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Factors affecting the performance of 5′ nuclease PCR assays for *Listeria monocytogenes* detection

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Abstract

The design and operating parameters affecting the performance of 5′ nuclease PCR (TaqMan) assays for the detection of *Listeria monocytogenes* was investigated. A system previously developed and based on the *hly*A gene was used as a model [Appl. Environ. Microbiol. 61 (1995) 3724]. A series of fluorogenic probes labeled with a reporter and a quencher dye was synthesized to explore the effect of probe position and sequence content on the efficiency of probe hydrolysis. In addition, a series of PCR primer pairs that altered the distance between the upstream primer and the interceding probe was examined. The effects of various assay parameters were evaluated by measuring the ratio of the fluorescence intensity of the reporter dye over the quencher dye (ΔRQ). For a given probe sequence, the ΔRQ was typically lower if the 5′ terminus was a G residue. Decreasing the probe concentration increased the ΔRQ, although this was at the expense of reproducibility in the assay readout. The distance between the upstream primer and the interceding probe has a significant effect on probe hydrolysis. Reducing the primer–probe distance from, for example, 127 to 4 nt increased the ΔRQ from 2.87 to 5.00. These general rules were used to develop a 5′ nuclease PCR (TaqMan) assay with enhanced signal output, providing higher and more reproducible ΔRQ values for *L. monocytogenes* detection.

Keywords: 5′ Nuclease; PCR; *Listeria monocytogenes*

1. Introduction

Various methods have been developed to decrease the time to obtain results in microbiology analysis. Recent rapid methods include those based upon the use of antibodies and nucleic acid sequences that uniquely recognize the microorganism of interest. The polymerase chain reaction provides a powerful format for designing nucleic acid-based assays that are highly specific, sensitive, as well as quantitative (Saiki et al., 1985; Erlich et al., 1991). Initially, detection of PCR products (and by inference, estimated target numbers) was accomplished by gel electrophoresis, typically by using ethidium bromide to visualize the amplification product(s). In an attempt to further the utility of PCR-based assays, a number of other for-

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Table 1
Listeria strains used in this study

Strain	Source	ΔRQ
L. monocytogenes		
Scott A	University of Minnesota	5.98
BR 1/93	Cornell University	6.02
BR 3/93	Cornell University	4.91
BR 17/93	Cornell University	6.34
BR 27/93	Cornell University	5.86
104035	Cornell University	5.22
L. ivanovii		
19119	ATCC[a]	0.04
L31	University of Vermont	0.03
L. innocua		
P5V5	University of Minnesota	0.00
LG5V5	USDA-ARS[b]	0.00

[a] ATCC=American Type Cultures Collection.
[b] USDA-ARS=US Department of Agriculture-Agricultural Research Service.

mats involving either post-PCR hybridization or direct homogenous detection have been reported. One type of format takes advantage of the 5′ nucleolytic activity (inherent in many thermostable DNA polymerases) to hydrolyze a probe that is located downstream of a primer. In addition to *Taq* DNA polymerase, a number of other DNA polymerases have 5′ nuclease activity including *Escherichia coli*, *Tth* and *Tfl* DNA polymerases (Lyamichev et al., 1993). Initially, 5′ nuclease-based formats used a 5′-^{32}P-labeled probe and the release of the ^{32}P-label was used to measure probe hydrolysis (Holland et al., 1991). Subsequently, dual-labelled fluorescent probes were used. The increase in fluorescence when the reporter fluorescent dye is released from a probe which also contains a second quenching dye was used as a measure of amplification (Lee et al., 1993). Design modifications altering the distance between the fluorogenic dyes revealed factors which affect not only the quenching of the reporter dye in the intact probe but also the hydrolysis of the probe during PCR (Livak et al., 1995a). The fluorogenic 5′ nuclease format has been used for assays to detect *Listeria monocytogenes* with both DNA and RNA as target sequences (Bassler et al., 1995; Nogva et al., 2000; Norton and Batt, 1999). Instrumentation which carries out simultaneous thermal cycling and

