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Qualitative and Quantitative Polymerase Chain Reaction Assays for an Alfalfa (*Medicago sativa*)-Specific Reference Gene To Use in Monitoring Transgenic Cultivars

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Genetically modified (GM) alfalfa (*Medicago sativa*) was marketed for the first time in 2005. For countries with established thresholds for GM plants, methods to detect and quantify their adventitious presence are required. We selected *acetyl CoA carboxylase* as a reference gene for the detection and quantification of GM alfalfa. Two qualitative polymerase chain reaction (PCR) assays (Acc1 and Acc2) were designed to detect alfalfa. Both were specific to alfalfa, amplifying DNA from 12 separate cultivars and showing negative results for PCR of 15 nonalfalfa plants. The limits of detection for Acc1 and Acc2 were 0.2 and 0.01%, respectively. A quantitative real-time PCR assay was also designed, having high linearity ($r > 0.99$) over alfalfa standard concentrations ranging from 100 to 2.0×10^5 pg of alfalfa DNA per PCR. The real-time PCR assay was effective in quantifying alfalfa DNA from forage- and concentrate-based mixed diets containing different amounts of alfalfa meal.

KEYWORDS: Genetically modified; GMO detection; real-time PCR; alfalfa; reference gene; *acetyl CoA carboxylase*

INTRODUCTION

During the 10 year period of 1996 to 2005, the global area of genetically modified (GM) crops increased more than 50-fold, reaching a total of 90 million hectares worldwide (*1*). Despite widespread use of GM crops and studies showing nutritional equivalence as compared to non-GM varieties (*2*), public acceptance varies (*3*, *4*). Some regulatory bodies have mandated labeling laws concerning approved GM foods and feeds in order to give consumers the option of selective purchasing (*5*, *6*). For unapproved GM crops, importation is generally banned if the GM trait is detected above a set threshold percentage. Therefore, companies exporting products containing GM constituents to nations with labeling legislations must abide by the laws of the importing countries. To meet these requirements, methods enabling detection and quantification of GM products are necessary.

Polymerase chain reaction (PCR) is capable of detecting and measuring the concentration of GM products at or below most regulatory thresholds (*7*, *8*). Event-specific tests can be developed by designing primers to amplify a DNA sequence spanning the junction between transgenic and plant genomic DNA (*9*). However, in addition to an event-specific test, a PCR assay for an endogenous species-specific gene needs to be available (*10*, *11*). In qualitative GM screening, amplification of the endogenous gene acts as a positive control, identifying the type(s) of plant species within the test sample. PCR quantification of GM products is performed by comparing the amount of transgenic DNA to a reference target (*11*), usually an endogenous reference gene.

In 2005, Roundup Ready (herbicide tolerant) alfalfa (*Medicago sativa*) was approved for release into the environment and for use as food and/or feed in the United States and Canada (*12*). In 2004, these two countries combined exported a total 436809 Mt of alfalfa meal and pellets with an estimated value of $U.S. 63 million (*13*). Alfalfa meal and pellets are widely used as feed for ruminants. While there are stipulations pertaining to the use and export of Roundup Ready alfalfa, quality assurance may require testing for GM alfalfa in shipments of seeds or feed. The purpose of this paper was to develop qualitative and quantitative PCR assays for an endogenous alfalfa reference gene that could be used for the estimation of adventitious presence of GM alfalfa in forage and feed shipments.

MATERIALS AND METHODS

Plant Materials. Twelve different cultivars of alfalfa (*M. sativa*; **Table 1**) were grown on adjacent fields at the Lethbridge Research Centre, Lethbridge, Alberta, in 2005. Fresh samples from each cultivar were collected in October, at harvest, and stored at −80 °C prior to DNA isolation. Nonalfalfa plants, used to validate primer specificity, were grown in an environment that precluded its contamination with alfalfa plant material (**Table 2**). Plant seeds were rinsed with ethanol (70% v/v) and then placed into 50 mL sterilized flasks containing agar (1.5% w/v). Plants were grown until there was adequate material for DNA extraction. The growth of cicer milkvetch (*Astragalus cicer*) and

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Table 1. List of Alfalfa (*M. sativa*) Plants

sample	cultivar	sample	cultivar
1	Rambler	7	SW LU8407
2	DS336	8	DS335
3	DS337	9	unknown[a]
4	Rangelander	10	Sperdor
5	AC Blue J	11	Geneva
6	Beaver	12	Gala

[a] Cultivar not reported.

