Research Report

Quantitative Real-Time PCR Assay for Determining Transgene Copy Number in Transformed Plants

BioTechniques 31:132-140 (July 2001)

David J. Ingham, Sandra Beer, Stephanie Money, and Geneviève Hansen
Syngenta, Research Triangle Park, NC, USA

ABSTRACT

The development of transgenic events can be limited by many factors. These include expression levels, insert stability and inheritance, and the identification of simple insertion events. All of the factors can be related to the copy number of the transgene. Traditionally, copy number has been determined by laborious blotting techniques. We have developed an alternative approach that utilizes the fluorogenic 5′ nuclease (TaqMan®) assay to quantitatively determine transgene copy level in plants. Using this assay, hundreds of samples can be analyzed per day in contrast to the low throughput encountered with traditional methods. To develop the TaqMan copy number assay, we chose to utilize our highly efficient Agrobacterium*-mediated transformation system of maize. This transformation procedure generates predominantly low copy number insertion events, which simplified assay development. We have also successful applied this assay to other crops and transformation systems.*

INTRODUCTION

Efficient transformation procedures for plants necessitate the development of rapid assays for the analysis of putative transformants. To determine whether the insertion of the transgene is simple (one copy) or complex (multiple and truncated copies), scientists have traditionally relied on labor-intensive nucleic acid blotting techniques (Southern blots) (16). To overcome this limitation, we have developed an assay for transgene insertion characterization that utilizes the speed, throughput, and quantitative accuracy of the fluorogenic 5′-nuclease assay known as TaqMan® (Applied Biosystems, Foster City, CA, USA) (4,7,8,10,12).

The TaqMan assay is a real-time PCR detection technique in which the accumulation of PCR product is monitored directly during the progress of the reaction. The details of the TaqMan assay have been described previously (4,8,10,12). Degradation of target-specific probe molecules by the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase during each cycle of amplification produces an accumulation of fluorescence. Increased levels of fluorescence are directly related to the accumulation of PCR product and are detected during each cycle of amplification through the use of specialized instrumentation (ABI PRISM™ 7700; Applied Biosystems, Foster City, CA, USA). Cycle thresholds (C_t) are assigned automatically to each sample according to the cycle at which the fluorescence exceeds a specific level above background (Figure 1). Samples with higher levels of template at the beginning of the reaction will amplify to detectable levels more quickly and yield a lower C_t. Because threshold values are determined during the logarithmic phase of amplification, PCR reagents are nonlimiting, and reactions will be most efficient (2). Therefore, the use of real-time (kinetic) detection techniques both simplifies and increases the accuracy of quantitative PCR assays (reviewed in Reference 2).

In the assay described here, we use duplexed TaqMan reactions to accurately quantitate the level of a transgene relative to an endogenous calibrator gene. This relative quantitation approach provides a simplified, accurate alternative to using standard curves and absolute quantitation. Because the endogenous calibrator sequence remains constant relative to total genomic DNA, any variation in the relative level of the transgene to the endogenous gene is indicative of a difference in copy number. As shown in Figure 1, 2-fold differences in copy number are easily distinguished using TaqMan technology. The TaqMan copy number assay allows the screening of hundreds of plants with greater accuracy within hours as compared to weeks using conventional methods. This allows greater numbers of independent transformation events to be screened for desired traits in shorter time and at significant cost savings. Advantages and limitations of the TaqMan assay over conventional procedures are also discussed.

MATERIALS AND METHODS

Plant Material

Transgenic maize plants from the inbred line A188 were obtained via *Agrobacterium*-mediated transformation. The selectable marker phosphomannose isomerase (*pmi*) under the

Table 1. Description of Primers and Probes

Target Gene	PCR Product Size (bp)	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Probe (5′→3′)
adh	71	GAACGTGTGT	TCCAGCAATCC	TGCAGCCTAACC
		TGGGTTTGCAT	TTGCACCTT	ATGCGCAGGGTA
pmi	60	CCGGGTGAA	GCCGTGGCCTT	TGCCGCCAACGA
		TCAGCGTTT	TGACAGT	ATCACCGG

control of the *Zea mays* ubiquitin promoter was used to recover transgenic plants as described by Negrotto et al. (13). Regenerated plants were transferred to soil and hardened off in a phytotron (16/8-h light). After 2–3 weeks, plants were transferred to the greenhouse and grown in commercial, soilless potting mixture. Greenhouse conditions for plant growth were 28°C days, 23°C nights, and a 16-h day length.

