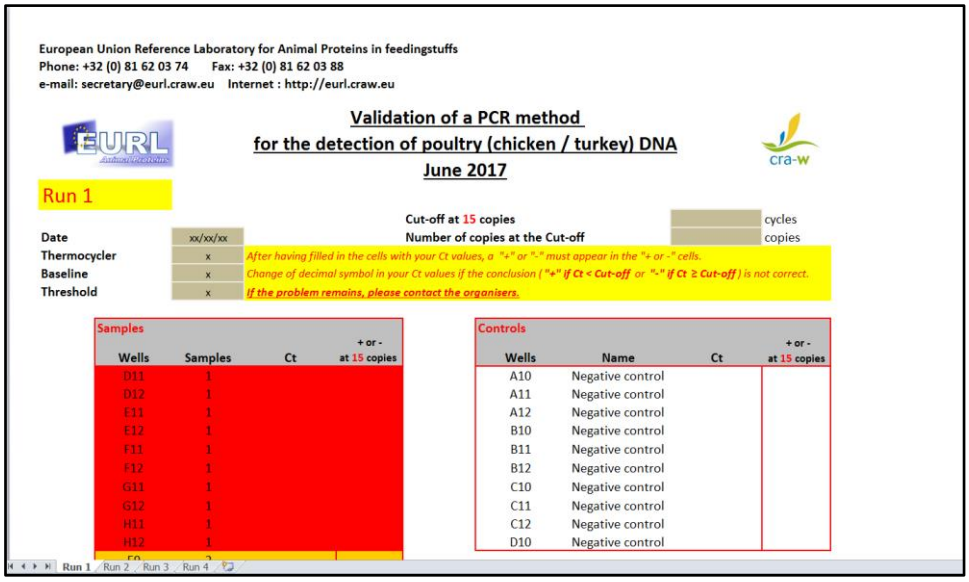


Figure 2 : Excel sheet for the reporting of the results

The complete protocol of the study is presented in Annex IV.

[1] This design could not be strictly followed by the participant using a Rotorgene (QIAGEN) but the runs were similar to the ones described for 96-well plates.

8. Description and preparation of test materials

The list of the material provided by the organisers is detailed in the protocol of the study provided in Annex IV. Besides the mastermix (Diagenode, Seraing, Belgium), the primers and probe (Eurogentec, Seraing, Belgium) already diluted at working concentrations, the delivered material consisted in the calibrants, the 10 blind samples and the PCR negative controls.

8.1. The calibrants

Three calibrants (each in vials containing 1000 µl of material per calibrant) were provided to the participants: PAP-484a - Cut-off calibration curve (640 copies) (515 copies / 5 µl*), PAP-484b - Cut-off calibration curve (160 copies) (120 copies / 5 µl*), PAP-484c - Cut-off calibration curve (40 copies) (40 copies / 5 µl*). They were prepared in a background of salmon sperm genomic DNA at the concentration of 50.5 ng / µl (to avoid loss of molecules by their sticking to the plastic walls of the vial). The calibrants are described in Annex V. The exact copy numbers were slightly different from the nominal ones and these exact figures (515 copies/5 µl, 120 copies/5 µl and 40 copies/5 µl respectively) were pre-introduced in the "Exact copy numbers" of the Excel file for the determination of the cut-off and the cells were locked to avoid any mistake from the participants (Figure 3).

* Unweighted mean value from 16 independent measurements obtained in 6 different laboratories by digital PCR.

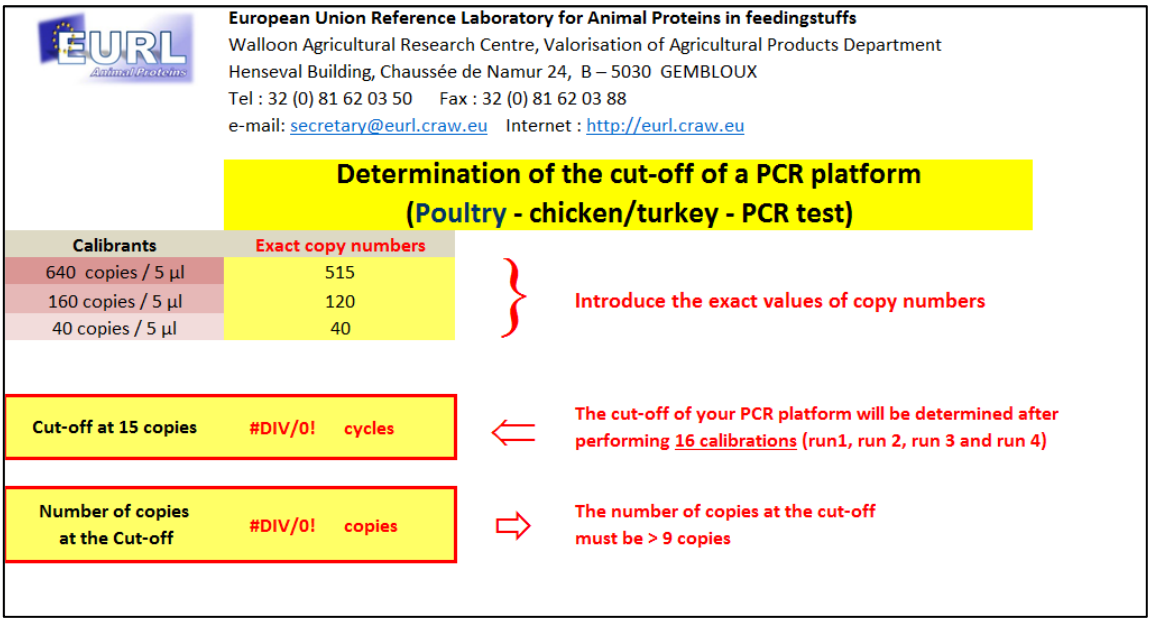


Figure 3 **:** "Exact copy numbers" of the Excel file for the determination of the cut-off

8.2. The ten blind samples

Two poultry feed were used as blank matrices. One poultry PAP processed according to method 7 was used to prepare the blind samples. In this case, the PAP was heated at a temperature of minimum 90°C during 30 minutes on the cooking side. With the drying treatment, the material was heated at approximately 95°C during 60 minutes. A set of blind samples was constituted with 4 concentrations of poultry PAP : 0.2 % in weight (~500 copies of the poultry target / 5µl), 0.1 % in weight (~250 copies of the poultry target / 5µl), 0.04 % in weight (~100 copies of the poultry target / 5µl) and 2 blanks at 0 % in weight (0 copy of the poultry target / 5µl) (Table 1).

***Table 1*:** Composition of the blind samples set used in the EURL-AP PCR
Validation study of a real-time PCR method for the detection of poultry DNA in feedingstuffs

Sample	Material	Number of vials and remarks	
DNA extracts			
1	Blank 1 : complete starter feed for fattening turkey	1	
2	0.2 % w/w poultry PAP in blank 1 (estimated target copy number in 5 µl of DNA : ~ 500 copies)	1	
3	0.04 % w/w poultry PAP in blank 1 (estimated target copy number in 5 µl of DNA : ~ 100 copies)	1	
4	0.1 % w/w poultry PAP in blank 1 (estimated target copy number in 5 µl of DNA : ~ 250 copies)	1	
5	Blank 2 : finishing feed for poultry	1	
6	Blank 2 : finishing feed for poultry	1	(replicate of sample #5)
7	0.1 % w/w poultry PAP in blank 1 (estimated target copy number in 5 µl of DNA : ~ 250 copies)	1	(replicate of sample #4)
8	Blank 1 : complete starter feed for fattening turkey	1	(replicate of sample #1)
9	0.04 % w/w poultry PAP in blank 1 (estimated target copy number in 5 µl of DNA : ~ 100 copies)	1	(replicate of sample #3)
10	0.2 % w/w poultry PAP in blank 1 (estimated target copy number in 5 µl of DNA : ~ 500 copies)	1	(replicate of sample #2)
Total		**10**	

The preparation scheme is illustrated in Figure 5.

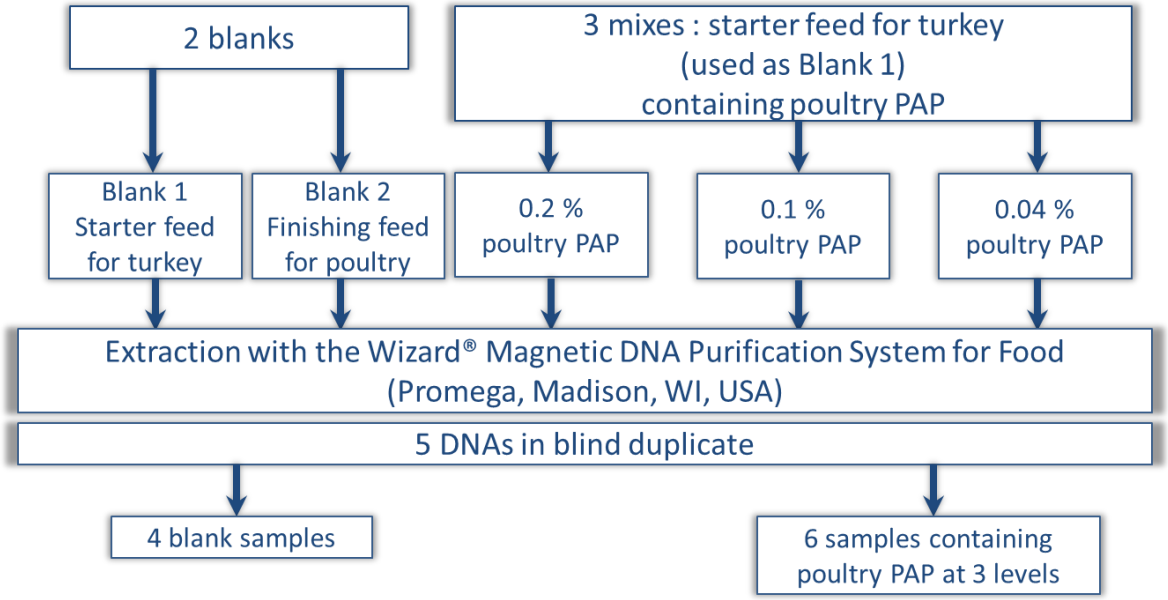


Figure 4 **:** Production of the blind samples set used in the EURL-AP PCR Validation study of a real-time PCR method for the detection of poultry DNA in feedingstuffs

A sample consisting of a blank complete starter feed for fattening turkey was adulterated at three levels (0.2 %, 0.1 % and 0.04 % w/w) with a poultry PAP processed according to method 7. Two blank samples were also extracted: the first one is the blank feed for turkey used in adulterated samples. The second one is a finishing feed for poultry.

