A molecular beacon-based real time NASBA assay for detection of *Listeria monocytogenes* in food products. Role of target mRNA secondary structure on NASBA design.

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ABSTRACT

A molecular beacon-based real-time NASBA (QNASBA) assay for detection and identification of *Listeria monocytogenes* has been developed. A correlation between targeting highly accessible mRNA sequences and QNASBA efficiency and sensitivity was demonstrated. The assay targets a sequence from the mRNA transcript of the *hly* gene which is specific for this bacterium; and includes an internal amplification control to disclose failure of the reaction. It was fully selective and consistently detected down to 100 target molecules and 40 *L. monocytogenes* exponentially growing cells per reaction. In addition, it was capable of accurate quantification of target RNA molecules independently of the presence of DNA in the sample. In combination with a short RNase treatment prior to nucleic acids extraction our QNASBA specifically detected viable *L. monocytogenes* cells. It was successfully applied to rapid detection of this pathogen in meat and salmon products, and is therefore a useful tool for the study of *L. monocytogenes* in food samples. We finally discuss considerations of target secondary structure with regard to development of NASBA assays.

Keywords: *Listeria monocytogenes*; real-time NASBA (QNASBA); RNA detection and quantification; secondary structure; viable microorganisms.

INTRODUCTION

Listeria monocytogenes is a facultative anaerobic gram-positive bacterial species widely distributed in the environment, capable of growth over a pH range of 4.39 to 9.40, and at refrigeration temperatures. It is the etiological agent of listeriosis, a severe infectious disease that predominantly affects certain risk groups, including pregnant women, newborns, elderly people and immunocompromised patients. Human listeriosis is associated with food products contaminated with *L. monocytogenes*, meat and salmon products being major sources of this food-borne pathogen (Peccio et al., 2003; Ryser 1999).

The classical approach for detection of *L. monocytogenes* in food involves growth in pre-enrichment medium, growth in enrichment medium and biochemical and serological confirmatory tests (Farber and Peterkin 1991), and takes around 10 days. DNA-based techniques such as PCR and, notably, real-time PCR (QPCR) offer a rapid, specific and sensitive alternative. QPCR assays have been developed for the quantitative detection of *L. monocytogenes* (Hough et al., 2002; Koo and Jaykus 2003; Liming et al., 2004; Nogva et al., 2000; Rodríguez-Lázaro et al., 2004b) and can exhibit limits of detection of around 100 cfu, and limits of quantification of around 1000 cfu, per g of food (Rodríguez-Lázaro et al., 2004c; Rodríguez-Lázaro et al., 2005). However, amplification of DNA from dead cells can overestimate the number of actual colony-forming units (Josephson et al., 1993). Efforts have been made to reduce amplification of DNA from dead cells through selective degradation by externally added DNases (Nogva et al., 2000) or intercalating dyes (Nogva et al., 2003; Pla et al., 2005). As messenger (m)RNA molecules generally possess shorter half-lives, they have been considered more suitable than DNA for viability assays (Bej et al., 1991; Cook 2003). Determination of viable *L. monocytogenes* has been reported by reverse-transcription (RT) coupled to PCR (Norton and Batt 1999), for which complete DNA digestion is required prior to the amplification reaction. Nucleic acid sequence-based amplification (NASBA) (Compton 1991) is a very promising alternative method, since it can selectively amplify mRNA even in the presence of genomic DNA (Simpkins et al., 2000). The NASBA product can be detected in real time using molecular beacons (Leone et al., 1998; Tyagi and Kramer 1996). This technology has been applied to the quantification of RNA viral particles (de Baar et al., 2001; Hibbitts et al., 2003; Moore et al., 2004). NASBA has the potential to detect viable microorganisms in various sample types including environmental and food matrices (Chan and Fox 1999; Cook 2003).

Although conventional NASBA has been reported (Blais et al., 1997; Uyttendaele et al., 1995), no real-time NASBA (QNASBA) assay has been published to date for *L. monocytogenes* . We describe a

QNASBA assay for selective and sensitive detection of viable *L. monocytogenes*, and its application to food samples. In addition, we present our assay as an illustrative example of the importance of target mRNA secondary structures for QNASBA optimization and discuss several technical issues that may be critical for the design of a QNASBA assay.

MATERIALS AND METHODS

Bacterial strains, culture media and growth conditions. Twenty *Listeria* isolates (15 *L. monocytogenes* and 5 non-*L. monocytogenes* isolates) and 9 non-*Listeria* spp. strains were used in this study (Table 1). The *L. monocytogenes* isolates were representative of the different serovars, as indicated in Table 1. Unless stated, *L. monocytogenes* strain CECT 4031 was used. *Listeria* strains were grown in brain heart infusion broth at 37°C, and non-*Listeria* strains were grown in MRS broth or tryptone soya broth at 30°C. For plate cultures, 1.5% (wt/vol) agar was added to these media. All media were purchased from Oxoid (Hampshire, United Kingdom).

Nucleic acid preparation and extraction. Overnight cultures were diluted in the same broth to exponential phase (approximately 10^5 ml^{-1}). Nucleic acids were extracted by the High Pure RNA Isolation kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's recommendations. Nucleic acid concentration was determined by spectrophotometry using the NanoDrop ND-1000 device (NanoDrop Technologies, Delaware, USA) and the 260/280 and 260/230 ratios were calculated (Sambrook and Russell 2001).

