Detection of Genetically Modified Food Products in a Commercial Laboratory

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ABSTRACT: Since genetically engineered agricultural plants entered the market, new challenges have been imposed on grain producers, food processors, and food testing laboratories. Service laboratories, especially in the United States, face a lack of standardization of testing methods for bioengineered crops. Food companies need a basis on which to choose a laboratory that can meet their specific analytical needs with regard to applicable regulations or commercial contracts by providing scientifically valid and reproducible results. This article outlines the prime technical aspects for laboratories providing analysis of food and agricultural products by PCR and ELISA.

Keywords: genetically modified crops, bioengineered crops, PCR, ELISA, genetically modified organism, GMO

Introduction

GENETICALLY MODIFIED (GM) CROPS are increasingly being introduced into the world’s food supply. Questions raised by consumers and regulatory agencies in various countries have demonstrated the need for the ability to determine if genetically engineered components are present and, often, the amount present.

Buyers and sellers at each step in the supply chain need to know if a shipment meets applicable labeling requirements as well as pertinent provisions of a commercial contract. Additionally, the question of whether a particular genetically engineered event has been approved for import into a country is an issue of considerable significance.

The challenge facing a testing laboratory is to be able to recognize a novel, artificially inserted sequence of DNA, determine the amount of that material, and determine exactly which specific modification is present.

A plant, such as corn or soy, is considered to be genetically modified when genetic material from outside that organism is introduced into its DNA sequence. In all cases, the series of base pairs which are inserted into the natural DNA sequence have some common elements. Each inserted artificial gene, or transgene, has a promoter, a protein encoding site, and a terminator. The promoter is an “on switch” that alerts the cell to begin making the novel protein. It is the starting point for the transcription of the inserted DNA into RNA. The terminator marks the end point for this gene transcription procedure. Figure 1 illustrates an example of the constructs typical of bioengineered insertions.

Types of testing

Detection of genetically engineered materials falls into two general testing methodologies. One method, enzyme-linked immunosorbent assay (ELISA), detects the presence of the novel protein that is the product of the information encoded by the modified DNA. Alternately, a technique called polymerase chain reaction (PCR) looks for the actual bioengineered DNA sequences that have been inserted into the plant’s naturally occurring DNA.

ELISA testing

ELISA techniques are widely accepted and used in many applications such as detecting pathogens, mycotoxins, and the widely recognized home pregnancy tests. In an ELISA test for genetically modified agricultural product, the kit manufacturer isolates the novel protein and raises antibodies against certain surface structures (epitopes) of this protein. In an assay, the proteins, if present, are bound by tagged antibodies. The presence of a genetically modified sample is indicated by a color reaction catalyzed by an enzyme linked to the antibodies.

In some commercial kits, it is possible to get a quantitative reading of the targeted protein by preparing a standard curve and using a reader to compare the degree of color change of the sample to the standard curve.

Even though this is a relatively simple analytical procedure, it does require several hours, laboratory equipment, and some training for the technicians. Therefore, it is not applicable for field situations or as a quick test for accepting/rejecting loads of incoming grain at a plant or elevator, especially during the high-volume harvest season when hundreds of trucks arrive per day.

To meet this need, the ELISA test concept has been incorporated into a field use application using lateral flow test strips. Using lateral flow test strips involves crushing a soybean or corn kernel, immersing it in a buffer solution, and then inserting a lateral flow test strip that has an area which has been treated with specific antibodies. A color change on the treated area indicates a positive reaction for the target protein.

Figure 1—Example of a bioengineered construct. This bioengineered sequence (RoundupReady® soybean) has a promoter (35s gene) from cauliflower mosaic virus and a terminator from the bacterium Agrobacterium tumefaciens. The herbicide resistance is encoded by the EPSPS gene, also from the A. tumefaciens. The chloroplast transit peptide from Petunia hybrida localizes the EPSPS protein to the plastids.
protein. A control reaction on another section of the strip indicates a successful test. These test strips are fast, cheap, and require minimal training and equipment.

**Applicability of ELISA testing**

ELISA assays are faster and less expensive than PCR analysis. It is relatively quick and easy to train technicians to perform these tests. The investment in equipment is much less than for PCR, especially quantitative PCR. However, there are several disadvantages that limit the applicability of ELISA testing for genetically engineered products.