These 5 samples were submitted to DNA extraction with the Promega protocol (Wizard Magnetic DNA Purification System for food) on 100 mg of matrix which is the extraction method used for routine analysis (EURL-AP, 2013 ; https://www.eurl.craw.eu/legal-sources-and-sops/method-of-reference-and-sops/). Due to the large volumes of DNAs to provide to the participants, 65 extracts of each sample were prepared. The DNA extracts were tested to check the amplifiability of DNA and the absence of PCR inhibition and contamination.

The samples were analysed with the poultry PCR target. For the blank samples, 5 series of 13 extracts were performed and the DNA extracts were pooled per series. The 5 pooled extract were tested (6 replicates by pooled extract) and then pooled in one. The blank samples were negative for poultry DNA; they did not give any amplification. Concerning the samples containing poultry PAP, a number of copies / 5 µl of extract was targeted for the 3 levels of PAP : +/- 500, 250 and 100 copies for the level 0.2, 0.1 and 0.04 % respectively. For that purpose, all DNA extracts obtained for each level were analysed. Given the low levels of adulteration, the signals obtained had a high variability from one extract to another one. That is why, for 0.1 and 0.04 % levels, the extracts close to the targeted copy numbers were selected to be pooled. Concerning the 0.2 % level, the number of copies being higher than 500 copies for the majority of extracts, the targeted level was obtained by an adequate dilution with DNA extract from the blank feed 1.

During the tests, all the DNA extracts were stored at 4 °C. The DNA extracts were aliquoted and stored at - 20 °C until shipment to the participants. The EURL-AP performed the full study with a set of samples chosen randomly just to check that the obtained data met what was expected but these data are not part of the validation study.

***Table 2*:** Mean Ct obtained during analysis of one sample set on the three samples containing poultry PAP on the LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Analysis mode: Second derivative and high confidence.

Level	**Mean Ct on Promega extracts (n = 40)**
0.2 % of poultry PAP in weight	31.51 cycles
0.1 % of poultry PAP in weight	32.81 cycles
0.04 % of poultry PAP in weight	33.86 cycles

The participants received vials of these three levels and 2 blanks in duplicates as blind samples giving thus a total of 10 vials containing the same volume of material (250 µl).

8.3. The PCR negative controls

The negative controls were made of PCR grade water. Six vials of 60 µl were provided to each participant.

9. Results

The Ct data provided by the participants to this inter-laboratory study generated two kinds of results :

1) For each participant, a cut-off value calculated at 15 copies was automatically generated by the Excel file when filled with the appropriate outcome of the calibration.

2) With the respective cut-off values, the replicates of the blind samples of each participant were automatically ranked as positive or negative.

9.1. Cut-off of the platforms

The cut-off value of a platform is defined as the upper value of the confidence interval for a set copy number of the target. It is calculated through inverse regression (Draper and Smith, 1998) between the logarithm of the copy number and the Ct measured in calibration curves obtained with plasmid solution at defined copy numbers. The assessment of the EURL-AP PCR assay on poultry showed that cut-off values calculated at 15 copies of the target could be suitable. The cut-off values and the corresponding copy numbers as determined for each participant are provided in Table 3. The copy numbers range between 8.83 and 11.52 copies.

Table 3 provides the cut-off figures calculated at 15 copies.

***Table 3*:** Cut-off values at 15 copies and corresponding number of copies of the participants.

Lab	Cut-off value at 15 copies	Corresponding number of copies
Lab 1	38.7610	11.16
Lab 2	36.1437	10.17
Lab 3	38.6351	10.23
Lab 4	36.5933	11.52
Lab 5	37.6936	8.83
Lab 6	38.1221	10.55
Lab 7	37.3097	9.26
Lab 8	39.3612	9.95
Lab 9	38.2608	9.88
Lab 10	38.1529	9.40
Lab 11	37.3415	10.52
Lab 12	37.4411	10.70
Lab 13	36.8346	10.55
Lab 14	37.4991	10.41
Lab 15	38.9642	9.76

The cut-off value obtained by Lab 5 (8.83 copies) corresponds to a number of copies below the quality criterion fixed by the organizers (9.00 copies).

9.2. Percentages of false results

Blind samples were tested forty times (10 replicates x 2 samples x 2 runs) for each of the three levels of poultry contaminated feedingstuffs or eighty times (10 replicates x 4 samples x 2 runs) for the blanks. A result for a well is considered as positive if the Ct value obtained for that well is smaller than the cut-off value determined for the platform of the considered laboratory. However, the signals obtained should be amplification curves. This explains why 7 results on blank samples of lab 7 were considered as negative, even if from a merely quantitative analysis of the data, they would have been positive. The operator of lab 7 did not interpret the obtained signals as amplification curves. Details on the obtained signals are shown in Figure 6.

The rates of false results are reported in Table 4.

Table 4: Rates of false results (in %)

Lab	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Mean
False positive results	0	0	**27.50**	0	0	0	0[a]	0	0	0	0	0	0	0	0	1.83
False negative results	0.83	0	0	0.83	0.83	0	0	0	0	0	0	0	0.83	0	0	0.22
0.2 % poultry PAP - 500 copies	2.50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.17
0.1 % poultry PAP - 250 copies	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00
0.04 % poultry PAP - 100 copies	0	0	0	2.50	2.50	0	0	0	0	0	0	0	2.50	0	0	0.50

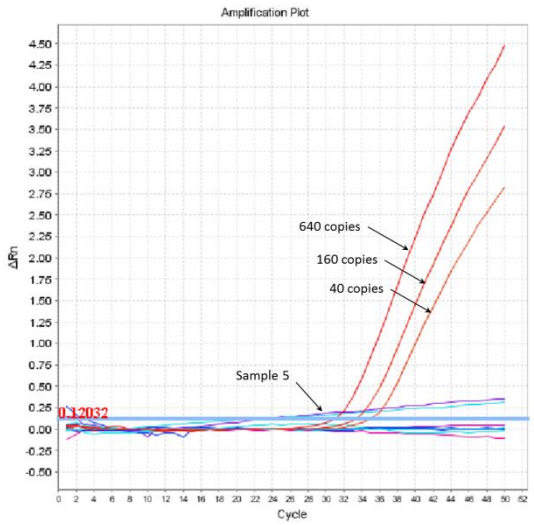
[a] The rate of false positive results reported by Lab 7 was originally of 8.75 % corresponding to 7 results before the cut-off on 80 reactions performed with the blank samples. From the indications of the participant, it appeared that these results would not be considered as positive results if the interpretation of the signal by an operator would be requested. The 7 cases are illustrated hereafter (Figure 6 a, b, c, d, e).

***Table 5*:** Rates of false positive results (in %) obtained with the negative PCR control using a cut-off at 15 copies

Lab	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Negative PCR controls															
False positive results (Cut-off at 15 copies)	0	0	0	0	0	0	0[a]	0	0	0	0	0	0	0	0

[a] The rate of false positive results reported by Lab 7 was originally of 7.50 % corresponding to 3 Ct values before the cut-off on 40 replicates. From the indications of the participant, it appeared that with the interpretation of the signals by an operator, these results would not be considered as positive. The 3 cases are illustrated hereafter (Figure 7 a, b and c).

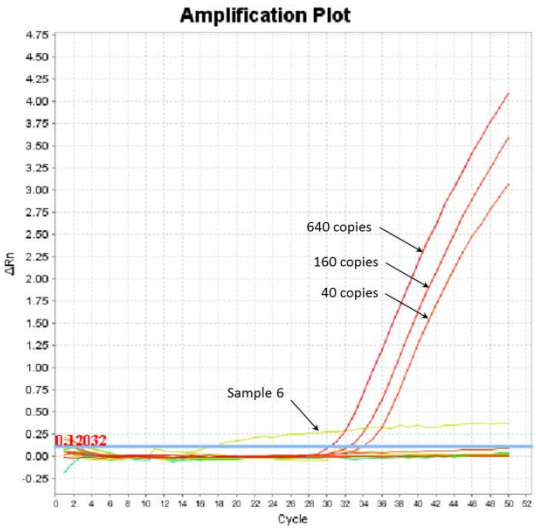
a)



Run 1 – curves of 10 replicates of the Sample 5 (Blank 2) and of one replicate of the 3 calibrants.

Two replicates of Sample 5 (purple and blue curves) gave a Ct value before the cut-off : 22.98 and 21.31 cycles respectively. The shapes of these 2 signals are not the ones of PCR amplifications.

