Evaluation for approval of the Brilliant II QPCR Low ROX Master Mix (Agilent technologies) for the detection of PAP in feed and feed materials by real-time PCR

Report

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TABLE DES MATIÈRES

1. Introduction

This report aims to provide evidence that the Brilliant II QPCR Low Rox Master mix of Agilent (Agilent technologies, Cedar Creek, TX, USA) gives comparable results and therefore is equivalent to the Universal mastermix of Diagenode (Diagenode s.a., Seraing, Belgium) currently recommended by EURL-AP. If so, the Brilliant II QPCR Low Rox Master mix of Agilent (Agilent technologies) could be used for real-time PCR detection of processed animal protein (PAP) in feed and feed materials.

The difficulty being to have a master mix not containing DNA of ruminant origin (e.g. due to the presence of bovine serum albumin - BSA), the first step of the evaluation of the master mix focused on the ruminant PCR test. Subsequent steps of the evaluation were carried out with the 3 official PCR tests to detect ruminant, porcine and poultry DNA.

2. Evaluation steps of a new master mix

2.1. Verification of the absence of Bovine Serum Albumin

The literature supports the hypothesis that bovine serum albumin (BSA) may stabilize the DNA polymerase (Farell and Alexandre, 2012). This is why BSA is often used by master mix manufacturers. This component induces the presence of bovine DNA which is a problem with ruminant PCR test.

The first test to evaluate a master mix for the detection of ruminant DNA by PCR is therefore to check that the master mix does not contain DNA of ruminant origin. Ten no template controls (NTC), reactions where sample's DNA is replaced by an equivalent volume of PCR grade water, were performed with the ruminant PCR test using the Brilliant II QPCR Low Rox Master mix of Agilent technologies (Table 1). In comparison, the reactions were also performed with the Universal mastermix of Diagenode. As with the recommended master mix, some NTC performed with the Brilliant II QPCR Low Rox Master mix of Agilent technologies give late amplification curves. Given the Ct values obtained, these signals are not a problem when compared to the cut-off values (see 2.2.) and allow to conclude on the negative character of these late amplification.

The analyses were performed on two thermocyclers: one LightCycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and one QuantStudio 6 Flex (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). Concerning the analytical parameters applied, Abs. quant/second derivative max and high confidence mode are used with the LightCycler 480. With the QuantStudio 6 Flex, the Automatic baseline mode is used. The threshold is set at 0.05 with the Universal mastermix of Diagenode and 0.06 with the Brilliant II QPCR Low Rox Master mix of Agilent Agilent technologies respectively.

Table 1: *Tests to verify the absence of DNA of ruminant origin in the master mix with no template controls performed with the Universal mastermix -Diagenode s.a.- and the Brilliant II QPCR Low Rox Master mix -Agilent technologies- on 2 real-time PCR thermocyclers (LightCycler 480 and QuantStudio 6 Flex).*

	C_t obtained on			
	LightCycler 480 with		QuantStudio 6 Flex with	
Replicate of no template control	Universal Mastermix of Diagenode	Brilliant II QPCR Low Rox Master mix of Agilent	Universal Mastermix of Diagenode	Brilliant II QPCR Low Rox Master mix of Agilent
1	40.13	40.27	39.60	39.21
2	40.49	40.27	40.65	40.68
3	40.56	40.84	43.99	40.79
4	40.84	41.01	48.29	40.89
5	43.63	45.00	No signal	48.62
6	No signal	45.00	No signal	No signal
7	No signal	No signal	No signal	No signal
8	No signal	No signal	No signal	No signal
9	No signal	No signal	No signal	No signal
10	No signal	No signal	No signal	No signal

2.2. Evaluation of master mix with ruminant PCR test

2.2.1. Determination of cut-off value for ruminant PCR test

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 (EURL-AP, 2017). The cut-off value for ruminant PCR test was determined using materials of calibration provided by the Joint Research Centre (Geel, Belgium) on the 4 PCR platforms : real-time thermocycler LightCycler 480 (Roche Diagnostics Ltd.) with the 2 master mixes to compare, the Universal mastermix (Diagenode s.a.) and the Brilliant II QPCR Low ROX Master Mix (Agilent technologies) and real-time thermocycler QuantStudio 6 Flex (Applied Biosystems) with the 2 same master mixes.

To determinate a cut-off value, four PCR plates were performed with each PCR platform according to the EURL-AP SOP (EURL-AP, 2017). Four calibrations were

realised per plate. One calibration consists of 3 replicates from 3 calibrant levels (9 wells). The cut-off value is calculated with the Excel file available on the EURL-AP website for the ruminant method (https://www.eurl.craw.eu). This cut-off value is defined as the upper limit of the interval confidence of Cq (or Ct) values for 15 copies of the target (EURL-AP, 2017). The quality criterion on the cut-off value must correspond to a number of copies greater than 9 copies.

