Report of a Single Laboratory Validation Study: Platform Extension of the Simplex Real-time PCR Method for Detection of Prohibited Materials in Animal Feed

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Abstract

A SmartCycler based real-time Polymerase Chain Reaction (rt-PCR) method is currently being utilized in the FDA Bovine Spongiform Encephalopathy (BSE) program to identify prohibited materials in animal feed. Here, we compared method performance on both the SmartCycler and the Applied Biosystems 7500 Fast (AB7500F) platforms. The purpose was to extend the instrument platform to the AB7500F so that FDA laboratories can continue to identify prohibited materials in animal feed using rt-PCR after the SmartCycler was discontinued. A total of 321 templates from three target species were tested. The false positive and false negative rates were slightly better using the rt-PCR on the AB7500F platform than the SmartCycler platform. For bovine, caprine, and ovine identification, the false positive rates were 2.9%, 0%, and 3.6% on the AB7500F with the number of DNA templates tested in this study, respectively; and 6.5%, 0.8%, and 8.0% on the SmartCycler, respectively. The false negative rates for the identification of all the target species were 0% on the AB7500F with the number of DNA templates tested in this study; and 1.7%, 1.3%, and 0% on the SmartCycler, respectively. The simplex rt-PCR method on both platforms was able to detect DNA of prohibited materials at the fractional levels for all the target species. Results of the McNemar's test showed that there was no significant difference in rt-PCR performance on the two platforms with 95% confidence level. In addition, AB7500F software versions v1.4 and v2.3 were compared using 141 of the 321 templates, and no significant difference was found between the two versions with 95% confidence level. Overall, this study supports the platform extension of the BSE simplex rt-PCR method from the SmartCycler to the AB7500F system. The Single Laboratory Validation (SLV) study has been reviewed and approved by the FDA Food and Veterinary Medicine (FVM) Chemistry Research Coordination Group (CRCG) and the FDA Molecular Identification Technical Advisory Group (MITAG).

Key words: platform extension, Bovine Spongiform Encephalopathy (BSE), simplex real-time PCR (rt-PCR), Applied Biosystems 7500 Fast (AB7500F), SmartCycler, Single Laboratory Validation (SLV)

Introduction

Transmissible Spongiform Encephalopathies (TSE) are a group of fatal neurological diseases characterized by tiny holes in the brain that give the brain a "spongy" appearance under a microscope. Bovine Spongiform Encephalopathy (BSE) is the bovine form of TSE, and scrapie is the form transmitted by goats and sheep (1). The causative agents are currently believed to be misfolded proteins called prions. Human and animals can be infected by BSE and certain other forms of TSE via consumption of prion contaminated meat and bone meals (MBM) (2). The first confirmed BSE case in cattle occurred in the United Kingdom in 1986, and BSE spread worldwide later. To date, over 184,500 BSE cases have been reported, resulting in devastating economic loss and posing a serious threat to public health (2). To prevent the transmission of TSE in our food supply, it is critical that the presence of ruminant-derived proteins be prohibited in animal feed. FDA introduced 21 CFR 589.2000 in 1997 and 21 CFR 589.2001 in 2008, which ban ruminant MBM in animal feed and feed ingredients used for food-producing animals in interstate commerce within the United States. In October 2003, the FDA compliance program #7371.009 on BSE/Ruminant Feed Ban Inspections was put into effect to assess the compliance of animal feed and animal feed producers with the two regulations (3).

Currently, there are three validated analytical methods that are being used in the BSE compliance program: a simplex rt-PCR method published in the Laboratory Information Bulletin (LIB) 4486 (4, 5), a multiplex rt-PCR method published in LIB 4544A (6), and an Animal Feed Microscopy (AFM) method published in AOAC's Official Methods of Analysis (7). The rt-PCR methods detect the presence of DNA from prohibited materials (bovine, caprine and ovine species), whereas the AFM method utilizes microscopy and certain chemical tests to visually and chemically ascertain whether feed contains suspect animal tissues like bone, muscle and hair. In most FDA laboratories, a combination of the simplex rt-PCR and the AFM methods are often used for screening and confirmation (8-10).

Both the simplex and multiplex rt-PCR methods utilize Invitrogen's Chargeswitch gDNA Rendered Meat Purification kit to extract DNA from feed and feed ingredients. Unlike the multiplex rt-PCR method which amplifies a homologous region of the ruminant genomes, the simplex rt-PCR method targets different genes in the bovine, caprine or ovine genome in separate reactions. Unfortunately, Cepheid Inc. discontinued the SmartCycler in December 2018 and will no longer service the existing instruments (11), which left FDA laboratories without a platform for running the simplex rt-PCR method published in LIB 4486.

To address this issue, a research project was recently accomplished to extend the instrument platform to the Applied Biosystems 7500 Fast (AB7500F) system (12). As the purpose was to extend the rt-PCR platform, no changes were made to the DNA extraction section of LIB 4486. Any changes to the rt-PCR cycling conditions and master mix recipes for method optimization were kept to a minimum on the AB7500F platform. In addition, a set of published internal amplification control (IAC) (13) was incorporated into the AB7500F based simplex rt-PCR assay. This IAC targets the green fluorescence protein (GFP) gene, which was first discovered in the jellyfish *Aequorea Victoria* and has been widely used as a PCR IAC to detect microbial pathogens (13, 14). Due to its unique sequence and function, GFP also serves as a transgenic marker in plants (15) and vertebrates (16).

To evaluate the AB7500F platform, a Single Laboratory Validation (SLV) study was designed following the FDA *Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products* (17). The design was approved by the FDA CRCG and the FDA MITAG. This SLV was performed and completed at ORA/ORS/Pacific Northwest Laboratory. Following the completion, the results were reviewed by CRCG and MITAG, and the platform extension

study was deemed successful. In this LIB, the approved SLV study is summarized and the results are reported.

