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(71) Applicant *(for all designated States except US)*: TECHNION RESEARCH AND DEVELOPMENT FOUNDATION LTD. [IL/IL]; Technion City, 32000 Haifa (IL).

(71)(72) Applicant and Inventor: KASHI, Yechezkel [IL/IL]; Dania Street 46, 34980 Haifa (IL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ZUR, Gideon [IL/IL]; 30099 Kibbutz Sarid (IL). SHARF, Rachel [IL/IL]; Herzl Street 14A, Kiryat Ata (IL). HALLERMAN, Eric [US/US]; 211 Primrose Drive, Blacksburg, VA 24060 (US).

(74) Agent: FRIEDMAN, Mark, M.; c/o Castorina, Anthony, Suite 207, 2001 Jefferson Davis Highway, Arlington, VA 22202 (US).

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(54) Title: NUCLEIC ACID–BASED ASSAY AND KIT FOR THE DETECTION OF *ALTERNARIA* CONTAMINATION IN FOOD PRODUCTS

(57) Abstract

A nucleic acid based method is provided for the detection of *Alternaria* contamination in a food product. The method is effected by (a) obtaining a sample of the food product; and (b) analyzing the sample of the food product for a nucleic acid sequence unique to *Alternaria*, wherein a detectable level of the nucleic acid sequence unique to *Alternaria* is indicative of *Alternaria* contamination in the food product.

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NUCLEIC ACID-BASED ASSAY AND KIT FOR THE DETECTION OF *ALTERNARIA* CONTAMINATION IN FOOD PRODUCTS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a nucleic acid-based assay and kit for the detection of *Alternaria* contamination in food products, and, more particularly, to a template dependent hybridization and/or synthesis assay for the detection of *Alternaria* contamination in food products.

Fungi of the genus *Alternaria* are ubiquitous saprophytes and economically important pathogens affecting a wide range of plants (37, 41). *Alternaria* sp. fungi are causal agents of black or brown spot diseases of Japanese pear (33), apples (53), tobacco (21), rough lemon (7), tangerines (22), strawberries (34), and many other fruits, vegetables and field crops. *A. alternaria* f. sp. *lycopersici* causes stem canker disease of tomato (10, 16). This fungus secretes at least seven (4) host-specific Alternaria alternata lycopersici (AAL) toxins, which elicit cellular necrosis patterns characteristic of the disease (48), and which appear to function as chemical determinants of stem canker disease (5). *Alternaria* infection causes serious damage to tomato products, causing rejection of fresh and processed products and decreasing storage and shelf life. Until recently, synthesis of AAL toxins was believed to be restricted to the genus *Alternaria* and the action of the toxins to be acute only to tomato cells. However, recent studies (3) have shown that fumonisin toxins structurally related to AAL toxins are secreted by *Fusarium moniliforme*. Fumonisin toxicity is relatively well studied. Fumonisin FB_1 induces cell death in tomato leaf cells, as do AAL toxins (12). More importantly, animal (8, 27) and human (28, 49) diseases have been linked with consumption of corn containing fumonisins produced by *F. moniliforme*. Consumption of fumonisin-contaminated corn has been reported to cause pathologies ranging from cancer to renal, neural, and hepatic necroses in several animal species and may be responsible for human esophageal cancer (39, 43, 52). Both FB_1 and AAL toxin TA inhibit cell proliferation in rat liver and dog kidney cells (31), and induced formation of DNA ladders, compaction of nuclear DNA, and appearance of apoptotic bodies in monkey kidney cells (56). There is considerable evidence that ingestion of AAL mycotoxins leads to disruption of lipid metabolism (40, 57, 58), perhaps by inhibition of ceramide synthase. Both FB_1 and AAL toxin TA inhibited ceramide synthase in rat hepatocyes (29) and in microsomal preparations from green tomato fruit (11). The

toxic properties and structural similarities of AAL toxins and fumonisins and the attendant threat to animal and human health (9) have caused concern about the presence of AAL and fumonisins, and hence the fungi that produced them, in the food chain (31, 42, 50, 52). Preliminary data indicate that both fumonisins and AAL toxins can be found in concentrations sufficient to induce death of monkey hepatocyte cells in field samples of processing tomato and corn (35, 55; B. Ward and D. Gilchrist, unpublished data cited in 55), although the effect of chronic exposure to these toxins on animal or human health is unresolved.

The presence or absence of *Alternaria* in a food product is regarded as an indicator of good agricultural practice (GAP) and good manufacturing practice (GMP). Action levels for mold counts for various processed tomato products have been promulgated by the U.S. Food and Drug Administration (Compliance Policy Guides 7114.30). The Howard Mold Count Method (17, 18) is used by food technologists to determine the presence of mold contamination in tomato products, and is based on microscopic observation of mold filaments in the comminuted finished product. The Howard method is not robust for definitive identification of *A. alternata*; further, the Howard method has been criticized as not being truly scientific and subject to wide variations in results among competent counters (13). The Howard method has been used by the tomato processing industry for over 70 years. More recently, polyclonal antibodies were used to develop enzyme-linked immunosorbent assays (ELISAs) for AAL compounds (51).

Over the past decade, morphological, biochemical, and immunochemical methods for detection of food microorganisms have been supplemented by a range of DNA-based methods (47). Development of techniques for detection of pathogenic and spoilage microorganisms in food have focused on application of the polymerase chain reaction (PCR, 47). Advantages of PCR-based assays include specificity, sensitivity, and speed. Hence, PCR assays have been developed for a range of pathogenic and food spoilage microbes ranging from bacteria and viruses (47), to yeasts (1) and fungi (26, 46). Albeit the need, among PCR-based systems shown to detect microbes in raw materials and processed food products, none target *Alternaria* sp.

Among regions of a genome that might be targeted for development of PCR primers, ribosomal RNA genes are ubiquitously distributed, but exhibit differences due to phylogenetic divergence (30, 59). DNA-based

screening tests can be designed with genus- to species- to strain-specificity (15). A significant advantage of using rRNA as the target DNA sequence is the high copy number, sometimes over 10^4/cell (47). PCR-based detection of rRNA sequences has been demonstrated for detection of lactococci and enterococci (2, 45), *Carnobacterium* spp. and *Leuconostoc* spp. (38), and the fungus *Aureobasidium pullulans* (26).

There is thus a widely recognized need for, and it would be highly advantageous to have, a nucleic acid-based assay and kit for the detection of *Alternaria* contamination in food products, which assay is devoid of the above mentioned limitations associated with the prior art.

SUMMARY OF THE INVENTION

Alternaria sp. are important contaminants of vegetable products, including *A. alternata*, a contaminant of tomato products. To date, the Howard method, based on microscopic observation of fungal filaments, has been the standard examination for inspection of tomato products. A nucleic acid-based method for specific detection of *A. alternata* DNA is disclosed herein for the first time. In one preferred embodiment, PCR primers were designed to anneal to the internal transcribed regions ITS1 and ITS2 of the 5.8s rRNA gene of *Alternaria*, but not to other microbial or tomato DNA.

Thus, according to one aspect of the present invention there is provided a nucleic acid based method for the detection of *Alternaria* contamination in a food product comprising the steps of (a) obtaining a sample of the food product; and (b) analyzing the sample of the food product for a nucleic acid sequence unique to *Alternaria*, wherein a detectable level of the nucleic acid sequence unique to *Alternaria* is indicative of *Alternaria* contamination in the food product.

According to another aspect of the present invention there is provided a kit useful for the detection of *Alternaria* contamination in a food product comprising a carrier being compartmentalized to receive in close confinement therein one or more containers comprising at least one oligonucleotide having a sequence hybridizable with a nucleic acid sequence unique to *Alternaria*.

According to further features in preferred embodiments of the invention described below, the kit further comprising a template dependent DNA polymerase, preferably a thermostable DNA polymerase.

According to still further features in the described preferred embodiments the food product is a tomato product, a grain or a spice.

According to still further features in the described preferred embodiments the food product is a dried powder.

According to still further features in the described preferred embodiments the food product is a mash.

According to still further features in the described preferred embodiments the step of analyzing the sample of the food product for the nucleic acid sequence unique to *Alternaria* is effected by a template-dependent assay.

According to still further features in the described preferred embodiments the template-dependent assay is a template-dependent synthesis assay.

According to still further features in the described preferred embodiments the template-dependent assay is a template-dependent hybridization assay.

According to still further features in the described preferred embodiments the template-dependent assay is a template-dependent hybridization and synthesis assay.

According to still further features in the described preferred embodiments the template-dependent assay is selected from the group consisting of primer extension, polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA) and strand displacement amplification (SDA).

According to still further features in the described preferred embodiments the template-dependent assay includes a step of primer extension effected by at least one oligonucleotide having a sequence hybridizable with the nucleic acid sequence unique to *Alternaria*.

According to still further features in the described preferred embodiments the at least one oligonucleotide is as set forth in SEQ ID NOs:6-15.

According to still further features in the described preferred embodiments the nucleic acid sequence unique to *Alternaria* is derived from a ribosomal RNA encoding gene of *Alternaria*.