The cut-off values at 15 copies and the copy numbers corresponding to the cut-off values are presented in the Table 2.

The quality criterion corresponding to the copy numbers at the cut-off values is reached for the 4 platforms.

Table 2: *Cut-off values for ruminant PCR test determined on 4 PCR platforms and copy numbers corresponding to the cut-off values.*

	Real-time PCR platforms			
	LightCycler 480 with		QuantStudio 6 Flex with	
	Universal mastermix of Diagenode	Brilliant II QPCR Low Rox Master mix of Agilent	Universal mastermix of Diagenode	Brilliant II QPCR Low Rox Master mix of Agilent
Cut-off at 15 copies (in cycles)	36.08	36.69	35.01	34.90
Copy number corresponding to the cut-off value (in copies)	10.72	11.61	9.75	11.46

By applying these cut-off on the results obtained with NTC replicates (Table 1), all no template control reactions are rightly considered as negative results. A ΔCt comprised between 3.58 and 4.59 cycles is observed between the cut-off values and the sooner NTC Ct values.

2.2.2. Comparison of results obtained on samples with a master mix recommended and the master mix proposed for evaluation

2.2.2.1. Results obtained on different feed samples

Eighteen feed samples, including 7 negative samples and 11 positive samples were analysed for the detection of ruminant DNA using the official PCR test with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on the LightCycler 480 thermocycler (Roche Diagnostics Ltd.). The

results obtained are presented in Table 3. The cut-off value being determined each year, the cut-off value applied therefore depends on the moment when the analysis is performed. This is why no cut-off value is indicated in the results tables. The interpretation of results is done according to the respective cut-off values.

Most samples analysed come from proficiency test organised by EURL-AP. Other samples of known composition were also analysed.

Table 3: *Comparison of results obtained on DNA extracts coming from samples known to contain no ruminant material with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on a LightCycler 480 thermocycler (Roche Diagnostics Ltd.).*

			LightCycler 480 with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
PT PCR 2015 sample 1 Fish meal	1	undiluted 10x diluted	50 42.02	-	43.32 44.09	-
	2	undiluted 10x diluted	50 42.00		43.49 50	
PT 2016 sample 6 Fish feed + 1% porcine PAP	1	undiluted 10x diluted	39.82 38.77	-	37.73 40.28	-
	2	undiluted 10x diluted	39.21 40.69		37.23 39.10	
PT 2017 sample 4 Fish feed containing haemoglobin meal (real world sample)	1	undiluted 10x diluted	38.38 40.72	-	37.74 39.10	-
	2	undiluted 10x diluted	39.81 40.14		37.22 40.67	
Fish feed containing haemoglobin powder	1	undiluted 10x diluted	39.82 44.44	-	38.25 50	-
	2	undiluted 10x diluted	40.03 41.83		38.24 41.03	
Fish feed (used in sample 3 of PT 2018)	1	undiluted 10x diluted	39.31 39.91	-	39.00 39.51	-
	2	undiluted 10x diluted	40.86 38.90		38.35 38.80	
PT 2020 sample 4 Aquafeed	1	undiluted 10x diluted	40.49 42.83	-	39.80 39.80	-
	2	undiluted 10x diluted	42.09 43.76		41.12 40.29	
Poultry feed 1	1	undiluted 10x diluted	37.17 40.03	-	36.68 41.49	-*
	2	undiluted 10x diluted	38.10 39.61		36.40 39.70	

* Ambiguous result in first analysis and negative result in second analysis, therefore the final result is reported as negative

Table 4: *Comparison of results obtained on DNA extracts coming from samples known to contain ruminant material with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on LightCycler 480 thermocycler (Roche Diagnostics Ltd.).*