Table 2. List of Nonalfalfa Plants

sample	plant	species	cultivar
1	nova sanfoin	*Onobrychis viciifola*	Nova
2	lentil	*Lens culinaris*	Laird
3	safflower	*Carthamus tinctorius*	Saffire
4	canola	*Brassica napus*	Westvar
5	birdsfoot trefoil	*Lotus corniculatus*	unknown[a]
6	triticale	*Triticosecale*	AC AHA
7	cicer milkvetch	*A. cicer*	Oxley
8	maize	*Zea mays*	unknown[a]
9	bean	*Phaseolus vulgaris*	NW63
10	pea	*Pisum sativum*	Trapper
11	Timothy	*Phleum pretense*	Richard
12	red clover	*Trifolium pretense*	Florex
13	sugar beet	*Beta vulgaris*	HM Bergen
14	wheat	*Triticum aestivum*	Superb
15	barley	*H. vulgare*	unknown[a]

[a] Cultivar not reported.

Table 3. Ingredients of Diets Containing High (HA) or Low (LA) Amounts of Alfalfa Meal

diet	HA	LA
	ingredients (kg t^{-1})	
alfalfa meal	352.51	80.7
barley (ground)	256.3	724.5
canola meal	150	150
beet pulp	203.17	0
calcium carbonate	0	7
sheep mineral[a]	7	7
vit ADE[b]	0.25	0.25
dry molasses	20	20
Maxi-Pel[c]	5	5
canola oil	5	5
Decox[d]	0.13	0.13

[a] Containing (%): NaCl (93.1), Mg (1.25), Zn (0.9), Mn (0.94), Cu (0.13), Se (0.003), K (1.25), and Fe (1.25). [b] Containing (IU g^{-1}): vitamin A (10000), vitamin D (1250), and vitamin E (10). [c] Feed pellet binder. [d] Decoquinate (60 g kg^{-1}).

barley (*Hordeum vulgare*) in the sterilized flasks was limited, and as a result, seeds rinsed with ethanol (70% v/v) were used for DNA extraction.

Alfalfa (cultivar Rambler), previously dried at 55 °C for 48 h, and canola meal were used to prepare standards for quantitative real-time PCR. Alfalfa DNA was quantified in two diets prepared at the Lethbridge Research Centre, containing high- (HA) and low-alfalfa meal (LA) content (35.3 and 8.1% as-fed basis, respectively; **Table 3**).

DNA Extraction and Quantification. Prior to DNA extraction, fresh plant tissue was ground in a mortar and pestle with liquid nitrogen or in the case of dried alfalfa, canola meal, cicer milkvetch, and barley seeds, ground to a fine powder using a planetary micro mill (Fritsch, Albisheim, Germany). A previously described CTAB procedure (*14*) was used to extract DNA from plant tissues, with the following modifications: For fresh plant material, 1.2 mL of CTAB lysis buffer was added to 300 mg of ground material. For all extractions, the samples were incubated for 90 min to lyse cells and the final DNA was resuspended in 100 μL of TE buffer. DNA was quantified fluorometrically using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Burlington, ON) with a VersaFluor fluorometer (BioRad).

Table 4. Description of Primers and Probe Used for PCR

fragment name and size[a]	primer or probe sequence (5′ to 3′)	gene position
	alfalfa-specific	
Acc1 (91 bp)	(F[b]) GATCAGTGAACTTCGCAAAGTAC	6813
	(R[c]) CAACGACGTGAACACTACAAC	6903
	(P[d]) TGAATGCTCCTGTGATCTGCCCATGC	6851
Acc2 (154 bp)	(F) GATCAGTGAACTTCGCAAAGTAC	6813
	(R) GAGGGATGCTGCTACTTTGATG	6966
	plant-specific	
Rbc (138 bp)	(F) CTTGGCAGCATTCCGAGTA	not reported
	(R) CCTTTGTAACGATCAAGACTGG	

[a] Primers for Acc1 and Acc2 were designed for this study specific for the alfalfa *acetyl CoA carboxylase* gene (*16*); Rbc specific for universal plant *rbcL* gene (*17*). [b] Forward primer. [c] Reverse primer. [d] Probe for real-time PCR.

