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Molecular Padlock Assay of Crude Plant Leaf Extracts for Detection of Listeria Monocytogenes

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MOLECULAR PADLOCK ASSAY OF CRUDE PLANT LEAF EXTRACTS FOR DETECTION OF *LISTERIA MONOCYTOGENES*

By

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An Abstract of the Thesis Presented in
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A molecular padlock assay was developed and assessed for detection of *Listeria monocytogenes* that operated in crude plant extracts. The molecular padlock assay was developed by Liu et al. (1996) and modified by Lizardi et al. (1998). We further modified and described a padlock probe that detected *L. monocytogenes* oligonucleotide, cDNA and genomic DNA containing a 16S rRNA sequence (GenBank Acc. No. X56153). This technique was effective in the presence of crude potato leaf extracts in contrast to PCR, which failed to detect the presence of *L. monocytogenes* targets in crude leaf extracts. Sensitivity of the padlock procedure was determined to be 0.02 ng using *L. monocytogenes* genomic DNA target templates and 0.0025 nM *L. monocytogenes* 40 nt oligonucleotide target sequences in an aqueous solution. Results also showed the efficacy of molecular padlocks to detect *L. monocytogenes* pathogens in a 5 μg potato leaf RNA background and crude leaf extracts, although sensitivity of the assay is insufficient to dispense with a pre-enrichment step for reliable detection of *L. monocytogenes*. The advantage of the molecular padlock assay over other FDA approved

serological and molecular-based assays, is that it may be possible to design the assay to be species or strain specific while still retaining the ability to be performed in crude plant extracts.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Listeria monocytogenes

L. monocytogenes is a food-borne pathogen that can survive and multiply in fresh and refrigerated foods. *L. monocytogenes* is a Gram positive bacteria. It is an intracellular parasite that binds to specific receptors on the surface of epithelial cells lining the intestinal walls as well as macrophage cells. Once bound to the receptors, *L. monocytogenes* invades and replicates inside the cell using several virulence factors. The bacteria invade the cell in order to evade the host immune system. Listerolysin O, a virulence factor, is used by *L. monocytogenes* to degrade the host cell phagosome membrane which allows the spread of the bacteria into the host cytosol. ActA, another virulence factor, plays a pivotal role in intracellular motility and virulence. ActA mutants were capable of escaping into the host cytoplasm but could not move intracellularly and accumulated as microcolonies within a host cell (Vasquez-Boland et al. 2001).

L. monocytogenes, the primary pathogenic species among the *Listeria* species, has thirteen serovars. Of these thirteen serotypes, serotypes 1 through 4 are the most commonly isolated from patients suffering from listeriosis (Jay, 2000). The thirteen serotypes are characterized by the possession of unique antigens. These unique antigens are determined using antisera raised against somatic and flagellar *Listeria* antigens in rabbits (Hofer et al. 2000).

Contamination by *L. monocytogenes* in food products is of great concern because infection causes meningitis, rhomboencephalitis and septicemia, and can lead to death in 25-30% of infected individuals (FSIS and USDA, 1999). Most affected by listeriosis are

pregnant women, newborns, and individuals with compromised immune systems. Symptoms of listeriosis can take from one to three weeks to appear in an infected individual (FSIS and USDA, 1999). Treatment for all affected individuals is a combination of antibiotics such as rifampicin and ampicillin, however death can still occur even with early treatment in compromised individuals because compromised hosts are more difficult to treat than hosts with competent immune systems.

L. monocytogenes can survive a wide range of environments which includes temperature ranges of 1 to 45°C and pH ranges of 4.1 to 9.6 and also have a long incubation period. This particularly places pre-cut fruits and vegetables (Johannessen et al. 2002), milk products (De Buyser et al 2001) and seafood (Jemmi et al 2002) and ready-to-eat foods at particular risk. *L. monocytogenes* is also widely spread in the environment and can be found on decaying vegetation, soils, animal feces, sewage and water. It can also be carried in animal intestines without affecting the host. The host can then shed the *L. monocytogenes* to meat and dairy products that are produced by the infected animals.

Thorough cooking of foods and refrigerating or freezing perishables within 2 hours of purchase can help to control the numbers of *L. monocytogenes* that may re-contaminate food products (FSIS and USDA, 1999). Most outbreaks of listeriosis are associated with ready-to-eat foods that become contaminated with *L. monocytogenes* after processing within the processing plant or after leaving the processing plant. The most recent outbreak of listeriosis was in 2002 with 46 confirmed cases, 7 deaths, and 3 stillbirths or miscarriages among the eight states that were affected by the outbreak. The multi-state outbreak was linked to individuals who ate sliceable turkey deli meat and

resulted in a nationwide recall of 27.4 million pounds of fresh and frozen ready-to-eat turkey and chicken products in October of 2002 (USDA, 2002).

The United States Department of Agriculture, Food Safety and Inspection Service and Food and Drug Administration oversees the safety of all food products. These agencies consider *L. monocytogenes* an adulterant bacterium and maintain a zero tolerance policy against it. Zero tolerance is defined as the absence of the organism in 25 gram samples. If foods are found to contain *L. monocytogenes* then those products are subject to recall and/or seizure (Jay, 2000). The United States has the most stringent policy governing *L. monocytogenes* in the world. Other countries have established legal limits in the numbers of organisms that are permissible in foods as compared to establishing a zero tolerance policy against *L. monocytogenes*.

Methods of Detection

Current methods of detecting *L. monocytogenes* include selective culture enrichment assays, ELISA assays, and PCR. The primary method of detection of *L. monocytogenes* is the selective culture enrichment assay which can isolate and enumerate viable bacteria starting with crude food extracts (Figure 1). The test material suspected of contamination with *L. monocytogenes* is first mixed with enrichment broth and incubated for 4 hours at 30°C (U.S. Food and Drug Administration, 2002). Selective agents such as acriflavin, nalidixic acid and cycloheximide are added and incubated for two days at 30°C. These selective agents are added because they inhibit the growth of anything other than *L. monocytogenes*. Acriflavine inhibits RNA synthesis and inhibits the growth of Gram positive cocci (Beumer et al. (2003). Nalidixic acid inhibits the

synthesis of DNA and suppresses the growth of Gram negative bacteria. Cycloheximide inhibits protein synthesis in eukaryotic cells which prevents the growth of most yeasts and molds. After incubation, the cultures are then streaked onto oxford agar (OXA), polymyxin acriflavine lithium chloride-ceftazidime agar modified (PALCAM) and lithium chloride phenylethanol moxalactam (LPM) agar plates. The plates are then examined for suspect colonies. On LPM agar plates, suspect colonies appear sparkling blue or white under a beamed white light. *L. monocytogenes* appear to have black haloes on OXA and PALCAM agar plates. Selective culture enrichment has been used primarily because it can be used to test food extracts, however, it is a time consuming process, taking up to 5 to 14 days to complete (Curtis et al 1995, Klein et al. 1997). Another disadvantage of selective culture enrichment assays is that it can be difficult to isolate pathogenic *L. monocytogenes* isolates because other *Listeria* spp. can grow together with *L. monocytogenes* (Cocolin et al. 2002).

In recent years, molecular-based and nucleic acid- based techniques have been developed to allow for more rapid detection of *L. monocytogenes.* Development of less time consuming assays makes it easier to detect non-persistent pathogens which are often found in very low concentrations in the host. However, like the selective culture enrichment assay, all methods have their own disadvantages. Enzyme-linked immunosorbent assay (ELISA) is one such diagnostic assay that is quicker than the selective culture enrichment assay (Figure 2). This immunological assay involves coupling enzymes to antigens or antibodies specific to a certain pathogen. The reaction between the antibody and antigen is visualized by the addition of a substrate that produces a colorimetric product, yielding either quantitative or qualitative results.