			LightCycler 480 with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
PT PCR 2015 sample 2 Fish meal not fortified with ruminant material but real world sample containing ruminant DNA at a low level	1	undiluted 10x diluted	35.26 37.81	+	33.77 36.39	+
	2	undiluted 10x diluted	35.23 37.55		34.16 37.58	
PT PCR 2015 sample 4 Fish feed not fortified with ruminant material but real world sample containing ruminant DNA at a low level	1	undiluted 10x diluted	31.21 33.91	+	30.33 33.67	+
	2	undiluted 10x diluted	30.46 33.34		29.76 33.09	
PT 2016 sample 5 Fish feed + 0.05% ruminant PAP	1	undiluted 10x diluted	29.77 29.55	+	28.05 28.48	+
	2	undiluted 10x diluted	32.03 32.47		30.22 31.30	
PT 2016 sample 7 Fish feed + 3% bovine plasma	1	undiluted 10x diluted	24.54 27.05	+	23.38 26.46	+
	2	undiluted 10x diluted	24.81 26.78		23.36 26.01	
PT 2016 sample 8 Fish feed + 0.1% milk powder	1	undiluted 10x diluted	28.86 32.01	+	28.16 31.57	+
	2	undiluted 10x diluted	28.58 31.67		27.62 30.96	
PT 2017 sample 2 Fish feed containing haemoglobin powder (real world sample) + 0.1% porcine PAP	1	undiluted 10x diluted	32.23 34.09	+	30.86 33.38	+
	2	undiluted 10x diluted	31.73 33.49		30.50 32.85	
PT 2017 sample 5 Fish feed + 0.05% ruminant PAP	1	undiluted 10x diluted	32.24 31.53	+	30.51 30.36	+
	2	undiluted 10x diluted	31.53 31.10		29.77 29.95	
PT 2015 sample 6 Fish feed + 0.05% ruminant PAP	1	undiluted 10x diluted	31.29 34.94	+	30.00 33.92	+
	2	undiluted 10x diluted	31.17 33.85		29.73 32.69	
Poultry feed 2 + 0,1% bovine blood meal	1	undiluted 10x diluted	33.87 34.78	+	32.52 34.32	+
	2	undiluted 10x diluted	35.09 35.66		33.61 34.35	
Poultry feed 1 + 0,1% bovine blood meal	1	undiluted 10x diluted	31.59 35.47	+	30.50 34.20	+
	2	undiluted 10x diluted	31.47 34.86		30.33 34.15	
PT 2020 sample 5 Aquafeed + 0.1% ruminant PAP	1	undiluted 10x diluted	28.48 32.49	+	27.55 31.87	+
	2	undiluted 10x diluted	27.90 31.80		26.91 31.16	

The Brilliant II QPCR Low Rox Master mix of Agilent technologies give the same results than the Universal mastermix from Diagenode. The samples known to contain no ruminant material are declared negative with both master mixes whereas the samples containing a low level of ruminant material are detected with both master mix.

In order to confirm that the 2 master mixes give equivalent results, some DNA extracts were analysed with both master mixes on the QuantStudio 6 Flex (Applied Biosystems). Tables 5 and 6 show that all the results are similar to those obtained on LightCycler 480 (Roche Diagnostics Ltd.).

Table 5: *Comparison of results obtained with DNA extracts coming from samples known to contain no ruminant material with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on a QuantStudio 6 Flex (Applied Biosystems).*

			QuantStudio 6 Flex with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
PT PCR 2015 sample 1 Fishmeal	1	undiluted 10x diluted	39.42 45.51	-	41.53 50	-
	2	undiluted 10x diluted	50 38.89		46.74 50	
PT 2016 sample 6 Fish feed + 1% porcine PAP	1	undiluted 10x diluted	39.16 41.52	-	36.74 38.41	-
	2	undiluted 10x diluted	38.22 38.66		37.53 38.96	
PT 2017 sample 4 Fish feed containing haemoglobin meal (real world sample)	1	undiluted 10x diluted	38.35 39.70	-	36.37 38.56	-
	2	undiluted 10x diluted	37.46 39.00		36.32 37.43	

Table 6: *Comparison of results obtained with DNA extracts coming from samples known to contain ruminant material with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on a QuantStudio 6 Flex (Applied Biosystems).*

			QuantStudio 6 Flex with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
PT PCR 2015 sample 2 Fish meal not fortified with ruminant material but real world sample containing ruminant DNA at a low level	1	undiluted 10x diluted	33.43 35.48	+	33.14 35.95	+
	2	undiluted 10x diluted	34.21 35.87		33.22 36.06	
PT PCR 2015 sample 4 Fish feed not fortified with ruminant material but real world sample containing ruminant DNA at a low level	1	undiluted 10x diluted	29.65 32.82	+	29.46 32.80	+
	2	undiluted 10x diluted	29.02 32.32		29.06 32.60	
PT 2016 sample 5 Fish feed + 0.05% ruminant PAP	1	undiluted 10x diluted	28.45 28.20	+	26.86 27.71	+
	2	undiluted 10x diluted	30.85 30.98		29.05 30.56	
PT 2016 sample 7 Fish feed + 3% bovine plasma	1	undiluted 10x diluted	23.13 25.32	+	22.57 25.29	+
	2	undiluted 10x diluted	23.41 25.80		22.46 25.74	
PT 2016 sample 8 Fish feed + 0.1% milk powder	1	undiluted 10x diluted	27.38 30.74	+	27.30 31.03	+
	2	undiluted 10x diluted	27.10 30.13		26.78 30.47	
PT 2017 sample 2 Fish feed containing haemoglobin powder (real world sample) + 0.1% porcine PAP	1	undiluted 10x diluted	30.76 32.93	+	29.65 33.08	+
	2	undiluted 10x diluted	30.59 32.29		29.45 31.72	
PT 2017 sample 5 Fish feed + 0.05% ruminant PAP	1	undiluted 10x diluted	30.10 30.26	+	28.69 29.86	+
	2	undiluted 10x diluted	30.97 34.18		29.50 29.20	