According to still further features in the described preferred embodiments the gene is a 5.8s rRNA gene of *Alternaria*.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a highly sensitive (20 fold as compared with prior art) assay for detecting *Alternaria* contamination in

food products, which is especially useful for powders, for which no assay is so far available.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG 1. shows comparison of DNA sequences for a portion of internal transcribed spacer 1 (lower case letters), the 5.8s rRNA gene (capital letters), and a portion of internal transcribed spacer 2 (lower case letters) for: *Alternaria alternata* (GCG accession number U05195), *Aspergillus flavus* (L76748), *Fusarium oxysporum* (X93902), *Saccharomyces cerevisiae* (Scu09327), and tomato *Solaneum lycopersicum* (X52265). Nucleotides matching the Alternaria sequence are shown as dots. Gaps introduced into an organism's DNA sequence to improve alignment with corresponding sequences of other organisms are indicated with hyphens. The sites for annealing of primers for the *Alternaria* detection assay according to the present invention are underlined.

FIGs. 2a-b show detection of mold using light microscopy. 2a - isolated *Alternaria hyphae* and *macroconidia*. Bar indicates 50 microns. 2b - observation of *Alternaria* sp. in tomato sauce using the Howard method. Bar indicates 18 microns.

FIG. 3 shows results of PCR-based assay of *Alternaria* DNA in tomato products. Lane A, 100 bp molecular weight size standard. PCR products of DNA extracted from: lanes B-E, serial dilution of 100 µl of *Alternaria*-infested fresh tomato sauce. B: undiluted, C: 10:1 dilution, D: 100:1, E: 1000:1; lane F: negative control - *Alternaria*-free tomato sauce; lanes G-J, serial dilution of 30 mg Alternaria-infested tomato powder in 200 µl water: G: 1:1 dilution, H: 10:1, I: 100:1, J: 1000:1; lanes K-M, serial dilution of *Alternaria*-infested commercial tomato powder: K: 1:1, L: 10:1. M: 100:1; N: *Alternaria*-free, commercially produced tomato powder, 1:1 dilution; O: positive control - *Alternaria* DNA. Lane P: 100 bp molecular weight size standards. Only the higher molecular weight, upper band at 217 bp should be read for each sample; the lower molecular weight band at less than 100 bp results from fluorescence of primer DNA.

FIG. 4 shows results of PCR-based assay of *Alternaria* DNA in a variety of products. The primers employed for PCR amplification were ALT13 and ALTR148 yielding a PCR product of 451 bp. (1) *Alternaria* grown in a petri dish; (2) *Alternaria* from cotton leaves; (3) *Alternaria* from

tomato leaves; (4) *Alternaria* from sesame grains; (5) *Alternaria* from wheat grains; (6) *Alternaria* from corn grains; (7) *Alternaria* from paprika powder; (8) water sample (negative control); and (9) size marker.

FIG. 5 shows a kit according to the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a nucleic acid-based assay and kit which can be for the detection of *Alternaria* contamination in food products. Specifically, the present invention can be used for the detection of *Alternaria* contamination in food products implementing any one of a variety of hybridization and/or synthesis molecular techniques which are template dependent.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions and examples.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Sensitive and rapid detection of *Alternaria* sp. in food products is important from the viewpoints of animal and human health. Supplementation of traditional microscopic examinations with molecular methods for detecting fungal DNA poses cost-effective improvement of the safety of food products.

Hence, according to one aspect of the present invention there is provided a nucleic acid based method for the detection of *Alternaria* contamination in a food product. The method is effected by implementing the following method steps, in which, a sample of the food product is obtained and is thereafter analyzed for a nucleic acid sequence unique to *Alternaria*, wherein a detectable level of the nucleic acid sequence unique to *Alternaria* is indicative of *Alternaria* contamination in the food product.

Any food product is amenable to the nucleic acid-based assay according to the present invention. Of particular importance are food

products derived from vegetables, fruits, grains and spices prone to *Alternaria* contamination.

While reducing the present invention to practice, contamination of *Alternaria* in tomato products prepared from tomato infested with *Alternaria* was successfully attempted and was shown to be at least 20 fold more sensitive and much more objective as compared with the prior art, microscope based, Howard method. Similar results were obtained for grains of various species and for spices.

However, as will be appreciated by one ordinarily skilled in the art, this example is not intended to and should not be considered as limiting. Still while reducing the present invention to practice both dried tomato powder and tomato mash were successfully screened for *Alternaria* contaminations. It will be appreciated that any other mashed or powdered food product is applicable for the nucleic acid based method for the *Alternaria* contamination according to the present invention.

The food product being analyzed for *Alternaria* contamination according to the present invention can be diluted prior to the nucleic-acid based analysis as described herein. In a preferred embodiment, nucleic acids, such as DNA and RNA are extracted from the sample and are analyzed in their extracted form. Methods of extracting nucleic acids from nucleic-acid containing samples are well known in the art and require no further description herein. One such, non limiting, method is further described in the Examples section that follows. Additional methods are described in, for example, Sambrook et al., molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989), which is incorporated by reference as if fully set forth herein.

The step of analyzing the sample of the food product for the nucleic acid sequence unique to *Alternaria* is effected according to preferred embodiments of the present invention by a template-dependent assay.

As used herein in the specification and in the claims section below, the phrase "template-dependent assay" refers to an assay in which template-dependent nucleic acid synthesis and/or template-dependent nucleic acid hybridization are effected.

In preferred embodiments of the present invention a template-dependent nucleic acid exponential amplification assay is employed. Such exponential amplification assays are either template-dependent hybridization assays and/or template-dependent synthesis assays. For example, polymerase chain reaction (PCR), nucleic acid sequence-based

amplification (NASBA) and strand displacement amplification (SDA) are all template-dependent hybridization and synthesis assays. On the other hand, a ligase chain reaction (LCR) is primarily a template-dependent hybridization assay. Other template-dependent hybridization assays include chemical amplification of nucleic acid sequences.

It will be appreciated that non-exponential nucleic acid amplification methods, such as a primer extension assay, e.g., a single nucleotide primer extension assay, is also within the scope of the present invention.

In fact, any of the above listed template-dependent synthesis assays call for a step of primer extension, which is effected by at least one oligonucleotide having a sequence hybridizable with the nucleic acid sequence unique to *Alternaria*.

As used herein the term "hybridizable" refers to specific base pairing under the following alternative stringent, moderate or mild hybridization conditions, wherein (i) stringent hybridization is effected by a hybridization solution of 6 x SSC and optionally 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C; (ii) moderate hybridization is effected by a hybridization solution of 6 x SSC and optionally 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C; and (iii) mild hybridization is effected by a hybridization solution of 6 x SSC and optionally 1 % SDS, hybridization temperature of 40 °C, final wash solution of 2 x SSC and final wash at 40 °C.

Thus, the term "hybridizable" refers to specific base pairing under hybridization solution of 6 x SSC and optionally 1 % SDS, hybridization temperature of 40-65 °C, final wash solution of 0.1-2.0 x SSC and final wash at 40-60 °C.

In addition, conventional hybridization assays with labeled probes can also be used for detection of contaminants according to the present invention.

The nucleic acid sequence unique to *Alternaria* according to preferred embodiments of the present invention is derived from a ribosomal RNA encoding gene of *Alternaria*, such as a 5.8s rRNA gene of *Alternaria*. Ribosomal RNA encoding gene unique sequences are presently preferred because of their high copy number (several thousands) in the genome of eukaryotes such as fungi. As such, ribosomal RNA encoding gene sequences are several fold more detectable as compared to other sequences. Yet, as shown in the Examples section that follows, sequence comparison or

alignment analysis enables one to select genera, species or strain unique sequences, so as to enable genera, species or strain specific template dependent assays to be conducted.

As shown in Figure 5, according to another aspect of the present invention there is provided a kit useful for detection of *Alternaria* contamination in a food product. The kit includes a carrier **20** which is compartmentalized to receive in close confinement therein one or more containers **22** (two are shown) which include at least one oligonucleotide having a sequence hybridizable with a nucleic acid sequence unique to *Alternaria*.

The kit according to the present invention preferably further includes a template dependent DNA polymerase, preferably a thermostable DNA polymerase, such as, but not limited to, *Thermophilus aquaticus* DNA polymerase, *Thermus thermophilus* DNA polymerase, *Pyrococcus furiosus* DNA polymerase, *Thermus flavus* DNA polymerase, *Bacillus stearothermophilus* DNA polymerase and *Thermococcus litoralis* DNA polymerase.

Thus, the nucleic acid-based assay described herein and exemplified for PCR in greater detail in the Examples section that follows can be easily adapted into a kit to facilitate industrial adoption as a method for screening tomato and other products for *Alternaria* contamination.

Use of the prior art Howard test is subject to confusion stemming from the gross similarity of *Alternaria* filaments and other (e.g., other mold and/or tomato) fibers (see Figure 2b). The nucleic acid-based assay described and exemplified herein is much more sensitive than the Howard test (see Table 1), and is not subject to variability among observers. The assay described herein offers advantages in terms of host specificity, sensitivity, speed, reliability, and the ability to detect viable or non-viable microbes.

There is a high degree of homology in ITS1 and ITS2 sequences among species and strains of *Alternaria* (19, 24). There are but 0-1 mismatches among *Alternaria* species in the DNA sequences to which the PCR primers employed in the Examples section that follows anneal, allowing these primers to support detection of many species and strains of *Alternaria* in the relevant range of foods. As already discussed, other regions of the ITS1 (19) and ITS 2 sequences could be targeted to develop species- or strain-specific primers or oligonucleotides. The PCR product can be analyzed for species- or strain-specific sequences via DNA

sequencing or hybridization of species- or strain-specific probes to DNA dot blots under stringent hybridization conditions.

