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Evaluating Precision and Accuracy When Quantifying Different Endogenous Control Reference Genes in Maize Using Real-Time PCR

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The agricultural biotechnology industry routinely utilizes real-time quantitative PCR (RT-qPCR) for the detection of biotechnology-derived traits in plant material, particularly for meeting the requirements of legislative mandates that rely upon the trace detection of DNA. Quantification via real-time RT-qPCR in plant species involves the measurement of the copy number of a taxon-specific, endogenous control gene exposed to the same manipulations as the target gene prior to amplification. The International Organization for Standardization (ISO 21570:2005) specifies that the copy number of an endogenous reference gene be used for normalizing the concentration (expressed as a % w/w) of a trait-specific target gene when using RT-qPCR. For this purpose, the copy number of a constitutively expressed endogenous reference gene in the same sample is routinely monitored. Real-time qPCR was employed to evaluate the predictability and performance of commonly used endogenous control genes (starch synthase, SSIIb-2, SSIIb-3; alcohol dehydrogenase, ADH; high-mobility group, HMG; zein; and invertase, IVR) used to detect biotechnology-derived traits in maize. The data revealed relatively accurate and precise amplification efficiencies when isogenic maize was compared to certified reference standards, but highly variable results when 23 nonisogenic maize cultivars were compared to an IRMM Bt-11 reference standard. Identifying the most suitable endogenous control gene, one that amplifies consistently and predictably across different maize cultivars, and implementing this as an internationally recognized standard would contribute toward harmonized testing of biotechnology-derived traits in maize.

KEYWORDS: Endogenous control; maize; quantitative PCR; ADH; SSIIb; zein; IVR; HMG

INTRODUCTION

Agricultural biotechnology has become an important venue for developing crop plants that are insect resistant, herbicide tolerant, and disease resistant and offer prospects of improved human and animal health. However, public acceptance of these genetically engineered (GE) crop varieties remains controversial. While the advantages of agricultural biotechnology continue to be debated, GE crops are becoming more common in both domestic and global commerce. Some government agencies, especially in Europe and Asia, have adopted legislative mandates requiring labeling when GE food products exceed specified threshold levels (*1*). These mandates significantly increase the complexity of trading grains and oilseeds in global markets, creating challenges for both exporting and importing countries. To comply with regulations and properly manage risks, both buyers and sellers must be able to accurately detect and measure amounts of GE traits present in raw commodities and finished food products. Although protein-based technologies are available, real-time quantitative PCR (RT-qPCR) is the most commonly used method for sensitive and accurate quantification to demonstrate compliance with regulatory mandates regarding GE traits in agricultural products (*2*−*5*).

An amplification profile from RT-qPCR is generated using a highly specific, fluorescent dye-based chemistry (*Taqman*) that, in effect, produces a lag phase, an exponential phase, and a plateau phase during the course of the reaction (*6*−*8*). Quantification occurs during the exponential phase of amplification when the fluorescence signal exceeds the detection threshold value, commonly referred to as a crossing threshold cycle (C_t). At an appropriate point in the exponential phase of amplification, C_t values are assigned to both reference standards and test samples. Using the relative standard curve method, analytical measurements on test samples are determined by interpolation (*9*). Because measurements are generated during the exponential phase of amplification, small fluctuations in C_t values translate into large variability in the final analytical results. Real-time qPCR theory stipulates that a measured ΔC_t value of "1"

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corresponds to a 2-fold difference in starting amount of DNA (i.e., $2^{\Delta C_t}$) (*10*).

European Union (EU) regulation 1829/2003 (Genetically Modified Food/Feed) mandates that life science companies submit RT-qPCR methods to the Community Reference Laboratory (CRL) for method validation through the Joint Research Commission (JRC). The CRL validates these methods through interlaboratory validation studies and, upon completion, makes dossiers available for public scrutiny. By following these methods, the content of a GE trait is quantified in percentage of trait DNA copy number in relation to a target taxon-specific DNA copy number, calculated in terms of haploid genomes (*11*). Because of a large number of both single and stacked transgenic traits registered for maize, a plethora of taxon-specific endogenous control reference genes have been validated by the JRC for trait-specific testing by RT-qPCR.

Quantification via RT-qPCR in plant species involves the measurement of the copy number of an endogenous control gene exposed to the same manipulations as the target gene prior to amplification (*12*). The International Organization for Standardization (ISO) specifies that the copy number of an endogenous reference gene in a sample must be used for normalizing the copy number of a trait-specific target gene when using RT-qPCR (*13*). For this purpose, the expression of a constitutively expressed endogenous gene (housekeeping gene or maintenance gene) in the same sample is routinely monitored. Detection of an endogenous control gene must be sensitive, accurate, reproducible, and species-specific and should amplify consistently across different cultivars. Due to the limited number of transgenic traits commercialized in grains, with the exception of maize, the JRC utilizes only one endogenous control gene for interlaboratory validation studies of most grains (*1, 13*).