2.2.2.2. Results obtained on feed samples known to show PCR inhibition

Different samples known to show PCR inhibition were analysed using the ruminant PCR test with both master mixes on the LightCycler 480 thermocycler (Roche Diagnostics Ltd.). The results obtained are presented on Table 7. The interpretation of results is done according to the respective cut-off value.

Table 7: *Comparison of results obtained on DNA extracts coming from samples presenting PCR inhibition with 2 master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on a LightCycler 480 thermocycler (Roche Diagnostics Ltd.).*

			LightCycler 480 with			
			Universal mastermix of Diagenode		Brilliant II QPCR Low Rox Master mix of Agilent	
Sample number	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
Poultry meal (real world sample)	1	undiluted	37.81	+	32.93	+
		10x diluted	34.45		31.83	
	2	undiluted	37.04		32.74	
		10x diluted	33.97		31.95	
Feed 1 - real world sample	1	undiluted	33.02	+	31.93	+
		10x diluted	34.81		33.88	
	2	undiluted	34.83		33.02	
		10x diluted	34.39		33.77	
Feed 2 - real world sample	1	undiluted	33.68	+	32.15	+
		10x diluted	33.22		32.73	
	2	undiluted	34.60		32.69	
		10x diluted	33.65		33.05	
Fish feed 1 - real world sample	1	undiluted	36.72	-*	34.59	+
		10x diluted	37.25		36.21	
	2	undiluted	35.70		33.76	
		10x diluted	36.50		35.35	
Fish feed 2 - real world sample	1	undiluted	35.57	+	33.59	+
		10x diluted	36.15		35.77	
	2	undiluted	35.23		33.38	
		10x diluted	36.34		35.19	
PT 2018 sample 3 Fish feed + 0.05 % ruminant PAP	1	undiluted	34.53	+	32.31	+
		10x diluted	31.05		29.48	
	2	undiluted	34.09		31.88	
		10x diluted	31.05		29.63	
PT 2019 sample 3 Porcine haemoglobin meal + 0.1 % ruminant PAP	1	undiluted	31.74	+	29.49	+
		10x diluted	32.97		32.30	
	2	undiluted	32.70		30.27	
		10x diluted	32.89		31.85	
Poultry feed 2	1	undiluted	45.00	-	50	-
		10x diluted	39.90		50	
	2	undiluted	45.00		40.78	
		10x diluted	38.97		40.59	

* ambiguous results also during a second analysis, therefore the final result is reported as negative

The Brilliant II QPCR Low ROX Master Mix (Agilent technologies) appears to be less susceptible to inhibitors. Indeed, the poultry meal sample gives a negative result with the undiluted DNA extracts using the Universal mastermix of Diagenode. The 10-fold dilution allows to remove the PCR inhibition and to have a positive result for the ruminant detection. The results obtained with the same sample using the Brilliant II QPCR Low Rox Master mix show also the presence of PCR inhibition but this one is less pronounced; both undiluted and diluted DNA extracts give positive results. The samples feed 1 and 2, fish feed 2 and sample 3 of PT 2019 show also a lower PCR inhibition with the Brilliant II QPCR Low Rox Master mix even if this has no impact on the final conclusion.

All samples give the same results whatever the master mix used excepted one sample. Indeed, the fish feed 1 gives different results depending on the master mix used. Declared positive with Brilliant II QPCR Low Rox Master mix, it gives ambiguous results in first analysis and is finally declared negative with the results of second analysis with the Universal mastermix (Diagenode s.a.).

