# The Use of 35S and T*nos* Expression Elements in the Measurement of Genetically Engineered Plant Materials

Marcia J. Holden[1*], Marci Levine[2],Tandace Scholdberg[3], Ross J. Haynes[1], and G. Ronald Jenkins[3]

[1]National Institute of Standards and Technology, Biochemical Science Division, 100 Bureau Dr. Gaithersburg MD 20899

[2]International Life Sciences Institute, ILSI International Food Biotechnology Committee, 1156 15th St., Washington DC, 20005

[4]US Department of Agriculture, Grain Inspection, Packers and Stockyard Administration, Technical Services Division, 10383 NW Ambassador Dr., Kansas City MO, 64153

*Corresponding author

**Abstract**

An online survey was conducted by the International Life Sciences Institute International, Food Biotechnology Committee, on the use of qualitative and quantitative Polymerase Chain Reaction (PCR) assays for Cauliflower Mosaic Virus 35S promoter and *Agrobacterium tumefaciens* T*nos* DNA sequence elements for the detection of genetically engineered crop plant material. Forty-four testing laboratories around the world completed the survey. The results showed that the wide-spread use of such methods, the multiplicity of published and in-house methods and the variety of reference materials and calibrants also in use. There was an interest on the part of respondents in validated quantitative assays relevant to all GE events that contain these elements. Data is presented using two variations of five published 35S assays on eight maize reference materials. The results showed that two of the five methods were not suitable for all the eight reference materials, showing poor linear regression parameters and multiple products with some of the reference materials. This preliminary study demonstrates that all 35S methods are not the same and the need for validation.

**Keywords:** Cauliflower mosaic virus, CaMV, 35S promoter, T*nos*, genetically engineered, qualitative polymerase chain reaction, PCR, quantitative real-time PCR

30 **Introduction**

31 Vector constructs for plant transformation contain sequences of DNA which are intended to be inserted into the
32 target organism, such as for the expression of a gene (1). In addition to the sequences which may be required
33 for insertion of the construct into the plans genome such as T DNA borders, a vector construct includes
34 promoter and terminator sequences that enable the plant to express the gene of interest. One source of such
35 promoters is the Cauliflower mosaic virus (CaMV). CaMV is a double stranded DNA virus affecting plants in the
36 Cruciferae, Resedaceae and Solanaceae families (2). The 35S promoter of CaMV is a functional, strong and
37 reliable promoter (3). Hence it has been incorporated into numerous constructs and used to produce many of
38 the genetically engineered (GE) crop plants that are in commercial production, such as maize, soy, canola and
39 rice. Similarly the RNA polyadenylation site (end of transcription) of the T*nos* sequence from the *Agrobacterium*
40 *tumefaciens* nopaline synthase gene has served as a polyadenylation site in some of the same constructs. Table
41 1 shows the number of GE events and GE products in 18 different taxa and how many have either or both 35S
42 promoter and T*nos* sequences (4). Maize has the largest number of GE products at 27. In recent years many of
43 the events have been crossed using normal breeding techniques to produce what are called stacked-trait
44 products. Searching the Agbios database (4) showed that there are 19 such double and triple stacked
45 maize products and to our knowledge there is no stacked product that is free from both 35S and T*nos*
46 sequences. The last column of Table 1 shows how few products have neither 35S nor T*nos* sequences.

47 Testing for the presence of CaMV 35S and T*nos* sequences has been used for years as a screening tool
48 for detection of GE plant material since, most or all GE events/products in commerce had contained
49 one or the other or both. Detection of either element would then lead to additional assays for
50 identification of specific elements or events (5). This might involve the use of qualitative PCR assays
51 with products separated by gel or capillary electrophoresis. Multiplex assays and microarrays are a
52 recent development (6,7) and provide a powerful alternative to identify GE products. Finally a
53 quantitative assay based on the identified product can be used to quantify GE material in food or grain.
54 This type of specific event assay may target the junction between the transgene construct and the
55 plant genomic DNA that is unique to any given event or may target unique junctions within the
56 construct.

57 One approach to quantification that has been used to some extent is the use of 35S or T*nos* as the
58 target sequence. Since these DNA sequences are still common to many crops in commerce, a validated

59 quantitative method would be useful and may, in some cases, substitute for quantification of unique
60 event assays. There are some important considerations. The method would have to be validated for
61 all products carrying 35S and T*nos*, if possible. The products for which quantitative screening assays
62 are not appropriate would have to be clearly understood. With some products there is more than one
63 copy of these elements, such as maize Bt11 that has two copies of the 35S sequence. This could lead
64 to an overestimate of the GE content. This could be problematic if the GE content is near a regulatory
65 threshold.