The chief disadvantage, especially for a food processing company, is that the target proteins are denatured relatively easily during processing. Therefore, for a processed food or ingredient, the target protein epitope may not be present in the condition detectable by the antibodies.

The second drawback to using an ELISA test is that commercial kits are available for only a few of the genetically engineered agricultural products now being grown. Also, at this time each kit can only detect one protein; so, if, as in corn, there is more than one possible modification or if more than one species of plant material is present, then a series of ELISA tests would be necessary, assuming a kit was available for each of the proteins in question. In real world applications for multi-event crops such as corn, ELISA on raw ingredients is used as a risk management tool rather than as a method to detect all possible GM events.

There are also questions related to the quantification capabilities of ELISA tests. Test results are reported in µg/ml of target protein to test solution. A conversion is then made to percent of genetically modified material present. It has been shown that the amount of protein expressed in genetically engineered corn varies from field to field, year to year, and between varieties. In one corn event, Bt176 (approximately 1% of 1999 plantings), the novel protein is primarily expressed in the green leaf and minimally in the kernel.

**PCR testing**

In the widely recognized double-helix DNA molecule, the two nucleotide strands are paired by complementary interactions between the bases adenine (A), thymine (T), cytosine (C), and guanine (G); these base pairs form the rungs of the ladder-like DNA molecule. Simplifying matters even further, and a fundamental factor in PCR testing, bonding only occurs between adenine and thymine or between cytosine and guanine. Thus, during replication, when the DNA molecule separates into two strands, each strand becomes a template that will construct, with the help of the enzyme DNA polymerase, an exact copy of the original molecule. Where there is an unpaired adenine, only its complementary base, thymine, can attach; an “open” guanine can only bond with a cytosine. As a result, the two individual strands are rebuilt into exact daughter duplicates of the original molecule. If these two molecules can then be induced to unzip into four single strands, these four strands would be rebuilt, in the same manner, into four exact copies of the original DNA molecule. The replication process, happening in nature whenever a cell divides, can be mimicked in a test tube using temperature shifts, “building block” chemicals, and a heat-resistant DNA polymerase—for example, Taq-polymerase. This in vitro process is called polymerase chain reaction (PCR).

In a laboratory, the use of thermocyclers allows several dozen PCR cycles to occur in about two hours. By programming the thermocycler to alternately raise and lower the temperature to specific levels for specific time periods, a single DNA molecule, in a chemical-rich solution, can replicate itself into millions upon millions of copies. As Table 1 shows, after 50 cycles a single molecule would theoretically yield 281 trillion copies. This increase is theoretical because, in actual practice, there is something less than an actual doubling of copies during each cycle, especially at higher number of cycles. Actual duplication efficiency is diminished by factors such as inhibiting chemicals, restricted amounts of “building block” chemicals, and increasing viscosity of the mixture.

**PCR applications**

PCR has been used to identify components in mixed products for purposes of determining if products have been adulterated. Using a primer that targets a nucleotide sequence unique to the species in question, PCR can be used to determine if that species is present in the sample. This type of ingredient identification was one of the first uses of PCR in the food industry.

More recently, PCR is being applied to questions concerning genetically engineered products in food products. Two general scenarios are most often presented to the testing laboratory. First, the question is whether a sample contains a genetically engineered component. The second type of request is for the lab to determine if a specific GM event is present in the sample. The response to both of these questions is elliptical. Positive-Negative answer or a quantitative determination of percentage amount of target DNA. In the first case, a test (or tests) would be performed looking for some component DNA sequence that is com-
Detection of Genetically Modified Food...

Concise Reviews in Food Science

Detection of Genetically Modified Food...

mon to all, or most, GM events. In the second case, the task is to detect a nucleotide sequence that is unique to the GM event in question. In the first case, the goal is to be as inclusive of GM events as possible; in the second, the aim is to be exclusive to one event.