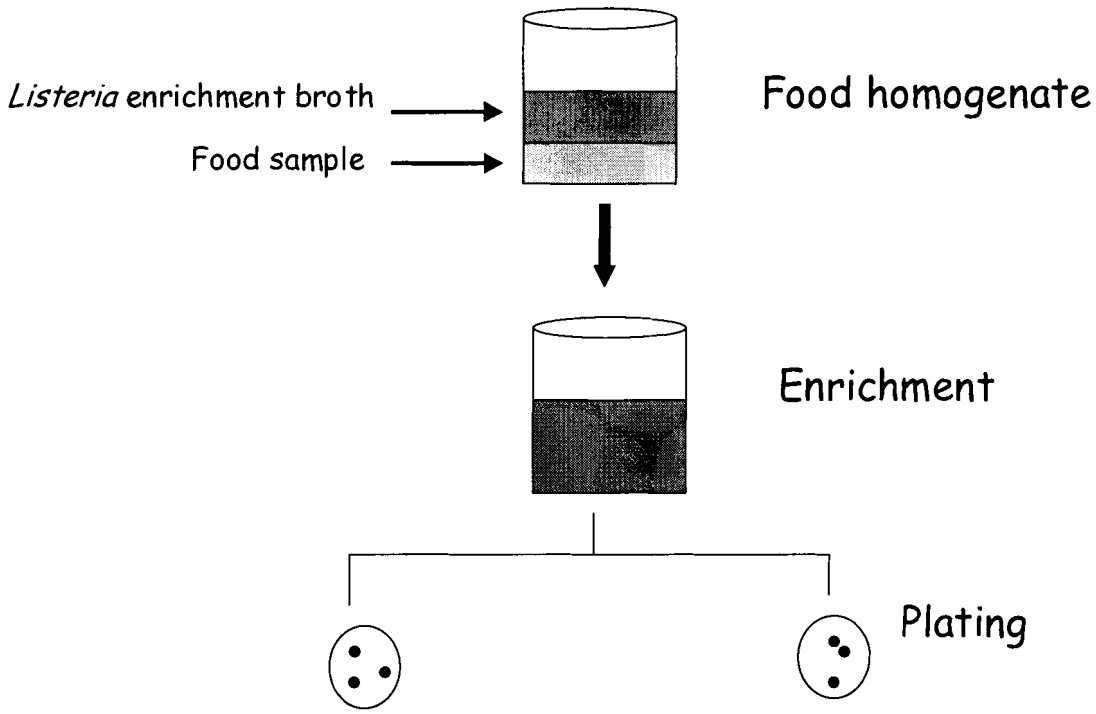
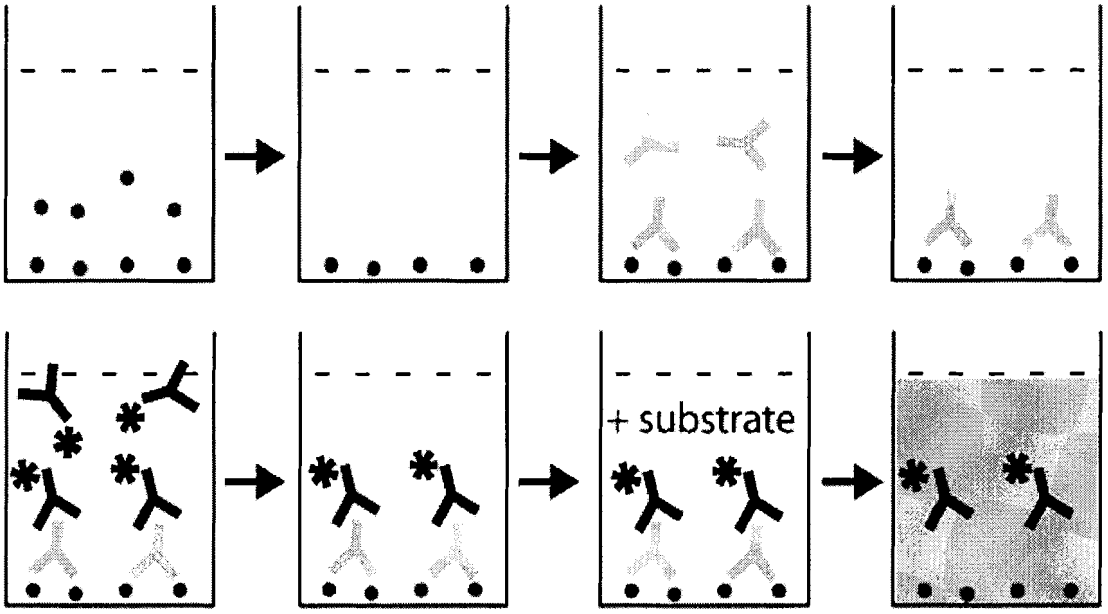


Figure 1. Diagram of selective culture enrichment. The food extract suspected of contamination with *L. monocytogenes* is mixed with enrichment broth for several hours. After which selective agents are added to inhibit the growth of anything besides *L. monocytogenes*. The broth is then streaked onto agar plates and colonies are visually inspected to determine if *L. monocytogenes* is present.



http://webmed.unipv.it/immunology/elisa.jpg

Figure 2. ELISA protocol. A specific antigen or antibody is coated on the bottom of a microtiter plate. Suspected sample is added and if the pathogen is present, it will bind to the antibody or antigen on the bottom of the well. A specific secondary antibody coupled to an enzyme is added to the wells. A substrate specific for the enzyme is added that allows for both visual and quantitative results.

ELISA assays also can be performed using crude food extracts but are not capable of distinguishing between *L. monocytogenes* and other *Listeria sp.* (Curiale et al 1994). However, recently Palumbo et al. (2003) developed an ELISA assay to detect the different serotypes of *L. monocytogenes* using monovalent and polyvalent antisera raised against somatic and flagellar antigens which are involved in virulence.

Polymerase chain reaction (PCR) is used to make large quantities of a nucleic acid sequence. PCR consists of three major steps: denaturation, annealing and extension or elongation (Figure 3) which allows for amplification of specific target sequences. PCR can discriminate between isolates differing in nucleic acid sequence which provides a high degree of specificity and is used for identification of food-borne pathogens. However, PCR assays are inhibited by components in crude food extracts, particularly those of fresh leaves called polyphenolics (Singh et al. 1998, Singh et al. 2002). Polyphenolics occur in different concentrations in leaves, bark and fruit of plants. Polyphenolics can form complexes with nucleic acids which causes the inhibition of molecular-based assays (Koonjul et al. 1999).

PCR can cause false positives even using partially purified nucleic acids due to the presence of DNA from nonviable cells (Starbuck et al 1992) because PCR cannot differentiate between viable and nonviable cells. Reverse-transcriptase PCR, which can differentiate between nonviable and viable cells because it targets RNA, has also been used for confirmation of *L. monocytogenes* (Klein and Juneia, 1997).

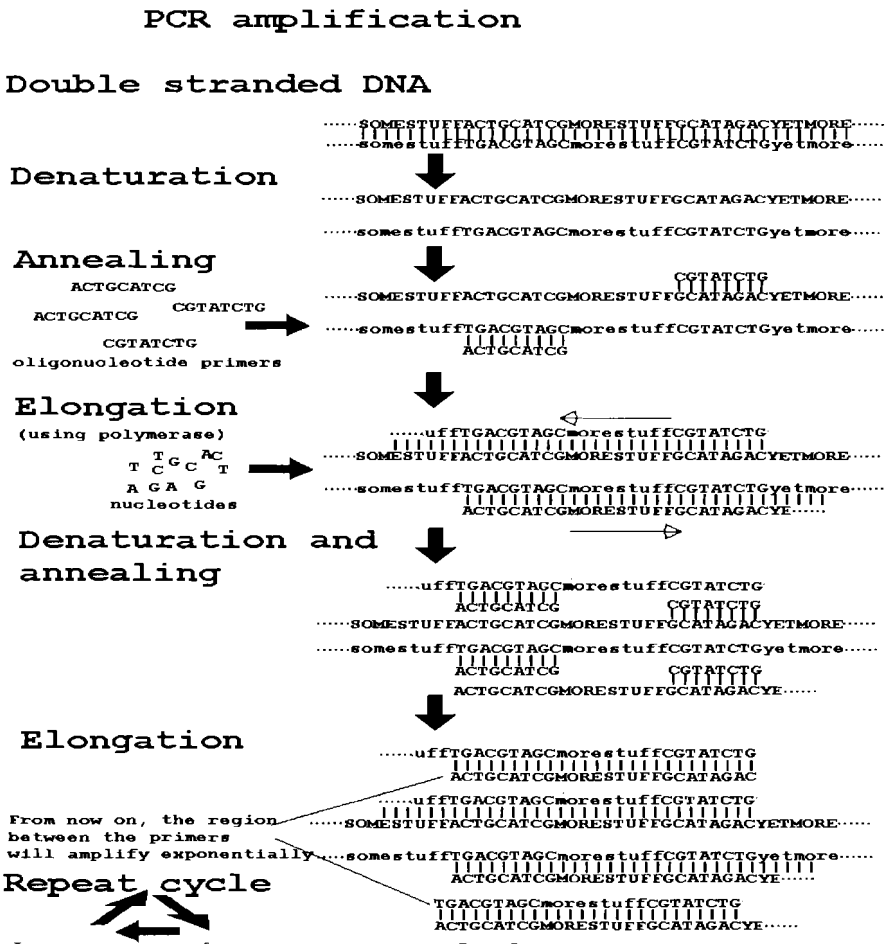


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Figure 3. Scheme for PCR amplification. The double stranded DNA is denatured which separates the strands. A specific primer hydrogen bonds to a target sequence on the template during annealing. The polymerase attaches to the annealed primer and starts copying the template during elongation. The polymerase adds dNTP's which are complementary to the template from 5' to 3', reading the template from 3' to 5'. The cycle is then repeated between 30 and 40 times to exponentially increase the number of templates.

Other nucleic acid-based techniques beyond RT-PCR and PCR have also been developed in recent years. Nucleic acid sequence-based amplification (NASBA) was developed for the detection of *L. monocytogenes* RNA and allows the specific detection of viable cells. NASBA uses two specific primers, one whose 3' end is complementary to the 3' end of the target sequence and the 5' end contains a promoter sequence that is recognized by T7 RNA polymerase (Figure 4). The second primer is complementary to the 5' end of the target sequence. NASBA assays are made of two phases, the non-cyclic phase and the cyclic phase. During the non-cyclic phase, the first primer anneals to the 3' end of the RNA target sequence. Avian myeloblastosis virus (AMV) reverse transcriptase extends the 3' end of the primer forming a cDNA copy of the template. RNase H is used to hydrolyze the RNA from the cDNA which is an RNA/DNA hybrid leaving only the single-stranded DNA copy. Reverse transcriptase synthesizes a second DNA strand creating a double-stranded promoter region. A third enzyme, T7 RNA polymerase transcribes RNA copies from the transcriptionally-active promoter, which can then be used as template for reverse-transcriptase during the cyclic phase. During the cyclic phase, the second primer binds to the template and is extended by reverse-transcriptase. This creates another RNA/DNA hybrid and the non-cyclic phase can begin again (Compton, 1991).

