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A Novel Real-Time PCR for *Listeria monocytogenes* That Monitors Analytical Performance via an Internal Amplification Control

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We describe a novel quantitative real-time (Q)-PCR assay for *Listeria monocytogenes* based on the coamplification of a target *hly* gene fragment and an internal amplification control (IAC). The IAC is a chimeric double-stranded DNA containing a fragment of the rapeseed *BnACCg8* gene flanked by the *hly*-specific target sequences. This IAC is detected using a second TaqMan probe labeled with a different fluorophore, enabling the simultaneous monitoring of the *hly* and IAC signals. The *hly*-IAC assay had a specificity and sensitivity of 100%, as assessed using 49 *L. monocytogenes* isolates of different serotypes and 96 strains of nontarget bacteria, including 51 *Listeria* isolates. The detection and quantification limits were 8 and 30 genome equivalents, and the coefficients for PCR linearity (R^2) and efficiency (E) were 0.997 and 0.80, respectively. We tested the performance of the *hly*-IAC Q-PCR assay using various broth media and food matrices. Fraser and half-Fraser media, raw pork, and raw or cold-smoked salmon were strongly PCR-inhibitory. This Q-PCR assay for *L. monocytogenes*, the first incorporating an IAC to be described for quantitative detection of a food-borne pathogen, is a simple and robust tool facilitating the identification of false negatives or underestimations of contamination loads due to PCR failure.

Many components of food products, culture media, and nucleic acid extraction reagents may inhibit PCR, leading to a dramatic decrease in sensitivity and even to false negative results (23, 26). In quantitative real-time (Q)-PCR, such inhibitors may cause underestimation of the contamination load in the sample, seriously compromising the applicability of this otherwise highly accurate technology (24). This is one of the major barriers to the systematic introduction of Q-PCR-based methods in routine food analysis. To tackle this problem, sample pretreatment procedures can be developed but, even if these are applied, it will always be necessary to assess PCR efficiency (or the performance of the sample pretreatment) in every reaction. The only way to achieve this is by the inclusion of an internal amplification control (IAC) (10, 20). A PCR IAC is a nontarget DNA fragment that is coamplified with the target sequence, ideally with the same primers used for the test reaction (6). In an IAC for Q-PCR, the forward and reverse target sequences are fused to both ends of a nontarget fragment, typically from an unrelated DNA, to which a second fluorescent probe (the IAC probe) hybridizes. The simultaneous use in a single reaction of two differently labeled fluorescent probes makes it possible to detect/quantify the target and to assess PCR efficiency at the same time. If negative results are obtained for the target PCR, the absence of a positive IAC signal indicates that amplification has failed (11).

A number of Q-PCR assays have been developed for the detection of food-borne pathogens, but few include an IAC. In particular, no IAC-containing assay has ever been developed for quantitative microbiological food analysis despite the generalized view that an IAC should be mandatory for PCR-based diagnostic tests (10). We report here the development and optimization of a novel Q-PCR assay for *L. monocytogenes* based on the simultaneous detection of *hly* gene target sequences, which we have shown to provide high specificity, sensitivity, and quantifiability (18), and an IAC sequence for the assessment of PCR inhibition. Using this assay, we show that some broth media widely used in the detection and enumeration of *L. monocytogenes* and certain food products commonly contaminated with these bacteria contain inhibitors that affect the analytical performance of the PCR.

