6060 *J. Agric. Food Chem.* **2007,** *55,* 6060–6066

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluating Homogeneity of LL601 Rice in Commercial Lots Using Quantitative Real-Time PCR

LARRY FREESE, TANDACE A. SCHOLDBERG, DAISHIA D. BURTON, TIMOTHY D. NORDEN, LUKE A. SHOKERE, AND G. RONALD JENKINS*

Grain Inspection, Packers and Stockyards Administration, Technical Services Division, U.S. Department of Agriculture, 10383 North Ambassador Drive, Kansas City, Missouri 64153

Homogeneity analysis was performed on four distinctive commercial lots, derived from the 2006 rice harvest in the United States. Lots that had previously been tested and suspected to have some level of LL601 were selected to determine lot homogeneity. LL601 infiltration in the lots was low and estimated to contain <0.01% ($\sigma = 0.026$), 0.014% ($\sigma = 0.020$), 0.054% ($\sigma = 0.043$), and 0.074% ($\sigma = 0.031$) LL601. Lots were analyzed statistically as a one-way classification, or one-factor experiment, to assess the presence of strata within the lot. A *p* value of 0.05 or lower is needed to declare statistical significance and would suggest significant differences among the samples. The data revealed *p* values ranging between 0.105 and 0.607. The calculated *p* values for all lots were greater than the critical value of 0.05. Samples taken from different locations throughout these four commercial lots did not show statistically significant stratifications within the lot.

KEYWORDS: Homogeneity; real-time Polymerase Chain Reaction; genetically modified organisms; LL601; long-grain rice; transgenic; DNA quantification; phospholipase D endogenous control gene; threshold cycle

INTRODUCTION

Through biotechnology, novel DNA sequences from unrelated species can be inserted into the chromosomes of plants, enabling them to express new traits that are not normally expressed in the plant (*1*). Advances in the agricultural biotechnology industry over the past 10 years have produced biotechnology-derived traits, the characteristics of which must be thoroughly investigated prior to authorization for commercialization (*2*). These innovations necessitated the implementation of control mechanisms such that no unauthorized biotechnology-derived traits exist in commercialized lots of grains or when legislative mandates implement compulsory labeling on grain (*2*). The principal analytical procedures used for detecting biotechnology-derived traits in grains and oilseeds include (i) antibody-based methods, which detect intact protein products (enzyme-linked immunosorbent assays and lateral flow strips) (*3*) and (ii) Polymerase Chain Reaction (PCR), which identifies the presence of a DNA sequence unique to the biotechnology-derived trait (*1*, *4*, *5*). Both protein- and DNA-based technologies are widely accepted methods to measure the presence of biotechnology-derived traits in consignments of grain (*4*, *6*, *7*). Quantitative Polymerase Chain Reaction (qPCR) is an established analytical method, accepted worldwide, as a means to measure amounts of biotechnology-derived traits in a bulk lot. However, this approach implies an inherent knowledge of the distribution of the trait and its uncertainty. Developing appropriate sampling plans can help ensure that the analytical sample is an accurate representation of the lot. Statistics and probability can be used to estimate the likely range that a sample deviates from true lot content, provided that minimal stratification exists within the lot. Although good sampling plans attempt to account for potential heterogeneity within a bulk lot of grain, sampling continues to be a ubiquitous source of error in the quantitative estimation of the trait characteristics of a lot. Statistical properties of an acceptance sampling plan are characterized by the operating characteristic curve representing the acceptance probability and attempts to allay risks of both buyers and sellers (*8*).

Stratification occurs when segments of a lot have different concentrations of a characteristic trait. Stratification, in a sampling context, is not limited to a definition of horizontal layers in a lot, such as a barge or a bin. Stratification can be viewed as high and low concentrations in a flowing grain stream as the grain passes a sampling device. Stratification can also be viewed as changes in concentration from one end of a barge to another. The stratification characteristics of a lot will likely change every time a lot is moved from one container to another. Despite statistical models of sampling, it is unknown to what extent stratification affects the accuracy of this process. Furthermore, stratifications within a lot can contribute to poor precision and a lower degree of confidence in the final analytical result (*7*, *9*). Adopting appropriate sampling plans that are

* Author to whom correspondence should be addressed [telephone (816) 891-0442; fax (816) 891-7314; e-mail g.ronald.jenkins@usda.gov].

10.1021/jf070665l This article not subject to U.S. Copyright. Published 2007 by the American Chemical Society
Published on Web 06/23/2007

flexible and economical can ensure that the sample is an accurate representation of the lot and complies with regulatory mandates (*10*).

Biotechnology-derived traits are present within the marketplace in various commodities. The inadvertent release of unapproved biotechnology-derived traits has become an increasing concern to grain-exporting countries because of the deleterious consequences with international trade and the devaluation of the commodity. Such an incident typically requires extensive sampling and testing for the biotechnology-derived trait (*11*). Stratification within lots has been of concern to countries that import large quantities of rice from the United States, due to the inadvertent release of low levels of Liberty Link 601 (LL601) into its commercial supplies.

LL601 contains a 35S promoter and a phosphinothricin-*N*-acetyl transferase (PAT/*bar*). The *pat*/*bar* genes, isolated from *Streptomyces hygroscopicus* (*bar*) and *Streptomyces viridochromogenes* (*pat*), respectively, have been inserted into plants to encode PAT protein so that these plants can tolerate the herbicide glufosinate (*12*).