Many cultivated crop plants contain a high number of gene duplications as a result of the breeding process. For example, the copy number of the zein gene can vary between different maize cultivars up to 15-fold (*14*). If the genome size of the target taxon is known and the DNA content in a pure DNA sample can be precisely determined, then a sample containing any relative percentage can be mixed by combining DNA from a non-GE plant specimen with a GE plant specimen, and the absolute copy numbers of the target taxon and GE-specific sequences can be estimated reliably in these samples (*15*). Estimates of genome sizes are available (*16*) and quantitative methods frequently refer to these estimates. However, as in the case of the zein endogenous control gene (and many other genes), the estimates are sometimes given as ranges, in which case an analytical uncertainty is introduced if genome size is a parameter in the quantification equation (*14, 15*). Reports identify at least five taxon-specific endogenous control reference genes for maize including alcohol dehydrogenase-1 (ADH), high-mobility group a (HMG), invertase-1 (IVR), zein, and starch-synthase-IIb (SSII-b), along with combinations of different primer/probe systems for several of these endogenous reference gene systems (*12, 17*−*19, 21, 22*). Some studies show that endogenous reference genes amplify inconsistently across different maize cultivars (*12*). The ADH gene, for example, shows a 2.9 cycle variation when C_t values of different maize cultivars are compared, possibly due to dissociation-induced mutants in this gene (*12, 20*). This difference in C_t values could conceivably translate into nearly a 10-fold difference in the final analytical result. These findings suggest that amplification efficiencies of different endogenous reference systems are dissimilar and thus cannot be used interchangeably among different maize cultivars with any substantial degree of confidence when analytical measurements are generated by RT-qPCR. Conversely, Shokere et al. reports highly predictable C_t values for the SSIIb-3 endogenous control reference gene, when six separate DNA extracts of Institute for Reference Materials and Measurements (IRMM) reference materials quantified by two fluorescent dye methods were used in RT-qPCR (using 50 ng of DNA per reaction with a mean C_t of 26.11 ± 0.10) (*23*). The complexity of detecting genetically engineered traits in maize continues to grow as new varieties are introduced into the marketplace. To avoid ambiguities and reduce the amount of experimental data that needs to be generated for method validation processes, the selection of a limited number of primer/probe reference systems for a maize endogenous control gene becomes paramount, especially in instances where stacked traits, from a number of different GE traits, have been developed. By assessing method variability, a mean quantitative value and the degree to which analytical measurements in a distribution are dispersed or clustered together can be determined. As a first step to identify variability among different endogenous control reference systems, this study evaluated the precision and accuracy of different primer/probe systems against IRMM reference materials and 23 different *Zea mays* lines. The primer/probe systems in this study included ADH, HMG, SSIIb-2, SSIIb-3, IVR, and zein.

MATERIALS AND METHODS

Sample Source. Finely ground certified reference maize flour (Sigma Aldrich, St. Louis, MO) consisted of individual vials of Bt-11, Bt-176, GA21, Mon810, Mon863, and NK603 fortified at 0.0−5.0% (% w/w). Cargill experimental samples consisted of 23 distinctive cultivars including waxy and dent varieties of identity preserved maize from the 2007 corn harvest that were kindly provided by Cargill (Minneapolis, MN). Additionally well-characterized Pioneer negative corn samples (PNC) that are distributed to participants in the GIPSA proficiency program were used in this study. These samples have been well characterized both internally and externally and shown to be negative for all GE traits currently on the marketplace. All maize samples were stored at 4 °C until used in the DNA extraction procedure.

DNA Extraction. DNA was isolated from approximately 200 mg of starting material of finely ground IRMM reference material or experimental samples using a hexadecyltrimethylammonium bromide (CTAB) (Sigma Aldrich) extraction method (*23, 24*). Briefly, maize flour was incubated in 700 μL of 1× CTAB extraction buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 M Tris, 20 mM Na_2EDTA, pH 8.0) and 300 μL of water at 65 °C to lyse the cells. Following cellular lysis, an extended 60 min treatment with 20 μL of RNase A at 37 °C (Fermentas, Hanover, MD), followed by a 20 μL proteinase K (Sigma Aldrich) digest was performed to divest the DNA product of contaminating RNA and proteins. The resulting digests were extracted with chloroform twice to eliminate PCR-inhibiting polysaccharides and polyphenols and were incubated in 1× CTAB precipitation buffer (5 g/L CTAB, 0.04 M NaCl, pH 8.0) at 25 °C overnight to allow selective precipitation of DNA (*25*−*27*). After precipitation, the samples were resolubilized into 175 μL of 0.5× TE (5 mM Tris and 0.5 mM EDTA) buffer and treated with 10 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 the sample was ethanol-precipitated overnight at −20 °C using twice the volume (700 μL) of 100% EtOH (Sigma Aldrich). After overnight precipitation, the samples were washed with 500 μL of 70% EtOH and dried in a vacuum microfuge (Eppendorf, Westbury, NY). The DNA pellets were dissolved in 100 μL of 0.5× TE, pH 8.0, buffer. The expected yield from maize flour was generally 20−50 μg of DNA from 200 mg of starting material of maize flour. Typically, DNA stock samples were solubilized in 100 μL of 0.5× TE buffer, pH 8.0, at a concentration of 200−300 ng/μL and stored at 4 °C until further use (*16*).

Table 1. Primers and Probe Sequences for the Taxon-Specific Endogenous Control Reference Genes

reference system name	endogenous control reference gene primer/probe sequence	amplicon (bp)
high-mobility group (HMG)	forward, 5′-TTGGACTAGAAATCTCGTGCTGA-3′ reverse, 5′-GCTACATAGGGAGCCTTGTCCT-3′ probe, 5′-FAM-CAATCCACAAACGCACGCGTA-TAMARA-3′	79
starch synthase (SSIIb-3)	forward, 5′-CCAATCCTTTGACATCTGCTCC-3′ reverse, 5′-GATCAGCTTTGGGTCCGGA-3′ probe, 5′-FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMRA-3′	114
invertase (IVR)	forward, 5′-CGCTCTGTACAAGCGTGC-3′ reverse, 5′-GCAAAGTGTTGTGCTTGGACC-3′ probe, 5′FAMCACGTGAGAATTTCCGTCTACTCGAGCCT-TAMARA-3′	104
alcohol dehydrogenase (ADH)	forward, 5′-CGTCGTTTCCCATCTCTTCCTCC-3′ reverse, 5′-CCACTCCGAGACCCTCAGTC-3′ probe, 5′-FAM-AATCAGGGCTCATTTTCTCGCTCCTCA-TAMARA-3′	136
starch synthase (SSIIb-2)	forward, 5′-CTCCCAATCCTTTGACATCTGC-3′ reverse, 5′-TCGATTTCTCTCTTGGTGACAGG-3′ probe, 5′-FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMARA-3′	151
zein	forward, 5′-GCCATTGGGTACCATGAACC-3′ reverse, 5′-AGGCCAACAGTTGCTGCAG-3′ probe, 5′-FAM-AGCTTGATGGCGTGTCCGTCCCT-TAMARA-3′	104