Experimental

DNA extraction and SmartCycler-based rt-PCR were performed following the procedures in LIB 4486 (4).

Equipment, supplies and reagents for the rt-PCR on the AB7500F platform

Please refer to the details in a separate LIB on the rt-PCR protocol (18).

The simplex rt-PCR on the AB7500F platform

As this is a simplex rt-PCR method, each set of master mix (bovine, caprine, ovine and IAC) was prepared separately. Briefly, the final concentration was 400 nM for all primers. The volume of each rt-PCR reaction was 25 µl, and 2 µl PCR template or control was used in a reaction (Table 1). The rt-PCR cycling conditions on the AB7500F platform are shown in Table 2. The acceptable melting temperature (Tm) ranges of target species are listed in Table 3. For detailed instruction of master mix preparation, template addition and setup on the AB7500F instrument, please refer to a separate LIB published by our group (18).

Table 1. The rt-PCR reactions on the AB7500F platform. NA = not applicable.

rt-PCR components	Stock concentration	Final concentration	Volume (µl)
iQ SYBR® Green SuperMix with ROX	2 ×	1 ×	12.5
Primer set for each species (forward and reverse)	5 µM	400 nM	2.0
Template or PCR controls	NA	NA	2.0
Molecular biology grade water (MGW)	NA	NA	8.5
Total volume	NA	NA	**25.0**

Table 2. The rt-PCR cycling conditions on the AB7500F platform.

Stage	Repetitions	Temperature	Time (min:sec)	Ramp Rate
1	1	94.0 °C	2:00	Auto
2	50	94.0 °C	0:10	Auto
		58.9 °C	0:15	Auto
		72.0 °C	0:40	Auto
3 (the **default** dissociation program)	1	95.0 °C	0:15	Auto
		60.0 °C	1:00	Auto
		95.0 °C	0:15	Auto
		60.0 °C	0:15	Auto
Mode: 7500 Fast **Data Collection:** Stage 2 Step 3 (72.0°C 40sec)				

Table 3. The acceptable Tm ranges of the simplex rt-PCR on the AB7500F platform.

Target	Acceptable Tm range (°C)
Bovine	81.8-83.3
Caprine	82.6-84.5
Ovine	78.5-80.3
GFP (IAC)	86.4-88.5

The templates tested in the SLV study

The reference materials used in this study included a bovine meat and bone meal (BMBM), a caprine meat meal (CMM), an ovine meat and bone meal (OMBM), and caprine gDNA. The BMBM, CMM, and OMBM were provided by Dr. Michael Myers from the FDA's Center for Veterinary Medicine (CVM). The caprine gDNA was purchased from Zyagen (San Diego, CA). DNA was extracted from the BMBM, CMM, and OMBM using the extraction method in LIB 4486, then diluted with MGW to three levels designated "high" (1.0% for all three species), "intermediate" (0.1% for all three species) and "fractional" (0.0001% for BMBM and OMBM, and 0.01% for CMM) to run the approved experiments. Thirty replicates of DNA at each level were tested on both platforms. Ten of the thirty replicates at each level were also tested to compare the AB software versions v1.4 and v2.3 (Table 4).

Table 4. DNA extracted from the BMBM, CMM and OMBM reference materials.

Reference material (species)	Levels	Replicates for platform comparison	Replicates for AB software comparison
BMBM (bovine)	High (1.0%)	30	10
	Intermediate (0.1%)	30	10
	Fractional (0.0001%)	30	10
CMM (caprine)	High (1.0%)	30	10
	Intermediate (0.1%)	30	10
	Fractional (0.01%)	30	10
OMBM (ovine)	High (1.0%)	30	10
	Intermediate (0.1%)	30	10
	Fractional (0.0001%)	30	10

As the CMM reference was not available at the beginning of the study, initially three different feed matrices were spiked with reference caprine gDNA at three levels per the approved study design. After spiking, DNA was extracted from each matrix and tested with rt-PCR (Table 5).

Table 5. Caprine gDNA spiked feed matrices.

Matrix #	Matrix texture	Spike levels (quantity of the reference caprine gDNA per 0.25 g matrix)	Replicates for platform comparison	Replicates for AB software comparison
808	Very fine, almost homogenous	Matrix control (0 µg)	3	3
		High (0.1 µg)	3	3
		Intermediate (0.01 µg)	3	3
		Fractional (0.001 µg)	8	8
824	Fine, relatively homogenous	Matrix control (0 µg)	3	3
		High (1.0 µg)	3	3
		Intermediate (0.25 µg)	3	3
		Fractional (0.05 µg)	8	8
2565	Coarse, heterogeneous	Matrix control (0 µg)	3	3
		High (0.1 µg)	3	3
		Intermediate (0.01 µg)	3	3
		Fractional (0.0001 µg)	8	8

Statistical evaluation

Median, mean, and standard deviation values were calculated using Microsoft Excel 2010. The false positive and false negative rates were calculated following the FDA *Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products* (17). The correct ID rates at the fractional levels were calculated with the true positive numbers divided by the total numbers at the respective levels. The McNemar's test (19) was used to determine if there was a significant difference between the results from the reference platform (SmartCycler) and the new platform (AB7500F). It was also applied to compare the two AB software versions.

Results and discussion

During the method development stage, rt-PCR efficiencies were determined to be similar between the two platforms with the number of reference gDNA templates tested in the study: 93.2%, 100.3%, and 94.9% on the AB7500F platform for bovine, caprine, and ovine identification, respectively; and 93.8%, 102.4%, and 93.4% on the SmartCycler platform, respectively. The linear coefficient (R^2) values of the standard curves were all greater than 0.99 on both platforms with the gDNA templates spanning from 2.0 to 2.0×10^4 pg per reaction.