Currently, no studies exist that evaluate lot homogeneity of U.S. commercial rice and seed supplies containing LL601 infiltration or whether stratifications occur. In the United States, samples can be taken from flowing or static lots (*13*). Various probing techniques are used to sample grain from static lots. Depending on the size and shape of the container, multiple probes of the lot will be combined to obtain the sample from the lot (*14*−*16*). Patterns for probing a lot are prescribed for various types of containers (*13*, *17*). The individual probe samples are taken sufficiently close to effectively sample across almost any stratification that may exist. Flowing grain streams are most often sampled with a mechanical sampler that periodically cuts the grain stream (*13*, *18*). Some manual methods are also used to sample flowing streams (*13*, *17*, *18*). The manual methods attempt to mimic the periodic sampling of the grain stream that occurs with the mechanical sampler. Dividers such as the Boerner, Cargo, and Gamet have demonstrated the ability to subdivide a bulk sample and have the resulting samples conform to distributions expected from a random process (*13*, *14*, *18*). In the present study, we analyzed 20 individual samples in duplicate from 4 separate lots of long-grain rice that previously tested positive for the trait LL601, using quantitative real-time PCR. Statistical analysis of the qPCR data revealed no significant heterogeneity within four distinctive lots that were tested, and the sampling approach appeared to be an accurate representation of the lot.

MATERIALS AND METHODS

Sample Acquisition. The bins located in Stuttgart, AR, consisted of lot 1 (1.8×10^5 kg) and lot 4 (4.5×10^5 kg) of rough rice. Lots 2 and 3 were in containers that are commonly referred to as lash barges. These barges were rejected at a European port because an unspecified level of LL601 was detected. The rice in these lots was new-crop rice (crop year 2006/2007). Lots 1 and 4 contained 40% Cheniere and 60% Clearfield 131 as declared by the grower. In the process of drying and binning the rice, there was significant comingling of material throughout processing, prior to export. No additional blending was performed on the lots. U.S. Department of Agriculture (USDA) employees sampled the bins by performing bin transfer and sampling of the rice by an Ellis cup (*19*). Twenty samples of approximately 2000 g each, systematically derived from 20 different locations throughout the lot, were sent to the USDA Grain Inspection, Packers and Stockyards Administration, Technical Services Division (GIPSA-TSD), for analysis. The samples were taken at approximately equally spaced increments during the movement of the lot. The lash barges were sampled as static lots using a 12 ft probe and a specified pattern to obtain 20 probings

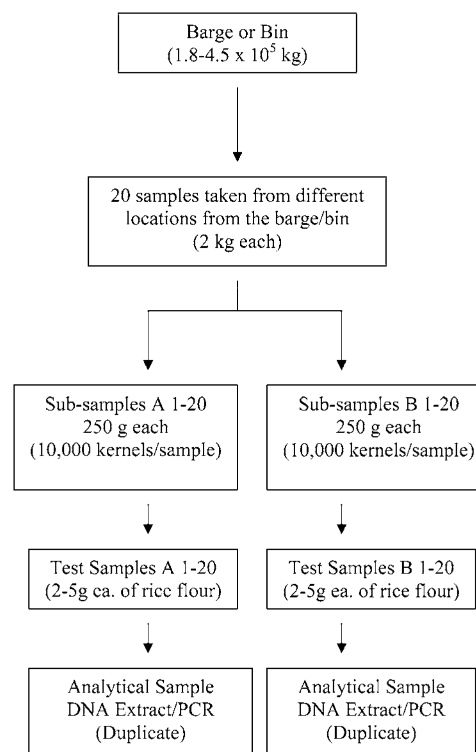

Figure 1. Schematic showing the procedure for sampling, grinding, extracting, and analyzing by qPCR.

from each lot (*15*). Two subsamples of approximately 10000 kernels (250 g) were cut from each bulk sample. Each of the 250 g subsamples were ground using an Osterizer blender for approximately 2 min or until the consistency of flour was obtained. The analytical sample contained 2−5 g of rice flour. DNA was extracted from the 2−5 g analytical samples of each 250 g replicate, and two PCR measurements were performed on each extraction. A schematic showing the procedure for sample processing is shown in **Figure 1**.

Pure LL601 seed, kindly provided by Bayer Crop Science, was ground to a fine powder, and DNA was extracted as described below. The extracted LL601 DNA was used as a positive control and to generate standard curves in subsequent qPCR experiments. Certified reference material for Liberty Link 62 (LL62) was obtained through the American Oil Chemists' Society (AOCS, Champaign, IL) (*20*) in the form of rice genomic DNA, derived from leaves (10 μg/vial).

DNA Isolation and Quantification. DNA was isolated from 2−5 g (test sample) of starting material of finely ground rice using a hexadecyltrimethylammonium bromide (CTAB) (Sigma-Aldrich, St. Louis, MO) extraction method (*21*). Briefly, rice flour was incubated in sterile water at 65 °C and CTAB buffer to lyse the cells. Following cellular lysis, an extended 60 min treatment with 50 μL of 10 mg/mL RNase A at 37 °C (Fermentas, Hanover, MD) followed by the addition of 100 μL of 31.5 mg of protein/mL of proteinase K (Sigma-Aldrich) was used to divest the DNA product of contaminating RNA and proteins. The resulting digests were twice extracted with chloroform to eliminate PCR-inhibiting polysaccharides and polyphenols, and then incubated in CTAB precipitation buffer at 25 °C to allow selective precipitation of DNA (*22*−*24*). After precipitation, the samples were resolublized into 175 μL of 0.5× TE (5 mM Tris and 0.5 mM EDTA) buffer and treated with 1 μL of RNase A at 37 °C for 1 h. An equal volume (175 μL) of 2.4 M NaCl was added to each sample, followed by a chloroform extraction and then ethanol precipitated overnight at −20 °C using twice the volume (700 μL) of 100% EtOH. After overnight precipitation, the samples were washed in 70% EtOH and dried in a vacuum microfuge (Eppendorf, Westbury, NY). The DNA pellets were dissolved in 50 μL of 0.5× TE, pH 8.0, buffer. The expected yield of DNA from rice flour was typically 5−15 μg of DNA from 2 g of starting material of rice flour. Typically, DNA stock samples

were solubilized in 50 μL of 0.5× TE buffer, pH 8.0, at a concentration of 100−300 ng/μL, and stored at 4 °C until further use (*25*).

