# Validation study of a real-time PCR method developed by CRA-W for the detection of pig DNA in feedingstuffs

**Olivier FUMIÈRE, Aline MARIEN, Gilbert BERBEN**

Department Knowledge and Valorisation of Agricultural Products

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

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

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## 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 most promising solution for this aim. CRA-W developed a real-time PCR method able to detect low levels of pig DNA. The fitness for purpose of this method was checked at the EURL-AP through an in-house validation study. A full validation through an interlaboratory study was conducted by the EURL-AP to provide evidence that the method is suitable for detection of processed animal proteins (PAP) in a network of laboratories.

As for the ruminant method, 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 potentially present at a high number of copies per cell and therefore accurate determination of the cut-off is of importance to obtain reliable results.

The study took place from end of December 2014 (date of the call for participants) up to end of February 2015 (return 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 5 copies per reaction was tested (in fact the upper 95% confidence limit of the Ct for 5 copies). Cut-off values calculated at 5 copies per reaction of the 15 platforms are in a range between 38.78 and 41.43 cycles, representing in terms of copy number a range of values from 3.11 to 3.76 (when limiting the analysis to the data kept for the conclusions). When using these cut-off values, the aimed 95% level of correct assignments with the blind samples included in the trial was met but results of two laboratories had to be excluded because of a too high rate of false positive results on the known negative PCR control. The rate of false positive results is 0.77 % and the rate of false negative results is 0.38 % at the level of 0.1 % w/w of pig PAP in feed. 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 fills, 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 the DNA to high temperatures and 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.*, 2021). 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 method, the EURL-AP completed the PCR assay for pig 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 CRA-W pig PCR assay targeting a region located in the mitochondrial DNA which is less abundant than the previously validated ruminant target (Marien *et al.*, 2021).

This full validation of the CRA-W pig PCR assay will mainly check the transferability of this analytical assay and its fitness to detect pig PAP at 0.1% (w/w) in feedingstuffs. The assessment of the assay by in-house validation at the EURL-AP already pointed out that the method was of interest by being able to meet acceptable performance parameters 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:

- 3 LC480 (Roche Diagnostics)
- 1 ABI 7300, 2 ABI 7500, 1 ABI 7900, 1 StepOnePlus and 1 ViiA7 (Applied Biosystems – Life Technologies)
- 3 CFX (Bio-Rad)
- 1 Mx3000P, 1 Mx3005P (Agilent)
- 1 RotorGene (QIAGEN)

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

## 5. Time schedule of the study

The 11th of December 2014, an invitation letter (Annex II) was sent to 20 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 9th of January 2015 by sending back a reply form indicating all important information about the laboratories and the models of thermocyclers participating to the study.

The 14th of January 2015, the instructions and the protocol of the study were sent to the participants.

The 2nd of February 2015, the experimental material was sent to all the participating laboratories which received the material within three days (between the 3rd and the 5th of February 2015).

The results were collected between the 6th and the 27th of February 2015.

## 6. Purpose of the study

The objective of this study was to evaluate the fitness of a protocol used to detect the presence of pig 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 to the protocol developed in 2009 and already successfully tested through the validation study of the ruminant PCR method in 2012 (Planchon *et al.*, 2010 ; Fumière *et al.*, 2010 ; Fumière *et al.*, 2016). The determination of the cut-off value was adapted to correspond to the upper limit of the confidence interval of a Ct value for 5 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.*, 2021). The copy number value targeted for the cut-off is smaller than that of the ruminant PCR assay because the pig target considered is a less abundant target in the cell genome than the ruminant one.

Ten DNAs extracted from feed samples containing 0, 0.0125, 0.025 and 0.1 % in mass fraction of pig PAP were tested in blind by the participants. Each DNA was analysed 20 times (10 replicates x 2 runs).

The present method will be considered as fit for the purpose if > 95 % of the reactions with DNA extracted from samples at 0.1 % in mass fraction of ruminants 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.

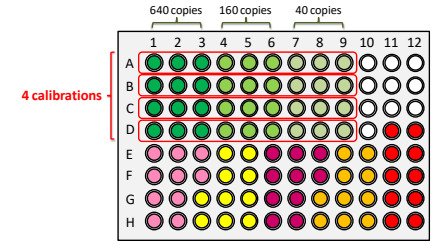
## 7. Design of the study

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 with low pig PAP content 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]

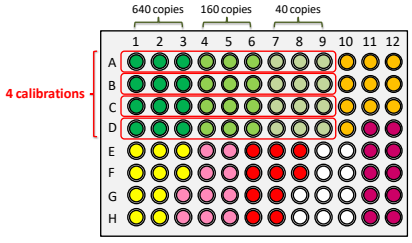
[1] This design could not be strictly followed by the participant using a Rotorgene (QIAGEN) but the runs were similar to the ones described for 96-well plates.

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 |

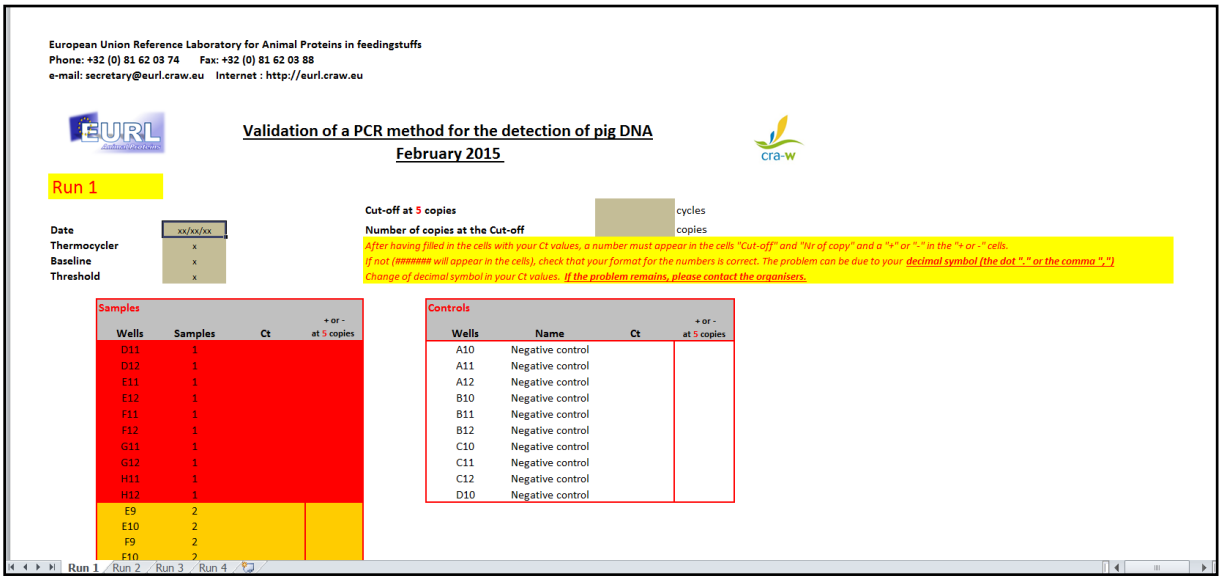
Run 2




| | | 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).