Table 2
Sequence of primers and probes used to amplify the *L. monocytogenes hly*A

Primer	Sequence (5′→3′)	Location
HLYP1	CCTAAGACGCCAATCGAAAAGAAA	1633–1656
HLYP2	TAGTTCTACATCACCTGAGACAGA	2467–2444
HLYP3	CACAATTAATTGCGAAATTTGG	2132–2153
HLYP4	CTTCTTCTTGCATTTTCCCTTC	2209–2188
HLYP5	TGTTAATGAACCTACAAGACCTTCC	2255–2279
HLYP6	GGCAGCATCAAAAGCAGCTT	2435–2416
HLYP7	GGATGCATCTGCATTCAATAAAGA	1563–1586
HLYP8	AGGATTGGATTACAATAAAAACAA	1686–1709
HLYP9	TTCCGAATTCGCTTTTACGAGAGC	1873–1850
HLYP10	CCTGGCAAATCAATGCTGAGTGTT	1944–1921
Probes		
HLYA4	**R**TGTAAA**Q**AATAGCTTGAATGTAAACTTCGG**p**	2169–2198
HLYA7	**R**CAAAGC**Q**GTTACTAAAGAGCAGTTGCA**p**	2301–2327
HLYA1	**R**ATGCAG**Q**GACAAATGTGCCGCCAA**p**	1721–1744
HLYA11	**R**GATGCAG**Q**GACAAATGTGCCGCCA**p**	1720–1743
HLYA12	**R**AGATGCAG**Q**GACAAATGTGCCGCC**p**	1719–1742
HLYA13	**R**GAGATGCAG**Q**GACAAATGTGCCGC**p**	1718–1741
HLYA14	**R**GGAGATGCAG**Q**GACAAATGTGCCG**p**	1717–1740
HLYA15	**R**CGGAGATGCAG**Q**GACAAATGTGCC**p**	1716–1739
HLYA16	**R**ACGGAGATGCAG**Q**GACAAATGTGC**p**	1715–1738
HLYA17	**R**CACGGAGATGCAG**Q**GACAAATGTG**p**	1714–1737
HLYA18	**R**CCACGGAGATGCAG**Q**GACAAATGT**p**	1713–1736

fluorometry has the potential to expand the dynamic range of these assays (Heid et al., 1996; Gibson et al., 1996; Wittwer et al., 1997).

Several factors in PCR can affect the amplification efficiency. Specifically, design parameters can alter the efficiency of 5′ nuclease-based assays. In general, these factors involve the residence of the probe on the target sequence with respect to the PCR primers. Both the concentration and spatial distance between the primers and the probes can dramatically affect the hydrolysis of fluorogenic probes and sensitivity of the assay. In extreme examples, the probe may not appear to be functional in a particular assay format with a given set of PCR primers. Some of these factors were explored in this study during the efforts to optimize a previously reported 5′ nuclease PCR assay (Bassler et al., 1995). As a result, an improved system was developed for the specific and sensitive detection of *L. monocytogenes*.

2. Material and methods

2.1. Bacterial strains

The bacterial strains used are listed in Table 1. *L. monocytogenes* Scott A was used in the optimization of the TaqMan assay. *L. ivanovii* and *L. innocua*, as well as other *L. monocytogenes* strains, were used for specificity testing of the optimized method. Growth of *Listeria* species and the isolation of the template DNA was described previously (Bassler et al., 1995).