NASBA assays can confirm *L. monocytogenes* down to species level and only takes 3 days to generate results as compared to culture enrichment assays. Uyttendaele et al. (1995) designed a NASBA assay to detect *L. monocytogenes* 16S rRNA sequences. The detection limit of the NASBA assay was determined to be 10^6 colony forming units (cfu) per ml of cell suspension. Blais et al. (1997), also devised a NASBA assay, which

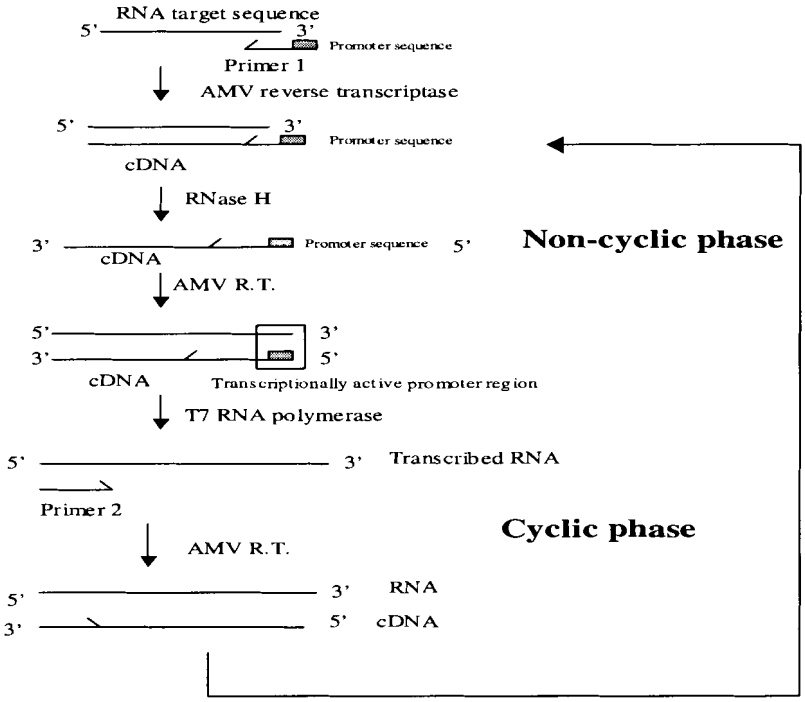

Figure 4. Scheme for the NASBA assay. NASBA uses two specific primers, one whose 3' end is complementary to the 3' end of the target sequence and the 5' end contains a promoter sequence that is recognized by T7 RNA polymerase. The second primer is complementary to the 5' end of the target sequence. During the non-cyclic phase, the first primer anneals to the 3' end of the RNA target sequence. AMV reverse transcriptase extends the 3' end of the primer forming a cDNA copy of the template. RNase H hydrolyzes the RNA from the cDNA. Reverse transcriptase synthesizes a second DNA strand creating a double-stranded promoter region. T7 RNA polymerase transcribes RNA copies from the transcriptionally-active promoter, which can then be used as template for reverse-transcriptase during the cyclic phase. During the cyclic phase, the second primer binds to the template and is extended by reverse-transcriptase. This creates another RNA/DNA hybrid and the non-cyclic phase begins again.

targets *L. monocytogenes* inducible hlyA mRNA. They found that the NASBA assay could detect *L. monocytogenes* cells that were inoculated into dairy and egg products and with a pre-enrichment step down to 0.2 cfu per gram and that the assay correctly identified 92.6% of inoculated samples.

An alternative to these other rapid detection methods is the molecular padlock assay developed by Liu et al. (1996) and modified by Lizardi et al. (1998). Lizardi et al. (1998) used rolling circle amplification (RCA) and hyperbranched RCA to detect point mutations in human genomic DNA. The molecular padlock assay is a nucleic acid-based technique that employs isothermal rolling circle amplification using a single oligonucleotide molecule whose termini are complementary to a target sequence. RCA generates concatemerized circles in a linear accumulation of product. Hyperbranched RCA uses a second primer with a sequence identical to a part of the circle which amplifies product exponentially producing double-stranded products. The strategy for the molecular padlock assay is shown in Figure 5. The 5'- and 3'- termini of the padlock probe were designed to hybridize to the antisense strand of a contiguous 40 nt sequence of the *L. monocytogenes* 16S rRNA gene (GenBank Accession No. X56153). A rRNA sequence was chosen as target because it has a high copy number in the cell. Hybridization of the padlock termini with an authentic target sequence allowed the padlock to be circularized by ligation. Addition of the RCA primer allowed rolling circle amplification to proceed around the ligated padlock. Presence of the HB primer allowed RCA amplified product to be converted to double stranded DNA that was detected by the intercalating fluorescent dye Picogreen. The purpose of the present study was to

determine whether the molecular padlock assay could be used to assess a crude plant extract for the presence of *L. monocytogenes* DNA targets.

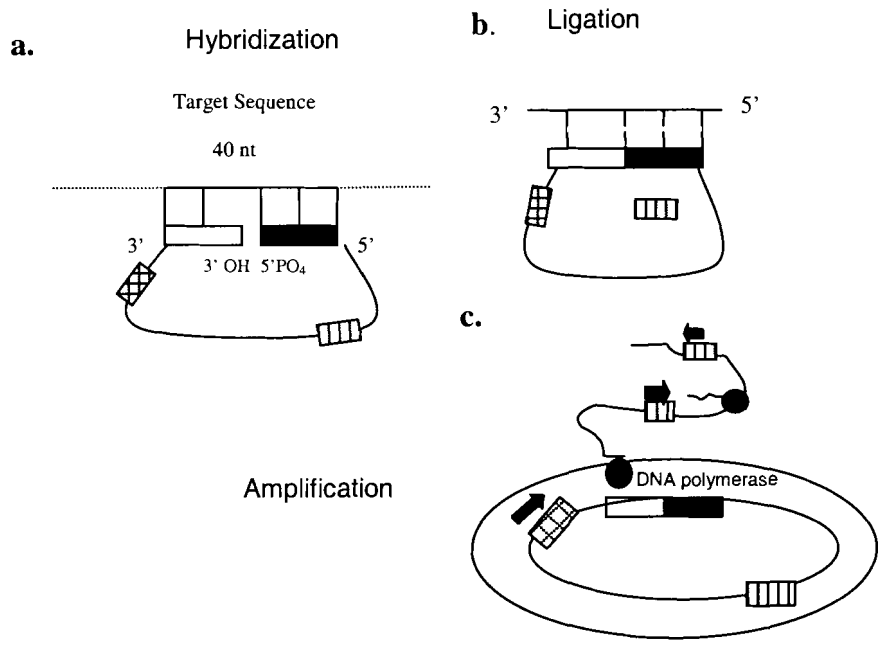

Figure 5. Strategy for molecular padlock amplification. **a.** Termini of the padlock hybridizes to a 40 nt sequence found within *L. monocytogenes* nucleic acid, bringing the 5' PO_4 and 3' OH ends of the padlock arms in juxtaposition. **b.** The 5' and 3' ends are covalently joined by ligation which results in a circular molecular padlock. **c.** The RCA primer binds to the RCA binding site in the molecular padlock and DNA polymerase proceeds around the circle creating a concatamer strand. The HB primers create double-stranded DNA product by priming opposite strand replication. Double-stranded DNA product is detected by intercalation of fluorescent dye, Picogreen.

METHODS AND MATERIALS

Preparation of Target Templates

An oligonucleotide target 5'-gcttcgcgacccttttgtactatccattgtagcacgtgtgt-3', complementary to nucleotides 1228 to 1268 of the *L. monocytogenes* 16S rRNA sequence (GenBank Acc. No. X56153), was synthesized by Sigma Genosys (Woodlands, TX). *L. monocytogenes* culture was provided by Dr. Alfred Bushway, Food Science and Human Nutrition, University of Maine, who originated the culture from a salmon meat contaminant. Total RNA was isolated from 250 µl of 1 x 10^7 cells/ml of *L. monocytogenes* packed cell lysate or 500 mg of potato leaf using either the Trizol protocol (Invitrogen, Carlsbad, CA) or the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was prepared from total RNA using 200 U Superscript II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA) and the 10 µM gene-specific complementary strand primer, 5'-attccggcttcatgtaggcgagtt-3' (GenBank Acc. No.X56153, nucleotides 1321-1344) designated Lmono16S2, by incubation in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 0.02 mM DTT, 200 µM dNTP's, 0.01M DTT at 42°C for 1 h.

A double-stranded target DNA was obtained by PCR using 0.2 µM of the *L. monocytogenes* gene-specific primers Lmono16S1, 5'-caacgagcgcaaccct-3' (GenBank Acc. No. X56153, nucleotides 1105-1120) and Lmono16S2 (see above sequence). PCR amplifications were performed in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.6 mM $MgCl_2$, 0.01% gelatin, 0.2 µM dNTP's, and 5 U Taq DNA Polymerase (Promega, Madison, WI) with 35 cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 1 min and

elongation at 72°C for 1 min. The 240 bp PCR product was isolated from agarose gels using the Qiagen Gel Extraction Kit according to the manufacturer's protocol, eluted with 30 µl dH_2O, and stored at -20°C. Identity of PCR products was confirmed by sequencing at the University of Maine DNA Sequencing Core Facility. *L. monocytogenes* genomic DNA was isolated using the Trizol protocol (Invitrogen, Carlsbad, CA), digested with *EcoRI,* then ethanol precipitated.