DNA Preparation from Plant Samples

Genomic DNA for TaqMan copy number assays was isolated from maize leaves of transgenic and nontransgenic plants. After collecting leaf samples of approximately 7 × 25 mm in wells of a 96-well plate (Corning, Corning, NY, USA) containing approximately 100 mg l-mm glass beads (BioSpec Products, Bartlesville, OK, USA), the plates were placed into a minus 80°C freezer for greater than 30 min and lyophilized overnight.

Tissues were ground by an adapted reciprocating saw for 30 s, rotated 180°, and ground for 30 s. Genomic DNA was extracted using the Puregene® Genomic DNA Extraction kit (Gentra Systems, Minneapolis, MN, USA) basically according to manufacturer's instruction, except all steps were conducted in 1.2-mL 96-well plates. The dried DNA pellet was resuspended in 500 µL TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

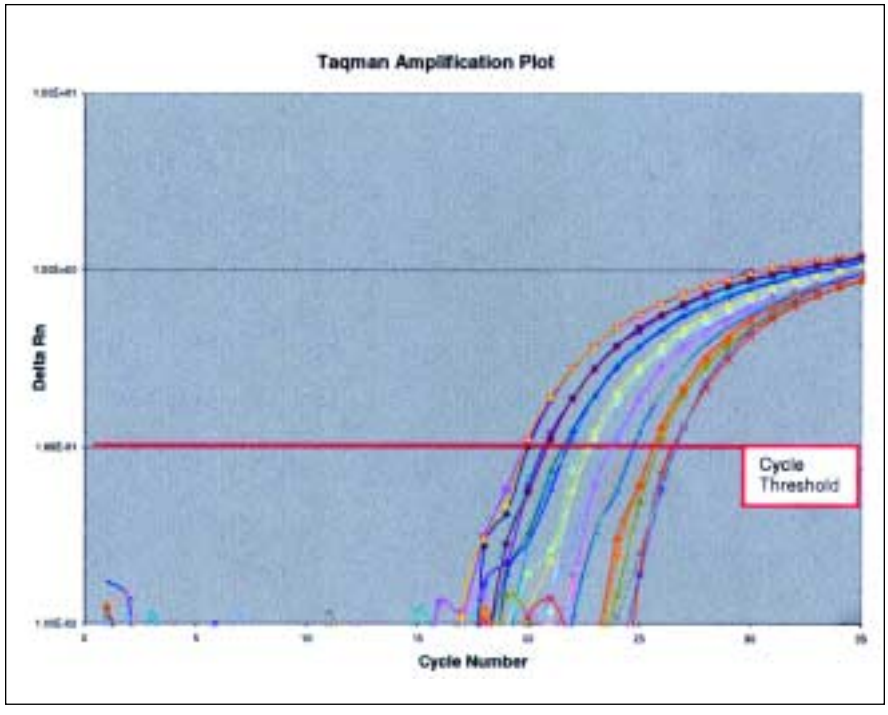

Figure 1. Example of TaqMan results representing amplification plots from serial dilutions of template DNA. Template DNA was serially diluted 2-fold and run in triplicate in standard TaqMan copy number assays. Data for the endogenous control (TET layer) extension phase were extracted and plotted. Each 2-fold dilution should be separated by 1 C_t. The cycle threshold (red horizontal line) provides an arbitrary cutoff at which a C_t value for each sample is assigned (see text).

Primers and Probes

PCR primers and probes were designed using Primer Express® software (Applied Biosystems). Primers and probes were purchased from Integrated DNA Technologies (Coraville, IA, USA). For the endogenous control, primers and probes were designed specific to the *Zea mays* alcohol dehydrogenase (*adh*) gene (GenBank® accession no. AF044295). For the transgene, primers and probes were designed specific to the selectable marker gene *pmi* (GenBank accession no. M15380) (13). Probes were labeled at their 5′ end with a reporter fluorophore [tetrachloro-6-carboxyfluorescein (TET) for *adh* and fluorescein (FAM) for *pmi*] and at the 3′ end with the quencher fluorophore tetramethylrhodamine (TAMRA). Sequences of primers and probes used in this work are shown in Table 1.

