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NEWS AND VIEWS

REPLY

# Resolution of the Mexican transgene detection controversy: error sources and scientific practice in commercial and ecological contexts

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Bernd Schoel and John Fagan (Vice-President and Founder/CEO, respectively, of Genetic ID, henceforth BS&JF) criticize and dismiss our recent publication in *Molecular Ecology* by focusing on our use of the Polymerase Chain Reaction (PCR) to detect specific DNA sequences. They raise important questions about the standards required to use PCR in various environmental conditions, pointing to the well-known fact that this delicate method may lead an unskilled operator to false results. They further suggest that our observations of transgenic DNA sequences in Mexican landrace maize should be attributed to false positives, i.e. a type I error. After considering their challenge and reviewing the evidence, we find their arguments seriously lacking in substance, and their practice permissive of false negatives, a type II error.

We seem to have attracted BS&JF's attention because, in an effort to corroborate our own results, we utilized the services of Genetic ID as full-paying customers. We established that Genetic ID failed on occasion to detect positive blind samples, which should not be surprising given the known vagaries of the PCR method. Yet for BS&JF this detection failure is not a factual possibility; instead, to explain our observations they would have us both (i) contaminating our samples and (ii) lying about the origin and nature of our materials. Specifically, BS&JF state:

1 'We contend that results such as these are incorrectly interpreted as positive and are more likely to be indicative of contamination in the laboratory.' and
2 'We would argue that the leaf sample provided by the authors did not contain the claimed NK603 event and, furthermore, does not contain material from any commercialized transgenic single plant.'

Other charges include an implication that we used false evidence and/or withheld inconvenient data (BS&JF, p.5, lines 5–11) to reach our conclusions.

All of these are indeed very serious challenges to our technical capacity and expertise, as well as our professional and personal integrity.

## PCR contamination or false negatives (type II error)?

BS&JF declare their suspicion that all of our PCR positive results arose from systematic contamination. They note the presence of bands in the PCR gels that are weaker than they would expect for a '100% (homozygous) or 50% (heterozygous) GMO level', the only evidence that they would take as a positive result. Such a view is based on the unwarranted expectation that an end-point PCR could be used as a quantitative method.

In our experience and that of other independent laboratories, the PCR amplification of transgenic sequences in landrace maize backgrounds tends to produce relatively faint bands of variable intensity in end-point reactions visualized on agarose gels, which so far has been the standard approach in the field (Quist & Chapela 2001; Alvarez-Morales 2002; Piñeyro-Nelson *et al.* 2009). Genetic ID's own gels (their standard to screen-out 'negatives') show this kind of variability, even for repeats of a single sample in a single assay, or for different assays performed for the same sample at different times [see Fig. S1 (Supporting information)].

Such results should not surprise anyone versed in the PCR method. Although early cycles in the PCR assay may reflect stoichiometric molecular relationships, end-point

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PCR should be expected to be variable for many reasons including the gradual inactivation of *Taq* polymerase, competition against primers through amplicon reannealing, and exponential increase in product to be amplified (Butler 2005). Because these are all stochastic processes that become exponentially amplified as the PCR cycling proceeds, variable plateau concentrations of amplicon (i.e. band intensity) should be expected even under the most strictly controlled conditions (Heid *et al.* 1996).

Nevertheless, BS&JF expect homogenous and strong bands in every context, and it is apparently their view that any sample with somewhat questionable intensity in gel bands should be declared a 'negative' and should not be studied further. We note that Genetic ID's procedures recognize but dismiss the existence of variability in samples. For example, a 'negative' sample for which there may be a weak but visible band in a duplicate PCR gel is reported as 'above detection level or detected', and in other cases where clear bands are present in PCR gels, results were at times reported as 'negative at the operational limit of quantitation of 0.1%'. BS&JF now choose to assign all the above-mentioned results as 'negatives', when in our experience such samples often proved clearly positive upon further molecular analyses. Thus Genetic ID has established standards which are permissive of, and indeed prone to, false negatives (type II error).

