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Is confidence in the monitoring of GE foods justified?

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Often the limits of detection of genetically engineered organisms (GEOs, LMOs, GMOs) determine what legislation sets as thresholds of allowable contamination of the human food chain with GEOs. Many countries have legislation that is triggered by certain thresholds of contamination. Importantly, international trade in food and animal feed is becoming increasingly vulnerable to interruptions caused by the ambiguity GEOs can create when shipments are monitored at the border. We examine the tools available for detection. Four key error-generating stages are identified with the aim of prompting a higher uniform standard of routine analysis at export and import points. Contamination of the New Zealand corn crop with GEOs is used as a case study for the application of monitoring tools and vulnerability to errors. These tools fail to meet emerging food safety requirements, but some improvements are in development.

Attention to food safety is growing in proportion to fears that food might be the target of terrorists or saboteurs, and concerns about the impacts of GEOs in different environments. Uninvited trans-border GEO contamination is demonstrating both how effective and porous food contamination monitoring can be. Is current monitoring capacity enough to prevent both the malicious and the accidental compromise of the food chain with illegal and/or harmful organisms or biomaterials?

We describe the regulatory 'food safety chain' in operation, highlighting the four procedural stages that are most susceptible to producing false confidence in data that regulators use to monitor GEOs. For illustrative purposes, we highlight the New Zealand experience with incursions of GE corn in 2003 [Team, N.P.P.R.L.I.I. (2003) *Suspect Genetically Modified Corn Investigation Report* (IIT-03/04-GM1–002) New Zealand Ministry of Agriculture and Forestry], and another putative incursion circa 2000 [1].

Monitoring the food chain has two goals. First is to detect and eliminate organisms or genes of potential threat. Second is to achieve detection limits that are relevant to the proportion of contamination that might cause harm. Monitoring capacity must be qualitatively and quantitatively relevant to the risk, where risk is a function of both the probability of a threatening event and the size of the effect should the event occur. GEOs pose specific challenges to monitoring because it is difficult if not impossible to determine how much GEO is enough to cause harm, especially as initially small numbers could reproduce into bigger numbers of organisms.

Monitoring GEOs can be seen as an extension of existing food safety frameworks developed for human pathogens and agricultural pests. However, the capacity of regulatory authorities can be pushed beyond routine limits by the relatively small changes in GEO genomes. Inability to make conclusive identification of agricultural goods might result in more frequent and costly disruptions to international trade (Box 1).

Box 1. Genes and trade

By 24 December 2003, just days after confirming that a cow in the United States suffered from bovine spongiform encephalopathy (BSE), 20 countries imposed bans on US meat exports, a move that could cost the USA $2 billion (http://www.nzherald.co.nz/storydisplay.cfm?thesection = newsandthesubsection = andstoryID = 3541061 andreportID = 1162607, accessed on 9 January 2004) [20]. The BSE incident demonstrates how important proper identification of materials in the food chain is for international trade.

Likewise, proper GEO monitoring must be as focused on detecting signs of unknown or undesirable organisms and genes as it is on monitoring known but prohibited commercial GEOs. Incomplete identification will more frequently result in pre-emptory rejection of imports at the border as countries increasingly take a precautionary stance. This has different impacts on different countries, because different countries have different ways of organizing their agricultural sectors. For example, from the view of the USA, a proportionately small number of producers is distributing their products across a much larger number of small markets, including a large domestic market. By contrast, New Zealand has many small producers that are concentrating their products into a few big markets (e.g. Japan, the USA and Europe) and a small domestic market.

For small exporters and small countries, even sporadic delays and occasional rejections of their products can be devastating to reputation and economy. This has been the lesson for New Zealand, where Japan reported it as a source of uninvited GEOs (sweet corn) and most recently blocked the import of bread dough manufactured using a product from a GEO.

While GEOs increase uncertainty in identifying organisms and genes, they increase the risks of and to international trade in agriculture. International best-practice standards for establishing the identity of any contaminating organism are urgently needed.

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This review was developed for both the scientific and regulatory communities. The broader scientific community might benefit from seeing how scientific information is presented to the regulatory community. It is they who must translate idealized laboratory protocols into effective environmental surveillance. Increasingly, effective regulation requires custom science.

Why monitor GEOs?