To date, the vast majority of GM products contain the 35S promoter from the cauliflower mosaic virus (CMV). So, by targeting and detecting 35S, a strong indication of the presence of a GM component can be inferred. It must be noted that the cauliflower mosaic virus does occur naturally, so a specific confirmation of the absence of cauliflower mosaic virus is advised on 35S positive samples. But not all GM products contain 35S. So a single screen test using 35S cannot detect all GM events currently on the market. Using corn as an example, all of the corn events approved for use, and commercially available, in the United States, except RoundupReady® (RUR), contain the 35S promoter. In order to determine for certain the presence or absence of GM corn in a sample, it would be necessary to run one qualitative test for 35S and another to determine if RUR corn is present. As more GM products become approved and commercially available, it is likely that they will contain promoters and terminators not currently being utilized. This will cause an increase in the number of individual PCR tests required to determine if GM materials are present. At this time, multiplex analyses have not been shown to be reliable enough for routine commercial use. Micro-DNA-arrays are discussed below.

To determine if a specific GM event is present in a sample, the lab will look for a nucleotide sequence that is unique to the GM event in question. As an example, in RoundupReady® soybeans, our company has developed primers that straddle the transition between the 35S promoter and the neighboring region encoding a transit peptide from the petunia plant. Both the 35S from the cauliflower mosaic virus and the chloroplast transit peptide occur in nature, but a contiguous sequence containing both of these together can reasonably be assumed to be a product of genetic engineering and is, in fact, unique to RoundupReady® soybeans. The bioengineered sequence for RUR soy is shown in Figure 2. In determining the presence of RUR, the sequence of base pairs, which straddle the transition of the CMV 35S and the petunia CTP, is multiplied using PCR. As will be discussed below, the PCR test can be either qualitative or quantitative.

Steps in PCR testing

1. Sampling: Strictly speaking, sampling is not a part of the test routine. But with PCR being theoretically sensitive enough to detect a single molecule, a sound sampling plan and the subsequent sample preparation is as critical a determinant to final sensitivity as any other step in a PCR test. GM material is randomly dispersed, especially in raw agricultural products. A comparison can be made to aflatoxin or low-level salmonella contamination in large bulk containers. A small amount of GM corn, randomly present in a 1700-ton river barge or in a farm silo, presents a statistical and mechanical problem that is not readily solved on a cost-effective basis, especially when compared to the unit commodity costs. With increased processing the homogeneity of the product increases, making sampling easier. But the increase in homogeneity comes at a price, in that the degree of processing usually damages and fragments the DNA, thus reducing the sensitivity of the analysis as illustrated in Figure 3.

Sample size is also a determining factor in the final sensitivity of the analysis. If a test on raw corn kernels is to have a chance of achieving a sensitivity of 0.01%, then there must be at least 10,000 kernels of corn in the sample taken for analysis. During grinding and sample preparation, the DNA in each of the 10,000 kernels must have a statistically equal chance of being in the portion actually analyzed.

2. Extraction. Extraction may well be the most exacting and delicate step in the PCR process. DNA in a plant cell is tightly wrapped, encased in layers of protective proteins, suspended in a solution containing hundreds of organic compounds and many inorganic ions, and protected by membranes and cell walls.

Several commercial DNA extraction kits are available, each with slightly different applications. It is often necessary to go through purification steps, especially in the case of mixed products, in order to isolate the DNA fol-

Figure 2—Detection of RoundupReady® soy by PCR

Figure 3—Sampling Effort - Crop to Processed Food
Qualitative original replicated into two exact copies of the ed base-pair sequence will have been of DNA.mentary strand for each single strand erased and heat-stable DNA-poly-
GM material may result.
neal to the target area, then a false tion of GM material will be report-
c手续费 again cannot an-
tration will separate the negatively
action products are analyzed by agar-
cs. For example, the official method used by the Swiss government specifies the use of 45 cycles.

5. Elongation. In the last step of the cycle, the temperature is again increased and heat-stable DNA-poly-
merase, using the primer as a starting point, begins building a new complementary strand for each single strand of DNA.

At the end of this step, each target ed base-pair sequence will have been replicated into two exact copies of the original. 