There is evidence arguing against BS&JF's PCR contamination hypothesis. We observe, for example, that the presence of positive bands in our samples is neither randomly nor homogeneously distributed as would be required by such a hypothesis. Specifically, at the inception of our study, maize ears were collected, seeds were subdivided from each ear and distributed independently to our separate laboratories (RR in Irapuato and EAB in Mexico City) by an outside researcher (S. Ortíz-García, a co-author with B.S. in Ortíz-García *et al.* 2005). Maintaining each laboratory in complete isolation from the other through double-blind coding, seeds from these subsamples were germinated, emergent leaf tissue lyophilized in facilities free of cloning or PCR products, extracted and PCR-amplified, after which we compared all results for congruence. As explicitly described in our original study, we took a highly conservative position before we would call a positive sample: samples were never scored as positive unless we had at least two repeated confirmatory results in each separate laboratory based on independent DNA extractions and amplifications.

Under these circumstances, the laboratory contamination implied by BS&JF should be expected to either appear in all samples or to be randomly distributed among families within laboratories, with a possible differentiation between the two laboratories reflecting their differing patterns of contamination. None of these scenarios occurred. Families and/or localities consistently appeared with positive individuals in both laboratories while others consistently failed to show positives. We have now subjected all our results to a statistical analysis showing that the distribution pattern of positive samples among seed families or localities is indistinguishable between the two independent laboratories; i.e., overall, the frequency of positives among families matches across laboratories. The probability of this pattern emerging from a contamination source is $<0.001$.

Naturally, there were instances where we suspected false positives and false negatives based on the performance of appropriate controls. On occasion, we also saw lack of reproducibility of results within or across laboratories for individual samples that were further analysed. Such results are to be expected in any normal laboratory procedure, and certainly in a PCR-based process, but we designed protocols and controls to deal with this expectation and never allowed questionable results to be included in our publications.

Thus we remain confident of our statements even if considered only from the partial perspective of PCR amplification. However, other evidence supports our claims.

### Inappropriate standardization for transgene detection in native maize landraces

We believe that while Genetic ID's methods may well be suited for commercial use on commercial crop varieties in the U.S., they seem inadequate for research-oriented and environmental applications. BS&JF's vague assertion that Genetic ID has 'standardized validation procedures using all transgenic maize events and 14 species commercialized to date' (Schoel and Fagan, p. 4) covers at best their proficiency in working with the limited genetics of commercial hybrid maize. Most commercial GMO assays (including Genetic ID's) have been optimized using maize varieties widely grown in the United States including *commercial* transgenic lines, all of them built upon a very homogenous genetic background, a cross of Northern Flint x Southern Dent. Both inbreds, and Northern Flint in particular, are quite divergent genetically from Mexican maize land races (Doebley *et al.* 1986, 1988). There are good reasons to believe that such limited focus may place Genetic ID's methods at a relative disadvantage for detecting transgenic DNA sequences in landrace maize. Using real-time PCR, we found that there are significant differences when comparing a hybrid transgenic commercial line against a landrace sample in the relative amplification of an internal control, a zein gene, included in the TaqMan® kit for the quantification of the 35S CaMV promoter sequence (see Fig. 1). This work was completed in 2005 using materials relevant to our publication (Piñeyro-Nelson *et al.* 2009). We also observed unexpected results such as high background levels when using ELISA kits for the Cry9c protein on landrace maize, but in this case open discussions with the manufacturer identified the problem and allowed subsequent kits to perform successfully in testing local landrace samples.

The challenges faced by a commercial approach to study landrace materials relate to: (i) the diversity of genetic backgrounds leading to expected inefficiencies and higher degree of variability in the PCR; (ii) uncertainty related to internal-standard variability; and (iii) sequence diversity even for expected target sequences.