Gene Selection. The Entrez Nucleotide database (*15*) was used to search for candidate alfalfa genes meeting the following criteria: The gene was previously reported to be present at less than five copies per genome by Southern blot analysis; a sequence of the gene showed no homology to nonalfalfa plant DNA when entered into NCBI's BLAST search; and a TaqMan primer and probe set could be designed within the nonhomologous region by Beacon Designer 5 (Premier Biosoft, Palo Alto, CA) with a default software rating greater than 50. From these criteria, the best candidate gene was *acetyl CoA carboxylase* (*16*; accession #L25042).

PCR Analyses. The primers and probe used for PCR analyses in this study are described in **Table 4**. Two primer sets were used for the detection of alfalfa *acetyl CoA carboxylase* by conventional PCR, amplifying fragments Acc1 (91 bp) and Acc2 (154 bp). Alfalfa DNA from cultivar Rambler served as a positive control for PCR. Universal plant-specific primers for the chloroplast *rbcL* gene (encoding ribulose bisphosphate carboxylase, Rubisco) were used as a positive control for the nonalfalfa plants, amplifying a 138 bp fragment (Rbc; *17*). Conventional PCR conditions for fragments Acc1, Acc2, and Rbc were as follows: 95 °C for 15 min; 40 cycles of 95 °C for 20 s and 60 °C for 1 min; and 72 °C for 10 min. Each PCR mixture (50 μL) contained (final concentrations) 1× HotStarTaq Master Mix (Qiagen Inc., Mississauga, Canada), 0.15 μM each primer, and 100 ng of template DNA. The PCR was performed with a DNA Engine Dyad (M.J. Research Inc., Watertown, United States). Following each PCR, 20 μL of product was visualized on a 2.0% (w/v) agarose gel, following electrophoresis and staining with ethidium bromide.

Real-time PCR was performed using an iCyler iQ system (BioRad, Mississauga, Canada) and primers and probe for fragment Acc1 (**Table 4**). Each PCR (50 μL) contained (final concentrations) 1× Universal Master Mix (Applied Biosystems, Foster City, CA), 0.15 μM each primer, and 0.05 μM probe. The probe (**Table 4**) was labeled at the 5′-end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3′-end with the quencher dye, 6-carboxytetramethylrodamine (TAMRA). Each real-time PCR reaction contained 200 ng of DNA. For quantitative real-time PCR, standards were prepared using DNA isolated from dried alfalfa (Cultivar Rambler) and concentrations of 2.0×10^5, 1.0×10^5, 2.0×10^4, 1.0×10^4, 2.0×10^3, 1.0×10^3, 200, and 100 pg per reaction. As the amounts of alfalfa DNA were decreased, canola meal DNA was substituted in its place so that the final concentration of DNA (200 ng per reaction) in each standard remained constant.

Sequencing. Alfalfa DNA fragments were amplified using primer sets Acc1 or Acc2, as described above. PCR products were purified using a QIAquick PCR purification kit (Qiagen Inc., Ontario, Canada) and eluted in water (pH 8) from the columns. Samples were sequenced at the Lethbridge Research Centre DNA Sequencing Lab (Alberta, Canada).

Limits of Detection (LODs). LOD assays were conducted to determine the sensitivity of conventional PCR for fragments Acc1 and Acc2. PCR contained 1.0×10^3, 500, 200, 100, 50, 25, 12.5, and 6.25 pg of alfalfa DNA (cultivar Rambler) diluted in canola meal DNA (100