TaqMan Reactions

PCRs were carried out in 96-well reaction plates (Marsh Bio Products, Rochester, NY, USA). Reactions were multiplexed to simultaneously amplify *pmi* and *adh*. For each sample, a master mixture was generated by combining 20 µL extracted genomic DNA with 35 µL 2× TaqMan Universal PCR Master Mix (Applied Biosystems) supplemented with primers to a final concentration of 900 nM each, probes to a final concentration of 100 nM each, and water to a 70 µL final total volume. This mixture was distributed into three replicates of 20 µL each in 96-well amplification plates and sealed with optically clear heat seal film (Marsh Bio Products). PCR was run in the ABI PRISM 7700 instrument using the following amplification parameters: 2 min at 50°C and 10 min at 95°C, followed by 35 cycles of 15 s at 95°C and 1 min at 60°C. Postrun manipulations of data were performed according to the manufacturer's instructions and as described below.

Calculation of Gene Copy Number

During the TaqMan reaction, the software accompanying the ABI PRISM 7700 instrument detects the accumulation of PCR product by the accumulation of fluorescence. Normalized fluorescence relative to established baseline levels (ΔRn) is plotted versus cycle number. A C_t value is obtained by drawing an arbitrary cutoff through the reactions so that the line passes through the log phase of each reaction (Figure 1). The sequence detection system software with the ABI PRISM 7700 instrument provides the cycle number at which the accumulation of fluorescence (PCR product) of a particular reaction crosses the threshold (C_t). The FAM C_t (*pmi*) value is compared to the TET C_t (*adh*) value to normalize the FAM C_t value of each reaction to the level of total nucleic acids present to yield ΔC_t [$\Delta C_t = C_t(FAM) - C_t(TET)$]. This calculation removes any variation contributed by unequal template input in reactions and therefore allows the assay to be conducted without prior determination and normalization of DNA concentrations. Because the number of copies of endogenous gene per genome remains constant, a change in ΔC_t corresponds to a change in quantity (or copy) of the gene of interest. By comparing the ΔC_t value of the unknown samples to the ΔC_t of a known control, $\Delta\Delta C_t$ is obtained [$\Delta\Delta C_t = \Delta C_t(Unknown) - \Delta C_t(Known)$]. Copy number can then be calculated using the $\Delta\Delta C_t$ value using the equation: Copy Number = $2^{(\Delta\Delta Ct)}$. For example, when normalized to a known one-copy control, a one copy insert should have a $\Delta\Delta C_t$ value of zero ($2^{(\Delta\Delta Ct = 0)} = 1$ copy), a two copy insert $\Delta\Delta C_t$ will be one ($2^{(\Delta\Delta Ct = 1)} = 2$ copies), a four copy insert will display a $\Delta\Delta C_t$ value of 2 ($2^{(\Delta\Delta Ct = 2)} = 4$ copies), etc. Through the inclusion of a known copy control sample in each assay, accurate relative copy number is calculated for each unknown sample.

Southern Blot Hybridization

For Southern blot analysis, total DNA was isolated from approximately 1 g leaf tissues from nontransformed control plants and putative transformants as described previously (3). DNA (5–10 μg) was digested with *Eco*RV. Digestion with this enzyme and subsequent hybridization with a *pmi*-specific probe allows the identification of the border fragment between the insert and the plant DNA and provides an estimate of transgene copy number and number of insertion loci (Figure 3, A and B). Following digestion, DNA fragments were separated on a 0.8% agarose gel and blotted to positively charged nylon membrane following standard procedures (15). A *pmi*-specific restriction fragment was labeled with [^{32}P]-dCTP by random priming using the ReadyPrime™ II DNA Labeling Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridization was carried out overnight at 65°C in hybridization buffer (500 mM sodium phosphate, pH 7.0, 1 mM EDTA, 7% SDS, 1% BSA) containing approximately 100 000 cpm of labeled probe. The first wash was carried out at 65°C with (40 mM sodium phosphate, pH 7.0, 1 mM EDTA, 5% SDS, 0.5% BSA) for 15 min. The final wash was carried out at 65°C with (40 mm sodium phosphate, pH 7.0, 1 mM EDTA, 1% SDS) for 15 min. The membrane was exposed to X-ray film at -80°C for two days.