Some DNA extracts were also analysed with the 2 master mixes on the QuantStudio 6 Flex thermocycler (Applied Biosystems). The choice fell on the positive samples given the latest Cq values. The results presented in Table 8 show that, in this case, fish feed 1 gives a positive result with both master mix tested. However, there is a difference between the results for the fish feed 2: with the Universal mastermix this sample gives ambiguous results and is finally declared as negative for presence of ruminant while it is positive with the Brilliant II QPCR Low Rox Master mix.

Table 8: *Comparison of results obtained with DNA extracts coming from samples presenting inhibition with the 2 master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on the QuantStudio 6 Flex (Applied Biosystems).*

			QuantStudio 6 Flex			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
Poultry meal (real world sample)	1	undiluted 10x diluted	37.47 33.22	+	31.97 30.99	+
	2	undiluted 10x diluted	36.34 33.09		31.89 30.99	
Feed 1 (real world sample)	1	undiluted 10x diluted	31.74 33.44	+	30.61 33.10	+
	2	undiluted 10x diluted	33.48 33.62		31.65 32.73	
Fish feed 1 (real world sample)	1	undiluted 10x diluted	35.26 35.61	+*	33.77 35.97	+
	2	undiluted 10x diluted	34.42 35.73		33.14 35.09	
Fish feed 2 (real world sample)	1	undiluted 10x diluted	35.08 35.28	-**	32.92 34.79	+
	2	undiluted 10x diluted	34.18 35.20		32.75 35.96	
Feed 2 (real world sample)	1	undiluted 10x diluted	32.88 32.08	+	31.25 31.56	+
	2	undiluted 10x diluted	33.43 32.36		31.73 31.68	

* ambiguous results in first analyze, additional dilutions were tested to check the inhibition. The final result takes into account the analysis of these additional dilutions.

** ambiguous results also during a second analysis, therefore the final result is reported as negative

2.2.2.3. Results obtained on positive controls

DNA extracts used as extraction and PCR positive controls were analysed with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on 2 real-time PCR thermocyclers. These samples contain less than 0.1% in mass fraction of ruminant PAP. The results obtained are presented in Tables 9 and 10. The interpretation of results is done according to the respective cut-off value.

Table 9: *Comparison of results obtained with DNA extracts coming from positive controls with the 2 master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on LightCycler 480 thermocycler (Roche Diagnostics Ltd.).*

			LightCycler 480 with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract identification**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
Sow feed + 0.05% ruminant PAP	180503	undiluted 10x diluted	31.34 29.72	+	29.78 29.00	+
	180424	undiluted 10x diluted	33.38 30.85		31.85 30.06	
Feed + 0.25% mix of ruminant and porcine PAP	180523	undiluted	32.38	+	30.86	+
	180524	undiluted	33.19		31.36	

Table 10: *Comparison of results obtained with DNA extracts come from positive controls with 2 master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on a QuantStudio 6 Flex (Applied Biosystems).*

			QuantStudio 6 Flex with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
Sow feed + 0.05% ruminant PAP	180503	undiluted 10x diluted	30.44 29.04	+	29.91 27.68	+
	170507	undiluted 10x diluted	31.76 29.12		30.13 28.55	
Feed + 0.25% mix of ruminant and porcine PAP	180523	undiluted	31.88	+	29.73	+
	180524	undiluted	32.08		30.64	

2.2.3. Conclusion of the evaluation of the Agilent technologies master mix with ruminant PCR test

The results obtained show that the Brilliant II QPCR Low Rox Master mix of Agilent technologies does not contain DNA of ruminant origin and gives similar results to those obtained with the Universal mastermix of Diagenode. This reagent allows to detect the presence of low level (< 0.1 % in mass fraction) of ruminant PAP.

Only 2 samples presenting a PCR inhibition effect, fish feed 1 and 2, give a different result depending of the master mix but also of the thermocycler used. For these 2 samples the results are positive with the Brilliant II QPCR Low Rox Master mix of Agilent technologies whatever the thermocycler used. With regard to the results obtained with the Universal mastermix of Diagenode, the result is not clearly negative as repeatable ambiguous results are obtained leading to a negative result as conclusion. The Brilliant II QPCR Low Rox Master mix of Agilent technologies seeming to be more resistant to PCR inhibitors, it allows to obtain a clearer result when PCR inhibition is present.

These data allow to conclude that the Brilliant II QPCR Low Rox Master mix of Agilent technologies can be used for the ruminant PAP detection by real-time PCR with feed and raw material samples.

2.3. Evaluation of the master mix with the porcine PCR test

2.3.1. Determination of the cut-off value for the porcine PCR test

Cut-off values for the porcine PCR test were determined using materials of calibration provided by the Joint Research Centre (Geel, Belgium) on 2 PCR platforms : real-time thermocycler LightCycler 480 (Roche Diagnostics Ltd.) with 2 master mixes to compare, the Universal mastermix (Diagenode s.a.) and the Brilliant II QPCR Low ROX Master Mix (Agilent technologies).