The Examples provided herein focus on *A. alternata* contamination of tomato products. However, as described above, other food products might also be examined for *Alternaria* contamination using the present invention. Such products include grains, spices, etc.

It will be appreciated that until the development of the assay described herein there was no test for detecting *Alternaria* contamination of powders. It is shown herein that a nucleic acid-based assay provides a sensitive alternative to the prior art Howard test for detection of *Alternaria* contamination of food products.

It will further be appreciated that a variety of methods can be employed to detect DNA or RNA of specific sequence in a sample. The following paragraphs describe such methods.

Thus, various methods are known in the art which may be used to detect and characterize specific nucleic acid sequences and sequence changes. Nonetheless, as nucleic acid sequence data of the human genome, as well as the genomes of pathogenic organisms accumulates, the demand for fast, reliable, cost-effective and user-friendly tests for specific sequences continues to grow. Importantly, these tests must be able to create a detectable signal from a very low copy number of the sequence of interest. The following discussion examines three levels of nucleic acid detection currently in use: (i) signal amplification technology for detection of rare sequences; (ii) direct detection technology for detection of higher copy number sequences; and (iii) detection of unknown sequence changes for rapid screening of sequence changes anywhere within a defined DNA fragment. Each one of these approaches can be used to implement the method of the present invention.

Signal amplification technology methods for amplification:

The "Polymerase Chain Reaction" (PCR) comprises the first generation of methods for nucleic acid amplification. However, several other methods have been developed that employ the same basis of specificity, but create signal by different amplification mechanisms. These methods include the "Ligase Chain Reaction" (LCR), "Self-Sustained Synthetic Reaction" (3SR/NASBA), and "Qβ-Replicase" (Qβ).

Polymerase Chain Reaction (PCR): The polymerase chain reaction (PCR), as described in U.S. Pat. Nos. 4,683,195 and 4,683,202 to Mullis and Mullis *et al.*, describe a method for increasing the concentration of a

segment of target sequence in a mixture of genomic DNA without cloning or purification. This technology provides one approach to the problems of low target sequence concentration. PCR can be used to directly increase the concentration of the target to an easily detectable level. This process for amplifying the target sequence involves introducing a molar excess of two oligonucleotide primers which are complementary to their respective strands of the double-stranded target sequence to the DNA mixture containing the desired target sequence. The mixture is denatured and then alto hybridize. Following hybridization, the primers are extended with polymerase so as to form complementary strands. The steps of denaturation, hybridization, and polymerase extension can be repeated as often as needed, in order to obtain relatively high concentrations of a segment of the desired target sequence.

The length of the segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and, therefore, this length is a controllable parameter. Because the desired segments of the target sequence become the dominant sequences (in terms of concentration) in the mixture, they are said to be "PCR-amplified."

Ligase Chain Reaction (LCR or LAR): The ligase chain reaction (LCR; sometimes referred to as "Ligase Amplification Reaction" (LAR) described by Barany, Proc. Natl. Acad. Sci., 88:189 (1991); Barany, PCR Methods and Applic., 1:5 (1991); and Wu and Wallace, Genomics 4:560 (1989) has developed into a well-recognized alternative method for amplifying nucleic acids. In LCR, four oligonucleotides, two adjacent oligonucleotides which uniquely hybridize to one strand of target DNA, and a complementary set of adjacent oligonucleotides, which hybridize to the opposite strand are mixed and DNA ligase is added to the mixture. Provided that there is complete complementarity at the junction, ligase will covalently link each set of hybridized molecules. Importantly, in LCR, two probes are ligated together only when they base-pair with sequences in the target sample, without gaps or mismatches. Repeated cycles of denaturation, hybridization and ligation amplify a short segment of DNA. LCR has also been used in combination with PCR to achieve enhanced detection of single-base changes. Segev, PCT Publication No. W09001069 A1 (1990). However, because the four oligonucleotides used in this assay can pair to form two short ligateable fragments, there is the potential for the generation of target-independent background signal. The use of LCR for mutant screening is limited to the examination of specific nucleic acid positions.

Self-Sustained Synthetic Reaction (3SR/NASBA): The self-sustained sequence replication reaction (3SR) (Guatelli *et al.*, Proc. Natl. Acad. Sci., 87:1874-1878, 1990), with an erratum at Proc. Natl. Acad. Sci., 87:7797, 1990) is a transcription-based in vitro amplification system (Kwok *et al.*, Proc. Natl. Acad. Sci., 86:1173-1177, 1989) that can exponentially amplify RNA sequences at a uniform temperature. The amplified RNA can then be utilized for mutation detection (Fahy *et al.*, PCR Meth. Appl., 1:25-33, 1991). In this method, an oligonucleotide primer is used to add a phage RNA polymerase promoter to the 5' end of the sequence of interest. In a cocktail of enzymes and substrates that includes a second primer, reverse transcriptase, RNase H, RNA polymerase and ribo-and deoxyribonucleoside triphosphates, the target sequence undergoes repeated rounds of transcription, cDNA synthesis and second-strand synthesis to amplify the area of interest. The use of 3SR to detect mutations is kinetically limited to screening small segments of DNA (e.g., 200-300 base pairs).

Q-Beta (Qβ) Replicase: In this method, a probe which recognizes the sequence of interest is attached to the replicateable RNA template for Q β replicase. A previously identified major problem with false positives resulting from the replication of unhybridized probes has been addressed through use of a sequence-specific ligation step. However, available thermostable DNA ligases are not effective on this RNA substrate, so the ligation must be performed by T4 DNA ligase at low temperatures (37 °C.). This prevents the use of high temperature as a means of achieving specificity as in the LCR, the ligation event can be used to detect a mutation at the junction site, but not elsewhere.

A successful diagnostic method must be very specific. A straightforward method of controlling the specificity of nucleic acid hybridization is by controlling the temperature of the reaction. While the 3SR/NASBA, and Qβ systems are all able to generate a large quantity of signal, one or more of the enzymes involved in each cannot be used at high temperature (i.e., > 55 °C). Therefore the reaction temperatures cannot be raised to prevent non-specific hybridization of the probes. If probes are shortened in order to make them melt more easily at low temperatures, the likelihood of having more than one perfect match in a complex genome increases. For these reasons, PCR and LCR currently dominate the research field in detection technologies.

The basis of the amplification procedure in the PCR and LCR is the fact that the products of one cycle become usable templates in all subsequent cycles, consequently doubling the population with each cycle. The final yield of any such doubling system can be expressed as: $(1+X)^n = y$, where "X" is the mean efficiency (percent copied in each cycle), "n" is the number of cycles, and "y" is the overall efficiency, or yield of the reaction (Mullis, PCR Methods Applic., 1:1, 1991). If every copy of a target DNA is utilized as a template in every cycle of a polymerase chain reaction, then the mean efficiency is 100 %. If 20 cycles of PCR are performed, then the yield will be 2^{20}, or 1,048,576 copies of the starting material. If the reaction conditions reduce the mean efficiency to 85 %, then the yield in those 20 cycles will be only 1.85^{20}, or 220,513 copies of the starting material. In other words, a PCR running at 85 % efficiency will yield only 21 % as much final product, compared to a reaction running at 100 % efficiency. A reaction that is reduced to 50 % mean efficiency will yield less than 1% of the possible product.

In practice, routine polymerase chain reactions rarely achieve the theoretical maximum yield, and PCRs are usually run for more than 20 cycles to compensate for the lower yield. At 50 % mean efficiency, it would take 34 cycles to achieve the million-fold amplification theoretically possible in 20, and at lower efficiencies, the number of cycles required becomes prohibitive. In addition, any background products that amplify with a better mean efficiency than the intended target will become the dominant products.

Also, many variables can influence the mean efficiency of PCR, including target DNA length and secondary structure, primer length and design, primer and dNTP concentrations, and buffer composition, to name but a few. Contamination of the reaction with exogenous DNA (e.g., DNA spilled onto lab surfaces) or cross-contamination is also a major consideration. Reaction conditions must be carefully optimized for each different primer pair and target sequence, and the process can take days, even for an experienced investigator. The laboriousness of this process, including numerous technical considerations and other factors, presents a significant drawback to using PCR in the clinical setting. Indeed, PCR has yet to penetrate the clinical market in a significant way. The same concerns arise with LCR, as LCR must also be optimized to use different oligonucleotide sequences for each target sequence. In addition, both

methods require expensive equipment, capable of precise temperature cycling.

Many applications of nucleic acid detection technologies, such as in studies of allelic variation, involve not only detection of a specific sequence in a complex background, but also the discrimination between sequences with few, or single, nucleotide differences. One method for the detection of allele-specific variants by PCR is based upon the fact that it is difficult for Taq polymerase to synthesize a DNA strand when there is a mismatch between the template strand and the 3' end of the primer. An allele-specific variant may be detected by the use of a primer that is perfectly matched with only one of the possible alleles; the mismatch to the other allele acts to prevent the extension of the primer, thereby preventing the amplification of that sequence. This method has a substantial limitation in that the base composition of the mismatch influences the ability to prevent extension across the mismatch, and certain mismatches do not prevent extension or have only a minimal effect (Kwok *et al.*, Nucl. Acids Res., 18:999, 1990)

A similar 3'-mismatch strategy is used with greater effect to prevent ligation in the LCR (Barany, PCR Meth. Applic., 1:5, 1991). Any mismatch effectively blocks the action of the thermostable ligase, but LCR still has the drawback of target-independent background ligation products initiating the amplification. Moreover, the combination of PCR with subsequent LCR to identify the nucleotides at individual positions is also a clearly cumbersome proposition for the clinical laboratory.