66 Complex mixtures with more than one product may be identified and could be due to the presence of a
67 stack-trait product, for example maize Mon 810 x Mon 88017, or to the independent presence of two
68 GE products. There is currently no good approach to distinguishing these two possibilities. But the
69 presence of trace amounts of both Mon 810 and Mon 88017, for example, would be suggestive of the
70 presence of a stack-trait product. Depending on the stack-trait product, quantification by 35S or Tnos
71 may or may not involve extra copies of these elements. The stack-trait product, Maize Mon 810 x
72 GA21, has 1 copy each of 35S and T*nos* elements. Two product-specific assays would estimate twice
73 the GE content as would a quantitative 35S assay. In the case of the stacked-trait maize Mon810 x
74 Mon 88017 there are two copies of 35S, therefore one might substitute a single assay for two product-
75 specific assays. Quantification by 35S qPCR is used by the Japanese government in their testing of
76 imported food and grain. [JAS handbook]. There are several advantages to this strategy. One validated
77 method could substitute in appropriate cases for event specific quantitative assays. The cost of
78 testing would be reduced and the efficiency of testing could be increased by combining diverse test
79 materials in a given assay run. Widespread adoption of such a method may lead to more consistency
80 of testing of materials upon export and subsequent import, reducing the number of trade disputes. In
81 addition, for those laboratories that would be using a 35S assay only for qualitative purposes, a real-
82 time quantitative method valid for all events would eliminate the need for post amplification
83 processing, such as detection using gels. Quantitation by 35S or T*nos* elements could be useful tools,
84 but knowledge of the products and regulatory requirements would be important.

85 In this manuscript we describe the results of a survey conducted by the International Life Science
86 Institute (ILSI) on the use of 35S and T*nos* methods by the international testing community to

determine the extent of use and interest in such methods. We then provide data on 35S measurements of 8 maize products using 5 published methods. The data show that all 35S methods are not suitable, thus that it is important to validate such a 35S detection method for each GE event.

**International Life Science Institute (ILSI) Survey on the use of the transgene elements 35S promoter and T*nos* for detection of genetically engineered plant materials.**

To assess the current status of 35S and Tnos PCR-based detection method use, ILSI sent out an email invitation to 150 testing laboratories around the world, requesting participation in a survey. The survey was done online using Survey Monkey (8). The scope of the survey was to collect information on the use of qualitative and quantitative PCR-based methods for 35S and T*nos* by the laboratories. Twenty five questions were asked about current and past use, the type of methods and detection strategies, the source of methods (published versus in-house) and types of reference materials. Each participant in the survey was allowed only one survey submission.

There were forty-six separate accessions to the survey and forty-four of these laboratories completed the survey. Identification was not obligatory, though twenty-six were willing to be identified in a participant list. Thirty-two of the participants identified at least their country. The geographic distribution of the laboratories is as follows: Fourteen from Europe (Germany, Poland, Portugal, France, Spain), ten from North America (Canada, USA, Mexico), three from South America (Argentina, Nicaragua, Brazil), and five from Asia (China, Thailand, India). The countries with the most respondents were Germany and the USA, both with seven.

Table 2 summarized usage of qualitative and quantitative assays for 35S and Tnos. Of the 44 laboratories, 40 currently use a qualitative only, a quantitative only, or a combination of qualitative and quantitative PCR assays for 35S, while 37 use some combination of assays for T*nos*. Similar numbers were seen when the question of past usage was asked. The use of qualitative methods has dropped from 37 to 33 labs for 35S and from 34 to 32 labs for T*nos*. In contrast the current use of quantitative assays has increased from past use: from 19 to 22 labs for 35S and from 12 to 16 for T*nos.* The laboratories that do not currently use quantitative assays for 35S and T*nos* were asked if they were

114 considering using a quantitative assay, 8 of 22 respondents said yes and 14 of 22 said no for 35S, while
115 8 of 25 said yes for T*nos* and 17 of 25 replied in the negative.