Unapproved material derived from a GEO is not permitted in the human food chain [2]. Although foods containing approved GEOs are more often being labelled, some exemptions are also common. The European Union (EU) exempts labels on foods contaminated with 0.9% or less the content from *authorized* GEOs, provided that the contamination was technically unavoidable or adventitious (Regulation No. 1829/2003). There is a transitional threshold of 0.5% for 'not yet authorized' materials containing, consisting of, or produced from GEOs. At or below that threshold labelling is not required provided that again this presence is adventitious or technically unavoidable; the genetically modified material received a favorable opinion from the Community Scientific Committee(s) or the Authority before 18 April 2004; the application for its authorization has not been rejected, and detection methods are publicly available. Thus, exceptions to labelling are granted provided that a conclusive identification of the contaminant has been made (Table 1 and Box 2) and that the contaminant can be assumed safe for human consumption (Box 3).

What is needed for proper identification?

Polymerase chain reaction (PCR) is a crucial tool in monitoring movements of food and animal feed both between and within countries [3,4]. PCR's reputation for amplifying DNA from very low initial concentrations in forensic police work has won it a place in environmental monitoring despite significant, under-appreciated differences in these two applications (Box 4). Addressing these limitations requires a holistic analysis of the monitoring process.

Monitoring has four stages that are most prone to producing false indications of contamination or estimates of the concentration of contaminants (Figure 1).

(i) Determining sample size and sampling technique.

(ii) Determining the size and homogeneity of the subsample from which DNA is to be extracted for analysis.

(iii) Determining the sensitivity, or resolution, of the PCR reaction.

(iv) Evaluating the data presented to regulatory authorities. Poor or incomplete reporting can impair proper decision-making and review by authorities and scientific advisors (Table 1 and Box 2).

Each of these stages contributes to 'sampling error', in other words, uncertainty of the extent to which any PCR result is representative of the tested material. Each of the first three stages (to be discussed in detail below) generates a theoretically quantifiable error. Overall uncertainty is a function of the error in each step and can be significantly larger than the single largest error of any single step in the process from material isolation to PCR result. The literature on this topic tends to discuss one or at most two stages of monitoring and neglects the error contributed by the other stages. The reason to consider the error holistically is to draw more responsible conclusions about the precision of the analysis. (Although stage 4 errors cannot be quantified, they are taken as self-evident and not discussed in detail).

Table 1. Establishing identities of approved GEOs suspected of causing adventitious contamination of food

Recommended standard	New Zealand Standard as of 2003
• Establish the 'chain of custody' from consumer through to farmer on to importer and finally to seed producer, including breeding/cultivation records and the results of any earlier testing for adventitious contamination, as well as how material was sampled	Achieved
• Confirm positives by Southern hybridization and sequencing	
• Use a battery of primers for all known DNA modifications	Able to detect a variety of modifications
• Develop or acquire custom primers specific to commonly used elements such as the CaMV promoter *P-35S* and *nos 3′* terminator. Event-specific primers should follow an analysis with primers spanning short sequence distances to avoid a false negative because of either rearrangements that occurred at time of integration or recombination events that might have separated the 5′ and 3′ ends of fragment to be amplified	?
• Develop customized primers from the sequence of any amplified fragments from earlier rounds of testing. Only custom primers make quantitative claims (e.g. to the 1% or 0.5% level) of resolution plausible	?
• Secure data from contracting organizations or suppliers in a form that can be interrogated by independent specialists. The results should include information on the three key sampling error stages (Figure 1 and below)	?
Test material for heterogeneity at other, unmodified, loci[a]	?
• Test parentage of organisms within a sample	
• Molecular profiles of approved varieties of GEOs, especially using a mix of markers that are heterozygous and homozygous, should be made available to the regulatory communities	?
• If the minority genome from food samples displays the profile expected of an approved GEO, identity has been established to a high standard	?
• If the minority genome instead produces a different profile, then more testing is required or the GEO should be considered unknown and untested	?

[a]Some techniques for parentage testing are DNA sequencing, random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) or multilocus strain typing (MLST) (e.g. Ref. [19]).