DNA Quantification. *Fluorescent Dye Assay.* DNA was quantified using a fluorescent dye 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. Stock DNA samples were diluted either 1:1000 or 1:2000 with 0.5× TE buffer to a target concentration of 30−250 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 either 1:2000 or 1:4000 final dilution and were compared with fluorometric measurements from a standard curve. Identical samples were quantified using a Hoescht dye (Fisher Scientific, Pittsburgh, PA) method. With the Hoescht dye method, diluted DNA samples (as described above) were mixed 1:1 with 2× Hoescht dye assay solution that was prepared from 0.2 μg/mL Hoescht dye dissolved into TNE buffer (0.2 M NaCl, 20 mM Tris-Cl, 1 mM EDTA, pH 7.4) solution prior to quantification. DNA samples had >85% concordance compared with PG prior to "accepting" an empirically determined concentration. A calibration curve was generated from λ phage DNA that was supplied by the manufacturer at a 100 μg/mL stock concentration and diluted to 250, 125, 62.5, 31.25, or 0.0 pg/μL with 0.5× TE buffer. The calibration slope error generally 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. The mean of DNA quantification values from both the PG and Hoescht dye methods provided an "accepted" concentration for a specified sample. After fluorometric quantification, the stock DNA samples were diluted to a working concentration of 20 ng/μL. Five microliters of the 20 ng/μL sample (equivalent to 100 ng of DNA/sample) was loaded onto an 0.8% agarose gel and size fractionated using electrophoresis. The 20 ng/μL samples were used for subsequent analysis in the RT-qPCR reaction setup as described below.

Gel Electrophoresis. The integrity of the DNA extracts was determined by electrophoresis in an 0.8% agarose gel stained with ethidium bromide. Five microliters of the 20 ng/μL working stock DNA per sample was added to individual lanes. Lambda *Hin*dIII was supplied in aqueous 1× TE solution. The presence of an intense, high molecular weight band indicated high-integrity, intact maize genomic DNA with minimal degradation and minimal RNA contamination (data not shown).

Quantitative Real-Time PCR. The method consisted of an event-specific, real-time quantitative *Taqman* PCR procedure for the endogenous control reference gene. Each endogenous control reference gene was taxon-specific and employed primers and a sequence-specific 6-carboxyfluorescein/carboxytetramethylrhodamine (FAM/TAMRA) probe. The RT-qPCR reactions for the endogenous reference genes were performed in separate wells in simplex format. *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 25 μL per reaction. Separate master mix preparations were required for each primer/probe system per plate using the method. RT-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 RT-qPCR reactions were performed on an ABI 7900 or ABI 7500 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 (containing 20 ng/μL) were loaded individually into a 96-well plate (Applied Biosystems), and each RT-qPCR was performed in triplicate for each DNA extract. Primers and probe sequences for the taxon-specific endogenous control reference genes used in this study are shown in **Table 1** (*28*).

RESULTS

Amplification Efficiency. The RT-qPCR efficiency of six different endogenous control genes was evaluated using 0.0% IRMM Bt-11 maize. Four serial dilutions of maize genomic DNA [containing 100 ng (36363 copies), 50 ng (18181 copies), 25 ng (9090 copies), 12.5 ng (4545 copies), or 6.25 ng (2273 copies), respectively, per well] were assayed in duplicate with each of the six endogenous primer/probe systems, and standard curves were generated (*16*). The copy number was based on the average size of the maize genome where one copy (the unreplicated haploid genome of maize) is equal to 2.75 pg of DNA (*16*). The corresponding amplification efficiency was calculated using the following equation: efficiency $= 10^{(-1/\text{slope})} - 1$, where slope is the value from the standard curve plot (*29*). The amplification efficiencies from the six primer/probe systems were calculated from the slopes and standard curves generated by the ABI 7500/ABI 7900 data analysis software over 5 orders

Table 2. PCR Amplification Efficiencies of IRMM 0% Bt-11 Maize Using Six Endogenous Control Genes[a]

endogene	slope	*Y*-intercept	R^2	RT-qPCR efficiency
ADH	−3.29	29.05	0.9982	98.6
HMG	−3.26	28.70	0.9984	97.2
SSIIb-3	−3.34	30.66	0.9988	99.1
IVR	−3.21	28.96	0.9963	95.4
zein	−3.21	29.62	0.9988	95.4
SSIIb-2	−3.19	31.67	0.9997	94.4

[a] The PCR efficiency data indicated that all endogenous control genes had an amplification efficiency of >94%.

of magnitude (*30*, *31*). As shown in **Table 2**, the RT-qPCR data revealed efficiencies from each of the six endogenous primer/probe systems to be between 94.4 and 99.1% ($-3.34 <$ slope < -3.19). The slope values suggested satisfactory efficiencies of the reactions with negligible inhibition. The six R^2 values were >0.996, showing the reaction's high linearity and suitability for quantification purposes.

