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Journal of Microbiological Methods 66 (2006) 206–216
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Evaluation of probe chemistries and platforms to improve the detection limit of real-time PCR

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Received 15 September 2005; received in revised form 28 September 2005; accepted 4 November 2005
Available online 20 December 2005

Abstract

A validated PCR-based *Salmonella* method targeting a 94-bp sequence of the *ttr* gene was used as a model to compare six different combinations of reporter and quencher dyes of a *Taq*Man probe, on three different instruments, to improve the detection limit in a real-time PCR assay with the aim of a same-day analysis. The use of locked nucleic acids (LNA) and Scorpion probes were also tested. The combination FAM–BHQ1 or Cy5–BHQ3, both dark quenchers, gave the best results (Cycle threshold (Ct) of 25.42 ± 0.65 and 24.47 ± 0.18 at 10^3 DNA copies). When comparing different probe technologies, the LNA probe (FAM–BHQ1) was the most sensitive with the strongest fluorescence signal (dR last 48 066), resulting in 0.6 to 1.1 lower Ct values than a DNA *Taq*Man probe, and 1.9 to 4.0 lower Ct than the Scorpion system (FAM–BHQ1). The RotorGene real-time PCR instrument gave 0.4–1.0 lower Ct values (more sensitive) than the Mx3005p, and 1.5–3.0 lower than the ABI 7700. Using the LNA in a RotorGene instrument, we detected the following *Salmonella* DNA copies in 1-ml pre-enriched samples: fishmeal (100 copies), chicken rinse (100 copies) and pig feces (10 copies). The detection probability of the final assay on inoculated fecal samples was 100% at 2×10^4 copies per ml. In conclusion, the LNA probe with annealing temperature of 65 °C could be useful for more sensitive detection limits.
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Keywords: PCR; Real-time; *Salmonella*; Probe; Diagnostic

1. Introduction

The increasing use of quantitative real-time PCR for the detection of pathogens and in other applications has provided the possibility of faster and more accurate diagnostics (Mackay, 2004). However, in sub-clinical samples, foods, feed or environmental samples with low amounts of target pathogens, there is a need for more sensitive probe technologies in order to detect very few target DNAs in the presence of large background flora in often PCR inhibitory sample matrices (Marlony and Hoorfar, 2005). In addition, the interaction of sample constituents with probe dyes can compromise fluorescence readings (Hoorfar et al., 2004).

The use of dark quenchers has the advantage over other conventional quenchers such as TAMRA, of being non-fluorescent, reducing the background by eliminating the risk of fluorescence interference by the quencher (Lukhtanov et al., 2001). Among the sensitive alternative probe technologies, the Scorpion system relies on the intramolecular probing. The probe is incorporated into the amplified DNA and binds internally with the advantage of the probe being in close proximity to the target sequence and thus increases the chance of a fluorescent signal. The locked nucleic acid (LNA) probes have a

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doi:10.1016/j.mimet.2005.11.006

higher melting temperature than *Taq*Man probes because of the locked nucleotides which are a nucleic acid analogues containing a bicyclic furanose unit locked in an RNA-mimicking sugar conformation (Kumar et al., 1998; Whitecombe et al., 1999). These modifications of LNA oligonucleotides provide stronger hybridisation between the two strands of a DNA molecule and thus, higher melting temperature. More sensitive probes can help by decreasing the enrichment time needed for the detection of a few DNA copies and thereby obtain faster diagnostic response. Rapid diagnostics methods with short enrichment times can help both diagnostic laboratories and food producers (Hagen et al., 2005; Rodriguez-Lazaro et al., 2004).

Thus, the purpose of the present study was to evaluate the detection sensitivity of different dark-quenchers and reporter dyes in a *Taq*Man assay and compare it to new promising technologies, such as LNA probes (Kumar et al., 1998) and the Scorpion system (Whitecombe et al., 1999). Although many probe chemistries are available with combinations of novel reporter and dark quenchers (Moreira et al., 2005), to our knowledge, no systematic study has been conducted on the comparison of various advanced probe dyes and quenchers in the same diagnostic PCR assay.