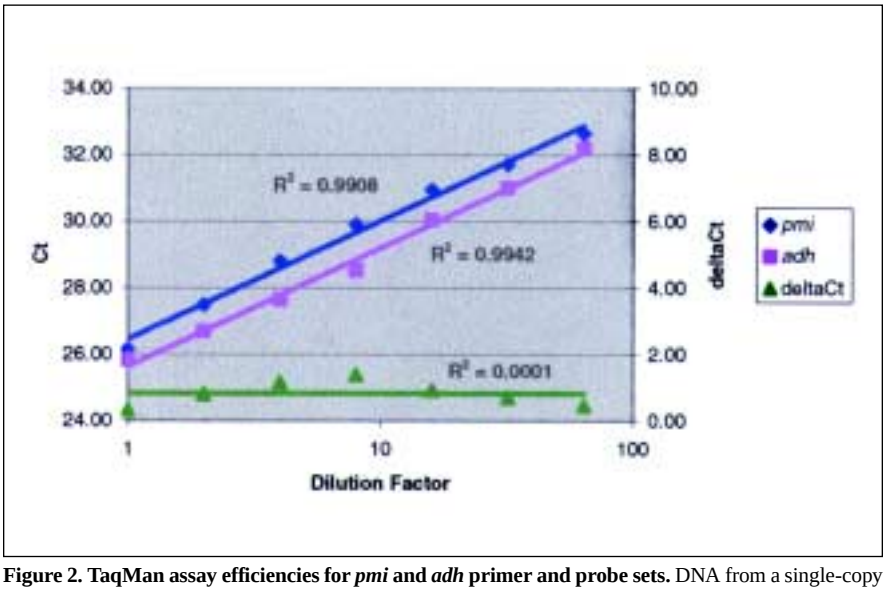

Figure 2. TaqMan assay efficiencies for *pmi* and *adh* primer and probe sets. DNA from a single-copy *pmi* transgenic *Zea mays* plant sample was serially diluted 2-fold and used in duplexed TaqMan copy number assays as discussed in Materials and Methods. Averaged C_t values of three replicates of each sample for the FAM (*pmi*) or TET (*adh*) layers were plotted versus dilution factor. In addition, the ΔC_t values obtained by subtracting average C_t values of *adh* from *pmi* were plotted on the secondary axis. A logarithmic curve was fitted to each data set, and the corresponding R^2 value was displayed on the plot.

RESULTS AND DISCUSSION

Development of the TaqMan Copy Number Assay

To estimate the copy number of *pmi* gene in transgenic plants, we have chosen the $\Delta\Delta C_t$ method for duplexed TaqMan reactions. In this approach, the transgene level is determined relative to an endogenous control gene to normalize all reaction variations that are due to initial template DNA concentration differences. For an endogenous control, one should select a sequence with few copies per genome, and the gene should be well conserved through different ecotypes or lines of the crop being used to allow the assay to be broadly applicable. This approach will simplify the assay development process (see below).

To analyze the efficiency of duplexed TaqMan assays, we conducted experiments similar to that in Figure 2. In these experiments, a dilution series of a transformed DNA sample is generated, and duplexed assays are run in triplicate for each dilution. By plotting the average C_t and ΔC_t values for each dilution and fitting a logarithmic curve to each set of data, one can determine the relative efficiencies of the reactions. A reaction having an efficiency of

100% would yield a C_t R^2 value of 1.0. Therefore, the closer a R^2 value is to 1.0, the more efficient the reaction (Figure 2). The R^2 values for *pmi* and *adh* TaqMan sets are 0.9908 and 0.9942, respectively; therefore, each reaction is highly efficient, and there is no evidence of competition for reagents. In addition, if both assays have approximately equal efficiency, then one would expect ΔC_t values to change little over the dilution range. Therefore, the expected ΔC_t R^2 value for reactions of equal efficiency would be zero. As shown in Figure 2, the R^2 value for the plotted ΔC_t values of this experiment was 0.0001, indicating little variation over the dilution series. This result is also an indication of the robustness of the assay. Because little or no variation is observed in ΔC_t over the dilution range, calculated copy number will also change little over that range. Therefore, the assay can accurately predict copy number among samples of varying DNA concentrations. This allows us to perform assays on samples without prior DNA quantitation and normalization. This reduces the turnaround time and cost of the assay.