Box 2. A case study in regulating a putative GE contamination event

Each stage of testing for GE contamination is vulnerable to uncertainties as a result of technical and human error, as well as uncertainties that arise from uncontrollable variables. Examples of the stage errors depicted in Figure 1 will be provided from a review of an investigation into an alleged contamination of New Zealand with GE corn [1] in the year 2000. That this example is from a first world country underscores the need for a greater global capacity in biosafety.

The first stage 1 error arose when New Zealand investigators were unable to confirm the chain of custody because some seed samples used in testing were taken from open seed bags, leading to controversy over whether the samples could have been contaminated by soil. When a consignment of seed is isolated for testing, a 'chain of custody' must be established. The chain should encompass all who had contact with, or control of, the seed, from consumer through to farmer on to importer and finally to the seed producer. Evidence establishing the chain should include breeding/cultivation records, results of any earlier testing for adventitious contamination, as well as how material was sampled. Original seed certificates provided by the exporter should be confirmed by tests conducted by independent laboratories contracted by the regulatory authority. Some data could not be required by regulatory authorities or even a special parliamentary investigatory committee because the testing was performed under contract to the seed producer. This latter oversight can lead to stage 4 errors.

A second stage 1 error came from sampling technique and size. Most laboratories used 1000 seed samples. Most testing laboratories reported to the New Zealand Ministry of Agriculture and Forestry that they could reliably detect as little as 0.1% contamination. However, this value is not an actual detection limit. According to the USDA tables, at a hypothetical contamination of 0.1%, 1000 seed samples would, at best (neglecting stage 2 and 3 error), lead to incorrect acceptance of a consignment nearly half the time.

The seeds sourced from New Zealand were tested by laboratories in three different countries, each following different protocols introducing stage 2 and 4 errors. The testing laboratories either failed to report their milling procedures or used vague terms such as 'coarse'. The mass of the subsamples was also either unreported or varied as a proportion of total sample by almost two orders of magnitude between laboratories (from 0.02–1%). Furthermore, testing laboratories reporting to New Zealand did not report primer sequences, specificity in experimental samples, or range of primers used.

Some laboratories did report contamination, but the putative amplified DNA was not further investigated, a source of stage 3 and 4 errors. On detection of a putative contaminant, the amplified DNA product should be isolated for sequencing and development of custom primers.

Sampling

The first step in any surveillance is determining how and how much to sample [2]. Sample sizes must be big enough to detect contaminants at concentrations that could cause harm. Inconsistency in sample sizes or improper sampling technique can result in 'jackpot' distributions of positive and false negative results which causes the contaminant to go undetected, or undermines efforts to quantify detectable material (Table 1 and Box 2). The USDA has published electronic spreadsheets for determining seed sample sizes (http://www.usda.gov/gipsa/biotech/samplingplan1.xls, Accessed on 9 January 2004). These spreadsheets calculate either sample size for a desired detection limit or the detection limit for a known sample size. The spreadsheets should be used as a rough guide

Box 3. Labelling thresholds as safety thresholds?

New Zealand Food Safety Standards (1.5.2) allow for adventitious contamination of food by GEOs, provided that they are identified in the Standard. There is evidence that this proviso to the labelling regulations has been misunderstood as an implicit indication of food safety [21]. In 2003, a GEO not approved for release in New Zealand, with a known modification, the 'Bt11 event', and no evidence of other known modifications, was assumed to be approved for consumption without labelling, despite the identity of the GEO not being confirmed beyond this description (http://www.maf.govt.nz/biosecurity/imports/plants/papers/gm-sweetcorn/sweetcorn-briefing-2003-08-01.pdf, Accessed on 2 November 2003). At that time, a second GEO was detected but not identified even to the level of a specific type of modification (http://www.maf.govt.nz/biosecurity/imports/plants/papers/gm-sweetcorn/test-results-gisborne-2003.pdf, Accessed on 2 November 2003). The Food Safety Authority concluded that because the 'Concentration of [the] GM organism [was] less than 0.05 percent... well below the Australia/New Zealand standard for unintentional presence of 1 percent... no further action' – that is, testing, monitoring or recalling food contaminated by the unknown organism – was required (http://www.nzfsa.govt.nz/publications/media-releases/2003/2003-07-07-corn2.htm, Accessed on 2 November 2003).