2.2. Fluorogenic probes and PCR primers

The fluorogenic probes and primers are listed in Table 2 and their locations within the hemolysin gene are depicted in Fig. 1. Probes and primers were synthesized as previously described (Bassler et al., 1995). All used FAM as the reporter dye on the 5′ and

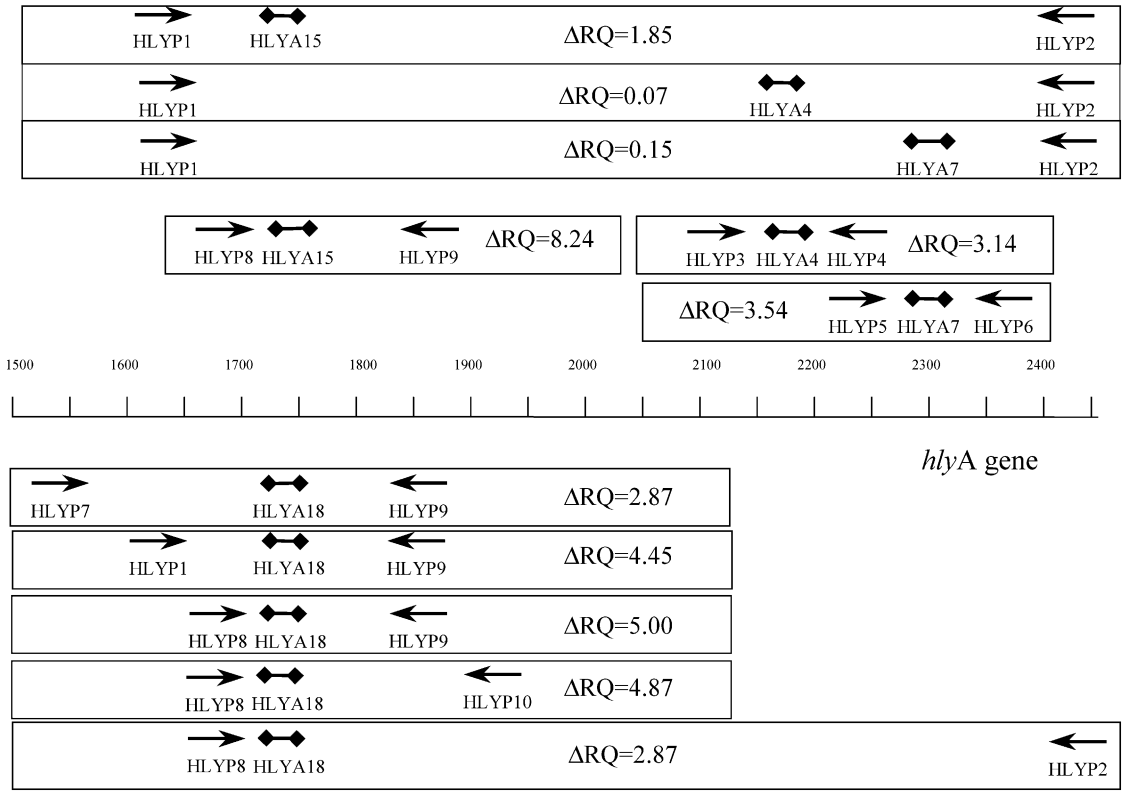

Fig. 1. Position of PCR primers and fluorogenic probes on *hly*A amplicon. ΔRQ values for various fluorogenic probes and pairs of primers are noted within each box.

TAMRA as the quencher dye. The quencher dye was placed at various positions within the probe, attached through a succinimidyl ester linkage. The 3′ end of each probe was blocked by attaching a phosphate (Applied Biosystems, Foster City, CA) to prevent its extension during PCR.

2.3. 5′ Nuclease PCR assays

The 5′ nuclease reactions were performed in 25-μl volumes as follows: 1 μl DNA template (10 ng was used in all the optimization assays), 5.0 mM $MgCl_2$, 460 nM each of primers, 1.6–200 nM fluorogenic probe, 200 μM each of dATP, dCTP, dGTP, 400 μM dUTP, 0.625 U AmpliTaq™ DNA polymerase (Perkin Elmer, Norwalk, CT) and 0.25 U uracil-*N*-glycosylase (UNG) (Perkin Elmer) in 1× PCR buffer. A Perkin Elmer 9600 thermocycler was used for amplification. All amplifications began with 2 min at 50 °C, then 2 min at 95 °C. Following these initial steps, the 30 cycles were 15 s at 95 °C, 30 s at 60 °C and 90 s at 72 °C. All samples were held at 72 °C once cycling was complete. All experiments were done in triplicate for each sample.