The instructions that accompany the TaqMan instrumentation detail the requirement for determining appropriate limiting primer concentrations for mul-

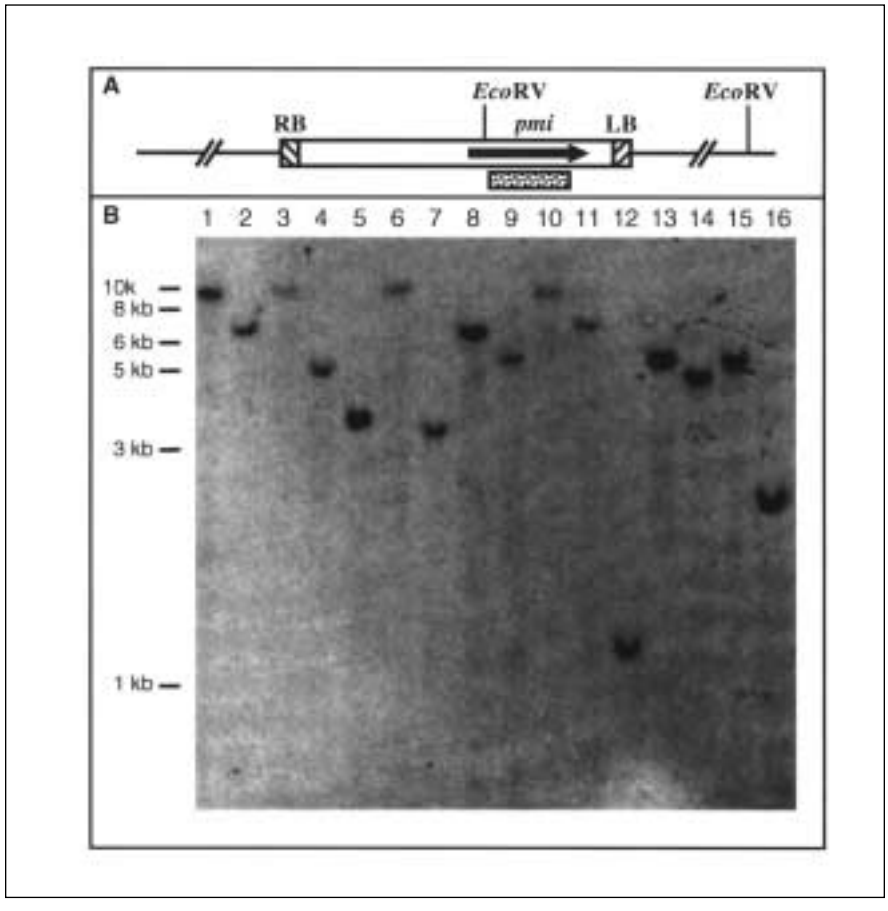

Figure 3. Southern blot showing the correlation of TaqMan copy number assay data with Southern analysis. (A) Graphic representation of the putative insertion event (not to scale). Open box indicates the *Agrobacterium* insertion cassette with left (LB) and right (RB) borders represented by hatched boxes. The position of the *pmi* gene is indicated by the filled arrow. Flanking plant genomic sequences are indicated by the solid lines. Positions of *Eco*RV restriction sites are indicated above the T-DNA insert and at an unknown position in the genomic DNA flanking the insert. The fragment used to generate *pmi*-specific probes is indicated by the speckle-filled box below the *pmi* gene. (B) As described in Materials and Methods, DNA samples from 16 single-copy events as determined by TaqMan copy number assay were digested with *Eco*RV, fractionated by agarose gel electrophoresis, blotted to nylon, and probed with a *pmi*-specific probe. Results confirm all 16 events as single copy for the *pmi* transgene. Position of molecular weight size markers is indicated to the left of the figure.