Direct detection technology:

When a sufficient amount of a nucleic acid to be detected is available, there are advantages to detecting that sequence directly, instead of making more copies of that target, (e.g., as in PCR and LCR). Most notably, a method that does not amplify the signal exponentially is more amenable to quantitative analysis. Even if the signal is enhanced by attaching multiple dyes to a single oligonucleotide, the correlation between the final signal intensity and amount of target is direct. Such a system has an additional advantage that the products of the reaction will not themselves promote further reaction, so contamination of lab surfaces by the products is not as much of a concern. Traditional methods of direct detection including Northern and Southern blotting and RNase protection assays usually require the use of radioactivity and are not amenable to automation. Recently devised techniques have sought to eliminate the use of radioactivity and/or

improve the sensitivity in automateable formats. Two examples are the "Cycling Probe Reaction" (CPR), and "Branched DNA" (bDNA).

Cycling probe reaction (CPR): The cycling probe reaction (CPR) (Duck *et al.*, BioTech., 9:142, 1990), uses a long chimeric oligonucleotide in which a central portion is made of RNA while the two termini are made of DNA. Hybridization of the probe to a target DNA and exposure to a thermostable RNase H causes the RNA portion to be digested. This destabilizes the remaining DNA portions of the duplex, releasing the remainder of the probe from the target DNA and allowing another probe molecule to repeat the process. The signal, in the form of cleaved probe molecules, accumulates at a linear rate. While the repeating process increases the signal, the RNA portion of the oligonucleotide is vulnerable to RNases that may carried through sample preparation.

Branched DNA: Branched DNA (bDNA), described by Urdea *et al.*, Gene 61:253-264 (1987), involves oligonucleotides with branched structures that allow each individual oligonucleotide to carry 35 to 40 labels (e.g., alkaline phosphatase enzymes). While this enhances the signal from a hybridization event, signal from non-specific binding is similarly increased.

Detection of unknown sequence changes:

The demand for tests which allow the detection of specific nucleic acid sequences and sequence changes is growing rapidly in clinical diagnostics. As nucleic acid sequence data for genes from humans and pathogenic organisms accumulates, the demand for fast, cost-effective, and easy-to-use tests for as yet unknown mutations within specific sequences is rapidly increasing.

A handful of methods have been devised to scan nucleic acid segments for mutations. One option is to determine the entire gene sequence of each test sample (e.g., a bacterial isolate). For sequences under approximately 600 nucleotides, this may be accomplished using amplified material (e.g., PCR reaction products). This avoids the time and expense associated with cloning the segment of interest. However, specialized equipment and highly trained personnel are required, and the method is too labor-intense and expensive to be practical and effective in the clinical setting.

In view of the difficulties associated with sequencing, a given segment of nucleic acid may be characterized on several other levels. At the lowest resolution, the size of the molecule can be determined by electrophoresis by comparison to a known standard run on the same gel. A

more detailed picture of the molecule may be achieved by cleavage with combinations of restriction enzymes prior to electrophoresis, to allow construction of an ordered map. The presence of specific sequences within the fragment can be detected by hybridization of a labeled probe, or the precise nucleotide sequence can be determined by partial chemical degradation or by primer extension in the presence of chain-terminating nucleotide analogs.

Restriction fragment length polymorphism (RFLP): For detection of single-base differences between like sequences, the requirements of the analysis are often at the highest level of resolution. For cases in which the position of the nucleotide in question is known in advance, several methods have been developed for examining single base changes without direct sequencing. For example, if a mutation of interest happens to fall within a restriction recognition sequence, a change in the pattern of digestion can be used as a diagnostic tool (e.g., restriction fragment length polymorphism [RFLP] analysis).

Single point mutations have been also detected by the creation or destruction of RFLPs. Mutations are detected and localized by the presence and size of the RNA fragments generated by cleavage at the mismatches. Single nucleotide mismatches in DNA heteroduplexes are also recognized and cleaved by some chemicals, providing an alternative strategy to detect single base substitutions, generically named the "Mismatch Chemical Cleavage" (MCC) (Gogos *et al.*, Nucl. Acids Res., 18:6807-6817, 1990). However, this method requires the use of osmium tetroxide and piperidine, two highly noxious chemicals which are not suited for use in a clinical laboratory.

RFLP analysis suffers from low sensitivity and requires a large amount of sample. When RFLP analysis is used for the detection of point mutations, it is, by its nature, limited to the detection of only those single base changes which fall within a restriction sequence of a known restriction endonuclease. Moreover, the majority of the available enzymes have 4 to 6 base-pair recognition sequences, and cleave too frequently for many large-scale DNA manipulations (Eckstein and Lilley (eds.), Nucleic Acids and Molecular Biology, vol. 2, Springer-Verlag, Heidelberg, 1988). Thus, it is applicable only in a small fraction of cases, as most mutations do not fall within such sites.

A handful of rare-cutting restriction enzymes with 8 base-pair specificities have been isolated and these are widely used in genetic

mapping, but these enzymes are few in number, are limited to the recognition of G+C-rich sequences, and cleave at sites that tend to be highly clustered (Barlow and Lehrach, Trends Genet., 3:167, 1987). Recently, endonucleases encoded by group I introns have been discovered that might have greater than 12 base-pair specificity (Perlman and Butow, Science 246:1106, 1989), but again, these are few in number.

Allele specific oligonucleotide (ASO): If the change is not in a recognition sequence, then allele-specific oligonucleotides (ASOs), can be designed to hybridize in proximity to the unknown nucleotide, such that a primer extension or ligation event can bused as the indicator of a match or a mis-match. Hybridization with radioactively labeled allelic specific oligonucleotides (ASO) also has been applied to the detection of specific point mutations (Conner *et al.*, Proc. Natl. Acad. Sci., 80:278-282, 1983). The method is based on the differences in the melting temperature of short DNA fragments differing by a single nucleotide. Stringent hybridization and washing conditions can differentiate between mutant and wild-type alleles. The ASO approach applied to PCR products also has been extensively utilized by various researchers to detect and characterize point mutations in ras genes (Vogelstein *et al.*, N. Eng. J. Med., 319:525-532, 1988; and Farr *et al.*, Proc. Natl. Acad. Sci., 85:1629-1633, 1988), and gsp/gip oncogenes (Lyons *et al.*, Science 249:655-659, 1990). Because of the presence of various nucleotide changes in multiple positions, the ASO method requires the use of many oligonucleotides to cover all possible oncogenic mutations.

With either of the techniques described above (i.e., RFLP and ASO), the precise location of the suspected mutation must be known in advance of the test. That is to say, they are inapplicable when one needs to detect the presence of a mutation of an unknown character and position within a gene or sequence of interest.

Denaturing/Temperature Gradient Gel Electrophoresis (DGGE/TGGE): Two other methods rely on detecting changes in electrophoretic mobility in response to minor sequence changes. One of these methods, termed "Denaturing Gradient Gel Electrophoresis" (DGGE) is based on the observation that slightly different sequences will display different patterns of local melting when electrophoretically resolved on a gradient gel. In this manner, variants can be distinguished, as differences in melting properties of homoduplexes versus heteroduplexes differing in a single nucleotide can detect the presence of mutations in the target

sequences because of the corresponding changes in their electrophoretic mobilities. The fragments to be analyzed, usually PCR products, are "clamped" at one end by a long stretch of G-C base pairs (30-80) to allow complete denaturation of the sequence of interest without complete dissociation of the strands. The attachment of a GC "clamp" to the DNA fragments increases the fraction of mutations that can be recognized by DGGE (Abrams *et al.*, Genomics 7:463-475, 1990). Attaching a GC clamp to one primer is critical to ensure that the amplified sequence has a low dissociation temperature (Sheffield *et al.*, Proc. Natl. Acad. Sci., 86:232-236, 1989; and Lerman and Silverstein, Meth. Enzymol., 155:482-501, 1987). Modifications of the technique have been developed, using temperature gradients (Wartell *et al.*, Nucl. Acids. Res., 18:2699-2701, 1990), and the method can be also applied to RNA:RNA duplexes (Smith *et al.*, Genomics 3:217-223, 1988).

Limitations on the utility of DGGE include the requirement that the denaturing conditions must be optimized for each type of DNA to be tested. Furthermore, the method requires specialized equipment to prepare the gels and maintain the needed high temperatures during electrophoresis. The expense associated with the synthesis of the clamping tail on one oligonucleotide for each sequence to be tested is also a major consideration. In addition, long running times are required for DGGE. The long running time of DGGE was shortened in a modification of DGGE called constant denaturant gel electrophoresis (CDGE) (Borrensen *et al.*, Proc. Natl. Acad. Sci. USA 88:8405, 1991). CDGE requires that gels be performed under different denaturant conditions in order to reach high efficiency for the detection of unknown mutations.