In the SLV study, a total of 321 DNA templates were tested for platform comparison, including 90 using the BMBM, 90 using the CMM, 90 using the OMBM and 51 using the caprine gDNA spiked feed matrices. In addition, 141 of these templates were tested to compare the AB7500F software versions v1.4 and v2.3 (Tables 4 and 5). Results of all SLV rt-PCR experiments are attached in Supplementary Information 1.

As summarized in Table 6, with the number of DNA templates tested in the SLV, rt-PCR false positive rates were slightly reduced on the new platform (2.9%, 0%, and 3.6% on the AB7500F with software v1.4 versus 6.5%, 0.8%, and 8.0% on the SmartCycler for bovine, caprine, and ovine identification, respectively). The false negative rates for bovine and caprine identification were also decreased on the new platform (0% for all targets on the AB7500F versus 1.7%, 1.3%, and 0% on the SmartCycler for bovine, caprine, and ovine identification, respectively). At the fractional levels, the simplex rt-PCR method was able to identify target DNA on both platforms (Table 6).

The performance of the two AB7500F software versions, v1.4 and v2.3, were also compared using 141 of the 321 templates. As shown in Table 6 (the last two columns on the right), the false positive rates for bovine, caprine, and ovine identification were 3.4%, 0%, and 0% with AB7500F software v1.4, respectively; and 3.4%, 0%, and 6.9% with software v2.3, respectively. With the number of DNA templates tested in this study, there were no false negative results for any of the target species using either software version. At the fractional levels, the target DNA was correctly identified using both AB7500F software versions (Table 6).

Table 6. The false positive and false negative rates using templates at the high and intermediate levels, and the correct ID rates using templates at the fractional levels.

Species identification	Result summary	rt-PCR instrument platforms		AB software versions	
		SmartCycler	AB7500F v1.4	AB7500F v1.4	AB7500F v2.3
Bovine	false positive rate	6.5% (9 out of 138 replicates)	2.9% (4 out of 138 replicates)	3.4% (2 out of 58 replicates)	3.4% (2 out of 58 replicates)
	false negative rate	1.7% (1 out of 60 replicates)	0% (0 out of 60 replicates)	0% (0 out of 20 replicates)	0% (0 out of 20 replicates)
	correct ID rate at the fractional level	6.7% (2 out of 30 replicates)	16.7% (5 out of 30 replicates)	10.0% (1 out of 10 replicates)	30.0% (3 out of 10 replicates)[$]
Caprine	false positive rate	0.8% (1 out of 120 replicates)	0% (0 out of 120 replicates)	0% (0 out of 40 replicates)	0% (0 out of 40 replicates)
	false negative rate	1.3% (1 out of 78 replicates)	0% (0 out of 78 replicates)	0% (0 out of 38 replicates)	0% (0 out of 38 replicates)
	correct ID rate at the fractional level	42.6% (23 out of 54 replicates)	61.1% (33 out of 54 replicates)	73.5% (25 out of 34 replicates)	64.7% (22 out of 34 replicates)
Ovine	false positive rate	8.0% (11 out of 138 replicates)	3.6% (5 out of 138 replicates)	0% (0 out of 58 replicates)	6.9% (4 out of 58 replicates)
	false negative rate	0% (0 out of 60 replicates)	0% (0 out of 60 replicates)	0% (0 out of 20 replicates)	0% (0 out of 20 replicates)
	correct ID rate at the fractional level	80.0% (24 out of 30 replicates)	76.7% (23 out of 30 replicates)	50.0% (5 out of 10 replicates)	70.0% (7 out of 10 replicates)

[$] One of the positive results was reported as "positive but under the limit of detection (LOD)" per LIB 4486 (4).

As in Table 7, the results of the McNemar's test showed that there was no significant difference between rt-PCR results obtained from the SmartCycler platform and the AB7500F platform (with software v1.4) using the cutoff values of 0.05 and 3.84 for the p and chi-square, respectively, with 95% confidence level. Calculated the same way, there was either no significant difference between results obtained with the two AB7500F software versions.

Table 7. Summary of the McNemar's test on rt-PCR results obtained between the two platforms and two AB software versions. P = two-tailed p-values. Chi^2 = chi-square values with 1 degree of freedom. NA = not applicable. For $p \geq 0.05$, $chi^2 \leq 3.84$ with 95% confidence level.

Summary of the McNemar's test	Platform comparison SmartCycler vs. AB7500F v1.4			AB software comparison AB7500F software v1.4 vs. v2.3		
	Bovine	Caprine	Ovine	Bovine	Caprine	Ovine
Sample size (n)	291	267	291	131	107	131
P	0.08	0.48	0.18	0.48	NA, identical rt-PCR results	0.13
Chi^2	3.12	0.50	1.79	0.50	NA, identical rt-PCR results	2.25

In SYBR Green based rt-PCR assays, the melting temperature (Tm) is a critical parameter for reporting results. Statistical analyses of the true positive Tm values in the SLV are summarized in Table 8. The standard deviations (SD) of Tm values obtained on the AB7500F platform were ≤ 0.3 °C for all target species, indicating that the Tm results were consistent across three different template concentrations in this study.

Table 8. Statistical analyses of the Tm values. Tm unit = °C. NA = not applicable.