tiplexed reactions. Because we are primarily interested in simple insertion events and *Agrobacterium*-mediated transformation produces predominantly simple insertion events (6), the duplexed targets (*pmi* and *adh*) would be at approximately the same starting concentration. Therefore, we reasoned there would be little need to use limiting primer concentrations for our assays because there would be little (if any) competition for reagents during the log phase of each reaction. This is in contrast to duplexed reactions in which one target is in large excess of the other (as in expression studies using 18S ribosomal RNA as a normalizing control), which may deplete reagents before the less abundant target reaction accumulates to detectable levels. This type of a result is detrimental to the efficiency (and therefore the accuracy) of the low-abundance target reaction (2). To test our reasoning, we performed reaction efficiency experiments similar to Figure 2 comparing limiting and nonlimiting primer concentrations for each target. We found that for all TaqMan assays tested, primer limitation increased the variability and decreased the dynamic range of the assays. For example, when we examined the TaqMan sets used in this report, we found the standard deviations for ΔC_t calculated over a dilution series to drop to 0.11 for nonlimited primer conditions, from 0.22 under limiting primer conditions. In addition, we found the ΔC_t R^2 values were reduced from a range of 0.1–0.2 under limiting conditions to less than 0.07 under nonlimiting conditions, indicating greater accuracy of copy number calculation when using nonlimiting primer concentrations (Figure 2). Therefore, we decided to use the maximal recommended primer concentration (900 nM) for each primer in the reaction. The elimination of the requirement to find limiting primer concentrations for each duplex reaction has significantly reduced our assay development time.

In addition to testing the TaqMan sets on transformed samples, the set for *pmi* was tested for background signal in samples from nontransgenic plants. For *pmi*, no detectable background signal was obtained (data not shown). Once all criteria were satisfactorily met, the assay was then run using samples in which the copy number of the gene of interest was already determined by Southern analysis. A high degree of correlation was observed between the TaqMan data and data from Southern analysis (Table 2 and Figure 3). This result indicated that the developed TaqMan copy assay was highly accurate; therefore, we implemented the assay on large numbers of samples with unknown copy number (see below).

Table 2. Correlation of TaqMan to Southern Blot Assay for Transgene Copy Determination

	TaqMan Copy Assay[a] (% of Total)	Correlation with Southern[b] (No. Analyzed)
1 Copy	201 (70%)	100% (27)
2 Copies	62 (21%)	75% (8)[c]
>2 Copies	24 (8%)	100% (2)
Total No. Assayed	289 95%	(37)

[a]Number of samples determined to have the indicated copy number by TaqMan assay.

[b]Percentage of samples in which Southern data confirmed TaqMan results (only a subset of samples were analyzed by Southern).

[c]Two samples called 2 copy by TaqMan assay displayed a single band on a Southern blot.

Copy Number of *pmi* in Transgenic Plants

Copy number was assessed for transgenic maize containing the *pmi*-selectable marker. To calculate the number of copies of *pmi* for each plant, DNA extracted from each plant was run, in triplicate, in a duplex TaqMan assay comparing *pmi* level to the endogenous control gene (*adh*). Copy number was calculated for each sample as described above by comparison of each unknown to a one-copy control sample included in each assay. In general, there was very little variation (<0.2 ΔC_t) between the triplicate reactions.

We evaluated 289 putative transformants and found that the *Agrobacterium*-mediated transformation system developed for *Zea mays* (13) produced a majority of simple transformants (Table 2). Transformants (70%) were found to contain only one copy of the insert, and only 21% had two copies. The remaining 8% of plants contained greater than two copies of the insert. These results are consistent with previous observations for *Agrobacterium*-transformed dicotyledonous and monocotyledonous plants (6).

Although developed in *Zea mays*, the TaqMan copy number assay discussed here is also applicable to other crops (both monocotyledonous and dicotyledonous plants) and diverse organisms. We have successfully adapted this assay for copy number determination in a variety of transgenic plants, including wheat, barley, rice, watermelon, tomato, cotton, and sugarbeet. In addition, similar assays have also been reported for the quantitative analysis of protooncogene amplification in cancer studies (1,5,7,9,11,14). Thus, it is not surprising for the TaqMan copy number assay to be widely applicable to any organism. Taking advantage of the optimized TaqMan copy number assay developed here, we are evaluating extensive numbers of transformation events for a variety of crops to better characterize the transformation process and to more precisely identify desirable events.