Detection—Qualitative
Detection of PCR products can be performed either qualitatively or quantitatively. In a qualitative system, the PCR is performed for a sufficient number of cycles to get maximum formation of replicated molecules. The increase in number of replicates reaches a plateau, usually after 20 to 45 cycles, depending upon the initial amount of target DNA. Since PCR works exponentially, slight changes in the number of cycles used can affect the detection of low numbers of target copies tremendously. Although in an ideal PCR reaction roughly 35-40 cycles may appear to be sufficient to detect single target molecules, in real life molecular biologists experience that this theoretical concept mostly fails. To ensure that the PCR procedure is able to detect lowest amounts of GM DNA, GeneScan labs have settled on 50 cycles as a standard in routine qualitative PCR analysis. It must be noted that extra care in validation of any primer system used in analytical PCR is mandatory to avoid artifacts that might be mistaken as positive results, especially if the PCR procedure is designed for maximum sensitivity. However, other laboratories or specified methods use various numbers of cycles. For example, the official method used by the Swiss government specifies the use of 45 cycles.

Following PCR amplification, the reaction products are analyzed by agarose gel electrophoresis. Applying a current will separate the negatively charged DNA fragments according to their size; that is, the number of base pairs. Using UV light, PCR product is detected as a fluorescent band on the gels after staining with ethidium bromide. The presence or absence of an appropriately sized band is reported as a positive or a negative. In Figure 4, the light bands in the agarose gel indicate the presence of targeted DNA sequence, in this case RUR soy.

Quantification by real-time PCR
PCR quantification is done by determining the ratio of target GM DNA to the total of the species DNA. This can be done using fluorescence-coupled real-time PCR machines such as the Applied Biosystem ABI PRISM™ 7700 detector instrument (TaqMan), which is a thermocycler incorporated with an optical system. It detects laser-activated fluorescence signals via a high performance cooled CCD (Charged Coupled Device) camera that can independently monitor each of the 96 wells. In each test solution, an additional oligonucleotide labeled with a fluorescent dye is added (TaqMan probe). This probe anneals, like an ordinary PCR primer does, to a specific region of the single-stranded, unzip DNA molecule. During the polymerization step, as the DNA polymerase builds the complementary strand, it encounters the annealed dye-labeled probe, hydrolyzes the probe, which causes a fluorescence emission. Fiber optic sensors over each well measure the change in fluorescence light intensity with the increasing number of cycles. Calibration curves are calculated from standards being run simultaneously and the percentage of target DNA versus total species DNA is calculated.

Table 2—Relative Amounts of DNA in Processed Foods

<table>
<thead>
<tr>
<th>Product</th>
<th>Amount of DNA Isolated</th>
<th>Product</th>
<th>Amount of DNA Isolated</th>
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<tbody>
<tr>
<td>Soy Beans</td>
<td>+++</td>
<td>Soy Flour</td>
<td>+++</td>
</tr>
<tr>
<td>Soy Meal</td>
<td>+++</td>
<td>Tofu</td>
<td>+++</td>
</tr>
<tr>
<td>Soy Drink</td>
<td>++</td>
<td>Natto</td>
<td>+</td>
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<tr>
<td>Soy Sauce</td>
<td>+</td>
<td>Cookies</td>
<td>+</td>
</tr>
<tr>
<td>Lecithin</td>
<td>+</td>
<td>Crude Oil</td>
<td>+</td>
</tr>
<tr>
<td>Refined Oil</td>
<td>(+)</td>
<td>Chocolate</td>
<td>+</td>
</tr>
<tr>
<td>Corn</td>
<td>+++</td>
<td>Canned Corn</td>
<td>+++</td>
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<tr>
<td>Corn Snacks</td>
<td>+++</td>
<td>Corn Grits</td>
<td>+++</td>
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<tr>
<td>Corn Starch</td>
<td>+</td>
<td>Corn Flakes</td>
<td>+</td>
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<tr>
<td>HiFruc Corn Syrup</td>
<td>(+)</td>
<td>Veggie Burgers</td>
<td>+</td>
</tr>
<tr>
<td>Tomato</td>
<td>+</td>
<td>Tomato Paste</td>
<td>+</td>
</tr>
<tr>
<td>Ketchup</td>
<td>+</td>
<td>Canola Honey</td>
<td>+</td>
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+ + + = > 100 ng/µl; + + = 5 to 100 ng/µl; + = < 5 ng/µl; (+) = DNA not always detectable.