Assessing Accuracy and Precision of the IRMM Bt-11 Cassette. To assess intrasample variability by RT-qPCR, triplicate extractions from an IRMM Bt-11 cassette consisting of 100 ng each of 0.0, 0.1, 0.5, 1.0, 2.0, and 5.0% reference material were analyzed in triplicate RT-qPCRs per extraction (a total of 9 RT-qPCRs per sample) as described under Materials and Methods. Average C_t values generated from the nine RT-qPCRs from each Bt-11 fortification level are shown in **Table 3**. The statistical analysis system (Base SAS software) output for the analysis of variance revealed that the test for differences among the endogenous genes was statistically significant (data not shown), even though each reaction contained the same 100 ng quantity of DNA. These data suggested that copy number variants exist for the endogenous control genes in maize and that a multitude of confounders contributed toward C_t variability with different primer/probe systems. However, within an individual primer/probe system, at different fortification levels of IRMM Bt-11, the data revealed low variability in C_t values, suggesting relatively low uncertainty in the analytical measurements when normalized to endogenous control reference genes by RT-qPCR. Data for the C_t range, mean C_t, standard deviation (SD), and percent trueness shown in **Table 3** were compiled from average C_t values generated by the nine replicate results per sample. The data revealed that SD calculations, computed from the C_t values, ranged from 0.07 (SSIIb-3) to 0.12 (IVR). SSIIb-3 provided the best precision (i.e., least intrasample variability) when average C_t values for the Bt-11 IRMM cassette samples were compared against the different primer/probe systems in this study. The closeness of agreement between the average C_t value obtained from the experimental samples and the average 0.0% Bt-11 reference C_t value represented a measure of trueness (*32*). The sample set with the trueness closest to zero indicated the best accuracy among the different primer/probe systems. As expected, irrespective of the IRMM fortification level, the various endogenous control primer/probe systems amplified predictably at all fortification levels (i.e., the average measured C_t value for the test sample was proximal to the C_t value of the reference). The primer/probe systems for HMG, zein, SSIIb-2, and ADH provided negligibly higher relative bias (+0.53, +0.35, +0.41, and +0.28%, respectively) compared to SSIIb-3 (−0.34%) and IVR (−0.47%), which provided negligibly lower relative bias.

Because experimental data from the previous section demonstrated >94% efficiency for all of the endogenous control genes used in this study, it was deemed appropriate to use C_t values and calculate a theoretical measured quantity using the following equation: theoretical measured quantity = 100 ng × $2^{\Delta C_t}$ (*33*). The ΔC_t value was generated from the difference between the average C_t of the experimental sample subtracted from the average C_t of the reference ($C_{t,\ ref} - C_{t,exptl}$). The average $C_{t,ref}$ in all instances was derived from 0.0% IRMM Bt-11, containing 100 ng of DNA (based on two fluorescent dye methods) per well, whereas the average $C_{t,exptl}$ was derived from 0.0, 0.1, 0.5, 1.0, 2.0, and 5.0% Bt-11, also containing 100 ng of DNA. **Figure 1** displays the mean ± one SD (σ) unit of the theoretical measured quantities generated from individual primer/probe systems for 100 ng of DNA from each of the fortification levels of the Bt-11 IRMM cassette, treated as "experimental" samples in RT-qPCR. Duncan's multiple-range test for tDNA (*34*) revealed that HMG (110.0 ± 7.5), SSIIb-2 (107.4 ± 6.6), ADH (105.2 ± 5.5), and zein (103.1 ± 8.1) were not statistically different and that SSIIb-3 (94.9 ± 4.8) and IVR (93.0 ± 7.5) were not statistically different from one another, but that the two groups [i.e., (HMG, SSIIb-2, ADH, zein) vs (SSIIb-3, IVR)] were different from one another. All samples generated theoretical measured quantities proximal (within ±10.0% or better) to the 100 ng target.

Evaluation of Precision and Accuracy of Various Endogenous Control Primer/Probe Systems Using 0.0% IRMM Reference Material and 100 ng of DNA/Well. To assess intersample variability generated by RT-qPCR using each of the six endogenous reference genes as described in this study, different certified 0.0% IRMM reference materials (i.e., Bt-11, Bt-176, GA21, Mon810, Mon863, and NK603) and GIPSA negative maize were extracted in triplicate and amplified by RT-qPCR in triplicate as previously described. The average $C_{t,ref}$ in all instances was derived by RT-qPCR from 100 ng of DNA (based on two fluorescent dye methods) of 0.0% IRMM Bt-11 per well, whereas average $C_{t,exptl}$ was derived from 100 ng of 0.0% IRMM maize treated as an "experimental" sample per well.

Precision and accuracy of different primer/probe systems were evaluated by comparing average C_t values generated by replicate RT-qPCRs derived from 0.0% IRMM Bt-11 reference to average C_t values generated by 0.0% IRMM experimental samples, as shown in **Table 4**. On the basis of average C_t values, the data revealed standard deviations ranging from 0.12 to 0.40, among the different primer/probe systems that were evaluated in this study. SSIIb-2 and IVR were the least precise, with SD values of 0.26 and 0.40, respectively, suggesting unacceptably high variability, whereas zein provided an intermediate SD value of 0.19. Clearly, precision data supersedes accuracy data within the context of this experimental design because all 0.0% IRMM reference samples were regarded as equivalent and selecting 0.0% Bt-11 as reference was done purely for consistency purposes. Regardless, when the trueness was assessed on the basis of C_t values, the data revealed that IVR serendipitously provided the value closest to the reference C_t value with a trueness of −0.04%. ADH, HMG, and SSIIb-3 provided intermediate values ranging from −0.3 to −0.7%, whereas zein and SSIIb-2 provided the highest relative bias (−0.93 to −2.1%), farthest from the reference C_t.