Target species	Tm statistics	SmartCycler (°C)	AB7500F v1.4 (°C)	AB7500F v2.3 (°C)
Bovine	Number of true positives (n)	61	65	23
	Minimum Tm	83.2	82.0	82.0
	Maximum Tm	83.6	83.1	82.7
	Median of Tm	83.4	82.7	82.3
	Mean of Tm	83.4	82.7	82.3
	Standard deviation of Tm	0.1	0.2	0.2
Caprine	Number of true positives (n)	100	111	60
	Minimum Tm	83.3	82.8	82.7
	Maximum Tm	84.6	84.2	83.6
	Median of Tm	84.3	83.6	83.3
	Mean of Tm	84.3	83.6	83.3
	Standard deviation of Tm	0.1	0.3	0.2
Ovine	Number of true positives (n)	84	83	27
	Minimum Tm	79.7	78.7	78.6
	Maximum Tm	80.6	80.1	79.3
	Median of Tm	80.0	79.3	79.1
	Mean of Tm	80.0	79.4	79.0
	Standard deviation of Tm	0.2	0.3	0.2
GFP IAC	Number of templates (n)	NA	321	141
	Minimum Tm	NA	87.0	86.9
	Maximum Tm	NA	88.2	87.6
	Median of Tm	NA	87.6	87.4
	Mean of Tm	NA	87.6	87.3
	Standard deviation of Tm	NA	0.3	0.1

The Tm ranges of target species were initially determined based on experimental true positive values during the method development. The initial ranges were approved in the SLV study design. Following the completion of this SLV, acceptable Tm ranges were re-calculated by combining the experimental values and the [mean ± 3SD] of Tm values in the SLV (Table 8). For example, during the method development, the minimum and maximum experimental bovine Tm values were 81.8 and 83.1 °C, respectively. After the SLV study, the mean and SD of bovine Tm on the AB7500F with software v1.4 were 82.7 and 0.2 °C, respectively (Table 8), resulting in a [mean ± 3SD] range of 82.1-83.3 °C. Combining the experimental range (81.8 – 83.1 °C) and the [mean ± 3SD] range (82.1-83.3 °C), the final bovine Tm range was recalculated to be 81.8-83.3 °C. Similarly, the caprine Tm range was recalculated to be 82.6 - 84.5 °C (82.8-84.4 °C previously); the ovine Tm range was recalculated to be 78.5-80.3 °C (78.5-80.1 °C previously); and the GFP Tm range was recalculated to be 86.4 - 88.5 °C (86.4-88.4 °C previously). The updated Tm ranges are summarized in Table 3. These adjustments did not affect the study results or the conclusions made regarding platform extension and software version comparison.

In this study, PCR inhibition was detected in 1 out of 54 DNA extractions. The DNA was extracted from matrix 824 spiked with 0.05 µg caprine gDNA (Table 9A). The matrix spiking, DNA extraction, and rt-PCR were repeated to obtain valid results with acceptable IAC (Table 9B). The difference in rt-PCR results may be due to variation in the feed ingredients, or spike of that particular feed matrix, and/or variation in DNA extraction.

Table 9A. PCR inhibition indicated by the IAC in 1 out of 54 DNA extractions tested in the study. Spp.=species. Ctl=PCR control. Neg=negative. Pos=positive.

Experiment		AB7500F with software v1.4, operator 2				AB7500F with software v2.3, operator 1			
Template ID	Primer spp.	Ct (cycles)	Tm (°C)	rt-PCR result	Ctl correct? Y/N	Ct (cycles)	Tm (°C)	rt-PCR result	Ctl correct? Y/N
824 0.05µg Caprine gDNA 1#	Bovine	**Undetermined**	**65.4**	**neg**	**N (IAC neg indicating PCR inhibition)**	**Undetermined**	**61.4**	**neg**	**N (IAC neg indicating PCR inhibition)**
824 0.05µg Caprine gDNA 1#	Caprine	**Undetermined**	**65.4**	**neg**		**Undetermined**	**62.1**	**neg**	
824 0.05µg Caprine gDNA 1#	Ovine	**Undetermined**	**83.9**	**neg**		**Undetermined**	**62.8**	**neg**	
824 0.05µg Caprine gDNA 1#	IAC	**Undetermined**	**67.4**	**neg**		**Undetermined**	**63.8**	**neg**	
Neg ctl	Bovine	36.2	74.5	neg	Y (neg ctls were correct)	Undetermined	63.0	neg	Y (neg ctls were correct)
Neg ctl	Caprine	32.8	79.8	neg		36.4	78.9	neg	
Neg ctl	Ovine	Undetermined	76.2	neg		Undetermined	60.9	neg	
Neg ctl	IAC	47.6	77.2	neg		Undetermined	62.7	neg	
B pos ctl	Bovine	23.7	82.3	pos	Y (pos ctls were correct)	25.8	82.3	pos	Y (pos ctls were correct)
C pos ctl	Caprine	23.7	83.9	pos		26.4	83.6	pos	
O pos ctl	Ovine	24.9	79.1	pos		26.9	78.9	pos	
IAC pos ctl	IAC	25.7	87.2	pos		24.4	87.2	pos	

Table 9B. Acceptable results of the repeated experiment. Spp.=species. Ctl=PCR control. Neg=negative. Pos=positive.