116 The laboratories were queried as to the type and source of their qualitative methods (Table 3). All of
117 the qualitative methods for both 35S and T*nos* elements are thermo cycling PCR. Published methods
118 for 35S were used by 24 of 38 respondents (63%) while 14 of 38 (37%) of the laboratories used in-
119 house developed methods. For T*nos*, 13 of 35 (37%) of laboratories use in-house methods while 22 of
120 35 laboratories use published methods. For qualitative assays, detection of the PCR product is done
121 using agarose gels by 66% (23 of 35 labs) of respondents for both 35S and T*nos* assays. The other 12
122 labs (34%) use fluorescent-based techniques, such as TaqMan real-time PCR (7 labs), the Agilent 2100
123 Bioanalyzer, SYBR Green, and polyacrylamide electrophoresis.

124 All of the labs that reported performing a quantitative assay for 35S or T*nos* use real-time PCR, 13 out
125 of 25 use published 35S methods and 8 out of 17 use published T *nos* methods (Table 3). For
126 quantitative assays, 85% of the respondents (19 labs) use probes labeled with fluorophores while the
127 rest used intercalating dyes.

128 The survey participants were queried as to the source of the published methods for both qualitative
129 and quantitative methods and Table 4 summarizes what was indicated. There were a few listings of
130 specific references but in some cases sources were general such as the Joint Research Center GMO
131 database and GMDD (9,10). The survey participants did not specify which method(s) within the
132 databases were used. Table 4 shows the variety of unique references for each of the four categories of
133 methods found in the named sources. Some of the references show up in more than one source so the
134 total number of unique methods is less than adding up the totals for each assay type, for example
135 there are not 27 unique methods for qualitative 35S assays.

136 The participants were asked to identify what endogenous gene they use when doing relative
137 quantification. Twenty-one labs identified gene targets in four taxa (Table 5). For maize 5 targets were
138 specified: alcohol dehydrogenase (Adh), invertase, high mobility group (Hmg), starch synthase, and
139 zein. For rape (canola) labs use cruciferin, fatty acid dehydrogenase; or phosphoenolpyruvate
140 carboxylase. One target, phospholipase, was indicated for rice and lectin for soy. In addition to the

141 variety of gene targets, there is likely even more variety in assays chosen because more than one assay
142 for some of these target sequences and they do not all behave the same (11). One laboratory
143 indicated that they use a chloroplast gene, but did not specify which taxa were relevant for use of that
144 target. One respondent stated that they use the validated species specific on the Community Research
145 Laboratories web site (12).

146 The next section of the survey concerned the use of reference materials (RM). The participants were
147 initially asked if they use commercial RMs for calibration and quality control. Of 35 total responses, 27
148 said that they use commercial RMs for calibration of 35S assays and 26 use such materials for quality
149 control. While 22 use commercial RMs for Tnos calibration and 23 for quality control. In a related
150 question, 25 of 30 labs reported using certified RMs from sources such as the Institute for Reference
151 Materials and Measurements (IRMM) (13) and the American Oil Chemists Society (AOCS) (14). Eleven
152 respondents indicated that they used other materials for calibration. These included in-house
153 developed RMs, plasmids, seeds, and materials from proficiency testing programs and inter-laboratory
154 trials. Then the respondents were asked to indicate what categories of materials that they use (Table
155 6). Individual respondents indicate more than one type of material being used, but the most popular
156 type is powder, such as certified RMs produced by IRMM. When asked if there were additional
157 comments on reference materials, 7 participants noted the lack of reference materials for some of the
158 events of interest. In some cases, laboratories have had problems obtaining the commercial RMs.
159 There was also concern on price and the shelf life of an opened vial of a CRM. One comment stated a
160 preference for powder materials, as this required extraction (unlike pure DNA RMs), thus covering the
161 whole process of DNA extraction and PCR assay.

162 The survey participants were asked if their qualitative and quantitative 35S and T*nos* assays were able
163 to detect all the events that the participants encounter in their testing. Twelve participants replied yes
164 and 20 said no for their 35S assays, while 12 said yes and 17 no for T*nos* assays. Some elaborated on
165 this saying that they test for events that do not have either of the two targets in the genome. Others
166 say the assays work for all the events that they test for and several pointed out the necessity for using
167 both assays. One lab noted not uncommon contamination of test samples with “spurious Roundup
168 ready soy”. Precautions noted by the respondents include the importance of confirming using event or

construct specific methods, false positives due to the presence of actual Cauliflower mosaic virus, the original source of the 35S promoter genetic element. No one mentioned that a similar problem exists for T*nos* due to contamination by *Agrobacterium tumefaciens* in the environment. There are PCR methods available that target other regions of the cauliflower mosaic virus that can serve as controls assays for virus contamination (15), 16). One lab noted that not all primer/probe combinations will successfully amplify all events, but did not indicate if that referred to 35S or T*nos* assays or both. The copy number of these elements in specific events is also of concern to some of the respondents with respect to quantitative assays. One lab noted that they thought the assays had low sensitivity and reproducibility.