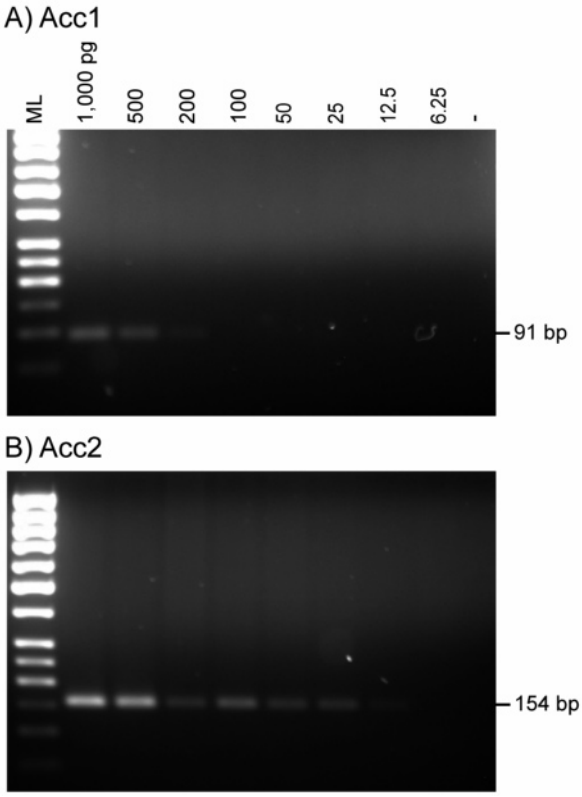

Figure 1. LOD for primer sets Acc1 (**A**) and Acc2 (**B**). Alfalfa DNA (6.25–1000 pg) was diluted in DNA extracted from canola meal so that the amount of DNA for each PCR (100 ng) remained constant.

ng DNA per PCR). Other PCR mixture components, thermocycling conditions, and resolution of PCR products were identical to those described earlier for fragments Acc1 and Acc2.

RESULTS AND DISCUSSION

Gene Selection. We chose *acetyl CoA carboxylase* (*16*; accession #L25042) as a reference gene for the detection and quantification of transgenic alfalfa. *Acetyl CoA carboxylase* is involved in the production of malonyl CoA for fatty acid synthesis. The gene has been reported to be present at two copies per haploid genome by Southern Blot analysis (*16*). Low-copy genes are generally considered to be less variable in copy number between cultivars and have lower mutation rates (*18*). Thus, *acetyl CoA carboxylase* is an ideal candidate for the quantification of GM alfalfa, as it limits the amount of error arising from cultivar variation.

LOD and Primer Specificity. Using the BLAST tool for comparison of homology of the *acetyl CoA carboxylase* gene to other DNA sequences in the NCBI database, a section of the gene appeared to be alfalfa-specific. Upon entering this region into Beacon Designer software for design of a TaqMan assay, the best-rated primer and probe set was for a 91 bp sequence of the gene (Acc1; **Table 4**). Initially, we were going to use the same primer pair used in real-time PCR for qualitative detection of alfalfa. However, smaller amplicons generally have greater LODs by ethidium bromide gel staining and visualization as a result of a reduction in the total DNA produced by PCR. Therefore, a second primer pair was designed, amplifying a 154 bp alfalfa DNA sequence with increased sensitivity for detection by conventional PCR (Acc2; **Table 4**).

As predicted, the LOD for Acc1 was higher as compared to Acc2 (200 and 12.5 pg, respectively; **Figure 1**). Considering the 2C (diploid) value of alfalfa is 3.44 pg (*19*), this represented

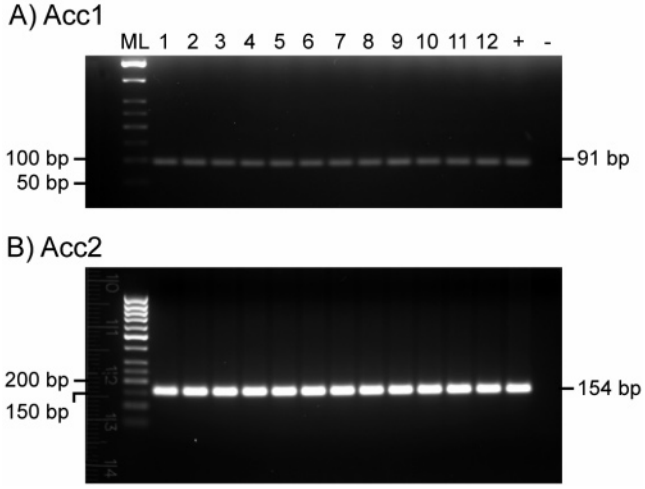

Figure 2. PCR of the alfalfa *acetyl CoA carboxylase* gene from 12 different alfalfa cultivars, using primer set Acc1 (**A**) or Acc2 (**B**). Alfalfa cultivars 1–12 correspond to the same cultivar number in **Table 1**. Positive control (+) contained DNA from cultivar Rambler. Negative control (–) contained water in place of DNA template.

the detection of approximately 58 and four genome equivalents for Acc1 and Acc2, respectively. As a percentage of total DNA in the PCR (100 ng), both primer sets were highly sensitive, detecting alfalfa DNA when it was present at 0.2 (Acc1) or 0.01% (Acc2). Plant DNA may be degraded to fragments after heat treatment of feeds and foods (*20*, *21*) or ensiling (*22*, *23*), potentially limiting the size of DNA that can be amplified by PCR. The small fragment sizes of both Acc1 and Acc2 should enable qualitative PCR detection of alfalfa from samples containing fragmented DNA as a result of heat processing.