2.4. Post-PCR analyses

The fluorescence intensities of the FAM reporter and TAMRA quencher dyes were quantified as previously described (Bassler et al., 1995). A Perkin Elmer LS50B equipped with a 96-well microtiter plate reader was used in conjunction with white flat-bottom microtiter plates. All data acquisition and analysis were handled by a PC-based system using the Fluorescence Data Manager-FLDM (Perkin Elmer) and the ΔRQ was calculated with an EXCEL (Microsoft, Redmond, WA) spreadsheet as described (Bassler et al., 1995).

3. Results

3.1. Factors influencing the 5′ nuclease PCR assay

The 5′ nuclease PCR assay is based upon the hydrolysis of a fluorogenic probe by the incoming DNA polymerase, an event which occurs only when the probe is hybridized to the target amplicon. Efficient hydrolysis requires that, as the DNA polymerase approaches, the probe is completely hybridized to the target sequence. Typically, the PCR is carried out with annealing and extension temperatures below the T_m of the probe. The probe's T_m can be estimated by computational methods, but these do not accurately account for effects of the fluorogenic dyes. Furthermore, the T_m of the fluorogenic probe does not increase during the PCR, since it cannot be extended due to the addition of a 3′ phosphate. During the optimization process of a TaqMan assay based on the *hly*A gene for detection of *L. monocytogenes* strains, a series of primers and probes, which span the amplicon, was tested. Various combinations of primers and probes were used to alter the relative probe and primer distances, as well as the amplicon size.

3.1.1. Probe concentration

The concentration of fluorogenic probe in the 5′ nuclease PCR assay would determine the fraction of the probe which was hydrolyzed during the reaction. As the probe concentration is decreased, the fraction hydrolyzed would increase. Reducing the probe concentration would lower the absolute fluorescence intensity, since the total amount of bound and released reporter dye decreases. The concentration of fluorogenic probe was varied from 1.6 to 200 nM (Fig. 2). HLYA1 probe at 200 nM with primers HLYP1 and HLYP2 gave ΔRQ values in the range of 2–3. As the probe concentration decreased, the ΔRQ increased, reaching a maximum of approximately 6 with a probe concentration of 8 nM. Below a probe concentration

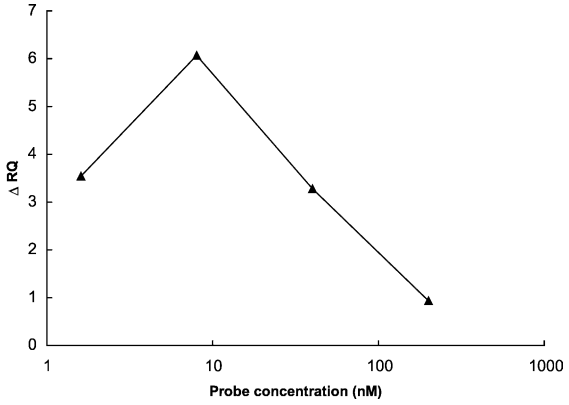

Fig. 2. Effect of fluorogenic probe concentration on the ΔRQ of the 5′ nuclease PCR assay for *L. monocytogenes*.

of 8 nM, the ΔRQ decreased. In general, the variability of the ΔRQ increased as the probe concentration was decreased (data not shown). A 40-nM concentration was used in the subsequent assays.