A thoroughly validated *Taq*Man PCR method (Marlony et al., 2004) on the detection of *Salmonella* was used as an evaluation model in combination with different detection technologies and three instrument platforms (RotorGene 3000, Mx3005p and ABI 7700) to assess the robustness of the assay.

2. Materials and methods

The overall experimental setup and overview of the comparative study is shown in Fig. 1.

2.1. Genomic DNA

DNA was purified previously from *Salmonella enterica* serovar Typhimurium 51K61 (BfR, Berlin, Germany) from an overnight culture with the DNeasy kit (Qiagen, Hilden, Germany). About 1 μg of a vacuum-dried DNA was diluted in 100 μl of Tris–EDTA buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). The DNA concentration was measured in a ND-1000 Nanodrop spectrometer (Nanodrop Technologies, DE, USA). The genomic DNA (gDNA) copy number was calculated from the genome size of *S. enterica* Typhimurium LT2 of 4,857,432 bp (McClelland et al., 2001) by the way of Josefsen et al. (2004). Tenfold dilution series of this DNA ($10–10^7$ copies) was used in the experiments where exogenous addition of gDNA in matrices was conducted.

2.2. Primers and probes

Primer and probe sequences were adopted from a previously reported real-time PCR method (10), targeting a 94-bp amplicon from the *ttr* locus in the *Salmonella* genome (GenBank Accession No. AF282268) encoding the gene required for tetrathionate respiration. Six *Taq*Man probes, all with the 5′–CACCGACGGCGA-

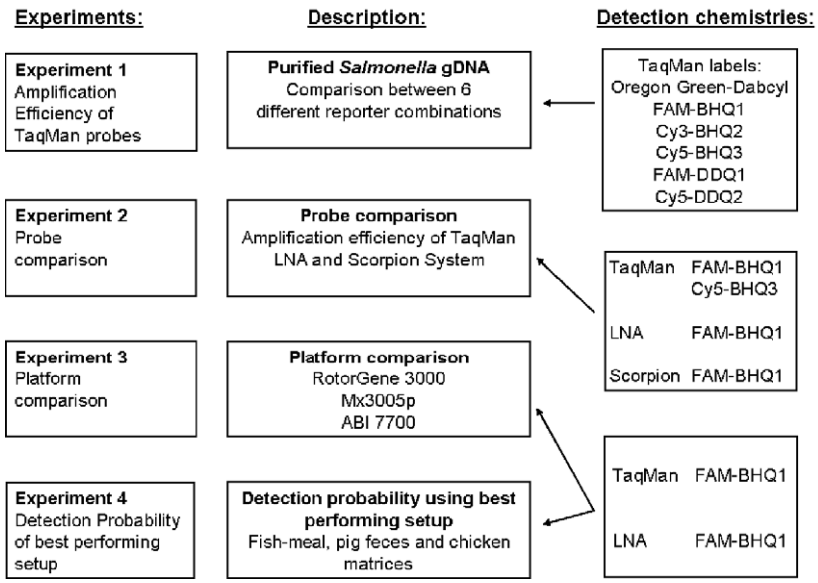

Fig. 1. The overall experimental setup of the *Salmonella* real-time PCR.

GACCGACTTT–3′ sequence with different dye labels were compared (Table 1). Additionally, a Scorpion primer with the 5′–TCCGCGACGGCGAGACCGCG-GA-que-heg-CTCACCAGGAGATTACAACATGG sequence and a locked nucleic acid probe (LNA) were designed from the *Taq*Man *Salmonella* probe sequence (Table 1). The underlined base pairs of the Scorpion represent the probe element. The lower letters in the LNA sequence represent the locked nucleotides. The Scorpion is a uni-probe and was designed by DxS Ltd (Manchester, UK) and the LNA by Proligo (Paris, France).

2.3. Real-time PCR assay

The PCR conditions were adopted from Malorny et al. (2004) with the following modifications: 25 μl reaction volume, the T*th* polymerase (one unit) and the relevant buffer (Roche Diagnostics, Basel, Switzerland), annealing time of 60 s and $MgCl_2$ concentration of 4.5 mM. About 5 μl of DNA sample was added to the PCR mixture. Internal amplification control (IAC) was omitted in the comparative experiments in order to avoid fluorescence interference with target probe-dyes. All runs included two wells with non-template controls (NTC), which contained all the reagents except for the sample.