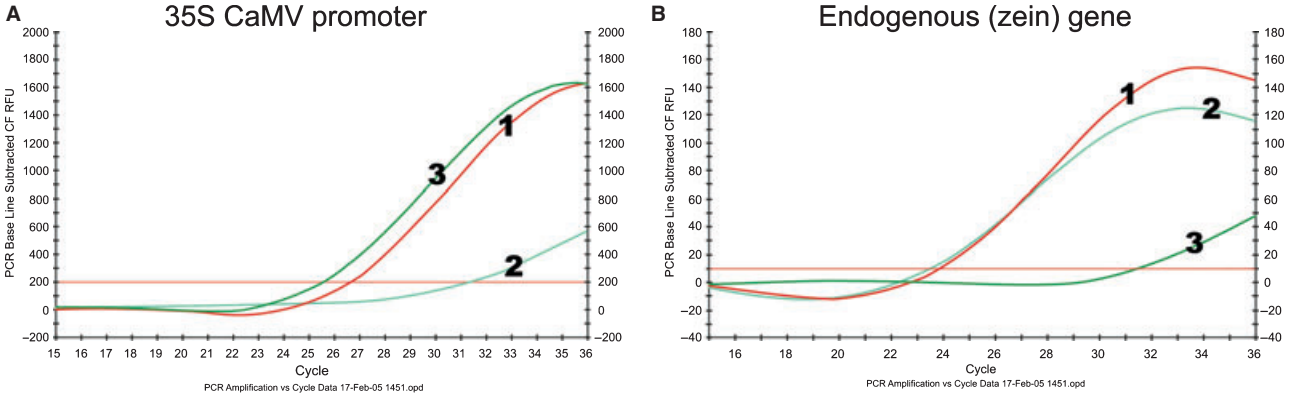


**Fig. 1** Amplification curves in qPCR assays using the TaqMan® GMO Maize 35S Detection Kit (Applied Biosystems). A. 35S CaMV amplification and quantification was efficient for both the positive control, a commercial transgenic line (1) and a transgenic landrace variety (3); whereas the negative control, a commercial non-transgenic maize line (2) did not produce a proper amplification signal. B. Amplification and quantification of an endogenous gene (believed to be a zein gene) was efficient in both commercial transgenic and non-transgenic maize lines (1, 2), whereas the quantification in the landrace variety (3) was not possible (even though a PCR product was visible in the gel) perhaps, according to the manufacturer, as a result of a faulty interaction of the PCR product and TaqMan® probe.

**1** We have already pointed out the expectation of much higher levels of molecular diversity in Mexican landrace samples with a diverse genetic background compared with hybrid, commercial varieties (Piñeyro-Nelson *et al.*2009). Significant genome size variation among landraces has been reported (1700 to 3300 megabases; see comment in Walbot 2008), while lack of genetic colinearity and pervasive gene duplication have been described (Fu & Dooner 2002; Wang & Dooner 2006). We stand by our expectation that such diversity could cause inefficiencies and variability in PCR results stemming from direct or indirect molecular effects on any of the components and conditions of PCR assays. In these conditions, a protocol with no flexibility for careful observation and follow-up of bands that are less than optimal would create ample opportunity for false negatives.

**2** BS&JF dismiss any discussion of PCR inconsistencies by vaguely invoking an undefined and unaccountable protocol, thus:

'[Genetic ID] includes at several points in its analytical procedures controls that would detect the kinds of problems cited by the authors and therefore ensure accurate reporting of results. For example, PCR inhibition tests are routinely conducted to rule out the presence of compounds ('metabolites') that could interfere with PCR amplification.'

Of particular interest is their claim of a standard, routine test for inhibition of the PCR assay, which should stand for any and all sources of inhibition possible from commercial *and* landrace materials that have a wide range of, for example, phenolic compounds in their constitution (Arnason *et al.* 1994); no details are given about the specific sequences used in such tests, gene dosage or specific genetic behaviour. Our own experimental routine shows this facile dismissal of the inhibition problem to be fallacious. Specifically, we showed as part of our careful method evaluation that there are indeed differences between commercial, hybrid maize varieties and landrace materials as far as their PCR performance is concerned.

**3** In their critique, BS&JF deride our expectation of sequence diversity in our target sequences by claiming that such an expectation violates 'the known and accepted norms of genetics' (Schoel & Fagan, p. 3). BS&JF's sole source of support is a general evaluation of the average rate of spontaneous mutation across broad taxonomic groups (Drake *et al.* 1998). This approach fails to recognize site-specific differences in mutation rates, especially well known in transgenic constructs where, for example, the borders of the transgenic construct are prone to sequence variation (Matsuoka *et al.* 2002). Maize itself has highly variable mutation rates at different loci, ranging from $<0.1 \times 10^{-5}$ to $49.2 \times 10^{-5}$ mutations per gamete, while the equivalent rate for retrotransposons is a much higher value of 0.1 per genome per replication (Drake *et al.* 1998).

Our sequencing data do confirm that there was indeed sequence diversity among the events of transgenic transformation that we were able to detect (Piñeyro-Nelson *et al.* 2009), which is also further evidence against the possibility of DNA contamination suggested by BS&JF.