3.1.2. Probe sequence

A variety of probes (HLYA1, 11–18) was synthesized which progressively extended from the 5′ end while maintaining the quencher position (Table 2). This effectively varies the distance between the reporter and the quencher by 7–15 bp. It also alters the 5′ nucleotide to test the effect of 5′ sequence content on the hydrolysis of the probe. The RQ^-, RQ^+ and ΔRQ were measured to examine the initial quenching of the reporter by the quencher dye (RQ^-) and subsequently, the extent of hydrolysis after thermocycling (RQ^+, ΔRQ; Table 3). Although the changes in both the sequence content of the probe and the T_m confound a fully objective analysis, a few clear trends can be observed. The RQ^- varies from 0.70 for HLYA11 to 1.35 for HLYA15. There does not appear to be a consistent effect of reporter–quencher distance on RQ^- in these sets of probes. The ΔRQ values varied from 1.14 for HLYA13 to 3.04 for HLYA1. HYLA1 has the shortest distance (six nucleotides) between the reporter and the quencher dye which should limit the opportunity for hydrolysis by the *Taq* DNA polymerase. When the HLYA1 probe is extended by the addition of a single G nucleotide on the 5′ terminus, the ΔRQ decreases from 3.04 to 1.66. In a similar fashion, when HLYA12 is extended by a single G nucleotide, the ΔRQ decreases from 2.72 to 1.14. In contrast, altering the 5′ terminus from a G by a single nucleotide extension as in the case of HLYA11 and HLYA12 increases the ΔRQ from 1.66 to 2.72. The effect of a G nucleotide on the 5′ end is, however, not the only cause for differences in probe hydrolysis. For example, when a single A nucleotide is added to HLYA15 to create HLYA16, the ΔRQ decreases from 2.93 to 1.85.

Table 3
Effect of probe sequence on RQ^-, RQ^+ and ΔRQ values for fluorogenic probes

Probe	RQ^-	RQ^+	ΔRQ
HLYA1	0.73	3.76	3.04
HLYA11	0.70	2.36	1.66
HLYA12	0.88	3.59	2.72
HLYA13	1.01	2.16	1.14
HLYA14	0.80	1.95	1.15
HLYA15	1.35	4.28	2.93
HLYA16	1.13	2.98	1.85
HLYA17	1.03	3.50	2.37
HLYA18	1.20	3.70	2.51

3.1.3. Distance between primers and probe

The 5′ nuclease activity of *Taq* DNA polymerase is a manifestation of its processivity. It is intuitive that the distance between the upstream primer and the fluorogenic probe would have an effect on the efficiency of the 5′ nuclease PCR assay. It was demonstrated previously that probe HLYA1 was efficiently hydrolyzed with the use of the primers HLYP1 and HLYP2, while the ΔRQs with more downstream probes (HLYA4 and HLYA7) were at least 10-fold lower when using the same primer pair (Bassler et al., 1995).

In a first approach to test the effect of primer–probe distance and fragment length over the entire *hly*A amplicon, three pairs of PCR primers (HLYP3/HLYP4, HLYP5/HLYP6 and HLYP8/HLYP9) were synthesized and used in a comparative analysis with the HLYP1/HLYP2 primers using the same three probes (HLYA4, HLYA7 and HLYA15). The ΔRQ for probes HLYA4, HLYA7 and HLYA15 were 0.07, 0.15 and 1.85, respectively, with HLYP1/HLYP2 primers. These primers amplify a 847-bp amplicon. When PCR primers HLYP1/HLYP2 were substituted for HLYP3/HLYP4 (the former of which lies 499 bp closer to the HLYA4 probe as compared to HLYP1), the ΔRQ increased from 0.07 to 3.14. Similarly, the ΔRQ increased from 0.15 to 3.54 when the HLYP5/HLYP6 primer set was used with the HLYA7 probe and from 1.8 to 8.24 when the HLYP8/HLYP9 primer set was used with the HLYA15 probe (Fig. 1).