DNA was quantified using a fluorometric assay with a TD-700 fluorometer instrument (Turner Biosystems, Inc., Sunnyvale, CA) in conjunction with a Quant-iT PicoGreen (PG) reagent kit (Invitrogen/ Molecular Probes, Eugene, OR). The PG reagent binds double-stranded DNA with high specificity (*26*, *27*). Stock DNA samples were diluted 1:250, 1:500, or 1:1000 with 1× TE buffer to a target concentration of 50−800 pg/μL. The PG reagent was prepared according to the manufacturer's protocol; the diluted DNA samples were mixed 1:1 with PG reagent to a final volume of 200 μL, to produce a 1:500, 1:1000 or 1:2000 dilution, and assayed in duplicate. A calibration curve was generated from λ phage DNA, supplied by the manufacturer at a stock concentration of 100 ng/μL, and diluted to 1000, 500, 250, and 0.0 pg/μL with 1× TE buffer. The calibration slope error ranged from 5 to 9% and was within the tolerance limit of 25% recommended by the instrument's manufacturer. Spectral processing was conducted using the TD-700 Hyperterminal Software Package. A 90% agreement between two diluents was required prior to the "acceptance" of an empirically determined concentration. The mean of replicates provided an "accepted" concentration for a specified sample. Stock DNA samples were diluted to a working concentration of 20 ng/μL.

Gel Electrophoresis. The integrity of the DNA extracts was determined by electrophoresis in an 0.8% agarose gel, stained with ethidium bromide (*28*). Approximately 100 ng of DNA, quantified by the aforementioned techniques, was added to each lane. λ phage was supplied in aqueous 1× TE solution. The presence of an intense, high molecular weight marker band, with minimal degradation, indicated high-integrity DNA with minimal RNA contamination.

Quantitative Real-Time PCR. The method as described consists of an event-specific, real-time quantitative *Taqman* PCR procedure for the content of LL601 relative to the *phospholipase* D (PLD) endogenous control gene (*29*). PLD is a rice-specific endogenous gene, and the method employs gene-specific primers and a sequence-specific 6-carboxyfluorescein/carboxytetramethylrhodamine (FAM/TAMRA) probe.

For amplification of the transgene, Bayer Crop Science provided GIPSA with long-grain (rough) rice seeds containing pure LL601 and an event-specific method for LL601 called "Real-time PCR for selected herbicide tolerant rice (HTR) for rice seed /grain samples" (*30*). The qPCR reactions for the target and reference genes were performed in separate wells (simplex). *Taq* DNA polymerase (Applied Biosystems, Foster City, CA) *TaqMan* Universal PCR Master Mix (containing passive reference ROX, 2× concentrate) and primers/probes were dissolved in sterile 0.5× TE to a concentration of 10 μM. The master mix contained *Taq* DNA polymerase at 1×, primers at 400 nM, and probe at 200 nM final concentrations. Nuclease-free water was added to adjust the final volume to 20 μL per reaction. Two separate master mix preparations were required (one for the endogenous control gene and one for the transgene) per plate using the method. qPCR products were measured during each cycle by means of a target-specific oligonucleotide probe, labeled with two fluorescent dyes: 6-FAM as a reporter dye at the 5′ end and TAMRA as a quencher dye at the 3′ end. All qPCR reactions were performed in either an ABI 7500 or ABI 7900 instrument (Applied Biosystems). Samples were heated to 95 °C for 10 min (activation of *Taq* DNA polymerase), cooled to 60 °C for 60 s (annealing/extension), and heated to 95 °C for 15 s (denaturation). Annealing/extension and denaturation steps were repeated for a total of 45 cycles.

Five microliter aliquots from DNA extracts (at 20 ng/μL) were loaded individually into a 96-well plate (Applied Biosystems), and each qPCR was performed in duplicate for each analytical sample. A standard curve was generated for the PLD endogenous control using 100 ng of pure LL601 DNA, serially diluted 1:10, 1:100, 1:1000, and 1:10000 in 0.5× TE, and analyzed in duplicate. The slope of the curve was between −3.05 and −3.20, and the linearity (R^2) was between 0.99 and 1.0. A standard curve was generated for the LL601 transgene using 10 ng of pure LL601 DNA that was serially diluted 1:10, 1:100, 1:1000, and 1:10000. The slope was between −3.05 and −3.40, and the linearity (R^2) was between 0.97 and 0.99. Primers and probes for both the reference-specific PLD and target-specific LL601 genes are shown below (*30*):

name	description	5′–3′ sequence
	LL601 Target Reaction	
SHA040	forward primer	TCT AGG ATC CGA AGC AGA TCG T
SHA041	reverse primer	GGA GGG CGC GGA GTG T
TM098	probe	6-FAM-CCA CCT CCC AAC AAT AAA AGC GCC TG-TAMRA
	Phospholipase D Reference System Reaction	
KVM159	forward primer	TGG TGA GCG TTT TGC AGT CT
KVM160	reverse primer	CTG ATC CAC TAG CAG GAG GTC C
TM013	probe	6-FAM-TGT TGT GCT GCC AAT GTG GCC TG-TAMRA