To better understand the context of C_t measurements in the RT-qPCR, theoretical measured quantities (based on 100 ng of DNA per each PCR reaction) were calculated from the average C_t values obtained from the primer/probe systems for the 0.0% IRMM experimental samples. As shown in **Figure 2**, theoretical measured quantities calculated for the 0.0% Bt-11 reference were proximal to the 100 ng target value as previously observed.

Table 3. Average C_t Values for the IRMM Bt-11 Cassette[a]

IRMM reference	endogenous control primer/probe system					
	ADH	HMG	SSIIb-3	IVR	zein	SSIIb-2
Bt-11 0.0% (reference)	24.90 ± 0.16	24.33 ± 0.22	23.75 ± 0.17	23.49 ± 0.20	22.78 ± 0.10	24.56 ± 0.09
Bt-11 0.1%	24.90 ± 0.17	24.31 ± 0.06	23.86 ± 0.11	23.61 ± 0.17	22.82 ± 0.08	24.47 ± 0.19
Bt-11 0.5%	24.80 ± 0.10	24.12 ± 0.08	23.77 ± 0.07	23.47 ± 0.21	22.65 ± 0.12	24.37 ± 0.13
Bt-11 1.0%	24.71 ± 0.09	24.12 ± 0.05	23.77 ± 0.07	23.57 ± 0.09	22.66 ± 0.12	24.34 ± 0.10
Bt-11 2.0%	24.79 ± 0.15	24.18 ± 0.09	23.88 ± 0.10	23.66 ± 0.20	22.61 ± 0.12	24.49 ± 0.19
Bt-11 5.0%	24.87 ± 0.12	24.11 ± 0.07	23.93 ± 0.04	23.79 ± 0.11	22.66 ± 0.10	24.53 ± 0.10
C_t range	24.71−24.90	24.11−24.33	23.75−23.93	23.47−23.79	22.61−22.82	24.34−24.56
mean C_t (n = 6)	24.83	24.20	23.83	23.60	22.70	24.46
SD (precision)	0.08	0.10	0.07	0.12	0.08	0.09
% trueness (accuracy)	+0.28	+0.53	−0.34	−0.47	+0.35	+0.41

[a] Average C_t ± SD data in the top section of the table were derived from triplicate extractions × three RT-qPCRs per test sample.

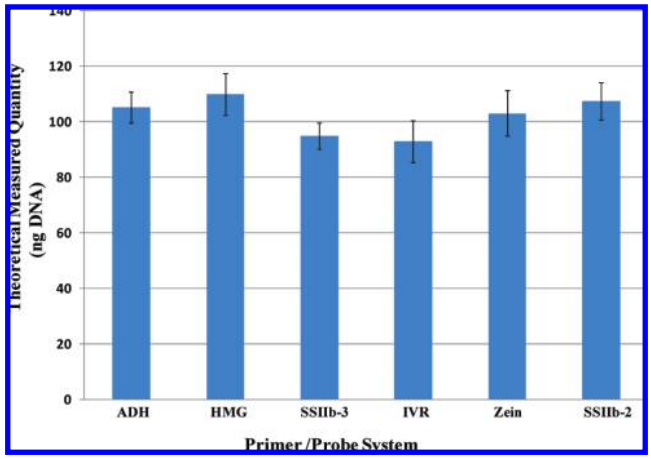

Figure 1. C_t measurements converted to theoretical measured quantities (100 ng of DNA per reaction) of various endogenous control genes using the IRMM Bt-11 cassette. The calculated mean ± 1σ unit is shown to estimate variability of each primer/probe system.

Duncan's multiple-range test for tDNA revealed that ADH (87.7 ± 7.7), HMG (95.2 ± 8.4), SSIIb-3 (93.3 ± 9.1), IVR (104.1 ± 32.9), and zein (85.8 ± 6.9) were not statistically different but that SSIIb-2 (66.2 ± 6.2) was statistically different from the preceding five primer/probe systems. All samples generated theoretical measured quantities proximal (within 14.2% or better) to the 100 ng target, except for SSIIb-2, which was within 43.8% of the theoretical value, suggesting that, in the absence of method standardization, the SSIIb-2 endogenous gene would contribute toward a higher level of bias in analytical measurements when using RT-qPCR, whereas IVR would contribute the greatest amount of variability of all of the primer/probe systems used in this study.

Evaluation of Precision and Accuracy of 0.0% IRMM Reference Samples using 50 ng of DNA per Well. One hundred nanogram samples, as described above, were diluted to 50 ng concentrations for each of the 0.0% IRMM reference materials and used in RT-qPCR to confirm precision and accuracy measurements of the primer/probes systems in this study. Data derived from C_t measurements at both 50 and 100 ng, along with their respective C_t values, were used to assess both the accuracy and precision of each primer/probe system under investigation. Precision calculations were shown to contain concordant values when the standard deviations at 100 ng were compared with 50 ng DNA concentrations in the RT-qPCR. All of the primer/probe systems were within a SD value of 0.04 except zein, which had a greater discordant result (i.e., 0.19_{100ng} vs 0.09_{50ng}). Accuracy measurements (% trueness) at both the 100 and 50 ng concentrations were shown to be consistent for ADH (-0.70_{100ng} vs -0.84_{50ng}), HMG (-0.30_{100ng} vs -0.28_{50ng}), SSIIb-3 (-0.40_{100ng} vs -0.46_{50ng}), and SSIIb-2 (-2.1_{100ng} vs -2.3_{50ng}). IVR, which gave the least precise σ_{100ng}, provided a greater difference in trueness measurements (-0.04_{100ng} vs $+0.27_{50ng}$), suggesting that the accuracy measurement of -0.04_{100ng} was fortuitous. The average ($C_{t50} - C_{t100}$) was used to assess ΔC_t values as shown in **Table 5**. With ideal RT-qPCR amplification efficiency, a 2-fold difference in concentration correlates to a ΔC_t value of 1.0 (*29*). The data revealed ΔC_t values ranging from 0.91 to 1.15, with the lowest ΔC_t value of 0.91 observed with ADH and the highest ΔC_t value of 1.15 observed with IVR. HMG and SSIIb-3 provided the most consistent ΔC_t, closest to 1.0.

