

EURL-AP Standard Operating Procedure

Detection of pig DNA in feed using real-time PCR

Method development:
CRA-W
Method assessment and validation:
EURL-AP

Experts for edition and revision	
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EURL-AP	

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1. SUMMARY

This SOP is a binding complement to point 2.2.5. of Annex VI to Commission Regulation (EC) No 152/2009 as lastly amended by Commission implementing Regulation (EU) No 2020/1560 and describes a pig PCR assay. The validation data linked to that assay are briefly outlined. A step by step description of the protocol is provided and starts from the DNA extracts that were obtained from two independent test portions taken from the sample. The protocol outlines the necessary controls to include in the analysis. It also provides guidance on how to integrate into one interpretation all the PCR results obtained on a sample.

One of the essential points of the assay is the use of a cut-off that will make it possible to decide whether a PCR is to be considered as positive or negative.

Before using the test for the detection of pig PAPs in feed samples for routine analysis, the laboratory has to set the cut-off of its PCR platform(s) (combination thermocycler-PCR reagents). The cut-off of a PCR platform is determined by performing 16 calibrations with dedicated calibrants dispatched on 4 independent runs. Each cut-off expressed in Ct is specific for a PCR platform and cannot be transferred to another thermocycler.

2. SCOPE AND PURPOSE

This protocol describes the real-time PCR procedure for the detection of pig DNA in a feed sample using a mitochondrial multicopy target developed by CRA-W and validated by the EURL-AP.

The PCR assay was optimised for use on block-heated real-time PCR instruments for 96-well plates. However it is applicable to other real-time PCR devices using plastic reaction vessels. This method must not be performed as such with thermocyclers using glass capillaries because the buffer composition and specially the MgCl₂ content must be adapted first.

For the detection of pig DNA, a mitochondrial DNA target of 83 base pairs based on the NAD gene (NCBI Accession Number NC_012095.1, *Sus scrofa domestica*) is amplified using two specific primers. PCR products are measured during each cycle by means of an oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct", "Cq" or "Cp" value. This Ct value is compared to a predetermined cut-off figure to establish if the PCR result is positive or negative. The way to determine the cut-off is also outlined.

As the analysis of the replicates of a sample may lead to inconsistent PCR results, the interpretation rules are also provided to be able to cope with such a situation in which there are different outcomes from the replicates.

This SOP is a binding complement to point 2.2.5. of Annex VI to Commission Regulation (EC) No 152/2009 as lastly amended by Commission implementing Regulation (EU) No 2020/1560.

3. DEFINITIONS

3.1. Cut-off

In an analytical process, a cut-off is a threshold value that makes the difference between what can be considered as a positive result and what is a negative result.

3.2. Controls

Definitions of the several controls to be used ("positive DNA target control", "amplification reagent control = no template control", "PCR inhibition control") are the same as the ones considered in ISO 24276:2006.

3.3. PCR platform

In this SOP the PCR platform is considered as the combination of a thermocycler and the reagents used to perform a PCR. The platform is machine-specific and cut-off values established for one machine cannot be transferred to another machine without re-determining the cut-off value.

3.4. Abbreviations used

- Ct : threshold cycle
- DNA : deoxyribonucleic acid
- JRC : Joint Research Centre (JRC) of the European Commission
- LOD : limit of detection
- NA : not applicable
- NCBI : National Center for Biotechnology Information (Rockville Pike, Bethesda MD, USA)
- NRL : national reference laboratory
- PAP : processed animal proteins
- PCR : polymerase chain reaction
- SD : standard deviation
- SOP : standard operating procedure

4. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS

4.1. Collaborative trials

The method was validated in a validation study organized by the EURL-AP. The study was undertaken with 15 laboratories.

Each participant received ten unknown samples consisting of DNA extracted from feedingstuffs adulterated with or without a pig PAP. The levels of adulteration were 0.0125, 0.025 and 0.1 % in mass fraction of pig PAP. Each sample was analysed 20 times (10 replicates spread on 2 plates).