European Union Reference Laboratory for Animal Proteins in feedingstuffs
Phone: +32 (0) 81 62 03 74 Fax: +32 (0) 81 62 03 88
e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

Validation of a PCR method for the detection of pig DNA
February 2015

Run 1

Date xx/xx/xx
Thermocycler x
Baseline x
Threshold x

Cut-off at 5 copies cycles
Number of copies at the Cut-off copies

*After having filled in the cells with your Ct values, a number must appear in the cells "Cut-off" and "Nr of copy" and a "+" or "-" in the "+ or -" cells.*
*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.*

Samples

| Wells | Samples | Ct | + or - at 5 copies |
|---|---|---|---|
| D11 | 1 | | |
| D12 | 1 | | |
| E11 | 1 | | |
| E12 | 1 | | |
| F11 | 1 | | |
| F12 | 1 | | |
| G11 | 1 | | |
| G12 | 1 | | |
| H11 | 1 | | |
| H12 | 1 | | |
| E9 | 2 | | |
| E10 | 2 | | |
| F9 | 2 | | |
| F10 | 2 | | |

Controls

| Wells | Name | Ct | + or - at 5 copies |
|---|---|---|---|
| A10 | Negative control | | |
| A11 | Negative control | | |
| A12 | Negative control | | |
| B10 | Negative control | | |
| B11 | Negative control | | |
| B12 | Negative control | | |
| C10 | Negative control | | |
| C11 | Negative control | | |
| C12 | Negative control | | |
| D10 | Negative control | | |

Run 1 Run 2 Run 3 Run 4

***Figure 2 :*** Excel sheet for the reporting of the results

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

## 8. Description and preparation of test materials

The list of the material provided by the organisers is presented in Annex IV. Besides the mastermix (Diagenode, Seraing, Belgium), the primers and probe (Eurogentec, Seraing, Belgium) already diluted at working concentrations, the material provided consisted in the calibrants, the 10 blind DNA 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: IRMM-483a - Cut-off calibration curve (640 copies) (635 copies / 5 µl*), IRMM-483b - Cut-off calibration curve (160 copies) (170 copies / 5 µl*), IRMM-483c - Cut-off calibration curve (40 copies) (45 copies / 5 µl*). They were prepared in a background of salmon sperm genomic DNA at the concentration of 51 ng / µl (to avoid loss of molecules by their sticking to the plastic walls of the vial). The calibrants are fully described in Annex V. The exact copy numbers were slightly different from the nominal ones and these exact figures (635 copies/5 µl, 170 copies/5 µl and 45 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).

* exact copy numbers determined with 14 independent measurements obtained in 7 different laboratories by digital PCR.

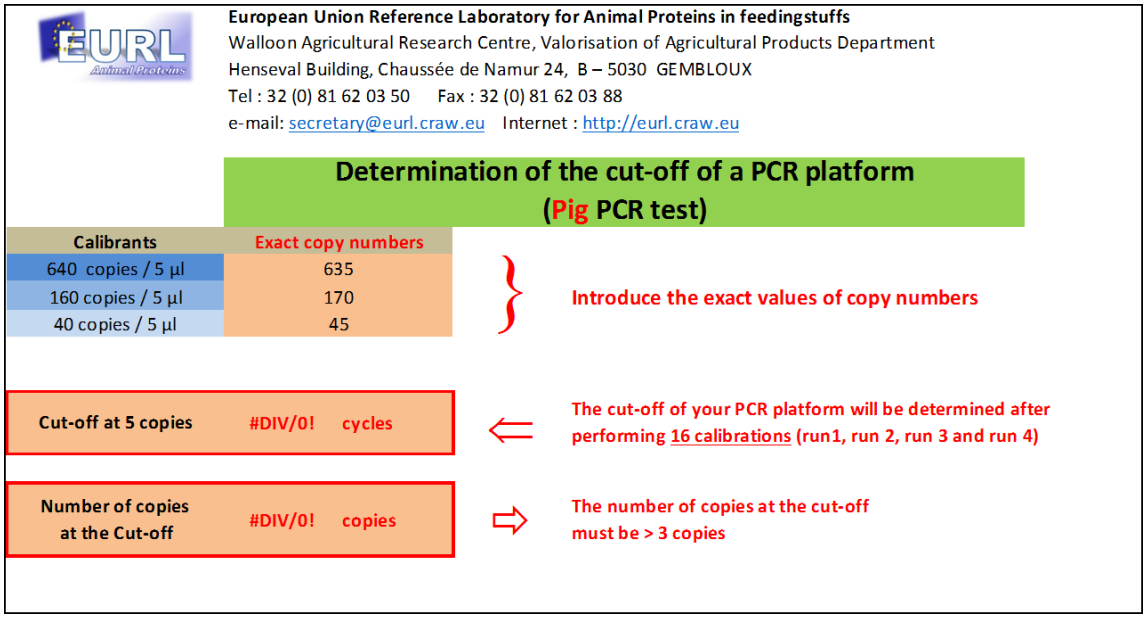



***Figure 3*** **:** "Exact copy numbers" of the Excel file for the determination of the cut-off

### *8.2. The ten blind samples*

A set of blind samples was constituted with 4 concentrations of porcine PAP (porcine PAP processed according to method 1 [heat treated at 133 °C under a pressure of 3 bars and during at least 20 minutes]) : 0.1 % in weight (~170 copies of the pig target / 5µl), 0.0250 % in weight (~35 copies of the pig target / 5µl), 0.0125 % in weight (~15 copies of the pig target / 5µl) and a blank at 0 % in weight (0 copy of the pig 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 pig DNA in feedinstuffs