Evaluation of 23 Different Cargill Maize Lines. One hundred nanogram aliquots of genomic DNA extracted from each of the 23 different Cargill maize lines were analyzed using the six primer/probe systems described previously. Precision and accuracy of different primer/probe systems were evaluated by comparing average C_t values generated by RT-qPCR from Cargill field corn samples (that all contained equal amounts of 100 ng of genomic DNA) to the C_t of the 0.0% Bt-11 reference sample, also containing 100 ng of genomic DNA. As shown in **Table 6**, average C_t values were obtained for GIPSA negative maize and 23 Cargill maize samples and were compared with 0.0% IRMM Bt-11 reference samples by performing concurrent RT-qPCR using respective primer/probe systems. On the basis of C_t values and standard deviations calculated from these values, the data revealed that zein (σ = 0.24) and HMG (σ = 0.25) primer/probe systems provided the most consistent measured quantities among the 23 different Cargill field corn samples, followed by SSIIb-3 (σ = 0.28), IVR (σ = 0.30), SSIIb-2 (σ = 0.33), and ADH (σ = 0.42). C_t measurements were converted to theoretical measured quantities as described previously and summarized in **Figure 3**. Duncan's multiple-range test for tDNA revealed that ADH (112.9 ± 39.8), HMG (117.7 ± 18.4), IVR (117.6 ± 22.5), and zein (118.5 ± 26.4) were not statistically different and that SSIIb-2 (90.4 ± 22.1) and SSIIb-3 (104.6 ± 18.3) were statistically different from the preceding four primer/probe systems, as well as from each other. All samples generated theoretical measured quantities proximal (within 18.5% or better) to the 100 ng target.

The precision and accuracy data were summed to provide overall assessments of performance as shown in **Table 7**. The HMG primer/probe system provided the best overall precision followed by SSIIb-3, ADH, zein, IVR, and SSIIb-2, whereas SSIIb-3 provided the best accuracy followed by IVR, HMG, ADH, zein, and SSIIb-2.

Table 4. Average C_t Values and Measured Quantities for GIPSA Negative Maize and 100 ng of DNA 0.0% IRMM Reference Samples/Well[a]

	endogenous control primer/probe system					
IRMM reference	ADH	HMG	SSIIb-3	IVR	zein	SSIIb-2
GIPSA NC	24.27 ± 0.15	23.46 ± 0.07	25.06 ± 0.09	24.60 ± 0.21	23.72 ± 0.08	25.46 ± 0.13
0% Bt-11 (reference)	24.21 ± 0.03	23.47 ± 0.07	25.10 ± 0.04	24.62 ± 0.11	23.55 ± 0.04	25.02 ± 0.03
0% Bt-176	24.55 ± 0.08	23.39 ± 0.02	25.12 ± 0.09	23.96 ± 0.08	23.71 ± 0.07	25.72 ± 0.08
0% GA21	24.37 ± 0.09	23.55 ± 0.05	25.21 ± 0.06	24.79 ± 0.12	23.67 ± 0.07	25.54 ± 0.06
0% Mon810	24.50 ± 0.08	23.63 ± 0.03	25.25 ± 0.10	24.30 ± 0.96	23.92 ± 0.12	25.64 ± 0.05
0% Mon863	24.48 ± 0.05	23.76 ± 0.07	25.47 ± 0.08	25.16 ± 0.07	24.11 ± 0.28	25.84 ± 0.02
0% NK603	24.26 ± 0.11	23.53 ± 0.12	25.20 ± 0.26	24.93 ± 0.11	23.70 ± 0.20	25.56 ± 0.13
C_t range	24.21−24.50	23.39−23.76	25.06−25.47	23.96−25.16	23.55−24.11	25.02−25.84
mean C_t $n = 7$	24.38	23.54	25.20	24.63	23.77	25.54
SD (precision)	0.13	0.12	0.14	0.40	0.19	0.26
% trueness (accuracy)	−0.70	−0.30	−0.40	−0.04	−0.93	−2.1

[a] Average C_t and SD data in the top section of the table were derived from triplicate extractions × three RT-qPCRs per test sample.

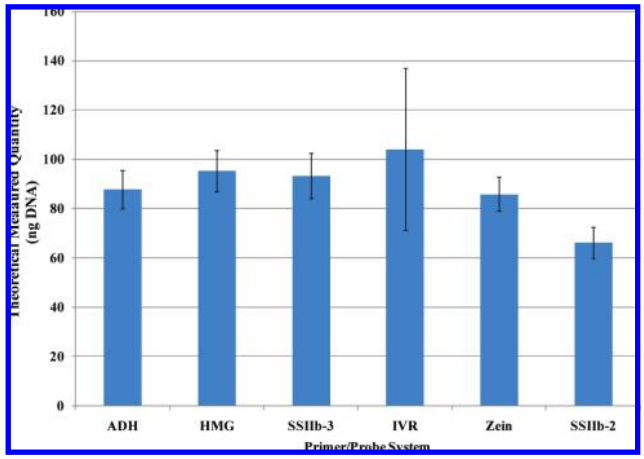

Figure 2. C_t measurements converted to theoretical measured quantities (100 ng of DNA per reaction) of various endogenous control genes using 0% IRMM material.