By contrast, the EU requires approval for deviations from approved strains, even for hybrids arising from crosses between GEOs, presumably because the hybrid might be sufficiently different from either parent to pose uniquely different implications for human and environmental health [14,22].

Moreover, the relationship between proportion of GEO DNA and the amount of GEO or gene-product is not absolute. DNA yields are not equivalent between cultivars, with some yielding up to 20% less DNA than expected from their weight-to-weight ratios [4]. This fact alone can cause significant underestimation of the proportion of GEOs in a mix. If in the New Zealand case the transgenic cultivar were on the low end of the DNA yield curve, the actual contamination frequency based on 0.05 ng of DNA would be 0.0625%. Similarly, a hypothetical detection in Europe reported to be at a frequency of 0.89% would correspond with an actual frequency of 1.1%.

Furthermore, if the transgenic were itself a hybrid, additional complications are introduced because the proportion of genomes from the genetically engineered parent will vary in the hybrid depending on the sex of the parent [4]. Kernels are composed of three different kinds of tissues: embryo, endosperm and teguments. These tissues vary in the quantity of DNA, the number of copies of the genome, and in the mix of DNA from the male (pollen) and female (egg) parent. Whereas teguments contribute negligible amounts of DNA to the kernel, the amount contributed by the endosperm varies over a large range, from 36–60%. This particularly complicates matters because the endosperm is triploid and two-thirds of the genomes are maternal [4]. A hybrid contaminant detected at 0.05% would actually be as high as 0.15% if its father was the transgenic and it was a low DNA yielding cultivar. In a 100 ng DNA sample taken from ground seeds, only 0.024–0.036 ng transgenic DNA would derive from the embryo and 0.016–0.024 ng from the endosperm, for a total of 0.048–0.052 ng of DNA representing the transgenic genomes. Regardless of the true contamination frequency, the PCR result can underestimate the quantity of an unknown transgenic by a factor of 3–4 because of this variable alone.

The evidence used by New Zealand regulators leaves open the possibility that the contaminating GEO is an uncharacterized pre-commercial hybrid of the same species, a novel organism that has more than one modification including the one detected, a novel hybrid that arose when an unintentional and uncharacterized DNA insert [9] on another chromosome of a GEO was acquired by a previously unmodified conspecific, or an unknown organism engineered to attract the Bt11 event-specific primers used in the PCR tests and thus tempt regulators to look no further. Thus, claims of safety based on the indirect measure of proportion of genomes should be treated with extreme caution.

Box 4. Comparison of PCR applied to forensic police work and environmental monitoring

Forensic scientists commonly use amplified fragment length polymorphism (AFLP) profiles generated by PCR to identify people and establish the origin of biological material found at crime scenes. They develop one or more primers designed to amplify regions of DNA with variable numbers of tandem repeat DNA sequences [23]. Forensic police have a large number of primers with which to generate profiles. The AFLP profile is based on generating DNA fragments of different length using primers with essentially perfectly complementary sequences, so reaction resolution is not a source of false negative results. As long as some pattern is obtained, it can be compared both for number and intensity of bands.

Criminal forensic work is usually limited by the amount of DNA; a negative result leads rightly to no conclusion. By contrast, food monitoring relies on the detection and either qualitative or quantitative amplification of a limited number of sequence targets from a DNA-rich sample, from a large number of potential GEOs belonging to an ever-growing list of species [9,15]. A PCR profile on environmental samples typically yields 'yes or no' results, that is, an amplified fragment is or is not there. Real-time PCR also helps quantify how much DNA is in a 'yes' result. Environmental PCR is limited not by DNA, but by the quality of the match between a small number of primers and the target, the size of the target genome, and the number of potential targets that can be effectively sought in each reaction [9,15]. DNA extraction methods yield DNA to certain maximum concentrations above which they become saturated [8]. Thus, the difficulty in applying PCR to environmental monitoring is in being able to survey a sufficiently large number of genomes from the DNA extracted from a sample. The larger a target genome is (e.g. corn versus soybean) significantly changes detection limits. A negative result using this profiling design should not be taken as evidence of absence of GE material.

only, and with the requisite caveat that they report minimum sample sizes assuming negligible error from all other sampling steps.