To determine the specificity of Acc1 and Acc2 primer sets, PCR analyses were conducted on 12 different alfalfa cultivars and 15 nonalfalfa plants. For DNA from each of the alfalfa plants, PCR resulted in amplified fragments corresponding to the predicted sizes (**Figure 2A**,**B**). Additionally, the sequences of Acc1 and Acc2 were identical to those published in the NCBI database (data not shown), and neither sequence showed any homology to plant species entered in the database. The nonalfalfa plants were chosen to represent commonly used forage- and concentrate-based animal feeds because mixed diets are frequently used in animal nutrition and may require testing for the presence of GM alfalfa. DNA from each of the plants was of PCR quality, indicated by amplification of a general plant Rbc sequence (**Figure 3A**). In contrast, none of the nonalfalfa plants tested positive for Acc1 or Acc2 (**Figure 3B**,**C**, respectively). Moreover, artifact bands were not present after PCR of any plant species examined, including alfalfa cultivars, suggesting a high degree of primer specificity to alfalfa *acetyl CoA carboxylase*.

Real-Time PCR. When analyzed by real-time PCR, no nonalfalfa plant produced a positive threshold cycle (C_T), supporting the high degree of specificity of the Acc1 primer set for alfalfa (data not shown). Alfalfa DNA (200 ng) extracted from fresh plant material was tested by real-time PCR to investigate variability in gene copy number between cultivars (**Figure 4**). Each of the cultivars showed similar amplification plots. Only slight variation in C_T was evident, ranging between 19.8 and 20.6 PCR cycles (mean, 20.3; standard error, 0.02). The difference in the range in C_T (0.8) was similar to values reported for single-copy reference genes for wheat (*10*), maize (*18*), and canola (*24*). An important quality of a reference gene to be used in the quantification of GM plants is that the copy

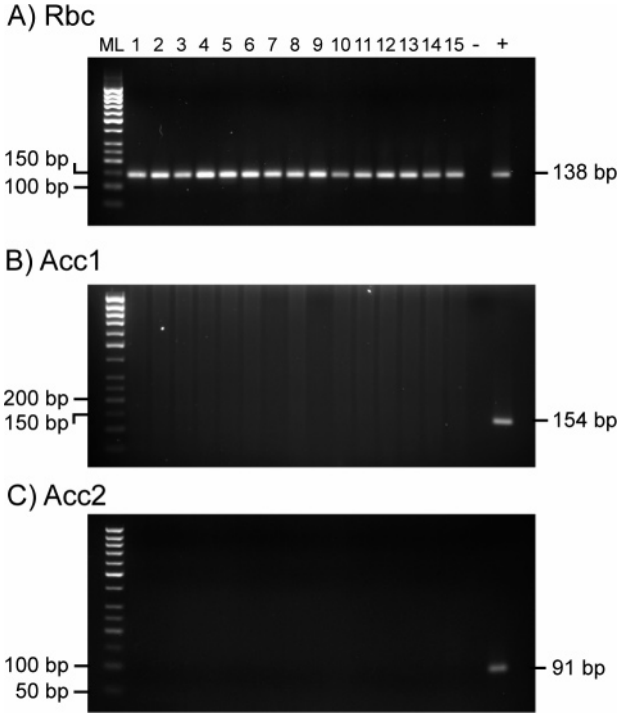

Figure 3. PCR of the universal plant chloroplast *rbcL* gene using primer set Rbc (**A**) or the alfalfa- specific *acetyl CoA carboxylase* gene using primer sets Acc1 (**B**) or Acc2 (**C**) from 15 nonalfalfa plants. The plant numbers correspond to the same species numbers in **Table 2**. Negative control (–) contained water in place of DNA template. Positive control (+) contained DNA from alfalfa cultivar Rambler.