DISCUSSION

This study was designed to assess variability among different endogenous control reference systems that might contribute to uncertainty when using RT-qPCR to measure levels of GE traits in maize. Previous studies have shown that amplification efficiencies of different endogenous reference systems are dissimilar and thus cannot be used interchangeably among different maize cultivars with any substantial degree of confidence in the generation of analytical measurements by RT-qPCR (*12*). Identifying the most appropriate primer/probe system from a plethora of choices remains a daunting endeavor. Absolute quantification employs an internal or external calibration curve to derive the input template copy number. Absolute quantification allows for the exact transcript copy number to be determined (*29*). A number of variables can affect the efficiency of RT-qPCR, including length of the amplicon, secondary structure, allelic polymorphisms, and primer quality (*29*, *35*−*37*). A series of six commonly used endogenous control reference systems, identified by various sources (*38*), were evaluated on the basis of each endogenous gene systems' respective PCR amplification efficiency, precision, and accuracy. The endogenous control reference genes were evaluated for precision and accuracy using a 0.0% IRMM Bt-11 reference sample against (1) an event Bt-11 IRMM cassette consisting of fortification levels ranging from 0.0 to 5.0% (w/w), (2) samples of 0.0% IRMM reference maize flour, and (3) 23 different 2007 field *Zea mays* lines. The ISO 21570 guidelines specify that the copy number of an endogenous reference gene for a sample must be used for normalizing the copy number of a trait-specific target gene when using RT-qPCR. The allelic and copy number stability must be considered for cultivars of different geographic or phylogenic origins when a suitable endogenous control gene is identified for RT-qPCR. An abundance of endogenous control reference genes exist for maize, and there are no requirements in the ISO 21570 guidelines specifying the use of one particular endogenous control gene. Evaluation of the IRMM Bt-11 cassette revealed that, irrespective of the IRMM fortification level, the various endogenous control primer/probe systems amplified predictably at all fortification levels (i.e., the average measured C_t value for the test sample was proximal to the C_t value of the reference). The data revealed moderate variability in C_t values within individual primer/probe systems, suggesting relatively low uncertainty in the analytical measurements normalized to endogenous control reference genes by RT-qPCR, but that each primer/probe system produced comparatively varying amounts of relative bias. Previous papers have revealed high variability using RT-qPCR with different primer/probe systems when various maize cultivars containing discriminating morphological traits were analyzed (*12*). The paper suggests that C_t variability can be attributed to unique DNA sequences or copy number differences among different maize cultivars (*12*). Commercially available 0.0% IRMM reference materials, recognized as the preeminent standard in RT-qPCR, were selected to evaluate variability of different primer/probe systems because these materials most likely contain minimal differences in morphological traits and DNA sequences between cultivars (*39*). Duncan's multiple-range test attempted to identify which primer/probe systems perform differently from one another. With this test the SSIIb-2 primer/probe system had significantly lower results than the other systems, indicating that its performance was significantly different from those of the other five primer/probe systems. No differences were identified among the other five primer/probe systems. In a separate test, variability among the different primer/probe systems was examined, and IVR had greater variability compared with the other primer/probe systems.

Precision data provide a degree of mutual agreement among individual measurements of the same property under prescribed similar conditions, such as replicate measurements of the same sample. To avoid an erroneously high or low estimation of DNA quantity in different cultivars assayed,

Table 5. Average C_t Values from RT-qPCR of 0.0% IRMM Samples[a]

	endogenous control primer/probe system					
IRMM reference sample	ADH	HMG	SSIIb-3	IVR	zein	SSIIb-2
GIPSA NC	25.23 ± 0.121	24.48 ± 0.142	25.93 ± 0.194	25.83 ± 0.242	24.68 ± 0.302	26.54 ± 0.184
0% Bt-11 (reference)	25.08 ± 0.042	24.54 ± 0.052	26.04 ± 0.056	25.85 ± 0.101	24.60 ± 0.067	26.09 ± 0.050
0% Bt176	25.34 ± 0.076	24.46 ± 0.080	26.03 ± 0.093	25.04 ± 0.078	24.64 ± 0.091	26.72 ± 0.095
0% GA21	25.30 ± 0.129	24.61 ± 0.056	26.20 ± 0.057	25.98 ± 0.158	24.66 ± 0.089	26.76 ± 0.073
0% MON810	25.42 ± 0.078	24.74 ± 0.124	26.24 ± 0.109	25.41 ± 0.117	24.79 ± 0.123	26.86 ± 0.145
0% MON863	25.46 ± 0.086	24.82 ± 0.061	26.45 ± 0.025	26.27 ± 0.034	24.86 ± 0.087	27.04 ± 0.043
0% NK603	25.24 ± 0.102	24.61 ± 0.123	26.20 ± 0.140	26.05 ± 0.061	24.63 ± 0.190	26.73 ± 0.161
C_t range	25.08−25.46	24.46−24.82	25.93−26.45	25.04−26.27	24.60−24.86	26.09−26.86
mean ($n = 7$)	25.29	24.61	26.16	25.78	24.69	26.68
SD (precision)	0.13	0.13	0.17	0.42	0.09	0.30
% trueness (accuracy)	−0.84	−0.28	−0.46	+0.27	−0.37	−2.3
ΔC_t ($C_{t50} - C_{t100}$)	0.91	1.07	0.96	1.15	0.92	1.14

[a] C_t values generated using RT-qPCR amplification of 0% IRMM reference material and GIPSA negative corn at 50 ng DNA/well. Average C_t and σ data in the top section of the table were derived from triplicate extractions and three RT-qPCRs per test sample. Nanograms of DNA based upon C_t values ($\pm\sigma$) using 0.0% IRMM Bt-11 as the reference. The measured quantity was obtained from the average of C_t values generated by the RT-qPCR reactions and compared with 0.0% IRMM Bt-11. C_t measurements were also made to assess the efficiency of the RT-qPCR.