A detailed validation report can be found under:

<https://www.eurl.craw.eu/publications/scientific-reports/>

The method was also used in an implementation study organized by the EURL-AP to assess the implementation in the NRL network of the method as validated (Table 1). Twenty-eight labs (27 NRLs + Norwegian NRL) participated but only 24 participants submitted results. The study was based on a set of 9 blind samples. A blank feed matrix consisting of a compound feed for pigs from an organic feed producer was fortified with pig processed animal proteins at levels of 0.05 and 0.1 % in mass fraction. All the samples were provided in triplicate.

The report of this implementation study can be found under:

<https://www.eurl.craw.eu/publications/scientific-reports/>

Table 1 : Results of the collaborative studies

	Validation study	Implementation study
Year of collaborative study	2015	2016
Number of participating NRLs	15	28
Number of NRLs submitting results	15	24
Number of samples per laboratory	10	9
Number of samples containing pig PAP	6	6
Number of replicates per sample	20	-
Number of reported results	1800	216
Number of accepted results	1560	216
False positive results	8/1040 (0.77%)	2/72 (2.78 %)
False negative results *	2/520 (0.38%)	2/72 (2.78 %)

* False negative results at the level of 0.1 % of pig PAP in mass fraction

4.2. Limit of detection

According to the results obtained during the assessment of the PCR step on a PCR platform by the EURL-AP, the absolute LOD valid for the PCR step is ≤ 20 copies (when the cut-off is calculated at 5 copies).

In-house validation at EURL-AP and the implementation study (<https://www.eurl.craw.eu/publications/scientific-reports/>) showed also that the limit of detection of the method (thus including sample preparation and extraction) is $< 0.1\%$ in mass fraction of pig PAP in feed as no false negative result was recorded at this level (when the cut-off is calculated for the upper confidence interval - in terms of Ct - at 5 copies of the target).

4.3. Specificity

The specificity of the primers and probe was tested in silico through comparisons with the NCBI sequence database. It showed no similarity with other animal and plant species. The validation showed that the primers and probe binding sites are pig-specific. The only exception is wild boar which cannot be distinguished from pig as it is too closely related as a taxon.

The specificity was also experimentally tested with success on a collection of reference DNAs (10 ng of DNA in the PCR) :

- pig (*Sus scrofa domesticus*) and wild boar (*Sus scrofa scrofa*) are detected,
- while non-target species are not detected. This was tested on :
 - ten terrestrial mammalian species (cattle - *Bos taurus*, sheep - *Ovis aries*, goat - *Capra hircus*, stag or red deer - *Cervus elaphus*, roe deer - *Capreolus capreolus*, horse - *Equus caballus*, donkey - *Equus asinus*, hare - *Lepus europaeus*, rat - *Rattus rattus* and human - *Homo sapiens*),
 - six sea mammals (*Stenella coeruleoalba*, *Tursiops truncatus*, *Grampus griseus*, *Ziphius cavirostris*, *Phocoena phocoena*, Seal - *Phocidae*),
 - nine bird species (chicken - *Gallus gallus*, turkey - *Meleagris gallopavo*, duck - *Cairina moschata*, guinea fowl - *Numida meleagris*, goose - *Anser spp.*, quail - *Coturnix japonica*, pheasant - *Phasianus colchicus*, pigeon - *Columba livia* and ostrich - *Struthio camelus*),
 - nineteen fish species (*Gadus morhua*, *Pollachius virens*, *Melanogrammus aeglefinus*, *Micromesistius poutassou*, *Sebastes spp.*, *Mallotus villosus*, *Scomber scombrus*, *Clupea harengus*, *Merluccius merluccius*, *Trachurus trachurus*, *Trisopterus minutus*, *Sardina pilchardus*, *Engraulis encrasicolus*, *Gadus ogac*, *Trisopterus esmarki*, *Ammodytes lancea*, *Sprattus sprattus*, *Salmo salar*, *Raja spp.*)

- one crab species (*Paralithodes camtschaticus*)
- seven vegetal samples (*Glycine max*, *Zea mays*, *Brassica napus*, *Triticum aestivum*, *Oryza sativa*, *Lycopersicon esculentum*, *Beta vulgaris*).