Experiment		AB7500F with v1.4 software, operator 1				AB7500F with v2.3 software, operator 1			
Template ID	**Primer spp.**	**Ct (cycles)**	**Tm (°C)**	**rt-PCR Result**	**Ctl correct? Y/N**	**Ct (cycles)**	**Tm (°C)**	**rt-PCR Result**	**Ctl correct? Y/N**
824 0.05µg Caprine gDNA repeat	Bovine	Undetermined	65.3	neg	Y (IAC correct, caprine pos)	Undetermined	63.0	neg	Y (IAC correct, caprine pos)
824 0.05µg Caprine gDNA repeat	Caprine	32.3	83.7	pos		34.5	83.1	pos	
824 0.05µg Caprine gDNA repeat	Ovine	Undetermined	89.3	neg		Undetermined	63.2	neg	
824 0.05µg Caprine gDNA repeat	IAC	21.9	87.8	pos		23.3	87.4	pos	
Neg ctl	Bovine	Undetermined	66.0	neg	Y (neg ctls were correct)	Undetermined	62.9	neg	Y (neg ctls were correct)
Neg ctl	Caprine	33.7	79.5	neg		35.3	78.5	neg	
Neg ctl	Ovine	Undetermined	88.4	neg		Undetermined	64.4	neg	
Neg ctl	IAC	42.5	75.3	neg		Undetermined	67.7	neg	
B pos ctl	Bovine	22.1	82.4	pos	Y (pos ctls were correct)	24.9	82.1	pos	Y (pos ctls were correct)
C pos ctl	Caprine	23.9	84.0	pos		25.8	83.5	pos	
O pos ctl	Ovine	25.1	79.2	pos		26.9	78.9	pos	
IAC pos ctl	IAC	21.3	87.1	pos		23.9	87.1	pos	

Per the approved study design, each sample melt curve was visually inspected when the sample Tm fell within the acceptable range for the respective species. If the melt curve was not acceptable, e.g., exhibiting a shoulder or multiple peaks at similar height, the rt-PCR was repeated using 2 µl of the same template to obtain additional data to draw a conclusion. Please see a separate LIB on the AB7500F rt-PCR protocol by our group (18) for detailed instructions. This procedure reduced the false positive rates from 6.5% to 2.9% for bovine identification, and from 5.1% to 3.6% for ovine identification using the AB7500F rt-PCR with software v1.4 in this study. Using the AB7500F rt-PCR with software v2.3, the false positive rates were also slightly reduced from 5.2% to 3.4% for bovine identification, and from 8.6% to 6.9% for ovine identification. The reduced false positive rates are summarized in Table 6. On the other hand, this procedure slightly decreased the correct identification rates of caprine templates at the fractional levels, from 63.0% to 61.1% on the AB7500F with software v1.4 (34 versus 33 true positives out of 54 templates tested), and from 67.6% to 64.7% on the AB7500F with software v2.3 (23 versus 22 true positives out of 34 templates tested). Melt curve inspection was not performed to the positive results obtained on the SmartCycler platform, as it was not in LIB 4486. The false positive rates on the SmartCycler platform would likely be reduced if the same procedure had been done.

Conclusions

This LIB summarizes results of a SLV study completed at Pacific Northwest Laboratory on the platform extension of the simplex rt-PCR method in LIB 4486 to detect prohibited materials in animal feed. The results include the false positive and false negative rates using templates at the high and intermediate levels, as well as the correct identification rates using templates at the fractional levels for each target species. The acceptable Tm ranges of all target species were determined for the rt-PCR method on the AB7500F platform. Comparison was performed between the reference platform (SmartCycler) versus the new platform (AB7500F), and also between the two AB7500F software versions (v1.4 and v2.3). The simplex rt-PCR method performed on the AB7500F platform was able to identify target DNA from bovine, caprine, and ovine species at three different levels. The rt-PCR performance on the AB7500F platform was at least equal to that on the SmartCycler platform, with some of the false positive and false negative rates slightly reduced on the AB7500F with the number of DNA templates tested here. Both the AB7500F software v1.4 and v2.3 worked for the simplex rt-PCR method in the SLV study.

After technical review, this study has been deemed successful by the FDA CRCG and MITAG. The platform of the simplex rt-PCR method in LIB 4486 has been approved to be extended to the AB7500F system.

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Supplementary Information 1. All rt-PCR results obtained in the SLV study.

BMBM, bovine meat and bone meal. CMM, caprine meat meal. OMBM, ovine meat and bone meal.

Pos, positive rt-PCR result. Neg, negative rt-PCR result. Fal pos, false positive. Fal neg, false negative. NA, not available.

False positive and false negative results are highlighted in blue and purple, respectively. Positive results at the fractional levels are highlighted in yellow boxes.

Results obtained with dilutions of DNA extracted from BMBM, CMM, and OMBM									
Platforms	**SmartCycler**			**AB7500F v1.4**			**AB7500F v2.3**		
Templates ID	**B primers**	**C primers**	**O primers**	**B primers**	**C primers**	**O primers**	**B primers**	**C primers**	**O primers**
BMBM 1% 1	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 1% 2	pos	neg	neg	pos	neg	neg	pos	neg	**pos (fal pos)***
BMBM 1% 3	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 1% 4	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 1% 5	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 1% 6	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 1% 7	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 1% 8	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 1% 9	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 1% 10	pos	neg	**pos (fal pos)***	pos	neg	neg****	pos	neg	neg
BMBM 1% 11	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 12	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 13	pos	neg	**pos (fal pos)***	pos	neg	neg	NA	NA	NA
BMBM 1% 14	pos	neg	neg	pos	neg	neg***	NA	NA	NA
BMBM 1% 15	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 16	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 17	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 18	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 19	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 20	pos	**pos (fal pos)****	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 21	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 22	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 23	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 24	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 25	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 26	pos	neg	neg	pos	neg	**pos (fal pos)***	NA	NA	NA
BMBM 1% 27	pos	neg	neg	pos&	neg	neg	NA	NA	NA
BMBM 1% 28	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 29	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 1% 30	pos	neg	neg	pos	neg	**pos (fal pos)***	NA	NA	NA
BMBM 0.1% 1	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 0.1% 2	pos	neg	**pos (fal pos)***	pos	neg	neg	pos	neg	neg
BMBM 0.1% 3	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 0.1% 4	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 0.1% 5	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 0.1% 6	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 0.1% 7	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 0.1% 8	pos	neg	neg	pos	neg	neg	pos	neg	neg
BMBM 0.1% 9	pos	neg	**pos (fal pos)***	pos	neg	neg	pos	neg	neg
BMBM 0.1% 10	pos	neg	neg	pos	neg	neg****	pos	neg	neg
BMBM 0.1% 11	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 12	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 13	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 14	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 15	pos	neg	**pos (fal pos)***	pos	neg	neg	NA	NA	NA
BMBM 0.1% 16	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 17	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 18	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 19	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 20	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 21	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 22	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 23	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 24	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 25	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 26	pos	neg	neg	pos	neg	neg****	NA	NA	NA
BMBM 0.1% 27	pos	neg	neg	pos	neg	neg***	NA	NA	NA
BMBM 0.1% 28	pos	neg	neg	pos	neg	neg	NA	NA	NA

