Optimization of a Positive Control for the Feed Extraction and Real-time PCR Method Used in the Bovine Spongiform Encephalopathy Program

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Abstract

Genomic DNA (gDNA) positive controls were optimized for feed extraction and the downstream real-time Polymerase Chain Reaction (rt-PCR) method used in FDA's Bovine Spongiform Encephalopathy (BSE) program. A matrix interference study was conducted to compare three solution-based reference materials in the first stage of this study: whole blood, serum, and gDNA from the target species. After feed samples were fortified, they were subjected to DNA extraction and rt-PCR. The extract from gDNA fortified feed (40, 2, and 4 ng gDNA per 0.25 g feed for bovine, caprine, and ovine, respectively) yielded positive rt-PCR results for all target species, whereas neither whole blood nor sera did so. As a result, feed fortification using reference gDNA was further evaluated for robustness. The target DNA species from fortified samples were detected in 41 out of 43 unique feeds, indicating the high compatibility with both feed extraction and rt-PCR procedures. The two negative feed samples were fortified with bovine meat and bone meal (BMBM) at 0.1% (w/w) to further explore a possible matrix effect on target DNA detection, and the bovine target was detected by rt-PCR. Taken together, this study demonstrates that feed fortification with a combination of bovine, caprine and ovine reference gDNA can serve as a positive control for feed extraction used upstream of the validated BSE rt-PCR analysis. Additionally, utilizing gDNA solutions in lieu of BMBM powder can reduce potential cross-contamination. In rare cases, when the reference gDNA positive controls cannot be detected by rt-PCR, BMBM may be used with caution as an alternative positive control.

Key words: Bovine Spongiform Encephalopathy, feed extraction, positive control, rt-PCR.

Introduction

Bovine Spongiform Encephalopathy (BSE) belong to a family of progressive neurological diseases that most likely result from prion infections. Because the disease can spread by feeding ruminants with prion-infected feed components, including ruminant meat and bone meals (MBM), the FDA compliance program 7371.009 banned the use of ruminant MBM in animal feeds and feed ingredients used for food-producing animals (1). Although annual numbers of BSE infections have dropped sharply after the epidemic peak in 1990's, BSE still poses a threat to public health. A CDC surveillance identified 26 BSE cases in North America in 2018, including 6 in the United States and 20 in Canada (2).

A validated simplex rt-PCR method (3) on the Applied Biosystems 7500 Fast (AB7500F) platform is currently being used by the FDA for feed testing in the BSE program (4,5). Upstream from the rt-PCR portion, feed DNA is extracted using Invitrogen's ChargeSwitch Purification Kit as the rt-PCR template. Due to the heterogenous nature of feeds and wide variety of feed ingredients, extracted feed DNA may contain lipids, salts, or molasses that can significantly affect the downstream rt-PCR (6). Therefore, a positive control is needed during feed extraction to ensure adequate quality control.

Reference MBM powders were previously provided by the Center for Veterinary Medicine (CVM) with limited quantity and may be used as a positive control; however, they are extremely difficult to find commercially in the USA. Additionally, the risk of cross contamination introduced by powders is usually greater than liquids for sample fortification due to the migration of airborne contaminants (7). To develop a suitable positive control for the feed extraction step of BSE analysis, three potential references in liquid format from the target species were compared in the first stage of study, including whole blood, sera, and gDNA. The best candidate was selected based on rt-PCR results and used in the next stage where a variety of feed samples were spiked to assess the overall performance of this positive control.

Experimental

1. Equipment and supplies

Purified reference gDNA from bovine, caprine, and ovine (Zyagen catalog number GB-110F, GG-150, and GS-190F, respectively);

Reference whole blood from bovine, caprine, and ovine (Rockland Immunochemicals catalog number R300-0050, R304-0050, and R311-0050, respectively);

Reference Sera from bovine, caprine, and ovine (ThermoFisher Scientific catalog numbers 16030074, 16210064, and 16070096, respectively);

Whatman FTA classic cards with a loading volume of 125 µl/spot and spot diameter of 25 mm (Millipore Sigma catalog number WHAWB120205);

Harris 3 mm micro-punch tool (Millipore Sigma catalog number WHAWB100038);

Archived regulatory feeds and locally purchased animal feeds as matrices;

Other necessary laboratory supplies used for feed extraction described in LIB 4486 (3) and AB7500F rt-PCR method described in LIB 4657 (4).

2. Candidate reference materials

2.1 Whole blood

Reference whole blood from each target species was spotted on Whatman FTA classic cards at 125 µl/spot, air dried, and stored at room temperature until use. As the diameter of a spot is 25 mm, and that of a micro punch is 3 mm excised with a Harris tool, there are about 70 micro punches

in an FTA spot area. The average concentration of bovine DNA in blood is reported to be 60 µg/ml (8). So, there is estimated to be 7.5 µg gDNA in 125 µl blood per FTA spot, and approximately 100 ng gDNA per punch. One to three standardized blood punches were excised from the FTA cards and put in a reaction tube containing 0.25 ± 0.01 g feed, yielding spike levels of approximately 100-300 ng gDNA per sample.

2.2 Sera

Similar to 2.1, reference serum from each target species was spotted on Whatman FTA classic cards at 125 µl/spot. The average gDNA concentration is approximately 30 ng/ml in serum (9). After spotting, the FTA cards were air dried and stored at room temperature prior to excising with a Harris tool. One standardized punch per species was tested in the study, yielding an estimated gDNA level of 0.05 ng per punch from serum.