Qualitative Protein Test (Lateral Flow Strip). Lateral flow strips, specifically designed to detect the PAT protein in bulk rice samples, were purchased from Envirologix (Portland, ME). On the basis of the binomial probability distribution, when sampling a lot with a 0.1% concentration of LL601, a 3000 kernel sample has a 95% probability of having one or more LL601 seeds in the sample (*31*). A sample of 4500 kernels has approximately a 99% probability of having one or more LL601 kernels. Because the lateral flow strips were demonstrated to reliably detect at a 2% concentration level, larger samples had to be divided into smaller subsamples so that single seeds of LL601 could be detected. Sixty subsamples of 75 kernels each were obtained from the excess seeds of the first lot sampled. Sixty replicates of 75 kernels each were ground into a fine powder using a mortar and pestle. The average weight of an individual rice kernel was calculated to be 0.02 g. The flour from the 75 kernel sample was placed into an extraction vial that was provided by the manufacturer, and 2.25 mL of extraction buffer, included in the kit, was added. The strip was placed into the extraction vial at room temperature and allowed to develop for 10 min. Any clearly discernible pink test line was deemed to be a positive result.

Parboiled Rice. A simulated laboratory procedure for mimicking parboiling of rice included soaking four separate 10000 kernel samples of rice that were manually fortified with 0, 1, 4, and 10 kernels of LL601 rice, in excess water for 3.5 h at 71 °C. The 10000 kernel samples were then autoclaved for 10 min at 15 psi and 121 °C. The samples were dried for 6 h at 60 °C and the husks removed using a McGill sheller (Rapsilver, Brookeshire, TX). The dehusked rice was ground into a fine powder by cryogenically grinding the entire sample for 10 min in a Spex Certiprep 6800 freezer mill (Spex Certiprep, Inc., Metuchen, NJ). DNA was extracted from a 2−5 g sample as previously described.

Statistical Analysis. A standard deviation among all subsamples within a lot was compared with the pooled standard deviation among replicate PCR measurements. Method variability was assessed by estimating the pooled standard deviation among duplicate PCR measurements on the same analytical sample within the lot (*32*, *33*).

RESULTS

Evaluating LL601 Method Specificity. GIPSA non-transgenic rice samples, derived from long-grain rice, were obtained from GIPSA file samples and used as the control blank. LL62 and LL601 rice varieties contain similar gene constructs that confer resistance to glufosinate but express different levels of protein. Both constructs express the same PAT protein, derived from the *bar* gene, and have nearly identical DNA sequences, but their integration sites are novel. Therefore, specificity studies were performed on long-grain rice samples that were fortified with either 1% LL62 or 1% LL601 or nonfortified. Each sample was amplified as a simplex format using (i) event-specific LL601 primers/probe and (ii) endogenous control PLD primers/ probe. Specificity testing resulted in suitable amplification of both the PLD endogenous control and taxon-specific (LL601) PCR products for the 1% LL601 gravimetrically fortified samples. As expected, the 1% LL62 sample exhibited suitable

Table 1. Fortification Levels of GIPSA and Bayer Crop Science Supplied Samples

sample	level of LL62 (%)	level of LL601 (%)	PCR result (LL601-specific method)
non-transgenic (GIPSA)	0.0	0.0	negative
1% LL62 (GIPSA)	1.0	0.0	negative
1% LL601 (GIPSA)	0.0	1.0	positive
P1 (Bayer Crop Science)	0.0	0.02	positive
P2 (Bayer Crop Science)	0.01	0.0	negative
P3 (Bayer Crop Science)	0.1	0.0	negative
P4 (Bayer Crop Science)	0.0	0.03	positive

amplification of the PLD endogenous control, but no amplification for the taxon-specific product was detected (**Table 1**). Furthermore, Bayer Crop Science provided GIPSA with samples P1, P2, P3, and P4 that contained 0.01−0.10% LL62 or 0.02−0.03% LL601 (**Table 1**). Samples P1 and P4 contained LL601, whereas samples P2 and P3 contained LL62. LL601 was detected in samples P1 and P4, whereas samples P2 and P3 did not amplify for the LL601 transgene, indicating specificity in the outlined method. These data demonstrated that the primers and probe used in this study distinguished between LL601 and LL62.

Evaluating LL601 Method Sensitivity (Limit of Detection, LOD). From three separate experiments, a LOD for the method was empirically estimated. First, 100% LL601 DNA was serially diluted into non-transgenic long-grain rice DNA at levels between 1.0 and 0.00%. The total rice DNA content was maintained at 100 ng per reaction and PCR amplified in triplicate. All three replicates at the 0.01% level met the criteria as described in the subsequent section for detection using the qPCR method. Second, 20 rice flour samples gravimetrically fortified to 0.01% (w/w) were analyzed, and 20 of 20 samples provided detections according to the criteria as outlined in the method. Two nonfortified control samples provided nondetection according to the criteria. Finally, a single kernel of LL601 was combined with 10000 non-transgenic kernels (i.e., 0.01%) and treated by a laboratory-simulated parboiling procedure. The 10000 kernel sample was dehusked and ground to a fine powder. DNA was extracted, and a 100 ng sample was used in qPCR to assess the sensitivity of the method. Sensitivity testing, performed in triplicate, resulted in suitable amplification of both the PLD endogenous control and taxon-specific LL601 qPCR products at the 0.01% LL601 level for parboiled rice samples. The reagent blank did not amplify for either the PLD endogenous gene or the LL601 specific gene. Non-transgenic control samples amplified sufficiently for the PLD endogenous control, but the LL601 transgene was not detected. Collectively, these data suggested that a 0.01% LOD was appropriate for the method. These data are summarized in **Table 2**.