IAC design and construction. The IAC consisted of a 104-bp DNA fragment containing a portion of the acetyl-coenzyme A carboxylase gene from rapeseed (*Brassica napus*), *BnACCg8* (GenBank accession no. X77576), flanked by the *L. monocytogenes*-specific *hly* gene sequences targeted by the previously described *hlyQF* and *-R* primers (18). This chimeric DNA fragment was generated by two rounds of PCR. The first used as template 100 ng of *B. napus* DNA and primers *hlyAccF* (5′-CATGGCACCACCAGCATCTGGTGAGCTGTATAATC) and *hlyAccR* (5′-ATCCGCGTGTTTCTTTTCGAGGCGCAGCATC), which contained the corresponding *BnACCg8* target sequences plus a 5′ tail with the *hlyQF*/*R* primer sequences. The second PCR round used the purified first-round PCR product (diluted 1:1,000) as a template and the *hlyQF*/*R* primers. PCR conditions were as previously described (9). The IAC PCR product was purified, quantified using PicoGreen (Molecular Probes, Eugene, OR) in a luminescence spectrometer LS50B (Perkin-Elmer, Norwalk, CT), and diluted to the working concentration in double-distilled water containing 5

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TABLE 1. Detection and quantification limits of the *hly*-IAC Q-PCR assay[a]

Approx. no. of *L. monocytogenes* DNA molecules/reaction	Confidence interval limit[b]		*hly* system (FAM)			IAC system (VIC)	
	Lower	Upper	Signal ratio[c]	C_T[d]	ΔR_n[e]	C_T[f]	ΔR_n
3×10^4	29,661	30,340	9	22.38 ± 0.11	0.98 ± 0.03	33.67 ± 0.79	0.10 ± 0.01
3×10^3	2,893	3,108	9	25.91 ± 0.10	0.92 ± 0.09	33.56 ± 0.75	0.12 ± 0.03
3×10^2	267	334	9	30.17 ± 0.15	0.91 ± 0.02	33.67 ± 0.57	0.26 ± 0.04
60	45	76	9	32.49 ± 0.19	0.81 ± 0.02	33.14 ± 0.47	0.43 ± 0.04
30	20	41	9	34.25 ± 0.16	0.78 ± 0.04	33.57 ± 0.66	0.53 ± 0.06
15	8	23	9	35.57 ± 0.57	0.75 ± 0.06	33.76 ± 0.74	0.65 ± 0.10
8	3	13	9	36.07 ± 0.59	0.73 ± 0.02	33.54 ± 0.47	0.70 ± 0.06
4	1	8	5	35.58 ± 0.76[g]	0.79 ± 0.05	33.36 ± 0.31	0.73 ± 0.09
1	0	3	4	35.56 ± 1.03[g]	0.85 ± 0.06	33.71 ± 0.75	0.70 ± 0.11

[a] Note that both the *hly* and IAC templates are amplified by the same primers and that the number of copies of the *hly* target is variable, whereas that of the IAC template is constant (100 copies). This is reflected in the IAC data, in which the C_T values remain constant, whereas the VIC fluorescence endpoints (ΔR_n values) gradually decrease with increasing numbers of *L. monocytogenes* DNA molecules in the reaction. See Fig. 1 for representative amplification profiles for *hly* and IAC.
[b] Calculated for the expected number of template molecules at each dilution with *P* as 0.05. The calculations were performed assuming a binomial distribution and confirmed by Monte Carlo simulations as previously described (18).
[c] Signal ratio means positive reactions respective to nine reactions.
[d] Cycle number at which fluorescence intensity equals a fixed threshold. FAM C_T values were calculated with a prefixed threshold at 0.035 and a baseline from cycles 3 to 15.
[e] ΔR_n is the difference between R^+n (reporter emission intensity/passive reference emission intensity) and R^-n (background reporter emission intensity/passive reference emission intensity [calculated in no. template controls]) (3).
[f] VIC C_T values were calculated with a prefixed threshold at 0.035 and a baseline from cycles 3 to 23.
[g] *hly*-negative amplifications were excluded from mean and SD calculations.

ng/μl tRNA as a blocking agent (to avoid binding of the negatively charged IAC DNA to the plastic microtubes).

With the exception of the *BnACCg8* sequence (nucleotide positions 9651 to 9755), the IAC did not show significant similarity to any DNA sequence deposited in public DNA databases, as shown by BLAST-N searches (National Center for Biotechnology Information, Bethesda, MD; http://www.ncbi.nlm.nih.gov). The IAC and *hly* amplicons are specifically detected with previously described VIC- (8) and 6-carboxyfluorescein (FAM)-labeled (18) TaqMan probes, respectively. The IAC amplicon, 143 bp, is longer than the 64-bp *hly*-specific amplicon (18), facilitating distinction between these two PCR products by gel electrophoresis.