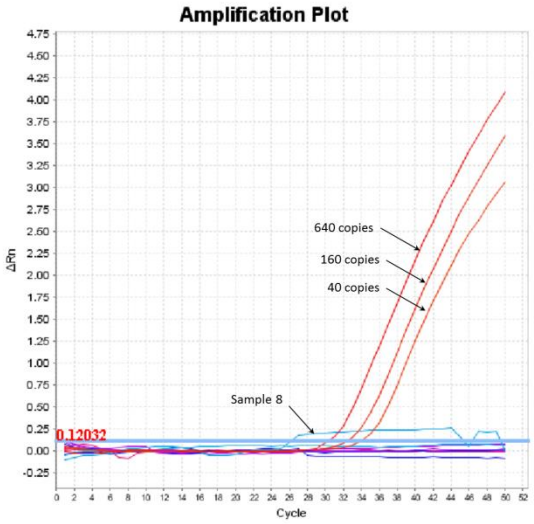
b)



Run 3 – curves of 10 replicates of the Sample 6 (Blank 2) and of one replicate of the 3 calibrants.

One replicate of Sample 6 (yellow curve) gave a Ct value of 17.52 cycles but the shape of the signal is too flat.

c)

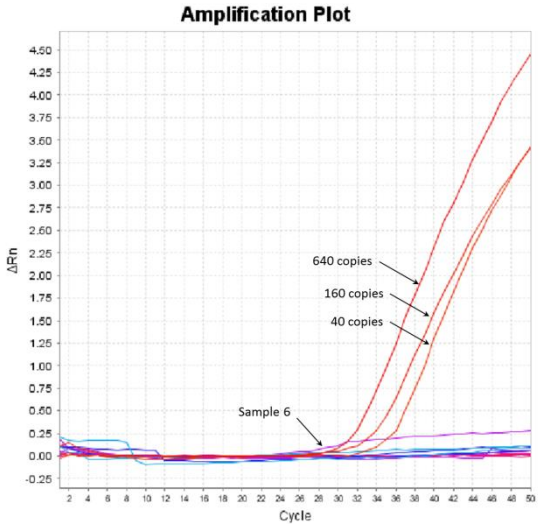


Run 3 – curves of 10 replicates of the Sample 8 (Blank 1) and of one replicate of the 3 calibrants.

One replicate of Sample 8 (blue curve) gave a Ct value of 26.21 cycles. The noise present in the reaction is quite high and the shape of the signal does not correspond to a PCR amplification.

***Figure 6 a), b), and c)*:** Signals obtained with blank sample replicates giving false positive results (source: results reported by Lab 7).

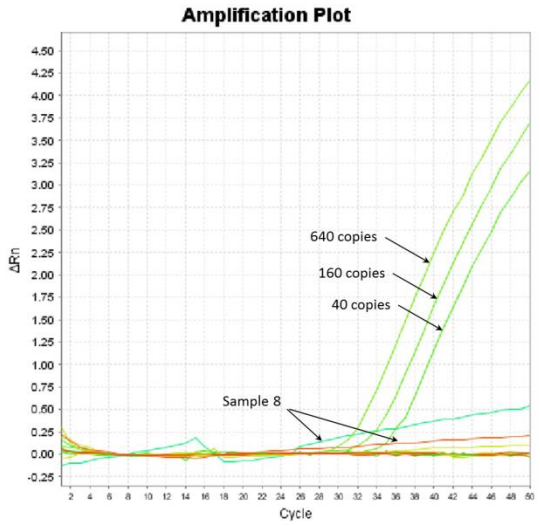
d)



Run 4 – curves of 10 replicates of the Sample 6 (Blank 2) and of one replicate of the 3 calibrants.

One replicate of Sample 6 (purple curve) gave a Ct value of 29.93 cycles and the shape of the signal is very flat.

e)



Run 4 – curves of 10 replicates of the Sample 8 (Blank 1) and of one replicate of the 3 calibrants.

Two replicates of Sample 8 (blue and red curves) gave a Ct value before the cut-off : 27.46 and 36.71 cycles respectively. The shapes of these 2 signals are not the ones of PCR amplifications.

***Figure 6 d) and e)*:** Signals obtained with blank sample replicates giving false positive results (source: results reported by Lab 7).

10. Statistical data treatment

10.1. Assessment of the performances of the labs based on the results obtained with the negative PCR control

The performance of the labs was qualitatively estimated from the percentages of false positive results with the negative PCR control made of milliQ water. These results were obtained on known samples and the laboratories could therefore do some plates again as enough reagents were provided to perform 2 additional plates.

Rates of false positive results on negative PCR controls are reported per participant in Table 5.

Here too, based on merely quantitative results, lab 7 would have false positive results for some of the negative PCR controls. However based on the shape of the signals, which cannot be considered as amplification curves (Figure 7) the results are negative.

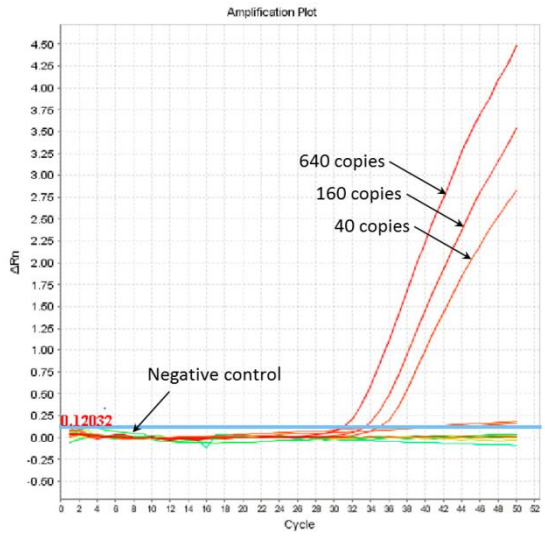
With the negative PCR control, a rate ≤ 5 % of false positive results was considered as acceptable. A rate of > 5 % of false positive results was assimilated to an underperformance. On this basis, no lab was considered as under-performant and was excluded from the statistical analysis of the results.

10.2. Rates of false positive and false negative results

The results of all the participating labs were considered. The global rate of false positive results reached 1.83 % (22 / 1200) while the false negative rate amounted to 0.22 % (4 / 1800) for a cut-off value calculated at 15 copies. It must be emphasized that all the false positive results were recorded by the same lab (Lab 3). As the results obtained by the participant with the negative control were excellent, there is no evidence that these false results can be due to problem of cross-contamination.

At the level of 0.2 % of poultry PAP in feedingstuffs, only 1 reaction out of 600 gave a negative result (0.17 %). For the level of 0.1 % of poultry PAP in feedingstuffs, no false negative result was recorded out of 600 reactions. By gathering the results of these two levels, only one reaction out of 1200 (0.08 %) did not detect the presence of poultry PAP at the minimum level of 0.1 % in mass fraction. These values are far below the rate of 5 % and the method can therefore be considered as fit for purpose.

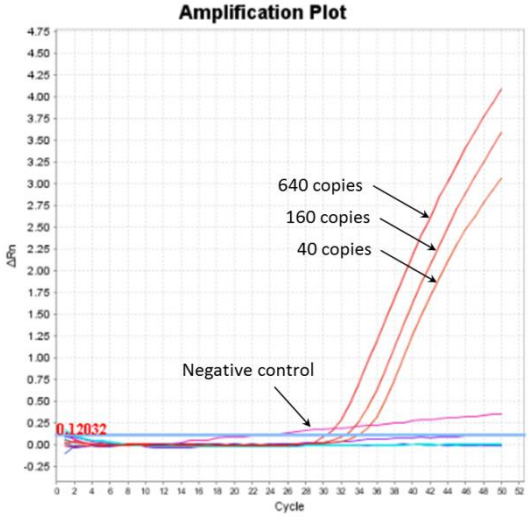
a)



Run 1 – curves of the 10 replicates of the negative control and of one replicate of the 3 calibrants.

A Ct of 2.96 cycles was recorded for the negative control (green curve). Such a Ct is an artefact due to noise during the first cycles of the run.

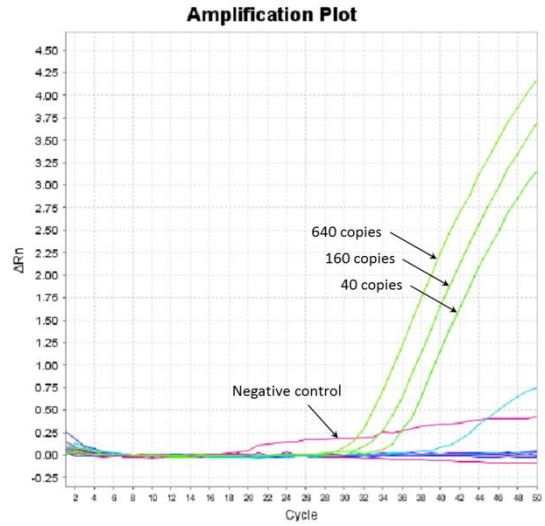
b)



Run 3 – curves of the 10 replicates of the negative control and of one replicate of the 3 calibrants.

One of the negative control replicates (pink curve) gave a Ct of 23.71 cycles. The observation of the curve shape shows clearly that the signal is not due to a PCR amplification.

c)



Run 4 – curves of the 10 replicates of the negative control and of one replicate of the 3 calibrants.

As during the run 3, one of the negative control replicate (pink curve) presents an increasing fluorescence. The Ct value obtained is 21.99 cycles. The examination of the signal allows to conclude that the fluorescence recorded is not due to a PCR amplification.

***Figure 7 a), b) and c)*:** Signals obtained with negative control replicates giving false positive results (source: results reported by Lab 7).

11. Conclusions

- The cut-off values calculated in cycles of the 15 platforms and determined using the proposed protocol showed differences between platforms (36.1437 < Cut-off value$_{15\ copies}$ < 39.3612).
- In terms of copy numbers, the cut-off values correspond to a range between 8.83 and 11.52 copies.
- The transfer of the protocol to new laboratories with thermocyclers from 5 companies (Thermo Fischer Scientific, Bio-Rad, Roche Diagnostics, Agilent and QIAGEN) was successful.
- Based on the rates of false results, the study can be considered as successful and the protocol of the assay as fit for purpose and validated.