2.3 Genomic DNA (gDNA)

The stock concentration of commercial bovine, caprine, and ovine reference gDNA were 1.0 µg/µl, and they were diluted to various concentrations with molecular biology grade water prior to experiments. A total of 6 µl diluted gDNA (2 µl per species) were directly pipetted into a reaction tube containing 0.25 ± 0.01 g feed. In the initial evaluation comparing reference blood, sera, and gDNA, the gDNA concentrations were 10, 1, and 1 ng/µl for bovine, caprine, and ovine, respectively. After optimization, the concentrations were 20, 1 and 2 ng/µl, respectively, to provide the most consistent rt-PCR results for all target species in various feed samples.

2.4 Bovine meat and bone meal (BMBM)

BMBM was kindly provided by Dr. Myers at FDA Center for Veterinary Medicine (CVM). Fortified feed samples were prepared by mixing 0.01 g BMBM per 10 g feed in a properly sealed reaction tube to reach the final concentration of 0.1% (w/w).

3. Feed extraction

The detailed procedure using ChargeSwitch gDNA Rendered Meat Purification Kit is described in LIB 4486 (3).

4. SYBR Green based rt-PCR on the AB7500F system

The detailed experimental procedure is described LIB 4657 (4).

Results and discussion

Three fluid-based reference materials from the target species, including whole blood, sera, and gDNA, were evaluated in the first stage of this study. When reference sera on three standardized FTA punches (one punch per species) were run through feed extraction and rt-PCR, there was no target signal detected even without feed fortification. Therefore, no further testing was conducted using sera in the next evaluation stage. An archived feed xxxx625 was arbitrarily chosen for spike because of the availability. After extraction and rt-PCR using the fortified feed, the reference gDNA was successfully identified for all target species; whereas target DNA from bovine and ovine blood could not be detected even with three FTA punches (Table 1). As a result, the reference gDNA was determined to be the best control candidate for feed extraction and was further evaluated in the next stage of our study.

Table 1. Real-time PCR results of an archived feed xxxx625 spiked with the reference blood and gDNA.

Spiked reference material	Spike level	rt-PCR results for the target species
whole blood	3 FTA punches of B blood	neg
	3 FTA punches of O blood	neg
	2 FTA punches of C blood	pos
	None (blank control)	neg (as expected)
gDNA	6ul B, C, and O gDNA* (2ul per species)	pos for all target species
	None (blank control)	neg (as expected)

* Concentrations of Bovine, Caprine, and Ovine reference gDNA solutions were 10, 1, and 1 ng/ul, respectively, resulting in the spike levels at 20, 2, and 2 ng gDNA per 0.25 g feed in the initial stage of this study.
Abbreviations: B, bovine; C, caprine; O, ovine; ID, identification; gDNA, genomic DNA; rt-PCR, real-time polymerase chain reaction; neg, negative; pos, positive; Ct, cycle threshold.

To determine the appropriate amount of reference gDNA to be used as the positive control, various concentrations of gDNA were tested. Feed xxxx625 was first spiked with bovine, caprine, and ovine gDNA at 20, 2, and 2 ng per 0.25 g feed, respectively. After extraction and rt-PCR, a positive bovine signal was observed but under the limit of detection (LOD), as the C_T came up too late to be quantified. The ovine result was positive but with a late C_T (46.2 cycles). To ensure positive rt-PCR results for all target species, the spike levels of bovine and ovine gDNA were doubled while caprine gDNA remained the same. Following this adjustment, positive results were obtained for all three species with the C_T falling in a range of 27.0-32.5 (Table 2). Based on these preliminary results, we decided to use 40, 2 and 4 ng gDNA per 0.25 g feed for bovine, caprine, and ovine, respectively, as the extraction positive control to be further evaluated for robustness in this study.

Table 2. Real-time PCR results of feed xxxx625 spiked with two different concentrations of reference gDNA.

Spike levels of B, C, and O reference gDNA (ng/0.25 g feed)	Feed matrix ID (brief description)	Target species	C_T	Tm	rt-PCR result
20, 2, and 2 ng	xxxx625 (beet pulp pellet)	B	Undetermined	82.5	pos but under LOD*
		C	25.6	83.2	pos
		O	46.2	79.0	pos
		IAC	25.6	87.1	pos (as expected)
40, 2, and 4 ng**	xxxx625 (beet pulp pellet)	B	32.5	82.9	pos
		C	27.0	84.2	pos
		O	27.7	79.4	pos
		IAC	19.7	87.6	pos (as expected)
None	xxxx625 (beet pulp pellet) matrix blank control	B	Undetermined	63.2	neg
		C	43.0	80.1	neg
		O	Undetermined	63.9	neg
		IAC	27.0	87.1	pos (as expected)

* The bovine result was reported as positive but under LOD per LIB 4486 (3).
** PCR inhibition was indicated by the internal amplification control (IAC) when using the extracted feed DNA without dilution in rt-PCR. The valid results shown here were obtained with 10-time diluted feed DNA with molecular biology grade water.
Abbreviations: B, bovine; C, caprine; O, ovine; ID, identification; gDNA, genomic DNA; rt-PCR, real-time polymerase chain reaction; neg, negative; pos, positive; C_T, cycle threshold; Tm, melting temperature; IAC, internal amplification control; LOD, limit of detection.