DNAse and RNase treatments. To confirm that the origin of the NASBA signals was indeed mRNA, prior to amplification, 500 ng *L. monocytogenes* nucleic acids were subjected to DNase or RNase enzymatic treatments. DNase treatment was performed using the RQ1 RNase-free DNase kit (Promega, Madison, USA), according to the manufacturer's instructions. RNase treatment was performed with 20 µg/ml ribonuclease A (kindly provided by M. Ribó, UdG), treated to inactivate any contaminating DNase, according to the manufacturer's instructions. After enzymatic treatment, purification of nucleic acids was performed according to Sambrook and Russell (2001). To confirm that the enzymatic treatments had been effective, the treated extracts were assayed by *hly* QPCR (Rodríguez-Lázaro et al., 2004b) and QRTPCR (i.e. conventional reverse transcription coupled to the same *hly* QPCR assay). The experiment was in triplicate and a total of 8 QNASBA reactions were performed per treatment.

Synthesis and quantification of target RNA transcript. We optimized the QNASBA using the RNA transcript hly-1339, a 605-nt molecule whose secondary structure was predicted by the *RNAstructure* program version 4.11 (Mathews et al., 2004). It corresponds to nucleotides 1339 to 1944 of the *L. monocytogenes hly* gene. We designed hly-1339 so that the QNASBA target sequence was placed in a central position, because the artificial 5'-and 3' termini of the RNA transcript may adopt secondary structures. hly-1339 was synthesized by *in vitro* transcription from a PCR product obtained with *L. monocytogenes* genomic DNA and primers T7hly-1339F and hly-1919R (Table 2). To obtain RNA target, 1 µg DNA template was transcribed *in vitro* for 1 h at 37ºC followed by a 5 min treatment with RNase-free DNase I at 37ºC. Cellulose CF11 chromatography was used to eliminate DNA fragments and non-incorporated nucleotides. The transcript was then quantified by spectrophotometry. *hly* specific QPCR was used to confirm the absence of DNA (Rodríguez-Lázaro et al., 2004b).

Synthesis of hly-1339 DNA for use as NASBA control. The dsDNA molecule DNA-hly-1339 was used as a control to assess the nature of the QNASBA template molecules. It corresponds to nucleotides 1339 to 1919 of the *L. monocytogenes hly* gene (the same as the RNA target hly-1339) and was synthesised by PCR with *L. monocytogenes* genomic DNA and primers DNA-hly-1339F (equivalent to T7hly-1339F except for the T7 sequence) and hly-1919R. Reactions were performed with TaqMan PCR core reagents (Applied Biosystems-Roche Molecular Systems Inc, Branchburg, NJ, USA) and 50-µL final volume including 1x bufferII, 1.5mM $MgCl_2$, 200 µM dNTPs, 900 nM of each primer and 1 U AmpliTaq Gold DNA polymerase. Reactions were run in a Master Cycler Gradient device (Eppendorf AG, Hamburg, Germany), using the following program: 10 min at 95ºC; 40 cycles of 20 s at 95ºC, 30 s at 56ºC and 1 min at 72ºC; and 7 min at 72ºC. The DNA-hly-1339 product was purified with the Qiaquick PCR purification kit (Qiagen Sciences Inc., Maryland, USA) and quantified by spectrophotometry and *hly* specific QPCR.

Oligonucleotide primers and probes. The oligonucleotide primers and probes used in this study are shown in Table 2. *L. monocytogenes* primers and molecular beacon corresponded to sequences from the *L. monocytogenes* virulence gene *hly* (GenBank accession no.M24199), encoding the hemolysin listeriolysin O (Mengaud et al., 1988). Several *L. monocytogenes* specific QPCR assays (Koo and Jaykus 2003; Nogva et al., 2000; Rodríguez-Lázaro et al., 2004b) and a *L. monocytogenes* NASBA (Blais et al., 1997) have been developed targeting *hly*. We previously reported that a 512-bp (nt 1346 to 1858) portion of this gene showed a high degree of interstrain sequence conservation in a panel of isolates of serovars representative of the three phylogenetic divisions of the species (Rodríguez-Lázaro et al., 2004b); GenBank accession numbers

AY174657 to AY174669), which makes it suitable for analysis of an *L*. *monocytogenes* strain regardless of its genetic background. To develop a NASBA applicable to the entire species *L*. *monocytogenes*, this conserved portion of *hly* was used as target. Subsequently, the *RNAstructure* program version 4.11 was used to predict regions with stable secondary structure within the *hly* RNA target; and the *oligowalk* tool (Mathews et al., 1999) of the same software was used to design primers hlyF and hlyR and molecular beacon hlyMB to target accessible sequences (i.e. break target ΔG values above -8.0 kcal mol^{-1}). As a control, hlyF2 and hlyR2 were designed in a highly structured region (i.e. break target ΔG value of -33.0 kcal mol^{-1}).

Primers hlyF and hlyR amplified a 125-bp fragment of *hly*. The BLAST-N v. 2.2.6 tool (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) was used to confirm that the selected oligonucleotides were specific for *L. monocytogenes hly*. Blast search was carried out by using default settings of the tool. All oligonucleotides were purchased from MWG Biotech AG (Germany).