LL601 Homogeneity. The lots were chosen to be analyzed because they were previously identified as containing LL601. To obtain an indication of the prevalence of LL601 infiltration and whether stratification occurrence is a routine phenomenon, samples from four distinctive lots were collected and analyzed for the presence of LL601 using qPCR as previously described. To properly assign a qPCR result, the following criteria were implemented: (i) LL601 detection = acceptable amplification of the PLD endogenous control gene [crossing threshold (Ct) value of 16.0−25.9] and acceptable amplification of the LL601 target gene (Ct value of 27.8−39.5); (ii) LL601 nondetection = acceptable amplification of the PLD endogenous control gene (Ct value of 16.0−25.9) and unacceptable amplification of the LL601 target gene (Ct value of >39.5); (iii) LL601 inconclusive = unacceptable amplification of the PLD endogenous control gene (Ct value of >25.9) and unacceptable amplification of the LL601 target gene (Ct value of >39.5). All detect and nondetect results were assigned a quantitative result based upon Ct values in comparison with a 100% LL601 reference (20 ng/μL DNA), serially diluted to generate a standard curve as described under Materials and Methods.

For purposes of evaluating the homogeneity of each lot, all valid measurements were retained. It is understood that some of these measurements were below an estimated LOD and may represent true positives or false positives. In either case, this additional variation was included in the statistical analysis. Inconclusive results were not included in subsequent statistical analyses. The first lot tested, lot 1, was a bin of long-grain rough rice. All 40 analytical samples from lot 1 were assessed as detects for the presence of LL601 with acceptable endogenous control amplification of the PLD gene (Ct_{endo} = 17.5−26.9, Ct_{trans} = 30.2−36.8). The data were analyzed as a one-way classification or one-factor experiment (*32*). Statistically declared differences among samples imply that samples were taken from different strata within the lot. A p value of ≤ 0.05 is needed to declare statistical significance and would suggest significant differences among the samples. **Table 3** provides an average calculated estimate of LL601 rice for individual analytical samples (analyzed in duplicate). Each alphanumeric result is the average of two qPCR reactions (i.e., analytical sample 1A, $n = 2$; 1B, $n = 2$, etc.). The test for differences among the samples had a p value of 0.61. As shown in analysis of variance (see **Table 4**), no differences among samples were declared. Therefore, the lot stratification was statistically insignificant and considered to be homogeneous.

The second lot, lot 2, consisted of a lash barge of long-grain brown rice. The lot was sampled with a probe using a systematic 20 point probe pattern (*15*). One analytical sample was deemed to be inconclusive, with low amplification for the endogenous control PLD gene (Ct_{endo} = 36.5, Ct_{trans} > 40.0). This datum was not included in the statistical analyses. Twenty-nine of 39 analytical samples were evaluated as nondetects for LL601 (Ct_{endo} = 16.3−20.4, Ct_{trans} > 40.0). Nine analytical samples tested as detects (Ct_{endo} = 18.0−20.4, Ct_{trans} = 30.9−39.5). The observed low levels of LL601 created a more challenging assessment of homogeneity because the data probably do not have a normal distribution. Skewed distributions can potentially produce too many significant test results or p values that are <0.05 (*33*). Because the associated statistical test was not significant, the conclusion that no differences exist among samples was considered to be a conservative estimate. A best approximation of a p value for differences among samples in lot 2 was determined to be 0.177 (see **Table 4**) and >0.05, suggesting homogeneity exists in lot 2. An analysis of variance for testing differences among samples from lot 2 was performed on the basis of a best approximation using statistics that were generated from the qPCR method. Regardless of the limitations of estimating a p value calculation, the qPCR results showed with 95% confidence that the LL601 content in lot 2 was between 0.00 and 0.018%.

The third lot tested, lot 3, was also a lash barge of long-grain brown rice. The lot was sampled in the same manner as the previous lash barge. Three analytical samples provided unacceptably low amplification for the PLD endogenous control gene (Ct_{endo} = 25.9−>40.0, Ct_{trans} > 40) and thus were eliminated from the analysis of variance calculations. Ten of 37 analytical samples were assessed as nondetects (Ct_{endo} = 19.1−21.7, Ct_{trans} > 40). Twenty-seven analytical samples tested as detects (Ct_{endo}= 16.21−21.5, Ct_{trans} = 29.1−36.4). Similar

Table 2. Estimation of Limit of Detection for LL601 Rice

serially diluted DNA sample ($n = 3$)	endogene (PLD) +	endogene (PLD) −	transgene (LL601) +	transgene (LL601) −	parboiled DNA sample ($n = 3$)	endogene (PLD) +	endogene (PLD) −	transgene (LL601) +	transgene (LL601) −	0.01% gravimetric sample	endogene (PLD) +	endogene (PLD) −	transgene (LL601) +	transgene (LL601) −
1% LL601	3	0	3	0	1% LL601	3	0	3	0	0.01% LL601 ($n = 20$)	20	0	20	0
0.1% LL601	3	0	3	0	0.1% LL601	3	0	3	0					
0.01% LL601	3	0	3	0	0.05% LL601	3	0	3	0	0.0% LL601 ($n = 2$)	2	0	0	2
0.005% LL601	3	0	1	2	0.01% LL601	3	0	3	0					
0.00% LL601	3	0	0	3	0.00% LL601	3	0	0	3					