To assess the robustness, the reference gDNA mixture was tested in 43 archived feed samples, each having different ingredients and texture attributes. The target DNA was extracted from the spiked feeds and subsequently detected in 41 of 43 (95.3%) samples by rt-PCR (Table 3), indicating that the addition of this gDNA mixture was highly compatible with both feed extraction and the downstream rt-PCR. Of the 43 samples tested, 16 were rt-PCR positive prior to the spiking of this gDNA mixture, and all C_T values of spiked samples were lower than the C_T of matrix blank controls (highlighted in bold purple, Table 3). These results demonstrate that the added gDNA mixture was successfully detected even in feeds that may have been contaminated with prohibited materials.

Table 3. Real-time PCR results of 43 archived feeds spiked with the reference gDNA.

Exp No.	Feed matrix ID (brief description if available)	Target species	C_T	T_m	rt-PCR result	Result summary
1	xxxx625 (beet pulp pellet) matrix blank control*	B	Undetermined	63.0	neg	B, C, and O neg
		C	38.9	79.4	neg	
		O	Undetermined	63.5	neg	
		IAC	23.9	87.1	pos	
	xxxx625 (beet pulp pellet) spiked with reference gDNA*	B	32.5	82.9	pos	B, C, and O pos (as expected)
		C	27.0	84.2	pos	
		O	27.7	79.4	pos	
		IAC	19.7	87.6	pos	
2	xxx824 (cattle mineral premix) matrix blank control	B	Undetermined	61.3	neg	B, C, and O neg
		C	36.9	79.2	neg	
		O	Undetermined	62.7	neg	
		IAC	24.4	87.2	pos	
	xxx824 (cattle mineral premix) spiked with reference gDNA	B	Undetermined	63.1	**neg**	**C pos, but B and O neg**
		C	33.4	83.4	pos	
		O	Undetermined	63.4	**neg**	
		IAC	23.5	87.4	pos	
3	xxx855 (cattle feed) matrix blank control	B	45.6	80.3	neg	B, C, and O neg
		C	36.6	78.9	neg	
		O	Undetermined	62.0	neg	
		IAC	27.3	86.7	pos	
	xxx855 (cattle feed) spiked with reference gDNA*	B	33.3	82.5	pos	B, C, and O pos (as expected)
		C	26.5	83.4	pos	
		O	26.3	79.1	pos	
		IAC	22.9	87.3	pos	
4	xxx503 (calf starter) matrix blank control	B	41.9	81.6	neg	B, C, and O neg
		C	34.9	78.7	neg	
		O	41.2	85.2	neg	
		IAC	28.0	86.9	pos	
	xxx503 (calf starter) spiked with reference gDNA	B	31.9	82.2	pos	B, C, and O pos (as expected)
		C	23.9	83.5	pos	
		O	32.0	78.7	pos	
		IAC	20.5	87.0	pos	
5	xxxx759 matrix blank control	B	48.1	74.0	neg	B, C, and O neg
		C	40.0	79.0	neg	
		O	Undetermined	62.9	neg	
		IAC	19.0	87.1	pos	
	xxxx759 spiked with reference gDNA**	B	37.4	82.2	pos	B, C, and O pos (as expected)
		C	31.2	83.7	pos	
		O	30.1	79.2	pos	
		IAC	17.4	87.6	pos	

Exp No.	Feed matrix ID (brief description if available)	Target species	C_T	T_m	rt-PCR result	Result summary
6	xxxx182 (steer) matrix blank control	B	36.1	74.5	neg	B, C, and O neg
		C	38.4	79.5	neg	
		O	40.0	70.9	neg	
		IAC	17.6	87.1	pos	
	xxxx182 (steer) spiked with reference gDNA	B	30.1	82.5	pos	B, C, and O pos (as expected)
		C	25.9	83.7	pos	
		O	26.1	79.0	pos	
		IAC	17.8	87.5	pos	
7	xxxx184 (dairy special B16 coarse) matrix blank control	B	35.9	74.5	neg	B, C, and O neg
		C	35.6	80.7	neg	
		O	43.3	80.0	neg	
		IAC	17.8	87.3	pos	
	xxxx184 (dairy special B16 coarse) spiked with reference gDNA	B	31.4	82.5	pos	B, C, and O pos (as expected)
		C	27.9	83.7	pos	
		O	26.8	79.0	pos	
		IAC	17.8	87.5	pos	
8	xxxx357 (bulk soybean meal for cattle) matrix blank control	B	33.9	75.0	neg	B, C, and O neg
		C	36.6	80.0	neg	
		O	42.9	80.5	neg	
		IAC	17.4	87.5	pos	
	xxxx357 (bulk soybean meal for cattle) spiked with reference gDNA	B	30.6	82.4	pos	B, C, and O pos (as expected)
		C	27.5	83.8	pos	
		O	25.6	79.0	pos	
		IAC	17.5	87.5	pos	
9	xxxx081 (corn meal) matrix blank control	B	39.3	81.3	neg	B, C, and O neg
		C	35.8	85.1	neg	
		O	Undetermined	61.8	neg	
		IAC	20.3	87.6	pos	
	xxxx081 (corn meal) spiked with reference gDNA	B	29.1	82.6	pos	B, C, and O pos (as expected)
		C	23.5	83.8	pos	
		O	24.4	79.2	pos	
		IAC	20.4	87.6	pos	
10	xxxx172 (swine feed) matrix blank control[#]	B	44.9	77.1	neg	**B and C neg; O pos**
		C	36.3	79.7	neg	
		O	**35.8**	**79.2**	**pos**	
		IAC	20.2	87.7	pos	
	xxxx172 (swine feed) spiked with reference gDNA	B	28.6	82.8	pos	**B, C, and O pos (O C_T was lower than that of the matrix blank control, as expected)**
		C	23.2	84.2	pos	
		O	**23.8**	**79.6**	**pos**	
		IAC	20.1	88.1	pos	
11	xxxx278 matrix blank control[*]	B	45.9	74.0	neg	B, C, and O neg
		C	35.2	79.5	neg	
		O	49.3	77.4	neg	
		IAC	23.8	87.2	pos	
	xxxx278 spiked with reference gDNA[*]	B	32.6	82.0	pos	B, C, and O pos (as expected)
		C	29.5	83.1	pos	
		O	27.4	78.7	pos	
		IAC	23.8	87.2	pos	
12	xxxx417 (canola meal) matrix blank control	B	Undetermined	62.3	neg	B, C, and O neg
		C	34.8	79.2	neg	
		O	Undetermined	73.9	neg	
		IAC	21.9	87.2	pos	
		B	29.6	82.4	pos	B, C, and O pos (as expected)