Potato Tissue Extraction

Potato leaf samples were collected from plots at Aroostook Farm in Presque Isle, ME in July 2000, flash frozen in liquid nitrogen, and stored at –80°C until use. Crude leaf lysates were prepared from 500 mg of potato leaf ground with a drill and centrifuged at 14,000 x g for 5 minutes. 200 µl of the supernatant was diluted in 800 µL of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 200 U RNase Inhibitor (Promega, Madison, WI), 1.2% citric acid, and 0.1% 2-mercaptoethanol (Sigma, St. Louis, MO) in a mortar and pestle. Leaf lysate was incubated at 37°C for 1 h, followed by a denaturation at 90°C for 10 minutes. Potato sap was clarified by centrifugation for 5 min at 14,000-x g at 4°C and the supernatant was stored at –80°C.

Molecular Padlock Assay

The molecular padlock assay is illustrated in Figure 5. A consensus sequence alignment was used to compare the target *L. monocytogenes* sequence (GenBank Acc. No. X56153) to other *Listeria* species nucleotide sequences. The alignment was used to

choose a padlock sequence at which a single nucleotide difference was built into the 5' arm of the padlock that would differentiate the *L. monocytogenes* target sequence from the other *Listeria sp.* sequences which provided species specificity. The alignment was done using the *L. monocytogenes* target sequence (GenBank Acc. No. X56153, nucleotides 1228-1268), *L. seeligeri* (GenBank Acc. No. X56148, nucleotides 1227-1267), *L. welshimeri* (GenBank Acc. No. X56149, nucleotides 1226-1266), *L. grayi* (GenBank Acc. No. X56150, nucleotides 1229-1269), *L. ivanovii* (GenBank Acc. No. X56151, nucleotides 1227-1267), *L. innocua* (GenBank Acc. No. X56152, nucleotides 1228-1268) and *L. murrayi* (GenBank Acc. No. X56154, nucleotides 1230-1270). The single nucleotide difference is located at the 3' end of the padlock sequence (GenBank Acc. No., nucleotide 1248) in which the *L. monocytogenes* target sequence has an A while the other *Listeria sp.* contain a G. The 5' and 3' terminal sequences of the sense padlock, 5'-agtacaaagggtcgcgaagcgactgcatggtcactcttctgccgattaaaaaatttggccggtctttaatcacacgtgctacaatg g-3' spanning nucleotides 1228 to 1268, were designed to hybridize to the antisense strand of 16S rRNA sequence of *L. monocytogenes* (GenBank Acc. No. X56153). Ligation was performed using 250 nM oligonucleotide target (5'-gcttcgcgacccttgtactatccattgtagcacgtgtgt-3'), or 2 μg cDNA, PCR product or genomic DNA with 250 nM 16S padlock, and 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, BSA, 25 μg/ml, pH 7.5) in a 25 μl reaction. 6.25 μl the prepared potato leaf extract was added to the ligation reaction for crude lysate experimentation. The reaction was denatured at 90°C for 10 min, cooled to 55°C for 2 min, followed by immediate addition of 400 U T4 DNA Ligase (New England Biolabs, Beverly, MA) and ligation at

37°for 1 h. Reactions were terminated by denaturation at 90°C for 10 min. Unligated material was degraded by addition of 20 U RNase A (Sigma, St. Louis, MO), 20 U exonuclease I (New England Biolabs, Beverly, MA), and 50 U exonuclease III (Promega, Madison, WI) in exonuclease III buffer at a final concentration of 33 mM Tris-acetate, 10 μM magnesium-acetate, 10 μM potassium-acetate, 50 μM DTT, 1 mg/mL BSA at a final reaction volume of 50 μl, incubated at 37°C for 30 min and 90°C for 10 min. Isothermal amplification was performed by addition of the rolling circle primer (RCA) 5'-acgaagagtgaccatgca-3' and the hyperbranching primer (HB) 5'-cggtctttaacacacg-3' directed to sites internal to the padlock probe, as indicated in Figure 5. Each 75 μl RCA reaction contained 1 μM RCA primer, 1 μM HB primer, 200 μM dNTP (Invitrogen, Carlsbad, CA), 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 7.5 mM DTT and the 50 μl exonuclease reaction. Amplification reactions were initiated by addition of T4 Gene 32 Protein, 40 μg/ml (Ambion, Austin, TX) and 5 U *E. coli* polymerase I Klenow fragment (New England Biolabs, Beverly, MA) and incubated at 37°C for 1 h. Reaction products were passed through Qiaquik PCR Purification Kit (Qiagen, Valencia, CA) filters to remove input oligonucleotides, according to the manufacturer's protocol. Padlock amplification products were analyzed qualitatively using agarose gels and quantitatively with Picogreen (Molecular Probes, Eugene, OR) using a Fusion Universal Microplate Analyzer (Packard Bioscience, Wellesley, MA). Molecular padlock assay products were measured in triplicate. A reaction was considered to be positive when it exhibited 3-fold higher emission at 520 nM than no target controls.

RESULTS

Modifications to the Procedure

The original protocol by Lizardi et al. (1998) was modified to optimize the procedure for use as a single tube assay that could easily be performed by technicians in working environments. Each step of the protocol, ligation, exonuclease degradation and amplification, had to be optimized and work correctly before combining the three separate reactions into a single tube. The ligation reaction was modified by substituting T4 DNA Ligase (NEB) for Ampligase (Epicentre). This substitution was made because T4 DNA Ligase catalyzes the formation of a phosphodiester bond between a 5' phosphate and a 3' hydroxyl termini in duplex DNA or RNA or DNA/RNA hybrids (Nilsson et al. 2001) whereas Ampligase only catalyzes the ligation of phosphate bonds in duplex DNA. The switch to T4 DNA ligase would allow the molecular padlock assay to be used to target either RNA or DNA sequences, making the molecular padlock assay a more versatile technique.

The exonuclease reaction was optimized using a combination of three exonuclease enzymes: exonuclease I (NEB), exonuclease III (Promega) and RNase A (Qiagen). Exonuclease digestion was used to remove the single-stranded molecules present in the molecular padlock assay. This modification of the original procedure (Lizardi et al. 1998) was essential to remove background resulting from input primers and non-target nucleic acids in both an aqueous solution and a potato leaf lysate. Ligated padlocks were protected from exonuclease digestion by the absence of a free 3' end. Thus, removal of the single-stranded molecules eliminated non-specific amplification

from unligated padlocks that did not hybridize to authentic target molecules (Zhang et al. 1998) and lowered background from input DNA target. Baner et al. (1998) reported that exonuclease digestion enhanced rolling circle amplification by removing target molecule after ligation and facilitating polymerase entry.

To demonstrate the necessity of the combination of different exonuclease enzymes in degradation of input molecules, 5 μg of potato leaf RNA and 250 nM of *L. monocytogenes* 16S rRNA padlock were degraded with each enzyme and a combination of all three enzymes at 37°C for 1 hour and results were determined using Picogreen fluorescence (Figure 6) in a single trial. Exonuclease I catalyzes the removal of nucleotides from single-stranded DNA from free 3' hydroxyl termini. It reduced potato leaf RNA 9-fold compared to fluorescence results from the undegraded material. Exonuclease III also catalyzes the removal of nucleotides from free 3' hydroxyl termini, is double-strand specific and reduced background 4-fold compared to undegraded potato leaf RNA. RNase A hydrolyzes phosphodiester bonds at the 3' termini adjacent to pyrimidine residues on single-stranded RNA. It reduced the background 4-fold compared to undegraded material. Each exonuclease separately reduced the RNA background but together the three enzymes degraded the background 13-fold compared to fluorescence results from undegraded nucleic acids. These results demonstrated the ability to reduce input background molecules and the need to use three separate exonucleases to achieve optimal degradation.

Picogreen fluorescence results further demonstrated that the padlock procedure, originally designed (Liu et al. 1996, Lizardi et al. 1998) to use Φ29 DNA polymerase,

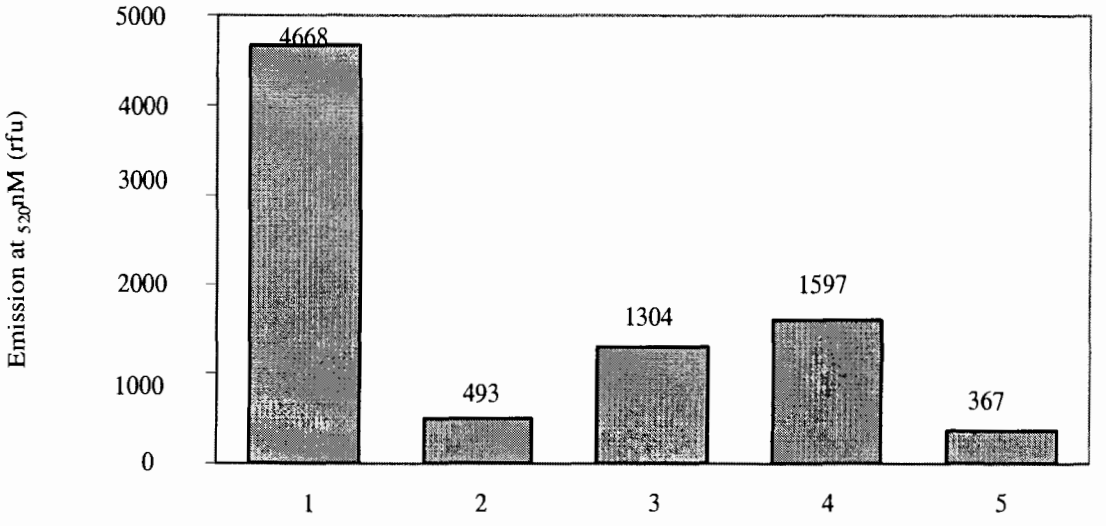

Figure 6. Exonuclease degradation of potato leaf RNA and *L. monocytogenes* padlock. Picogreen results were determined for potato leaf RNA and padlock alone (1). Potato leaf RNA and padlock were then degraded with exonuclease I (2), exonuclease III (3), RNase A (4) and a combination of all three exonucleases (5). rfu: relative fluorescent units.

could operate effectively by using the Klenow fragment of *E. coli* DNA polymerase I (Fire et al. 1995, Baner et al. 1998, Liu et al. 1996). The Klenow fragment of *E. coli* DNA polymerase I catalyzes the transfer of nucleotides from to the 3' hydroxyl terminus of a DNA primer. Until recently only the Klenow fragment of *E. coli* DNA polymerase I was commercially available where as Φ29 DNA polymerase was not.