| Sample | Material | Number of vials and remarks | |
|---|---|---|---|
| *DNA extracts* | | | |
| 1 | 0.0125 % w/w porcine PAP in blank 2 | 1 | |
| 2 | Blank 2 : feed for pig | 1 | |
| 3 | 0.1 % w/w porcine PAP in blank 2 | 1 | |
| 4 | Blank 1 : feed for horse with 0.5 % milk powder | 1 | |
| 5 | 0.025 % w/w porcine PAP in blank 2 | 1 | |
| 6 | 0.1 % w/w porcine PAP in blank 2 | 1 | (replicate of sample #3) |
| 7 | 0.025 % w/w porcine PAP in blank 2 | 1 | (replicate of sample #5) |
| 8 | Blank 1 : feed for horse with 0.5 % milk powder | 1 | (replicate of sample #4) |
| 9 | Blank 2 : feed for pig | 1 | (replicate of sample #2) |
| 10 | 0.0125 % w/w porcine PAP in blank 2 | 1 | (replicate of sample #1) |
| **Total** | | **10** | |

The preparation scheme is illustrated in Figure 4.

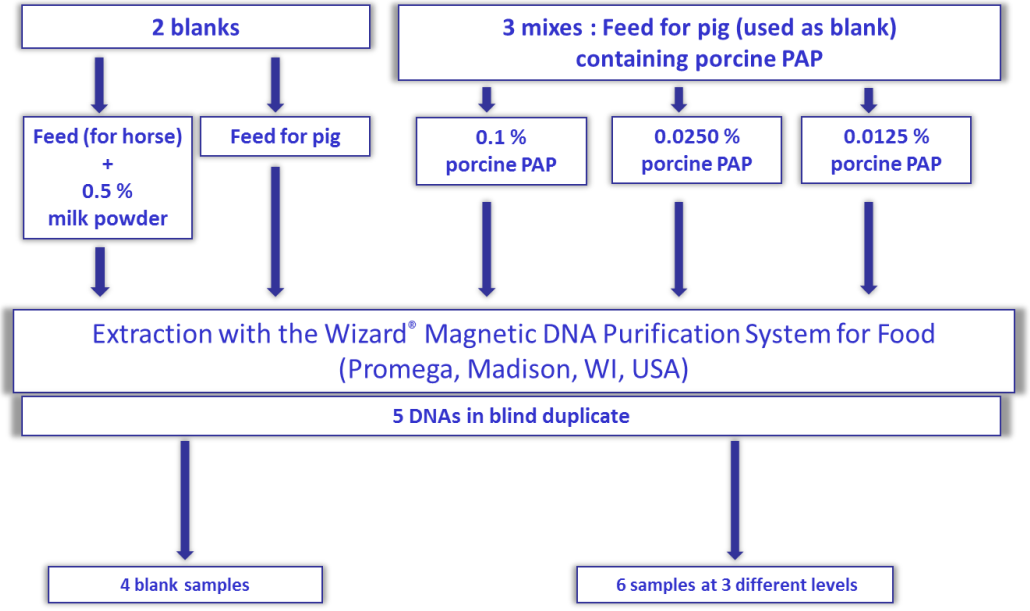



***Figure 4*:** Production of the blind samples set used in the EURL-AP PCR
Validation study of a real-time PCR method for the detection of pig DNA in feedingstuffs

A sample consisting of a blank feed for pig was adulterated at three levels (0.1 %, 0.025 % and 0.0125 % w/w) with a porcine PAP processed according to method 1 (heat treated at 133 °C under a pressure of 3 bars and during at least 20 minutes). Two blank samples were also extracted: the first one is the blank feed for pig used in adulterated samples. The second one is a feed for horses spiked with 0.5 % of milk powder.

These 6 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, 52 extracts of each sample were prepared. At the end, the 52 extracts were pooled and homogenised. The DNA extracts were tested to check the absence of PCR inhibition and contamination.

The samples were analysed with the porcine PCR target. The blank samples were tested with 40 replicates. The blank samples were negative for porcine DNA; they do not give any amplification or if there was a signal, it appeared after the cut-off except for one signal on 40 replicates that was recorded before the cut-off value (blank Ct at 40.20 cycles, cut-off value at 40.35 cycles) for the blank 1.

The 0.1 % level of porcine heat treated material is equivalent to +/- 170 copies of the ruminant target per 5 µl. The ΔCt between the levels at 0.1 and 0.025 % is a slightly higher than the 2 cycles expected. This result can be explained by the low level of contamination and a higher variability of the signals. Moreover the two samples were prepared independently. A ΔCt of +/- 1 cycle as expected was nevertheless observed between the samples at 0.025 and 0.0125 % w/w.

***Table 2*:** Mean Ct obtained on the three samples containing porcine PAP on the LC480 (Roche Diagnostics Ltd.) with the Universal Mastermix (Diagenode s.a.). Analysis mode: Abs. quant/Fit points with parameters: background 2-15, noiseband 2.5 and threshold 3.2.

| Level | Mean Ct on Promega extracts (n = 5) |
|---|---|
| 0.1 % of porcine PAP in weight | 33.97 cycles |
| 0.025 % of porcine PAP in weight | 36.62 cycles |
| 0.0125 % of porcine PAP in weight | 37.83 cycles |

During the tests, all the DNA extracts were stored at 4 °C. The DNA extracts were aliquoted and stored at - 20 °C until shipping 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.

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 5 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 CRA-W PCR assay on pig showed that cut-off values calculated at 5 copies of the target could be suitable. The copy numbers corresponding to the cut-off values expressed in terms of Ct for each participant are provided in Table 3. They range between 3.00 and 3.76 copies and even less (from 3.11 to 3.76) if labs that were discarded (see later on) are not considered.

Table 3 provides the cut-off figures calculated at 5 copies.