Figure 4—Qualitative detection of RoundupReady soy-DNA (128 bp amplicon). The samples RR soy, whole meal, lecithin, raw oil, and cakes contain genetically modified DNA. Conventional soy, extraction control and PCR control each show a negative result.
**Reporting of Results**

It has been our experience that analytical reports showing quantitative results for GM products are often misunderstood or misinterpreted. Unlike the reporting of most chemical tests, which are based on an absolute weight percentage, quantitative, real-time PCR results are reported as ratio of target GM DNA relative to the total amount of species DNA present, for example, the number of copies of RUR soy DNA vs the number of copies of total soy DNA (RUR + non-GM).

Quantitative PCR results are reported as a percentage where the amount of species DNA makes up the denominator and GM DNA makes up the numerator. For each quantitative analysis, a detection limit and a quantification limit would be calculated based on the amount of species DNA present at the start of the PCR process.

**Detection limit (DL)**

Theoretically, a qualitative PCR analysis can detect a single molecule of DNA. In the routine testing in the various GeneScan laboratories, we have proof for each individual sample DNA that the detection limit is ten copies of target DNA or less. However, a specific detection limit, expressed as a percentage, can only be determined by counting the number of copies of species DNA molecules present using a real-time quantitative PCR analysis. Daily routine testing in our various laboratories, using real-time PCR, has shown that amounts less than 0.1% of GM soy can be reliably detected in soybeans. In some matrices, a detection limit in the range of 0.01% can be achieved.

The detection limit of a quantitative PCR, given as a percentage of GM material, depends upon the amount of DNA molecules from the plant species present in the sample. In Figure 5a, as an example, if 10,000 copies of DNA had been extracted from the sample, then the theoretical detection limit (a single copy of GM target DNA) would be 0.01%. (In reality, with real samples, the DL could range from the theoretical 0.01% to 0.1%.) If another sample only had 1000 copies of DNA, then the theoretical detection limit would be 0.1% for that sample (again, in reality it could range up to 1%). It is axiomatic to say that the DL, given as a percentage, cannot be stated unless a quantitative PCR is performed on that particular sample since the number of plant species DNA molecules extracted from the sample is not known until the completion of a quantitative analysis.

In Figure 5a, a sample with 10,000 copies of species DNA, the DL would be 0.01% to 0.1% and the QL would be 0.1%. Twenty copies of GM target DNA would be reported as 0.2%, which is above the QL for this sample.

In Figure 5b, the sample with 1000 copies would have a DL of 0.1% to 1% and a QL of 1%. Using our 10-copy rule, we could not report a quantified result of less than 1% for this sample. In our experience, the ratio of 2 copies of target GM DNA to 1000 copies of plant species DNA would not be reproducible at a statistically valid level. So the 2 molecules of GM DNA found would not be reported as 0.2%; instead they would be reported as below the quantification limit of 1% for that sample.

**Quantification limit (QL)**

Similarly, the amount of DNA present is a factor in determining the limit of quantification for each real-time PCR analysis. The QL for a sample is the lowest level at which reproducible results can be calculated. In our experience, over many tens of thousands of samples, real-time PCR, using the TaqMan system, can reliably quantify target DNA molecules down to a lower limit of about ten (10) copies. Although the TaqMan system can easily detect single copies of target DNA, it is virtually impossible to obtain reproducible data in triplicate or quadruplicate with such very low copy numbers. Consequently, in the calculations used in GeneScan labs, if the sample DNA showed 10,000 copies of species DNA in a real-time PCR reaction, then the limit of quantification would be 0.1% (10 molecules of GM DNA out of 10,000 molecules of species DNA). If another sample had only 1000 copies of species DNA, then the QL would be 1% for that sample.

**Semiquantitative methods**

Before the availability of fluorescence-coupled real-time PCR devices, an attempt at quantification was made by amplifying serial dilutions of standards and of the sample DNA and subsequent agarose gel electrophoresis. A comparison of the intensities of the PCR products in the agarose gel was used to estimate the amount of GM DNA in the sample. The many technical and subjective issues with this procedure were made moot by the introduction of more modern, reliable equipment.