IAC synthesis. The QNASBA IAC was an *in vitro* synthesized chimeric RNA molecule containing an internal non-target sequence flanked by sequences complementary to the target QNASBA primers hlyF and hlyR. The non-target sequence was a 105-bp fragment derived from *Zea mays*. The construction strategy was that of (Rodríguez-Lázaro et al., 2004a). The IAC sequence was 5'-UGCGCAACAAACUGAAGCAAAAGCAACCUCUACCAAAUCUACGCUGAGAGCUUCCGCGAGUGGGAG GCCGACCCCACUAACCCAGCUCUCCGCGAGGAGAUGCGCAUCCAGUUCAACGACAUGAACAUCGAA AAGAAACACGCGGAUG-3' and did not show any significant homology to any sequence deposited in public databases when analyzed using the BLAST-N v. 2.2.6 software (cutoff E-value, 0.1).

QNASBA. Reactions were carried out using the NucliSens® Basic Kit (bioMérieux bv, The Netherlands). The primers and probe concentrations were initially optimized with hly-1339 (data not shown) to give the lowest time to positivity (T_p, equivalent to threshold cycle (C_T) in real time PCR assays) and the highest normalized fluorescence intensity ($\triangle R_n$) (Perkin-Elmer Applied Biosystems User Bulletin 2 [ABI PRISM 7700 Sequence Detection System], 1997). The IAC concentration per reaction was subsequently optimised to the lowest copy number giving consistent T_P (i.e. standard deviation S.D. below 10%) in order to avoid any interference with target amplification. This was performed using 10^3 copies of hly-1339 and 10^5, 10^4, 3×10^3 and 10^3 copies of IAC.

In the optimised assay, 1.25 µl of target template solution was added to 6.25 µl NASBA pre-mix containing 10^3 copies IAC in 80 mM Tris-HCl (pH 8.5), 24 mM $MgCl_2$, 140 mM KCl, 30% v/v DMSO, 2 mM

each dNTP, 4 mM each NTP, 200 nM hlyF primer, 500 nM hlyR primer and 200 nM hlyMB (FAM and DABCYL dual-labelled) and IACMB (HEX and DABCYL dual-labelled) molecular beacons. The mixtures were incubated at 42°C for 5 min; and 2.5 µl of enzyme mixture (0.08 U/µl RNase H, 32 U/µl T7 RNA polymerase, 6.4 U/µl AMV reverse-transcriptase per reaction) was subsequently added. Reactions were incubated at 42°C for 90 min in an ABIPrism® 7700 Sequence Detection System (Applied Biosystems, Foster City, USA) and fluorescence was monitored in real-time. Unless otherwise stated, all reactions were performed in a total of 8 replicates in 3 different QNASBA runs that corresponded to 3 independent experiments for *hly* transcript or 3 biological replicates for *L. monocytogenes* cells. Non-template controls (NTC) were included to determine the threshold T_P and ΔRn values to consider a positive result.

Initial reactions for the design and optimization of QNASBA either included 5-min incubation at 65ºC (i.e. standard reaction conditions recommended by the NucliSens® Basic Kit manufacturer) or 95ºC prior to 42ºC for 5 min and addition of the enzyme mix; or direct incubation at 42ºC for 5 min before addition of the enzyme mix.

Limit of detection and quantification capacity of *hly* QNASBA. The limit of detection (LOD) was established using the hly-1339 RNA transcript. In three independent experiments, serial dilutions of hly-1339 RNA transcript were prepared and 4 replicates of samples containing 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1 target copies were analyzed. For each dilution, the expected numbers of target molecules per reaction were calculated at the 95% confidence level by Monte Carlo simulations taking into account the experimental design (i.e. decimal dilutions in a final volume of 0.1ml). In parallel, three replicates of exponentially growing *L. monocytogenes* cell cultures were quantified by the standard plate count technique (Anonymous 1998b) and ten-fold serial dilutions were prepared in phosphate-buffered saline (PBS, Dulbecco's, Gibco, Paisley, Scotland), corresponding to 4×10^5, 4×10^4, 4×10^3, 4×10^2, 40, 4 and 0.4 CFU/ml. From each dilution, nucleic acids were extracted and subjected to QNASBA. The number of positive signals obtained were expressed as a percentage, and compared with the number of target molecules / cells in each dilution (Knutsson et al., 2002).

Quantification capacity was assessed by plotting the T_P values obtained against the logarithm of the number of target molecules or *L. monocytogenes* CFU in each dilution and calculating a linear regression. Both the slope and R^2 values are given.

Sample treatment for comparison of NASBA signals from viable and non-viable cells. In three replicates, *L. monocytogenes* cells were grown in brain heart infusion broth at 37°C to exponential phase

187 (approximately 10^5 ml^{-1}). Two 5-ml aliquots (#1 and #2) were taken from the culture. Aliquot #1 was
188 immediately processed (see below) and aliquot #2 was incubated at 65ºC in a heated water bath for 5 min,
189 to kill cells prior to processing. Monitoring of the sample temperature was carried out in a separate 5-ml
190 aliquot that was treated in parallel and discarded: the time of incubation was considered to start when the
191 sample temperature was 65ºC. Colony forming units of aliquots #1 and #2 were quantified by plate counting
192 or most probable number techniques (MPN) (Anonymous 1998b). One ml of each aliquot was digested with
193 20 µg/ml ribonuclease A for 40 min at 37ºC; and subsequently RNA was extracted and QNASBA was
194 performed as previously indicated.

195 Preliminary experiments were conducted to assess the persistence of *hly* mRNA target in non-
196 viable cells. Heat-treated cells were kept at RT for 5, 15, 30, 60, 120 and 180 min. then analyzed by MPN
197 and QNASBA. In all cases QNASBA was found to overestimate cell countings around 1 logarithmic unit. To
198 achieve full and quick degradation of *hly* mRNA from non-viable cells a ribonuclease A treatment was
199 included before nucleic acid extraction. Control experiments were carried out to demonstrate that
200 ribonuclease A digestion did not affect the QNASBA signal from exponentially growing cells.