Exp No.	Feed matrix ID (brief description if available)	Target species	C_T	T_m	rt-PCR result	Result summary
	xxxx417 (canola meal) spiked with reference gDNA	C	27.8	83.4	pos	
		O	26.6	78.9	pos	
		IAC	24.1	87.2	pos	
13	xxxx024 (cattle feed) matrix blank control	B	45.5	81.4	neg	B, C, and O neg
		C	34.5	79.5	neg	
		O	Undetermined	63.2	neg	
		IAC	21.2	87.2	pos	
	xxxx024 (cattle feed) spiked with reference gDNA	B	29.1	82.4	pos	B, C, and O pos (as expected)
		C	25.6	83.6	pos	
		O	23.9	79.0	pos	
		IAC	24.1	87.3	pos	
14	xxxx275 (bulk cow) matrix blank control	**B**	**45.9**	**81.8**	**pos**	**B pos; C and O neg**
		C	35.4	79.3	neg	
		O	Undetermined	63.0	Neg	
		IAC	21.8	87.2	Pos	
	xxxx275 (bulk cow) spiked with reference gDNA	**B**	**29.2**	**82.4**	**pos**	**B, C, and O pos (B C_T was lower than that of the matrix blank control, as expected)**
		C	26.7	83.6	pos	
		O	25.7	78.9	pos	
		IAC	24.9	87.3	pos	
15	xxxx276 (custom mash) matrix blank control	B	47.0	77.9	neg	B, C, and O neg
		C	34.7	79.2	neg	
		O	38.0	81.6	neg	
		IAC	21.7	87.2	pos	
	xxxx276 (custom mash) spiked with reference gDNA	B	28.7	82.3	pos	B, C, and O pos (as expected)
		C	26.3	83.4	pos	
		O	24.6	78.9	pos	
		IAC	24.5	87.2	pos	
16	xxxx426 (mill run) matrix blank control	B	43.2	80.9	neg	B, C, and O neg
		C	34.5	78.5	neg	
		O	46.7	86.4	neg	
		IAC	21.3	87.1	pos	
	xxxx426 (mill run) spiked with reference gDNA	B	31.6	82.3	pos	B, C, and O pos (as expected)
		C	28.9	83.3	pos	
		O	27.2	78.7	pos	
		IAC	23.7	87.2	pos	
17	local matrix I (17% textured goat feed) matrix blank control	B	43.8	81.7	neg	B, C, and O neg
		C	34.3	86.6	neg	
		O	Undetermined	65.9	neg	
		IAC	20.1	87.3	pos	
	local matrix I (17% textured goat feed) spiked with reference gDNA	B	31.3	82.1	pos	B, C, and O pos (as expected)
		C	25.9	83.5	pos	
		O	26.5	78.9	pos	
		IAC	26.9	87.3	pos	
18	local matrix II (nutritionally balanced goat feed) matrix blank control	B	47.7	78.3	neg	B, C, and O neg
		C	33.1	86.3	neg	
		O	Undetermined	65.3	neg	
		IAC	20.1	87.6	pos	
	local matrix II (nutritionally balanced goat feed) spiked with reference gDNA	B	33.6	82.3	pos	B, C, and O pos (as expected)
		C	27.7	83.5	pos	
		O	28.0	78.9	pos	
		IAC	26.9	87.0	pos	
19	local matrix III (cattle feed with 14% protein) matrix blank control	**B**	**42.4**	**82.3**	**pos**	**B pos; C and O neg**
		C	31.9	86.9	neg	
		O	44.3	86.0	neg	