On the Mx3005p instrument (Strategene, La Jolla, CA, USA), eight-tube strips or 96-microwell plates with optical caps were used and the fluorescence data were analysed with the Mx3005p v2.02 Build 268 software. On the ABI 7700, 96-microwell plates were closed with MicroAmp optical caps (N801-0935; Applied Biosystems, USA) and data were analysed with the SDS software (version 1.6.3.; Applied Biosystems, USA). Individual 0.2 -ml optical microtubes were used in the RotorGene 3000 instrument (Corbett Research, Sydney, Australia) and the analysis was done with the rotor-gene 6 software (version 6.0, Build 19).

Table 1
Probes used in the comparative real-time PCR experiments to detect a 94-bp amplicon within the *ttr* locus (GenBank Accession No. AF282268) of *Salmonella typhimurium*

Type of probe	Reporter dye[a]	Quencher[b]	T_m[c]	Producer[d]
*Taq*Man	Oregon Green (488)	Dabcyl	72	MWG
*Taq*Man	FAM	BHQ1	72	MWG
*Taq*Man	Cy3	BHQ2	72	MWG
*Taq*Man	Cy5	BHQ3	72	Eurogentec
*Taq*Man	FAM	DDQ1	72	Eurogentec
*Taq*Man	Cy5	DDQ2	72	Eurogentec
Locked nucleic acids (LNA)	FAM	BHQ1	79	Proligo
Scorpion	FAM	BHQ1 + HEG	53	Proligo

[a] FAM: 6-carboxyfluorescein, Cy3: indocarbocyanine, Cy5: indodicarbocyanine.
[b] BHQ1–3: black hole quencher 1–3, DDQ1–2: deep dark Quencher 1–2, HEG: PCR inhibitory monomer.
[c] T_m: melting temperature. The T_m values for the probes were obtained by Proligo. The T_m presented by the Scorpion sequence is of the probe element. The entire Scorpion has a T_m of 87 °C.
[d] MWG-BIOTECH AG (Ebersberg, Germany), Eurogentec (Seraing, Belgium), Proligo (Paris, France).

2.4. Determination of cut-off

The reporter dye threshold was assigned to a fixed value intersecting the amplification curves in the linear range of the logarithmic plot for standardization between runs. The threshold for normalized reporter signal was manually set to 200 ΔRn (adaptive baseline normalization of the fluorescence signal) on the ABI Prism and Mx3005p instruments, and a normalized fluorescence of 0.02 on the RotorGene instrument. The fluorescence was normalized to the background signal. Any sample showing a fluorescence signal above this value was regarded as positive and registered for Ct value.

2.5. Experiment 1: amplification efficiency of TaqMan probes

Optimization of the probe concentration in the PCR reaction was done in the Mx3005p instrument, using 50 nM, 100 nM, 150 nM, 240 nM and 350 nM final probe concentrations. Tenfold standard dilution concentrations of gDNA were prepared corresponding to 10^1–10^6 gDNA copies per reaction. Three replicates in one PCR run were used. For the data analysis, the threshold line in the Mx3005p software was manually set to 200 dR (baseline-corrected fluorescence). The parameters used to evaluate the probes were Ct values, amplification efficiency, linear range and signal strength (dR).

When the different probes were run parallel at 240 nM and the gain setting was set to 4, the fluorescence reading from the Cy5–DDQ2 probe was above the recommended baseline fluorescence range of 5000 to 35 000 in fluorescence (R) according to the Mx3005p manual (data not shown), which would influence both Ct value and fluorescence signal. The gain settings were fitted to each probe in order to have baseline fluorescence in the optimal range for the instrument.

2.6. Experiment 2: probe comparison

Optimization of the Scorpion probe were as follows: two-step PCR (same setup as above) was compared to a

3-step PCR setup with 95 °C denaturation for 1 min, followed by 40 cycles with 95 °C for 30 s, 58 °C for 15 s and 72 °C for 30 s. A PCR with the LNA probe was run in parallel for comparison in the new setup.