Acknowledgements

The authors would like to thank the laboratories which participated in this study. The authors are grateful to Philippe Corbisier, Janka Mátrai and Stéphane Mazoua (EC-JRC-IRMM, Geel, Belgium) for the production of the calibrants and Christoph von Holst (JRC Geel, Belgium) for statistics and fruitful discussions. The authors would like to thank also Julien Maljean for his efficient technical assistance.

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13. Annexes

a. Annex I: List of participating laboratories

Organization name	Country
Animal and Plant Health Agency (APHA-Penrith)	Penrith, UK
Cyprus Veterinary Services	Nicosia, Cyprus
Danish Veterinary and Food Administration	Ringsted, Denmark
Darling Ingredients Nederland BV	Son, The Netherlands
Federal Institute for Risk Assessment (BfR)	Berlin, Germany
Agroscope	Posieux, Switzerland
Instituto Nacional de Investigação Agrária e Veterinária (INIAV)	Oeiras, Portugal
Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZSTO – CreAA)	Torino, Italy
National Diagnostic Centre of Food and Veterinary Service	Ljubljana, Slovenia
National Diagnostic Research Veterinary Medical Institute	Sofia, Bulgaria
National Institute of Nutrition and Seafood Research (NIFES)	Bergen, Norway
NutriControl BV	Veghel, The Netherlands
Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES GmbH)	Linz, Austria
RIKILT Wageningen University & Research	Wageningen, The Netherlands
Service Commun des Laboratoires du MINEFI	Rennes, France

b. *Annex II: Invitation mail to participate in the validation study*

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
Henseval Building, Chaussée de Namur 24, B – 5030 GEMBLOUX

☎32 (0) 81 62 03 74 🖷32 (0) 81 62 03 88
e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

Gembloux, 19 May 2017

Invitation to participate to a validation study
of a PCR method for the detection of poultry (chicken and turkey) PAPs

Introduction and objective of the study

As planned in the TSE roadmap 2[1] and the revision of the Regulation (EC) No 999/2001, the 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 is a measure that would be acceptable only if validated analytical techniques to determine the species origin of PAP are available. Presently, Polymerase Chain Reaction (PCR) and the optical microscopy remain the reference methods for the detection of PAPs to be applied for official control.

In that framework, the present inter-laboratory study aims to validate a real-time PCR method developed by EURL-AP (Gembloux, Belgium) for the detection of poultry (chicken/turkey) PAPs.

The organizer team

The test will be coordinated by the European Union Reference Laboratory for animal Proteins in feedingstuffs (EURL-AP).
E-mail: secretary@eurl.craw.eu

Material provided

The organiser will provide :

- Ready-to-use primers and probe.
- The master mix and the passive reference (ROX) separately. The participant will add the correct amount of passive reference according to their thermocycler.
- Supplied material : a set of calibrants, a negative PCR control and a set of blind samples to test.

A list describing in details all the material provided will be in the protocol.

Walloon Agricultural Research Centre

[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.
http://www.fsai.ie/uploadedFiles/Legislation/FSAI_-_Legislation/2010/07_jul2010/EU_Communication_TSE.pdf

Wallonie

Material and equipment required but not provided

- Platform for real-time PCR with a heating block of 96 usable wells and analysis software. Please be sure that all 96 wells are functional.
- Optical 96-Well Reaction plates + Optical covers (adhesive films or caps)
- Micropipettes + Pipette tips with filter plugs
- Vortexer
- Standard centrifuge with 1.5 ml reaction tubes rotor
- Centrifuge with PCR Plate rotor
- Rack for reaction tubes
- DNAse free reaction tubes
- Ice
- PCR grade water
- Disposable gloves

General outline of the exercise

- The protocol of the study describing in details the experimental work to do will be sent to the participants 1 week (the 30th of May) before the materials. Please read it carefully and if anything remains unclear ask your question to the organizer in order to be ready to start the work after reception of the material.
- All the DNA samples to test will be provided by the organiser. No extraction will be needed.
- The work will consist in 4 PCR runs with full 96 wells plates. The 4 PCR runs should be performed within 2 or 3 consecutive days.
- An Excel file will be provided by the organiser for reporting the results. For practical reasons, the results will be reported only through this Excel file. A word file will also be provided by the organiser to the participant in order to allow them to describe any deviation from the protocol.
- At the end of the study, the participant will provide to the organiser a copy of the raw data (PCR run files) on a CD-rom or a USB key.
- It is emphasised that the purpose of this validation is to test the performance of the protocol and not that of the laboratory. Therefore, each laboratory should carry out the experiments exactly as described in the protocol. Any deviation from the procedure described should be reported.
- A participant must perform all the study on the same thermocycler.
- As there is no financial interest to participate, the participants will be associated as co-authors to any publication of the results.

Time schedule

- The study will take place in **June 2017**
- The samples will be sent to the participants the **6th of June**
- The deadline for returning of results to the organizer is **29th of June 2017 at noon**

Wallonie

Further information

- Dr Gilbert BERBEN
  ☎ +32 (0)81 62 03 63
  🖷 +32 (0)81 62 03 88
  e-mail: g.berben@cra.wallonie.be
- Dr Olivier FUMIERE
  ☎ +32 (0)81 62 03 51
  🖷 +32 (0)81 62 03 88
  e-mail: o.fumiere@cra.wallonie.be

We would very much appreciate a confirmation of your interest to participate by returning your signed reply form via e-mail to secretary@eurl.craw.eu by **29th of May 2017 at noon**.

Please indicate in your rep y form :

- your interest to participate.
- your equipment : brand(s) and model(s) of your thermocycler(s) on which the study could be performed (the organisers will select which ones to balance the equipments tested in the study).
- the name(s) of the person(s) to whom the material should be directed as well as the detailed shipping address plus phone number and e-mail.

We thank you very much in advance for your support in this task.

Yours sincerely,

Dr Gilbert Berben
European Union Reference Laboratory for Animal Proteins in Feedingstuffs

c. Annex III: Reply form

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
Henseval Building, Chaussée de Namur 24, B – 5030 GEMBLOUX

☎32 (0) 81 62 03 74 🖷32 (0) 81 62 03 88
e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

Inter-laboratory study for the validation of a real-time PCR method for the detection of poultry (chicken/turkey) PAPs

Reply form
to send via e-mail to secretary@eurl.craw.eu
and/or Fax (+32 (0)81 62 03 88) by 29th of May 2017 at noon

Name and address of the participant
e.g. Walloon Agricultural Research Centre (CRA-W)
Valorisation of Agricultural Products Department
Chaussée de Namur 21
B-5030 Gembloux
Belgium

Are you interested to participate to the study ? ☐ Yes ☐ No

Equipment of the lab
(model and brand of thermocyclers able to participate to the study*)
* only heating block thermocyclers with 96 functional wells
e.g. LightCycler LC480 (Roche Diagnostics)
ABI 7500 (Applied Biosystems)

Name(s), detailed shipping address, phone number and e-mail of person(s) to whom the material should be directed
e.g. Olivier Fumière
Valorisation of Agricultural Products Department
Chaussée de Namur 21
B-5030 Gembloux
Belgium
+32 (0)81 62.03.51
o.fumiere@cra.wallonie.be

Date, name and signature of the responsible

Page 1/1

Wallonie

d. Annex IV: Protocol of the study

PROTOCOL FOR THE VALIDATION OF A PCR METHOD FOR THE DETECTION OF POULTRY (CHICKEN / TURKEY) DNA

BEFORE STARTING THE EXPERIMENTS OF THE STUDY, PLEASE READ CAREFULLY ALL THE INSTRUCTIONS

INTRODUCTION

This inter-laboratory study aims to validate the protocol of a real-time PCR method for the detection of poultry (chicken/turkey) DNA designed by EURL-AP (Gembloux, Belgium). It will give a special focus on the following items :

1. The transferability of the qualitative PCR method on any platform (combination of thermocycler and mastermix) and the determination of a cut-off value using plasmids as calibrants are examined. The cut-off value of a platform is a C_t value above which a signal has a high probability to be due to a non-specific amplification. In a qualitative test, the cut-off value is the value delimiting positive results (C_t < cut-off) from negative results (C_t > cut-off). So the setting of an accurate cut-off value is essential to have a low rate of false positive results. The developed protocol determines this cut-off value of the platform on the basis of a statistical rationale.
2. The capacity of the method to detect the presence of 0.1 % (w/w) of poultry PAP in a feedingstuff will be evaluated.
3. Based on the results provided by the participants, a limit of detection in copy number of the target will also be determined.

BEFORE STARTING THE EXPERIMENTS OF THE STUDY, PLEASE READ CAREFULLY ALL THE INSTRUCTIONS

MATERIAL PROVIDED BY THE ORGANIZERS (CAN BE USED AS CHECKLIST)

AT THE RECEPTION OF THE MATERIAL, PLEASE STORE EVERYTHING AT -20 °C UNTIL USE.
THE MATERIAL PROVIDED ALLOWS TO PERFORM 6 PLATES INSTEAD OF THE 4 PLATES DESIGNED IN THE STUDY.