Exp No.	Feed matrix ID (brief description if available)	Target species	C_T	T_m	rt-PCR result	Result summary
		IAC	20.0	87.6	pos	
	local matrix III (cattle feed with 14% protein) spiked with reference gDNA	**B**	**31.4**	**82.3**	**pos**	**B, C, and O pos (B C_T was lower than that of the matrix blank control, as expected)**
		C	26.2	83.7	pos	
		O	25.8	79.2	pos	
		IAC	27.0	87.3	pos	
20	local matrix IV (textured feed for meat goat) matrix blank control	B	38.3	74.5	neg	B, C, and O neg
		C	36.1	85.9	neg	
		O	46.9	71.1	neg	
		IAC	26.0	87.3	pos	
	local matrix IV (textured feed for meat goat) spiked with reference gDNA	B	31.6	82.1	pos	B, C, and O pos (as expected)
		C	26.0	83.5	pos	
		O	26.1	79.0	pos	
		IAC	27.1	87.3	pos	
21	local matrix V (textured animal feed) matrix blank control	B	Undetermined	65.8	neg	B, C, and O neg
		C	35.6	80.1	neg	
		O	Undetermined	65.8	neg	
		IAC	26.9	87.3	pos	
	local matrix V (textured animal feed) spiked with reference gDNA	B	32.2	82.5	pos	B, C, and O pos (as expected)
		C	26.4	83.7	pos	
		O	26.1	79.0	pos	
		IAC	26.8	87.3	pos	
22	local matrix VI (corn, oats, and barley) matrix blank control	B	45.3	74.3	neg	B, C, and O neg
		C	36.3	80.1	neg	
		O	Undetermined	62.0	neg	
		IAC	26.3	87.2	pos	
	local matrix VI (corn, oats, and barley) spiked with reference gDNA	B	31.2	82.6	pos	B, C, and O pos (as expected)
		C	26.3	83.9	pos	
		O	26.0	79.2	pos	
		IAC	27.3	87.3	pos	
23	xxxx565 matrix blank control	**B**	**40.7**	**82.1**	**pos**	**B pos; C and O neg**
		C	34.4	79.0	neg	
		O	48.7	63.1	neg	
		IAC	26.1	87.2	pos	
	xxxx565 spiked with reference gDNA	**B**	**37.3**	**82.2**	**pos**	**B, C, and O pos (B C_T was lower than that of the matrix blank control, as expected)**
		C	32.7	83.3	pos	
		O	32.2	79.0	pos	
		IAC	26.7	87.4	pos	
24	xxxx568 matrix blank control	**B**	**41.8**	**82.8**	**pos**	**B pos; C and O neg**
		C	33.9	79.4	neg	
		O	47.1	86.1	neg	
		IAC	26.4	87.0	pos	
	xxxx568 spiked with reference gDNA	**B**	**38.5**	**82.4**	**pos**	**B, C, and O pos (B C_T was lower than that of the matrix blank control, as expected)**
		C	32.1	83.4	pos	
		O	31.7	78.8	pos	
		IAC	27.1	87.0	pos	
25	xxxx571 matrix blank control	**B**	**39.2**	**81.9**	**pos**	**B pos; C and O neg**
		C	35.1	79.4	neg	
		O	46.9	71.3	neg	
		IAC	25.4	87.4	pos	
	xxxx571 spiked with reference gDNA	**B**	**38.4**	**82.2**	**pos**	**B, C, and O pos (B C_T was earlier than the matrix blank control, as expected)**
		C	32.2	83.4	pos	
		O	31.5	79.0	pos	
		IAC	26.3	87.4	pos	
26		**B**	**42.5**	**82.0**	**pos**	**B pos; C and O neg**

Exp No.	Feed matrix ID (brief description if available)	Target species	C_T	T_m	rt-PCR result	Result summary
	xxxx824 (wheat middlings with ground wheat screenings) matrix blank control[$]	C	34.1	79.4	neg	
		O	Undetermined	80.1	neg	
		IAC	26.9	87.2	pos	
	xxxx824 (wheat middlings with ground wheat screenings) spiked with reference gDNA	**B**	**34.9**	**82.5**	**pos**	**B, C, and O pos (B C_T was lower than that of the matrix blank control, as expected)**
		C	29.8	83.6	pos	
		O	29.2	79.0	pos	
		IAC	27.6	87.2	pos	
27	xxxx825 (canola meal for animal feed) matrix blank control[$]	B	36.0	78.5	neg	**B and C neg; O pos**
		C	35.1	79.1	neg	
		O	**47.0**	**79.6**	**pos**	
		IAC	26.3	87.2	pos	
	xxxx825 (canola meal for animal feed) spiked with reference gDNA	B	31.1	82.2	pos	**B, C, and O pos (O C_T was lower than that of the matrix blank control, as expected)**
		C	27.6	83.6	Pos	
		O	**27.8**	**79.0**	**pos**	
		IAC	25.9	87.4	pos	
28	xxx803 (Medicated calf feed) matrix blank control[$]	**B**	**45.1**	**81.8**	**pos**	**B pos; C and O neg**
		C	35.8	79.4	neg	
		O	45.3	71.3	neg	
		IAC	26.1	87.2	pos	
	xxx803 (Medicated calf feed) spiked with reference gDNA	**B**	**36.4**	**82.5**	**pos**	**B, C, and O pos (B C_T was lower than that of the matrix blank control, as expected)**
		C	32.9	83.6	pos	
		O	32.2	79.1	pos	
		IAC	27.3	87.4	pos	
29	xxxx428 matrix blank control	**B**	**39.8**	**82.4**	**pos**	**B pos; C and O neg**
		C	33.3	79.6	neg	
		O	Undetermined	61.7	neg	
		IAC	25.9	87.4	pos	
	xxxx428 spiked with reference gDNA	**B**	**37.1**	**82.2**	**pos**	**B, C, and O pos (B C_T was lower than that of the matrix blank control, as expected)**
		C	32.0	83.4	pos	
		O	31.2	79.1	pos	
		IAC	26.4	87.4	pos	
30	xxxx433 matrix blank control[$]	**B**	**42.0**	**82.2**	**pos**	**B pos; C and O neg**
		C	34.9	79.6	neg	
		O	Undetermined	62.6	neg	
		IAC	25.8	86.8	pos	
	xxxx433 spiked with reference gDNA	**B**	**37.6**	**82.5**	**pos**	**B, C, and O pos (B C_T was lower than the matrix blank control, as expected)**
		C	32.2	83.6	pos	
		O	32.3	79.0	pos	
		IAC	25.9	87.4	pos	
31	xxxx149 (wheat mill run) matrix blank control	**B**	**45.4**	**82.6**	**pos**	**B pos; C and O neg**
		C	35.8	90.1	neg	
		O	37.1	81.3	neg	
		IAC	23.7	87.1	pos	
	xxxx149 (wheat mill run) spiked with reference gDNA	**B**	**34.8**	**82.2**	**pos**	**B, C, and O pos (B C_T was lower than the matrix blank control, as expected)**
		C	29.2	83.4	pos	
		O	30.9	79.0	pos	
		IAC	25.9	87.3	pos	
32	xxxx270 (sheep mineral mix) matrix blank control[&]	B	42.6	74.6	neg	B, C, and O neg
		C	35.9	79.6	neg	
		O	Undetermined	62.7	neg	
		IAC	23.6	87.3	pos	
		B	47.0	82.5	pos	B, C, and O pos (as expected)
		C	34.2	83.2	pos	