Perhaps the most interesting point from the survey is that there is interest in adopting a standardized method, if available. Of 32 respondents, 16 were "highly interested', 13 were "somewhat interested" and 3 were not interested in a standardized 35S method. The equivalent numbers for T*nos* were 16,13 and 4. Some respondents were of the opinion that there were already sufficiently standardized methods available. Some noted that a standardized method would be an improvement and could lead to better inter-laboratory results. Possible problems noted by participants could be the regulatory requirements in specific countries, the flexibility of a standardized method, such as core reagent selection (fixed versus open) and the cost of validating a new method in-house by a lab looking to adopt the standardized method.

It is clear from the survey that there are a large variety of 35S and Tnos methods in use along with a variety of reference materials used for calibration and quality control in use in laboratories around the world. This could lead to problems for the food/grain production and trade industry. Use of standard methods by laboratories at export and import sites could reduce the possibility of trade disputes.

**Method screening experiments**

Based on the survey results, we conducted preliminary experiments to assess and compare the performance of five 35S qPCR methods in the literature. The test materials were eight CRMs for maize.

196 **Materials and Methods**

197 Testing materials for this study consisted of certified reference materials (CRM). IRMM has produced
198 CRMs for many GE crops. The IRMM CRMs (13) included in this study are matrix materials, ground
199 corn seed, with a percentage of GE material in a background of isogenic conventional corn up to ~ 10%
200 (100 g/kg, W/W). We used seven of these CRMs in this study at the highest concentration available for
201 a specified event. The eighth material was a pure DNA preparation isolated from leaves of
202 homozygous transgenic T25 maize and thus was 100% GE material. The T25 material was certified by
203 the American Oil Chemists Society (14). Table 7 identifies the specific CRMs used in this study.

204 DNA was extracted using a publicly available CTAB method validated for maize TC1507 (EC Joint
205 Research Center, Community Reference Laboratory) [web site reference]. The method calls for two
206 clean up steps. In this study, only one method was used, the S-300 HR Microspin columns (Amersham-
207 Pharmacia). [1] In our hands the second cleanup step resulted in DNA absorbance scans that were of
208 poorer quality (smaller 260 nm / 280 nm ratios) suggesting an impurity was introduced. Extractions
209 were done with 100 mg maize flour. Seven to eight extractions were done from each material and
210 were pooled after doing a wavelength scan of each one. The 260 nm / 280 nm ratio ranged from 1.92
211 to 1.97 and the 260 nm / 230 nm ratio was over 2.0 for all samples. The few DNA samples that did not
212 meet these criteria were discarded. The absorbance at 260 nm was measured with the DNA in 0.2 x TE
213 buffer and after the addition of 2M NaOH. The calculation of the alkali denatured DNA concentration
214 (μg/mL) was on average 11% lower than DNA in buffer. The alkali denatured DNA value was used in
215 subsequent calculations and the DNA was adjusted to approximately 20 μg/mL (17.5 μg/mL to 22.0 μg/mL),
216 except for the T25 DNA, which was adjusted to 1 μg/mL . This adjustment was done to bring the GE DNA copy
217 number for the PCR assays into the same range for all materials. The extracted DNA was size separated using
218 agarose gel electrophoresis and stained with ethidium bromide. All DNA was observed to be intact with minimal
219 degradation, with the observed band in the range of 25 kilo base pair (kbp) to 50 kbp.

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[1] Certain commercial equipment and materials are identified to specify the experimental procedure. This does not imply recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material or equipment is the best available for the purpose

220 From the concentration of DNA, the number of copies per assay was calculated using a 1C value
221 derived from several references. The estimates from four references ranged from 2.57 to 2.8 pg per
222 haploid genome (18-20). In our calculations, one haploid genome was considered to be approximately
223 2.6 pg, and each ng of DNA equivalent to 385 haploid genome copies. The calculation of copies / ng for
224 the DNA of any specific event took into consideration the mass fraction of GE corn in the CRM and
225 zygosity (T25 is homozygous, the others are hemizygous). Bt11 has two copies of 35S in its transgene
226 construct.