A technique analogous to DGGE, termed temperature gradient gel electrophoresis (TGGE), uses a thermal gradient rather than a chemical denaturant gradient (Scholz, *et al.*, Hum. Mol. Genet. 2:2155, 1993). TGGE requires the use of specialized equipment which can generate a temperature gradient perpendicularly oriented relative to the electrical field. TGGE can detect mutations in relatively small fragments of DNA therefore scanning of large gene segments requires the use of multiple PCR products prior to running the gel.

Single-Strand Conformation Polymorphism (SSCP): Another common method, called "Single-Strand Conformation Polymorphism" (SSCP) was developed by Hayashi, Sekya and colleagues (reviewed by Hayashi, PCR Meth. Appl., 1:34-38, 1991) and is based on the observation

that single strands of nucleic acid can take on characteristic conformations in non-denaturing conditions, and these conformations influence electrophoretic mobility. The complementary strands assume sufficiently different structures that one strand may be resolved from the other. Changes in sequences within the fragment will also change the conformation, consequently altering the mobility and allowing this to be used as an assay for sequence variations (Orita, *et al.*, Genomics 5:874-879, 1989).

The SSCP process involves denaturing a DNA segment (e.g., a PCR product) that is labeled on both strands, followed by slow electrophoretic separation on a non-denaturing polyacrylamide gel, so that intra-molecular interactions can form and not be disturbed during the run. This technique is extremely sensitive to variations in gel composition and temperature. A serious limitation of this method is the relative difficulty encountered in comparing data generated in different laboratories, under apparently similar conditions.

Dideoxy fingerprinting (ddF): The dideoxy fingerprinting (ddF) is another technique developed to scan genes for the presence of unknown mutations (Liu and Sommer, PCR Methods Appli., 4:97, 1994). The ddF technique combines components of Sanger dideoxy sequencing with SSCP. A dideoxy sequencing reaction is performed using one dideoxy terminator and then the reaction products are electrophoresed on nondenaturing polyacrylamide gels to detect alterations in mobility of the termination segments as in SSCP analysis. While ddF is an improvement over SSCP in terms of increased sensitivity, ddF requires the use of expensive dideoxynucleotides and this technique is still limited to the analysis of fragments of the size suitable for SSCP (i.e., fragments of 200-300 bases for optimal detection of mutations).

In addition to the above limitations, all of these methods are limited as to the size of the nucleic acid fragment that can be analyzed. For the direct sequencing approach, sequences of greater than 600 base pairs require cloning, with the consequent delays and expense of either deletion sub-cloning or primer walking, in order to cover the entire fragment. SSCP and DGGE have even more severe size limitations. Because of reduced sensitivity to sequence changes, these methods are not considered suitable for larger fragments. Although SSCP is reportedly able to detect 90% of single-base substitutions within a 200 base-pair fragment, the detection drops to less than 50 % for 400 base pair fragments. Similarly, the

sensitivity of DGGE decreases as the length of the fragment reaches 500 base-pairs. The ddF technique, as a combination of direct sequencing and SSCP, is also limited by the relatively small size of the DNA that can be screened.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for DNA isolation, amplification and purification. These techniques and various other techniques are generally performed according to Sambrook et al., Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

MATERIALS AND EXPERIMENTAL METHODS

Food products: Tomato products used for development of the detection assay according to the present invention were produced in a pilot plant using methods representative of industrial tomato processing. The tomato sauce was heat treated as in commercial operations at 110 °C for 10 minutes. Two tomato powders were tested, one produced commercially and one produced in the pilot plant by heat treatment and spray-drying of tomato sauce. *Alternaria*-infested tomatoes were used for certain tomato sauce and powder preparations made in the pilot plant. In such cases, raw materials

included one tomato with a 10-20 % surface area coverage of *Alternaria* mold among the 10 tomatoes used per run. Dry grains and spices were of commercial stocks.

Howard test: Use of the Howard test followed standard methods (13). Tomato sauce was placed into a Howard mold counting chamber, a cover glass was placed over it, and the cover glass was pressed down to produce a film of the product, approximately 0.1 mm thick. The slide was examined at 90x magnification. Twenty-five fields were examined, fifty if to confirm absence of *Alternaria*. The presence or absence of mold filaments in each field was scored; a field was considered positive for mold if the aggregate length of mold filaments exceeded one-sixth the diameter of the field. The percentage of fields scored positive was calculated to obtain the results of the Howard test for a particular tomato product.

Molecular genetics methods: The 5.8s rRNA gene sequences for 24 strains representing 12 *Alternaria* species including *A. alternata* strain AA6 (19, 24, 32, SEQ ID NO:1) were compared with the corresponding sequences for: *Aspergillus flavus* ATCC 10124 (36, SEQ ID NO:2), *Fusarium oxysporum* ATCC 52434 (SEQ ID NO:3) and CBS 171.31 (6, 54), yeast *Saccharomyces cerevisiae* (14, SEQ ID NO:4), and tomato *Solaneum lycopersicum* (20, 25, SEQ ID NO:5).

As shown in Figure 1, a high level of homology was observed among DNA sequences encoding the 5.8s rRNA gene, and a low level at the ITS1 and ITS2 regions thereof; sequences at which *Alternaria* sp. and the other organisms differed were targeted for development of *Alternaria*-specific PCR primers. The two primers and their nucleotide sequences are as follow:

Primer 1 (ITS1): 5'-ATTGCAATCAGCGTCAGTAAC-3' (SEQ ID NO:6)
Primer 2 (ITS2): 5'-CAAGCAAAGCTTGAGGGTACA-3' (SEQ ID NO:7)

DNA purification followed (44) and the universal DNA isolation procedure of (46). Briefly, 100 µl of tomato sauce was diluted with 100 µl of water, to which was added 500 µl of guanidine thiocyanate dissolved in 50 mM Tris pH 8.3 mixed with an equal volume of phenol. After mixing, the material was incubated 15 min in boiling water. After addition of 250 µl of chloroform, the mixture was centrifuged, and the supernatant was removed to a clean tube. Isopropanol was added, and the mixture was held at -20 °C for one hour. The precipitate was washed in 70 % ethanol, air dried, and resuspended in water. For tomato and spices powder, 30 mg powder were added to 200 µl water, and DNA extraction proceeded as for

tomato sauce. Dry grains were imbibed in water, vortexed, and the supernatant collected. Solid particles present in the supernatant were collected by centrifugation and were subsequently subjected to nucleic acid purification as further detailed hereinabove.

Polymerase chain reaction was carried out using an MJ Research, Inc. thermocycler using the following protocol: 5 min 94 °C; five cycles of 45 seconds at 94 °C, 45 seconds at 58 °C, 45 seconds at 72 °C; 20 cycles of 45 seconds at 94 °C, 45 seconds at 55 °C, 45 seconds at 72 °C; followed by 5 minutes at 72 °C; and cooling to room temperature. PCR products were observed using UV light following electrophoresis in 1.5 % TBE agarose minigels prepared with ethidium bromide.

Sensitivity assay: Fresh tomato sauce was prepared from *Alternaria*-infested tomatoes, and diluted sequentially with *Alternaria*-free tomato sauce. Each dilution was divided into two aliquots, one analyzed by the Howard test, and one by the PCR-based assay.

Specificity analysis: The specificity of the assay for *Alternaria* DNA was tested by attempting PCR amplification of purified DNA from the molds *Aspergillus niger* and *Fusarium oxysporum* under the conditions specified above.

Additional primers: While the previous pair of primers is specific for all *Alternaria* species, new primer pairs were designed for specifically identifying *Alternaria alternata* strains but not other *Alternaria* strains and/or other molds (primers identified by SEQ ID NOs:8-13). In addition, two additional primers that specifically amplify the strain of *Alternaria solani* (SEQ ID NOs:14-15) were designed. All primers were derived from the 5.8s rRNA gene sequence of the respective *Alternaria* species and tested for specificity as described above for the first pair of primers.

Thus, the following primer pairs were designed for, and were found to have, specificity for *Alternaria alternate*: ALT1 - 5'-TTCGCCCACCACTAGGACA-3' (forward primer, SEQ ID NO:8); and ALT2 - 5'-AGGCTTAATGGATGCTAGAC-3' (reverse primer, SEQ ID NO:9). ALT91 - 5'-CTTCTTGTTTCCTTGGTGGG-3' (forward primer, SEQ ID NO:10); and ALTR96 - 5'-GCGCTCCGAAACCAGTAGGC-3' (reverse primers, SEQ ID NO:11). ALT13 - 5'-GGCGGGCTGGAACCTCTCGG-3' (forward primer, SEQ ID NO:12); and ALTR148 - 5'-AATGGATGCTAGACCTTTGC-3' (reverse primer, SEQ ID NO:13). The following primer pairs were designed for, and were found to have, specificity for *Alternaria solani*: ASO114 - 5'-

CGCCCACCACAAGGACCAACCC-3' (forward primer, SEQ ID NO:14); and ASOR135 - 5'-TCGGAGAACCTTGGGGCTGGAA-3' (reverse primers, SEQ ID NO:15).

EXPERIMENTAL RESULTS

The use of a PCR-based assay for amplifying the 5.8s rRNA gene of *Alternaria* sp. to detect the presence of *Alternaria* sp. in tomato products is demonstrated herein and its sensitivity is compared to the prior art microscopy-based test (Howard test).