Exp No.	Feed matrix ID (brief description if available)	Target species	C_T	T_m	rt-PCR result	Result summary
	xxxx270 (sheep mineral mix) spiked with reference gDNA	O	34.8	79.0	pos	
		IAC	24.7	87.5	pos	
33	xxx212 (cake beef feed) matrix blank control	B	28.1	73.0	neg	B, C, and O neg
		C	35.8	79.6	neg	
		O	24.5	83.0	neg	
		IAC	22.0	87.3	pos	
	xxx212 (cake beef feed) spiked with reference gDNA	B	32.8	82.3	pos	B, C, and O pos (as expected)
		C	28.6	83.6	pos	
		O	30.6	79.2	pos	
		IAC	21.6	87.5	pos	
34	xxxx380 (with trace mineral salt) matrix blank control	B	43.3	74.6	neg	B, C, and O neg
		C	36.0	79.8	neg	
		O	Undetermined	65.5	neg	
		IAC	23.3	87.4	pos	
	xxxx380 (with trace mineral salt) spiked with reference gDNA	B	37.2	82.3	pos	B, C, and O pos (as expected)
		C	31.0	83.6	pos	
		O	32.4	79.2	pos	
		IAC	25.2	87.5	pos	
35	xxxx984 (mixed cattle feed) matrix blank control	B	46.8	81.6	neg	B, C, and O neg
		C	36.5	78.7	neg	
		O	47.1	64.5	neg	
		IAC	23.8	87.3	pos	
	xxxx984 (mixed cattle feed) spiked with reference gDNA	B	31.3	82.2	pos	B, C, and O pos (as expected)
		C	25.4	83.6	pos	
		O	26.7	79.0	pos	
		IAC	25.1	87.3	pos	
36	Local feed VII (horse feed) matrix blank control	B	Undetermined	63.2	neg	B, C, and O neg
		C	36.9	79.4	neg	
		O	Undetermined	63.2	neg	
		IAC	22.7	87.1	pos	
	Local feed VII (horse feed) spiked with reference gDNA	B	35.0	82.0	pos	B, C, and O pos (as expected)
		C	24.9	83.1	pos	
		O	39.0	78.6	pos	
		IAC	28.0	87.1	pos	
37	xxxx547 ("beef starter" with salt) matrix blank control*	B	Undetermined	74.4	neg	B, C, and O neg
		C	36.1	79.6	neg	
		O	Undetermined	69.4	neg	
		IAC	18.9	87.3	pos	
	xxxx547 ("Beef Starter" with salt) spiked with reference gDNA*	B	39.9	74.2	**neg**	**B, C, and O neg**
		C	35.9	79.7	**neg**	
		O	Undetermined	63.4	**neg**	
		IAC	19.0	87.5	pos	
38	xxxx489 (ground canola) matrix blank control	B	36.8	74.9	neg	B, C, and O neg
		C	32.0	79.8	neg	
		O	47.1	80.4	neg	
		IAC	19.7	87.5	pos	
	xxxx489 (ground canola) spiked with reference gDNA	B	31.1	82.1	pos	B, C, and O pos (as expected)
		C	23.3	83.5	pos	
		O	25.2	78.9	pos	
		IAC	19.6	87.5	pos	
39	xxxx490 (mill run) matrix blank control	B	40.9	79.1	neg	**O pos; B and C neg**
		C	30.5	81.6	neg	