The fact stands that Genetic ID was unable to detect clear positive samples in various forms. Their standard methods failed to detect positives not only in landrace materials, but also in a non-commercial hybrid variety produced by CIMMYT and even in the well-documented Monsanto transgenic event NK603. Our own positive detection of transgenic sequences in the same samples through a variety of methods shows that they were present, so we must assume that some or all of the possibilities discussed above may be responsible for Genetic ID's false

negatives. As stated in our previous manuscript (Piñeyro-Nelson *et al.* 2009, p.758), specified on Genetic ID's webpage (Verified on July 16th, 2009; http://www.genetic-id.com/About-Us.aspx), and commented to one of us by B.S. himself (personal communication to APN), the CIMMYT event was likely not detected by GID as a result of variation in the 35S CaMV promoter sequence; the very suggestion BS&JF now dismiss. Finally, it is clearly strange that BS&JF would decide to question the positive nature of our sample #5, which by their own standards showed specific PCR amplification of the 35S CaMV promoter with 100% or close to 100% in duplicate assays from independent DNA extractions. In the latter case, BS&JF appropriately call for non-PCR methods to strengthen the hypothesis that such a sample is positive, a surprising call as this standard does not seem to carry value anywhere else in their discussion of our study.

### Alternative molecular assays: overwhelming evidence of transgenic sequences in Mexican maize landrace varieties

While BS&JF focus on a critique of our PCR methods to entirely dismiss our study, we are convinced that the only way to resolve the basic quandary of whether transgenic sequences are found in Mexican landraces of maize or not, is not hypothetical argumentation, but independent evidence. We have produced non-PCR evidence which makes us feel strongly that our conclusions are fully warranted: (i) the sequencing of PCR products; (ii) DNA–DNA hybridization (Southern Blot); (iii) immunological detection of proteins derived from a transgenic sequence; and (iv) physiological tests of transgenic sequence expression, such as herbicide resistance tests.

**1** We have systematically confirmed the presence of 35S CaMV sequences in our samples, a strong sign of transgenic DNA presence. From sequence data we conclude again that contamination with a laboratory DNA sequence is highly unlikely because the sequences obtained over time and through an independent sequencing laboratory were not identical to each other.

**2** Southern Blotting is well known for its propensity to deliver false negatives, providing stringent, independent confirmation of PCR-based results. Our very consistent match between PCR and Southern Blot results leaves little reasonable doubt about the validity of our statements.

**3** In a recent study, independent samples were collected by researchers (George Dyer and Hugo Perales) different from the collector for Piñeyro-Nelson *et al.* 2009 (2001 samples collected by S. Ortíz-García). These new samples were assayed independently by another researcher (Antonio Serratos) in a different facility using another type of method; an ELISA immunoassay targeted at detecting Cry1Ab/Ac and CP4 EPSPS recombinant proteins. The results from this study (Dyer *et al.* 2009) again support our conclusion that transgenes are present in Mexican maize landraces.

**4** We provide here further ELISA data for 347 seedlings analysed for the 2001 maize seed collection reported in Piñeyro-Nelson *et al.* (2009). Thirty-three out of the 347 plants assayed were positive for Cry1Ab/Ac; interestingly, 20 of these positives were distributed among the 10 families previously scored as PCR positives for the 35S CaMV promotor. [see Table S1, Supporting information and examples in Fig. S2 (Supporting information)]. In these assays, positive individuals for Cry1Ab/Ac were detected in localities 7 (1 in 50), 11 (1 in 29) and 23 (4 in 41), among others. We also performed BASTA resistance assays and found positive individuals for these three localities [see examples in Fig. S3 (Supporting information)]. The latter data had not been included in the study under discussion (Piñeyro-Nelson *et al.* 2009) because in this experiment we focused on multiple assays at the DNA level that could be performed on the same ten putative 'positive' and 'negative' individuals, for which we lacked enough tissue for ELISA (and live plants for BASTA analyses).

All the evidence quoted above consistently contradicts the critique by BS&JF.

### Validity of the NK603 positive control

BS&JF state:

'We would argue that the leaf sample provided by the authors did not contain the claimed NK603 event and, furthermore, does not contain material from any commercialized transgenic single plant.' (Schoel and Fagan, 2009, p. 2).

Against such a challenge to our integrity and competence, we can only restate that the sample in question represented leaf-tissue obtained from a certified NK603 maize variety, grown by us but extracted and PCR-processed by Genetic ID and for which positive results for SB and PCR were presented in Piñeyro-Nelson *et al.* (2009).