The presence of *Alternaria* DNA in fungus-infested tomato sauce was detected microscopically using the well-established Howard test (Figures 2a and 2b). Detection of *Alternaria* using the PCR-based assay is shown in Figure 3. A PCR product at 217 bp was taken as indicative of amplification of *Alternaria* rRNA sequences. The sensitivity and semi-quantitative potential of the assay is shown by amplification of target DNA in a series of dilutions of fresh tomato sauce (lanes B-E). The intensity of fluorescence was directly related to the concentration of *Alternaria* DNA. DNA of *Alternaria*-free tomato sauce also was assayed and proved negative for amplification (lane F).

A comparison of sensitivity (Table 1 below) indicated that the microscopy-based Howard test could detect *Alternaria* filaments to a 5:1 dilution of the tomato sauce, while the PCR-based test could detect *Alternaria* DNA to a dilution of 100:1. Amplification of *Alternaria* DNA occurred also after storage of two months.

TABLE 1
Comparison of sensitivity of Howard and PCR-based assays for detection of Alternaria sp.

	Tomato sauce		Tomato powder
Dilution	Howard method[1]	PCR[2]	PCR[2]
Undiluted	100 %	+	NA[3]
5:1	25 %	+	+
10:1	0 %	+	+
100:1	0 %	+	+
1000:1	NA	-	NA
Distilled water	NA	-	-

[1]Reported as percentage of microscopic fields positive for *Alternaria* filaments; [2]Four replicate reactions were assayed. [3] Not attempted.

The presence of *Alternaria* sp. in tomato powder was also searched. The Howard test was not a viable screening method, as the drying process disrupts any fungal filaments in the starting materials. Indeed, fungal filaments were not visible in either dry or wetted powder. Results of the PCR-based assay (Figure 3, Table 1) for pilot plant-produced tomato powder experimentally contaminated with *Alternaria* DNA exhibited amplification to a dilution of 100:1 (Figure 3, lanes G-J). An *Alternaria*-infested commercial product also exhibited amplification (lanes K-M). No amplification was seen for an *Alternaria*-free commercial product (lane P). Storage time did not affect results of the PCR assay of tomato powders. The specificity of the primer pair employed herein was assessed by attempting amplification of DNA from other microbes. No amplification products were observed following PCR of DNA from the molds *Aspergillus niger* and *Fusarium oxysporum*.

To test whether the method according to the present invention is applicable to other food products, including grains, such as barley, wheat, corn, sorghum, chickpea, lentil or different varieties (yellow, orange, green and gray) and spices such as paprika powder, these food products, which were derived from commercial stocks, were tested for the presence of *Alternaria* nucleic acid sequences using the PCR approach described herein. Positive results were obtained with corn and yellow lentil, whereas all other food products were tested negative. These results were confirmed using the Howard test.

While the previous pair of primers is specific for all *Alternaria* species, new primer pairs were designed for specifically identifying *Alternaria alternata* strains but not other *Alternaria* strains and/or other molds (primers identified by SEQ ID NOs:8-13). In addition, two additional primers that specifically amplify the strain of *Alternaria solani* (SEQ ID NOs:14-15) were designed. All primers were derived from the 5.8s rRNA gene sequence of the respective *Alternaria* species and tested for specificity as described above for the first pair of primers.

Figure 4 shows exemplary results of PCR-based assay of *Alternaria* DNA in a variety of food products. The primers employed for PCR amplification were ALT13 (SEQ ID NO:12) and ALTR148 (SEQ ID NO:12) specifically yielding a PCR product of 451 bp in the presence of

Alternaria alternata strains. (1) *Alternaria* grown in a petri dish; (2) *Alternaria* from cotton leaves; (3) *Alternaria* from tomato leaves; (4) *Alternaria* from sesame grains; (5) *Alternaria* from wheat grains; (6) *Alternaria* from corn grains; (7) *Alternaria* from paprika powder; (8) water sample (negative control); and (9) size marker.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A nucleic acid based method for the detection of *Alternaria* contamination in a food product comprising the steps of:

(a) obtaining a sample of the food product; and

(b) analyzing said sample of the food product for a nucleic acid sequence unique to *Alternaria*, wherein a detectable level of said nucleic acid sequence unique to *Alternaria* is indicative of *Alternaria* contamination in the food product.

2. The method of claim 1, wherein said food product is selected from the group consisting of a tomato product, a grain and a spice.

3. The method of claim 1, wherein said food product is a dried powder.

4. The method of claim 1, wherein said food product is a mash.

5. The method of claim 1, wherein said step of analyzing said sample of the food product for said nucleic acid sequence unique to *Alternaria* is effected by a template-dependent assay.

6. The method of claim 5, wherein said template-dependent assay is a template-dependent synthesis assay.

7. The method of claim 5, wherein said template-dependent assay is a template-dependent hybridization assay.

8. The method of claim 5, wherein said template-dependent assay is a template-dependent hybridization and synthesis assay.

9. The method of claim 5, wherein said template-dependent assay is selected from the group consisting of primer extension, polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA) and strand displacement amplification (SDA).

10. The method of claim 5, wherein said template-dependent assay includes a step of primer extension effected by at least one oligonucleotide having a sequence hybridizable with said nucleic acid sequence unique to *Alternaria*.

11. The method of claim 10, wherein said at least one oligonucleotide is as set forth in SEQ ID NOs:6-15.

12. The method of claim 1, wherein said nucleic acid sequence unique to *Alternaria* is derived from a ribosomal RNA encoding gene of *Alternaria*.

13. The method of claim 12, wherein said gene is a 5.8s rRNA gene of *Alternaria*.

14. A kit useful for the *Alternaria* contamination in a food product comprising a carrier being compartmentalized to receive in close confinement therein one or more containers comprising at least one oligonucleotide having a sequence hybridizable with a nucleic acid sequence unique to *Alternaria*.

15. The kit of claim 14, further comprising a template dependent DNA polymerase.

16. The kit of claim 15, wherein said template dependent DNA polymerase is thermostable.

17. The kit of claim 14, wherein said at least one oligonucleotide is as set forth in SEQ ID NOs:6-15.

18. The method of claim 14, wherein said nucleic acid sequence unique to *Alternaria* is derived from a ribosomal RNA encoding gene of *Alternaria*.

19. The method of claim 18, wherein said gene is a 5.8s rRNA gene of *Alternaria*.

FIG. 1

SEQIDNO:

```
                    351                                                 400
A. alternata        gtacttcttg tttccttggt gggttcgccc accactagga caaacataaa  1
A. flavus           ccg.cgggg. cg..agcccc ...cc...g. c.gc.gga.. c.ccacg..c  2
F. oxysporum        .at.ag.cc. ctc..ggtaa aacggga.gg c..g.c..ag g.cc.c....  3
S. cerevisiae       aat.ggggcc cagagg.aac aaaca.aaa. .attt..ttt attcat....  4
S. lycopersicum     .cg.ggaaa. cgc.aag.aa tac.a.aat. ga..gccctc .ccctcgcgc  5

                    401                                                 450
A. alternata        ccttttgta- --attgcaat cagcgtcagt aaca--aatt aat-aattaC  1
A. flavus           t..g.ctg.t ct.g..a.g. .t.a..tga. tgt.--tcgc ...c.g...a  2
F. oxysporum        .tc.g.t.ct at..gta.c. tctgagt.aa .c..--t.-- ...a.a.c.a  3
S. cerevisiae       tt...gtc.a aa.caag... ttt...a.c. gga.attt.a ..at.t.a.a  4
S. lycopersicum     ..cg..cgcg gatcgtgcgg gg.gaagc.c gctgctctg. t.ac.caa..  5

                    451                                                 500
A. alternata        AACTTTCAAC AACGGATCTC TTGGTTCTGG CATCGATGAA GAACGCAGCG  1
A. flavus           .......... ..T....... .......C.. .......... ..........  2
F. oxysporum        .......... .......... .......... .......... .........A  3
S. cerevisiae       .......... .......... ........C. .......... ..........  4
S. lycopersicum     G...C..GG. .......A.. .C..C...C. .......... .....T....  5

                    501                                                 550
A. alternata        AAATGCGATA AGTAGTGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT  1
A. flavus           .......... .C........ .......... ..C....... .....G....  2
F. oxysporum        .......... ....A..... .......... .......... ..........  3
S. cerevisiae       .......... C...A..... .......... ..C....... ..........  4
S. lycopersicum     .......... CT.G...... .......... CCC.....C. .....G....  5

                    551                                                 600
A. alternata        TGAACGCACA TTGCGCCCTT TGGTATT--C CAAAGGGCAT GCCTGTTCGA  1
A. flavus           .......... ........CC ........--. .GGG...... ..........  2
F. oxysporum        .......... ........GC CA.....--. TGGC...... ..........  3
S. cerevisiae       .......... ........C. ........--. ..GG...... ..........  4
S. lycopersicum     ........AG ........GA A.CC...TGG .CG......C .TCTG..T.G  5

                    601                                                 650
A. alternata        GCGTCATTTg taccctcaag ctttgcttgg tgttgggcgt cttgtctcta  1
A. flavus           ........Gc .g...atc.a gcac.gc.t. ...gtt.g.. .g.cgtc.cc  2
F. oxysporum        .........c a......... .aca...... .......ac. .gc..taa.-  3
S. cerevisiae       .........c ctt.tca..c a..ctg..t. gtagt.agtg a.ac...t.g  4
S. lycopersicum     ......CGAt cg.gtcgccc ..cgcacgcc gcaa..ctt. agc.cggggg  5

                    651                                                 700
A. alternata        gctttgctgg agactcgcct taaagtaatt ggcagccggc ctactggttt  1
A. flavus           t..cc.gggg g...gg...c c....gc.gc ...g..accg .gt.c.a.cc  2
F. oxysporum        ---------- tcg.gtt... c...t.g... ...g.t.acg -.cga.c..c  3
S. cerevisiae       .ag..aactt ga.a.t..tg gcctt.tca. t.g.tgtttt t.ttccaaag  4
S. lycopersicum     cggaa..... cct.c..tgc gccccg.cgg c..g...... ...aat.cga  5

                    701                                                 750
A. alternata        cggagcgca- --gcacaagt cgcactctct atcagcaaag gtctagcatc  1
A. flavus           tc.....t.t gg.gcttt.. .a.c.g.... g.ag..                 2
F. oxysporum        .at....t.g ta.t.a..cc .t.gt.actg g.a.t.gtc. cggcca.gc.  3
S. cerevisiae       a.ag.tttct ct..gtgct. gaggtataa. gca..t.cg. tcg.ttt.gg  4
S. lycopersicum     gtcca..tcg ac.g..gtcg ..gcaag.gg tggttg...c tcaact.tct  5
```

