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

Walloon Agricultural Research Centre

Validation study of a real-time PCR method developed by TNO Triskelion bv for the detection of ruminant DNA in feedingstuffs

Olivier FUMIÈRE, Aline MARIEN, Gilbert BERBEN

Department Valorisation of Agricultural Products

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

Contact information
Olivier Fumière
Authentification and Traceability Unit (U16)
Valorisation of Agricultural Products Department
Walloon Agricultural Research Centre - CRA-W
European Union Reference Laboratory for Animal Protein in feedingstuffs – EURL-AP
Building "Henseval"
Chaussée de Namur, 24
5030 Gembloux (Belgium)
Tél : +32(0)81 62 03 51
Fax : +32(0)81 62 03 88
Mail : o.fumiere@cra.wallonie.be
Website : http://www.cra.wallonie.be

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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. TNO Triskelion bv developed a real-time PCR method able to detect low levels of ruminant DNA. This method was always used by their developers and a validation through an interlaboratory study has to be conducted by the EURL-AP to provide evidence that the method would be suitable for detection of processed animal proteins (PAP) in a network of laboratories.

The EURL-AP had to adapt its protocol for the determination of a cut-off value whatever the PCR platform used. The transfer protocol is based on the use of plasmid calibrations combined with statistical considerations to set an accurate cut-off value specific of the PCR platform (thermocycler + master mix) used. 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 very high number of copies per cell and therefore accurate determination of the cut-off is of even more importance to obtain reliable results.

The study took place from end of December 2011 (date of the call) up to end of February 2012 (return of the results). Twelve institutes agreed to participate to the study. A total of 12 thermocyclers dispatched between 4 major companies were tested. Taking into account the high sensitivity of the method and the risk to have false positive results, two cut-off values calculated at 10 and 15 copies per reaction were simultaneously tested as both seemed suitable according to the assessment of the TNO Triskelion PCR method for ruminants that had been made by the EURL-AP. The cut-off values calculated at 15 copies per reaction of the 12 platforms are in a range between 35.9 and 38 cycles. The cut-off values calculated at 10 copies per reaction on the 12 same PCR platforms are in a range between 36.6 and 38.6 cycles. When using these cut-off values, the 95% level of correct assignments with the blind samples included in the trial was aimed but results of three laboratories had to be excluded because of a too high rate of false positive results on the negative PCR control. The rate of false positive results is 0.42 % and the rate of false negative results is 0 % at the level of 0.1 % w/w of ruminant PAP in feed. When doing this same exercise with a cut-off value calculated at 10 copies these criteria become respectively 1.39 % and 0 %. Based on the overall conclusions of the study, the method can be considered as fit for purpose and validated with the advice to use a cut-off calculated at 15 copies to avoid as much as possible false positive results while sensitivity is still fine.

2. Introduction

Since the outbreak of bovine spongiform encephalopathy (BSE), the use of PAPs including meat and bone meal as feed ingredients for farmed animals is drastically controlled within the European Union through several regulations (Regulation (EC) 999/2001, Regulation (EC) 1774/2002 and Regulation (EC) 1234/2003). Presently, classical optical microscopy is the only official method for the detection of PAPs in compound feed in the European Union (Commission Regulation (EC) 152/2009) but the method offers very limited species discrimination perspectives. The Regulation (EC) 152/2009 however stated that alternative methods can be used to gain more information about the origin of the PAPs.

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 TNO Triskelion b.v. and has been fully assessed by the EURL-AP. Conclusions of the study were that the method was fit for a validation in the sense that such a validation would have a high chance to be successful (Fumière *et al.*, 2012). This however requested from the EURL-AP to complete the TNO Triskelion PCR assay for ruminants with a protocol to define the cut-off value of any PCR platform (thermocycler and master mix) that would be used.

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 (Fumière *et al.*, 2010). By means of plasmids carrying the PCR target, calibration curves were built and through inverse regression (Draper and Smith, 1998) between the logarithm of the copy number and the Ct a cut-off value is defined. The way to define this value had to be adapted slightly for the TNO Triskelion PCR assay for ruminant because of the high number of copies of the target per cell.

This full validation of the TNO Triskelion ruminant PCR assay will mainly check if transferability of this analytical assay in order to detect ruminant PAP at 0.1% (w/w) in feedingstuffs. The assessment of the assay by in-house validation at the EURL-AP already pointed out the method was of interest by being able to meet acceptable performance 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 twelve institutes (named for the study "Lab #") from the European Union using thermocyclers from 4 major companies:

- 3 LC480 (Roche Diagnostics)
- 1 ABI 7300, 2 ABI 7500, 1 ABI 7500 Fast, 1 ABI 7900 and 1 StepOnePlus (Applied Biosystems)
- 2 CFX (Bio-Rad)
- 1 Mx3000P, 1 Mx3005P (Agilent)

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

5. Time schedule of the study

The 21th of December 2011, an invitation letter (Annex II) was sent to several potential participants with already some experience in PCR 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 13th of January 2012 by sending back a reply form indicating all important information about the laboratories, the models of thermocyclers participating to the study.

The 16th of January 2012, the study was officially announced to the participants. The instructions and the protocol of the study were sent to the participants by post mail.

The 31th of January 2012, the experimental material was sent to all the participating laboratories which received the material in good conditions within two days.

The results were collected between the 6th of February and the 2nd of March 2012.

6. Purpose of the study

The objective of this study was to evaluate the fitness of a protocol used to detect the presence of ruminant DNA in feedingstuffs. A cut-off value able to delimit signals due to the presence of the target from unspecific signals was determined according the protocol validated in 2009 (Fumière *et al.*, 2010). In reason of the abundance of the target and consequently of the sensitivity of the method, the determination of the cut-off value was adapted to correspond to the upper limit of the confidence interval of a Ct value for more than 1 copy in the reaction. Cut-off values focussed on the confidence interval at 10 and 15 copies per reaction were tested independently. These adapted cut-off values were determined during the evaluation of the method to keep a good sensitivity and to minimize the rate of false positive results.

Ten blind samples containing 0, 0.0125, 0.025 and 0.1 % in weight of ruminant DNA were tested by the participants. Each sample was analysed 20 times (10 replicates x 2 runs).