Platforms	SmartCycler			AB7500F v1.4			AB7500F v2.3		
Templates ID	B primers	C primers	O primers	B primers	C primers	O primers	B primers	C primers	O primers
BMBM 0.1% 29	neg (fal neg)	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.1% 30	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.0001% 1	neg	neg	neg	neg	neg	neg	pos (under LOD)[$]	neg	neg
BMBM 0.0001% 2	neg	neg	neg	pos	neg	neg	neg	neg	neg
BMBM 0.0001% 3	neg	neg	neg	neg	neg	neg	neg	neg	neg
BMBM 0.0001% 4	neg	neg	neg	neg	neg	neg	pos	neg	neg
BMBM 0.0001% 5	neg	neg	neg	neg	neg	neg	neg	neg	neg
BMBM 0.0001% 6	neg	neg	neg	neg	neg	neg	pos	neg	neg
BMBM 0.0001% 7	neg	neg	neg	neg	neg	neg	neg	neg	neg
BMBM 0.0001% 8	neg	neg	neg	neg[****]	neg	neg	neg	neg	neg
BMBM 0.0001% 9	neg	neg	neg	neg	neg	neg[****]	neg	neg	neg
BMBM 0.0001% 10	neg	neg	neg	neg	neg	neg	neg	neg	neg
BMBM 0.0001% 11	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 12	neg	neg	neg	neg	neg	neg[****]	NA	NA	NA
BMBM 0.0001% 13	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 14	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 15	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 16	neg	neg	neg	neg[****]	neg	neg[****]	NA	NA	NA
BMBM 0.0001% 17	pos	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.0001% 18	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 19	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 20	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 21	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 22	pos	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 23	neg	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.0001% 24	neg	neg	neg	neg	neg	neg[****]	NA	NA	NA
BMBM 0.0001% 25	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 26	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 27	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 28	neg	neg	neg	pos	neg	neg	NA	NA	NA
BMBM 0.0001% 29	neg	neg	neg	neg	neg	neg	NA	NA	NA
BMBM 0.0001% 30	neg	neg	neg	pos	neg	neg	NA	NA	NA
CMM 1% 1	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 1% 2	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 1% 3	neg	pos	neg	pos (fal pos)[*]	pos	neg	neg	pos	neg
CMM 1% 4	pos (fal pos)[*]	pos	pos (fal pos)[**]	neg	pos	neg	neg	pos	neg
CMM 1% 5	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 1% 6	neg	pos	pos (fal pos)[*]	neg	pos	neg	neg	pos	neg
CMM 1% 7	neg	pos	neg	neg	pos	neg	pos (fal pos)[*]	pos	pos (fal pos)[**]
CMM 1% 8	neg	pos	pos (fal pos)[**]	neg	pos	neg	neg	pos	pos (fal pos)[*]
CMM 1% 9	neg	pos	neg	neg	pos	neg	neg	pos	neg[***]
CMM 1% 10	neg	pos	neg	neg[****]	pos	neg	neg	pos	neg
CMM 1% 11	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 12	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 13	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 14	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 15	neg	pos	pos (fal pos)[**]	neg	pos	neg	NA	NA	NA
CMM 1% 16	neg	pos	pos (fal pos)[**]	neg	pos	neg	NA	NA	NA
CMM 1% 17	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 18	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 19	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 20	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 21	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 22	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 23	neg	pos	neg	neg	pos	pos (fal pos)[*]	NA	NA	NA
CMM 1% 24	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 25	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 26	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 27	neg	pos	pos (fal pos)[*]	neg	pos	neg	NA	NA	NA
CMM 1% 28	neg	pos	neg	neg	pos	pos (fal pos)[**]	NA	NA	NA
CMM 1% 29	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 1% 30	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 1	neg	pos	neg	neg	pos	neg	neg	pos	neg