227 Five published quantitative real-time PCR methods (21-25) for the 35S element were selected and
228 labeled as Method 1 to Method 5 for purposes of this work. All of these methods were selected
229 because of the use of TaqMan probe technology and the small size of the amplicons, which ranged
230 from 68 base pairs (bp) to 101 bp. All methods used Applied Biosystem real-time PCR platforms, but
231 not all the same model. All but one used ABI Taqman® Universal PCR master mix. The cycling
232 parameters were as recommended for that type of assay and master mix. There were a few
233 modifications to the cycling parameters in two methods: a shorter extension time in one method (30 s
234 versus 60 s, Method 2); a longer denaturation time in second method (30s versus 15s, Method 1).
235 Figure 1. shows the location of the primers for the five methods on the sequence of the CaMV 35S
236 promoter sequence. The entire promoter sequence is not shown, only the part relevant to this study.

237 Quantitative real-time PCR assays were conducted at GIPSA and NIST. Assays conducted in the GIPSA
238 laboratory were as described in the published methods. Assays were run on an ABI 7900 instrument
239 using Taqman® Universal PCR master mix at 1x final concentration and the following standard cycling
240 parameters recommended for the ABI universal master mix: 2 min at 50 $^{o}$C (UNG activation), 95 $^{o}$C for
241 10 min (activation of Taq DNA polymerase), followed by 45 cycles of 95 $^{o}$C for 15 s (denaturation)and
242 60 $^{o}$C for 60 s (annealing.extension). A series of four 1:2 dilutions of the DNA were made from the
243 ~20 μg/mL stock DNA and 5 μL of DNA were added to the reaction mix. Each of the five DNA
244 concentrations per product were assayed in triplicate and the log transform of the copy number was
245 plotted against the Ct value and the linear regression curve parameters were calculated. The number
246 of genome copies in the assays ranged from 60 to 2000.

247 The NIST laboratory conducted assays on an ABI Prism 7000 with the primers at the recommended
248 concentration but used SYBRGreen intercalating dye as the fluorescent detection agent. SYBRGreener
249 Universal master mix for ABI Prism (Invitrogen) was used. NIST used the same preparation of primers
250 as did GIPSA. In the SYBRGreen assays the concentration of the primers was the same as described in
251 the published methods. The assays were conducted with the following cycling parameters
252 recommended for the master mix: 50 $^{o}$C for 2 min; 95 $^{o}$C for 10 min; 40 cycles of annealing and
253 extension at 95 $^{o}$C for 15 s and 60 $^{o}$C for 60 s. At the end of 40 cycles a melting curve analysis was
254 performed.

255 The complete experimental procedure was conducted twice with respect to DNA extraction, DNA
256 characterization and SYBRGreen assays with very similar results. Data is not shown for the first set of
257 extractions and extra extractions were done on TC1507 and additional assays performed. The TaqMan
258 assays were performed once at GIPSA on the second complete set of DNA extractions.

259

260 **Experimental results.**

261 Experimental data were produced using 5 published quantitative real-time PCR methods for 35S DNA
262 sequences to test eight GE maize events. Data were not generated for T*nos* sequences. Since we
263 utilized CRMs for this work with certified mass fractions of GE maize, we could compute the copy number of
264 genomes containing the transgene construct. We made a series of dilutions of each of the extracted DNAs and
265 assayed each of the five dilutions in triplicate. We then plotted the data (Ct value versus the log of the
266 transgene copy number) and conducted a linear regression. The slope of the linear regression provided
267 information on the efficiency of amplification with a slope -3.32 being ideal and equivalent to and efficiency of
268 100% (26, 27). The correlation coefficient ($R^2$) indicates how closely the data points approximate the regression
269 line. The intercept indicates the Ct value that would be expected starting with a single transgene copy in the
270 assay.

271 The data for the TaqMan assays are summarized in Table 8. In this table is also recorded the average Ct value
272 for the highest and lowest DNA concentration. This is an easy way to compare the data from one method to
273 another for a given DNA batch. The mean of the slopes across the events were calculated and ranged from -3.16
274 to -3.62, with an exception, TC1507 discussed below. Only one curve of 39 had a correlation coefficient below

275 0.95 and most were 0.98 and above The copy number for the GE product was calculated based on the mass
276 fraction of event DNA, the zygosity and copy number in the genome. Events 176, Mon810, NK603 had about
277 half as many copies per assay as did the equivalent DNA quantity for TC1507, Mon 863, 59122 because of mass
278 fraction differences of the certified RM (~5% versus ~10%). Bt11 was present at the 5% level in the certified RM
279 but it has two copies of 35S per transgene construct. T25 (100% transgene) was diluted to be equivalent
280 toTC1507, Mon863, 59122, and Bt11. Therefore we should expect to see a lower Ct values (~ 1 Ct) with those
281 samples with the2x higher level copy number for 35S. On average the Ct values of TC1507, Mon 863, 59122 and
282 T25 copy number tended to be lower (28.2, 28.6, 29.1, 28.5) than Bt176, Mon810 and NK603 (29.6, 29.6,29.5).
283 No statistically significant difference is claimed here as there is insufficient data for a proper treatment, but the
284 trend was generally in the correct direction.