5. HEALTH AND SAFETY WARNINGS

Take into consideration all safety measures advised by the manufacturer of an equipment for its use. Keep track of safety data sheets of all reagents involved in the process and take into consideration the safety warnings they contain.

6. EQUIPMENT AND MATERIALS

6.1. General instructions and precautions

- The procedures requires an experienced staff.
- Laboratory organisation should follow the guidelines given by relevant authorities like EN ISO 24276 (General requirements and definitions).
- Exposure of the work surface to UV-radiation for a period of time may be a helpful complementary way to decontaminate the bench area.
- Air conditioning shall be stopped before starting the manipulation. Windows shall be closed to avoid air movements in the room.
- The work surface is decontaminated by using e.g. HCl 0.1N or 10 % sodium hypochlorite solution (bleach with 3% of active chlorine) before to starting. Eventually this step can be followed by a cleaning of the surface with denatured ethanol (allows a more rapid drying).
- All handling of reagents and controls shall conform to ISO 9001:2000 or ISO 17025 standards or equivalent.
- PCR reagents shall be stored and handled in a separate freezer and in equipment where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment – especially pipettes.
- All the equipment used must be cleaned prior to use for instance with DNA Erase or an equivalent treatment to remove any residual DNA (e.g. pipettes, robot, balances,...). All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and shall not absorb proteins or DNA.
- In order to avoid contamination, filter pipette tips protected against aerosol shall be used.
- Use only powder-free gloves and change them frequently.
- All handling steps on reagents used for PCR – unless specified otherwise – will preferably be carried out at 0 - 4°C (e.g. ice bath, cooling blocks). Notice however that hot start polymerase can stand for a while at room temperature.
- In order to avoid repeated freeze/thaw cycles aliquots of reagents should be prepared.

6.2. Equipment

- Real-time PCR instrument for plastic vessels (or glass capillaries but only after suitable modification of buffer composition – especially MgCl₂ concentration)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for evaluating data

- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes (also called microcentrifuge tubes or vials)

6.3. Reagents

6.3.1. Primers and probe sequences

Primer A : 5'-ACA ACA TAA TCT GAA TCA ATG C-3'

Primer B : 5'-TTC GCC TAG TTG GTT TAG TAG-3'

Probe : 5'-AGT ACA TAG TCT CCT CAT TAG CCT GAT C-3'

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

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

6.3.2. Master mix

Be careful that the master mix used is fit for the purpose.

Examples of master mixes that are fit for purpose are available at <https://www.eurl.craw.eu/legal-sources-and-sops/method-of-reference-and-sops/>.

Notes :

1. The use of a master mix containing dUTPs is recommended to reduce the risk of contamination by former PCR products.
2. An alternative master mix may be used if comparable performance has been evidenced by a pre-test. A dossier describing the experiments done to prove the equivalence of the alternative master mix must be communicated for approval to the EURL-AP via the NRL.

6.3.3. PCR grade water

7. STEP BY STEP PROCEDURE

7.1. Protocol

7.1.1. Real-time PCR mix

After complete thawing of the reagents, in a DNase free microfuge tube, the reagents are mixed in the following order for a final volume of 35 µl :

PCR grade water, 8.75 picomoles of primer A and primer B, 8.75 picomoles of probe, mastermix (volume depending on the concentration of the mastermix).

The examples of mixes are given in Table 2:

Table 2 : Examples of mixes

	1 reaction	96 reactions	104 reactions (1 plate)*
PCR grade water	8.75 µl	840.00 µl	910.00 µl
Primer A (7 µmole/l)	1.25 µl	120.00 µl	130.00 µl
Primer B (7 µmole/l)	1.25 µl	120.00 µl	130.00 µl
Probe (7 µmole/l)	1.25 µl	120.00 µl	130.00 µl
Master Mix 2x	17.50 µl	1680.00 µl	1820.00 µl
Total PCR mix volume/reaction	30.00 µl		
DNA or calibrant to be added in each PCR	5.00 µl		
Total reaction volume = 35 µl / well			

7.1.2. Thermal program

The thermal program to follow is outlined in Table 3.