Exp No.	Feed matrix ID (brief description if available)	Target species	C_T	T_m	rt-PCR result	Result summary
		O	**38.2**	**80.1**	**pos**	
		IAC	19.8	87.2	pos	
	xxxx490 (mill run) spiked with reference gDNA	B	33.6	82.5	pos	**B, C, and O pos (O C_T was lower than the matrix blank control, as expected)**
		C	26.1	83.5	pos	
		O	**27.7**	**79.1**	**pos**	
		IAC	19.9	87.4	pos	
40	xxxx491 ("Corn DDG") matrix blank control	B	34.9	75.0	neg	**O pos; B and C neg**
		C	31.9	79.9	neg	
		O	**49.0**	**78.8**	**pos**	
		IAC	19.8	87.5	pos	
	xxxx491 ("Corn DDG") spiked with reference gDNA	B	30.9	82.5	pos	**B, C, and O pos (O C_T was lower than the matrix blank control, as expected)**
		C	21.7	83.7	pos	
		O	**23.2**	**79.1**	**pos**	
		IAC	19.6	87.5	pos	
41	xxxx138 ("Soybean Meal") matrix blank control	B	46.0	81.0	neg	**O pos; B and C neg**
		C	36.5	79.6	Neg	
		O	**46.9**	**80.1**	**Pos**	
		IAC	18.9	87.4	Pos	
	xxxx138 ("Soybean Meal") spiked with reference gDNA	B	30.9	82.5	pos	**B, C, and O pos (O C_T was lower than the matrix blank control, as expected)**
		C	21.7	83.7	pos	
		O	**23.2**	**79.1**	**pos**	
		IAC	19.6	87.5	pos	
42	xxxx997 ("Breeder Cubes") matrix blank control	B	35.4	75.0	neg	B, C, and O neg
		C	34.6	86.0	neg	
		O	38.8	76.1	neg	
		IAC	18.4	87.7	pos	
	xxxx997 ("Breeder Cubes") spiked with reference gDNA	B	33.6	82.4	pos	B, C, and O pos (as expected)
		C	29.7	84.0	pos	
		O	28.3	79.3	pos	
		IAC	18.5	87.9	pos	
43	xxxx015 ("Special Supplement for Beef Cattle") matrix blank control	B	36.6	74.5	neg	**O pos; B and C neg**
		C	34.9	86.3	neg	
		O	**38.2**	**80.1**	**pos**	
		IAC	18.6	87.5	pos	
	xxxx015 ("Special Supplement for Beef Cattle") spiked with reference gDNA	B	30.0	82.8	pos	**B, C, and O pos (O C_T was lower than the matrix blank control, as expected)**
		C	25.6	84.4	pos	
		O	**24.3**	**79.8**	**pos**	
		IAC	18.4	88.3	pos	

[*] PCR inhibition was detected by the internal amplification control (IAC) when using extracted feed DNA without dilution. The valid results shown here were obtained with template diluted with molecular biology grade water at 1:10 to 1:20 ratio.

[**] The initial rt-PCR result was inconclusive due to a slight Tm shift, but the amplification was relatively strong (CT<37). After rt-PCR was repeated with 4 ul template at the original concentration, and 2 ul template diluted with molecular biology grade water at 1:10 to 1:50 ratio, all the results were B positive without Tm shift. Therefore, the result was determined to be B positive. The representative data shown here was obtained with 1:10 diluted template.

[$] The initial rt-PCR result was inconclusive due to multiple peaks per LIB 4657, or positive but under LOD per LIB 4486. To improve the signal-to-noise ratio, 4 ul template was used to repeat the rt-PCR and conclusive results were subsequently obtained.

[#] Previous analysis of the feed using the SmartCycler multiplex rt-PCR method (LIB 4544A) were inconclusive due to negative IAC even with template dilution. The AB7500F simplex rt-PCR yielded conclusive results as shown here, suggesting that the simplex rt-PCR may be more resistant to PCR inhibition than the multiplex rt-PCR for this feed.

[&] Previously, bovine blood (one FTA punch) was used in the feed as an extraction control, but the SmartCycler multiplex rt-PCR (LIB 4544A) yielded negative result. When the reference gDNA mixture was used as an extraction control and subsequently tested by the AB7500F simplex rt-PCR method (LIB 4657), the results were conclusive, suggesting that the reference gDNA may be more adaptable than the reference blood as an extraction control for this feed.

Abbreviations: B, bovine; C, caprine; O, ovine; ID, identification; gDNA, genomic DNA; rt-PCR, real-time polymerase chain reaction; neg, negative; pos, positive; C_T, cycle threshold; Tm, melting temperature; IAC, internal amplification control.

Of the 43 feed samples tested, spiked xxx824 and xxxx547 exhibited negative rt-PCR results on some or all target species (Exp No. 2 and 37 in Table 3, highlighted in bold brown). The negative results were likely due to inefficient feed extraction and/or degradation of the target DNA, suggesting that the reference gDNA was not a suitable positive control for these two samples. As an alternative positive control, 0.1% (w/w) BMBM was tested and the target bovine DNA was successfully detected by rt-PCR post feed extraction (Table 4). Therefore, the BMBM worked as an extraction positive control for xxx824 and xxxx547 when the reference gDNA did not serve this purpose.

Table 4. Real-time PCR results of the two feed samples spiked with 0.1% (w/w) BMBM.