Platforms	SmartCycler			AB7500F v1.4			AB7500F v2.3		
Templates ID	B primers	C primers	O primers	B primers	C primers	O primers	B primers	C primers	O primers
CMM 0.1% 2	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 3	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 4	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 5	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 6	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 7	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 8	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 9	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 10	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.1% 11	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 12	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 13	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 14	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 15	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 16	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 17	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 18	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 19	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 20	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 21	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 22	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 23	neg	**neg (fal neg)**	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 24	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 25	neg	pos	neg	neg	pos	**pos (fal pos)****	NA	NA	NA
CMM 0.1% 26	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 27	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 28	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 29	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.1% 30	neg	pos	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 1	neg	neg	neg	neg	neg	neg	neg	pos	neg
CMM 0.01% 2	neg	neg	neg	neg	neg	neg	neg	neg	neg
CMM 0.01% 3	neg	neg	neg	neg	neg	neg	neg	pos	neg
CMM 0.01% 4	neg	neg	neg	neg	pos	neg	neg	neg	neg
CMM 0.01% 5	neg	pos	neg	neg	neg	neg	neg	neg	neg
CMM 0.01% 6	neg	neg	neg	neg	pos	neg	neg	neg	neg
CMM 0.01% 7	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.01% 8	neg	pos	neg	neg	pos	neg	neg	pos	neg
CMM 0.01% 9	neg	neg	neg	neg	neg	neg	neg	neg	neg
CMM 0.01% 10	neg	neg	neg	neg	pos	neg	neg	neg	neg
CMM 0.01% 11	neg	neg	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 12	neg	neg	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 13	neg	neg	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 14	neg	neg	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 15	neg	neg	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 16	neg	neg	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 17	neg	neg	neg	neg	neg***	neg	NA	NA	NA
CMM 0.01% 18	neg	neg	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 19	neg	neg	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 20	neg	neg	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 21	neg	neg	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 22	neg	pos	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 23	neg	pos	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 24	neg	neg	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 25	neg	neg	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 26	neg	neg	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 27	neg	neg	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 28	neg	neg	neg	neg	neg	neg	NA	NA	NA
CMM 0.01% 29	neg	neg	neg	neg	pos	neg	NA	NA	NA
CMM 0.01% 30	neg	neg	neg	neg	neg	neg	NA	NA	NA
OMBM 1% 1	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 1% 2	**pos (fal pos)***	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 1% 3	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 1% 4	neg	neg	pos	neg	neg	pos	neg	neg	pos

Platforms	SmartCycler			AB7500F v1.4			AB7500F v2.3		
Templates ID	B primers	C primers	O primers	B primers	C primers	O primers	B primers	C primers	O primers
OMBM 1% 5	neg	neg	pos	neg***	neg	pos	neg***	neg	pos
OMBM 1% 6	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 1% 7	neg	neg	pos	neg***	neg	pos	neg	neg	pos
OMBM 1% 8	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 1% 9	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 1% 10	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 1% 11	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 12	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 13	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 14	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 15	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 16	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 17	pos (fal pos)*	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 18	pos (fal pos)*	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 19	pos (fal pos)*	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 20	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 21	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 22	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 23	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 24	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 25	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 26	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 27	neg	neg	pos	neg***	neg	pos	NA	NA	NA
OMBM 1% 28	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 29	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 1% 30	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 1	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 2	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 3	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 4	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 5	neg	neg	pos	pos (fal pos)*	neg	pos	neg	neg	pos
OMBM 0.1% 6	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 7	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 8	pos (fal pos)*	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 9	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 10	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.1% 11	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 12	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 13	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 14	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 15	neg	neg	pos	pos (fal pos)*	neg	pos	NA	NA	NA
OMBM 0.1% 16	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 17	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 18	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 19	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 20	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 21	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 22	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 23	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 24	pos (fal pos)*	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 25	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 26	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 27	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 28	neg	neg	pos	pos (fal pos)*	neg	pos	NA	NA	NA
OMBM 0.1% 29	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.1% 30	neg	neg	pos	neg***	neg	pos	NA	NA	NA
OMBM 0.0001% 1	neg	neg	pos	neg	neg	neg	neg	neg	pos
OMBM 0.0001% 2	neg	neg	pos	neg	neg	neg	neg	neg	neg
OMBM 0.0001% 3	neg	neg	pos	neg	neg	neg	neg	neg	pos
OMBM 0.0001% 4	neg	neg	pos	neg	neg	neg	neg	neg	neg
OMBM 0.0001% 5	neg	neg	neg	neg	neg	pos	neg	neg	pos
OMBM 0.0001% 6	neg	neg	neg	neg	neg	pos	neg	neg	neg
OMBM 0.0001% 7	neg	neg	pos	neg	neg	pos	neg	neg	pos

Platforms	SmartCycler			AB7500F v1.4			AB7500F v2.3		
Templates ID	B primers	C primers	O primers	B primers	C primers	O primers	B primers	C primers	O primers
OMBM 0.0001% 8	neg	neg	pos	neg	neg	neg	neg	neg	pos
OMBM 0.0001% 9	neg	neg	pos	neg	neg	pos	neg	neg	pos
OMBM 0.0001% 10	neg	neg	neg	neg	neg	pos	neg	neg	pos
OMBM 0.0001% 11	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 12	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 13	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 14	neg	neg	pos	neg	neg	neg	NA	NA	NA
OMBM 0.0001% 15	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 16	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 17	neg	neg	neg	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 18	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 19	neg	neg	neg	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 20	neg	neg	pos	neg	neg	neg	NA	NA	NA
OMBM 0.0001% 21	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 22	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 23	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 24	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 25	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 26	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 27	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 28	neg	neg	neg	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 29	neg	neg	pos	neg	neg	pos	NA	NA	NA
OMBM 0.0001% 30	neg	neg	pos	neg	neg	pos	NA	NA	NA