201 **Artificial contamination of meat and salmon with *L. monocytogenes* and sample treatment prior to**
202 **QNASBA.** In three replicates, samples of 25 g of cooked ham or smoked salmon slices obtained in local
203 markets were artificially contaminated with exponentially growing *L. monocytogenes* cells diluted in peptone
204 water at the following approximate concentrations: 10^4, 10^3, 10^2, 10, 1, 0.1 and 0 (control) UFC/g. *L.*
205 *monocytogenes* was analyzed in all samples by standard microbiological methods (according to document
206 ISO 11290 (Anonymous 1998a) and QNASBA. Briefly, slices were diluted (1:10) with half-Fraser broth
207 (Oxoid), homogenized for 1 min in stomacher bags (125-µm pore size; BioChek, Gouda, Holland) and
208 incubated at 30ºC for 24h. One ml of each pre-enrichment was used for nucleic acid extraction and
209 QNASBA. Pre-enrichment cultures were further diluted (1:100) with Fraser broth (Oxoid) and incubated at
210 30ºC. One-ml aliquots were taken after 24 h and 48 h enrichment for nucleic acids extraction and QNASBA.
211 After 48 h enrichment, 0.1-ml aliquots were plated on Palcam agar plates and a number of colonies were
212 confirmed by API *Listeria* (bioMérieux, France).

213 **Statistical analyses.** Statistical treatment of the data was performed using SPSS for windows v13.0 (SPSS
214 Inc, Chicago, IL). Descriptive analyses, one-way ANOVA and (when applicable) pair-wise comparison of
215 mean values by Tukey's test were used, with a significant level of $P<0.05$.

216

RESULTS AND DISCUSSION

Role of the secondary structure in the design of primers and probes for NASBA.

The strategy used to design oligonucleotides suitable for *L. monocytogenes*-specific QNASBA was based on target sequence specificity and *in silico* predicted secondary structure. The former is a classical approach for nucleic acid amplification-based techniques e.g. PCR. In contrast, the importance of the role of secondary structures in the design of NASBA has not been emphasised to date. RNA molecules tend to adopt secondary structures whose formation and stability can be theoretically predicted on a sequence basis using secondary structure prediction algorithms. Accessibility to the target sequence is a crucial requirement for successful interaction of nucleic acid molecules (e.g. (Branch 1998; Nadal et al., 2005; Scherr et al., 2000). This is especially relevant for NASBA due to its isothermal nature where typically no denaturing steps are included which could destabilise target secondary structures.

We designed two different primer pairs. They targeted either predictably accessible sequences (oligonucleotides hlyF/R/MB, Table 2, Figure 1) or sequences involved in a predicted strong secondary structure (oligonucleotides hlyF2/R2/MB, Table 2, Figure 1). For the NASBA amplicon and the 600-nt RNA hly-1339, break target ΔG values were -1.3 to -6.9 kcal mol^{-1}; and -17.4 to – 32.3 kcal mol^{-1} respectively.

In combination with hlyMB probe (Table 2) and in the standard reaction conditions, the performance of QNASBA targeting accessible sequences was significantly higher than the amplification obtained when the primers were placed in predictably structured sequences (Table 3). When 100 target copies (i.e. 100-fold less than the former reactions) were subjected to NASBA they could only be detected using the primer pair which targets accessible sequences (100% *vs.* 0% of the replicates).

We further confirmed that target structural impediments were the source of such differences by either denaturing the target RNA before NASBA was performed or preserving any secondary structure (i.e. direct 42ºC incubation). As shown in Table 3, RNA incubation at 95ºC prior to NASBA allowed primers targeting poorly accessible sequences to consistently prime target amplification, whereas preservation of target secondary structures resulted in lower percentages of positive amplification (50% and 75% at the respective lower temperatures). Amplification of the sequences with strong secondary structure was not completely inhibited; a possible explanation is that they are partially accessible as a function of “RNA breathing”. Such breathing occurs temporally randomly (and increases with the temperature). This might cause the NASBA reaction to slow down (higher Tp values) and increase the SD values. Primers targeting accessible sequences performed equally well after 95ºC, 65ºC and 42ºC pre-treatments (higher