To determinate the cut-off value, four PCR plates were performed with each PCR platform according to the EURL-AP SOP (EURL-AP, 2021). Four calibrations were realised per plate. One calibration consisting of 3 replicates from 3 calibrant levels (9 wells). The cut-off value is calculated with the Excel file available on the EURL-AP website for the porcine method (https://www.eurl.craw.eu). This cut-off value is defined as the upper limit of the interval confidence of Cq (or Ct) values for 5 copies of the target

(EURL-AP, 2021). A quality criterion has been set on the cut-off value. This one must correspond to a number of copies greater than 3 copies.

The cut-off values at 5 copies and the copy numbers corresponding to the cut-off values were determined on the 2 PCR platforms and are presented in the Table 11.

The quality criterion corresponding to the copy numbers at the cut-off values is reached for the 2 platforms.

Table 11: *Cut-off values for the porcine PCR test determined on 2 PCR platforms and copy numbers corresponding to the cut-off values.*

	Real-time PCR platforms	
	LightCycler 480 with	
	Universal mastermix of Diagenode	**Brilliant II QPCR Low Rox Master mix of Agilent**
Cut-off at 5 copies (in cycles)	39.56	39.10
Copy number corresponding to the cut-off value (in copies)	3.85	3.51

2.3.2. Comparison of results obtained on samples with a master mix recommended and the master mix proposed for validation

Sixteen samples, including 5 negative samples and 11 positive samples were analysed for the detection of porcine DNA using the official PCR test with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on the LightCycler 480 thermocycler (Roche Diagnostics Ltd.). The results obtained are presented in Table 12. The interpretation of results is done according to the respective cut-off value.

Table 12: *Comparison of results obtained on DNA extracts coming from samples known to contain no porcine material with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on LightCycler 480 thermocycler (Roche Diagnostics Ltd.).*

			LightCycler 480 with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
Poultry PAP 1	1	undiluted	42.06	-	43.25	-
		10x diluted	50		42.92	
	2	undiluted	40.94		43.40	
		10x diluted	50		50	
Bovine PAP 1	1	undiluted	50	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	
Fish meal	1	undiluted	50	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	
Maize	1	undiluted	50	-	50	-
		10x diluted	50		42.07	
	2	undiluted	50		50	
		10x diluted	50		50	
Fish feed 3	1	undiluted	43.88	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	

Table 13: *Comparison of results obtained on DNA extracts coming from samples known to contain porcine material with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on the LightCycler 480 thermocycler (Roche Diagnostics Ltd.).*

			LightCycler 480 with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
Porcine PAP 1	1	undiluted 10x diluted	22.22 25.19	+	21.78 25.74	+
	2	undiluted 10x diluted	21.74 25.38		22.13 25.80	
Porcine PAP 2	1	undiluted 10x diluted	24.39 25.28	+	22.87 25.63	+
	2	undiluted 10x diluted	24.18 25.26		22.86 25.56	
Porcine PAP 3	1	undiluted 10x diluted	26.20 28.89	+	25.99 29.29	+
	2	undiluted 10x diluted	26.14 29.14		26.01 29.33	
Maize + 0.1% porcine PAP 3	1	undiluted 10x diluted	33.65 37.47	+	33.48 37.25	+
	2	undiluted 10x diluted	32.29 35.93		32.13 35.90	
Fish meal + 0.1% porcine PAP 3	1	undiluted 10x diluted	37.04 39.31	+	36.71 40.23	+
	2	undiluted 10x diluted	36.12 38.26		35.67 38.73	
Fish feed 3 + 0.1% porcine PAP 3	1	undiluted 10x diluted	33.99 36.49	+	33.19 35.96	+
	2	undiluted 10x diluted	32.81 35.65		32.55 35.96	
Pig feed 1 + 0.1% porcine PAP 3	1	undiluted 10x diluted	35.19 37.21	+	33.99 36.59	+
	2	undiluted 10x diluted	32.66 36.76		34.98 36.34	
Pig feed 1 + 0.1% porcine PAP 1	1	undiluted 10x diluted	31.43 33.60	+	31.13 33.57	+
	2	undiluted 10x diluted	29.61 34.22		30.82 34.32	
Fish meal + 0.1% porcine PAP 1	1	undiluted 10x diluted	33.52 37.32	+	33.89 37.04	+
	2	undiluted 10x diluted	33.53 36.94		33.62 36.97	
Fish feed 3 + 0.1% porcine PAP 2	1	undiluted 10x diluted	31.22 33.74	+	30.13 33.56	+
	2	undiluted 10x diluted	30.07 32.71		29.45 32.62	
Poultry PAP 1 + 0.1% porcine PAP 2	1	undiluted 10x diluted	35.85 38.91	+	36.41 40.17	+
	2	undiluted 10x diluted	36.41 39.79		37.21 38.98	