The final modification to the original procedure by Lizardi et al. (1998) to optimize and increase the sensitivity of detection by the molecular padlock assay was to purify the amplified padlock product using a PCR purification kit manufactured by Qiagen in a single trial. Purification of the amplified padlock products helped to eliminate input molecules such as primers from the background. This helped reduce the risk that Picogreen may bind non-specifically to the input molecules and increase product fluorescence. This is demonstrated by testing the molecular padlock with and without using the PCR purification kit in Figure 7. The molecular padlock assay was tested using a synthetic oligonucleotide target sequence and a padlock. Without purification, Picogreen fluorescence was 2-fold higher than with purification of amplified products. Background results were decreased with purification of no target and no padlock controls, with a 45-fold and an 84-fold reduction respectively in Picogreen fluorescence over the no target and no padlock controls that were not purified before Picogreen fluorescence.

After multiple trials of the three separate reactions to ensure that each reaction was working correctly with the modifications that were made, the separate reactions were combined into a single tube assay. This change was made to reduce the amount of time, money and materials needed for the assay. It also helped to get the molecular padlock

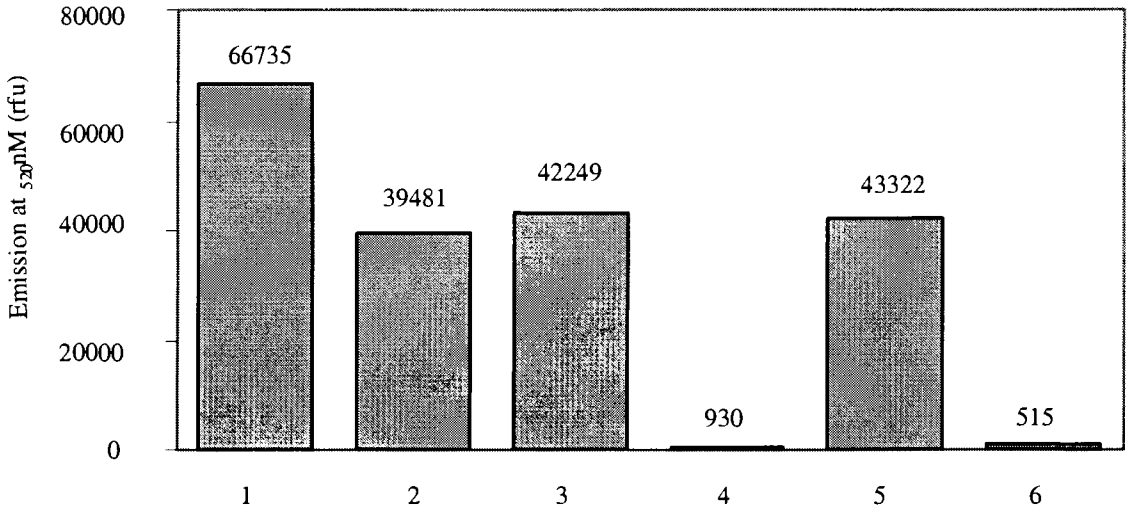

Figure 7. Effect of Qiagen kit during the molecular padlock assay. Picogreen results demonstrate the need for the PCR purification kit when the molecular padlock assay was tested with an oligonucleotide target sequence and not purified with the PCR purification kit (1) and with the Qiagen PCR purification kit (2) in a single trial. Background results were determined using no target that was not purified (3) and was purified (4) as well as no padlock without purification (5) and with purification (6). rfu: relative fluorescent units.

assay closer to the objective of making a molecular-based assay that could be used in working environments.

Detection of *L. monocytogenes* Target Sequences

The feasibility of the molecular padlock assay was first tested in an aqueous solution with a variety of target templates. Figure 8 illustrates that the sense molecular padlock efficiently recognized several *L. monocytogenes* nucleic acid target molecules. The padlock was tested with a 40 nt *L. monocytogenes* oligonucleotide target molecule to ensure that the padlock could detect the target sequence. The padlock detected 2 μg of *L. monocytogenes* first strand cDNA to demonstrate that the padlock could detect target sequence amongst other nucleic acid material. The padlock was shown to detect 50 ng PCR product to show that the padlock probe could detect the target sequence among double-stranded DNA molecules. It was also demonstrated that the padlock could recognize endogenous target sequence among 2 μg *L. monocytogenes* genomic DNA. Padlock amplification products were detected qualitatively by agarose gel electrophoresis (data not shown), or quantitatively by Picogreen fluorescence (Figure 8). Each of these targets yielded products detected by Picogreen fluorescence that was 100-fold over no target controls. By contrast, no product was evident when a non-target oligonucleotide sequence was provided (potato PVY target) to ensure that the padlock did not detect non-*L. monocytogenes* target. No product was also evident when either no target sequence or no padlock was provided.

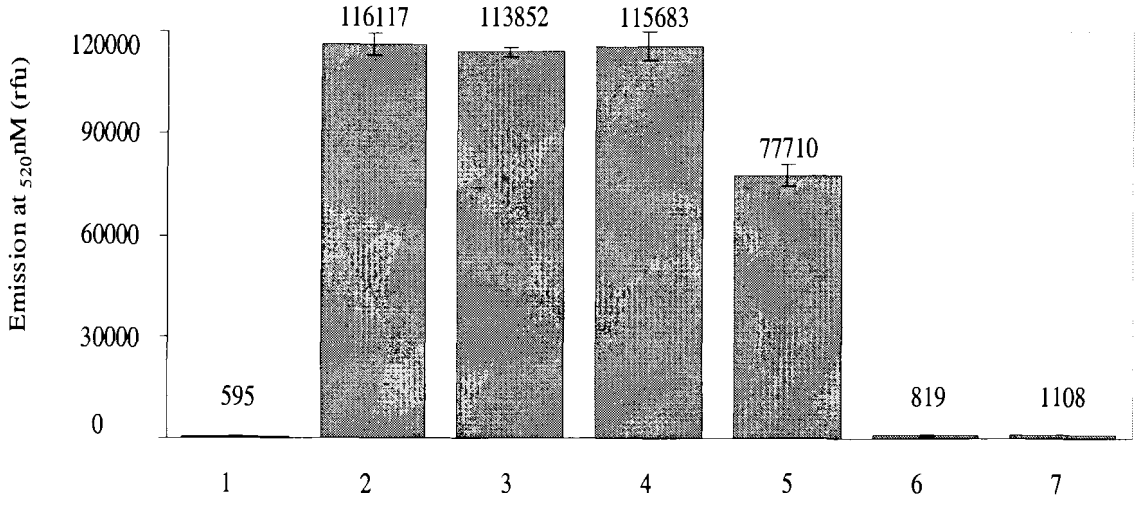

Figure 8. Ability of molecular padlock assay to detect *L. monocytogenes* nucleic acid targets in an aqueous solution. Nucleic acid targets used in the molecular padlock assay were 250 nM PVY oligonucleotide target (1), 250 nM *L. monocytogenes* oligonucleotide target (2), 2 μg *L. monocytogenes* cDNA (3), 50 ng double-stranded PCR product (4), and 2 μg genomic DNA (5). No target (6) and no padlock (7) controls were included in three independent trials to determine the background results for the molecular padlock assay. Results shown are the average of three independent trials and the standard error of means is indicated with error bars. rfu: relative fluorescent units.

A serial dilution of a *L. monocytogenes* 40 nt oligonucleotide target sequence was made in order to determine the threshold of the padlock for single-stranded target sequences in an aqueous solution in a single trial. Half of the amplified products were analyzed using Picogreen fluorescence (Figure 9a), while the other half was analyzed on a 1.5 % agarose gel stained with 10 mg/ml of ethidium bromide (Figure 9b). Results were linearly correlated with target concentration over the range of 250 nM to 0.0025 nM. Target concentrations greater than 250 nM yielded similar Picogreen fluorescence which indicates saturation of the assay (data not shown). However, target concentrations below 0.0025 nM yielded results that were 3-fold less than background results indicating the threshold of the assay (Figure 9a). Figure 9b demonstrates that quantification of the molecular padlock assay products by Picogreen fluorescence was found to be more useful than analysis by gel electrophoresis. A positive sample will appear as a smear during gel electrophoresis while no smear indicates a negative result.

Figure 9b demonstrates the ability of the molecular padlock to detect the oligonucleotide target sequence and indicated that the threshold was approximately 0.0025 nM.