Description	Number of vials	Volume
CALIBRATORS (SET OF REFERENCE MATERIAL PAP 484)		
PAP-484a - Cut-off calibration curve (640 copies)	1	1000 µl
PAP-484b - Cut-off calibration curve (160 copies)	1	1000 µl
PAP-484c - Cut-off calibration curve (40 copies)	1	1000 µl
PRIMERS AND PROBE (READY TO USE)		
Primer A	1	850 µl
Primer B	1	850 µl
Probe	1	850 µl
Negative control	6	60 µl
SAMPLES		
#1	1	250 µl
#2	1	250 µl
#3	1	250 µl
#4	1	250 µl
#5	1	250 µl
#6	1	250 µl
#7	1	250 µl
#8	1	250 µl
#9	1	250 µl
#10	1	250 µl
UNIVERSAL MASTERMIX (DIAGENODE S.A., LIÈGE, BELGIUM – REF DMMLD2D600)		
2x Reaction Buffer (mastermix)	6	1400 µl
Passive reference (ROX – Pink cap)	1	500 µl
Adjust the concentration of passive reference in the mastermix according to your thermocycler (see the requirements of provider in Annex 1)		

MATERIAL NEEDED BY THE PARTICIPANTS FOR THE STUDY

- Platform for real-time PCR (usable with 96 well plates) and analysis software
- Optical 96-Well Reaction plates + Optical covers (adhesive films or caps) or equivalent
- Micropipettes + Pipette tips with filter plugs
- Vortexer
- Standard centrifuge with 2 ml reaction tubes rotor
- Centrifuge with PCR Plate rotor
- Rack for reaction tubes
- 1.5 ml DNAse free reaction tubes
- 2.0 ml DNAse free reaction tubes
- 5.0 ml DNAse free reaction tubes
- PCR grade water
- Ice
- Disposable gloves

PROTOCOL

1. REAL-TIME PCR MIX

To prepare one plate:

- Take one vial of mastermix and defreeze it. Add if needed, the correct amount of passive reference (see Annex 1) and vortex them.
- Before using these reagents and samples, vortex them and centrifuge all vials for a short time.
- In a DNAse free 5 ml microfuge tube, mix in the following order :

	1 reaction	96 reactions	105 reactions (1 plate)
PCR grade water	4.80 µl	460.80 µl	504.00 µl
Primer A	0.90 µl	86.40 µl	94.50 µl
Primer B	0.90 µl	86.40 µl	94.50 µl
Probe	0.90 µl	86.40 µl	94.50 µl
Mastermix 2X	12.50 µl	1200.00 µl	1312.50 µl
Total PCR mix volume/reaction	20.00 µl	1920.00 µl	2100.00 µl

Template DNA to be added in each well of the plate : 5.00 µl

Total reaction volume = 25 µl / well

Check that the correct reaction volume is encoded in the PCR program.

IMPORTANT REMARKS :

1. **THE NUMBER OF REACTIONS PREPARED (105) IS CLOSE TO 96. PAY ATTENTION TO PIPETTING ERRORS AND FINISH THE DISPATCHING OF THE PCR MIX WITH THE WELLS DEDICATED TO THE NEGATIVE CONTROLS.**
2. **20 µL OF THE PCR MIX CONTAINING H_2O, PRIMERS, PROBE AND MASTERMIX IS FIRST DISPATCHED IN ALL THE WELLS. AFTER CHECKING OF THE CORRECT FILLING OF THE WELLS WITH THE PCR MIX, THE DNAS OR THE NEGATIVE CONTROL CAN BE ADDED.**
3. **WHEN THE DNA IS ADDED, HOMOGENIZE THE MIX BY PIPETTING.**
4. **WHEN THE PLATE IS READY FOR THE PCR, BE SURE THAT ALL THE MIX IS IN THE BOTTOM OF THE WELLS WITH NO BUBBLE AT THE BOTTOM OF THE WELLS (POSSIBLE INTERFERENCE DURING FLUORESCENCE MEASUREMENTS). BEFORE TO PUT THE PLATE IN THE THERMOCYCLER, THE PLATE IS CENTRIFUGED DURING 2 MINUTES AT 500 RPM.**

2. PCR THERMAL PROGRAM

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**

REMARKS :

1. **DO NOT USE ANY FAST PCR PROTOCOL. MAXIMUM RAMPING RATE ~1.5 °C / SEC.**
2. **THE ACTIVATION OF DNA POLYMERASE STEP (10 MIN AT 95°C) IS MANDATORY.**
3. **CHECK THAT THE REACTION VOLUME OF 25 µl IS ENCODED IN THE PROGRAM.**

3. MEASUREMENTS OF THE SIGNALS

The probe used for the test is a hydrolysis probe labelled with FAM as reporter dye and TAMRA as quencher dye.

CHOOSE THE CORRECT CHANNEL FOR THE MEASUREMENT OF THE FLUORESCENCE (FAM).

4. CALIBRATION OF A PLATFORM

3 vials of calibrants labelled "PAP-484a", "PAP-484b" and "PAP-484c" are used to calibrate the platform (thermocycler + mastermix). They contain 640 copies of the target / 5 µl (or 128 copies / µl), 160 copies / 5 µl (32 copies / µl) and 40 copies / 5 µl (8 copies / µl) respectively [2]. For routine analysis, 1 calibration is made with 3 replicates from the 3 levels (9 wells) but a calibration of a new platform needs more data.

ALL THE CALIBRATIONS AND SAMPLE ANALYSES MUST BE PERFORMED ON THE SAME THERMOCYCLER.
DO NOT USE DIFFERENT THERMOCYCLERS EVEN FROM THE SAME BRAND AND SAME MODEL!

[2] *For the sake of easiness, we kept the figures 640, 160 and 40 copies but calibrations have to be carried out with the exact copy number of the calibrants obtained by means of digital PCR. These figures can vary from batch to batch. Calculations in this study will be done automatically with the exact copy numbers. They are already encoded in the file for the determination of the cut-off.*

For the study, we ask to the participants to perform 4 runs and 4 calibrations per run as described in the Figure 1.
In the wells highlighted in green in Figure 1, the template DNA is made of the plasmid solution (calibrants).

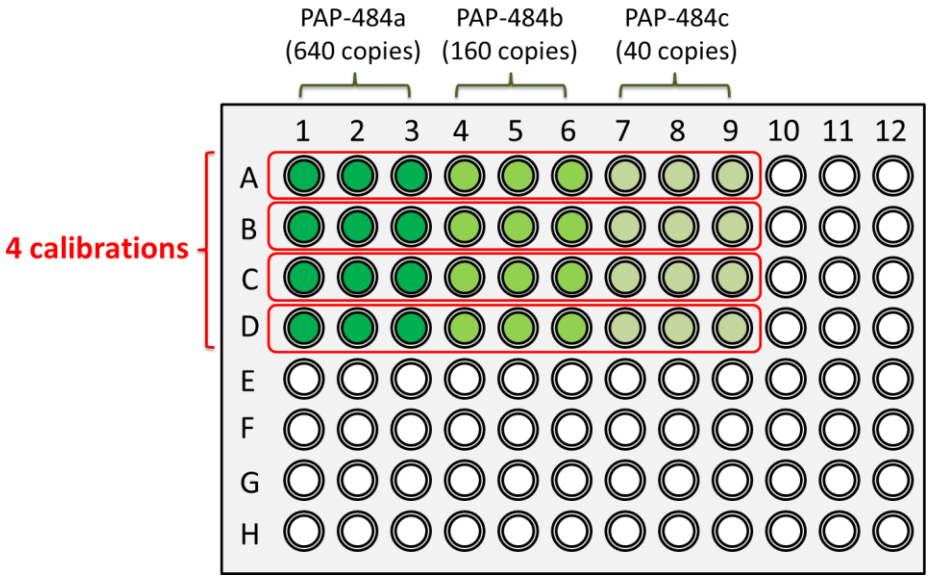


Figure 1 : Location of the wells used for the calibration of the platform.

5. Samples and PCR negative controls

There are 10 samples to be analysed. They consist of DNA extracted from feedingstuffs adulterated or not with a poultry PAP (processed animal proteins).
For some samples, not all the replicates of these samples will give a signal. This is normal and expected by the organizers.
Each sample must be analysed 20 times (10 replicates spread on 2 plates).
The complete schemes of the 4 plates of the study are presented in Annex 2.
For practical reasons, it is asked to stick strictly to the design of the plates as given in Annex 2.

Remarks :

1. **Cycles of freezing and thawing must be avoided. Thaw the material for the plate that you are preparing. Once they are thawed, keep them on ice or at 4°C and sheltered from light until the end of the study.**
2. **During the preparation of the plate, the samples, the calibrants, the PCR controls and the reagents (probe & primers and mastermix) must be kept on ice.**
3. **The material provided allows to perform 6 plates. In case of mistake, you can do your plate again.**

6. Analysis of the results

The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

A fixed fluorescence threshold can be set above the baseline and within the exponential increase phase (which looks linear in the log transformation of the Y-axis linked to fluorescence measurement). The parameter C_t (threshold cycle) is defined as the fractional

cycle number at which the fluorescence passes the fixed threshold. The C_t value is directly related to the amount of PCR product and, therefore, related to the original amount of target present in the PCR. A low C_t value means a high level of initial number of targets, and a high C_t value means a low level thereof.

The C_t value and the cut-off value are relative parameters directly influenced by the level of the threshold. The baseline influences also the shape of the signal and the C_t calculated. For these reasons, it is requested to set the baseline and the threshold at the same value for all 4 plates.

For the determination of the threshold, please analyse carefully the signals. Set the threshold in the exponential increase phase and at a level higher than any fork effect as illustrated in the Figure 2 (the threshold level in green is correct, not the one in red).