Table 3 : Thermal program of the pig PCR assay

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

7.1.3. Fluorescence threshold

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 Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The Ct 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 Ct value means a high level of initial number of targets, and a high Ct value means a low level thereof.

The Ct 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 Ct calculated. For these reasons, it is mandatory to set the baseline and the threshold at the same value for all 4 plates used for cut-off determination (and during subsequent analyses of samples).

For the determination of the threshold, careful analysis of the signals is required. Set the threshold in the exponential increase phase and at a level higher than any fork effect as illustrated in Figure 1 (the threshold level in green is correct, not the one in red).

* A larger volume than the one required to fill the wells has to be prepared (add ~ 10 % more)

† Optional treatment with uracyl-n-glycosylase if the used nucleotide mix also integrated dUTP in previous PCR.

‡ Once the cut-off is known, the number of cycles may be reduced to the Ct of the cut-off + 10 cycles because the outcome of this sum should be smaller than 50.

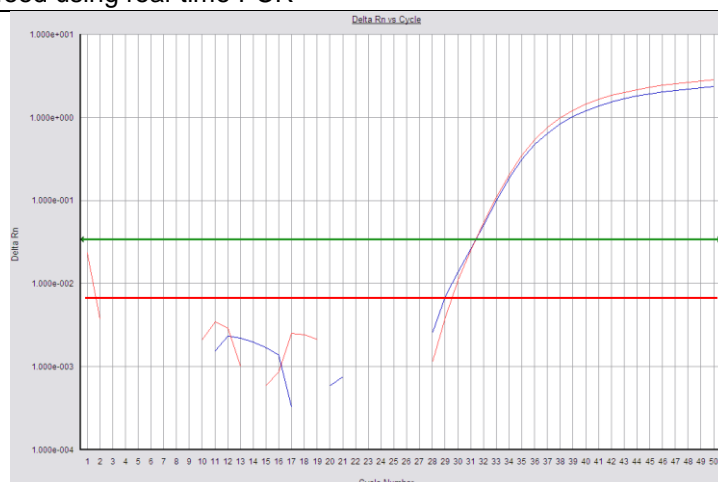


Figure 1 : Setting of the threshold level for the correct determination of the Ct values

The use of different procedures (automatic or manual) for the determination of the threshold and of the baseline was tested with thermocyclers from different brands (LC 480, ABI 7000 and ABI 7500).

With ABI thermocyclers, the best repeatability of the results is obtained when the operator fixes the threshold. The EURL-AP recommends to fix the baseline automatically and to set the threshold manually.

With a LightCycler LC 480, it was observed, during the preparation of the inter-laboratory study, that the use of the automatic algorithm “Abs. Quant./2nd Derivative” could give erroneous values on late signals showing low amplification efficiency. There is no problem for the signals with good amplification efficiency. For that reason, the EURL-AP recommends to analyze the results in manual mode “Abs Quant/Fit Points” (the parameters chosen on the EURL-AP platform are: Background at 2-15, Noiseband (Fluoresc) at 2.5 and Threshold at 3.2).

Keep the same parameters for all plates (for the calibration of the platform but also for the following runs of routine analysis).

7.2. Cut-off determination

7.2.1. Plate lay-out for cut-off determination

One calibration is made of 3 replicates from 3 calibrant levels (9 wells) but the whole calibration process of a new PCR platform needs more data. Perform 4 calibration runs as described in Figure 2 (the design has to be adapted accordingly for thermocyclers using a rotor – e.g. Rotor-Gene from QIAGEN).

In the wells highlighted in green in Figure 2, the template DNA is made of the plasmid solution (calibrants are produced and distributed by JRC). Negative controls must be tested on each plate to check the absence of contamination.