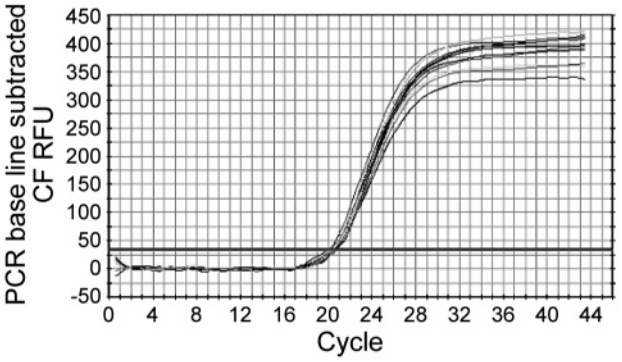

Figure 4. Real-time PCR of the alfalfa *acetyl CoA carboxylase* gene from 12 different cultivars. Each PCR contained 200 ng of alfalfa DNA.

number is stable among cultivars (*24*). The C_T values obtained imply that *acetyl CoA carboxylase* was conserved and the numbers of copies per genome among the alfalfa cultivars tested were similar. Error inherent in DNA extraction, quantification, and dilution may have added to the C_T variation.

Eight DNA standards ranging from 100 to 2.0×10^5 pg of alfalfa DNA (per PCR reaction) were created for quantitative real-time PCR analysis of two mixed diets (**Table 3**). Matrix effects can cause variation in the amplification of DNA. Ideally, the standards and unknown test samples should have similar matrices to avoid errors due to matrix effects. In an attempt to mimic the DNA from the diets, we used DNA from dried alfalfa in our standards and diluted the alfalfa DNA with DNA extracted from canola meal, which was present in both diets (15% w/w). Drying the alfalfa did appear to result in degradation of the number of copies of Acc1 isolated. The C_T, which is inversely related to copy number, increased from a mean value of 20.3 to 22.2 when 200 ng of DNA extracted from fresh

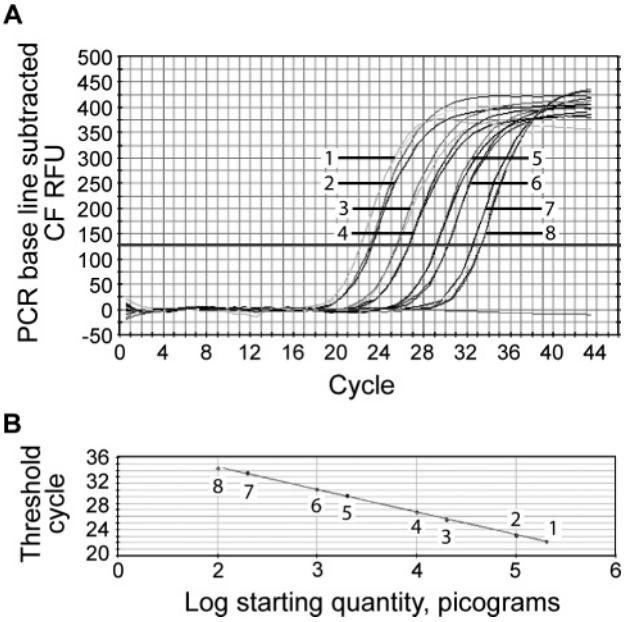

Figure 5. Real-time PCR amplification plots of the *acetyl CoA carboxylase* gene in eight alfalfa DNA standards (**A**) and the standard curve generated from DNA amplification (**B**). Alfalfa DNA content: 2.0×10^5 (1); 1.0×10^5 (2); 2.0×10^4 (3); 1.0×10^4 (4); 2.0×10^3 (5); 1.0×10^3 (6); 200 (7); and 100 (8) pg per reaction. As the amounts of alfalfa DNA were decreased, canola meal DNA was substituted in its place so that the final concentration of DNA (200 ng per reaction) in each standard remained constant.

(**Figure 4**) vs dried (**Figure 5A**) alfalfa was compared by PCR. This highlights the importance of extracting DNA for use as standards from alfalfa that is processed in a manner similar to unknown test samples.

The PCR efficiency, calculated by iCycler software, was consistently between 93 and 99% (representative standard amplification plots and curve shown in **Figure 5**). This implied that alfalfa DNA was amplifiable to a similar extent in each of the standards, regardless of starting concentration (**Figure 5A**). Additionally, these data demonstrated that the primer and probe concentrations were suitable for efficient amplification and that PCR inhibitors did not adversely affect efficiency, regardless of the concentration of alfalfa or canola meal DNA in the standard. The standard curve generated from the amplification plots confirmed efficient amplification for each DNA standard (**Figure 5B**). High linearity was evident across each of the alfalfa DNA concentrations, and the correlation coefficient was always greater than 0.99. The concentration of alfalfa DNA in the lowest standard (100 pg of alfalfa DNA) was 0.05% of total DNA. The range of quantification therefore included thresholds (0.5−5%) set by regulatory bodies that mandate the allowable adventitious levels of approved GM products or those given favorable risk assessments (*5*, *6*).