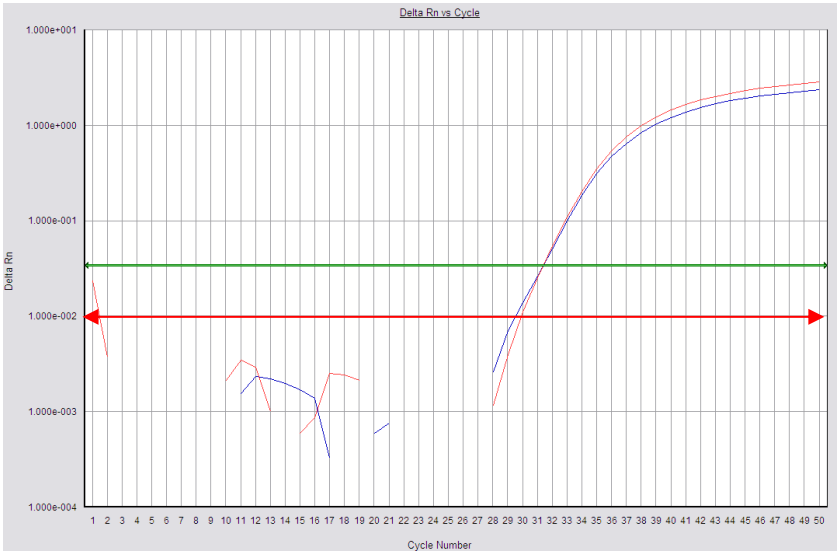


Figure 2 : Amplification signals and threshold levels.

REMARK :

THE USE OF DIFFERENT PROCEDURES (AUTOMATIC OR MANUAL) FOR THE DETERMINATION OF THE THRESHOLD AND OF THE BASELINE WAS TESTED WITH DIFFERENT THERMOCYCLERS (LC 480, ABI 7000 AND ABI 7500). THE STATISTICAL ANALYSIS OF THE RESULTS SHOWS CLEARLY THAT, WITH ABI THERMOCYCLERS, THE BEST REPEATABILITY OF THE RESULTS IS OBTAINED WHEN THE OPERATOR FIXES HIMSELF THE THRESHOLD.

THAT IS WHY THE ORGANISERS ASK TO THE PARTICIPANTS TO FIX THE BASELINE AUTOMATICALLY AND TO SET THE THRESHOLD MANUALLY.

WITH A LIGHTCYCLER, THE BEST REPEATABILITY OF THE RESULTS IS OBTAINED WHEN THE THRESHOLD AND THE BASELINE ARE FIXED AUTOMATICALLY.

KEEPING THE SAME PARAMETERS ALONG THE 4 PLATES IS ALSO REQUESTED.

7. DOCUMENTATION OF THE RESULTS AND REPORTING

The organisers provide 2 Excel files : "Poultry cut-off at 15 copies - validation" and "Reporting file poultry validation study". Both file contain areas to fill in.

7.1. Determination of the cut-off at 15 copies

One run corresponds to one sheet named "Run 1", "Run 2", "Run 3" and "Run 4".

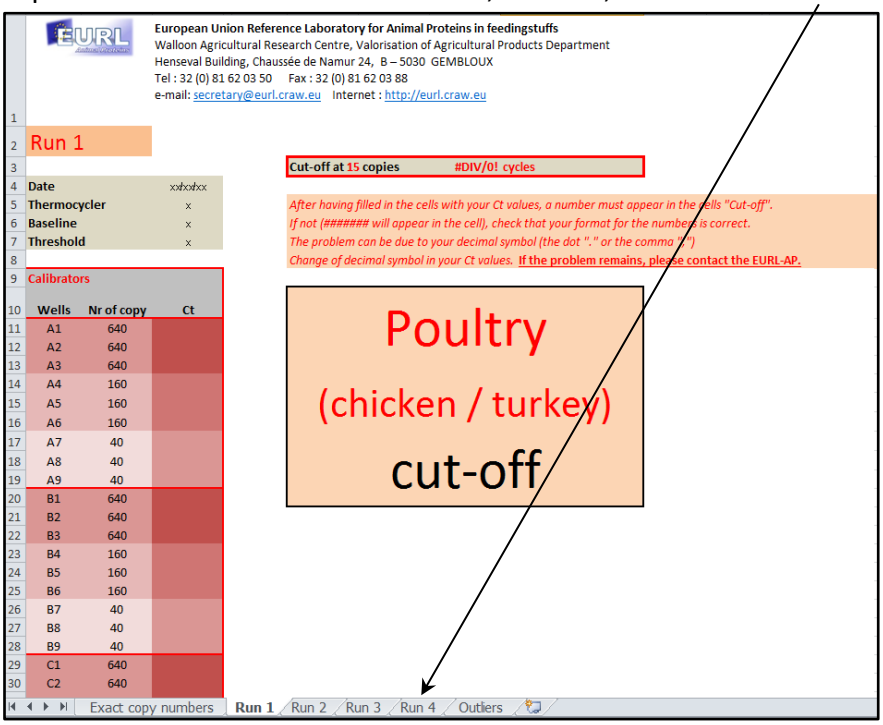


Figure 3 : Image of the sheets in the "Poultry cut-off at 15 copies - validation" Excel file.

1. THE PARTICIPANTS ARE ASKED TO DELIVER THE RESULTS (CT VALUES) IN THE EXCEL FILE PROVIDED BY THE ORGANISERS.
2. CUT-OFF DETERMINATION: AFTER HAVING FILLED IN THE CELLS WITH YOUR CT VALUES, A NUMBER MUST APPEAR IN THE CELLS "CUT-OFF AT 15 COPIES". IF NOT (####### WILL APPEAR IN THE CELLS), CHECK THAT YOUR FORMAT FOR THE NUMBERS IS CORRECT. THE PROBLEM CAN BE DUE TO YOUR DECIMAL SYMBOL (THE DOT "." OR THE COMMA ","). CHANGE OF DECIMAL SYMBOL IN YOUR CT VALUES.
   IF THE PROBLEM REMAINS, PLEASE CONTACT THE ORGANISERS.
3. IF OUTLIERS ARE OBSERVED WITHIN CALIBRATION DATA (RED CELLS IN THE "OUTLIERS" SHEET), THE CORRESPONDING CT VALUES CAN BE REMOVED (NO MORE THAN 5% OF THE DATA. IF MORE THAN 5% OF THE DATA ARE DETECTED AS OUTLIERS, PERFORM NEW CALIBRATIONS). PLEASE DO NOT WRITE ANYTHING IN THE CORRESPONDING CELL (E.G. 0, 50, NOT DETERMINED, NC, ...) BUT LEAVE IT EMPTY.
4. THE CUT-OFF VALUE IS DETERMINED ONLY AT THE END OF THE 4 RUNS EVEN IF A FIGURE APPEARS BEFORE.
5. THE PARTICIPANTS ONLY HAVE TO FILL IN THE CELLS WITH THE CT VALUES.
6. A WORD FILE IS ALSO PROVIDED TO THE PARTICIPANTS TO ALLOW THEM TO DESCRIBE ANY DEVIATION FROM THE INITIAL PROTOCOL.
7. EVEN IF ADDITIONAL PLATES ARE PERFORMED BY A PARTICIPANT, IT IS ASKED TO REPORT ONLY THE RESULTS OF 4 PLATES CORRESPONDING TO THE DESIGN OF THE STUDY.
8. THE RAW DATA OF THE RUNS MUST BE RECORDED ON A CD-ROM AND SENT TO THE ORGANISERS.

7.2. Reporting of samples results

One run corresponds to one sheet named “Run 1”, “Run 2”, “Run 3” and “Run 4”.

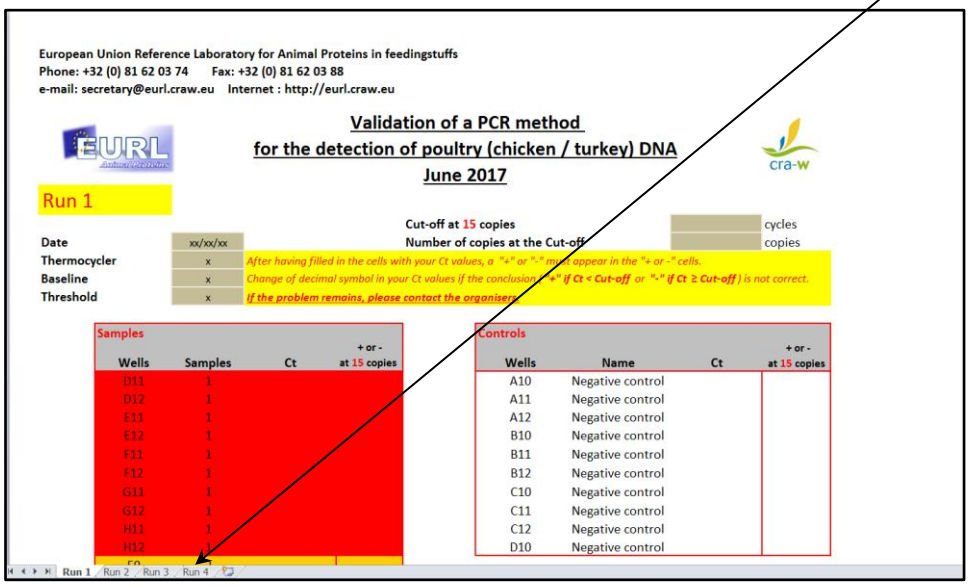


Figure 4 : Image of the sheets in the “Report file pig validation study” Excel file.

Within each sheet, the cells to fill in with the Ct values are pre-defined.
The result of each reaction (“+” or “-”) is automatically interpreted.