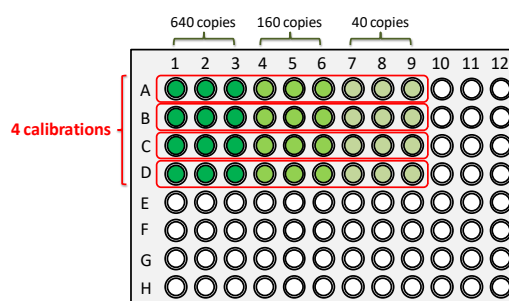


Figure 2 : Location of the wells used for the calibration of the platform

In order to avoid repeated freeze/thaw cycles for the calibrants, the runs for the determination of the cut-off will be performed within 2 or 3 consecutive days. Once thawed, the calibrants will be kept at 1 - 4 °C.

Remark : 16 calibrations spread on 4 runs is the minimum requirement to set the cut-off. However, when data are identified as outliers, they are removed from the calibration dataset. If the number of outliers is high (i.e. loss of data exceeding 5 % for a complete calibration) additional calibrations or runs shall be performed to replace the removed data and to calculate an accurate cut-off.

7.2.2. Calculation of the cut-off (with the Excel file available on the EURL-AP website)

When the laboratory receives a new batch of calibrants, the exact copy number values[§] must be encoded in the “Exact copy numbers” sheet of the Excel file called “Pig Cut-off determination with exact copy number” as presented in Figure 3 and available on the website of the EURL-AP (<https://www.eurl.craw.eu/legal-sources-and-sops/method-of-reference-and-sops/>). At the production of these batches, it is not possible to reach exactly the nominal figures of 40, 160 and 640 copies in 5 µl. However estimated figures (e.g. 38 copies per 5 µl) determined by digital PCR will be indicated. It is this exact copy number instead of the nominal one that has to be introduced in the Excel file.



	A	B	C	E	F	G	H	I	J
			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 Tel : 32 (0) 81 62 03 50 Fax : 32 (0) 81 62 03 88 e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu						
1	<div>Determination of the cut-off of a PCR platform (Pig PCR test)</div>								
2									
3									
4									
5									
6	Calibrants	Exact copy numbers	} Introduce the exact values of copy numbers						
7	640 copies / 5 µl	xxx							
8	160 copies / 5 µl	xxx							
9	40 copies / 5 µl	xxx	← The cut-off of your PCR platform will be determined after performing <u>16 calibrations</u> (run1, run 2, run 3 and run 4)						
10									
11	Cut-off at 5 copies #DIV/0! cycles		⇒ The number of copies at the cut-off must be > 3 copies						
12	Number of copies at the Cut-off #VALEUR! copies								

Figure 3 : View of the “Exact copy numbers” sheet of the “Pig Cut-off determination with exact copy number” Excel file

When the Ct values of the replicates from the 16 calibrations are determined, report the Ct values in the correct cells of the “Run 1”, “Run 2”, “Run 3” and “Run 4” sheets of the file (as presented in Figure 4).

	A	B	C	D	E	F	G	H	I	J	K	L
--	---	---	---	---	---	---	---	---	---	---	---	---



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Run 1

Date

xx/xx/xx

Thermocycler

x

Baseline

x

Threshold

x

Cut-off at 5 copies

#DIV/0! cycles

After having filled in the cells with your Ct values, a number must appear in the cells "Cut-off". If not (passage will appear in the cell), 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 EURL AP.

Calibrators

	Wells	Nr of copy	Ct
A1	640		
A2	640		
A3	640		
A4	160		
A5	160		
A6	160		
A7	40		
A8	40		
A9	40		
B1	640		
B2	640		
B3	640		
B4	160		
B5	160		
B6	160		
B7	40		
B8	40		
B9	40		

Pig

cut-off

Cut-off at 5 copies #DIV/0! cycles

After having filled in the cells with your Ct values, a number must appear in the cells “Cut-off”. If not (##### will appear in the cell), 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 EURL-AP.

Pig cut-off

Figure 4 : View of the “Run1” sheet of the “Pig Cut-off determination with exact copy number” Excel file

[§] Take care that copy number values are indicated per µl in the JRC certificate and must be recorded in copies/5 µl in the Excel file.

Pay particular attention to the correspondence between Ct values and copy numbers to ensure the exact value of the platform cut-off is calculated automatically.