Table 3. Representative Quantitative LL601 Measurements for 20 Analytical Samples (Lot 1)[a]

sample	A measurement	Ct ($n = 2$) endo	Ct ($n = 2$) trans	B measurement	Ct ($n = 2$) endo	Ct ($n = 2$) trans
1	0.105	20.28	30.10	0.088	20.83	30.90
2	0.024	21.21	33.47	0.021	22.90	35.30
3	0.072	21.07	31.52	0.051	21.09	32.05
4	0.101	20.50	30.40	0.056	20.26	31.04
5	0.092	20.50	30.54	0.064	20.45	31.04
6	0.157	20.93	30.17	0.087	20.69	30.83
7	0.103	21.46	31.37	0.032	21.45	33.16
8	0.078	22.41	32.78	0.100	20.15	30.05
9	0.057	20.61	31.37	0.050	20.89	31.86
10	0.069	22.03	32.18	0.071	21.09	31.19
11	0.094	21.77	31.47	0.073	20.53	30.58
12	0.102	20.60	30.20	0.040	20.47	31.43
13	0.065	21.72	31.97	0.140	21.27	30.43
14	0.034	22.08	33.27	0.077	21.36	31.34
15	0.128	20.95	30.18	0.045	22.19	32.94
16	0.084	22.47	32.32	0.036	22.13	33.24
17	0.089	26.96	36.76	0.069	17.54	29.42
18	0.076	23.28	33.29	0.057	23.49	33.91
19	0.066	21.15	31.78	0.072	22.24	32.71
20	0.038	21.69	33.11	0.094	24.70	34.03

[a] Each measurement was obtained from the mean of two qPCR results.

Table 4. Analysis of Variance for Testing Differences among Samples on Lots 1–4

lot	source of variation	SS	df	MS	*F*	*p* value
1	between samples	0.016345	19	0.000860	0.88	0.6079
	within sample	0.019545	20	0.000977		
	total	0.035890	39			
2	between samples	0.015048	19	0.000792	1.54	0.1765
	within sample	0.009755	19	0.000513		
	total	0.024803	38			
3	between samples	0.009941	19	0.000523	1.75	0.125259
	within sample	0.005077	17	0.000299		
	total	0.015018	36			
4	between samples	0.044514	19	0.002343	1.78	0.105085
	within sample	0.026347	20	0.001317		
	total	0.070862	39			

to lot 2 results, the observed low levels of LL601 created a more challenging assessment of homogeneity because the data were probably not normally distributed. As shown in **Table 4**, the *p* value for the test of differences among samples was 0.125 and >0.05, suggesting homogeneity existed in lot 3. The qPCR results showed with 95% confidence that the content of LL601 in lot 3 was between 0.008 and 0.020%.

The fourth lot tested, lot 4, was a bin of long-grain rough rice. This lot was sampled in the same manner as lot 1. All 40 analytical samples from lot 4 provided detects for the presence of LL601 ($Ct_{endo} = 18.9-26.6$, $Ct_{trans} = 28.5-37.3$). The test for differences among the samples had a *p* value of 0.105. As shown in the analysis of variance (see **Table 4**), no differences among samples were declared. Therefore, the lot stratification was shown to be statistically insignificant and considered to be homogeneous.

Table 5. Average Lot Content and Standard Deviations for Each Lot As Estimated by qPCR

lot	av	pooled standard deviation: all subsamples[a]	within sample[b]	method[c]
1	0.074	0.031	0.031	0.017
2	0.008	0.026	0.023	0.017
3	0.014	0.020	0.017	0.004
4	0.054	0.043	0.036	0.042

[a] Standard deviation among all subsample measurements within a lot ($n = 40$). [b] Pooled standard deviation among subsamples within a sample. [c] Pooled standard deviation among duplicate PCR measurements on the same analytical samples within a lot.

Lateral Flow Strip Testing. The qPCR data were corroborated on a single sample from lot 1 (for which qPCR revealed a level of 0.074% LL601) using lateral flow strip technology. Testing was conducted as described under Materials and Methods. Detecting the presence of LL601 at the 0.1% detection level with a 95% confidence required 60 replicate analyses from a subsample (i.e., 10000 kernels) that was divided into 75 rice kernels per subsample. The data revealed that 3 of 60 samples (or a minimum of 3/4500 kernels) tested positive for the LL601 trait. The estimated probability of a positive is 5% (3/60). A lot concentration of 0.068% has a 5% probability of testing positive with a 75 kernel sample. The 95% confidence interval for the estimated probability of a positive, based on 60 tests, is from 1.8 to 13.9%. The corresponding lot concentrations for the confidence interval are from 0.024 to 0.199% (*34*). The qPCR results clearly fall within the expected range of these data (*34*).

Concentration of LL601 in Lots 1−4. Table 5 provides a summary of the average concentration and standard deviation of LL601 in lots 1−4. The concentration of LL601 ranged between 0.008 and 0.074%. Two of the four lots contained average concentration levels that were in close proximity to the LOD for the method. Because no heterogeneity was confirmed within the lots, the standard deviation for samples was computed across all subsample measurements for a lot. A pooled standard deviation for the variability among PCR measurements on replicates was computed and is shown in **Table 5** as method variability.