FIG. 2A

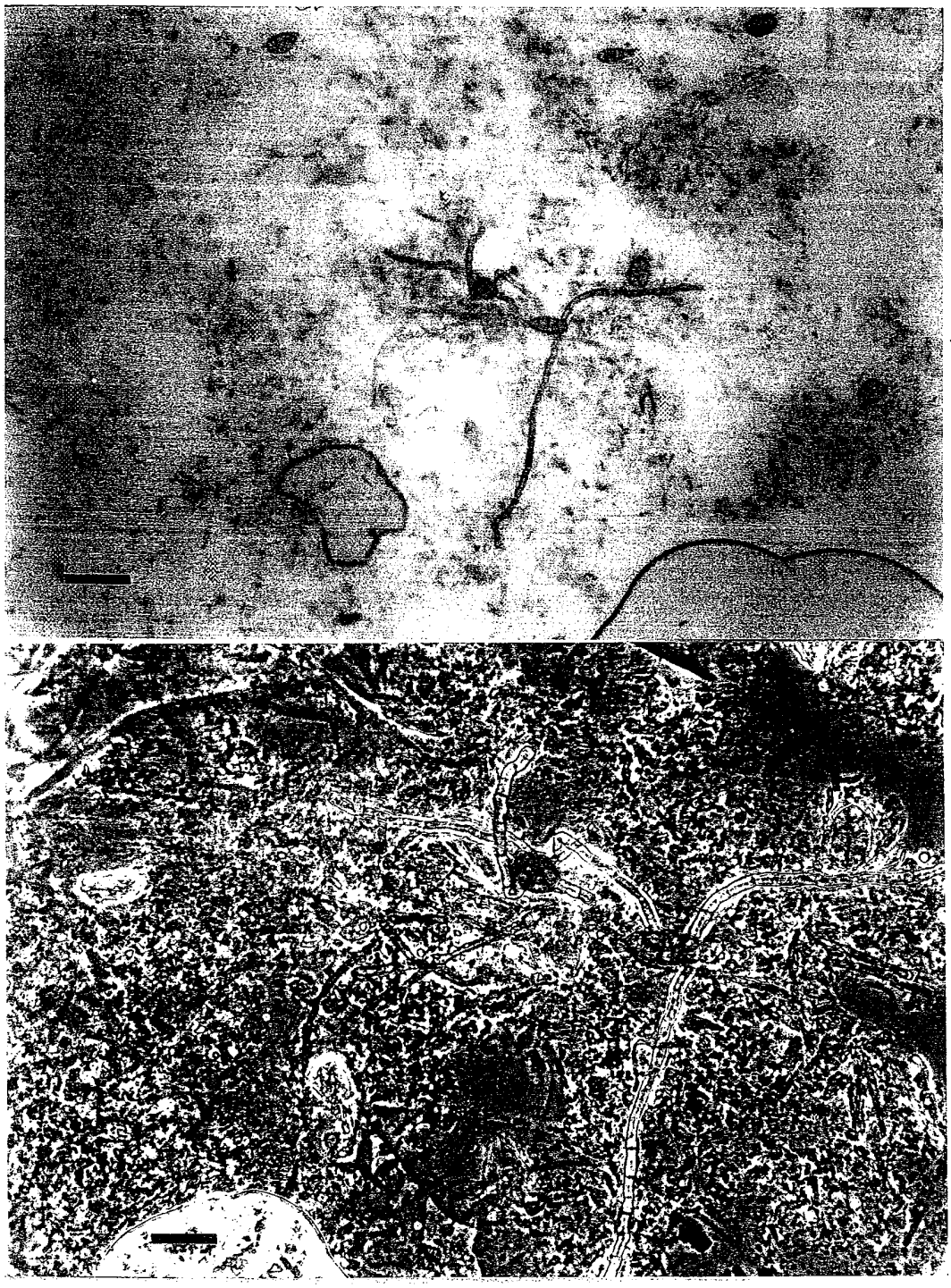


FIG. 2B

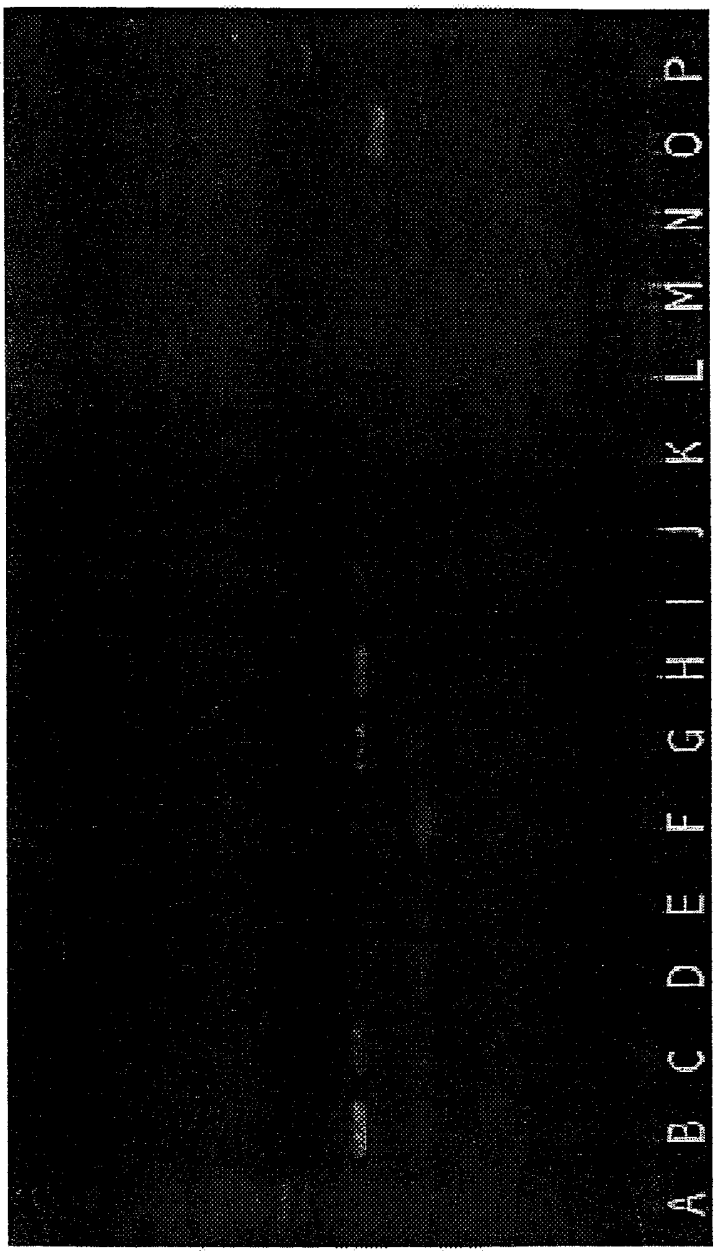


FIG. 3

FIG. 4

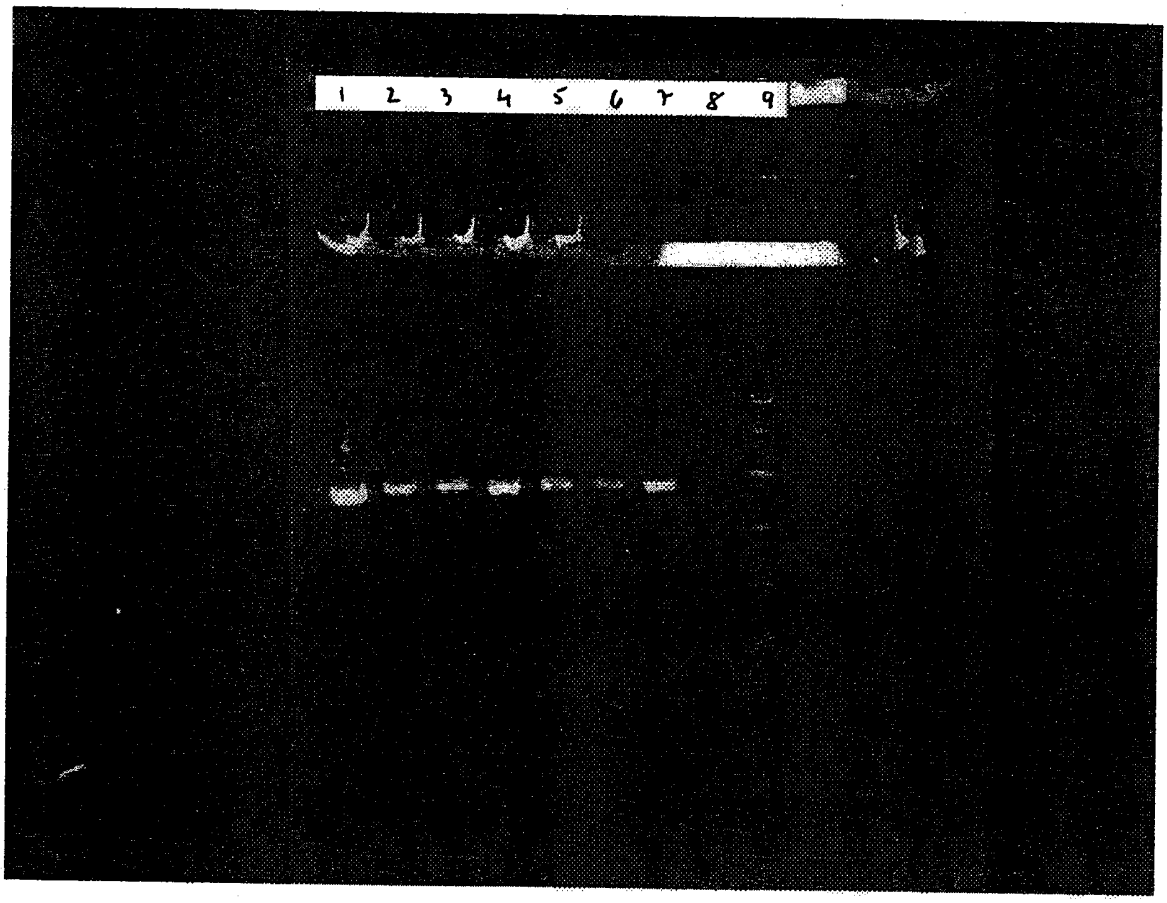
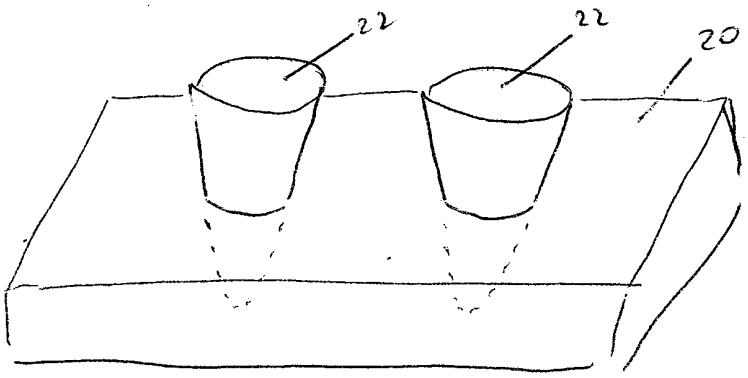


FIG. 5



SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Yechezkel Kashi et al.

(ii) TITLE OF INVENTION: NUCLEIC ACID-BASED ASSAY AND KIT FOR THE DETECTION OF *ALTERNARIA* CONTAMINATION IN FOOD PRODUCTS

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Mark M. Friedman c/o Anthony Castorina

(B) STREET: 2001 Jefferson Davis Highway, Suite 207

(C) CITY: Arlington

(D) STATE: Virginia

(E) COUNTRY: United States of America

(F) ZIP: 2202

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 1.44 megabyte, 3.5" microdisk

(B) COMPUTER: Twinhead® Slimnote-890TX

(C) OPERATING SYSTEM: MS DOS version 6.2, Windows version 3.11

(D) SOFTWARE: Word for Windows version 2.0

an ASCI file

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: U.S. 09/241,427

(B) FILING DATE: February 2, 1999

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Friedmam, Mark M.

(B) REGISTRATION NUMBER: 33,883

(C) REFERENCE/DOCKET NUMBER: 74/86

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 972-3-5625553

(B) TELEFAX: 972-3-5625554

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 389

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

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CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC 150

GATAAGTAGT GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG 200

CACATTGCGC CCTTTGGTAT TCCAAAGGGC ATGCCTGTTC GAGCGTCATT 250

TGTACCCTCA AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TAGCTTTGCT 300

GGAGACTCGC CTTAAAGTAA TTGGCAGCCG GCCTACTGGT TTCGGAGCGC 350

AGCACAAGTC GCACTCTCTA TCAGCAAAGG TCTAGCATC 389

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 382

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

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AACGCACATT GCGCCCCCTG GTATTCCGGG GGGCATGCCT GTTCGAGCGT 250
CATTGCTGCC CATCAAGCAC GGCTTGTGTG TTGGGTCGTC GTCCCCTCTC 300
CGGGGGGGAC GGGCCCCAAA GGCAGCGGCG GCACCGCGTC CGATCCTCGA 350
GCGTATGGGG CTTTGTCACC CGCTCTGTAG GC 382

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 382
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

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TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCAAAAT 150
GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA 200
CGCACATTGC GCCCGCCAGT ATTCTGGCGG GCATGCCTGT TCGAGCGTCA 250
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CCCTCGTTAC TGGTAATCGT CGCGGCCACG CC 382

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 398
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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AAATGCGATA CGTAATGTGA ATTGCAGAAT TCCGTGAATC ATCGAATCTT 200
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

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TGAACGCAAG TTGCGCCCGA AGCCATTTGG CCGAGGGCAC GTCTGCCTGG 250

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CGGAAGCTGG CCTCCCGTGC GCCCCGACGC CGCGGCCGGC CTAAATGCGA 350
GTCCACGTCG ACGGACGTCG CGGCAAGTGG TGGTTGAAAC TCAACTCTCT 400

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(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
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(2) INFORMATION FOR SEQ ID NO:7:
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(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
CAAGCAAAGC TTGAGGGTAC A 21

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