Optimization of *hly*-IAC Q-PCR assay. The optimal IAC probe concentration (3, 21) was determined by performing Q-PCRs in the presence of 1,000 IAC molecules, no *L. monocytogenes* DNA, 100 nM FAM-labeled *hly* probe, and various amounts (from 25 to 250 nM) of the VIC-labeled IAC probe. The PCR conditions were those previously established for the *hly*-specific assay (18). The minimum probe concentration not resulting in an increase in cycle threshold (C_T) was 100 nM. An excess of IAC may inhibit the target-specific reaction (5). To determine the optimal IAC concentration, we first performed Q-PCRs in the presence of various IAC amounts (1,000, 300, 100, 30, and 10 molecules per reaction) to determine the minimum required to give positive amplification. Ten IAC molecules were consistently detected, but the variation in VIC C_T values was excessive (standard deviation [SD], >1.0). We then tested the three next lowest IAC amounts (30, 100, and 300 molecules) in the presence of *L. monocytogenes* CTC1010 (18) DNA corresponding to the quantification limit of the *hly* assay, previously determined to be 30 genome equivalents (GE) (note that the *hly* gene is in monocopy in the *L. monocytogenes* genome so that 1 GE corresponds to 1 bacterium or CFU in stationary phase) (16). The maximum IAC amount with no inhibitory effect on the *hly*-specific FAM signal was established at 100 copies.

Specificity and sensitivity of the *hly*-IAC Q-PCR assay. We evaluated the specificity of the assay with 1 ng of genomic DNA (purified using the Wizard genomic DNA purification kit

TABLE 2. Performance of the *hly*-IAC Q-PCR with various media commonly used for *Listeria*

Medium	Value obtained with:				
	hly system (FAM)			IAC system (VIC)	
	C_T[a]	ΔR_n[b]	Relative accuracy[c]	C_T[d]	ΔR_n[b]
Double-distilled water	30.15 ± 0.15	0.95 ± 0.02	94.76	33.55 ± 0.61	0.55 ± 0.26
BPW	30.05 ± 0.16	0.98 ± 0.03	100.50	33.95 ± 0.52	0.60 ± 0.19
BHI	30.22 ± 0.12	0.96 ± 0.02	90.94	33.79 ± 0.49	0.65 ± 0.22
Half-Fraser	36.55 ± 1.05	0.70 ± 0.05	2.20	38.20 ± 1.20	0.13 ± 0.04
Fraser	37.25 ± 1.20	0.72 ± 0.05	1.45	38.52 ± 1.05	0.11 ± 0.04

[a] Cycle number at which fluorescence intensity equals a fixed threshold. FAM C_T values (mean plus or minus standard deviation) were calculated with a prefixed threshold at 0.035 and a baseline from cycles 3 to 15.
[b] ΔR_n is the difference between R^+n (reporter emission intensity/passive reference emission intensity) and R^-n (background reporter emission intensity/passive reference emission intensity [calculated in number of template controls]) (3).
[c] Degree of correspondence between the response obtained by the reference method (2) and the response obtained by the alternative (Q-PCR) method.
[d] VIC C_T values were calculated with a prefixed threshold at 0.035 and a baseline from cycles 3 to 23.