1. **THE PARTICIPANTS ARE ASKED TO DELIVER THE RESULTS (CT VALUES) IN THE EXCEL FILE PROVIDED BY THE ORGANISERS.**
2. **THE PARTICIPANTS ONLY HAVE TO FILL IN THE CELLS WITH THE CT VALUES. THE QUALITATIVE (POSITIVE OR NEGATIVE) RESULTS ARE DETERMINED AUTOMATICALLY. ". IF NOT (####### WILL APPEAR IN THE CELLS), CHECK THAT YOUR FORMAT FOR THE NUMBERS IS CORRECT. THE PROBLEM CAN BE DUE TO YOUR DECIMAL SYMBOL (THE DOT "." OR THE COMMA ","). CHANGE OF DECIMAL SYMBOL IN YOUR CT VALUES.**
   IF THE PROBLEM REMAINS, PLEASE CONTACT THE ORGANISERS.
3. **A WORD FILE IS ALSO PROVIDED TO THE PARTICIPANTS TO ALLOW THEM TO DESCRIBE ANY DEVIATION FROM THE INITIAL PROTOCOL.**
4. **THE RAW DATA OF THE RUNS MUST BE RECORDED ON A CD-ROM AND SENT TO THE ORGANISERS.**

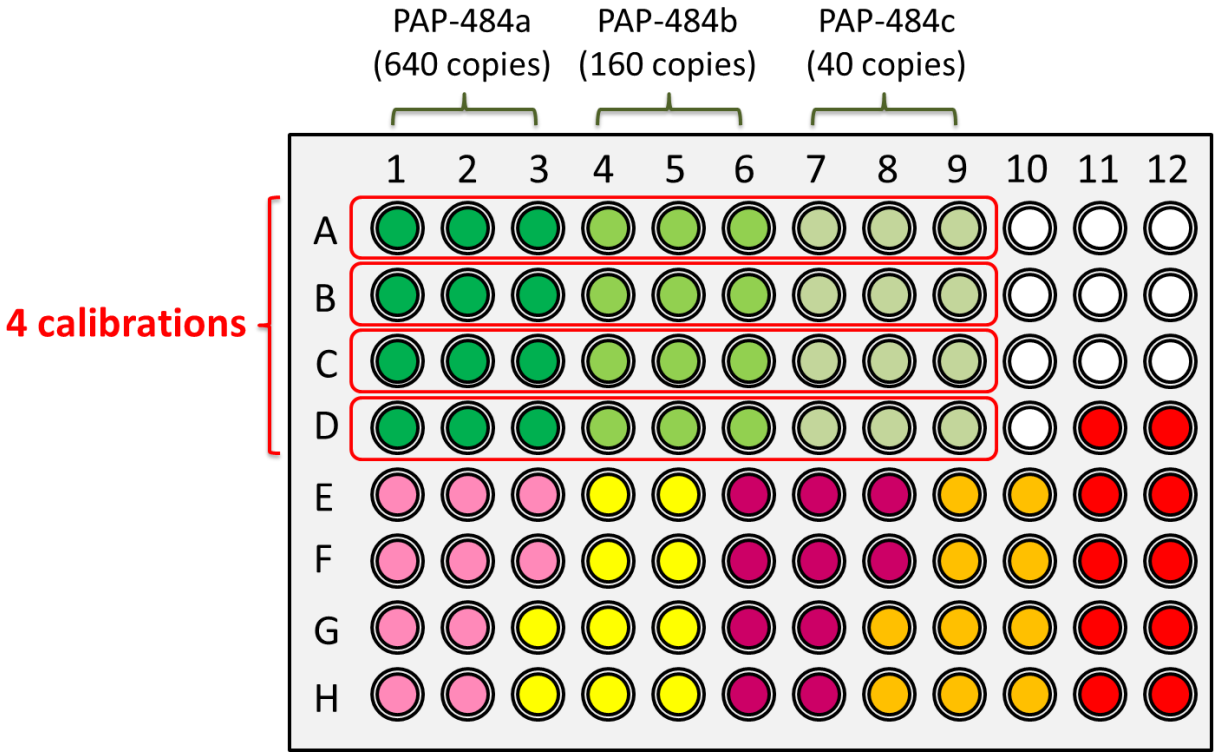
ANNEX 1 : AMOUNTS OF PASSIVE REFERENCE (ROX) TO ADD TO THE MASTERMIX 2X

SYSTEM	MASTERMIX 2X	PASSIVE REFERENCE
7000 – 7300 – 7900 (ABI)	1000 µL	40 µL
7500 (ABI)	1000 µL	2.8 µL
LC480 (ROCHE)	1000 µL	0 µL
ICYCLER (BIORAD)	1000 µL	0 µL
ROTORGENE 6000 (CORBETT)	1000 µL	0 µL
MX3000P/3005P (STRATAGENE – AGILENT)	1000 µL	0 µL

SOURCE: UNIVERSAL MASTERMIX 7.5ML, TECHNICAL DATA SHEET, DIAGENODE (LIÈGE, BELGIUM)

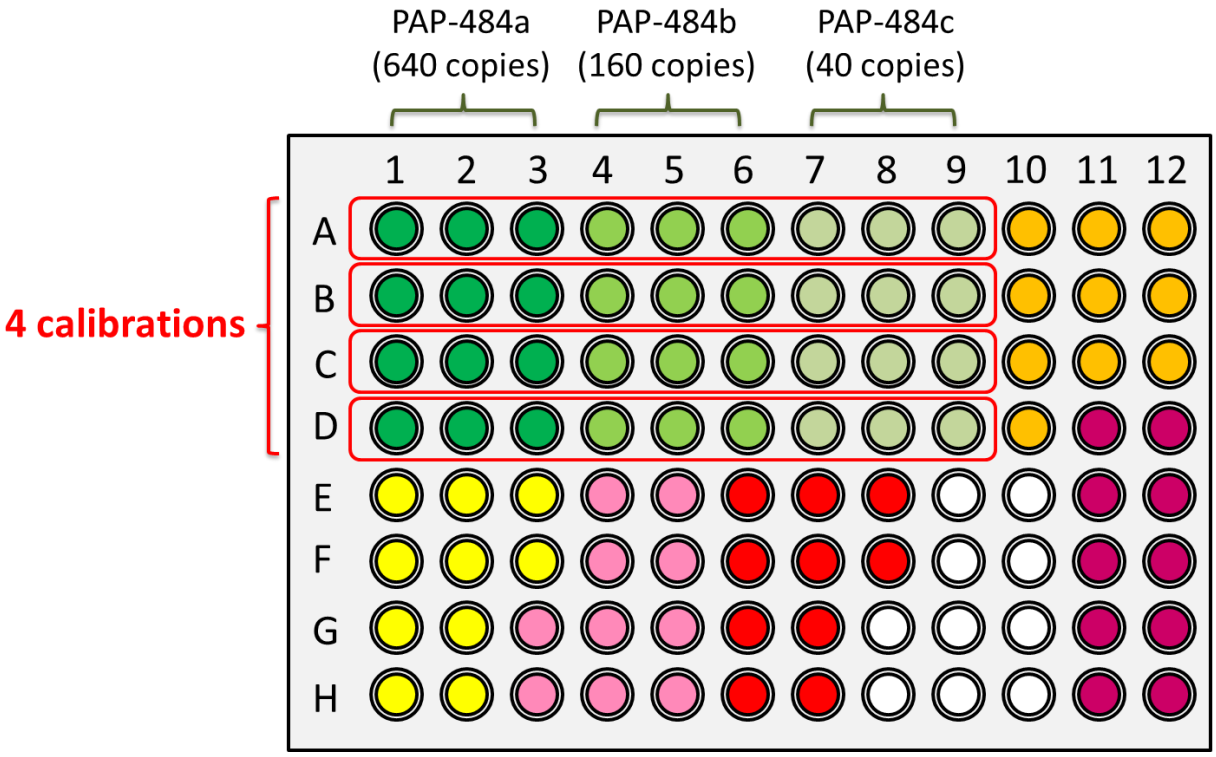
ANNEX 2 : SCHEMES OF THE 4 PLATES OF THE STUDY

RUN 1



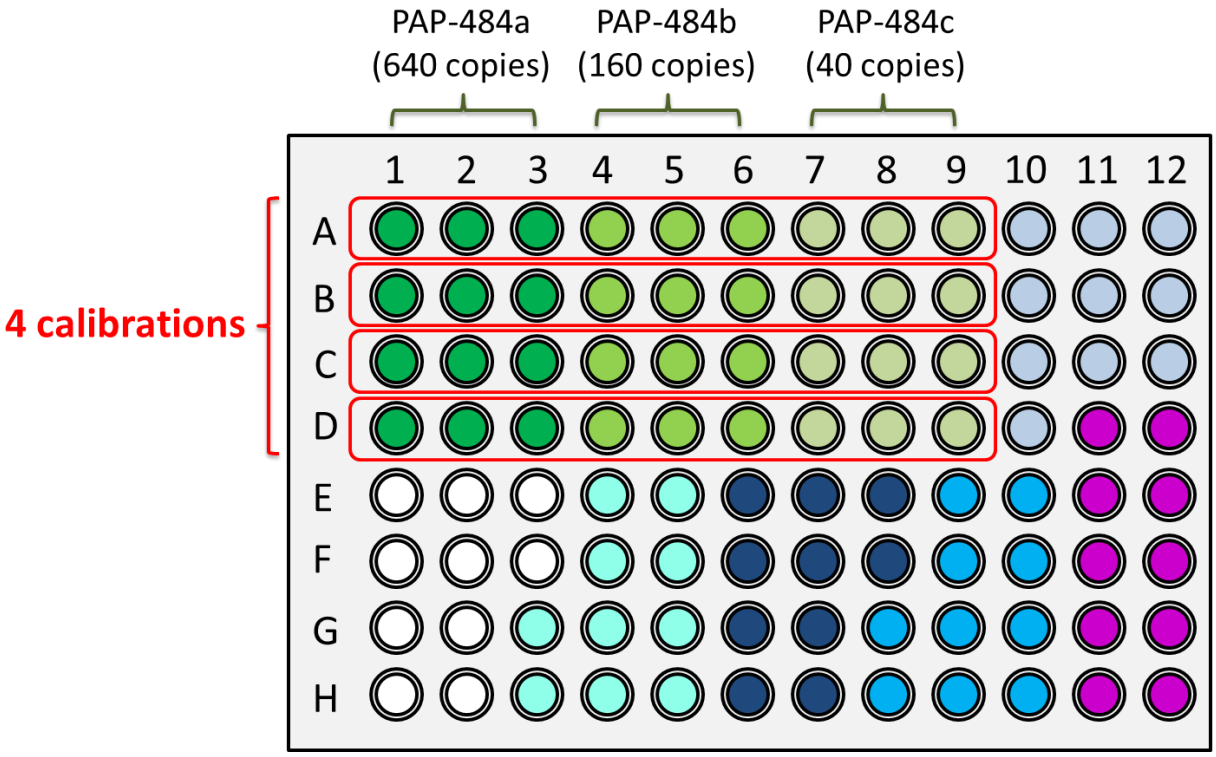
		Wells
Calibrants		
	640 copies	A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3
	160 copies	A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6
	40 copies	A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9
Control		
	Negative PCR control	A10, A11, A12, B10, B11, B12, C10, C11, C12, D10
Samples		
	# 1	D11, D12, E11, E12, F11, F12, G11, G12, H11, H12
	# 2	E9, E10, F9, F10, G8, G9, G10, H8, H9, H10
	# 3	E6, E7, E8, F6, F7, F8, G6, G7, H6, H7
	# 4	E4, E5, F4, F5, G3, G4, G5, H3, H4, H5
	# 5	E1, E2, E3, F1, F2, F3, G1, G2, H1, H2