DISCUSSION

Innovations and production of biotechnology-derived crops offer potential to provide a safe, wholesome, and unadulterated food product for future generations. As life science companies continue to develop biotechnology-derived traits in grains and oilseeds to provide a more manageable crop for farmers and food processors, an international consensus on sampling and

testing procedures is necessary to demonstrate compliance with distinctive regulatory mandates for each individual importing country. This continues to be a major challenge for researchers, growers, producers, manufacturers, regulators, and legislators. Organizations within the scientific community, including government and manufacturers, have a responsibility to ensure a premiere quality food supply, maintain consumer confidence, and educate the public as the biotechnology industry advances these products.

This study evaluated the homogeneity of four unique lots of rice that previously tested positive for LL601. When random samples are not practical, a sampling procedure known as systematic sampling is often used. The sample is taken by advancing through the lot systematically and selecting items on equal intervals (*13*, *18*). The diverter sample is very similar to the systematic sample. Systematic samples are usually assumed to be equivalent to random samples (*13*, *17*, *18*). If kernels are randomly distributed in the lot, the systematic sample is a random sample. When kernels are randomly distributed throughout the lot, almost any method of sampling will produce a random sample. Concern arises when the lot may not be thoroughly mixed (*35*).

For detection purposes, PCR has distinct advantages over protein-based testing in that DNA-based methods can distinguish between two traits (i.e., LL601 vs LL62), irrespective of the fact that they express the same protein and contain nearly identical inserted gene sequences. European Union regulations require detailed information concerning the specificity and competency of PCR-based methods that are used in the detection and quantification of biotechnology-derived traits in grains and oilseeds (*2*, *36*). Clearly, detecting these biotechnology-derived traits requires not only primer/probe pairs and DNA with an appropriate method but also internationally recognized reference materials as calibrants (*16*, *37*). The quantitative assays performed in this study consist of primers and probe that show high specificity for LL601.

The bin lots comprised rice suspected to have LL601 contamination. One of the assumptions underlying the test in the analysis of variance is that the data are normally distributed. With many samples assessed as nondetects in lots 2 and 3, the assumption of normality was not ideal using statistical analysis. However, even without normality, the F test can often provide a reasonable approximation for equality among the samples (*33*). Because the p value was significantly >0.05, the conclusion of no differences among samples was still reasonable. These variability data were consistent with the conclusion that insignificant stratifications existed within these four commercial lots of long-grain rice. Thus, a systematic sampling plan such as those recommended by USDA-GIPSA and referenced in this paper was a practical and appropriate approach to estimating the content of biotechnology-derived traits in these four lots. Any observed differences among sample results were due to random effects. No study of a limited number of lots can ensure that stratification will not occur in some U.S. commercial lots. This study suggested that, for the lots that were sampled, significantly different strata do not exist. The sampling procedure was shown to be unbiased because the average of all possible sample estimates was essentially equivalent to the lot content. These results are likely to be useful for developing sampling plans, thereby facilitating international trade.

SAFETY

Ethidium bromide is a known human carcinogen, and proper precautions should be utilized during the use and disposal of this reagent.

ABBREVIATIONS USED

qPCR, quantitative PCR; Ct, crossing threshold; *bar*/*pat*, phosphinothricin-*N*-acetyl transferase; USDA-GIPSA-TSD, U.S. Department of Agriculture Grain Inspection, Packers and Stockyards Administration, Technical Services Division; PLD, phospholipase D; LL601, Liberty Link 601; LL62, Liberty Link 62; FAM/TAMRA, 6-carboxyfluorescein/carboxytetramethylrhodamine; LOD, limit of detection; CTAB, hexadecyltrimethylammonium bromide; EU, European Union; HTR, herbicide-tolerant rice; df, degrees of freedom; PG, PicoGreen reagent; SS, sum of squares; MS, mean squares; endo, endogenous; trans, transgene.

ACKNOWLEDGMENT

We thank Ray Shilito with Bayer Crop Science for providing pure LL601 rough rice, Eddie Clark and Lynn Polston for technical assistance, and Steve Tanner, Don Kendall, and Dave Funk for editorial advice.

LITERATURE CITED

(1) Namuth, D. J. R. Real-time PCR—some basic principles. *J. Nat. Resour. Life Sci. Educ.* **2005**, *34*, 124−125.

(2) 1138/98 ACREN. Commission Regulation (EC), 2000; pp 13−14.

(3) Urbanek-Karlowska, B.; Jedra, M.; Badowski, P. Detection of genetic modification in maize and maize products by ELISA-test. *Rocz. Panstw. Zakl. Hig.* **2003**, *54* (4), 345−353.

(4) Grothaus, D. G.; Bandla, B.; Currier, T.; Giroux, R.; Jenkins, G. R.; Lipp, M.; Shan, G.; Stave, J. W.; Pantella, V. Immunoassay as an analytical tool in agricultural biotechnology. *J. AOAC Int.* **2006**, *89* (4), 913−928.

(5) Ciabatti, I.; Gatto, F.; Amaddeo, D.; Marchesi, U. In-house validation and quality control of real-time PCR methods for gmo detection: a practical approach. *Dev. Biol.* **2006**, *126*, 79−86.

(6) Lipp, M.; Anklam, E.; Stave, J. W. Validation of an immunoassay for detection and quantification of a genetically modified soybean in food and food fractions using reference materials. *J. AOAC Int.* **2000**, *83* (4), 919−927.

(7) Lipton, C. R.; Dautlick, J. X.; Grothaus, G. D.; Hunst, P. L.; Magin, K. M.; Mihaliak, C. A.; Rubio, F. M.; Stave, J. W. Guidelines for the validation and use of immunoassays for determination of introduced proteins and biotechnology enhanced crops and derived food ingredients. *Food Agric. Immunol.* **2000**, *12*, 153−164.