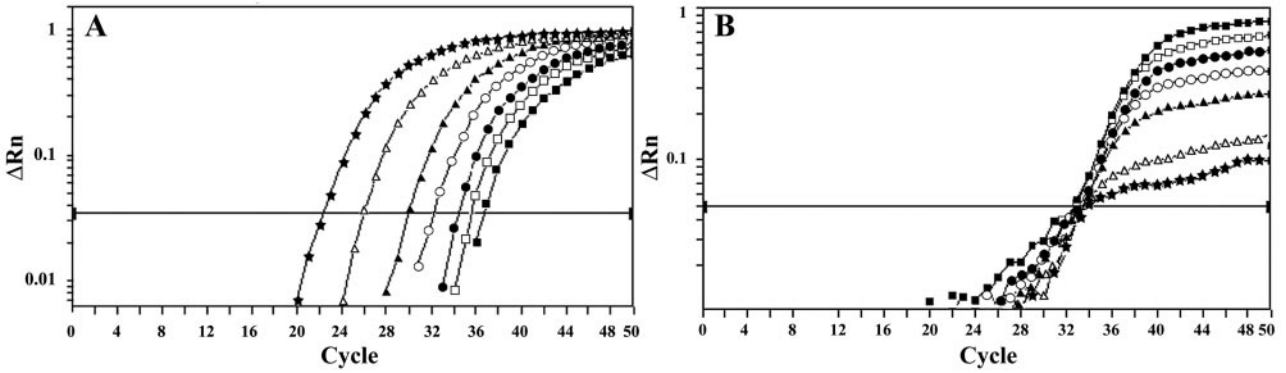

FIG. 1. Representative amplification plots for *hly* (A) and IAC (B) templates obtained in the experiments shown in Table 1. Each reaction contained 100 IAC molecules and decreasing amounts of *L. monocytogenes* CTC1010 genomic DNA, equivalent to 3×10^4 (⋆), 3×10^3 (△), 3×10^2 (▲), 60 (○), 30 (●), 15 (□), and 8 (■) target molecules.

[Promega, Madison] and quantified with PicoGreen as above) from each of 49 *L. monocytogenes* strains, including representative strains of the different serovars of the species, and 96 nontarget bacteria, including 51 *Listeria* strains (17 *L. innocua*, 7 *L. grayi*, 10 *L. seeligeri*, 5 *L. welshimeri*, and 12 *L. ivanovii*) and 45 non-*Listeria* strains. The complete list of strains used can be found in Tables 1 and 2 of reference 18. The *hly*-IAC Q-PCR unequivocally distinguished *L. monocytogenes* isolates from nontarget bacteria. All reactions generated a positive IAC (VIC) signal, indicating that the lack of *hly* (FAM) signal that was obtained with non-*L. monocytogenes* isolates was not due to failure of the PCR.

To ensure maximum analytical sensitivity, the *L. monocytogenes*-specific signal should not be inhibited by the simultaneous coamplification of the IAC, particularly if small numbers of target molecules are expected. The detection limit of the *hly*-IAC assay was assessed by conducting Q-PCRs in the presence of 100 molecules of IAC and various amounts of genomic DNA from *L. monocytogenes* CTC1010 (equivalent to approximately 30, 15, 8, 4, and 1 GE per reaction). Table 1 shows FAM (*hly*) and VIC (IAC) C_T and ΔR_n values obtained in a total of nine replicates of three independent experiments. The Q-PCR assay detected as few as eight *L. monocytogenes* DNA molecules in 100% of the replicates and one to four target molecules in at least four out of the nine replicates. These results are similar to those previously reported for *hly*-specific uniplex assays (12, 16, 18). The IAC was coamplified in all reactions with overall C_T values of 33.59 ± 0.68 and ΔR_n values of 0.66 ± 0.11. Thus, the addition of 100 initial IAC molecules to the PCR mixture did not markedly decrease the sensitivity of the assay.