Results obtained with caprine gDNA spiked feed matrices (FACTS No. partially masked)									
Platforms	SmartCycler			AB7500F v1.4			AB7500F v2.3		
Templates ID	B primers	C primers	O primers	B primers	C primers	O primers	B primers	C primers	O primers
808 non-spike 1	neg	neg	neg	neg	neg	neg	neg	neg	neg
808 non-spike 2	neg	neg	neg	neg	neg	neg	neg	neg	neg
808 non-spike 3	neg	neg	neg	neg	neg	neg	neg	neg	neg
808 0.1µg 1	neg	pos	neg	neg	pos	neg	neg	pos	neg
808 0.1µg 2	pos (fal pos)*	pos	neg	neg	pos	neg	pos (fal pos)*	pos	neg
808 0.1µg 3	neg	pos	neg	neg	pos	neg	neg	pos	neg
808 0.01µg 1	neg	pos	neg	neg	pos	neg	neg	pos	neg
808 0.01µg 2	neg	pos	neg	neg	pos	neg	neg	pos	neg
808 0.01µg 3	neg	pos	neg	neg	pos	neg	neg	pos	neg
808 0.001µg 1	neg	neg	neg	neg	pos	neg	neg	pos	neg
808 0.001µg 2	neg	neg	neg	neg	pos	neg	neg	pos	neg
808 0.001µg 3	neg	pos	neg	neg	neg	neg	neg	pos	neg
808 0.001µg 4	neg	neg	neg	neg	pos	neg	neg	pos	neg
808 0.001µg 5	neg	pos	neg	neg	pos	neg	neg	pos	neg
808 0.001µg 6	neg	neg	neg	neg	pos	neg	neg	neg	neg
808 0.001µg 7	neg	pos	neg	neg	neg	neg	neg	neg	neg
808 0.001µg 8	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 non-spike 1	neg	neg	neg	neg	neg	neg	neg	neg	neg
824 non-spike 2	neg	neg	neg	neg	neg	neg	neg	neg	neg
824 non-spike 3	neg	neg	neg	neg	neg	neg	neg	neg	neg
824 1.0µg 1	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 1.0µg 2	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 1.0µg 3	neg	pos	neg	neg	pos	neg	neg	pos	pos (fal pos)*
824 0.25µg 1	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 0.25µg 2	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 0.25µg 3	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 0.05µg 1#	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 0.05µg 2	neg	neg	neg	neg	pos	neg	neg	pos	neg
824 0.05µg 3	neg	pos	neg	neg	pos	neg****	neg	pos	neg
824 0.05µg 4	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 0.05µg 5	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 0.05µg 6	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 0.05µg 7	neg	pos	neg	neg	pos	neg	neg	pos	neg
824 0.05µg 8	neg	pos	neg	neg	pos	neg	neg	pos	neg
2565 non-spike 1	neg	neg	neg	neg	neg	neg	neg	neg	neg

Platforms	SmartCycler			AB7500F v1.4			AB7500F v2.3		
Templates ID	B primers	C primers	O primers	B primers	C primers	O primers	B primers	C primers	O primers
2565 non-spike 2	neg	neg	neg	neg	neg	neg	neg	neg	neg
2565 non-spike 3	neg	neg	neg	neg	neg	neg	neg	neg	neg
2565 0.1µg 1	neg	pos	neg	neg	pos	neg	neg	pos	neg
2565 0.1µg 2	pos (fal pos)*	pos	neg	neg	pos	neg	neg	pos	neg
2565 0.1µg 3	neg	pos	neg	neg	pos	neg	neg	pos	neg
2565 0.01µg 1	neg	pos	neg	neg	pos	neg	neg	pos	neg
2565 0.01µg 2	neg	pos	neg	neg	pos	neg	neg	pos	neg
2565 0.01µg 3	neg	pos	neg	neg	pos	neg	neg	pos	neg
2565 0.0001µg 1	neg	pos	neg	neg***	pos	neg	neg	pos	neg
2565 0.0001µg 2	neg	pos	neg	neg	pos	neg	neg	neg	neg
2565 0.0001µg 3	neg	pos	neg	neg	pos	neg	neg	neg	neg
2565 0.0001µg 4	neg	pos	neg	neg	pos	neg	neg	neg***	neg
2565 0.0001µg 5	neg	pos	neg	neg	pos	neg	neg	pos	neg
2565 0.0001µg 6	neg	neg	neg	neg	neg	neg	neg	pos	neg
2565 0.0001µg 7	neg	pos	neg	neg	pos	neg	neg	neg	neg
2565 0.0001µg 8	neg	pos	neg	neg	neg	neg	neg	pos	neg

* A false positive result with the mis-priming Ct≥10 cycles later than that of the true positive Ct. The melt curve was acceptable.

** A false positive result with the mis-priming Ct approximately 5 cycles later than that of the true positive Ct. The melt curve was acceptable.

*** The rt-PCR was repeated with 2 µl template, because although initially Ct>0 with an acceptable Tm, the melt curve was unacceptable, e.g., with a shoulder or multiple peaks. Because the repeated Tm fell outside of the acceptable Tm range, the result was determined negative regardless of the Ct and melt curve. The melt curve inspection and repeat procedure has been approved in the study design.

**** The rt-PCR was repeated with 4 µl template, because when 2 µl template was initially tested, the Tm was acceptable but Ct=0. The template was doubled in the repeated run. As the repeated Tm was unacceptable, the result was determined negative regardless of the Ct. This procedure is established in LIB 4486.

& The rt-PCR was repeated with 2 µl 1% BMBM DNA, as the Tm was just 0.1 °C outside the initial range but the amplification was strong (Ct=27.1) with an acceptable melt curve. The repeated result was positive because the Tm was acceptable, Ct>0 and the melt curve was acceptable.

$ Result of a BMBM template at the fractional level was positive but under the Limit of Detection (LOD), according to the established procedure in LIB 4486. The initial Tm was acceptable but Ct=0. After 4 µl template was repeated, the result was positive because Ct>0, Tm fell in the acceptable range, and the melt curve was acceptable.

For one template from 824 spiked with 0.05 µg caprine gDNA, the presence of PCR inhibition was indicated by the GFP IAC because GFP Ct=0. There was no target amplification as all Ct=0. Spiking, DNA extraction, and rt-PCR were repeated. Valid results with correct IAC were obtained from the repeated experiment.