To detect outliers present in the calibrations, the mean and the SD of the data for each calibrant level will be determined automatically in the sheet "Outliers". The data out of the range Mean \pm 3 SD (red cells with Ct values in bold) must be considered as outliers and will be removed from the calibration data by the user.

Outliers= data outside [Mean \pm 3 SD]

A quality control of the cut-off value will be automatically provided by determining to which copy number the obtained cut-off corresponds. The quality criterion to meet for this is: **number of copies corresponding to the cut-off value > 3 copies**.

This quality control is calculated on the basis of the data recorded for the determination of the cut-off (calibration data) and is displayed below the cut-off value on the "Exact copy numbers" sheet of the "Cut-off determination with exact copy number" Excel file.

7.3. PCR plate lay-out for sample analysis

7.3.1. General rules

PCR shall be performed on at least two different dilutions of the DNA extract of a test portion and each sample is analysed with two replicate test portions. The extract shall be obtained using the "Promega Wizard® Magnetic DNA Purification system for food" kit (Promega, Madison, WI USA www.promega.com) as described in the EURL-AP SOP for the DNA extraction available on the EURL-AP website. The EURL-AP recommends to test first the undiluted extract and a 10-fold diluted extract. Hereafter, we will call "PCR test samples" the extracts, diluted or not, that are submitted to PCR.

Each plate shall contain per target under analysis :

- at least one well with a positive DNA target control (also called positive PCR control)
- at least one well with an amplification reagent control (also called no template control or negative PCR control)

For each DNA extraction series, it is mandatory to analyse at least on one plate in at least one well per target under consideration :

- the extraction blank control (also called negative extraction control)
- the positive DNA extraction control

The positive DNA extraction control can be made by the laboratory by using small amounts of PAP (0.1% in mass fraction or less) of a known animal species in a blank feed matrix.

7.3.2. Preparation of the plate

The layout of the plate is defined before PCR begins. This layout must indicate for each well used what is the sample, the test portion and the dilution tested. An example realised with the Excel software is presented in Figure 5 (the design has to be adapted accordingly for thermocyclers using a rotor – e.g. Rotor-Gene from QIAGEN).

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	1	2	3	4	5	6	7	8	9	10	11	12
A	CRL 1647.1 3X	CRL 1647.2 3X	CRL 1648.1 3X	CRL 1648.2 3X	CRL 1649.1 3X	CRL 1649.2 3X	CRL 1650.1 3X	CRL 1650.2 3X		Neg Ext Ctl 120312	Pos Ext Ctl 120312	Pos PCR Ctl
B	CRL 1647.1 30X	CRL 1647.2 30X	CRL 1648.1 30X	CRL 1648.2 30X	CRL 1649.1 30X	CRL 1649.2 30X	CRL 1650.1 30X	CRL 1650.2 30X	Neg Ext Ctl 120313.1	Neg Ext Ctl 120313.2	Neg PCR Ctl	Neg PCR Ctl
C	CRL 1651.1 3X	CRL 1651.2 3X	CRL 1652.1 3X	CRL 1652.2 3X	CRL 1653.1 3X	CRL 1653.2 3X	CRL 1654.1 3X	CRL 1654.2 3X	CRL 1655.1 3X	CRL 1655.2 3X		
D	CRL 1651.1 30X	CRL 1651.2 30X	CRL 1652.1 30X	CRL 1652.2 30X	CRL 1653.1 30X	CRL 1653.2 30X	CRL 1654.1 30X	CRL 1654.2 30X	CRL 1655.1 30X	CRL 1655.2 30X		
E	CRL 1656.1 3X	CRL 1656.2 3X	CRL 1657.1 3X	CRL 1657.2 3X	CRL 1658.1 3X	CRL 1658.2 3X	CRL 1659.1 3X	CRL 1659.2 3X	CRL 1660.1 3X	CRL 1660.2 3X		
F	CRL 1656.1 30X	CRL 1656.2 30X	CRL 1657.1 30X	CRL 1657.2 30X	CRL 1658.1 30X	CRL 1658.2 30X	CRL 1659.1 30X	CRL 1659.2 30X	CRL 1660.1 30X	CRL 1660.2 30X		
G												
H												

Figure 5 : Example of a PCR plate scheme realised in an Excel file

The real-time PCR mix prepared as described at point 7.1.1. is pipetted into the wells and afterwards the samples to be tested (DNA extract, control or water) are added in their respective wells.