**Sources of error**

At this point in time, from the client’s point of view, testing for GM material can be like playing laboratory rou-
Concise Reviews in Food Science

lett. Unlike other types of testing, which have had many years of collaborative trials, check sample accreditation programs, and referenced methods, this area of analytical work is still so new that efforts at standardization are just beginning. For example, the American Oil Chemists Society (AOCS) in early 2001 sent first samples of soy to participating laboratories as part of their initial GM check sample series; the Grain Inspectors, Packers and Stockyards Administration (GIPSA) of the USDA will implement a PCR laboratory accreditation program in the near future; other industry groups such as the American Association of Cereal Chemists (AACC) and the Association of Official Analytical Chemists (AOAC) have similar programs planned. Choosing a capable and competent GM testing laboratory takes some extra effort on the part of clients.

Beyond the variability in test results that will result from the lack of standardized methods, there are other, more fundamental, sources of analytical variation. Issues such as number of inserted transgenic cassettes and the polyploid status of the chromosomes affect the copy number of transgenes within a sample. As an example, some GM corn varieties have multiple insertions containing 35S, so using 35S as a basis for quantification could lead to overestimating the amount of GM material present.

Few reference materials and only a very few certified reference materials are available. Virtually none are available for mixed and processed samples. Further complicating the issue of reference materials are questions as to whether the reference materials, and even the reference genomes themselves, behave the same as the target GM genomes under PCR conditions.

PCR and ELISA testing are often done by laboratories that also perform other types of tests on grain and food samples. The presence of these other samples, whether for traditional testing or for GM testing, is a potential source of GM contamination. Even the tiniest amount of grain dust in a sample can lead to false positive results. Strict attention to the movement of equipment, samples, personnel, and even the air flow is needed to minimize this potential source of error.

Biochips

In the coming year, the number of GM products on the market will undoubtedly increase. It is also quite likely that, like current GM products, these new events will not be uniformly approved and accepted in all countries. Further complications will be introduced to the analytical mix as the molecular biologists creating the upcoming generations of bioengineered products use an increasing variety of promoters and terminators. One result will be an increasingly complex testing scenario for laboratories. The number of PCR tests that would be required to test for the presence of this increasing range of products will no doubt also increase, with resulting increase in cost and turnaround times.

As a consequence, new analytical

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Figure 6—Schematic of biochip test grid. Detection of different GM maize traits on a DNA array. Reticule fields contain the following DNA probes specific for the detection of different transgenic maize events: A1: Dt-Xtra, B1: Bt-176, RR-corn, C1: Mond-810, D1: Bt-11. In addition, positive and negative controls are displayed: D7: Maize species control, D8: negative control E8: detection control. Dots outside the reticule show fluorescent coupling controls. All probes are dotted in duplicate. DNA arrays were exposed to four different food samples (I-IV).

Figure 7—Microarray biochip. Positive results are shown as fluorescent dots on a biochip. Each sample here was run in triplicate.
methods are being developed, one of which would employ DNA micro-arrays. The arrays, or biochips, are small surfaces (about 1 cm × 1 cm) onto which many different DNA sequences can be immobilized and analyzed in parallel. Hundreds of tests could thus be run simultaneously and in a cost-effective manner. The presence or absence of the whole panel of relevant genetic modifications could be determined at once. Subsequent quantification could be made on those specific positive reactions where a threshold concentration applied. The first commercial biochips for GM materials are now on the market and available commercially.

Biochips would speed up PCR analysis and reduce costs for testing multiple events. But sophisticated laboratory equipment and skills would still be needed, and the time needed would still be measured in hours. To meet the need for rapid, on-site detection of multiple GM events in something close to real time, GeneScan has partnered with Motorola to develop a hand-held GM detection device that will utilize microcircuits of DNA fragments and not need PCR amplification. The goal is for a farmer or an elevator manager to be able to test in real-time the samples of grain they are working with. But that goal is a few years away.

Conclusions

Detection and quantification of bioengineered food products by PCR is a relatively new area of analytical service, especially when compared to the older and more established methods of traditional food chemistry and food microbiology.

At this time, there is a lack of consistency and lack of standardization amongst the laboratories providing PCR analysis in this field. Some first steps towards performance checking by means of check sample proficiency programs are now underway. Until such time that accreditation and certification programs are in place, potential users of PCR testing laboratories will have to carefully examine the procedures and capabilities of laboratories offering services in this field.

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