(8) Kobilinsky, Y. B. Minimum cost acceptance sampling plans for grain control, with application to gmo detection. *Chemom. Intell. Lab. Syst.* **2005**, *75*, 189−200.

(9) Ahmed, F. E. Detection of genetically modified organisms in foods. *Trends Biotechnol.* **2002**, *20* (5), 215−223.

(10) Whitaker, T. B.; Freese, L. F.; Giesbrecht, F. G.; Slate, A. B. Sampling grain shipments to detect genetically modified seed. *J. AOAC Int.* **2001**, *84* (6), 1941−1946.

(11) Paoletti, C.; Heissengerber, A.; Mazzara, M.; Larcher, S.; Grazioli, E.; Corbisier, P.; Hess, N.; Berben, G.; Lubeck, P. S.; De Loose, M.; Moran, G.; Henrey, C.; Brera, C.; Folch, I.; Ovesna, J.; Van den Eede, G. Kernel lot distribution assessment (KELDA): a study on the distribution of gmo in large soybean shipments. *Eur. Food Res. Technol.* **2006**, *224* (1), 129−139.

(12) Tan, S.; Singh, B. Herbicidal inhibitors of amino acid biosynthesis and herbicide tolerant crops. *Amino Acids* **2006**, *30* (2), 195−204.

(13) USDA Grain Inspection Packers and Stockyards Administration. Grain Inspection Handbook 1; 1995; available at http://www.usda.gov.

(14) Grain Inspection Packers and Stockyard. Grain Sampling Procedures; 2001; available at www.gipsa.usda.gov/GIPSA/documents/GIPSA_Documents/sampling.pdf.

(15) American National Standard Institute. ISO, 13690 Cereals, pulses and milled products—sampling of static batches; 1999.
(16) Trapmann, S.; Schimmel, H.; Kramer, G. C.; Van Den Eede, G.; Pauwels, J. Production of certified reference materials for the detection of genetically modified organisms. *J. AOAC* **2002**, *85* (3), 775–779.
(17) Ahmed, F. E. *Testing of Genetically Modified Organisms in Foods*; Food Products Press: New York, 2004; Vol. 1, pp 324–350.
(18) Lischer, P. Sampling procedures to determine the proportion of genetically modified organisms in raw materials. Part II. *Mitt. Lebensm. Hyg.* **2001**, *92*, 305–311.
(19) Grain Inspection Packers and Stockyards. Sampling for the Detection of Biotech Grain; available at http://archive.gipsa.usda.gov/biotech/sample2.htm.
(20) Trapmann, S. H.; Kramer G. N.; Van den Eede, G.; Pauwels, J. Production of certified reference materials for the detection of genetically modified organisms. *J. AOAC Int.* **2002**, *85* (3), 775–779.
(21) Majchrzyk, P. In *Current Protocols in Molecular Biology*, *Unit 2.4*; J.W.A. Sons: 2002.
(22) Demeke, T.; Adams, R. P. The effects of plant polysaccharides and buffer additives on PCR. *BioTechniques* **1992**, *12*, 332–334.
(23) Wilson, I. G. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **1997**, *63*, 3741–3751.
(24) Do, N.; Adams, R. P. A simple technique for removing plant polysaccharide contaminants from DNA. *BioTechniques* **1991**, *10*, 162–164.
(25) Arumuganathan, K.; Earle, E. D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep.* **1991**, *9*, 208–218.
(26) Georgiou, C. D. Assay for the quantification of intact/fragmented genomic DNA. *Anal. Biochem.* **2006**, *358* (2), 247–256.
(27) Singer, V. L.; Jones, L. J.; Yue, S. T.; Haugland, R. P. Characterization of picogreen reagent and development of a fluorescence-based solution assay for double stranded DNA quantitation. *Anal. Biochem.* **1997**, *249*, 228–238.
(28) Sambrook, R. A. *Molecular Cloning, A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbory, NY, 2001.
(29) Ueki, J.; Komari, T.; Kumashiro, T. Purification and characterization of phospholipase D (PLD) from rice (*Oryza sativa* L.) and cloning of cDNA for PLD from rice and maize (*Zea mays* L.). *Plant Cell Physiol.* **1995**, *36* (5), 903–914.
(30) Bayer Crop Science. http://gmo-crl.jrc.it/LLRice601update.htm.
(31) http://www.seedtest.org/en/content---1--1143.html Volume.
(32) Box, G. E. P.; Hunter, W. G.; Hunter, J. S. *Statistics for Experimenters*, 1st ed.; Wiley: New York, 1978.
(33) Snedecor, G. W.; Cochran, W. G. *Statistical Methods*, 6th ed.; Iowa State University Press: Ames, IA, 1967.
(34) Remund, K.; Dixon, D. A.; Wright, D. L.; Holden, L. R. Statistical considerations in seed purity testing for transgenic traits. *Seed Sci. Res.* **2001**, *11* (2), 101–120.
(35) Bayer Crop Science. Application for an extension of the determination of nonregulated status for glufosinate-tolerant rice (98-329-01p): transformation event LLRICE601; 2006.
(36) Amending Council Regulation (EC) No 1138/98 concerning the compulsory indication on the labeling of certain foodstuffs produced from genetically modified organisms of particulars other than those provided for in directive 79/112/EEC. *Off. J. Eur. Communities* **2000**.
(37) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Nobutaro, A.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC* **2002**, *85* (5), 1077–1089.

Received for review March 7, 2007. Revised manuscript received May 15, 2007. Accepted May 20, 2007.

JF070665L