247 percentages of positive reactions and lower T_P and SD values). These results evidence a correlation
248 between strong *in silico*-predicted secondary structure of target sequences and the reduced yield of RNA
249 amplification by QNASBA. Therefore, careful *in silico* analysis of the predicted structure of the target is
250 recommended as a preliminary step in the development of NASBA. It should also be remembered that
251 applying high temperatures to denature any mRNA secondary structure prior to NASBA, could result in
252 denaturation of the genomic DNA; subsequent primer annealing would then facilitate amplification of the
253 DNA sequences, which would be especially undesirable in a viability assay. Omission of a high-temperature
254 step shortens the assay in comparison to including DNase treatment.
255
256 **Quantification of *L. monocytogenes* target RNA transcripts by QNASBA.**
257 Table 4 shows the detection probability against target copy number. Down to 100 copies of target
258 RNA could be detected in all replicates. A T_P threshold was established at 35 (i.e. the highest expected T_P
259 [with 95% probability] for the lowest number of target molecules giving consistent QNASBA signal). The IAC
260 was correctly amplified in all reactions containing up to 1000 RNA target molecules (mean T_P value, 44.5;
261 SD, 1.6); thus proving that not only hly-1339 positive but also negative QNASBA replicates performed
262 adequately.
263 Regression curves were calculated for T_P values obtained in QNASBA reactions corresponding to
264 10^6 to 10^3 target copies (i.e. they displayed T_P relative S.D. values below 5 %). The number of RNA target
265 copies correlated with T_P in a highly linear manner as demonstrated by R^2 values above 0.998. The slopes
266 (i.e. -3.61) were similar to the theoretical optimal ones calculated for real-time PCR assays (Higuchi et al.,
267 1993) and in particular to the ones obtained in the *hly* based QPCR which targets the same gene region (nt
268 1603 to 1667, (Rodríguez-Lázaro et al., 2004b). Thus, the reaction proceeded efficiently.
269 These results indicate that our QNASBA assay has the potential for accurate quantification of the
270 RNA target. The use of target RNA transcripts as calibrants for quantification by QNASBA has been
271 reported (e.g. (Moore et al., 2004; Polstra et al., 2002). Our results stress the importance of the secondary
272 structure in NASBA and therefore highlight the need for calibrants that adopt secondary structures similar to
273 those in the target.
274 One critical parameter for the validation of an alternative method is the relative accuracy, i.e. the
275 closeness of agreement between a test result and the accepted reference value (according to document
276 ISO 16140 (Anonymous 2002). The quantification capacity of our *hly* QNASBA was further tested by

assessing the degree of correspondence between the quantitative data obtained by spectrophotometry and the QNASBA assay using hly-1339 transcript. QPCR was used to confirm that the amount of DNA in the transcript RNA samples was residual (data not shown). Four hly-1339 transcript solutions at different concentrations (i.e. around 1×10^6, 1×10^5, 1×10^4 and 1×10^3) were quantified by the two methods (Table 5). The T_P values obtained were extrapolated to the corresponding standard regression curve, previously calculated experimentally using hly-1339 as calibrator. The obtained values differed by less than 21%, indicating good correspondence along a 4-logarithmic range e.g. (Rodríguez-Lázaro et al., 2004b). Quantitative values obtained with QRTPCR produced similar results (data not shown).

hly QNASBA detects _L. monocytogenes_ RNA but not genomic DNA.

NASBA has been shown to selectively mediate the detection of RNA. This should eliminate the necessity for DNase treatment, which is required when using RT-PCR to try to ensure that the signal comes only from amplification of RNA (Klein and Juneja 1997). But our previous results with a *M. avium* subsp. *paratuberculosis* NASBA (Rodríguez-Lázaro et al., 2004e) indicated that in this bacterium NASBA can detect DNA. To assess the use of our *hly* QNASBA to detect *L. monocytogenes* RNA we verified the nucleic acid origin of our QNASBA signal. To do this, in 3 independent experiments *L. monocytogenes* nucleic acid extracts were used directly in the QNASBA or used after treatment with DNAse I or RNAse A. Equal amounts of *hly* nucleic acids as determined by *hly* QRTPCR and *hly* QPCR were subsequently subjected to QNASBA. The quantitative results obtained by extrapolating T_P values to a hly-1339 transcript based standard curve demonstrate that the QNASBA substrate was indeed RNA. Samples with mainly RNA (i.e. those treated with DNAse I) displayed the highest readings: $T_P = 24.9 \pm 0.3$, corresponding to $4.8\times10^3 \pm 1.2\%$ *hly* mRNA molecules per reaction. As expected, non-treated samples containing RNA but also DNA exhibited significantly lower ($p<0.05$) values ($T_P = 27.3 \pm 0.4$, $1.3\times10^3 \pm 1.5\%$ *hly* mRNA molecules per reaction). On the contrary, RNase A=treated samples did not produce any positive QNASBA signal. This proved that genomic DNA is not a substrate for amplification in our QNASBA.

Specific and sensitive detection of _L. monocytogenes_ by _hly_ QNASBA.

The *hly* QNASBA detected only the *L. monocytogenes* isolates (Table 1). Positive IAC signals showed that the negative results were true (data not shown). This proved the target specificity of the assay.

306 Table 4 shows the QNASBA results obtained with serial dilutions of a *L. monocytogenes* overnight
307 culture. *hly* RNA could be consistently detected in cultures containing 400 CFU / ml of *L. monocytogenes* as
308 determined by plate cell counting (i.e. limit of detection LOD, 400 CFU / ml). Samples containing 40 cfu were
309 positive in 25 % of analyses. These values are similar to other published NASBA e.g. (Rodríguez-Lázaro et
310 al., 2004d).

311 The number of *L. monocytogenes* CFU / ml (from 4×10^5 to 4×10^2 CFU / ml) correlated with T_P in a
312 highly linear manner as established by R^2 values of the calculated linear regression curves above 0.97. The
313 slopes (i.e. -3.57) showed that the amplification was very efficient. These values were especially relevant
314 taking into account that they were obtained from serial dilutions of exponentially growing cells and each
315 sample was independently subjected to nucleic acid extraction prior to QNASBA. Consequently, deviation
316 from the linear relationship of T_P and CFU / ml was probably due to the difference in RNA extraction
317 efficiencies at various RNA concentrations. It should be remarked that all samples derived from a single
318 exponentially growing culture; and therefore the mean levels of *hly* RNA per CFU were similar.

319 The *hly* copy numbers calculated with hly-1339 transcript calibrators indicated that, under the
320 conditions used in the tests, *L. monocytogenes* cells harbored around 2.3 *hly* mRNA molecules per cell.