The sensitivities and amplification efficiencies of the *Taq*Man (FAM–BHQ1 and Cy5–BHQ3), LNA and Scorpion probes were compared on the Mx3005p instrument. Because of the different properties of Scorpion primers (Thelwell et al., 2000; Whitecombe et al., 1999) in comparison to the *Taq*Man chemistry, a preliminary study was carried out to see whether the timing of data collection in the real-time PCR (in the annealing and extension steps) would have influence on the results; that is, whether data collection should be set early or late in the annealing step. Data collection was set after 6, 30 and 60 s in the annealing/elongation step, with 10^3 *Salmonella* equivalents per reaction.

In the probe comparison trial, the same thermal settings and PCR setup as previously described were used. The Scorpion primer was run at 400 nM concentrations, the same concentration as the forward and reverse primers in the *Taq*Man PCR. The same tenfold DNA standard dilution concentrations of gDNA as described above were used. One PCR with triplicates was used for this experiment.

2.7. Experiment 3: comparison of platforms

The final PCR assay (FAM–BHQ1 labelled LNA) was performed in triplicate on three platforms: Mx3005p, RotorGene 3000 and ABI 7700 (Applied Biosystems, Foster City, USA). On the Mx3005p, gain settings were optimized manually for each probe (gain setting at 8 for the FAM–BHQ1 LNA probe) but in RotorGene, automatic calibration of Gain-Setting was done by the instrument software before the first data acquisition (the gain setting was automatically set to 9.33). The ABI 7700 did not have any gain-setting function. The gain settings were determined using a 10^3 gDNA copies per PCR reaction.

2.8. Sample preparation for Experiment 4

Three different matrices were prepared: fishmeal (Danish plant directorate sample mandatory control of feed stuffs), pig feces (negative diagnostic reference sample from our laboratory) and chicken neck skin (retail shop). The lack of *Salmonella* was confirmed using the NMKL reference method (Anonymous, 1999). In the present study, the *Salmonella* culturing was done as follows: 25 g of each sample matrix was added to 225 g buffered peptone water (BPW) in a stomacher bag and shaken gently by hand for one min and incubated aerobically non-shaking at 37 °C for same working day. About 10 ml from each enriched sample were aliquoted into micro-tubes and kept at −20 °C within 3 weeks. Seven aliquots with 1 ml from each matrix were spun down at 16 000 rpm for 5 min at 4 °C. A tenfold gDNA serial dilution was prepared to inoculate into each matrix giving inoculated samples containing 2 to 2×10^6 genomic equivalents per ml. *Salmonella typhimurium* gDNA and 200 µl lysis buffer (Promega, see below) were added to the pellet, vortexed and finally processed in KingFisher magnetic beads automatic DNA isolation (Thermo Labsystems, Waltham, USA) using Promega Magnesil KF, Genomic system (MD1460) DNA isolation kit (Promega Corporation, Madison, USA). The lysed samples were dispensed in three wells (in a 96-well plate) containing the magnetic beads where the DNA was bound to the magnetic beads, transferred to a salt buffer and where a washing solution is included in the kit. Finally, the purified DNA was dissolved in Tris–EDTA buffer.

2.9. Experiment 4: detection probability of the best performing setup

The PCR assay with the best performance from Experiment 3 was evaluated in triplicate on magnetic purified DNA from pig feces, fishmeal and chicken neck skin samples on a RotorGene and with the LNA probe in the PCR mixture. The probability of detecting *Salmonella* in a fecal sample of known concentration was assessed as described previously (Josefsen et al., 2004). The analysis range was 2 to 2×10^6 genomic equivalents/ml. After the DNA purification step, a 5-µl aliquot of each dilution was added to five separate PCR mixtures. The cycle conditions were the same as those described above. The experiment was repeated four times, resulting in 20 PCRs for each concentration. The PCR results are presented as either positive or negative (Ct=40). The detection probability was obtained by plotting the relative number of positive PCRs (Ct<40) against the concentration of the cell suspension. A sigmoidal line fitting was performed with the ORIGIN program (version 4.0; Microcal Software, Northampton, MA, USA).