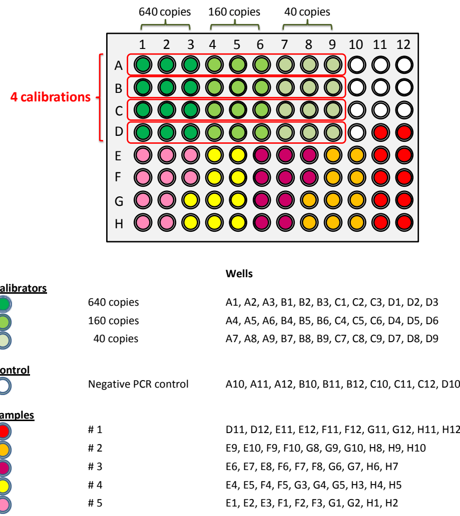
The present method will be considered as fit for the purpose if ~ 95 % of the reactions with DNA extracted from samples at 0.1 % in weight of ruminants PAPs are positive and if the rate of false positive results does not exceed 5%. As the use of the cut-off in that perspective is absolutely crucial, 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 which will nevertheless be taken into account afterwards if the method is considered fit for purpose.

7. Design of the study

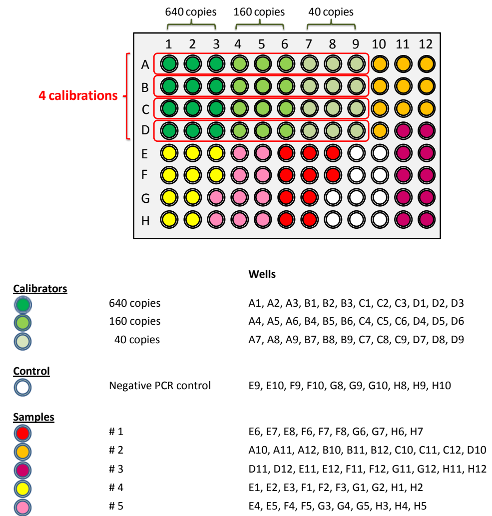
The participation of the laboratories consisted in 4 PCR runs to perform within 2 or 3 consecutive days. On each plate, 4 calibrations were realized and 5 samples with low ruminant PAP content were tested in blind (10 replicates / sample) on two consecutive runs. The position of the samples on the plates 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 the Figures 1 and 2.

Run 1



Run 2



Figures 1 and 2: 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 calibrators as on the blind samples (Figure 3).

Run 1

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

Cut-off at 15 copi #DIV/0!
Cut-off at 10 copi #DIV/0!

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.***

	Calibrators			Samples					Controls				
	Wells	Nr of cop	Ct	Wells	Samples	Ct	at 10 copies	at 15 copies	Wells	Name	Ct	at 10 copies	at 15 copies
11	A1	640		D11	1		#DIV/0!	#DIV/0!	A10	Negative control		#DIV/0!	#DIV/0!
12	A2	640		D12	1		#DIV/0!	#DIV/0!	A11	Negative control		#DIV/0!	#DIV/0!
13	A3	640		E11	1		#DIV/0!	#DIV/0!	A12	Negative control		#DIV/0!	#DIV/0!
14	A4	160		E12	1		#DIV/0!	#DIV/0!	B10	Negative control		#DIV/0!	#DIV/0!
15	A5	160		F11	1		#DIV/0!	#DIV/0!	B11	Negative control		#DIV/0!	#DIV/0!
16	A6	160		F12	1		#DIV/0!	#DIV/0!	B12	Negative control		#DIV/0!	#DIV/0!
17	A7	40		G11	1		#DIV/0!	#DIV/0!	C10	Negative control		#DIV/0!	#DIV/0!
18	A8	40		G12	1		#DIV/0!	#DIV/0!	C11	Negative control		#DIV/0!	#DIV/0!
19	A9	40		H11	1		#DIV/0!	#DIV/0!	C12	Negative control		#DIV/0!	#DIV/0!
20	B1	640		H12	1		#DIV/0!	#DIV/0!	D10	Negative control		#DIV/0!	#DIV/0!
21	B2	640		E9	2		#DIV/0!	#DIV/0!					
22	B3	640		E10	2		#DIV/0!	#DIV/0!					
23	B4	160		F9	2		#DIV/0!	#DIV/0!					
24	B5	160		F10	2		#DIV/0!	#DIV/0!					
25	B6	160		G8	2		#DIV/0!	#DIV/0!					
26	B7	40		G9	2		#DIV/0!	#DIV/0!					
27	B8	40		G10	2		#DIV/0!	#DIV/0!					
28	B9	40		H8	2		#DIV/0!	#DIV/0!					
29	C1	640		H9	2		#DIV/0!	#DIV/0!					
30	C2	640		H10	2		#DIV/0!	#DIV/0!					
31	C3	640		E6	3		#DIV/0!	#DIV/0!					
32	C4	160		E7	3		#DIV/0!	#DIV/0!					
33	C5	160		E8	3		#DIV/0!	#DIV/0!					
34	C6	160		F6	3		#DIV/0!	#DIV/0!					
35	C7	40		F7	3		#DIV/0!	#DIV/0!					
36	C8	40		F8	3		#DIV/0!	#DIV/0!					
37	C9	40		G6	3		#DIV/0!	#DIV/0!					
38	D1	640		G7	3		#DIV/0!	#DIV/0!					
39	D2	640		H6	3		#DIV/0!	#DIV/0!					
40	D3	640		H7	3		#DIV/0!	#DIV/0!					
41	D4	160		E4	4		#DIV/0!	#DIV/0!					
42	D5	160		E5	4		#DIV/0!	#DIV/0!					

Run 1 / Run 2 / Run 3 / Run 4

Figure 3: 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 calibrators, the 10 blind samples and the PCR negative controls.

8.1. The calibrators

Three calibrators (1 vial containing 450 µl of material per calibrator) were provided to the participants: 640 copies/5 µl (680 copies/5 µl*), 160 copies/5 µl (177 copies/5 µl*), 40 copies/5 µl (46 copies/5µl*). They were prepared directly and independently from a stock solution at 639 copies/µl* and always in a background of maize DNA at the concentration of 12 ng/µl (to avoid loss of molecules by their sticking to the plastic walls of the vial). The exact copy numbers were pre-introduced in the hidden sheets (used for the automatic determination of the cut-off) of the Excel reporting file.

* exact copy numbers determined by digital PCR (“BioMark™ HD System”, Fluidigm Corporation, South San Francisco, CA, USA) in the facilities of JRC-IRMM (Geel, Belgium).