***Table 3***: Cut-off values at 5 copies / 5 µl and corresponding number of copies of the participants. Data in red belong to labs discarded from the final analysis

| Lab | Cut-off value at 5 copies/5 µl | Corresponding number of copies |
|---|---|---|
| **Lab 1** | 38.7797 | 3.75 |
| **Lab 2** | 39.1153 | 3.64 |
| **Lab 3** | 39.9907 | 3.68 |
| **Lab 4** | 38.6760 | 3.40 |
| **Lab 5** | 40.2264 | 3.00 |
| **Lab 6** | 40.3438 | 3.11 |
| **Lab 7** | 39.5654 | 3.66 |
| **Lab 8** | 41.0227 | 3.77 |
| **Lab 9** | 39.9459 | 3.15 |
| **Lab 10** | 39.8106 | 3.73 |
| **Lab 11** | 41.4319 | 3.27 |
| **Lab 12** | 38.7948 | 3.60 |
| **Lab 13** | 40.5997 | 3.14 |
| **Lab 14** | 39.7220 | 3.76 |
| **Lab 15** | 40.0368 | 3.43 |

### *9.2. Percentages of false results*

Blind samples were tested either forty times (10 replicates x 2 samples x 2 runs) for each of the three levels of pig 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.

***Table 4:*** Rates of false results (in %)

| Lab | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cut-off at 5 copies** | | | | | | | | | | | | | | | | |
| False positive results | 1.25 | 0 | 0 | 0 | **26.25** | 1.25 | **18.75** | 0 | 1.25 | 0 | 1.25 | 1.25 | 2.50 | 1.25 | 0 | 3.17 |
| False negative results | | | | | | | | | | | | | | | | |
| 0.1 % | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.50 | 0 | 0 | 0 | 0 | 2.50 | 0.33 |
| 0.0250 % | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.00 |
| 0.0125 % | 2.50 | 0 | **32.50** | 0 | 2.50 | **27.50** | 0 | 0 | 2.50 | 0 | 0 | 2.50 | 0 | 0 | **10.00** | **5.33** |

## 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.

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

| **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 5 copies) | 2.50 | 0 | 0 | 0 | **15.00** | 2.50 | **17.50** | 0 | 0 | 0 | 5.00 | 2.50 | 2.50 | 0 | 0 |

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. Labs 5 and 7 were considered as under-performant and were excluded from the statistical analysis of the results.

### *10.2. Rates of false positive and false negative results*

If results of labs 5 and 7 are not considered, the global rate of false positive results reached 0.77 % (8 / 1040) while the false negative rate amounted to 2.12 % (33 / 1560) for a cut-off value calculated at 5 copies. These values are far below the rate of 5 % and the method can therefore be considered as fit for purpose the more as for the level of 0.1 % of pig PAP in feedingstuffs the false positive rate is even 0.38% (2 / 520).

## 11. Conclusions

- The cut-off values of the 15 platforms determined using the proposed protocol showed differences between platforms (38.6760 < Cut-off value $_{5\ copies}$ < 41.4319) but were extremely close to each other in terms of copy numbers.
- The transfer of the protocol to new laboratories with thermocyclers from 5 companies (Applied Biosystems, 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 (EC-JRC-RMM, Geel, Belgium) for statistics and fruitful discussions. The authors would like to thank also Julie Hulin and Julien Maljean for their 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 |
| Danish Veterinary and Food Administration | Ringsted, Denmark |
| Darling Ingredients Nederland | Son, The Netherlands |
| Department of Agriculture, Food and Marine | Celbridge, Ireland |
| Institute of Animal Health, Food Safety and Environment (BIOR) | Riga, Latvia |
| Institute of Livestock Sciences (Agroscope) | Posieux, Switzerland |
| Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZSTO – CreAA) | Torino, Italy |
| Joint Research Centre – Institute for Reference Material and Measurement (JRC-IRMM) | Geel, Belgium |
| National Institute of Nutrition and Seafood Research (NIFES). | Bergen, Norway |
| National Diagnostic Centre of Food and Veterinary Service | Ljubljana, Slovenia |
| NutriControl BV | Veghel, The Netherlands |
| Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES) | Linz, Austria |
| RIKILT-Institute of Food Safety | Wageningen, The Netherlands |
| Service Commun des Laboratoires du MINEFI | Rennes, France |
| TNO Quality of Life | Zeist, The Netherlands |

## *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, 11 December 2014

**Invitation to participate to a validation study**
**of a PCR method for the detection of pig 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 however 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 would aim to validate a real-time PCR method developed by CRA-W (Gembloux, Belgium) for the detection of pig 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 :

- Primers and probe ready-to-use.
- 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 : calibrators, 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.

[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 2 weeks 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.
- 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 **January and February 2015**
- The samples will be sent to the participants between **the 26th of January and the 6th of February**
- The deadline for returning of results to organizers is **3 weeks (15 working days) after reception of the samples**

**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 **9th of January 2015 at noon**.

Please indicate in your reply form (see next page):

- 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



Wallonie

## *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 pig PAPs**

**Reply form**
**to send via e-mail to secretary@eurl.craw.eu**
**and/or Fax (+32 (0)81 62 03 88) by 9th of January 2015 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
fumiere@cra.wallonia.be

Date, name and signature of the responsible

Walloon Agricultural Research Centre

Wallonie

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### *d. Annex IV: Protocol of the study*

# PROTOCOL FOR THE VALIDATION OF A PCR METHOD FOR THE DETECTION OF PIG 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 pig DNA designed by CRA-W (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 pig 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 IRMM 483) | | |
| IRMM-483a - Cut-off calibration curve (640 copies) | 1 | 1000 µl |
| IRMM-483b - Cut-off calibration curve (160 copies) | 1 | 1000 µl |
| IRMM-483c - 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 7.5 ML (DIAGENODE S.A., LIÈGE, BELGIUM) | | |
| 2 boxes | | |
| Box content: | | |
| 2x Reaction Buffer (mastermix) | 1 | 7500 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 the vial of mastermix, defreeze it and dispatch 1500 µl in 5 tubes. Add if needed, the correct amount of passive reference in each tube (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 | 104 reactions (1 plate) |
|---|---|---|---|
| PCR grade water | 8.75 µl | 840.00 µl | 910.00 µl |
| Primer A | 1.25 µl | 120.00 µl | 130.00 µl |
| Primer B | 1.25 µl | 120.00 µl | 130.00 µl |
| Probe | 1.25 µl | 120.00 µl | 130.00 µl |
| Mastermix 2X | 17.50 µl | 1680.00 µl | 1820.00 µl |
| Total PCR mix volume/reaction | 30.00 µl | 2880.00 µl | 3120.00 µl |

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

**Total reaction volume = 35 µl / well**

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

**IMPORTANT REMARKS :**

1. **THE NUMBER OF REACTIONS PREPARED (104) 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. **30 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 A FEW SECONDS (SPIN).**

## 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 35 µl IS ENCODED IN THE PROGRAM.**
   **IF THE MAXIMUM VOLUME ALLOWED BY THE SOFTWARE IS 30 µL, ENCODE 30 µL.**

## 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 "IRMM-483a", "IRMM-483b" and "IRMM-483c" 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).