321

322 **QNASBA amplification of mRNA distinguished viable from non-viable *L. monocytogenes*.**

323 In 3 independent experiments, *L. monocytogenes* cells were either incubated at 65ºC or left
324 untreated prior to MPN enumeration and QNASBA (Table 6). QNASBA quantification of untreated aliquots
325 was possible by using a regression curve constructed with serial dilutions of the same cell culture (i.e. the
326 *hly* expression levels per cell were the same). As expected, the obtained cell numbers correlated with CFU
327 shown by standard microbiological methods. Heat-treated aliquots showed survival rates of around 0.3 % as
328 determined by MPN technique. QNASBA analyses of heat-treated samples produced inconsistent results
329 (RSD values above 10%), with T_P readings significantly ($P<0.05$) higher than untreated cells and above the
330 LOD. In contrast, *hly* QPCR-based quantification of *L. monocytogenes* produced the same results in
331 untreated and heat-treated cells. This demonstrates the suitability of our QNASBA method to detect viable
332 *L. monocytogenes* cells.

333

334 **Application of *hly* QNASBA in food matrices.**

Many countries have a zero tolerance policy regarding the presence of *L. monocytogenes* in food (European Commission 2005; FDA FSIS 2003). Examples of food products considered to be at risk for transmission of human listeriosis are smoked salmon and processed meat products because of the frequent occurrence of *L. monocytogenes* in food processing plants (Gravani 1999), and since this bacterium can survive the cold-smoking process (Farber and Peterkin 1991; Rijpens and Herman 2002). In three replicates, cooked ham and smoked salmon slices were artificially contaminated with decreasing amounts of *L. monocytogenes* cells and analyzed by standard microbiological methods (Anonymous 1998a). One-ml aliquots were taken after pre-enrichment and after 24 and 48 h enrichment and subjected to nucleic acids extraction and *hly* QNASBA. As shown in Table 7, QNASBA detected *L. monocytogenes* immediately after pre-enrichment in samples containing down to 10^2 CFU per g of cooked ham; and down to 10^3 CFU per g of smoked salmon. Interestingly, consistent positive QNASBA signals were obtained from all food samples artificially contaminated with down to approximately 10^{-1} *L. monocytogenes* per g of product (i.e. around 2.5 CFU / 25 g) after 48 h and also 24 h enrichment in Fraser broth. Moreover, T_P values obtained after 48 and 24 h enrichment were not statistically different ($p<0.05$) and proved to be highly consistent i.e. overall T_P mean = 17.64 ± 0.89 (RSD, 5.0%) for salmon and overall T_P mean = 19.88 ± 2.78 (RSD, 13.9%) for cooked ham samples. The same results were obtained through conventional microbiological analyses (Table 7); and by *hly* QPCR (data not shown). This proved that our *hly* QNASBA assay in combination with a standard pre-enrichment and enrichment protocol can provide reliable *L. monocytogenes* detection in around 3 working days.

As expected, control samples inoculated with 0 CFU *L. monocytogenes* / 25g product produced QNASBA negative results (IAC positive) in all tested conditions.

We describe the first QNASBA assay for selective and sensitive detection of *L. monocytogenes*. It targets *hly* mRNA; and includes an IAC to disclose failure of the reaction (Hoorfar et al., 2004) . Our QNASBA is capable of accurate quantification of RNA molecules present in a sample using a long RNA transcript containing the target as calibrator. This makes our QNASBA a useful tool for RNA quantification with a range of different purposes. As an example, *hly* NASBA results linearly correlated with the number of *L. monocytogenes* cells along a 4-logarithmic range.

Our *hly* QNASBA is an illustrative example of a number of technical issues critical for most favorable design and optimization of a molecular beacon-based QNASBA assay. The formation of intramolecular structures has been reported to have an effect on intramolecular interactions involving

nucleic acids such as small interfering RNAs (Bohula et al., 2003; Lee et al., 2002), antisense oligonucleotides (Kretschmer-Kazemi and Sczakiel 2003; Vickers et al., 2000), *trans*-cleaving ribozymes (Amarzguioui et al., 2000; Campbell et al., 1997; Nadal et al., 2003) and triplex-forming clamps (Nadal et al., 2005). Therefore, target mRNA secondary structures may pose a substantial but as yet unaddressed problem regarding interaction with primers and probes for NASBA. Our results show a correlation between the design of NASBA primers and probe targeting highly accessible sequences (as determined by *in silico* analyses and experimentally confirmed) and excellent NASBA efficiency and sensitivity. In contrast, primers designed against strongly structured sequences resulted in poor amplification efficiencies and sensitivities. Therefore, we strongly recommend accessibility mapping of the target RNA (e.g. by *in silico* prediction of secondary structure) aimed at the design of primers and probes targeting accessible sequences. Moreover, we demonstrate that an adequate design can obviate the 65ºC incubation commonly performed prior to NASBA in order to destabilize the secondary structure of RNA. This may have important implications e.g. (i) it converts NASBA into a real isothermic reaction, thus simplifying the experimental procedure; and (ii) it may avoid short or partially degraded DNA molecules denaturing and becoming substrates for NASBA, which is especially important for viability issues. Finally, our results show that quantification of mRNA is possible by using QNASBA and the corresponding *in vitro* synthesized transcript to construct a standard curve. However, it is important to remark that target sequences within such calibrants should adopt secondary structures similar to the ones of the mRNA target. In our *hly* QNASBA example this was achieved by using a long RNA *hly* transcript (i.e. 589 nt) in which the QNASBA target sequences were placed at the central positions 211 to 315.