8.2. The ten blind samples

A set of blind samples was constituted of samples at the 4 concentrations of ruminant PAP (bovine PAP heat treated at 141 °C under a pressure > 3 bars and during at least 20 minutes) : 0.1 % in weight (~400 copies of the ruminant target/5µl), 0.0250 % in weight (~100 copies of the ruminant target/5µl), 0.0125 % in weight (~50 copies of the ruminant target/5µl) and a blank at 0 % in weight (0 copy/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 ruminant DNA in feedinstuffs**

Sample	Material	Remark	
DNA extracts			
1	0.1 % w/w bovine PAP in blank 1	1	
2	Blank 1 : Compound feed 100 % plant material	1	
3	0.0125 % w/w bovine PAP in blank 1	1	
4	Blank 2 : Fish meal	1	
5	0.025 % w/w bovine PAP in blank 1	1	
6	0.0125 % w/w bovine PAP in blank 1	1	(replicate of sample #3)
7	0.025 % w/w bovine PAP in blank 1	1	(replicate of sample #5)
8	Blank 3 : Rapeseed oilcake	1	
9	Blank 4 with non-target PAP : Maize with 5 % in weight of pig PAP	1	
10	0.1 % w/w bovine PAP in blank 1	1	(replicate of sample #1)
Total		**10**	

The preparation scheme is illustrated in Figure 4.

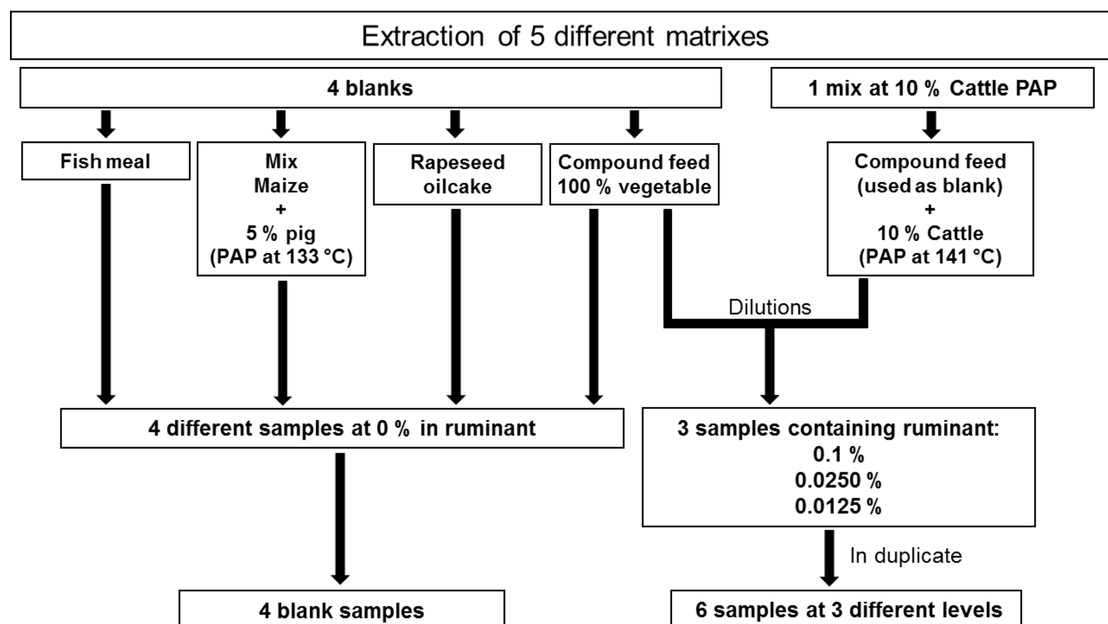


Figure 4: Production of the blind samples set used in the EURL-AP PCR
Validation study of a real-time PCR method for the detection of ruminant DNA in feedinstuffs

A sample consisting of blank compound feed 1 with 10 % w/w of bovine PAP (heat treated at 141 °C under a pressure > 3 bars and during at least 20 minutes) was used for the production of the primary DNA extract containing the ruminant target (Table 1 and Figure 4). In parallel 4 blank samples with two of them containing non target PAP (fish meal and pig PAP) were also submitted to DNA extraction. The primary DNA extract with the 10% of ruminant DNA was afterwards diluted in the DNA extract of pure blank 1 compound feed to reach the three levels of ruminant contents (0.1%, 0.025% and 0.0125%).

Due to the large volumes of DNAs to provide to the participants, the CTAB method on several replicates of 2 g of matrix was used to obtain large quantities of DNA extracts. As the Promega protocol (Wizard Magnetic DNA Purification System for food) on 100 mg of matrix will be the extraction method to be used for routine analysis, the samples were adjusted to mimic DNAs extracted from similar samples according the Promega extraction method (~ Ct values).

The CTAB extracts from the 4 blanks were tenfold diluted with PCR grade water to obtain samples #2, #4, #8 and #9. This dilution factor was determined by comparison between Promega extracts and CTAB extracts.

The analysis of vegetal compound feed (sample #2) with a flax PCR target (Genetic ID, 2009) showed a Ct difference (ΔCt between the CTAB extract and the Promega extract) of 4.09 cycles which is equivalent to a dilution factor of 17 -fold. In other words to obtain an

equivalent number of the flax targets contained in the Promega extract the CTAB extract should be diluted by a factor 17. A difference of 3.3 cycles between the Promega and the CTAB extracts was observed for fish meal (sample #4) when analysing the extracts through a fish target PCR assay of the EURL-AP, this difference is equivalent to a tenfold dilution factor. The mix composed of maize with 5 % w/w pig PAP (sample #9) was analysed with the EURL-AP pig PCR target. The dilution factor between the both extraction methods is of 7 fold, the difference of Ct values is 2.79 cycles. The last blank, rapeseed oilcake (sample #8) was analysed with a rapeseed PCR target (Mazzara *et al.*, 2007) and the results showed a difference of 2.99 cycles between the Promega and CTAB extracts equivalent to a dilution factor 8 fold. Even though the dilution factors vary between the several matrices under analysis, a tenfold dilution was applied to them to mimic as much as possible a Promega extract.

These 4 blank samples were analysed with the ruminant PCR target. Similar results were obtained with both extraction methods. The blank samples are negative for ruminant DNA; they do not give an amplification or if there is a signal, it appears after the cut-off values in terms of Ct.

The DNA extracted from the mix at 10 % (w/w) of cattle PAP according to the CTAB protocol was diluted with DNA from blank 1 (sample #2) extracted according the same protocol to obtain samples #1, #3, #5, #6, #7 and #10 of the set.