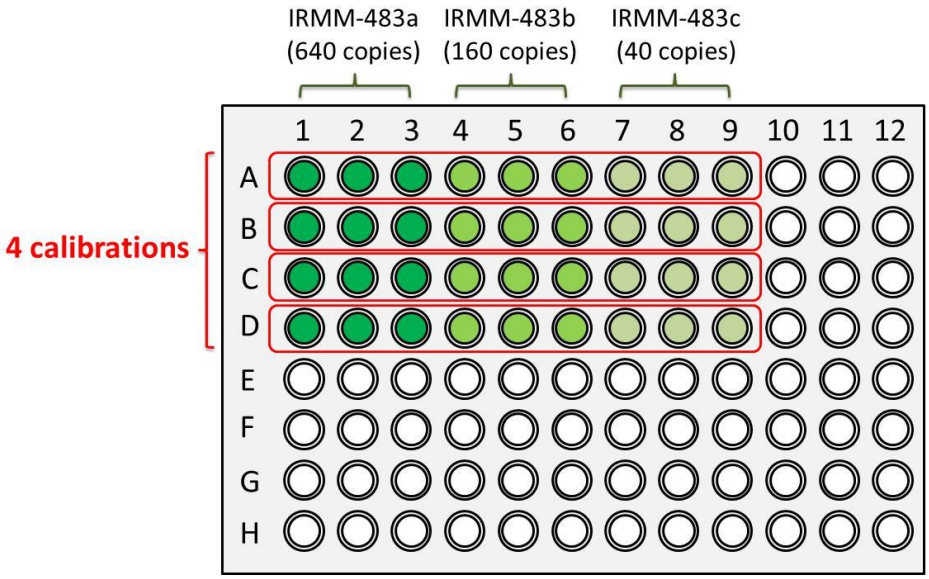



**Figure 1 :** Location of the wells used for the calibration of the platform.

## 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 pig 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).

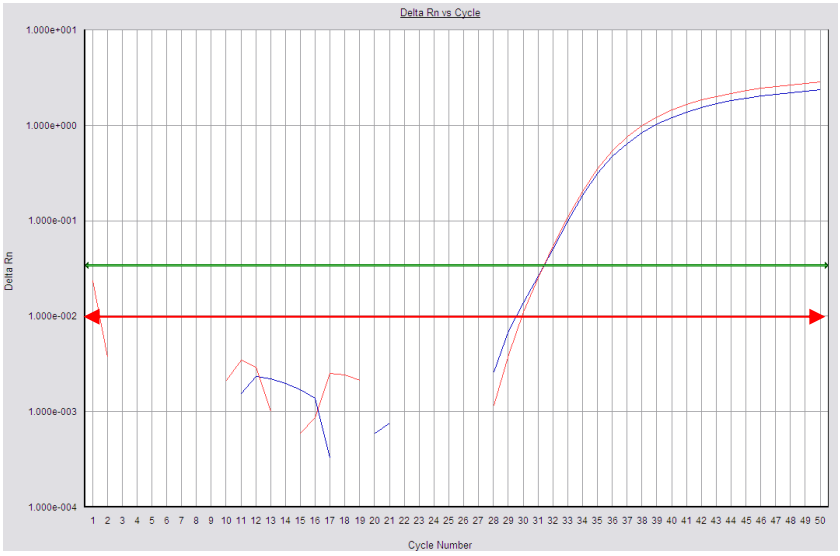



**Figure 2 :** Amplification signals and threshold levels.

**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 : “Pig cut-off at 5 copies - validation” and “Reporting file pig validation study”. Both file contain areas to fill in.

## 7.1. Determination of the cut-off at 5 copies

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

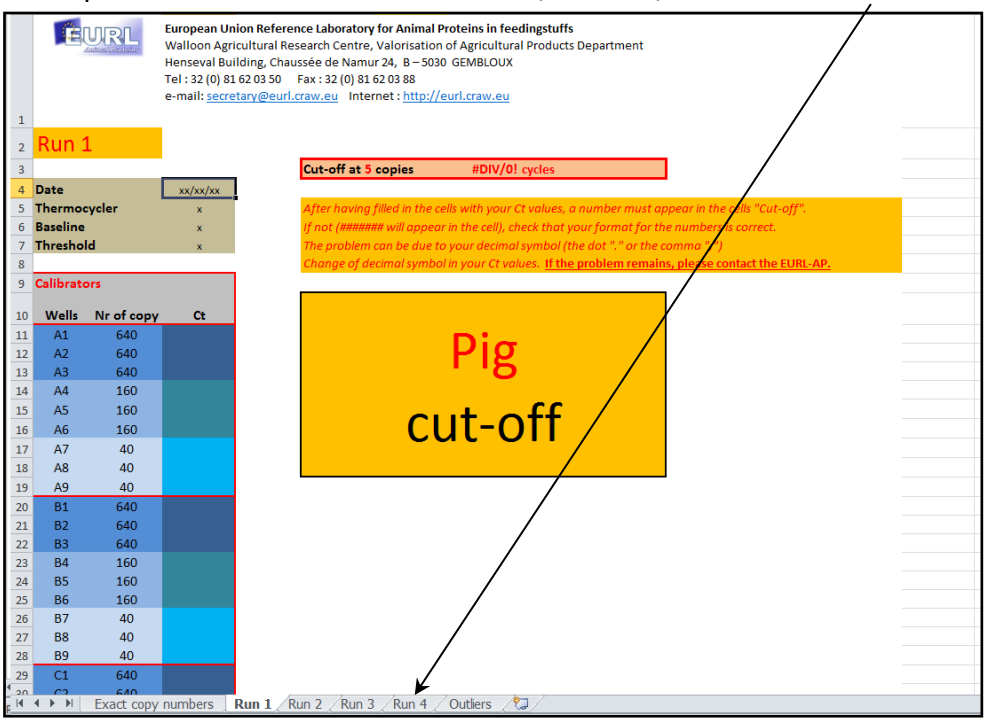



**Figure 3** : Image of the sheets in the “Pig cut-off at 5 copies - validation” Excel file.

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 5 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”.