Accuracy of TaqMan Copy Number Assay

The accuracy of the assay developed here can be assessed in two ways. First, by comparing results obtained from TaqMan assays to those from Southern blot analysis, we can determine the rate of correlation. We compared TaqMan and Southern results for a total of 37 samples for this report. These results are summarized in Table 2 and exem-

Table 3. TaqMan Assay Copy Discrimination Capability

Copy No.[a]	*n*[b]	SD[c]	CV[d]
1	47	0.10	10%
2	41	0.24	12%
3	10	0.43	14%
4	8	0.32	8%
5	18	0.53	11%
6			
7			
8	11	1.22	15%
9	13	1.39	15%
10			
11			
12	13	1.82	15%

[a]Transgene copy number as determined by TaqMan and/or Southern assay

[b]Total number of samples analyzed.

[c]Standard deviation of calculated copy numbers.

[d]Coefficient of variation (standard deviation/copy number).

plified in Figure 3. We found a 95% overall correlation rate between Southern blot and TaqMan assay data for these transgenic plants. The two noncorrelative samples were identified as two copy insertion events by TaqMan copy number assay but displayed a single band when analyzed by Southern blot. We have not determined which assay result is correct. It is possible that the single hybridizing band observed in the Southern blot could be generated by the insertion of two T-DNA copies in a tandem inverted orientation. Such a two-copy insertion event would yield a single hybridizing band that would vary in size depending on the amount of intervening sequence. This scenario, although speculative, illustrates the potential pitfalls of well-established procedures such as Southern analysis.

These results illustrate both the high levels of accuracy of the TaqMan assay and the limitations of both TaqMan and Southern blot analysis. Because TaqMan assays are specific to a small sequence of the gene, it is not possible to rule out the presence of truncated or mutated copies of the transgene that may not be detected by the assay. Therefore, both TaqMan and Southern blot analyses are important assays for comprehensive analysis of transformation events. Currently, every sample that passes the copy number criteria by the TaqMan assay is independently confirmed by additional assays including nucleic acid blotting before extensive field testing.

A second approach to determining the level of accuracy of the TaqMan assay involves the comparison of results of replicate samples. The C_t difference between consecutive copy numbers diminishes as the copy number increases. Therefore, the most accurate differentiation is obtained between one and two copy events with discrimination capability diminishing as copy number increases. To test these predicted limitations, we conducted replicate assays on samples of various copy number (Table 3). The standard deviation for the calculated copy numbers of replicate samples increases from 0.1 copy variation for one-copy samples to greater than 1.0 copy deviations for samples greater than or equal to eight copies. These results are consistent with our observation of ΔC_t standard deviations in the range of 0.1–0.2 for most assays (unpublished observations). If we also look at the calculated coefficient of variation (CV) for each copy number in this assay, they fall in the range of 8%–15% (average 12%), illustrating the high level of consistency of the assay over a broad range of target copy number. If we use an average CV of 12% to predict the ability of the assay to differentiate sequential copy numbers, we find the discrimination ability of the assay will stop around four copies of a target (12% of 4 copies = about 0.5 copy variation). The observed standard deviation shown for a four-copy sample in Table 3 illustrates the assay's discrimination ability to be slightly better than this prediction. For these reasons, we limit assigning exact copy values to results of four copies and below. This limitation was not problematic for our studies because simple insertion events were the desired product.

Similar limitations for high copy number samples can also be observed for copy number analysis by Southern blot (unpublished observation). Complex banding patterns create added subjectivity and therefore reduce the confidence level for copy determination.

Although the TaqMan copy assay does have some limitations, the high degree of accuracy and robustness combined with throughput and assay cost provides numerous advantages over traditional Southern blot techniques. These benefits should establish TaqMan copy assay as standard assay for high-throughput plant biotechnology research and development. Each TaqMan copy number assay plate can analyze, in triplicate, 30 unknown and two control samples in less than 2 h. Currently, the only other limitations to the number of samples that can be run in the assay are determined by the number of ABI PRISM 7700 instruments available and the rate/throughput of sample preparation. In addition to speed and throughput, the TaqMan copy number assay also has an advantage over Southern blot analysis in the size of sample required for each assay. Because TaqMan is a PCR-based assay, much less sample is required. Currently, we use about 1/20th the amount of sample required for Southern analysis. Theoretically, this could be reduced much further because each sample preparation for TaqMan analysis yields enough DNA for about 25 assays. In addition, because of the small sample size required, the TaqMan assay can be conducted much earlier in the production of transgenic events. This allows undesirable events (those with greater than one copy) to be eliminated before lengthy and expensive greenhouse cultivation procedures.