In order to mimic DNA extracts at 0.1, 0.025 and 0.0125 % obtained according the Promega extraction method starting from CTAB extract of mix at 10 % Cattle PAP, 10 Promega extracts performed on blank 1 feed with 0.1, 0.025 and 0.0125 % of ruminant PAP were analysed with ruminant PCR target. The mean Ct values obtained are presented in Table 2.

The 0.1 % level of ruminant heat treated material is equivalent to +/- 300 copies of the ruminant target per 5 µl. The ΔCt between the levels at 0.1 and 0.025 % is +/- 2 cycles as expected. A ΔCt of more than 1 cycle is however observed between the levels at 0.025 and 0.0125 % in the Promega extracts while on their diluted equivalents the ΔCt is almost exactly 1.

***Table 2*:** Comparison of mean Ct obtained

Level	**Mean Ct on Promega extracts (n = 20)**	**Mean Ct on the dilutions of 10 % Cattle PAP CTAB extracts (n = 10)**
0.1 % of ruminant PAP in weight (bovine PAP)	31.39 cycles	31.77 cycles
0.025 % of ruminant PAP in weight (bovine PAP)	33.47 cycles	33.82 cycles
0.0125 % of ruminant PAP in weight (bovine PAP)	35.29 cycles	34.79 cycles

The detection of ruminant target is slightly more difficult on the dilutions of the CTAB extracts (based on an initial mix at 10 % of cattle PAP) than on the samples directly extracted with the Promega method except for the level at 0.0125 % of ruminant PAP.

Once prepared, all the materials to be provided to the participants were stored on ice. Within the day, they were aliquoted and tested by PCR on one aliquot chosen randomly to check if the concentrations were correct. After that, all the materials were stored at -20 °C until shipping to the participants.

The participants received vials of these three levels in duplicates and 4 blanks 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 MilliQ 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 thermocycler, a cut-off value calculated at 10 copies and 15 copies per reaction was automatically generated by the excel file when filling the appropriate data generated by the calibrants.

2) With the obtained cut-off values of each thermocycler, each replicate of the blind samples was 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 TNO Triskelion PCR assay on ruminant showed that cut-off values calculated at 10 and 15 copies of the target could be suitable.

Table 3 provides the cut-off figures calculated at 10 and 15 copies.

***Table 3*: Cut-off values at 10 and 15 copies/5 µl and corresponding number of copies of the participants**

Lab	Cut-off value at 10 copies/5 µl	Corresponding number of copies	Cut-off value at 15 copies/5 µl	Corresponding number of copies
Lab1	37.8470	6.4	37.2272	9.7
Lab2	38.0137	6.8	37.3510	10.3
Lab3	36.6750	6.4	36.0411	9.8
Lab4	37.7729	6.7	37.1313	10.1
Lab5	36.6285	6.4	35.9889	9.7
Lab6	37.6783	7.2	37.0493	10.9
Lab7	38.6630	2.2	37.9607	3.6
Lab8	37.1292	5.6	36.4803	8.5
Lab9	38.3973	4.1	37.7058	6.4
Lab10	38.1265	4.3	37.4567	6.6
Lab11	37.6323	6.5	36.9926	9.8
Lab12	36.8532	5.1	36.2699	7.8

9.2. Percentages of false results

Blind samples were tested fourty times (10 replicates x 2 samples x 2 runs) for each of the three levels of ruminant 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

Lab	1	2	3	4	5	6	7	8	9	10	11	12
Cut-off at 15 copies												
False positive results	0	0	0	0	0	0	0.0125	0	0	0.3125	0.025	0.3125
False negative results												
0.1%	0	0	0	0	0	0	0	0	0	0	0	0
0.0250%	0	0	0	0	0	0	0.025	0	0	0	0	0
0.0125%	0	0	0	0	0	0	0	0	0.025	0	0	0
Cut-off at 10 copies												
False positive results	0	0	0	0	0.0125	0	0.05	0	0.075	0.425	0.0625	0.4875
False negative results												
0.1%	0	0	0	0	0	0	0	0	0	0	0	0
0.0250%	0	0	0	0	0	0	0.025	0	0	0	0	0
0.0125%	0	0	0	0	0	0	0	0	0.025	0	0	0

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 15 and 10 copies

Lab	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Negative PCR controls												
False positive results (Cut-off at 15 copies)	0	0	0	0	0.05	0	0	0	0.1	0.075	0	0.425
False positive results (Cut-off at 10 copies)	0	0	0	0	0.05	0	0.025	0.225	0.25	0.175	0.075	0.6

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 9, 10 and 12 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 9, 10 and 12 are not considered, the global rate of false positive results reached 1.38% (10/720) or 0.41% (3/720) respectively for cut-off values calculated at 10 and 15 copies. While the false negative rate amounted to 0.55% (2/360) for both cut-off figures. 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 ruminant PAP in feedingstuffs the false positive rate is even 0%.

11. Conclusions

- The cut-off values of the 12 platforms determined using the proposed protocol showed differences between platforms (35.99 < Cut-off $value_{15\ copies}$ < 37.96). The obtained range is however smaller than what was expected by comparison with former studies (Fumière *et al.,* 2010) but it can probably be explained by a smaller dispersion of the results due to the number of copies at which the cut-off was determined which is no longer at a single copy
- Both cut-off values calculated respectively at 10 and 15 copies of the target are fit for purpose but the rate of false positive results is somewhat improved when using the cut-off calculated at 15 copies while this had no visible impact on the detection of the considered positive samples. It is therefore advised to use the cut-off value calculated at 15 copies instead of 10 copies.
- The transfer of the protocol to new laboratories with thermocyclers from 4 companies (Applied Biosystems, Bio-Rad, Roche Diagnostics and Stratagene) was succesfull. This is the major conclusion of this study as it had to be tested that the cut-off protocol was really effective for the transfer of the PCR method. Like already said above that is also why it was crucial to work on DNA extracts during this validation study in order to avoid any interference with the extraction step.
- As can be seen from the samples prepared for the study, the method with inclusion of the cut-off protocol is method extraction dependent. The method was assessed with DNA extracts obtained with the Promega Wizard magnetic beads for food kit. For practical reasons we had to use here CTAB extracts but diluted in such a way as to mimic Promega extract. It is therefore essential that the method be used together with DNA extracts obtained with the Promega Wizard magnetic beads for food kit as long as suitability of other DNA extraction methods has not been evaluated.
- Based on the overall previous conclusions, the study can be considered as successful and the protocol of transfer 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 and Stéphane Mazoua (EC-JRC-IRMM, Geel, Belgium) for their help in the determination of the exact copy numbers of the calibrants by digital PCR and Christoph von Holst (EC-JRC-RMM, Geel, Belgium) for statistics and fruitful discussions. The authors would like to thank also Julie Hulin and Alison Pire for their efficient technical assistance.