BS&JF not only discredit us actively, but also several other scientists by omission. When BS&JF state that their '…conclusion from both publications on this topic is that results obtained to date are not sufficient to ascertain whether introgression of transgenic traits into the Mexican maize population has or has not taken place', as if there were only two publications –Piñeyro-Nelson *et al.* (2009) and the one where Bernd Schoel is the main methodological author (Ortíz-García *et al.* 2005)– they unexplicably choose to dismiss the work of many others, and ignore the fact that the list of published studies confirming the presence of transgenic DNA sequences in landrace maize varieties in Mexico is long and growing. It includes studies by Quist & Chapela (2001), Serratos-Hernández *et al.* (2007), Dyer *et al.* (2009), as well as a long list of public statements, presentations and governmental reports (for a review of the former until 2007, see Mercer & Wainwright 2008). A few of these publications include samples from the same Oaxaca region considered in our study for 2001 and 2004 and are thus confirmatory of our results. Except

for the one study for which Bernd Schoel is an author and in which Genetic ID is again implicated, all of this considerable body of evidence contradicts BS&JF's statements. We understand that science is not made by simple addition of papers but rather by reasoned and tested evidence, but BS&JF do not contemplate the evidence and reasoning of many experienced scientists, and this we find as unusual as it is unacceptable.

## Conclusion

An overwhelming body of evidence has accumulated from many experienced researchers, using a diversity of independent methods and often published under the most stringent (even hostile, see Delborne 2008) standards to state that transgenic DNA sequences are present in collections of Mexican maize landraces. BS&JF are not loath to stand against such overwhelming evidence yet provide nothing but the face value of their statements to support their position.

It is impossible to test BS&JF's statements using the standard methods of science because they have not provided full, transparent disclosure of many details in their methods as because of intellectual property claims by their company, Genetic ID. Even with limited information, we have shown here that BS&JF base their statements on a system biased at many levels in favour of type II error, i.e. a system which is overly permissive of false negatives.

BS&JF seem honestly convinced of the infallibility of their proprietary system at Genetic ID, and do not see the need to account for it transparently. However, the value of a scientific procedure lies not in its infallibility but rather in the qualities that make it falsifiable by independent confirmation or refutation based on the transparent description of methods and procedures. It bears stating that although science can be used for commercial purposes (and Genetic ID may have very good science applied in their practices, although we cannot attest to this), the converse is not easily achieved.

The detection and monitoring of transgenic DNA in commercial and non-commercial living organisms is of great environmental importance. We learn from the experience of this discussion that such monitoring is extremely difficult under the conditions of high variability characteristic of the most delicate environmental situations. In the case of maize, we see that methods that may be acceptable in highly homogenous situations should not be expected to work in the diverse conditions found, for example, in the centres of origin and ongoing diversification of crops. Most importantly, we conclude that, given the challenges demonstrated here, monitoring for transgenic DNA in the environment should be performed by independent, non-commercial, transparent institutions with a clear mandate of public good instead of profit.

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## Supporting information

Additional supporting information may be found in the online version of this article.

**Fig. S1** PCR assays conducted in different moments by Genetic ID on same samples. A. Samples analysed on June 2004 (edited from figure 1d published in Piñeyro-Nelson *et al*. 2009). B. Samples analysed in November 2005. C. Samples analysed on September 2006. Labelling in panels B and C corresponds to the same samples as in panel A. Samples sent for analysis at Genetic ID comprised lyophlilized leaf tissue from maize landraces of the 2001 maize collection; samples were DNA extracted at this company. Note significant amplification variability both between duplicates of a given sample (see samples 4 and 6 in panels A and C), and among replicates of a positive control (see 0.01% control in all panels).

**Fig. S2** ELISA tests performed on 2001 maize seedlings. A. Lateral flow ELISA strips for LL (Pat/Bar) and Bt (Cry1Ab/AC). The two bands in the 'Bt' strip are a positive result for Cry1Ab/AC. In the foreground is the locality code. B. ELISA plate assay for detection of Cry1Ab/Ac in maize landrace samples. First column on the left shows progressive dilutions of the positive control; second column shows wells with extraction buffer. Samples are in quintuplicates and comprise both clearly negative samples (see wells in the sixth column, positions B to F) and clearly positive samples (see wells in sixth column, positions G, H and seventh column, positions A to C).

**Fig. S3** BASTA herbicide resistance assays. Panel A shows a leaf from a plant from locality 17 which is BASTA -resistant; Panel B shows a susceptible plant from locality 20. BASTA herbicide was applied by hand within the painted circle marked in each leaf.

**Table S1** Results of ELISA assays for Cry1Ab/Ac expression, conducted on leaf tissue of seedlings from the 2001 maize collection

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