A serial dilution of *L. monocytogenes* genomic DNA was made in order to determine the threshold of the padlock for an endogenous target sequence in an aqueous solution among double-stranded material. Figure 10 illustrates that the padlock assay could effectively detect *L. monocytogenes* target in 0.02 ng genomic DNA. Picogreen fluorescence was linearly correlated with target concentration over the range of 13.8 ng to 0.02 ng. Target concentrations greater than 13.8 ng yielded similar fluorescence indicating saturation of the assay (data not shown). Conversely, target concentrations

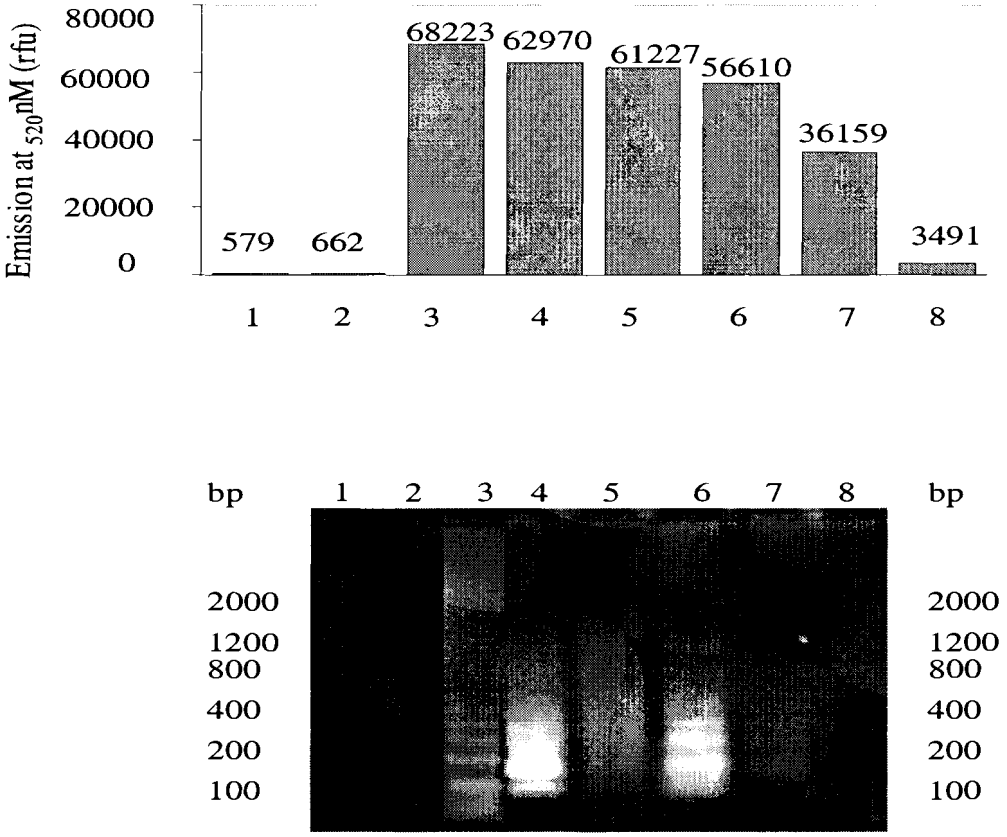

Figure 9. Detection limit of the molecular padlock assay using a *L. monocytogenes* 40 nt oligonucleotide target sequence. Background results were determined using no padlock (1) and no target (2). Theshold of the molecular padlock assay was determined using the following concentrations: 250 nM (3), 25 nM (4), 2.5 nM (5), 0.25 nM (6), 0.025 nM (7) and 0.0025 nM (8). Results were analyzed by **A.** Picogreen fluorescence and **B.** 1.5% agarose gel stained with 10 mg/ml ethidium bromide.

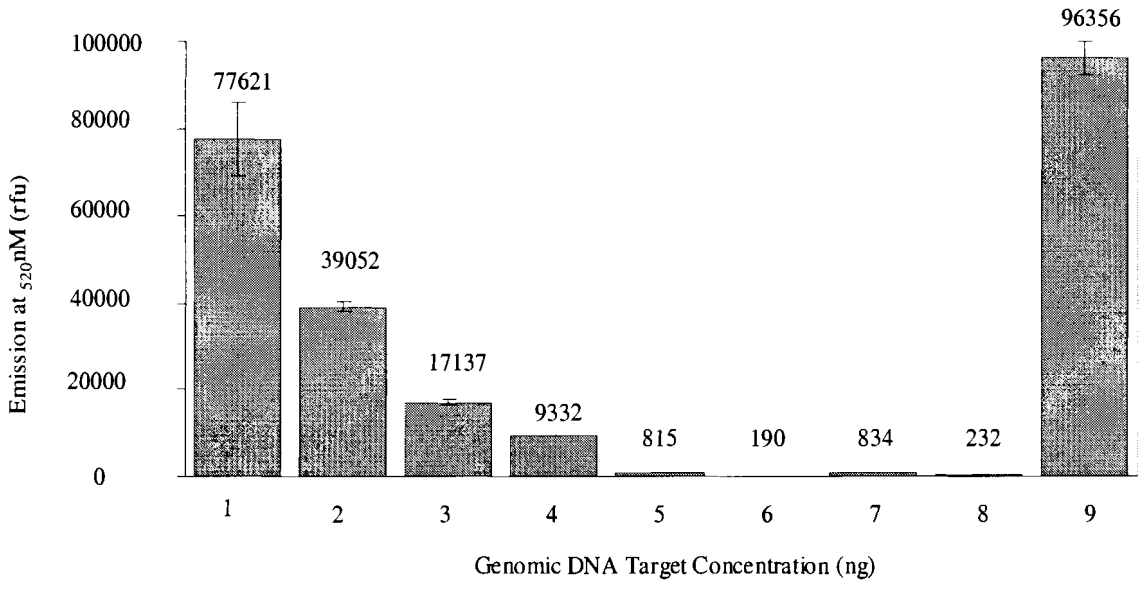

Figure 10. Detection limit of the molecular padlock assay using genomic DNA. Threshold of the molecular padlock assay was determined using the following concentrations of genomic DNA: 13.8 ng (1), 8.1 ng (2), 2.5 ng (3), 0.02 ng (4) and 0 ng (5) in a single trial. Background results for the trials were determined by including no padlock (6), no target (7), 250 nM PVY oligonucleotide (8), and 250 nM *L. monocytogenes* oligonucleotide target (9) controls. The error bars indicate the standard error of means and rfu is the relative fluorescent units.

less than 0.02 ng (3.7 x 10^6 molecules) provided a fluorescent signal that was less than 3-fold that of the no template control.

The molecular padlock assay was tested in a 5 μg potato leaf RNA background to determine if the molecular padlock assay could recognize and amplify target sequences among background molecules and to simulate detection of *L. monocytogenes* in food extracts (Figure 11). The padlock detected 2 μg of 16S first-strand cDNA with product fluorescence similar to that of 16S first-strand cDNA target in aqueous solution. This indicates that the molecular padlock assay could efficiently locate target sequence amongst the other nucleic acids present.

The molecular padlock assay also worked effectively when target sequences were presented in the form of a crude leaf extract, simulating detection of pathogen on leafy vegetables. Potato leaves have been routinely tested in the laboratory for detection of potato viruses and was readily available in the laboratory. Potato leaves cannot be ingested by humans because they are toxic, however this was just a simulation of detection in a crude lysate of a leafy vegetable. After multiple trials, target was detected in 6.25 μl, 2.5 μl, 1.25 μl and 0.5 μl of crude potato leaf extract (data not shown). Figure 12 shows that padlock products were amplified from *L. monocytogenes* targets in the presence of 6.25 μl of potato leaf extract. The results show that the padlock was not inhibited by components of the crude leaf lysate as product fluorescence was similar to that of oligonucleotide target in aqueous solution (Figure 12). No product was detected with the non-*L. monocytogenes* oligonucleotide target.

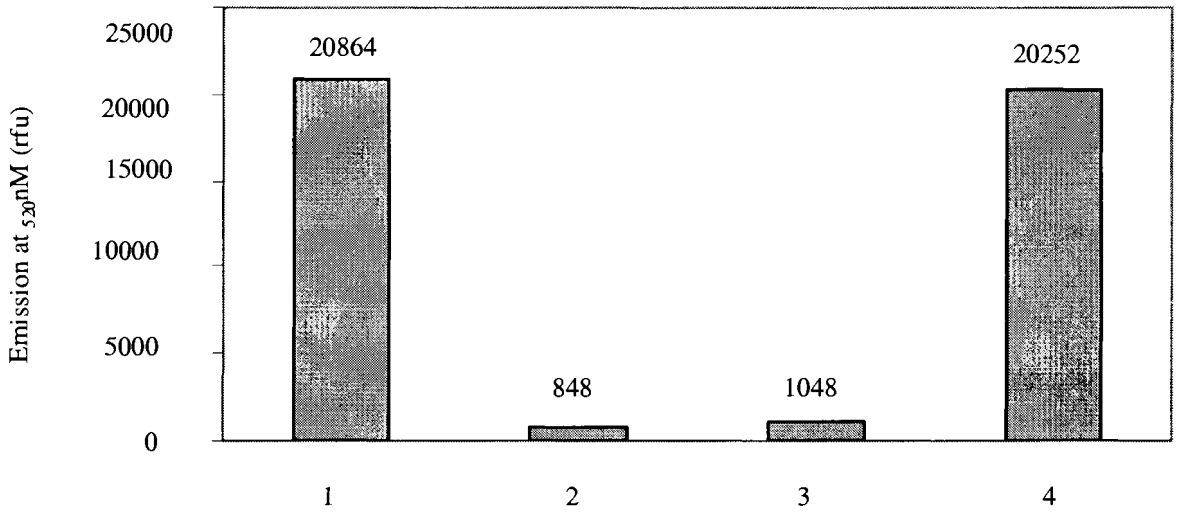

Figure 11. Ability of the padlock to detect nucleic acid target in potato leaf RNA. The molecular padlock assay was tested in a 5 μg potato leaf RNA background in a single trial. Nucleic acid target used in the molecular padlock assay was 2 μg of 16S first-strand cDNA (1). Background results were determined by testing with no target (2), and RNA only (3). Amplified products produced from 16S first-strand cDNA in an aqueous solution is shown in (4). rfu: relative fluorescent units.