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13. Annexes

a. Annex I: List of participating laboratories

Organization name	Country
Animal Health and Veterinary Laboratory Agency (AHVLA)	Newcastle, UK
Bundesinstitut für Risikobewertung (BfR)	Berlin, Germany
Danish Veterinary and Food Administration	Lyngby, Denmark
Department of Agriculture, Marine and Food	Celbridge, Ireland
Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZSTO – CreAA)	Torino, Italy
Joint Research Centre – Institute for Health and Consumer Protection (JRC-IHCP)	Ispra, Italy
National Diagnostic Centre of Food and Veterinary Service	Riga, Latvia
National Veterinary Institute	Ljubljana, Slovenia
National Veterinary Research Institute	Pulawy, Poland
Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES)	Linz, Austria
RIKILT-Institute of Food Safety	Wageningen, The Netherlands
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

Walloon Agricultural Research Centre

Gembloux, 21th December 2011

Invitation to participate to a validation study of a PCR method for the detection of ruminant PAPs

Introduction and objective of the study

As planned in the TSE roadmap II[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) is the only method being helpful for that purpose besides the optical microscopy that remains the reference method 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 TNO Triskelion bv (Zeist, The Netherlands) for the detection of ruminant PAPs.

The organizer team

The study 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.

Participants to the validation study agree to use the provided material for the sole purpose of this validation.

[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 **by normal mail two weeks before the sending of the material**. TNO Triskelion bv requires that you send back to the EURL-AP this document **and all its copies** upon completion of the participation to the study. Please read this protocol 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 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.

Time schedule

- The study will take place in **January and February 2012**
- The protocol of the study will be sent to the participants in January (two weeks before the samples)
- The samples will be sent to the participants between **the 16th of January and the 3rd 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: berben@cra.wallonie.be
- Dr Olivier FUMIERE
  ☎ +32 (0)81 62 03 51
  🖷 +32 (0)81 62 03 88
  e-mail: 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 and/or Fax (+32 (0)81 62 03 88) by **13th of January 2012 at noon** as well as its original hard copy by normal mail.

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.

A non disclosure agreement will also have to be signed by each participant laboratory prior to its participation to the study. Please return it signed **as soon as possible** via e-mail to secretary@eurl.craw.eu and/or Fax (+32 (0)81 62 03 88). As soon as the EURL-AP will be in possession of the signed document, it will send you the protocol of the study.

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

Yours sincerely,

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

c. Annex III: Reply form

European Union Reference Laboratory for Animal Proteins in feedingstuffs

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

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

Inter-laboratory study for the validation of a real-time PCR method for the detection of ruminant PAPs

Reply form
to send via e-mail to secretary@eurl.craw.eu
and/or Fax (+32 (0)81 62 03 88) by 13th of January 2012 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.wallonie.be

Hereby, I agree to use the material for the sole purpose of the validation study and to send back to the EURL-AP the protocol that will be provided once the participation to the study is completed.

Date, name and signature of the responsible

Walloon Agricultural Research Centre

Wallonie

d. Annex IV: Protocol of the study

PROTOCOL FOR THE VALIDATION OF A PCR METHOD DEVELOPED BY TNO TRISKELION BV FOR THE DETECTION OF RUMINANT DNA

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

THIS DOCUMENT CAN BE USED FOR THE SOLE PURPOSE OF THE VALIDATION STUDY.
THE ORIGINAL AND ALL THE COPIES MUST BE RETURNED TO THE EURL-AP (REQUEST OF TNO TRISKELION BV).

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INTRODUCTION

This inter-laboratory study aims to validate the protocol of a real-time PCR method for the detection of ruminant DNA designed by TNO Triskelion (Zeist, The Netherlands). 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 master mix) and the determination of a cut-off value using plasmids as calibrators 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. Based on the results provided by the participants, a limit of detection in copy number of the target will be determined.
3. The capacity of the method to detect the presence of 0.1 % (w/w) of ruminant PAP in a feedingstuff will also be evaluated.

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

MATERIAL PROVIDED BY THE ORGANISERS (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		
Cut-off calibration curve (640 copies)	1	450 µl
Cut-off calibration curve (160 copies)	1	450 µl
Cut-off calibration curve (40 copies)	1	450 µl
PRIMERS AND PROBE		
Primer A	1	750 µl
Primer B	1	750 µl
Probe	1	500 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
MASTER MIX + PASSIVE REFERENCE (ROX)		
Adjust the concentration of ROX in the master mix according to your	6	1300 µl
thermocycler (see the prescription of provider in Annex 1)	ROX: 1	500 µl

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)
- 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 master mix, defreeze it and add the correct amount of ROX (see Annex 1).
- Before using these reagents and samples, vortex them and centrifuge all vials for a short time.
- In a DNAse free 5 ml microfuge tube, mix in the following order :

	1 reaction	96 reactions	105 reactions (1 plate)
PCR grade water	5.00 µl	480.00 µl	525.00 µl
Primer A	1.10 µl	105.60 µl	115.50 µl
Primer B	1.10 µl	105.60 µl	115.50 µl
Probe	0.73 µl	70.08 µl	76.65 µl
Master Mix	12.07 µl	1158.72 µl	1267.35 µl
Total PCR mix volume/reaction	20.00 µl	1920.00 µl	2100.00 µl

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

Total reaction volume = 25 µl / well

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

IMPORTANT REMARKS :

1. **THE NUMBER OF REACTIONS PREPARED (105) IS CLOSE TO 96. PAY ATTENTION TO PIPETTING ERRORS AND FINISH THE DISPATCHING OF THE PCR MIX WITH THE WELLS DEDICATED TO THE NEGATIVE CONTROLS.**
2. **20 µL OF THE PCR MIX CONTAINING H_2O, PRIMERS, PROBE AND MASTER MIX 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 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	**60**

REMARKS :

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

3. Measurements of the signals

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

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

4. CALIBRATION OF A PLATFORM

3 vials of calibrators labelled "640 copies", "160 copies" and "40 copies" on the cap are used to calibrate the platform (thermocycler + master mix). 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 [1].

For routine analysis, 1 calibration is made of 3 replicates from the 3 levels (9 wells) but a calibration of a new platform needs more data.

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 (calibrators).