285 The results of the SYBRGreen assays are shown in Table 9. The data is plotted as for the TaqMan assays with the
286 addition of the melting curve analysis. The intercepts for the SYBRgreen assays were at a lower Ct value than
287 the TaqMan assays across the board. Consistent with lower Ct value for the intercept were lower Ct values for
288 the highest and the lowest DNA concentrations. The slopes were on shallower on average than those of the
289 TaqMan assays, the overall average slope equal to -3.16 for SYBR Green assays versus -3.36 for the TaqMan
290 assays. The range for the averaged SYBRGreen slopes was -2.80 to -3.45, not including maize TC1507.

291 The SYBRGreen assays were a modification of the original published TaqMan method. The primary use
292 of this modified method is to ascertain whether or not a single amplified product is produced in the
293 assay as indicated by the presence of one peak in the melting curve analysis. This analysis revealed
294 that the only (or major) peak had the same melting temperature for each method across the eight
295 event DNAs. However, there were additional peaks seen in some assays (Table 9). Fig. 2 a & b shows
296 melting curves for Bt11 assays where a single peak is seen with the Method 4 assays, while three peaks
297 are seen with Method 5.

298 The biggest anomaly was seen with maize TC1507 assayed with methods two and five, see Table 9,
299 Table 10, Fig. 3. The SYBRGreen assays for method 2 and 5 showed a very shallow slope, much larger
300 Ct values (low and high concentrations) and very poor correlation coefficients. In addition, for method
301 five, evidence for two products was seen with the observation of a second peak with a lower melting
302 temperature (70 C). The TaqMan assays for methods two and five with TC1507 also showed larger Ct
303 values for the high and low DNA concentrations as compared to the other three methods see Table 8.

304 The slopes of TaqMan assays were less extreme than the SYBRGreen assays (-3.156 TM versus –1.12 SG
305 and –2.729 TM versus –2.01 SG).

306 Additional anomalies included multiple products seen when SYBRGreen assays were run on Bt11 DNA
307 with method five. There were three peaks, the expected and two others (Fig. 2). The TaqMan assay on
308 Bt11 for method five gave Ct values that were about 1 Ct value later than methods one to four. The
309 SYBRGreen assays for Bt11 and method two showed a small shoulder on the peak that is the expected
310 product. SYBRGreen assays on NK603 with method five also showed an additional product with a
311 higher melting temperature than the peak of the expected product.

312 **Discussion**

313 The survey showed that a large variety of methods are in use for qualitative and quantitative detection
314 of the 35S and T*nos* elements. While a number of sources were cited for published methods, a
315 significant percentage of laboratories (37 to 53%) are using in-house developed PCR assays for these
316 elements. Some laboratories are using real-time TaqMan assays for 35S and T*nos* as a qualitative tool.
317 Further they are using a variety of calibrants and quality control materials, including CRMs, plasmids,
318 and proficiency testing samples. The survey showed that there is interest in a standardized method.

319 This led us to do a preliminary screening of some published 35S quantitative PCR methods using
320 certified RMs at test material. Amplification of different regions of the 35S promoter, as defined by the
321 primer binding sites and using two different fluorescent detection strategies, TaqMan and SYBRGreen,
322 had utility in the initial screening of methods for 35S detection and quantification. Methods one, three,
323 and four gave consistent results with all GE certified reference materials. The primer binding sites for
324 these three methods are in the same region of the 35S promoter element and produce amplicons
325 related in sequence (see Fig. 1). Methods one and four share the same reverse primer and have
326 overlapping forward primers. The results suggest that the region covered by these three methods is
327 conserved in the DNA sequences of at least these eight events.