Samples taken from what is assumed to be a homogenous mixture of GE and conventional seeds, for example, will vary around the average of the actual proportion of GE seeds, as can be described by a simple binomial distribution [5]. Individual subsamples of seedlots contaminated with, on average, 0.1% GE seeds will have actual GE seed content across the range 0.07–0.14% with 95% confidence. Seed sample sizes should be chosen based on the lower range of the distribution. If the detection confidence sought is 95% at 0.1% contamination, then seed samples should be ≥4300 seeds and ≥6600 for 99% confidence. Any sample smaller than these cannot claim detection down to 0.1% contamination, regardless of the PCR resolution. It is therefore crucial that regulators do not confuse reaction amplification limits with sample detection limits.

However, these calculations of minimum sample size depend crucially on the assumption of a homogenous mixture of the material. Yet 'raw materials are often not systematically mixed during harvest, storage, etc., resulting in strata that can seriously invalidate assumptions associated with simple random sampling' [2]. This observation raises the additional issue of determining the number of samples (of the size recommended in the calculations described above) to quantify confidence limits associated with any heterogeneity because of incomplete mixing.

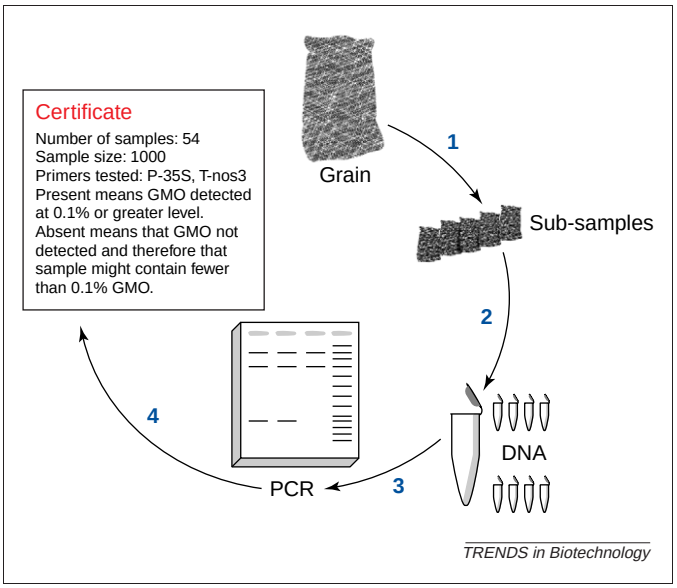

Figure 1. Main error-prone stages in monitoring for GEOs by PCR. Depicted is a skeleton version of the stages of material monitoring, from material isolation to result interpretation. The 'Certificate' is a stylized version of authentic certificates released by a New Zealand Parliamentary Select Committee in 2004. The skeleton is based on the process of investigating reports of actual and possible GE contamination of sweet corn seed, as conducted by New Zealand authorities in the years 2000 and 2003. The contamination was presumed to be by GE corn, but the possibility remains that the contaminant was not corn. Stage 1 errors result from poor sampling technique including improper seed sample sizes and assumptions of random GE seed distribution. Stage 2 errors result from poor sample preparation including milling that does not guarantee homogeneity of material in the sample and DNA extraction from too small or too large a subsample. Stage 3 errors result from reaction conditions in the PCR that are not comparable in the DNA from the monitored material and controls. Sources of these errors include reaction inhibitors carried through DNA extraction, DNA sheared to too small an average fragment size during extraction, variability in target and primer sequences and different than expected structure in the region to be amplified. The latter occurs when well-characterized constructs change because of mutation, recombination, insertions or deletions at the site during cultivation. Stage 4 errors result from poor data reporting technique including inconsistency in testing laboratory seed sample sizes, reporting of milling and DNA quality control procedures, primer sequences and method for following-up any amplified products, or indicating a detection limit based on the PCR control reactions.

Sample homogeneity and DNA extraction

Another homogeneity issue arises when seeds are milled to flour (Table 1 and Box 2). Milling ensures that DNA extracted from a subsample of the seeds will have a representative collection of genomes. Most DNA extraction procedures yield extracts of ~50 μl–100 μl [6], and the PCR is typically based on 100–200 ng of DNA [5].