Quantifiability of the *hly*-IAC Q-PCR assay. The capacity of the Q-PCR method to determine accurately the number of targets present in the sample depends upon the linearity and efficiency of the PCR. Linearity is the ability of the method to generate results proportional to the amount of analyte present in the sample and is represented by the regression coefficient. Efficiency is the capacity of the PCR to duplicate the amplicon molecules in each cycle and is calculated from the slope of the linear regression curve (s) from the equation $E = 10^{-1/s} - 1$ (14). These two parameters were assessed by carrying out PCRs with decreasing amounts of *L. monocytogenes* CTC1010 genomic DNA (equivalent to 3×10^4, 3×10^3, 3×10^2, 60, and 30 target DNA molecules per reaction). Figure 1 shows the typical amplification profiles obtained for each template. Table

TABLE 3. Detection of PCR-inhibitory activity in different food matrices using the *L. monocytogenes hly*-IAC Q-PCR assay[a]

L. monocytogenes contamination (CFU/g)	CFU/reaction	Results for:					
		Fermented pork sausage			Frankfurter sausage		
		hly[e]		IAC	*hly*[f]		IAC
		C_T values[b]	Relative accuracy[c]	C_T values[d]	C_T values	Relative accuracy	C_T values
3×10^7	3×10^3	26.05 ± 0.30	94.34	33.75 ± 0.71	25.94 ± 0.23	103.98	33.42 ± 0.35
3×10^6	3×10^2	29.26 ± 0.31	113.15	33.52 ± 0.45	29.68 ± 0.37	93.06	33.69 ± 0.54
3×10^5	3×10^1	33.01 ± 0.62	94.98	33.65 ± 0.54	29.43 ± 0.59	104.67	33.55 ± 0.75

[a] NA, not applicable; ND, amplification not detected.
[b] FAM C_T values (mean plus or minus standard deviation) were calculated with a prefixed threshold at 0.035, and a baseline from cycles 3 to 15.
[c] Degree of correspondence between the response obtained by the reference method (2) and the response obtained by the alternative (Q-PCR) method. Note that in those samples where there was PCR inhibition as detected by the absence of IAC signal the relative accuracy values dropped dramatically.
[d] VIC C_T values (mean plus or minus standard deviation) were calculated with a prefixed threshold at 0.035, and a baseline from cycles 3 to 15.
[e] Efficiency, 0.94; linearity, 0.9981.
[f] Efficiency, 0.93; linearity, 0.9983.
[g] Efficiency, 1.04; linearity, 0.9991.
[h] Efficiency, not applicable; linearity, not applicable.

1 shows FAM (*hly*) and VIC (IAC) C_T and ΔR_n values for nine replicates of three independent experiments.

The relationship between the initial number of *L. monocytogenes* DNA molecules and FAM C_T values was linear down to 30 target molecules, as indicated by the regression coefficient obtained ($R^2 = 0.997$). At optimal efficiency ($E = 1.00$), the slope is −3.322 (15). The calculated slope for our *hly*-IAC PCR assays, −3.916, corresponds to an E value of 0.80, only slightly lower (12.6%) than that previously obtained for the uniplex *hly* assay (0.916) (18). These data, together with the small SD values for both replicates and independent experiments (Table 1), indicate that our *hly*-IAC Q-PCR assay accurately quantifies *L. monocytogenes*. The experimental quantification limit of the assay, 30 GE, coincided with the theoretical limit. The theoretical quantification limit was determined through the calculation of the expected number of template molecules at each dilution with the P value as 0.05 (the calculations were performed assuming a binomial distribution and confirmed by Monte Carlo simulations) and establishing as the theoretical quantification limit the lowest sample dilution in which the 95% confidence interval does not overlap with that of the next dilution (Table 1). This value is identical to that previously reported for the corresponding uniplex assay (18) and similar to that reported for other quantitative Q-PCR systems (4, 12, 13, 16, 21).

Performance of the *hly*-IAC assay. The capacity of our assay to detect PCR inhibition was tested using four different broths typically employed for the culture, detection, or counting of *L. monocytogenes*: brain-heart infusion (BHI), buffered peptone water (BPW) (2), Fraser medium, and half-Fraser medium (7). The last two of these media are specified in ISO norms as enrichment media for the detection of *L. monocytogenes* in foodstuffs (1) and have been reported to inhibit PCR (23). We added 1 μl of broth medium or double-distilled water (control) to the standard *hly*-IAC Q-PCR mix containing 300 copies of genomic DNA from *L. monocytogenes* CTC1010.