328 The data also show that not all 35S PCR methods are likely to give accurate quantitative results with all
329 the GE products tested. There were problems associated with methods two and five, including
330 multiple amplicons in some assays and problems with PCR efficiency, poor correlation coefficient and
331 larger Ct values with TC1507 maize. Since the problems were seen in the SYBRGreen assays, the

332 TaqMan probe is unlikely to be the source of the problem. The reason for this result is unknown but
333 candidates include DNA inhibitors or sequence heterogeneity in primer binding sites. Additional
334 extractions of TC1507 DNA and repeat of the assays showed the same result, making DNA inhibition a
335 less likely candidate for the results.

336 The ILSI on-line survey showed that among the 44 laboratories that accessed and completed the
337 survey, 33 / 32 use a qualitative 35S / Tnos assay and 22 / 16 use a quantitative 35S / Tnos assay. Fifty
338 percent of the labs included in the survey are using a quantitative 35S method, but how many of the
339 laboratories use this as a quantification tool could not be ascertained in this survey. There is
340 continuing interest in using 35S and Tnos as targets for amplification. Table 1 shows that a large
341 percentage of GE products that are in the Agbios GM Database have either or both 35S and T*nos*
342 sequences in their transgene constructs. Maize has the largest number of GE events by far (27) and it
343 is increasingly more common for farmers to plant maize seed that are stacked-trait products. There
344 are 18 of those in the database. This increases the likelihood that 35S or T*nos* sequences are going to
345 be in the genome of the harvested grain. While some recent products and others under development
346 have transgene constructs that use alternative promoter and terminator sequences, 35S and T*nos* are
347 not likely to go away soon from commercial products.

348 The laboratories in the survey run a variety of assays, some of these assays were developed in-house
349 while others came from the literature, ISO standards, databases and official sources (Table 4). A
350 number of methods have gone through a validation process and then an interlaboratory study, such as
351 in references 23 and 28. Some methods have been tested on a variety of events after extensive
352 validation with one event. But there is no method in the literature that has been checked with the
353 multiplicity of events that are available in commerce. Alterations, often proprietary, to the 35S and
354 T*nos* sequences made during the construction of the promoter-gene-poly A site junctions can be the
355 source of error as the primers may be targeted to sequences that do not exist in the construct, and so
356 a given assay may be rendered non-functional. Alternatively, as in the case of TC1507 and Methods 2
357 and 5 gave a positive signal but the quantitation was incorrect.

358 Quality measurements depend on validated methods, determination of uncertainty and the availability
359 of reference materials as well as on equipment, operators, reagents and DNA quality. The use of

multiple methods and issues around availability of reference materials contribute to interlaboratory variability. This variability has ramifications for world trade in food and grain and can lead to trade disputes. A contribution could be made to international harmonization of testing by the availability of standardized methods for 35S and T*nos* that has been shown to work with all events/products in commerce.

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**About ILSI** The International Life Sciences Institute (ILSI) is a nonprofit, worldwide foundation established in 1978 to advance the understanding of scientific issues relating to nutrition, food safety, toxicology, risk assessment, and the environment. ILSI also works to provide the science base for global harmonization in these areas.

By bringing together scientists from academia, government, industry, and the public sector, ILSI seeks a balanced approach to solving problems of common concern for the well-being of the general public.

ILSI is headquartered in Washington, D.C. ILSI branches include Argentina, Brazil, Europe, India, Japan, Korea, Mexico, North Africa and Gulf Region, North America, North Andean, South Africa, South Andean, Southeast Asia Region, the Focal Point in China, and the ILSI Health and Environmental Sciences Institute. ILSI also accomplishes its work through the ILSI Research Foundation (composed of the ILSI Human Nutrition Institute, the ILSI Risk Science Institute, and the ILSI Center for Health Promotion). ILSI receives financial support from industry, government, and foundations.

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454 **Figure Legends**

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456 Fig. 1 CaMV 35S sequence, V00141 from GenBank, showing primer placement for methods 1-5

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458 Fig. 2. Melting curves for SYBRGreen assays of Bt11 showing a single peak for method 4 assays (2A) and three
459 peaks for method 5 assays (2B).

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461 Fig. 3. Plot of SYBRGreen assays of TC1507 using methods 1 and 2 with linear regressions. Assay parameters for
462 methods 1: $R^2$ = 0.982, and slope of -2.94, method 2: $R^2$ = 0.74 and slope of -1.12.