3. Results

3.1. Optimizations of probe concentration and Gain Settings

Before comparison trials, the raw fluorescence of probes at different concentrations and with different

gain settings was evaluated. Different probe concentrations had some effect on the Ct value regardless of the labeling, although at 240 nM and 350 nM, lower Ct values were obtained (data not shown). Optimization of primer concentrations was done previously (Burkhard Malorny, personal communication). Since the present study was focused on the effects of different probe technologies in a real-time PCR reaction, the primer concentration was kept constant through all experiments.

3.2. Experiment 1: amplification efficiency of TaqMan probes

When comparing the probe quality, all PCRs were run in parallel and with the probes at the same concentrations. Raw baseline fluorescence reading varied between the probes and was corrected with adjusting the gain setting for each dye on the instrument so that the raw fluorescence baseline reading would lie in the optimal fluorescence range. The amplification efficiency and the linear range for the detection of nucleic acid with the different probes were tested with serial dilutions of a DNA standard.

The efficiency was satisfactory (between 95% and 105%) for all probes in the range of 10 to 1×10^6 *Salmonella* equivalents per PCR reaction, except for the FAM–DDQ1 which gained linearity and full efficiency in the range of 100 to 1×10^6 copies per reaction. The difference in Ct values was considerable between probes where the Cy5–BHQ3 and FAM–BHQ1 generally showed lowest Ct values and strongest