The Brilliant II QPCR Low Rox Master mix of Agilent technologies give the same results than the Universal mastermix from Diagenode. The samples known to contain no porcine material are declared negative with both master mixes. The pure porcine PAP and samples containing a low level of porcine material are detected with both master mixes.

2.3.3. Conclusion of the evaluation of the master mix Agilent technologies with the porcine PCR test

The results obtained show that the Brilliant II QPCR Low Rox Master mix of Agilent technologies gives similar results to those obtained with the Universal mastermix of Diagenode. This reagent allows to detect the presence of a low level (0.1 % in mass fraction) of porcine PAP.

These data allow to conclude that the Brilliant II QPCR Low Rox Master mix of Agilent technologies can be used for the porcine PAP detection by real-time PCR in feed and raw material.

2.3. Evaluation of the master mix with the poultry (chicken and turkey) PCR test

2.3.1. Determination of the cut-off value for the poultry PCR test

Cut-off values for the poultry PCR test were determined using materials of calibration provided by the Joint Research Centre (Geel, Belgium) on 2 PCR platforms : the real-time thermocycler LightCycler 480 II (Roche Diagnostics Ltd.) with 2 master mixes to compare, the Universal mastermix (Diagenode s.a.) and the Brilliant II QPCR Low ROX Master Mix (Agilent technologies).

To determinate the cut-off value, four PCR plates were performed with each PCR platform according to the EURL-AP SOP (EURL-AP, 2021). Four calibrations were realised per plate. One calibration consisting of 3 replicates from 3 calibrant levels (9 wells). The cut-off value is calculated with the Excel file available on the EURL-AP website for the poultry method (https://www.eurl.craw.eu). This cut-off value is defined as the upper limit of the interval confidence of Cq (or Ct) values for 15 copies of the target (EURL-AP, 2021). A quality criterion has been set on the cut-off value. This one must correspond to a number of copies greater than 9 copies.

The cut-off values at 15 copies and the copy numbers corresponding to the cut-off values were determined on the 2 PCR platforms and are presented in the Table 14.

The quality criterion corresponding to the copy numbers at the cut-off values is reached for the 2 platforms.

Table 14: *Cut-off values for the poultry PCR test determined on 2 PCR platforms and copy numbers corresponding to the cut-off values.*

	Real-time PCR platforms	
	LightCycler 480 II with	
	Universal mastermix of Diagenode	Brilliant II QPCR Low Rox Master mix of Agilent
Cut-off at 15 copies (in cycles)	37.45	37.57
Copy number corresponding to the cut-off value (in copies)	11.56	11.73

2.3.2. Comparison of results obtained on samples with a master mix recommended and the master mix proposed for validation

Twenty-one samples, including 7 negative samples and 14 positive samples were analysed for the detection of poultry DNA using the official PCR test with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on the LightCycler 480 II thermocycler. The results obtained are presented in Table 15. The interpretation of results is done according to the respective cut-off value.

Table 15: *Comparison of results obtained on DNA extracts coming from samples known to contain no poultry material with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on a LightCycler 480 II thermocycler (Roche Diagnostics Ltd.).*

			LightCycler 480 II with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
Porcine PAP 3	1	undiluted	50	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	
Fish feed 4	1	undiluted	50	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	
Pig feed 2	1	undiluted	50	-	50	-
		10x diluted	39.77		50	
	2	undiluted	50		50	
		10x diluted	50		50	
Bovine PAP 2	1	undiluted	50	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	
Hens feed	1	undiluted	50	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	
Broilers feed	1	undiluted	50	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	
Turkey feed	1	undiluted	50	-	50	-
		10x diluted	50		50	
	2	undiluted	50		50	
		10x diluted	50		50	

Table 16: *Comparison of results obtained on DNA extracts coming from samples known to contain poultry material with both master mixes (Universal mastermix -Diagenode s.a.- and Brilliant II QPCR Low Rox Master mix -Agilent technologies) on a LightCycler 480 II thermocycler (Roche Diagnostics Ltd.).*