The plate is sealed with adhesive foil or closed with plastic caps for real-time PCR and is centrifuged at low speed to spin down liquid and avoid air bubbles at the surface of the mix.

8. INTERPRETATION OF RESULTS

8.1. General rules

The result interpretation must not be based exclusively on the Ct value. A careful analysis of the shape of the signal is also required and must show a clear increase of the fluorescence (exponential amplification phase).

Before analyzing any PCR result for a defined target on a sample, the PCR controls must deliver the expected results in all replicates that were analyzed (Table 4).

Table 4 : Results expected with controls

Control	Result	Conclusion	Purpose of the control
Positive DNA extraction control	Ct < cut-off value	+	Check of DNA extraction efficiency
Extraction blank control	Ct ≥ cut-off value or no amplification	-	Check of the absence of contamination during DNA extraction
Positive DNA target control (for the pig target)	Ct < cut-off value	+	Check of PCR efficiency
No template control	Ct ≥ cut-off value or no amplification	-	Check of the absence of contamination during PCR

Should one of the controls not meet the criteria outlined in Table 4, then the PCR run has to be repeated. If the problem remains, perform new extractions (if false results are obtained with the positive DNA extraction control and/or with the extraction blank control) or new PCR runs (if false results are obtained with the positive DNA target control and/or the no template control) using new reagents.

Generally, the two PCR to be run per test portion are enough to define if the test portion is positive or negative: if a PCR result is positive at least for one of the dilutions, the test portion will be considered as positive (Table 5).

Table 5 : Possible results positive test portion

Dilution 1 (e.g. 1-fold)	Dilution 2 (e.g. 10- fold)	Conclusions
+ ¹	+ ¹	Test portion positive
+ ¹	- ²	Test portion positive
- ²	+ ¹	Test portion positive

¹ Ct < cut-off value² Ct ≥ cut-off value or no amplification

However, if both PCR results are negative (Table 6), it is not possible to conclude directly that the test portion is negative and it is necessary to provide evidence that absence of the signal is not due to total PCR inhibition. This can be done via the use of an inhibition control (see section 8.2) or through a positive PCR result on the extracts of the same test portion with another target (e.g. with a plant universal target or another animal target).

Table 6 : Results for negative test portion or in case of PCR inhibition

Dilution 1 (e.g. 1-fold)	Dilution 2 (e.g. 10- fold)	Conclusions
- ²	- ²	Absence of PCR inhibition to be checked before concluding

¹ Ct < cut-off value² Ct ≥ cut-off value or no amplification

In any case, if there are indications of an inhibition, the operator shall repeat the PCR at other dilution rates (e.g. 2x, 20x, 30x). If, for one of these other dilution rates, the test portion delivers a positive result, the test portion has to be considered as positive.

When considering the Ct figures obtained on a PCR test sample, ensure amplification curves are checked because automatic determination of the Ct by the software can sometimes provide a Ct that is due to a local fluorescence peak that has not to be considered as a PCR amplification (Figure 6).

**Figure 6** : Example of a signal that must not be considered for the results (red curve)

The final interpretation of results for a sample with a PCR target shall take into consideration the two determinations performed i.e. PCR results on both test portions (Table 7).

If both test portions are positive with a PCR target the sample is positive for that target.

If both test portions are negative with a PCR target, then the sample has to be considered as negative for that PCR target after it has been demonstrated that there is no PCR inhibition. In case total PCR inhibition is demonstrated, an additional purification of the DNA extract must be performed (from the experience of the EURL-AP, this case is exceptional with the DNA extraction method to use).

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If results from the two test portions are inconsistent, the genetic amplification for the target under consideration shall be repeated (once again two dilutions per test portion). If by doing so both results are consistent, it becomes the final PCR result for the target under consideration, while if results on both test portions remain conflicting, then the result will be considered as negative.