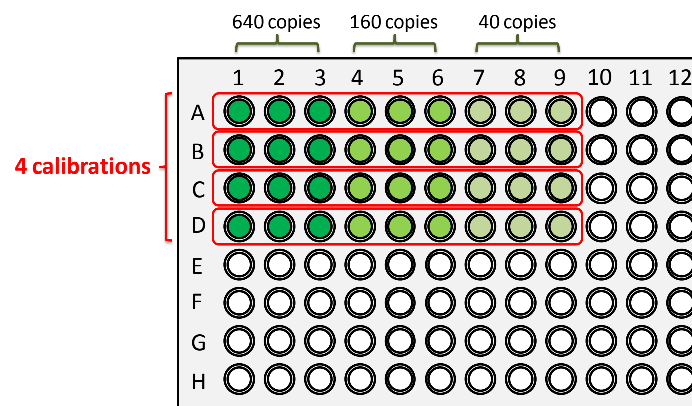


Figure 1 : Location of the wells used for the calibration of the platform.

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 ruminant PAP.

For some samples, not all the replicates of these samples will give a signal. This is normal and expected by the organisers.

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 UNTIL THE END OF THE STUDY AND SHELTERED FROM LIGHT.**
2. **DURING THE PREPARATION OF THE PLATE, THE SAMPLES, THE CALIBRATORS, THE PCR CONTROLS AND THE REAGENTS (PROBE & PRIMERS AND MASTER MIX) MUST BE KEPT ON ICE.**

---

[1] *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 calibrators 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.*

3. THE MATERIAL PROVIDED ALLOWS TO PERFORM 6 PLATES. IN CASE OF MISTAKE, YOU CAN DO YOUR PLATE AGAIN.

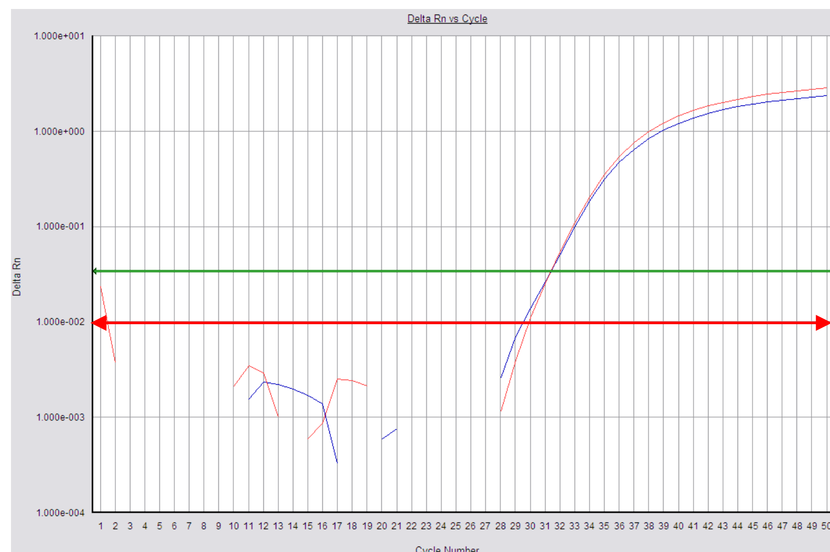
6. ANALYSIS OF THE RESULTS

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 related directly 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 5 (the threshold level in green is correct, not the one in red).

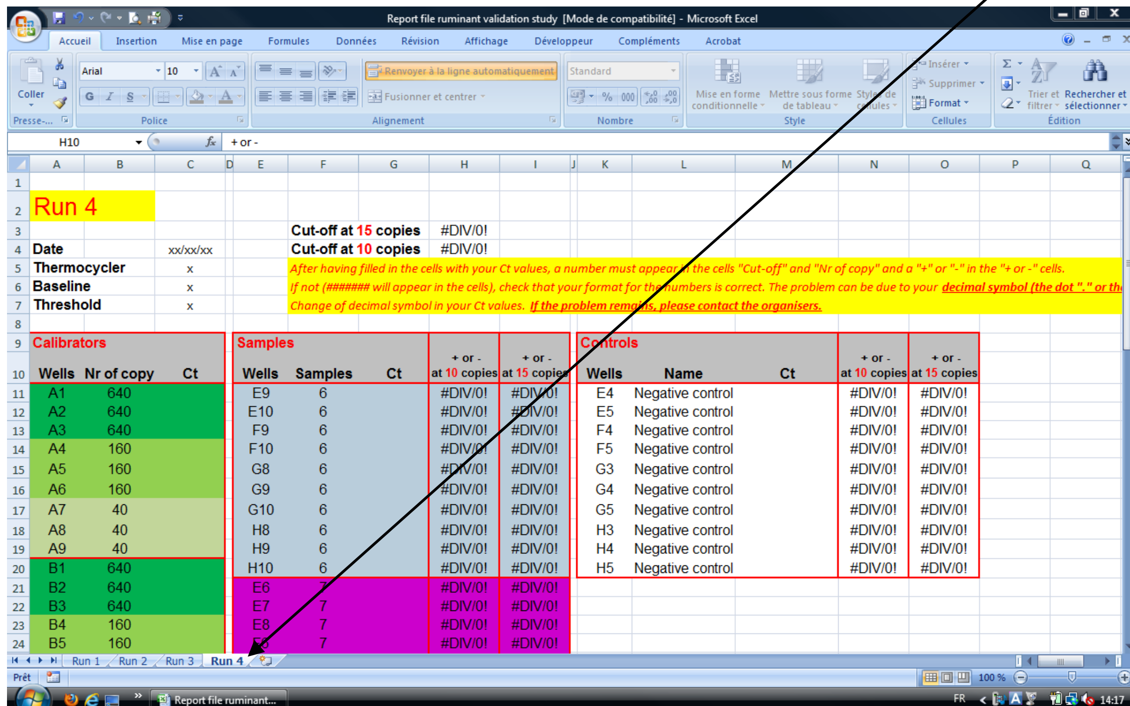


REMARK :
THE USE OF DIFFERENT PROCEDURES (AUTOMATIC OR MANUAL) FOR THE DETERMINATION OF THE THRESHOLD AND OF THE BASELINE WAS TESTED WITH DIFFERENT THERMOCYCLERS (LC 480, ABI 7000 AND ABI 7500). THE STATISTICAL ANALYSIS OF THE RESULTS SHOWS CLEARLY THAT, WITH ABI THERMOCYCLERS, THE BEST REPEATABILITY 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 OF 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 an Excel file with defined areas :

- One run corresponds to one sheet named “Run 1”, “Run 2”, “Run 3” and “Run 4”.
- Within each sheet, the cells to fill in with the Ct values are pre-defined.