The effect of distance on probe hydrolysis for both the upstream and downstream primers was further examined with additional sets of primers (Fig. 1). The upstream primer would measure the primer–probe distance, while the downstream primer would affect the amplicon size. A single reverse primer HLYP9 was tested in combination with a series of forward primers HLYP7, HLYP1 and HLYP8. In addition, a single forward primer HLYP8 was tested in combination with a series of reverse primers HLYP9, HLYP10 and HLYP2. For all of these 5′

nuclease PCR assays, a single probe, HLYA18, was employed. As the forward primer–probe distance was decreased from 127 to 4 bp, the ΔRQ increased proportionally from 2.87 to 5.00. With a constant forward primer–probe distance reducing the size of the amplicon from 781 to 187 bp, the ΔRQ increased from 2.87 to 4.87. Unlike the forward primer–probe distance, which had a consistently proportional effect on ΔRQ, no difference in the ΔRQ was observed when the amplicon size was decreased by using the HLYP8/HLYP9 as compared to HLYP8/HLYP10.

The ΔRQs obtained for a number of PCR primers and fluorogenic probes reveal the effect of amplicon size and forward primer–probe distance on ΔRQ (Fig. 1). In general, as either the amplicon size or the primer–probe distance is increased, the ΔRQ decreases. The effect of primer–probe distance does appear to be more significant as compared to the amplicon size. At a primer–probe distance of <100 bp, there is a great degree of variation in the ΔRQ values, which range from <2 to >8. The differences are probably a function of the variation in the primer and probe's T_m and the efficiency at which the individual probes are hydrolyzed. There is, however, a significant drop in the ΔRQ as the primer–probe distance increased above 500 bp.

3.2. Optimized 5′ nuclease PCR assay sensitivity and specificity

Based on the results described above, HLYP8/HLYP9 primers and HLYA15 probe were selected to perform specificity and sensitivity tests aiming at the development of an optimized 5′ nuclease PCR assay for *L. monocytogenes* detection.

The specificity of the 5′ nuclease PCR assay was obtained by using DNA from approximately 10^5 CFU of *L. monocytogenes*, *L. innocua* and *L. ivanovii* strains. The ΔRQ values varied from 4.91 to 6.34 for *L. monocytogenes* strains while all ΔRQ values for other *Listeria* species were below 0.05 (Table 1). In the positive/negative decision rendered on the basis of the significance of the ΔRQ for a given template preparation, all *L. monocytogenes* were positive where all other strains were negative.

Sensitivity studies were performed on pure cultures of *L. monocytogenes* Scott A to test the lower detection limit of the optimized assay. Results showed that the ΔRQ values were greater than the detection threshold of 0.20 when more than 10 CFU was present (Fig. 3).

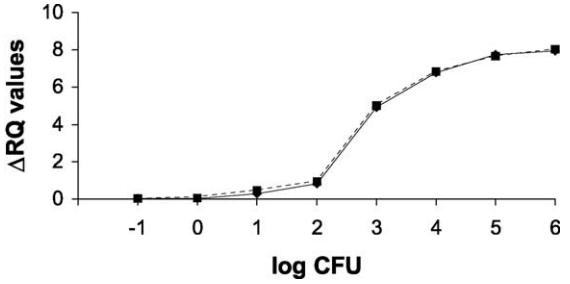

Fig. 3. Sensitivity of the optimized TaqMan assay for detecting *L. monocytogenes*. Tenfold dilutions of strain Scott A were made as previously described (Bassler et al., 1995). Assays were performed in triplicate in different days.

4. Discussion

The readout (ΔRQ) in 5′ nuclease PCR assays is the ratio of the fluorescence intensity of the reporter dye over the fluorescence intensity of the quencher dye. The dyes are located on a single oligonucleotide probe which is designed to hybridize to an amplicon, as defined by a pair of PCR primers. The reporter/quencher fluorescence ratio in the intact probe (RQ^-) is dependent upon the quenching of the former by the latter. The position of the quencher dye in the probe is critical and previous efforts have documented this effect (Livak et al., 1995a). The RQ^- of the various probes ranged from 0.7 to 1.35 with no consistent overall relationship between quencher position and RQ^-.