Exp No.	Feed matrix ID (brief description if available)	Target species	C_T	T_m	rt-PCR result	Result summary
2	xxx824 (strip 10% P mineral) matrix blank control	B	Undetermined	61.3	neg	B, C, and O neg
		C	36.9	79.2	neg	
		O	Undetermined	62.7	neg	
		IAC	24.4	87.2	pos	
	xxx824 (strip 10% P mineral) spiked with **0.1% BMBM**	**B**	**44.9**	**82.3**	**pos**	B pos, C and O neg (as expected)
		C	41.5	79.0	neg	
		O	Undetermined	62.0	neg	
		IAC	23.2	87.3	pos	
37	xxxx547 ("Beef Starter" with salt) matrix blank control*	B	Undetermined	74.4	neg	B, C, and O neg
		C	36.1	79.6	neg	
		O	Undetermined	69.4	neg	
		IAC	18.9	87.3	pos	
	xxxx547 ("Beef Starter" with salt) spiked with **0.1% BMBM**	**B**	**44.9**	**82.3**	**pos**	B pos, C and O neg (as expected)
		C	32.4	79.9	neg	
		O	Undetermined	61.7	neg	
		IAC	33.8	87.3	pos	

* PCR inhibition was detected by the internal amplification control (IAC) when using extracted feed DNA without dilution. The valid results shown here were obtained with 10-time diluted template.

Generally, BMBM is only available in powered form. As such, it must be handled with care during feed fortification to minimize dust formation and potential cross-contamination of unfortified samples. It should also be noted that under ideal circumstances, bovine, caprine and ovine MBM would be all spiked when the reference gDNA mixture does not work reliably in certain feeds. However, authenticated caprine and ovine MBM are almost impossible to obtain commercially in the USA. As a result, we were only able to evaluate BMBM in this study.

Lastly, the stability of reference gDNA as the extraction positive control was determined in four feed samples (described as # 17, 18, 19, and 23 in Table 3). Real-time PCR results indicated that all target species were successfully detected even after the spiked feeds were stored at -20 °C for up to 42 days without significant reduction in C_T values (Figure 1).

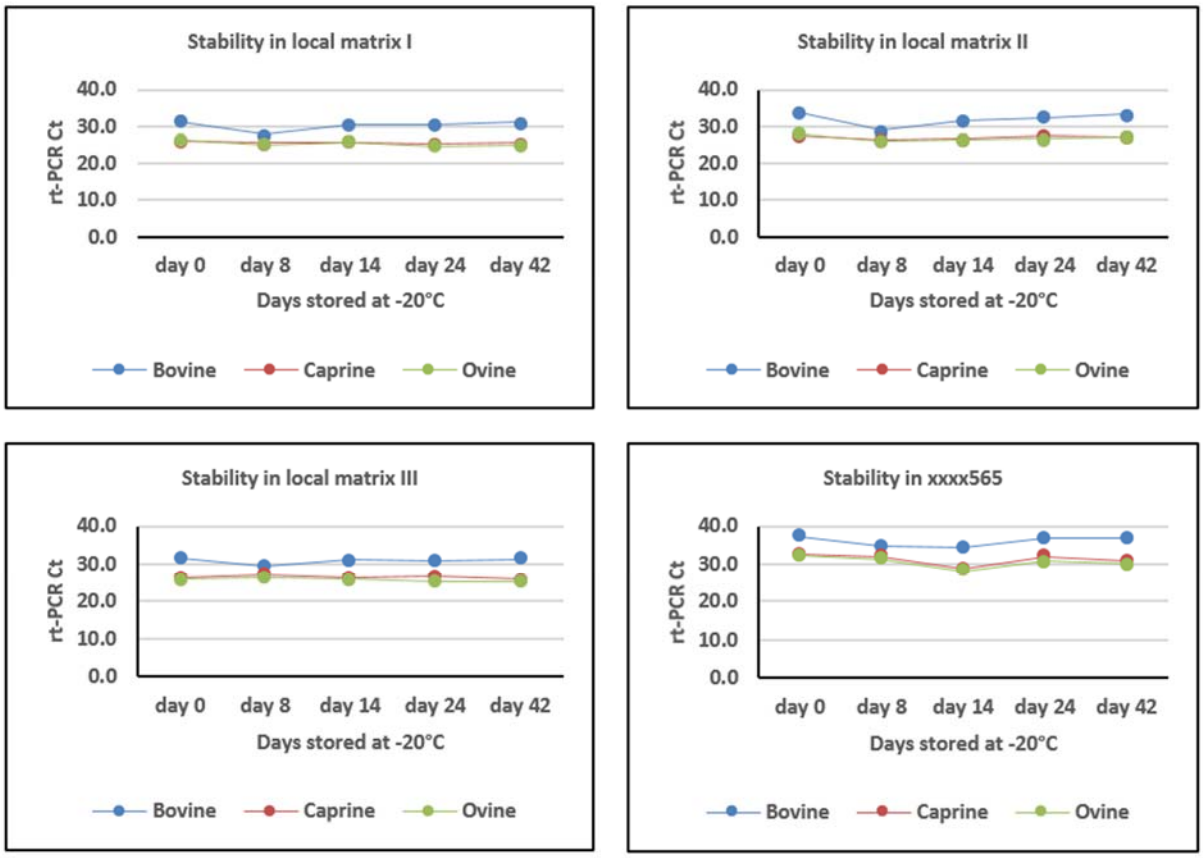

Figure 1. Time course of the rt-PCR results obtained from four feed samples spiked with the reference gDNA and stored at -20 °C for up to 42 days before feed extraction.

Taken together, results from this study indicate that the reference gDNA mixture from bovine, caprine, and ovine (40, 2, and 4 ng per 0.25 g feed, respectively) can be used as a positive control for feed extraction in the BSE program, and that 0.1% (w/w) BMBM may be used in feeds with caution as an alternative positive control when the reference gDNA cannot be detected reliably.

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