1. **The participants are asked to deliver the results (Ct values) in the Excel file provided by the organisers.**
2. **After having filled in the cells with your Ct values, a number must appear in the cells "Cut-off at 10 copies" and "Cut-off at 15 copies". A "+" or "-" must also appear in the "+ or – at 10 copies" and "+ or – at 15 copies" 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.**
   <u>If the problem remains, please contact the organisers</u>.
3. **If outliers are observed within calibration data, the corresponding Ct values can be removed. <u>Please do not write anything in the corresponding cell (e.g. 0, 50, Not determined, NC, …) but leave it empty</u>. You can however mention in the deviation report why you did not fill the cell.**
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. The qualitative (positive or negative) results are determined <u>automatically</u>.**
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, he will 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 with the original of this protocol and all the copies of it.**

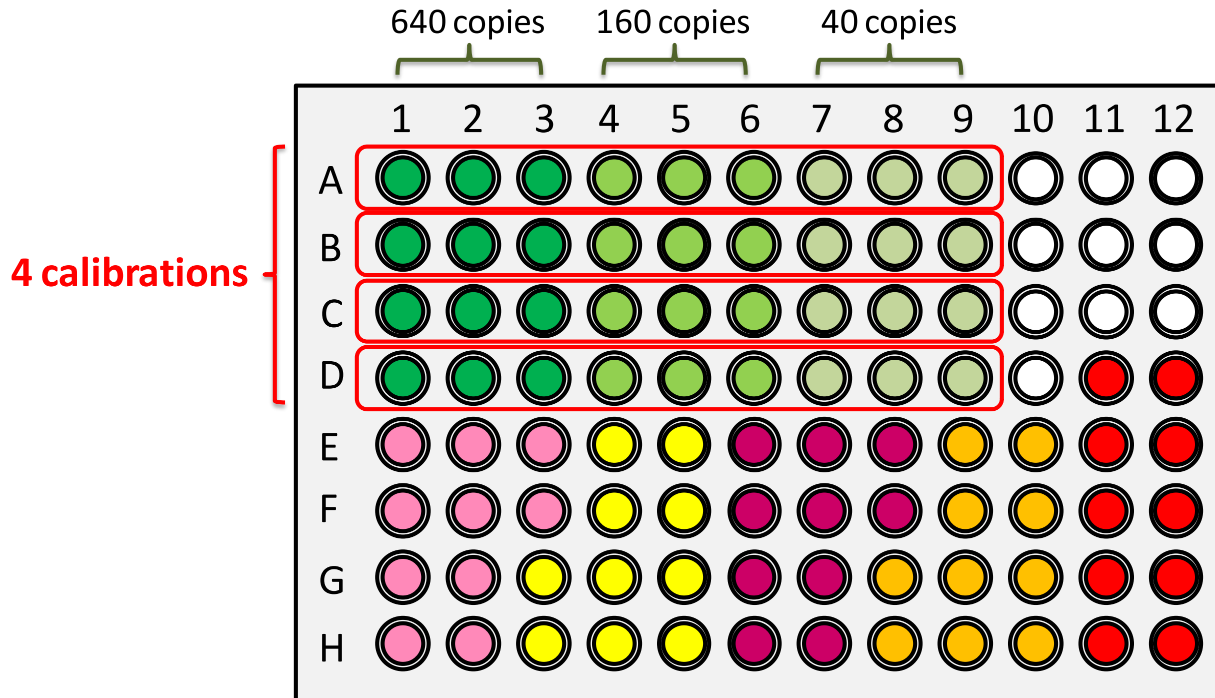
ANNEX 1 : AMOUNTS OF ROX (PASSIVE REFERENCE) TO ADD TO THE MASTER MIX 2X

SYSTEM	MASTER MIX 2X	ROX
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

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

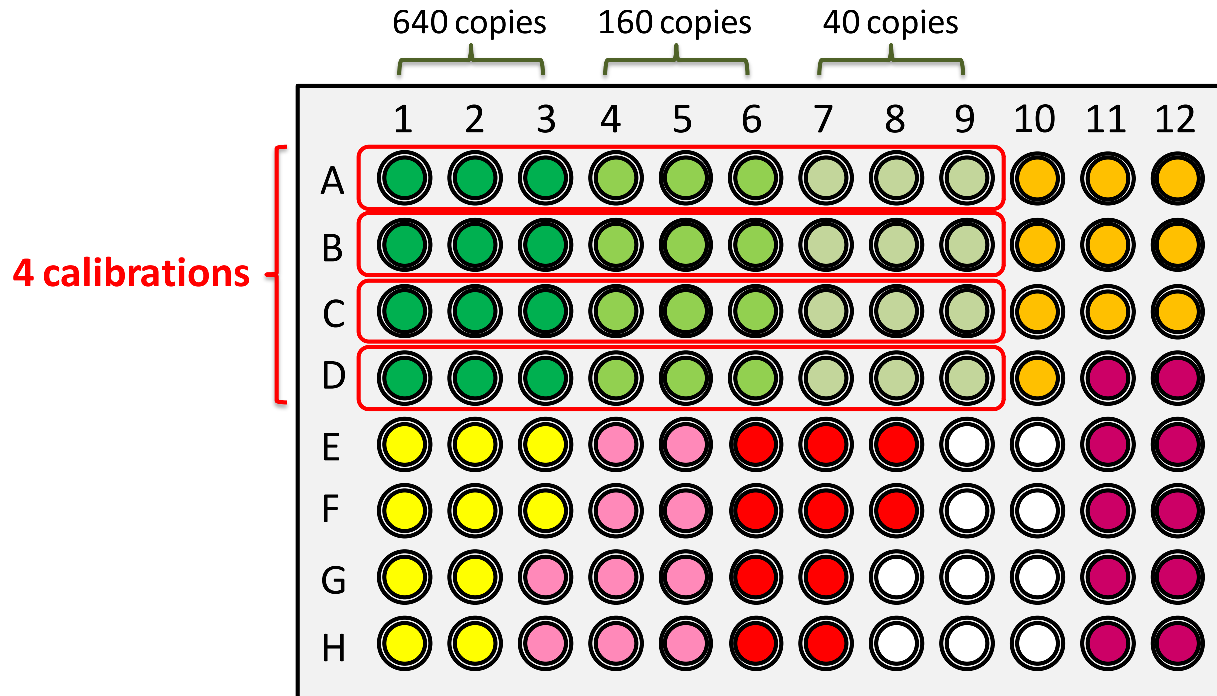
2 — E9, E10, F9, F10, G8, G9, G10, H8, H9, H10