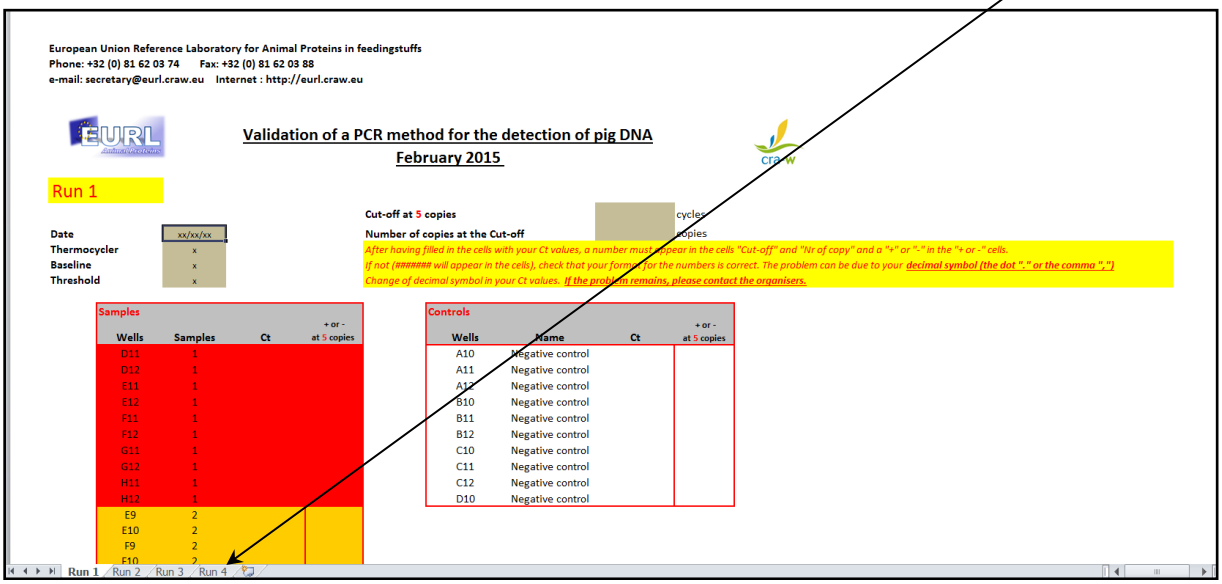



**Figure 4 :** Image of the sheets in the “Report file pig validation study” Excel file.

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.**

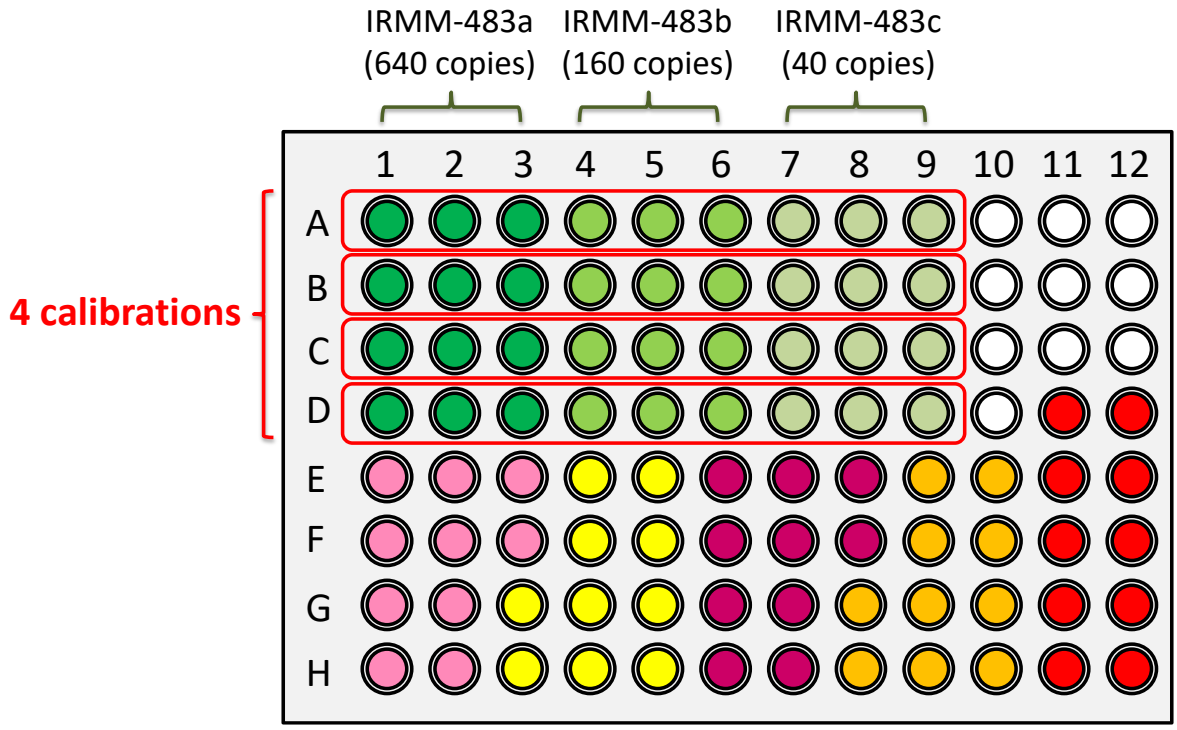
## ANNEX 1 : AMOUNTS OF PASSIVE REFERENCE (ROX) TO ADD TO THE MASTERMIX 2X

| 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)