Regulators may test processed food and feed products for the presence of GM constituents to ensure that producers are complying with labeling laws (*25*). Consequently, complex diets may need to be analyzed. The diets chosen to quantify alfalfa DNA varied in the concentration of alfalfa (35.3%, HA; 8.1%, LA) and other ingredients, therefore representing two types of complex matrices. The mean values for alfalfa DNA quantified were 4.40×10^4 (standard error 3.23×10^3, diet HA) and 1.04×10^4 pg (standard error 1.27×10^3, diet LA), representing 22 and 5.2% of total PCR DNA (200 ng), respectively. Although the quantity of plant DNA does not necessarily reflect plant weight (*11*), the ratios of DNA:weight should be similar for a

Table 5. Alfalfa Meal Weight and DNA Proportions in Mixed Diets

diet[a]	weight ratio (r_1)	DNA ratio (r_2)	r_2/r_1
LA	0.081	0.052	0.64
HA	0.353	0.22	0.62

[a] Diets containing low (LA) or high (HA) amounts of alfalfa meal are described in **Table 3**.

single plant source. The diets in this study were formulated using the same source of alfalfa meal. The alfalfa DNA:weight percentage ratios were 0.62 and 0.64 for diets HA and LA, respectively (**Table 5**). This suggested that the PCR assay worked well to quantify alfalfa DNA in two processed mixed diets.

In summary, the PCR assays that we developed were highly sensitive and specific for alfalfa. *Acetyl CoA carboxylase* is a suitable reference gene for the detection of alfalfa and quantification of GM cultivars. A real-time PCR assay specific to GM alfalfa needs to be designed and validated in combination with the quantitative *acetyl CoA carboxylase* assay presented in this paper.

ABBREVIATIONS USED

C_T, threshold cycle; FAM, 6-carboxyfluorescein; GM, genetically modified; HA, diet containing high alfalfa meal content; LA, diet containing low alfalfa meal content; LOD, limit of detection; Rubisco, ribulose bisphosphate carboxylase; TAMRA, 6-carboxytetramethylrodamine.

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LITERATURE CITED

(1) James, C. Executive summary of global status of commercialized biotech/GM crops: 2005. *ISAAA Briefs* **2005**, *34*.

(2) Flachowsky, G.; Chesson, A.; Aulrich, K. Animal nutrition with feeds from genetically modified plants. *Arch. Anim. Nutr.* **2005**, *59*, 1−40.

(3) Lusk, J. L.; Roosen, J.; Fox, J. A. Demand for beef from cattle administered growth hormones or fed genetically modified corn: A comparison of consumers in France, Germany, the United Kingdom, and the United States. *Am. J. Agric. Econ.* **2003**, *85*, 16−29.

(4) Gaskell, G.; Allum, N.; Bauer, M.; Durant, J.; Allansdottir, A.; Heinz, B.; Boy, D.; de Cheveigne, S.; Fjaestad, B.; Gutteling, J. M.; Hampel, J.; Jelsoe, E.; Jesuino, J. C.; Kohring, M.; Kronberger, N.; Midden, C.; Nielsen, T. H.; Przestalksi, A.; Rusanen, T.; Sakellaris, G.; Torgersen, H.; Twardowski, T.; Wagner, W. Biotechnology and the European market. *Nat. Biotechnol.* **2000**, *18*, 935−938.

(5) ISAAA. International service for the acquisition of agri-biotech applications, international approaches to labeling, 2006; [online] available: http://www.isaaa.org/kc/bin/isaaa_briefs/index.htm (September 23, 2006).

(6) Jia, H. GM labeling in China beset by problems. *Nat. Biotechnol.* **2003**, *21*, 835−836.

(7) Huang, C.-C.; Pan, T.-M. Event-specific real-time detection and quantification of genetically modified Roundup Ready soybean. *J. Agric. Food Chem.* **2005**, *53*, 3833−3839.

(8) Germini, A.; Zanetti, A.; Salati, C.; Rossi, S.; Forre, C.; Schmid, S.; Marchelli, R. Development of a seven-target multiplex PCR for the simultaneous detection of transgenic soybean and maize in feeds and foods. *J. Agric. Food Chem.* **2004**, *52*, 3275−3280.