3 — E6, E7, E8, F6, F7, F8, G6, G7, H6, H7

4 — E4, E5, F4, F5, G3, G4, G5, H3, H4, H5

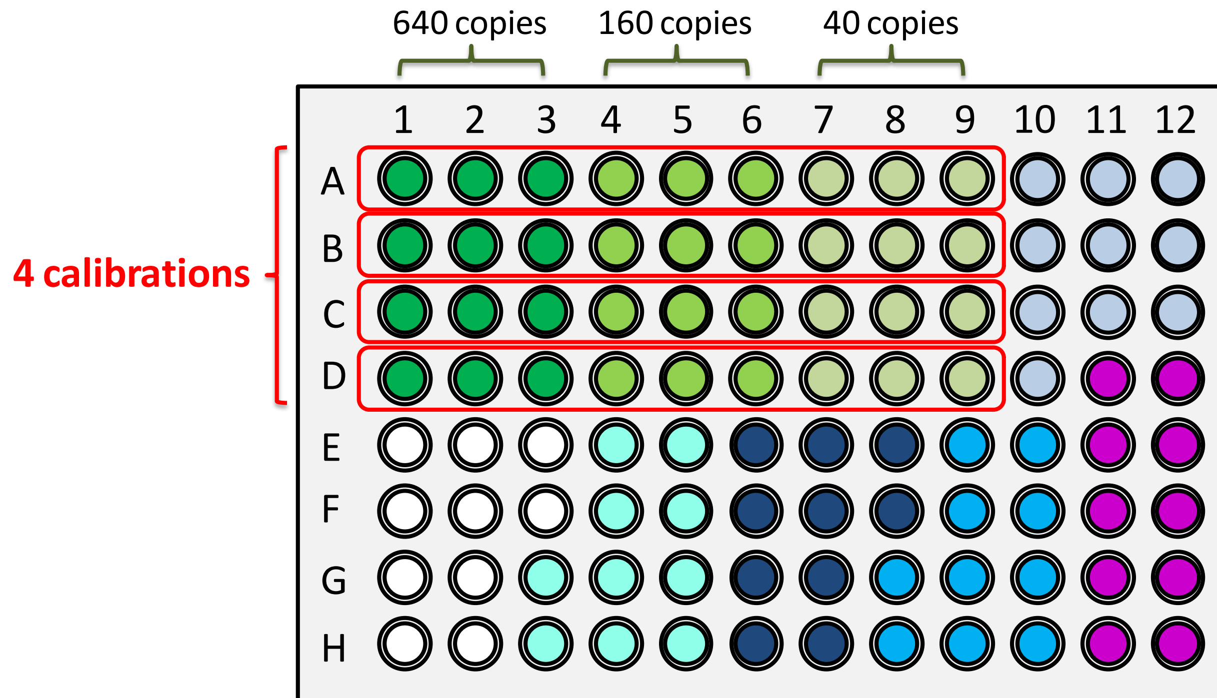
5 — E1, E2, E3, F1, F2, F3, G1, G2, H1, H2

RUN 2



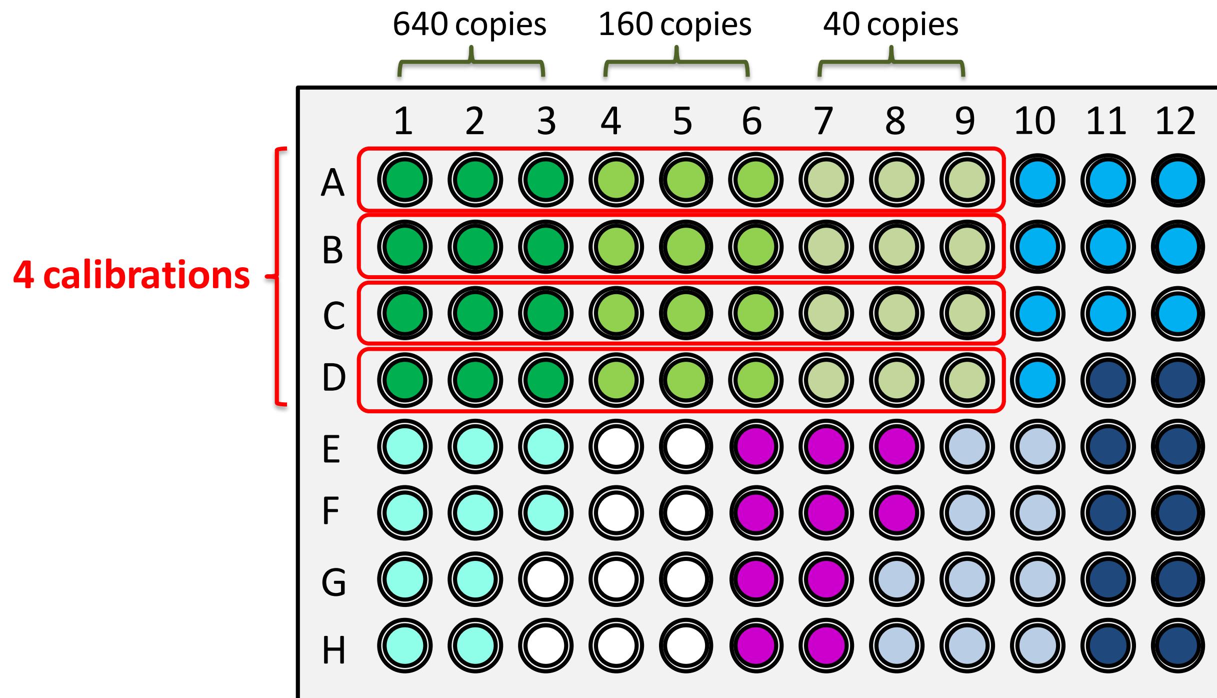
		Wells
Calibrators		
	640 copies	A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3
	160 copies	A4, A5, A6, B4, B5, B6, C4, C5, C6, D4, D5, D6
	40 copies	A7, A8, A9, B7, B8, B9, C7, C8, C9, D7, D8, D9
Control		
	Negative PCR control	E9, E10, F9, F10, G8, G9, G10, H8, H9, H10
Samples		
	# 1	E6, E7, E8, F6, 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

RUN 3



		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	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
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	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