Even an extract of 200 ng of corn DNA will contain on average only 0.2 ng target (GE) genomes at 0.1% contamination. This equates to an average of 36 target molecules per PCR test tube (Table 2) [6]. Of course, the actual number of genomes in a test tube will vary according to a normal distribution [5,7]. Moreover, the number of target genomes in a 200 ng sample will vary inversely with size, and average sizes are not representative of some GEOs [2]. Unfortunately, adding more DNA is not always possible because more concentrated environmental samples can saturate the isolation matrix [8] and inhibit the reaction [7].

The absolute detection limit of laboratory-idealized protocols can be as low as one to six target genomes per reaction [7,9], within range of the theoretical threshold for a PCR with a 20 pg resolution (Table 2). The PCR

Table 2. Limits of detection

Sample	1	2	3	4
% GEO[a]	2	1	0.1	0.02
GEO DNA ($\times 10^{-9}$ g)	4	2	0.2	0.02
Number of targets	730	365	36	4
Resolution ($\times 10^{-9}$ g DNA)[b]	10	1	0.2	0.02
Minimum number of targets required for amplification	1800	182	36	4
% GMO detected with 95% probability	5	0.6	0.12	0.03

[a]Numbers of targets as a function of contamination level based on 200 ng samples and 5.0×10^9 bp/corn (diploid) genome [6] and molecular weight of 660 g/m × nucleotide; [b]Limits of detection as a function of reaction sensitivity based on 100 ng samples and diploid genomes.

resolution is the minimum concentration of a target template in a mixed sample that can be reliably amplified. Under practical conditions, a minimum of two parallel reactions by real-time PCR is required for samples with a minimum of 30 target genomes [7]. Laboratories can need up to 10 ng of target DNA to yield a detectable signal from a reaction [10] (i.e. a minimum of 1800 target genomes, Table 2). This is 50 times the number of target corn genomes that would be in a standard sample at 0.1% actual contamination and five times the number at 1%. Thus, it is crucial that the resolution be determined for all reactions, not just control reactions.

The chances of a false negative will increase as the average number of genomes in a PCR test approaches the absolute detection limit for the resolution limit of the reaction [7]. This is because, in part, at the absolute detection limit, any given test tube might have fewer than the minimum necessary number of genomes. This particular problem cannot be countered by simply increasing the number of replicate PCR tests on samples. In the example that follows, we demonstrate the limited increase in confidence from replicates assuming that the reaction resolution is not limiting for detection.

The cumulative probability of detecting GEOs in a single sample, $p_{(1)}$, can be calculated using the binomial distribution [5,11]. Assuming (i) a 100 ng sample of diploid maize DNA containing 36 670 genome copies, and (ii) a PCR detection lower limit of 36 copies (Table 2), a single sample would detect the presence of GEOs with 95% confidence if the GEO content were 0.129% or greater. The cumulative probabilities of detection from replicate PCR samples can then be calculated using the equation:

$$1 - p_{(n)} = (1 - p_{(1)})^n$$

where $p_{(n)}$ is the probability of detection in at least one of n samples, each with the probability distribution $p_{(1)}$. With increasing sample replication, the 95% confidence limit for GEO detection for increasing number of samples $n = 2$, 3, 4 and 5 decreases non-linearly as 0.113%, 0.106%, 0.101% and 0.098%, respectively.

Reaction sensitivity

As indicated above, high-resolution reactions are reliable detectors around the absolute average of 30 target genomes. Regulators need to beware that reliability decreases dramatically when resolution is affected by reaction inhibitors in the extract, quality of the extracted DNA, match between primer- and target-sequences, and structure of the fragment the primers are meant to bracket. These variables effectively decrease the apparent number of genomes in a reaction and could yield falsely low quantitative levels or false negative GE-free results.

The effect of an inhibitor and the quality of DNA are easily determined. The influence of inhibitors can be measured by attempting to amplify a conserved target in the environmental sample separate from putative modifications [3] or by using mixing controls, that is, adding back the extract from an environmental sample to the positive control reaction involving known and perfectly matching primer and target sequences. The quality of the DNA, which should consist of molecules that are a minimum average size of 400 bp [2], can be verified with standard techniques. However, it is target sequence and the structure of the GEO genome that are the most crucial determinants for establishing the amplification limit, and unfortunately these are the most variable and unverifiable determinants [3].