ACKNOWLEDGMENTS

The authors would like to thank John Arbuckle for technical advice on DNA preparation procedure and the Greenhouse and Transformation groups for generating and maintaining the plants used in this study. In addition, we would like to acknowledge Tina Hammer and Nadine Carozzi for helpful discussions. We would also like to thank Carla Thomas, Mary Fielder, Jim Beck, and Henry-York Steiner for careful review of the manuscript.

REFERENCES

1. **Bieche, I., M. Olivi, M.H. Champeme, D. Vidaud, R. Lidereau, and M. Vidaud.** 1998. Novel approach to quantitative polymerase chain reaction using real-time detection: application to the detection of gene amplification in breast cancer. Int. J. Cancer *78*:661-666.
2. **Bustin, S.A.** 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. *25*:169-193.
3. **Dellaporta, S.L.** 1994. Plant DNA miniprep and microprep: version 2.1a-2.3. *In* F.M.a.V. Walbot (Ed.), The Maize Handbook. Springer-Verlag, New York.
4. **Gelfand, D.H., P.M. Holland, R.K. Saiki, and R.M. Watson, inventors; Hoffman-LaRoche, assignee.** Homogeous assay system using the nuclease activity of a nucleic acid polymerase. US Patent no. 5 210 015. 1993 May 11.
5. **Gelmini, S., C. Orlando, R. Sestini, G. Vona, P. Pinzani, L. Ruocco, and M. Pazzagli.** 1997. Quantitative polymerase chain reaction-based homogeneous assay with fluorogenic probes to measure c-erbB-2 oncogene amplification. Clin. Chem. *43*:752-758.
6. **Hansen, G. and M.D. Chilton.** 1999. Lessons in gene transfer to plants by a gifted microbe. Curr. Top. Microbiol. Immunol. *240*:21-57.
7. **Heid, C.A., J. Stevens, K.J. Livak, and P.M. Williams.** 1996. Real time quantitative PCR. Genome Res. *6*:986-994.
8. **Holland, P.M., R.D. Abramson, R. Watson, and D.H. Gelfand.** 1991. Detection of specific polymerase chain reaction product by utilizing the 5′→3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA *88*:7276-7280.
9. **Laurendeau, I., M. Bahuau, N. Vodovar, C. Larramendy, M. Olivi, I. Bieche, M. Vidaud, and D. Vidaud.** 1999. TaqMan PCR-based gene dosage assay for predictive testing in individuals from a cancer family with INK4 locus haploinsufficiency. Clin. Chem. *45*:982-986.
10. **Lee, L.G., C.R. Connell, and W. Bloch.** 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. *21*:3761-3766.
11. **Lehmann, U., S. Glockner, W. Kleeberger, H.F. von Wasielewski, and H. Kreipe.** 2000. Detection of gene amplification in archival breast cancer specimens by laser-assisted microdissection and quantitative real-time polymerase chain reaction. Am. J. Pathol. *156*:1855-1864.
12. **Livak, K.J., S.J. Flood, J. Marmaro, W. Giusti, and K. Deetz.** 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. *4*:357-362.
13. **Negrotto, D.M., M. Jolley, S. Beer, A.R. Wenck, and G. Hansen.** 2000. The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. Plant Cell Rep. *19*:2798-2803.
14. **Raggi, C.C., M.L. Bagnoni, G.P. Tonini, M. Maggi, G. Vona, P. Pinzani, K. Mazzocco, B. De Bernardi, M. Pazzagli, and C. Orlando.** 1999. Real-time quantitative PCR for the measurement of MYCN amplification in human neuroblastoma with the TaqMan detection system. Clin. Chem. *45*:1918-1924.
15. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. CSH Laboratory Press, Cold Spring Harbor, NY.
16. **Southern, E.M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*:503-517.

Received 25 August 2000; accepted 25 January 2001.

Address correspondence to:

Dr. David J. Ingham
Syngenta
3054 Cornwallis Road
Research Triangle Park, NC 27709, USA
e-mail: david.ingham@syngenta.com