RUN 2



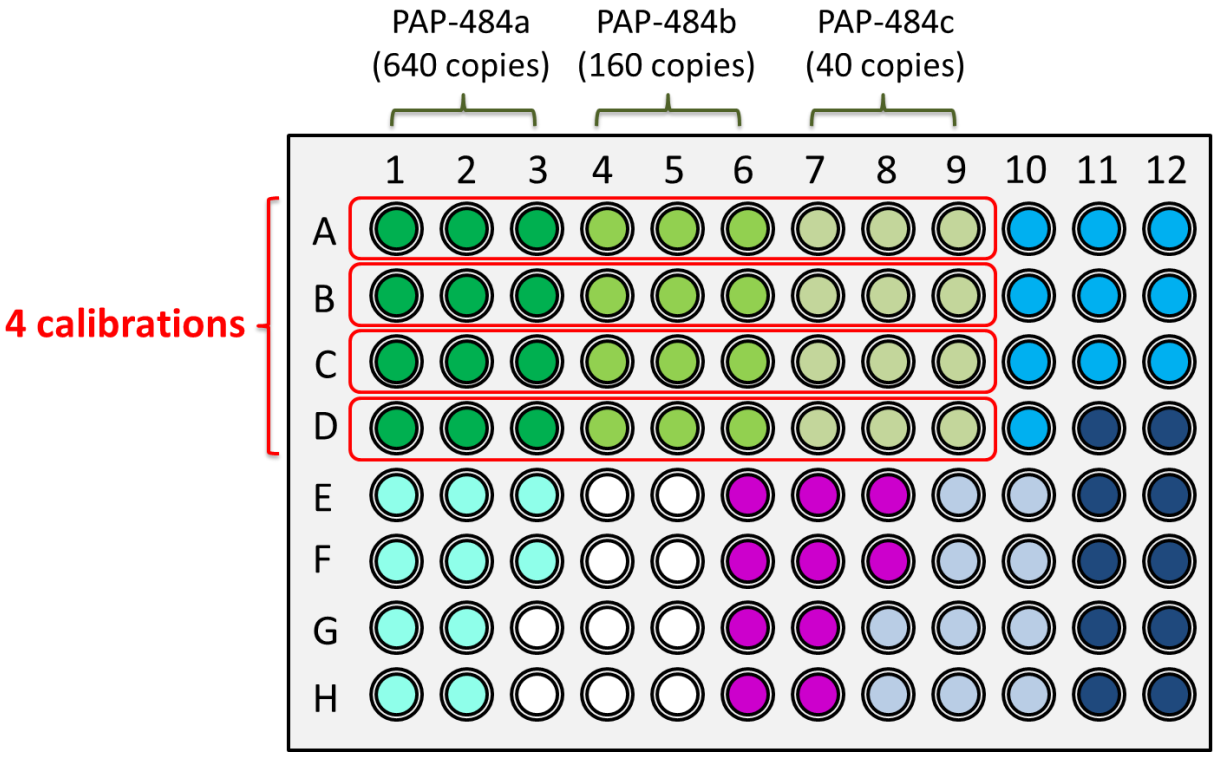
		Wells
Calibrants		
	640 copies	A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3
	160 copies	A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6
	40 copies	A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9
Control		
	Negative PCR control	E9, E10, F9, F10, G8, G9, G10, H8, H9, H10
Samples		
	# 1	E6, E7, E8, F6, F7, F8, G6, G7, H6, H7
	# 2	A10, A11, A12, B10, B11, B12, C10, C11, C12, D10
	# 3	D11, D12, E11, E12, F11, F12, G11, G12, H11, H12
	# 4	E1, E2, E3, F1, F2, F3, G1, G2, H1, H2
	# 5	E4, E5, F4, F5, G3, G4, G5, H3, H4, H5

RUN 3



		Wells
Calibrants		
	640 copies	A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3
	160 copies	A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6
	40 copies	A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9
Control		
	Negative PCR control	E1, E2, E3, F1, F2, F3, G1, G2, H1, H2
Samples		
	# 6	A10, A11, A12, B10, B11, B12, C10, C11, C12, D10
	# 7	D11, D12, E11, E12, F11, F12, G11, G12, H11, H12
	# 8	E9, E10, F9, F10, G8, G9, G10, H8, H9, H10
	# 9	E6, E7, E8, F6, F7, F8, G6, G7, H6, H7
	# 10	E4, E5, F4, F5, G3, G4, G5, H3, H4, H5

RUN 4



		Wells
Calibrants		
	640 copies	A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3
	160 copies	A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6
	40 copies	A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9
Control		
	Negative PCR control	E4, E5, F4, F5, G3, G4, G5, H3, H4, H5
Samples		
	# 6	E9, E10, F9, F10, G8, G9, G10, H8, H9, H10
	# 7	E6, E7, E8, F6, F7, F8, G6, G7, H6, H7
	# 8	A10, A11, A12, B10, B11, B12, C10, C11, C12, D10
	# 9	D11, D12, E11, E12, F11, F12, G11, G12, H11, H12
	# 10	E1, E2, E3, F1, F2, F3, G1, G2, H1, H2

e. Annex V: Calibrant product information sheet

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate D - Institute for Reference Materials and Measurements
Standards for Innovation and sustainable Development

PRODUCT INFORMATION SHEET
PAP-484a, PAP-484b, PAP-484c

Set of reference materials for poultry target detection by PCR

DESCRIPTION

The PAP-484 set consists of three different vials containing plasmid solutions with copy number concentrations of about 103 cp/µL, 24 cp/µL and 8 cp/µL. The plasmid bears one copy of a poultry specific DNA fragment. The vials contain at least 1 mL of a solution of this linearised, double stranded plasmid in Tris-EDTA buffer containing salmon sperm genomic DNA at a concentration of 50.5 ng/µL. The DNA copy number concentration of the starting material has been estimated by UV measurements, the solutions have been prepared by gravimetric dilution and the final copy number concentration was measured by digital PCR.

The following copy number concentrations and provisional expanded uncertainties were assigned for the three concentration levels.

SET OF PLASMID SOLUTIONS		
	Copy number concentration of the plasmid [a]	
	Value [cp/µL] [b]	Uncertainties [cp/µL] [c]
PAP-484a	103	18
PAP-484b	24	6
PAP-484c	8	2

[a] Copy number concentration of the double stranded plasmid as measured by a droplet digital PCR method amplifying one DNA fragment specific for poultry, using primers and probe described by the Standard Operating Procedures of EURL-AP [1].
[b] Unweighted mean value of 16 independent measurements obtained in 6 different laboratories. The value is traceable to the International System of units (SI).
[c] The provisional uncertainty is the expanded uncertainty, estimated on the basis of homogeneity, characterisation and 6 months stability studies, with a coverage factor $k = 2$ corresponding to a level of confidence of approximately 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2009.

INSTRUCTION FOR USE AND INTENDED USE

To make the plasmid solution ready for use, the content of the vial has to be thawed completely and mixed gently by inverting the vial several times at ambient temperature. The vial should be opened and handled under a laminar flow to reduce the risk of contamination. The material is intended to be used to discriminate a positive from a negative sample using the Standard Operating Procedures (SOPs) edited by the EURL-AP [1] for technical implementation of Commission Regulation (EC) No 152/2009 [2].

The minimum sample intake is 4 µL.

STORAGE

The materials shall be stored at -20 °C ± 5 °C prior to use. It is advisable to close the vials with the original screw caps after use. The plasmid solutions should not be exposed to direct sun light.

Under the condition that contamination during handling of opened vials has been excluded, the solutions can be used for several experiments. Based on the results of freeze-thaw studies done on a similar material [3], we recommend that PAP-484 does not pass more than 2 freeze-thaw cycles and to aliquot the solutions to reduce the number of freeze-thaw cycles. The materials can also be stored at +4 °C for 4 weeks as it was verified that no significant change to the assigned concentration was observed during that period.

However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened vials.

DISCLAIMER

The calibration kit for poultry target detection by PCR is not a certified reference material because the material has only been tested for a 6 months term stability. Consequently, provisional expanded uncertainties are calculated using only the uncertainty contributions from the homogeneity and characterisation studies. A shelf life of the material is not yet available.

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REFERENCES

[1] http://eurl.craw.eu/index.php?page=187
[2] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed.
[3] http://publications.jrc.ec.europa.eu/repository/bitstream/JRC97915/jrc97915%20erm%20ad483abc.pdf

Geel, June, 2016
Validity: 6 months

Signed: _____

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European Commission, Joint Research Centre
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