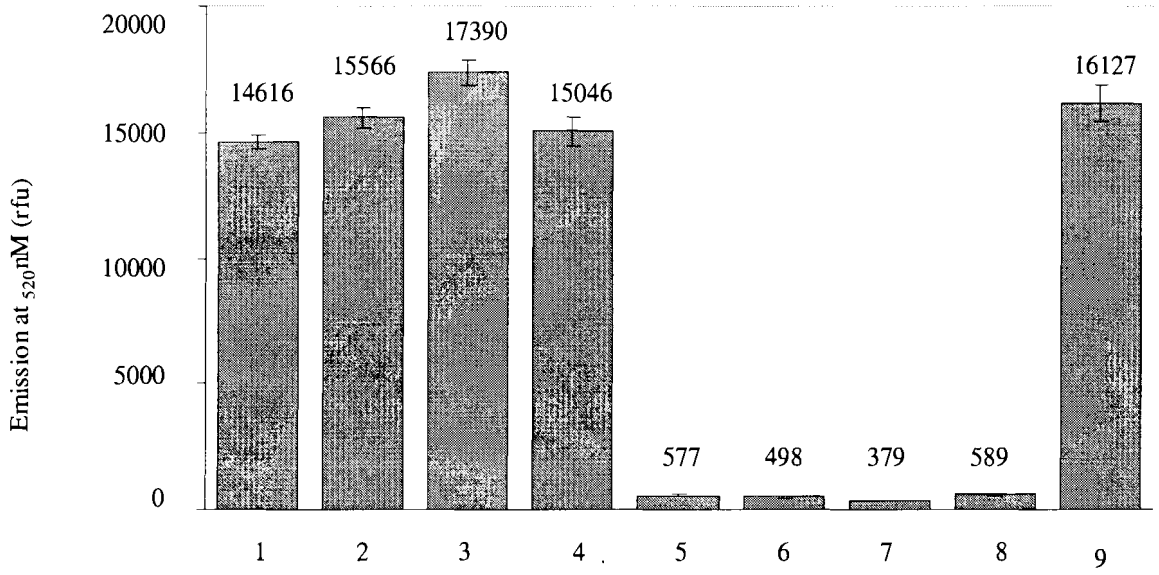

Figure 12. Ability of the padlock to detect *L. monocytogenes* nucleic acids in a crude leaf lysate. The molecular padlock assay was tested in 6.25 µl of potato leaf crude extract with nucleic acid targets for the padlock probe. Nucleic acid targets used in the molecular padlock assay in 6.25 µl potato leaf extract were 250 nM *L. monocytogenes* oligonucleotide target (1), 2 µg *L. monocytogenes* cDNA (2), 50 ng PCR product (3), and 2 µg *L. monocytogenes* genomic DNA (4). Background results of the molecular padlock assay were determined using lysate only (5), no target (6), and no padlock (7), and 250 nM PVY oligonucleotide target (8). Products produced from 250 nM *L. monocytogenes* oligonucleotide target in an aqueous solution is shown in (9). Results shown are the average of three independent trials and the error bars indicate the standard error of means, rfu: relative fluorescent units.

DISCUSSION

I have shown that the molecular padlock assay can be used to recognize and amplify 16S rRNA targets of *L. monocytogenes*. Rolling circle amplification has been used previously to amplify small DNA circles (Liu et al. 1996) and other padlock probes (Baner et al. 1998, Lizardi et al. 1998, Kuhn et al. 2002, Thomas et al. 1999) but has not been explored for the ability to operate in crude food extracts. I have demonstrated the ability of the molecular padlock to detect various *L. monocytogenes* target molecules in an aqueous solution (Figure 8), a 5 μg potato leaf RNA background (Figure 11), and a plant extract, a leaf lysate (Figure 12).

The detection limit of the padlock probe was determined to be 0.02 ng or 3.7 x 10^6 molecules of *L. monocytogenes* genomic DNA (Figure 10) or 0.0025 nM of a 40 nt *L. monocytogenes* oligonucleotide target sequence (Figure 9). The difference between the two thresholds may be due to the different target sizes used in the molecular padlock assay. The 40 nt oligonucleotide target molecules may be too small to recruit the T4 DNA ligase to the circularized padlock compared to the circularized padlock hybridized to the long strand of genomic DNA. This threshold indicates that the molecular padlock assay is less sensitive than RT-PCR at detecting purified nucleic acid targets but is as sensitive as the NASBA assays performed by Uyttendaele et al. (1995), who determined that the detection limit of the NASBA assay to be 10^6 cfu. The adavantage of the molecular padlock assay is the ability to conduct a nucleic-acid based diagnostic test in a crude plant extract. By contrast, phenolics and other components of crude plant extract severely inhibit PCR reactions (Singh et al. 1996; Singh et al. 1998; Singh et al. 2002).

I have also demonstrated the necessity of modifications made to the original protocol by Lizardi et al. (1998). The use of exonuclease degradation (Figure 6) and a Qiagen PCR purification kit (Figure 7) was necessary to remove input molecules from the molecular padlock assay. This helped to reduce background molecules that could undergo non-specific amplification. The ligation, exonuclease and amplification reactions of the molecular padlock assay were also combined to make a single tube assay. This reduces the amount of money, time and materials needed to run the molecular padlock assay. Molecular padlock assay products were analyzed by gel and Picogreen fluorescence (Figure 9), and in both cases, the sensitivity of either method of detection was improved by reducing background after the completion of the molecular padlock assay. Quantification of the molecular padlock assay products by Picogreen fluorescence was found to be more useful than analysis by gel electrophoresis which yielded a smear of reaction products.

Greater sensitivity might be achieved using molecular beacons (Tyagi and Kramer, 1996) to detect the amplified padlock (Figure 13). Molecular beacons fluoresce only after hybridization with their target and undergo a conformational change that spatially separates a fluorophore from a quenching residue (Leone et al. 1998). Molecular beacons included in a reaction mixture in which nucleic acids are synthesized allow the reaction progression to be followed in real time (Tyagi and Kramer, 1996). The ability to monitor the reaction progression in real time would allow a quantitative measurement of the number of circles used as template in the rolling circle amplification (Nilsson et al. 2002). Molecular beacons have also been used in conjunction with NASBA (Leone et al. 1998) and RCA (Nilsson et al. 2002) assays to generate fluorescent

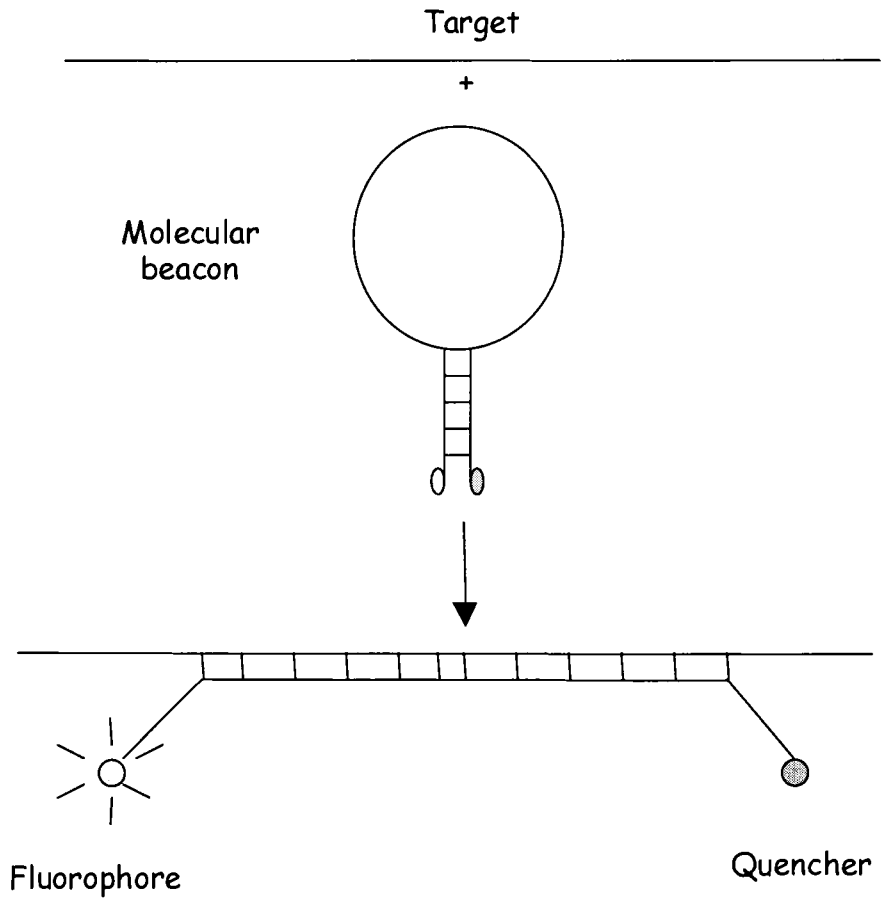

Figure 13. Scheme for molecular beacons. Molecular beacons fluoresce after hybridization with their target and undergo a confirmational change that causes the bound probe to fluoresce brightly.

signal that can be used for real-time monitoring during amplification. However the typical cost of molecular beacons is prohibitive for adoption of this technique for routine diagnostic testing. For new assays to be used for routine application, they should be rapid, sensitive, species-specific, reliable, easy to use and cost effective.