Primers 'specific' for target elements of already approved GEOs, such as the CaMV-derived promoter sequence *P-35S*, are often used, but potential problems can arise from sequence differences in commercial variants of these regulatory elements. For example, 'There are at least eight variants of *P-35S* used in GM crops' [2], and even commercial constructs with identical names have been shown to have different DNA sequences [12]. Small sequence differences between target and primer significantly affect reaction conditions [12]. Therefore, unless a battery of primers is used, it is misleading to state claims of resolution (Figure 1), and associated detection limits, based only on the optimized controls.

The regulatory community came to know of this variability in the *P-35S* element through a combination of publications from researchers seeking to describe the DNA sequences in GEOs and the slow release of such information from the bio-engineering community [3]. Given the variability in these elements, it cannot be generally assumed that primers will match all commercial varieties of GEOs.

P-35S and other commonly used sequence elements must be used as only a guide, not to be substituted for custom primers. For example, a group in Taiwan found that by using a combination of custom primers and those developed by others, they could detect varieties of GE corn approved only for animal consumption in the human food chain [13]. In fact, they could detect varieties of corn in human foodstuffs ranging from tortillas to processed potato products [13].

Complete knowledge of neither DNA sequence nor the structure and stability of transgenic regions can be assumed *a priori* for proper monitoring of GEOs. Long-term testing on the stability of GEO genomes and behavior of transgenes is lacking [14]. Therefore, the possibility exists that the indicator DNA sequences can become separated from the other components of the original construct. Even genetically small separations could lead to sub-optimal detection in samples containing transgenic DNA. Subsequent instability is not the only source of unexpected structures. The original transgenic routinely suffers more than one insertion (e.g. Ref. [9]), but often

only those insertions that are full length and expressed are characterized and reported. Hybrids and other derivatives of GEOs might have these and not the registered insertion.

It is incumbent on regulatory authorities to test for the unexpected in the food chain and thus be innovative in designing primers. In all cases, it is recommended that primers be designed for all known indicator sequences and that the primer pairs be used to amplify small fragments to reduce the likelihood of a recombination event having interrupted the expected DNA fragment [2,6]. Ignoring these precautions could have serious implications for regulators who need to detect registered GEOs, but even more serious consequences could derive if contamination by unauthorized GEOs that result from cross-fertilization [3], accident or with intent to evade detection, were overlooked.

Conclusions

We have identified the four stages of standard verification and monitoring procedures that are the source of greatest error in important regulatory decisions. The error, or level of uncertainty, at each of the first three stages is not additive and is significantly larger than the error of any single step. We have found no formal treatment of the effect of systematic error in evaluating protocols and few methodical tests of the error range at each stage for GEO monitoring (but see Refs [7,8]). Therefore, we cannot suggest how large the uncertainty is in a conclusion that a consignment of food or seed is free of GEOs. Pending the results of such research, we suggest that ~7000 corn seeds per sample must be used to approximate the confidence level called for by European labelling laws, based on stage one error analysis alone. In New Zealand, regulators were frequently supplied with data from samples of only 1000 seeds (Figure 1). Therefore, when combined with stage two and three errors, it is possible that detection limits are, sometimes and possibly relatively frequently, closer to 100% than 1%, much less 0.1% as often claimed based on PCR reactions using completely described template DNA for positive controls.

Stage four errors are generally a function of the way data are presented to the regulatory community and its advisors. Stage four errors are the easiest to eliminate because the solution is simply to require that GEO producers and testing laboratories make all data available to independent scrutiny, to the same standard required for the publication of scientific research.

New techniques being developed for monitoring generally attack the need to efficiently detect a variety of known GEOs at once or using a small number of primer sets, usually through multiplex reactions (e.g. Refs [9,15–17]). Other approaches are emerging, such as the use of biosensors [18]. Although the latter might reduce the costs of monitoring, it is still prone to some of the same sensitivity limits as conventional approaches.

Increased monitoring capacity is required of all countries, especially with the adoption of the Cartagena Protocol on Biosafety, as international law (http://www.biodiv.org/biosafety/default.aspx, Accessed on 11 January 2004). Monitoring must be improved to avert environmental harm, more and more sporadic seizures and rejections of imports, and the knock-on effects of further undermining public confidence in science (Box 1).

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