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(2) INFORMATION FOR SEQ ID NO:9:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
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(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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GCGCTCCGAA ACCAGTAGGC 20

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
GGCGGGCTGG AACCTCTCGG 20

(2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
AATGGATGCT AGACCTTTGC 20

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
CGCCCACCAC AAGGACCAAC CC 22

(2) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
TCGGAGAACC TTGGGGCTGG AA 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/01466

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/48, 1/68; C12N 9/12; C12P 19/34; C07H 21/02, 21/04

US CL :435/6, 15, 91.2, 194, 810; 536/23.1, 23.74, 24.32, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 15, 91.2, 194, 810; 536/23.1, 23.74, 24.32, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 5,106,727 A (HARTLEY et al) 21 April 1992 (21/4/92), see entire document, especially column 4, lines 18-32, column 9, lines 15-29, 40-52, claim 5.	14-15, 18-19 ----- 16
Y	US 5,324,632 A (WEISBURG ET AL) 28 June 1994 (28/6/94), see entire document, especially column 1, lines 12-15, column 3, lines 63-68, Tables 1-3.	1-19
Y	US 5,534,280 A (WELCH) 09 July 1996 (9/7/96), see entire document, especially column 20, line 54-column 21, line 48.	3
Y	US 4,031,264 A (AROLSKI ET AL) 21 June 1977 (21/6/77), see entire document, especially column 1, lines 33-39.	4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 MARCH 2000	Date of mailing of the international search report 05 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DIANA B. JOHANNSEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/01466

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ERLICH et al. Recent advances in the polymerase chain reaction. Science. June 1991, Vol. 252, pages 1643-1650, especially page 1643.	15-16
Y	JASALAVICH et al. Comparison of nuclear ribosomal DNA sequences from Alternaria species pathogenic to crucifers. Mycol. Res. June 1995, Vol. 99, No. 5, pages 604-614, especially pages 604-606, 613, Figure 1.	1-13
Y	LOVIC et al. Sequence analysis of the ITS regions of rDNA in Monosporascus spp. to evaluate its potential for PCR-mediated detection.MOLECUKAR PLANT Phytopathology. 1995, Vol. 85, No. 6, pages 655-661, especially pages 656, 658-660.	1-19
Y	TIETJEN et al. Nature and extent of losses in fresh repacked tomatoes. Phytopathology. 1982, Vol. 72, No. 2, pages 266-67, see entire abstract.	2-4
Y	PRYOR et al. Detection of Alternaria radicina and A. dauci using a PCR-based assay. Phytopathology. 1994, Vol. 84, No. 10, page 1087, see entire abstract.	1, 5-10, 13-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/01466

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 11 and 17
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The computer readable form of the Sequence Listing contained errors and could not be entered into the database. Claims 11 and 17 were searched in part, based on a search of SEQ ID NOs 6-7 from U.S. priority application no. 09/241,427.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/01466

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

USPT, JPAB, EPAB, DWPI, MEDLINE, AGRICOLA, CAPLUS, LIFESCI, SCISEARCH, EMBASE, BIOSIS, GENEMBL, GENESEQ 36, EST, ISSUED, PENDING
search terms: Alternaria, rRNA, rDNA, 5,8s, PCR, amplification, hybridization, detection, food, tomato, grain, spice, powder, mash, kits; SEQ ID NOs 1-7 of U.S. application no. 09/241,427