As our assay can accurately quantify *hly* mRNA molecules, and *hly* is an important virulence factor (Vazquez-Boland et al., 2001b; Vazquez-Boland et al., 2001a) and the principal target in molecular methods for *L. monocytogenes* analyses (Hough et al., 2002; Koo and Jaykus 2003; Nogva et al., 2000; Rodríguez-Lázaro et al., 2004b), our QNASBA is a useful tool to assess *hly* expression under different *in vivo* and *in vitro* conditions. As an example, our preliminary assays suggested that *hly* levels slightly rose with temperature (from 37 to around 50ºC) and were significantly lower ($p<0.05$) at 10ºC. Changes in the extracellular medium composition (e.g. activated charcoal) have also been reported to induce the expression of *hly* (Ermolaeva et al., 1999; Ripio et al., 1996).

NASBA reactions for viability purposes commonly require a post-killing incubation to allow degradation of target mRNA from dead cells. It has been demonstrated that mRNA degradation can be

dependent on the susceptibility of the transcript (Alifano et al., 1994), or regions thereof (Norton and Batt 1999). As an example, a 15 min post-pasteurization treatment is enough to achieve complete abolishment of a *Salmonella* spp. specific NASBA signal (Simpkins et al., 2000); although positive NASBA was still obtained up to 24 h and 30 h after *E. coli* cell death (Birch et al., 2001; Min and Baeumner 2002). Although our *L. monocytogenes* QNASBA primers and probe were complementary to regions of the target mRNA predictably susceptible to enzymatic degradation as recommended (Cook 2003), up to 3 h post-heat treatment could not fully abolish the QNASBA signal originating from dead cells. To avoid long post-killing treatments, we coupled our QNASBA assay to an RNase A treatment which rapidly and fully degraded target mRNA from dead cells. Using this method, our QNASBA assay was capable of specifically detecting viable *L. monocytogenes* cells.

It should be remarked that the two methods (microbiological and QNASBA after 24- or 48-h enrichment) correctly detected *L. monocytogenes* in all tested artificially contaminated food samples, with no false positive or false negative results at any contamination level nor in any of the tested food matrices. Our *L. monocytogenes* QNASBA assay provides a useful additional analytical tool for analysis of clinical and environmental samples. It would be especially useful for analysis of products in which high loads of inactive *L. monocytogenes* cells are expected, producing false positive QPCR results. As with QPCR,e.g. (Rodríguez-Lázaro et al., 2005), quantification of *L. monocytogenes* in food products could be envisaged by a combination of MPN method and QNASBA based confirmation of liquid cultures.

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TABLES

TABLE 1. Bacterial strains used in this study and specificity of our *L. monocytogenes* QNASBA.

L. monocytogenes strain	serovar	QNASBA result[a]	Non-*L. monocytogenes* species	strain	serovar	QNASBA result[a]
CECT[b] 932 [d]	1/2a	+	*L. grayi*	CECT 931 [d]		-
CECT 4031 [d]	1/2a	+	*L. innocua*	CECT 910 [d]	6a	-
CECT 936 [d]	1/2b	+	*L. ivanovii*	CECT 913 [d]	5	-
CECT 911 [d]	1/2c	+	*L. seeligeri*	CECT 917 [d]	1/2b	-
UdG[c] 1010 [e]	1/2c	+	*L. welshimeri*	CECT 919 [d]	6a	-
UdG 1011 [e]	1/2c	+	*Brochothrix thermosphacta*	UdG 1510 [e]		-
CECT 933 [d]	3a	+	*Enterococcus malodoratus*	UdG 7007 [e]		-
CECT 937 [d]	3b	+	*Lactobacillus murinus*	UdG 7004 [e]		-
CECT 938 [d]	3c	+	*Lactobacillus reuteri*	UdG 7010 [e]		-
CECT 934 [d]	4a	+	*Lactococcus garviae*	UdG 7001 [e]		-
CECT 935 [d]	4b	+	*Leuconostoc carnosum*	UdG 747 [e]		-
CECT 4032 [d]	4b	+	*Pediococcus pentosaceus*	UdG 745 [e]		-
UdG 1034 [e]	4b	+	*Salmonella enterica*	CECT 702 [d]		-
CECT 5725 [d]	4c	+	*Staphylococcus aureus*	CECT 4520 [d]		-
CECT 940 [d]	4d	+				

[a] All reactions were performed with 1 ng nucleic acids extracted from a 10^5 CFU / ml culture. Two biological replicates were analyzed per species, giving in all cases the same results.

[b] CECT: Colección Española de Cultivos Tipo

[c] UdG: Universitat de Girona

[d] source: collection

[e] source: food plant (meat)

573 TABLE 2. Oligonucleotides used in the QNASBA and QRTPCR assays for *L. monocytogenes*.

Use	Name	Type	Sequence	Position
QNASBA	hlyF	Forward primer	5'- TGC GCA ACA AAC TGA AGC AAA -3'	1542
	hlyR	Reverse primer	5'- aat tct aat acg act cac tat agg gag aag gCA TCC GCG TGT TTC TTT TCG A -3'	1646
	hlyMB	Molecular Beacon	5'- FAM – CGA TCG CCT GCA AGT CCT AAG ACG CCA CGA TCG - DABCYL -3'	1624
	lACMB	Molecular Beacon	5'- HEX – CGC AGG CCA CTA ACC CAG CTC TCC GCG ACC TGC G - DABCYL -3'	
	hlyF2	Forward primer	5'- CAT GGC ACC ACC AGC ATC T -3'	1624
	hlyR2	Reverse primer	5'- aat tct aat acg act cac tat agg gag aag gGC ACA TTT GTC ACT GCA TCT C -3'	1727
Construction hly-1339	T7 hly-1339F	Forward primer	5'- taatacgactcactataGGGACAGCAGGACTAGAA -3'	1339
	hly1919R	Reverse primer	5'- AATGAATCACGTTTTACAGGGAGAA -3'	1919

574 Lowercase: T7 promoter sequence.

575 Underlined: stem sequences for molecular beacons

576 TABLE 3. QNASBA amplification of hly-1339 RNA transcript (10^4 copies) with different primer pairs and pre-

577 treatments.