The FAM (*hly*) and VIC (IAC) C_T values obtained in the presence of BHI and BPW were similar to those for the control ($P > 0.001$) (Table 2). A mean of 287.16 ± 20.29 *L. monocytogenes* DNA molecules was detected on the basis of FAM C_T values (95.72 ± 6.76%, quantification accuracy), with no inhibition of PCR, as shown by VIC C_T values. In contrast, reactions containing Fraser or half-Fraser medium gave C_T values that were significantly higher ($P < 0.001$) than those for the controls for both FAM and VIC signals, indicating that these media do indeed inhibit PCR. Significantly, although the *hly* target was amplified, the estimated number of copies, based on C_T values, was below the quantification limit. Thus, in the absence of the corresponding IAC amplification profile, an underestimation by more than 2 orders of magnitude of the listerial contamination load would have passed unnoticed.

We also assessed the performance of the *hly*-IAC Q-PCR assay using foods in which *L. monocytogenes* is frequently found (25). Twenty-five-gram samples of raw pork meat, fermented pork sausage, cooked ham, frankfurter sausage, and raw or cold-smoked salmon were artificially contaminated with various amounts (approximately 3×10^7, 3×10^6, and 3×10^5 CFU/g) of *L. monocytogenes* CTC1010, as previously described (19, 22). These relatively high bacterial loads were used to enable accurate determination of the impact and scale of PCR inhibition on *L. monocytogenes* detection and quantification (something that would have been impossible with low bacterial numbers). The contaminated samples were immediately homogenized 1:10 (wt/vol) in BPW, and 1 μl of the homogenate was added to the standard *hly*-IAC Q-PCR mixture. In parallel, the number of *L. monocytogenes* CFU present in the samples was determined by standard plate counting (2). The results obtained are shown in Table 3.

The FAM and VIC C_T values obtained for fermented pork sausage, cooked ham, and frankfurter sausage samples were very similar ($P > 0.001$) to those obtained with purified DNA (Tables 1 and 3), indicating that our *hly*-IAC Q-PCR system accurately detects and quantifies *L. monocytogenes* DNA in processed meat products. However, the *L. monocytogenes*-specific *hly* (FAM) signal was not detected in any of the raw pork meat and raw or cold-smoked salmon samples. This lack of FAM signal was accompanied by a lack of IAC (VIC) signal, indicating that the failure to detect *L. monocytogenes* DNA was a false negative result due to inhibition of the PCR.

Conclusions. We have developed a Q-PCR assay with an IAC to facilitate monitoring of PCR inhibition and thus the identification of false negative results or target DNA underes-

TABLE 3—*Continued*

Results for:											
Cooked ham			Raw pork meat			Raw salmon			Smoked salmon		
hly[g]		IAC	*hly*[h]		IAC	*hly*[h]		IAC	*hly*[h]		IAC
C_T values	Relative accuracy	C_T values	C_T values	Relative accuracy	C_T values	C_T values	Relative accuracy	C_T values	C_T values	Relative accuracy	C_T values
26.05 ± 0.30	105.64	33.45 ± 0.64	ND	NA	ND	ND	NA	ND	ND	NA	ND
29.43 ± 0.17	90.19	33.62 ± 0.69	ND	NA	ND	ND	NA	ND	ND	NA	ND
32.49 ± 0.57	106.33	33.81 ± 0.45	ND	NA	ND	ND	NA	ND	ND	NA	ND

timation due to PCR failure. This assay presents the same specificity, sensitivity, and quantification characteristics as the uniplex assay, demonstrating that the inclusion of an IAC does not compromise Q-PCR performance. The application of this assay to samples containing various broth media or food matrices relevant to *Listeria* demonstrated the presence of PCR inhibitors in some of these. Our data indicate that the *hly*-IAC Q-PCR assay here reported is a robust technique that can be routinely applied to the direct detection and quantification of *L. monocytogenes* DNA in food products.

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