If however the laboratory suspects that the DNA extracts can be the cause of the inconsistency (e.g. a too large difference between Ct's on both test portions analysed at a same dilution rate – more than 3 Ct units), a new DNA extraction and a subsequent genetic amplification shall be performed on two test portions before interpreting the results.

Table 7 : Final results interpretation rules for a sample

Test portion	Dilution 1 (e.g. 1-fold)	Dilution 2 (e.g. 10- fold)	Conclusions	
#1 #2	+ ¹ + ¹	+ ¹ + ¹	Test portion 1 positive Test portion 2 positive	Target detected
#1 #2	+ ¹ + ¹	+ ¹ - ²	Test portion 1 positive Test portion 2 positive	Target detected
#1 #2	+ ¹ - ²	+ ¹ + ¹	Test portion 1 positive Test portion 2 positive	Target detected
#1 #2	+ ¹ + ¹	- ² - ²	Test portion 1 positive Test portion 2 positive	Target detected
#1 #2	- ² - ²	+ ¹ + ¹	Test portion 1 positive Test portion 2 positive	Target detected
#1 #2	+ ¹ - ²	- ² + ¹	Test portion 1 positive Test portion 2 positive	Target detected
#1 #2	- ² - ²	- ² - ²	Both test portion negative after control of the absence of PCR inhibition (see Table 6)	Target not detected
#1 #2	+ ¹ - ²	+ ¹ - ²	Test portion 1 positive Test portion 2 negative	Inconsistent result ⇒ further investigations (PCR repetition and/or new DNA extraction)
#1 #2	+ ¹ - ²	- ² - ²	Test portion 1 positive Test portion 2 negative	Inconsistent result ⇒ further investigations (PCR repetition and/or new DNA extraction)
#1 #2	- ² - ²	+ ¹ - ²	Test portion 1 positive Test portion 2 negative	Inconsistent result ⇒ further investigations (PCR repetition, PCR inhibition and/or new DNA extraction)

¹ Ct < cut-off value

² Ct ≥ cut-off value or no amplification

8.2. Inhibition test

When a given test portion does not deliver positive results with any of the tested PCR targets, then PCR inhibition has to be checked by the use of an inhibition control test.

Procedure to follow:

A known amount of target not exceeding 100 copies per well shall be analyzed in two different conditions (each at least in one well).

- 1) Without any other DNA source than the added target
- 2) In presence of the DNA extract (different dilutions may be tested) that is analyzed for inhibition.

The calibrant at 40 copies/5 µl provided for the determination of the cut-off can be used to that aim.

Table 8 : Interpretation rules for an inhibition control

Test conditions	Result	Conclusion
1) Inhibition control alone (e.g. calibrant)	- ² + ¹	Test invalid Test valid ⇒ check result of inhibition control + DNA sample
2) Inhibition control + DNA sample tested for inhibition	- ² + ¹	PCR inhibition (if test in 1 is valid) No inhibition or no total inhibition

¹ Ct < cut-off value

² Ct ≥ cut-off value or no amplification

To interpret results of the inhibition test, it is mandatory that the PCR performed on the inhibition control DNA is positive (conditions under 1 in Table 8). Should it be negative, then the test is not valid.

Once the validity of the inhibition control is checked, test conditions under 2) (Table 8) can be interpreted. If the result is positive, there is absence of inhibition (or at least no total inhibition). Conversely, if it is a negative result, this means that absence of amplification is due to an inhibitory effect on PCR of the DNA extract at the dilution at which it was tested.

8.3. Recommendations

If a positive DNA extraction control yielded a positive result in PCR, it can be considered as a valid positive DNA target control for future PCR assays with the considered target. However the content of the positive DNA extraction control should be close to the limit of detection of the method.

9. REFERENCES

Annex VI of Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed as lastly amended by Commission Regulation (EU) No 2020/1560 of 27 October 2020. Official Journal of the European Union, L357, 27.10.2020, 17-23.

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