463 Figure 1.

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466 Fig. 2 A & 2 B

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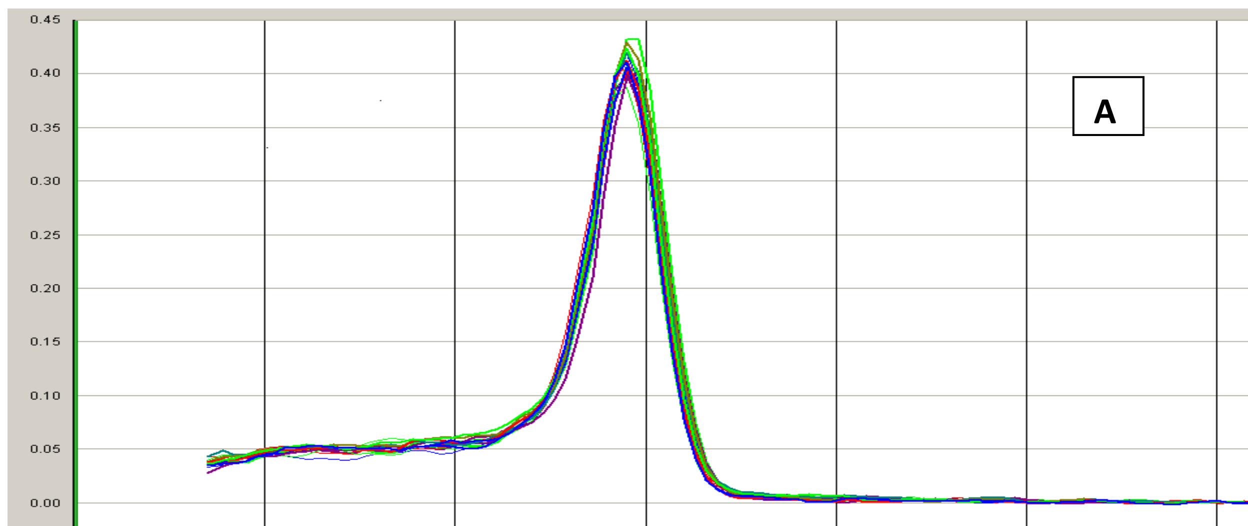


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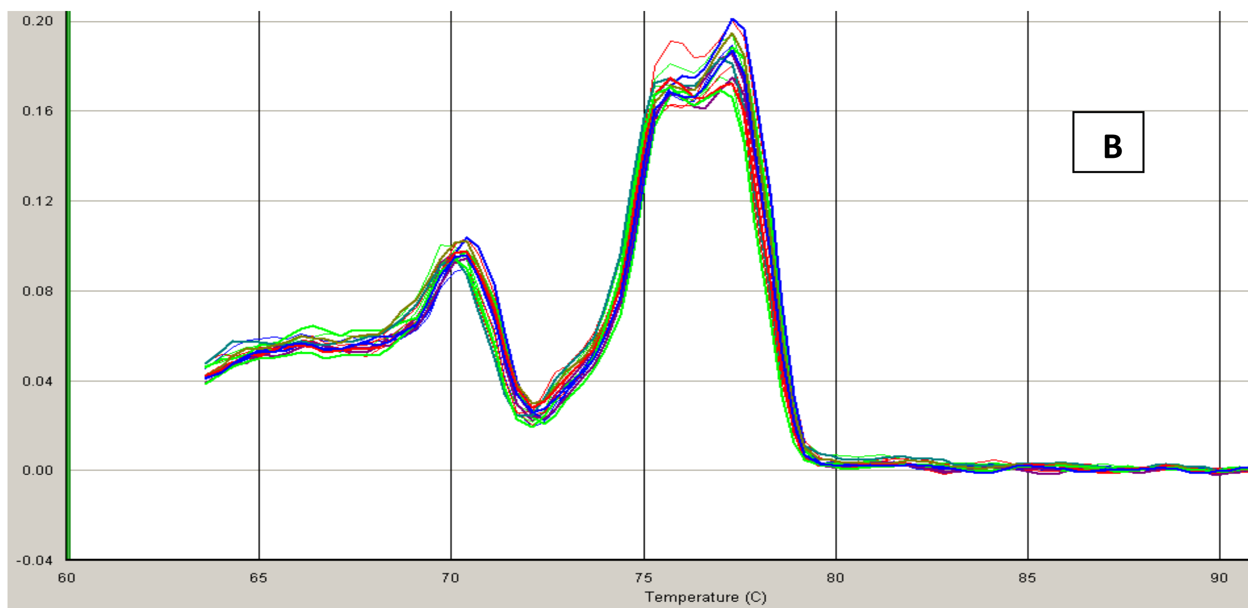


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476 Fig. 3

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