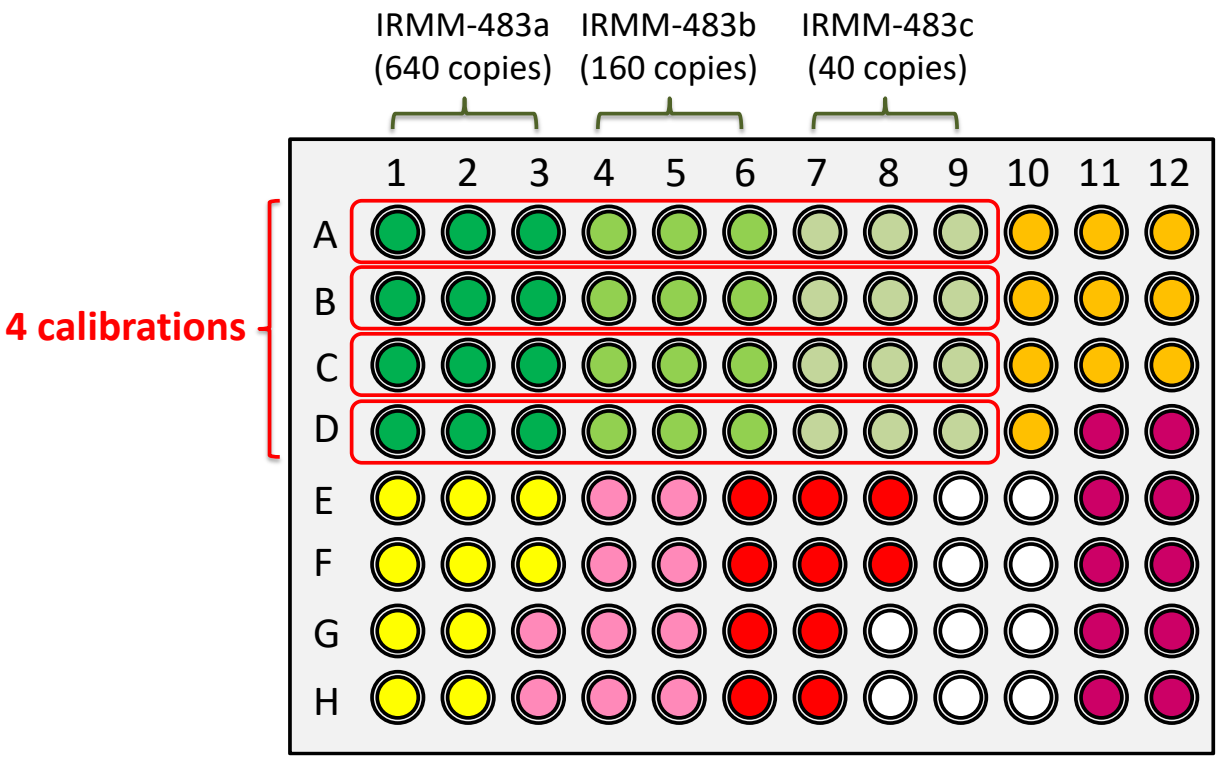
## ANNEX 2 : SCHEMES OF THE 4 PLATES OF THE STUDY

# RUN 1



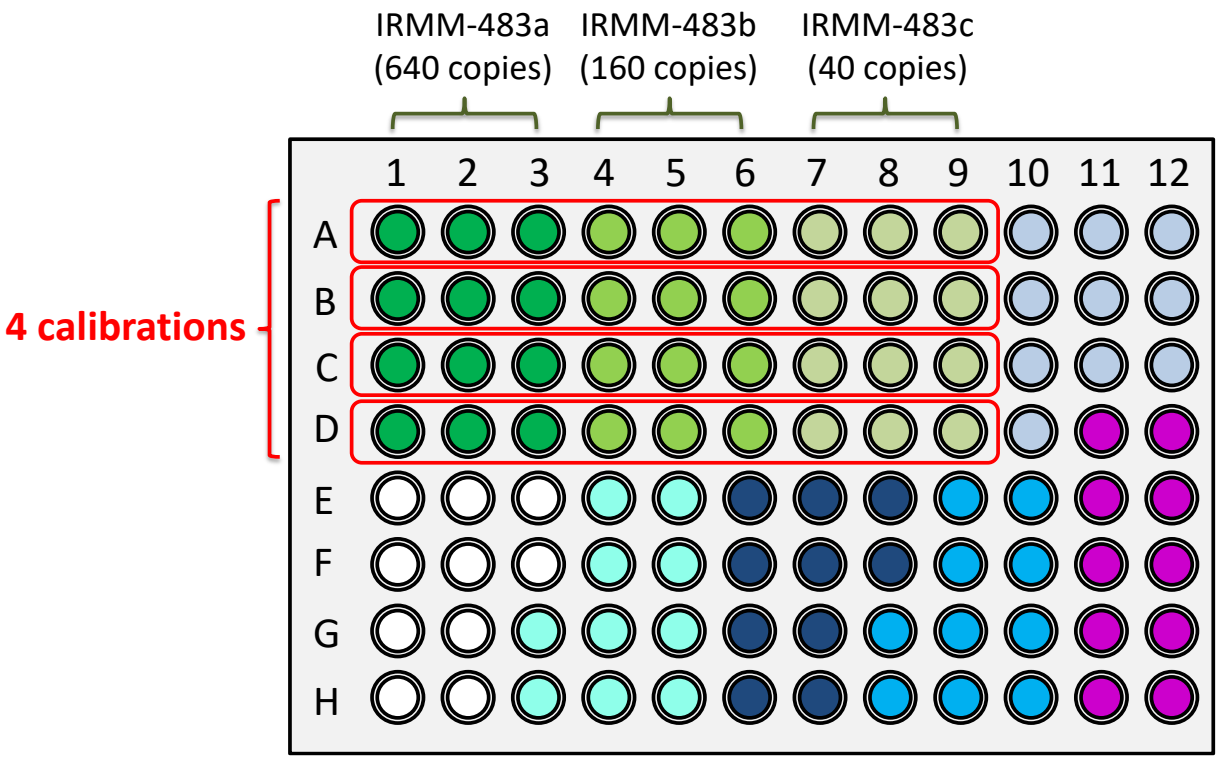

| | | Wells |
|---|---|---|
| **Calibrants** | | |
| | 640 copies | A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3 |
| | 160 copies | A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6 |
| | 40 copies | A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9 |
| **Control** | | |
| | Negative PCR control | A10, A11, 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