Primer pair	hlyF/R			hlyF2/R2		
Temperature pre-treatment	42ºC	65ºC	95ºC	42ºC	65ºC	95ºC
Percentage of positive results	100	100	100	50	75	100
T_P mean ± S.D.[a]	21.7[b] ± 0.4	21.6[b] ± 1.1	22.2[b] ± 0.2	59.2[c] ± 10.2	36.7[c] ± 3.4	57.3[c] ± 9.2

578 Results were obtained in a total of 8 reactions corresponding to 3 independent experiments. In each experiment, a new aliquot of 10^4

579 target RNA molecules / 5 µl was used.

580 [a] Calculated only for the positive results.

581 [b/c] Different letters indicate statistically significant differences between groups ($P<0.05$).

582 TABLE 4. QNASBA results using different initial amounts of RNA target and *L. monocytogenes* cells.

hly-1339 copy number	10^6	10^5	10^4	10^3	10^2	10	1
% of positive results	100	100	100	100	100	25	0
T_P (Mean ± S.D.) [a]	16.7 ± 0.6	19.9 ± 0.3	23.7 ± 0.1	27.5 ± 0.3	32.4 ± 2.5	33.1 ± 3.6	
% of IAC positive results	0	0	0	100	100	100	100
L. monocytogenes CFU/ml	4×10^5	4×10^4	4×10^3	4×10^2	40	4	0.4
% of positive results	100	100	100	100	25	0	0
T_P (Mean ± S.D.) [a]	17.4 ± 0.1	20.3 ± 0.5	22.9 ± 1.0	28.2 ± 1.4	34.8 ± 4.5		
% of IAC positive results	0	0	0	0	100	100	100

583 All reactions were performed in the presence of 1000 copies of IAC.

584 Results were obtained in a total of 8 reactions corresponding to 3 independent experiments (hly-1339 transcript) or biological replicates

585 (*L. monocytogenes* cells).

586 [a] Calculated only for the positive results.

587 TABLE 5. Quantification of *hly-1339* RNA transcript by QNASBA and spectrophotometry.

	Calculated *hly-1339* copy number			
Spectrophotometry	1×10^{6}	1×10^{5}	1×10^{4}	1×10^{3}
QNASBA (Mean ± S.D.)	1.1×10^{6} ± 2%	1.2×10^{5} ± 17%	1.0×10^{4} ± 5%	0.9×10^{3} ± 18%
% of correspondence	110%	121%	104%	92%

588 Results were obtained in a total of 8 reactions corresponding to 3 replicates.

589 Spectrophotometry values were calculated from the 3 independent stock solutions. Triple lectures showed S.D. below 5%.

590 TABLE 6. MPN count and QNASBA analysis of viable and heat-treated *L. monocytogenes* cells.

		Treatment	
		Non-treated	Heat-treated
MPN	CFU/ml	4.3×10^4	125
QNASBA	T_P	20.4^a	29.5^b
	CFU/ml	3.9×10^4	< LOD
QPCR	C_T	17.7^c	18.6^c
	CFU/ml	2.9×10^4	1.6×10^4

591 Results are mean of a total of 8 reactions corresponding to 3 biological replicates.

592 Except for QNASBA of heat-treated cells, R.S.D. was always below 10%.

593 $^{a/b, c}$ Different letters indicate statistically significant differences between groups ($P<0.05$).

TABLE 7. Conventional and QNASBA determination of *L. monocytogenes* cells in cooked ham and smoked salmon samples.

	L. monocytogenes CFU/g	10^4	10^3	10^2	10	01	0.1	0
Cooked ham	Microbiological method (ISO)	+	+	+	+	+	+	-
	hly QNASBA (pre-enrichment)	+	+	+	-	-	-	-
	hly QNASBA (24 h enrichment)	+	+	+	+	+	+	-
	hly QNASBA (48 h enrichment)	+	+	+	+	+	+	-
Smoked salmon	Microbiological method (ISO)	+	+	+	+	+	+	-
	hly QNASBA (pre-enrichment)	+	+	-	-	-	-	-
	hly QNASBA (24 h enrichment)	+	+	+	+	+	+	-
	hly QNASBA (48 h enrichment)	+	+	+	+	+	+	-

All reactions were performed in the presence of 1000 copies of IAC. All *hly* negative QNASBA showed positive IAC amplification.

Results were obtained in a total of 8 reactions corresponding to 3 independent biological replicates.

562 **FIGURE LEGEND**

563 **FIGURE 1.** *In silico* predicted secondary structure of *hly*-1339 RNA transcript (only nt 1539 to 1781 portion
564 is shown) and position of QNASBA primers and molecular beacon.

598 **FIGURES**

599

600 **Figure 1**

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