ELISA assays are a routine diagnostic tool for the detection of *L. monocytogenes*, and can be used in crude food extracts. However, most commercially available ELISA assays are generally sensitive only to the genus level and there is a limited availability and a high cost for commercially produced antisera for assays that can detect specific *Listeria* isolates. One advantage of the molecular padlock technique over the ELISA assay is that nucleic acid-based probes can be easily designed to detect specific serotype isolates of *L. monocytogenes*. However, Palumbo et al. (2003) also recently developed an ELISA assay that can be used to detect *L. monocytogenes* serotype specific isolates. There still remains the problem that polyvalent and monovalent antisera based on reactions of somatic and flagellar antigens have a limited commerical availability and a high cost. Padlock probes have already been used to differentiate single nucleotide differences (Nilsson et al. 1997, Lizardi et al. 1998). The requirement of precise base pairing at the ligation junction imparts high specificity of the padlock and allows the molecular padlock assay to detect pathogenic isolates of *Listeria* species.

The molecular padlock assay has the potential for use in the detection of *L. monocytogenes* in crude food extracts which provides quantitative results (Figure 11). I have demonstrated a new strategy for the detection of *Listeria* pathogens that is sensitive, quick and can be used in plant extracts. The detection limit of the padlock was established to be 0.02 ng of *L. monocytogenes* genomic DNA (Figure 10). I have also

demonstrated the ability of the molecular padlock to detect either *L. monocytogenes* cDNA or genomic DNA target molecules in crude leaf lysate backgrounds. The major advantage of the molecular padlock assay is that it can target a specific target sequence of a pathogenic *Listeria* isolate unlike current serological FDA testing methods that are used in crude extracts.

REFERENCES

Baner, J., Nilsson, M., Mendel-Hartvig, M., and Landegren, U. (1998) Signal amplification of padlock probes by rolling circle replication. *Nuc. Acids Res.* 22: 5073-5078.

Beumer, R.R., and Hazeleger, W.C. (2003) *Listeria monocytogenes*: diagnostic problems. *FEMS Immun. and Med. Microbiol.* 35: 191-197.

Blais, B.W., Turner, G., Sooknanan, R., and Malek, L.T. (1997) A nucleic acid sequence-based amplification system for detection of *Listeria monocytogenes hly A* sequences. *Appl. Environ. Microbiol.* 63: 310-313.

Cocolin, L., Rantsiou, K., Iacumin, L., Cantoni, C., and Comi, G. (2002) Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. *Appl. Environ. Microbiol.* 68: 6273-6282.

Compton, J. (1991) Nucleic acid sequence-based amplification. *Nature*. 350: 91-92.

Cook, N. (2003) The use of NASBA for the detection of microbial pathogens in food and environmental samples. *J. Virol. Methods.* 53: 165-174.

Curiale, M.S., Lepper, W., and Robison, B. (1994) Enzyme-linked immunoassay for detection of *Listeria monocytogenes* in dairy products, seafoods, and meats: collaborative study. *J. AOAC Int.* 77: 1472-1489.

Curtis, G.D.W., and Lee, W.H. (1995) Culture media and methods for the isolation of *Listeria monocytogenes. Int. J. Food Microbiol.* 26: 1-13.

De Buyser, M.L., Dufour, B., Maire, M., and Lafarge, V. (2001) Implication of milk and milk products in food-borne diseases in France and in different industrialized countries. *Int. J. Food Microbiol.* 67: 1-17.

Fire, A., and Xu, S.Q. (1995) Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci. U.S.A.* 92: 4641-4645.

Food Safety and Inspection Service and US Department of Agriculture. (1999) Listeriosis and Food Safety Tips. FSIS, USDA. Washington DC. (http://www.fsis.usda.gov/OA/pubs/lmtips.htm).

Hofer, E., Ribeiro, R., and Feitosa, D.P. (2000) Species and serovars of the genus *Listeria* isolated from different sources in Brazil from 1971 to 1997. *Mem. Inst. Oswaldo Cruz.* 95: 615-620.

Jay, J.M. (2000) Modern Food Microbiology. Aspen Publishers Inc.: Maryland.

Jemmi, T., Pak, S.I., and Salman, M.D. (2002) Prevalence and risk factors for contamination with *Listeria monocytogenes* of imported and exported meat and fish products in Switzerland. *Prev. Vet. Med.* 54: 25-36.

Johannessen, G.S., Loncarevic, S., and Kruse, H. (2002) Bacteriological analysis of fresh produce in Norway. *Int. J. Food Microbiol.* 77: 199-204.

Klein, P.G., and Juneja, V.K. (1997) Sensitive detection of viable Listeria monocytogenes by reverse transcription-PCR. *Appl. Environ.Microbiol.* 63: 4441-4448.

Koonjul, P.K., Brandt, W.F., Farrant, J.M., and Lindsey, G.G. (1999) Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. *Nuc. Acids Res.* 27: 915-916.

Kuhn, H., Demidov, V.V., and Frank-Kamenetski, M.D. (2002) Rolling circle amplification under topological constraints. *Nuc. Acids Res.* 30: 574-580.

Leone, G., van Schijndel, H., van Gemen, B., Kramer, F.R., and Schoen, C.D. (1998) Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nuc. Acids Res.* 26: 2150-2155.

Liu, D., Daubendiek, S.L., Zillman, M.A., Ryan, K., and Kool, E.T. (1996) Rolling circle DNA synthesis: small circular oligonucleotides as efficient templates for DNA polymerases. *J. Am. Chem. Soc.* 118: 1587-1594.

Lizardi, P.M., Huang, X., Zhu, Z., Bray-Ward, P., Thomas, D.C., and Ward, D.C. (1998) Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat. Gen.* 19: 225-232.

Nilsson, M., Krejci, K., Koch, J., Kwiatkowski, M., Gustavsson, P., and Landegren, U. (1997) Padlock probes reveal single-nucleotide differences, parent of origin and in situ distribution of centromeric sequences in human chromosomes 13 and 21. *Nat. Gen.* 16: 252-255.

Nilsson, M., Antson, D-O., Barbany, G., and Landegren., U. (2001) RNA- templated DNA ligation for transcript analysis. *Nuc. Acids Res.* 29: 578-581.

Nilsson, M., Gullberg, M., Dahl, F., Szuhai, K., and Raap, A.K. (2002) Real-time monitoring of rolling-circle amplification using a modified molecular beacon design. *Nuc. Acids Res.* 30: 1-7.

Palumbo, J.D., Borucki, M.K., Mandrell, R.E., and Gorski, L. (2003) Serotyping of *Listeria monocytogenes* by enzyme-linked immunosorbent assay and

identification of mixed-serotype cultures by colony immunoblotting. *J. Clin. Microbiol.* 41: 564-571.

Singh, R.P., Singh, M., and King, R.R. (1998) Use of citric acid for neutralizing polymerase chain reaction inhibition by chlorogenic acid in potato extracts. *J. Virol. Methods.* 74: 231-235.

Singh, R.P., Nie, X., Singh, M., Coffin, R., and Duplessis, P. (2002) Sodium sulphite inhibition of potato and cherry polyphenolics in nucleic acid extraction for RT-PCR. *J. Virol. Methods.* 99: 123-131.

Starbuck, M.A., Hill, P.J., and Stewart, G.S. (1992) Ultra sensitive detection of Listeria monocytogenes in milk by the polymerase chain reaction (PCR). *Lett. Appl. Microbiol.* 15: 248-252.

Thomas, D.C., Nardone, G.A., and Randall, S.K. (1999) Amplification of padlock probes for DNA diagnostics by cascade rolling circle amplification or the polymerase chain reaction. *Arch Pathol. Lab. Med.* 123: 1170-1176.

Tyagi, S., and Kramer, F.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnology.* 14: 303-308.

U.S. Food and Drug Administration and Center for Food Safety and Applied Nutrition. (2002) *Listeria monocytogenes. Bacteriological Analytical Manual.*

Uyttendaele, M., Schukkink, R., van Gemen, B., and Debevere, J. (1995) Development of NASBA, a nucleic acid amplification system, for identification of *Listeria monocytogenes* and comparison to ELISA and a modified FDA method. *Int. J. Food Microbiol.* 27: 77-89.

Van Gemen, B., Kievits, T., Schukkink, R., van Strijp, D., Malek, L.T., Sooknanan, R., Huisman, H.G., and Lens, P. (1993) Quantification of HIV-1 RNA in plasma using NASBA during HIV-1 primary infection. *J. Virol. Methods.* 43: 177-187.

Vasquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominiquez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., and Kreft, J. (2001) *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* 14: 584-640.

Zhang, D.Y., Brandwein, M., Hsuih, T.C.H., and Li, H. (1998) Amplification of target-specific, ligation-dependent circular probe. *Gene.* 211: 277-285.

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