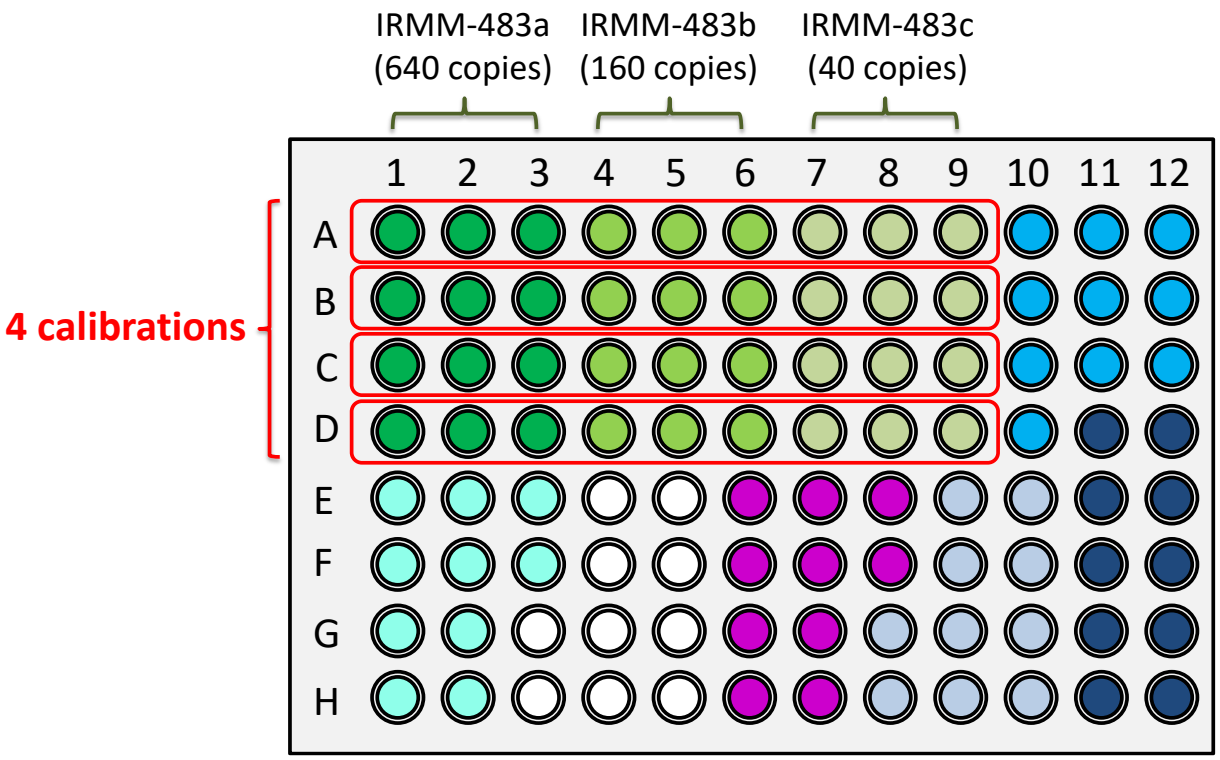

| | | Wells |
|---|---|---|
| **Calibrants** | | |
| | 640 copies | A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3 |
| | 160 copies | A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6 |
| | 40 copies | A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9 |
| **Control** | | |
| | Negative PCR control | E9, E10, 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 certificate

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

# PRODUCT INFORMATION

# IRMM-483a, IRMM-483b, IRMM-483c

# Set of reference materials for porcine target detection by PCR

### DESCRIPTION

The IRMM-483 set consists of three different vials containing plasmid solutions with copy number concentrations of plasmids of about 127 cp/µL, 34 cp/µL and 9 cp/µL. Each plasmid bears one copy of a porcine specific DNA fragment. The vials contain at least 1 mL solution of the linearized, double stranded plasmid in Tris-EDTA buffer containing salmon sperm genomic DNA at a concentration of 51 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 uncertainty values were assigned for the three concentration levels.

| SET OF PLASMID SOLUTIONS | | |
|---|---|---|
| | Copy number concentration of the plasmid [a] | |
| | Value [cp/µL] [b] | Uncertainty [cp/µL] [c] |
| IRMM-483a | 127 | 14 |
| IRMM-483b | 34 | 5 |
| IRMM-483c | 9 | 2 |

[a] Copy number concentration of the double stranded plasmid as measured by a digital PCR method amplifying one DNA fragment specific for porcine, using conditions, primers and probe described by the Standard Operating Procedures of the EURL-AP [1].
[b] Unweighted mean value of 14 independent measurements obtained in 7 different laboratories. The value and its uncertainty are traceable to the International System of units (SI).
[c] The provisional uncertainty is an expanded uncertainty 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 solutions ready for use, the content of the vials has to be thawed completely and mixed gently by inverting the vials several times at ambient temperature. The vials should be opened and handled under a laminar flow to reduce the risk of contamination. The material is intended to be used to determine the cut-off values as described by the Standard Operating Procedures (SOPs) of the EURL-AP [1] for technical implementation of Commission Regulation (EC) No 152/2009 [2].

The minimum sample intake is 4 µL.

All following pages are an integral part of the product information sheet.
Page 1 of 2

## 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 is excluded, the solutions could be used for several experiments. Based on the results of freeze-thaw studies done on a similar material [3], we recommend that IRMM-483 does not pass more than 5 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 1 week as it was verified that no significant change of the assigned concentration had been 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

This kit of reference materials for porcine target detection by PCR is not a certified reference material because the material has not yet been tested for long term stability. Consequently, provisional expanded uncertainty values are calculated using the uncertainties assigned only by the homogeneity and characterisation studies. A shelf-life period 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/111111111/29780/1/jrc%2085135%20final%20complete%20report.pdf

Geel, November, 2014

**Signed:** 27/11/2014

Prof. Dr. Hendrik Emons
European Commission, Joint Research Centre
Institute for Reference Materials and Measurements

European Commission – Joint Research Centre
Institute for Reference Materials and Measurements (IRMM)
Retieseweg 111, B - 2440 Geel (Belgium)
Telephone: +32-14-571.722 - Fax: +32-14-590.406