			LightCycler 480 II with			
			Universal mastermix of Diagenode		**Brilliant II QPCR Low Rox Master mix of Agilent**	
Sample number and Description	**Extract**	**Dilution**	**C_t (in cycles)**	**Interpretation results according to cut-off value**	**C_t (in cycles)**	**Interpretation results according to cut-off value**
Poultry PAP 1	1	undiluted 10x diluted	20.00 23.24	+	19.88 23.55	+
	2	undiluted 10x diluted	19.77 23.16		19.51 23.56	
Poultry PAP 2	1	undiluted 10x diluted	24.75 29.34	+	25.26 29.67	+
	2	undiluted 10x diluted	24.97 29.82		25.29 30.15	
Poultry PAP 3	1	undiluted 10x diluted	22.08 26.69	+	22.53 26.90	+
	2	undiluted 10x diluted	22.04 26.67		22.33 27.07	
Poultry PAP 4	1	undiluted 10x diluted	19.63 22.83	+	19.27 23.27	+
	2	undiluted 10x diluted	19.79 23.01		19.48 23.23	
Poultry PAP 5	1	undiluted 10x diluted	21.73 26.31	+	21.53 26.67	+
	2	undiluted 10x diluted	21.72 25.92		22.04 26.08	
Broilers feed + 0.1% poultry PAP 1	1	undiluted 10x diluted	28.29 31.14	+	28.47 30.93	+
	2	undiluted 10x diluted	28.04 30.78		28.66 30.67	
Broilers feed + 0.1% poultry PAP 3	1	undiluted 10x diluted	29.59 32.08	+	29.92 31.37	+
	2	undiluted 10x diluted	29.04 31.75		29.17 31.15	
Broilers feed + 0.1% poultry PAP 4	1	undiluted 10x diluted	28.29 31.09	+	28.80 30.93	+
	2	undiluted 10x diluted	28.59 31.17		29.20 31.06	
Broilers feed + 0.1% poultry PAP 2	1	undiluted 10x diluted	32.26 33.25	+	33.40 32.53	+
	2	undiluted 10x diluted	30.79 33.00		30.80 32.36	
Broilers feed + 0.1% poultry PAP 5	1	undiluted 10x diluted	30.40 33.06	+	30.59 32.29	+
	2	undiluted 10x diluted	30.56 33.23		30.78 32.56	
Turkey feed + 0.1% poultry PAP 2	1	undiluted 10x diluted	35.28 37.25	+	34.79 36.49	+
	2	undiluted 10x diluted	34.14 37.59		34.15 35.59	
Hens feed + 0.1% poultry PAP 2	1	undiluted 10x diluted	30.96 34.37	+	31.91 33.04	+
	2	undiluted 10x diluted	30.93 33.98		31.72 33.14	
Pig feed 2 + 0.1% poultry PAP 2	1	undiluted 10x diluted	29.15 32.34	+	29.60 31.77	+
	2	undiluted 10x diluted	29.74 33.01		30.16 32.04	
Fish feed 4 + 0.1% poultry PAP 2	1	undiluted 10x diluted	30.68 32.94	+	30.56 32.65	+
	2	undiluted 10x diluted	32.59 34.18		32.12 33.80	

The Brilliant II QPCR Low Rox Master mix of Agilent technologies gives the same results than the Universal mastermix from Diagenode. The samples known to contain no poultry material are declared negative with both master mixes. The pure poultry PAP and samples containing a low level of poultry material are detected with both master mix.

2.3.3. Conclusion of the evaluation of the master mix Agilent technologies with the porcine PCR test

The results obtained show that the Brilliant II QPCR Low Rox Master mix of Agilent technologies gives similar results to those obtained with the Universal mastermix of Diagenode. This reagent allows to detect the presence of a low level (0.1 % in mass fraction) of poultry PAP.

These data allow to conclude that the Brilliant II QPCR Low Rox Master mix of Agilent technologies can be used for the poultry PAP detection by real-time PCR in feed and raw material.

3. Conclusion

The Brilliant II QPCR Low Rox Master mix of Agilent technologies meets all criteria. This reagent :

- does not contain DNA of ruminant origin,
- allows to determinate cut-off values meeting the quality criteria for the ruminant, porcine and poultry real-time PCR tests,
- gives similar results to those obtained with Universal mastermix of Diagenode,
- allows to detect the presence of a low level (≤ 0.1 % in mass fraction) of ruminant, porcine or poultry PAP,
- is more resistant to PCR inhibitors and allows to obtain clearer results when inhibition is present.

These data allow to conclude that the Brilliant II QPCR Low Rox Master mix of Agilent technologies can be used for the ruminant, porcine and poultry PAP detection by real-time PCR in feed and raw material.

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