(9) Holst-Jensen, A.; Ronning, S. B.; Lovseth, A.; Berdal, K. G. PCR technology for screening and quantification of genetically modified organisms (GMOs) *Anal. Bioanal. Chem.* **2003**, *375*, 985−993.

(10) Iida, M.; Yamashiro, S.; Yamakawa, H.; Hayakawa, K.; Kuribara, H.; Kodama, T.; Furui, S.; Akiyama, H.; Maitani, T.; Hino, A. Development of taxon-specific sequences of common wheat for the detection of genetically modified wheat. *J. Agric. Food Chem.* **2005**, *53*, 6294−6300.

(11) Lipp, M.; Hillito, R.; Giroux, R.; Spiegelhalter, F.; Charlton, S.; Pinero, D.; Song, P. Polymerase chain reaction technology as analytical toll in agricultural biotechnology. *J. AOAC Int.* **2005**, *88*, 136−155.

(12) AGBIOS. GM database, 2006; [online] available: http://www.agbios.com/dbase.php (September 23, 2006).

(13) FAO. Key statistics of food and agriculture external trade, 2006; [online] available: http://www.fao.org/es/ess/toptrade/trade.asp?dir=exp&disp=countrybycomm&resource=862&ryear=2004 (October 3, 2006).

(14) Lipp, M.; Brodmann, P.; Pietsch, K.; Pauwels, J.; Anklam, E.; et al. IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder. *J. AOAC Int.* **1999**, *82*, 923−928.

(15) NCBI. Entrez Nucleotide Database, 2006; [online] available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar (September 23, 2006).

(16) Shorrosh, B. S.; Dixon, R. A.; Ohlrogge, J. B. Molecular cloning, characterization, and elicitation of acetyl-CoA carboxylase from alfalfa. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4323−4327.

(17) Reuter, T.; Aulrich, K. Investigations on genetically modified maize (BT-maize) in pig nutrition: Fate of feed-ingested foreign DNA in pig bodies. *Eur. Food Res. Technol.* **2003**, *216*, 185−192.

(18) Hernandez, M.; Duplan, M.-N.; Berthier, G.; Vaitilingom, M.; Hauser, W.; Freyer, R.; Pla, M.; Bertheau, Y. Development and comparison of four real-time polymerase chain reaction systems for specific detection and quantification of *Zea mays* L. *J. Agric. Food Chem.* **2004**, *52*, 4632−4637.

(19) Blondon, F.; Marie, D.; Brown, S. Genome size and base composition in *Medicago sativa* and *M. truncatula* species. *Genome* **1994**, *37*, 264−270.

(20) Alexander, T. W.; Sharma, R.; Okine, E. K.; Dixon, W. T.; Forster, R. J.; Stanford, K.; McAllister, T. A. Impact of feed processing and mixed ruminal culture on the fate of recombinant EPSP synthase and endogenous canola plant DNA. *FEMS Microbiol. Lett.* **2002**, *214*, 263−269.

(21) Lipp, M.; Bluth, A.; Eyquem, F.; Kruse, L.; Schimmel, H.; Van den Eede, G.; Anklam, E. Validation of a method based on polymerase chain reaction for the detection of genetically modified organisms in various processed foodstuffs. *Eur. Food Res. Technol.* **2001**, *212*, 497−504.

(22) Aulrich, K.; Pahlow, G.; Flachowsky, G. Influence of ensiling on the DNA-degradation in isogenic and transgenic corn. *Proc. Soc. Nutr. Physiol.* **2004**, *13*, 112.

(23) Hupfer, C.; Mayer, J.; Hotzel, H.; Sachse, K.; Engel, K.-H. The effect of ensiling on PCR-based detection of genetically modified Bt maize. *Eur. Food Res. Technol.* **1999**, *209*, 301−304.

(24) Hernandez, M.; Rio, A.; Esteve, T.; Prat, S.; Pla, M. A rapeseed-specific gene, acetyl-CoA carboxylase, can be used as a reference for qualitative and real-time quantitative PCR detection of transgenes from mixed food samples. *J. Agric. Food Chem.* **2001**, *49*, 3622−3627.

(25) Stave, J. W. Protein immunoassay methods for detection of Biotech crops: Applications, limitations, and practical considerations. *J. AOAC Int.* **2002**, *85*, 780−786.

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