Table 6. Mean C_t Values for Cargill Maize Field Samples

	endogenous control primer/probe system					
sample	ADH	HMG	SSIIb-3	IVR	zein	SSIIb-2
GIPSA NC	24.98 ± 0.08	24.37 ± 0.14	23.73 ± 0.08	23.37 ± 0.08	22.82 ± 0.07	24.96 ± 0.07
0% Bt-11 (reference)	24.95 ± 0.09	24.42 ± 0.04	23.72 ± 0.04	23.48 ± 0.06	22.75 ± 0.08	24.53 ± 0.03
Cargill samples 1−23						
C_t range	23.70−25.68	23.91−24.88	23.44−24.03	22.85−23.87	22.33−22.94	24.19−24.97
mean C_t	24.84	24.21	23.69	23.28	22.58	24.76
SD (precision)	0.42	0.25	0.28	0.30	0.24	0.33
% trueness (accuracy)	+0.44	+0.86	+0.13	+0.85	+0.75	−0.94

Table 7. Compilation of Standard Deviation and Trueness Values for All Endogenous Control Systems Analyzed[a]

	primer/probe system											
	ADH		HMG		SSIIb-3		IVR		zein		SSIIb-2	
sample set	σ	absolute trueness (%)	σ	absolute trueness (%)	σ	absolute trueness (%)	σ	absolute trueness (%)	σ	absolute trueness (%)	σ	absolute trueness (%)
Bt-11 IRMM cassette	0.08	0.28	0.10	0.53	0.07	0.34	0.12	0.47	0.08	0.35	0.09	0.41
0.0% IRMM 100 ng	0.13	0.70	0.12	0.30	0.14	0.40	0.40	0.04	0.09	0.93	0.30	2.1
0.0% IRMM 50 ng	0.13	0.84	0.13	0.28	0.17	0.46	0.42	0.27	0.38	0.37	1.1	2.3
Cargill field samples	0.42	0.44	0.25	0.86	0.28	0.13	0.30	0.85	0.24	0.75	0.33	0.94
total	0.76	2.26	0.60	1.97	0.66	1.33	1.24	1.63	0.79	2.40	1.82	5.75
relative ranking	3	4	1	3	2	1	5	2	4	5	6	6

[a] A summary of standard deviation (variability) and % trueness (accuracy) values for the series of four experiments as generated in this study. For all assays the samples were analyzed in triplicate for each of the six endogenous control reference systems.

RT-qPCR systems appropriate for species-specific DNA quantification should display identical efficiencies in the analysis of different cultivars. The data shown in this study indicated acceptable amplification efficiencies for the different primer/probe systems as described. A higher degree of variability in Cargill field corn samples was observed compared with IRMM reference materials. On the basis of C_t values and standard deviations calculated from these values, the data revealed that HMG and SSIIb-3 primer/probe systems provided the most consistent measured quantities among the 23 different Cargill field corn samples, followed by ADH, zein, IVR, and SSIIb-2.

The data in **Table 7** revealed that, collectively, HMG and SSIIb-3 provided the best overall precision and accuracy of all primer/probe systems in this study. The data suggested that the lack of standardization of endogenous control primer/probe reference systems contributes to measurement uncertainty in the final analytical result. For purposes of standardization and harmonization,

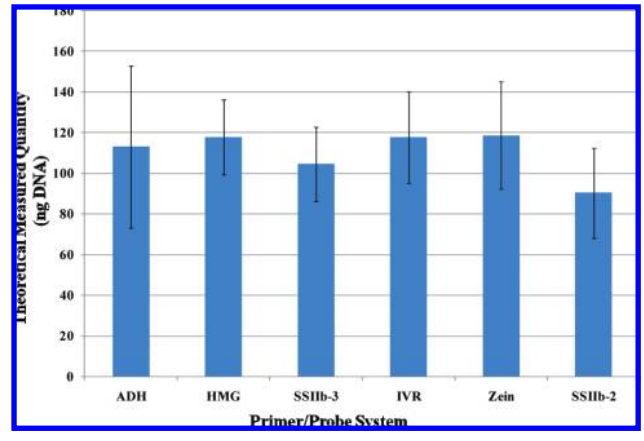

Figure 3. C_t measurements converted to theoretical measured quantities (100 ng of DNA per reaction) of various endogenous control genes using 23 distinctive Cargill maize field samples.

it is in the best interest of the grain trade industry to identify the most appropriate endogenous control system that could be used for normalization in real-time RT-qPCR applications.

ABBREVIATIONS USED

RT-qPCR, real-time quantitative PCR; Ct, crossing threshold; USDA-GIPSA-TSD, U.S. Department of Agriculture−Grain Inspection, Packers and Stockyards Administration−Technical Services Division; FAM/TAMRA, 6-carboxyfluorescein/carboxytetramethylrhodamine; CTAB, hexadecyltrimethylammonium bromide; EU, European Union; PG, picogreen reagent; SSIIb-2, SSIIb-3, starch synthase; ADH, alcohol dehydrogenase; HMG, high-mobility group; IVR, invertase; IRMM, Institute for Reference Materials and Measurements; JRC, Joint Research Centre; CRL, Community Reference Laboratory; ISO, International Standards Organization; GE, genetically engineered.

SAFETY

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

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