Upon hydrolysis of this fluorogenic probe, the reporter dye is released, and as a consequence, the ratio of the fluorescence intensities increases, since the reporter dye is no longer held in a proximate location to the quencher. Considerable evidence exists that the 5′ nuclease activity of DNA polymerases is dependent upon the recognition of a forked structure by the enzyme as it approaches the probe (Lyamichev et al., 1993; Holland et al., 1991; Robins et al., 1994). One or more nucleotides are cleaved from the probe, and the size of the cleavage product depends upon the degree of complementarity between the probe and the target sequence. Holland et al. (1991) and Lyamichev

et al. (1993) observed that a variety of synthetic substrates which contained mismatched nucleotides on their 5′ end was not cleaved, generating progressively longer products as the size of the noncomplementary region increased. This failure to efficiently cleave when a mismatch is present has been exploited to develop allele-specific 5′ nuclease PCR assays (Livak et al., 1995b).

In the series of *hly*A probes created to test the effect of sequence content, a few trends were observed. Primarily, there is an apparent large decrease in probe hydrolysis that occurs when the 5′ terminal nucleotide is a G. This effect is documented with, for example, a comparison of probes HLYA11 and HLYA16 (ΔRQ 1.66 and 1.85) and HLYA1 and HLYA15 (ΔRQ 3.04 and 2.93) which are, respectively, their nearest neighbors. The pronounced negative effect of a 5′ G on the fluorogenic probe may reflect the higher quenching of the fluorophore by the guanine base and/or the enzyme's difficulty in displacing a 5′ end with a relatively higher melting temperature as compared to other nucleotides (Tables 2 and 3).

The nucleolytic cleavage of the 5′ end of the probe by the approaching DNA polymerase does not require extension. Substrates that abut an upstream oligonucleotide primer with only a nick between them can be hydrolyzed even in the absence of deoxynucleotides in the PCR (Holland et al., 1991). Gaps of increasing size do not completely abolish 5′ nucleolytic cleavage of the probe, but without the addition of deoxynucleotides, the efficiency decreases dramatically. The increase in hydrolysis of the probe as the distance is decreased between it and the upstream primer is therefore not surprising. In other systems that target different amplicon sequences, a similar trend is observed (Witham et al., 1996). The effect is probably a function of the efficiency at which DNA polymerase can replicate the smaller target. The longer amplicons provide more sites for nucleation, the initial stages of re-annealing which would compete with binding of the fluorogenic probe. The effect of primer–probe distance may also be due to the processivity of the enzyme. DNA polymerases and their truncated derivatives (i.e. Stoffel's fragments) differ in their processivity with *Taq* DNA polymerase having a processivity of approximately 50–75 nucleotides (Abramson, 1995). It is possible that if the fluorogenic probe lies beyond a specific limit as defined by the processive extent of the enzyme, the polymerase may find it difficult to easily reassociate with the target sequence due to the presence of the probe. As a general rule, the smaller the primer–probe distance, the more efficiently the probe is cleaved.

There may be, however, some practical limits to the primer–probe distance. In certain cases, sequence similarity between targets, which must be distinguished by the assay, may compromise specificity, forcing the use of a more divergent upstream primer. Secondly, primer design consideration and the avoidance of regions that would produce either primer–primer dimers or primer–probe dimers affect the choice of primers and probes. In general, however, most target sequences contain sufficient diversity to accommodate a wide latitude in primer and probe design for 5′ nuclease PCR assays.

The use of the principles studied here allowed the development of an optimized TaqMan system for the specific and sensitive detection of *L. monocytogenes* strains. In comparison with the previously developed system (Bassler et al., 1995), we obtained a better discrimination between *L. monocytogenes* and other *Listeria* strains as well as a higher sensitivity. These principles will probably help in the development of 5′ nuclease systems for detection and quantitation of other microorganisms.

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