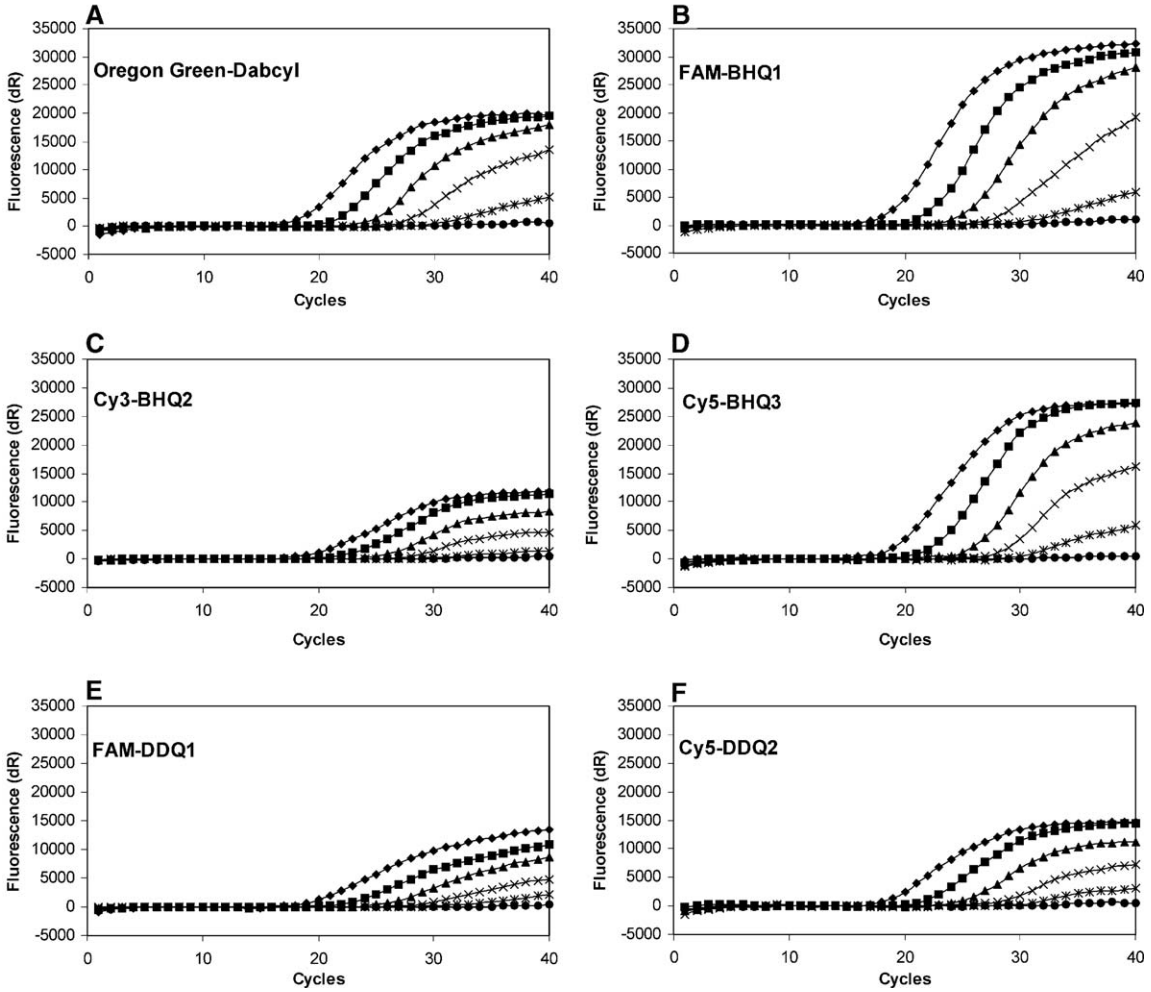

Fig. 2. Amplification plots of different labeled probes tested in Experiment 1. About 5 μl of 10-fold dilution concentrations of genomic *Salmonella* DNA was added to each PCR. Each graph displays amplification plot generated in a real-time PCR assay with three replicates at each concentration. See Table 1 for keys.

Table 2
Results of Experiment 1

Copies/reaction	Oregon–Dabcyl	FAM–BHQ1	Cy3–BHQ2	Cy5–BHQ3	FAM–DDQ1	Cy5–DDQ2
10^6	16.07 ± 0.54	15.70 ± 0.22	16.82 ± 0.46	15.13 ± 0.38	17.45 ± 0.20	16.50 ± 0.36
10^5	19.90 ± 0.22	18.88 ± 0.22	20.28 ± 0.29	18.06 ± 0.47	20.39 ± 0.65	19.59 ± 0.50
10^4	22.64 ± 0.34	21.91 ± 0.35	23.63 ± 0.29	21.31 ± 0.05	24.16 ± 0.26	22.95 ± 1.68
10^3	25.57 ± 0.73	25.42 ± 0.65	26.92 ± 0.41	24.47 ± 0.18	27.56 ± 0.28	25.65 ± 1.56
10^2	29.10 ± 0.83	27.88 ± 0.50	30.08 ± 0.46	28.98 ± 0.56	30.71 ± 0.59	29.44 ± 1.11
10^1	33.01 ± 2.29	31.35 ± 1.03	32.87 ± 1.60	31.68 ± 0.33	36.50 ± 0.19	33.83 ± 0.83
Efficiency (%)	101.2	109.7	104.2	101.1	88.3	97.0

Ct values and amplification efficiency of real-time PCR in Mx3005p. Different reporter–quencher dyes of the same *Taq*Man probe were tested on *Salmonella typhimurium* DNA. The threshold line was placed at 200 dR (baseline corrected fluorescence) with all probes.
See keys in Table 1.
Values are means ± S.D.

fluorescence signal (Fig. 2 and Table 2) at all DNA concentrations.

3.3. Experiment 2: comparison of probes

When data were collected after 6, 30 and 60 s in the annealing/elongation step, only a minor difference of 0.18–0.62 Ct values could be detected where the lowest Ct was after 60 seconds with all probe chemistries, *Taq*Man, LNA and Scorpions. Likewise, the signal generated was 5–35% stronger after 60 s than after 6 s with the three different probe techniques. This was tested because it has been reported that the Scorpion probe has a maximum fluorescence early in the elongation process, when the Scorpion hybridizes to the amplified DNA (Whitecombe et al., 1999). Since no improvements were observed when data were collected earlier in the annealing/extension step, no additional adjustments were made to the PCR with Scorpion primer in later experiments and collection of data was kept after the 60-s annealing step.

In general, the LNA probe showed the strongest PCR response. As can be seen in Fig. 3, the fluorescence plateau from the LNA probe was higher than with the *Taq*Man probe. Likewise, the LNA probe showed a lower Ct value than the other chemistries at all gDNA concentrations (Table 3) tested. The standard deviation (S.D.) for LNA was smaller than with *Taq*-

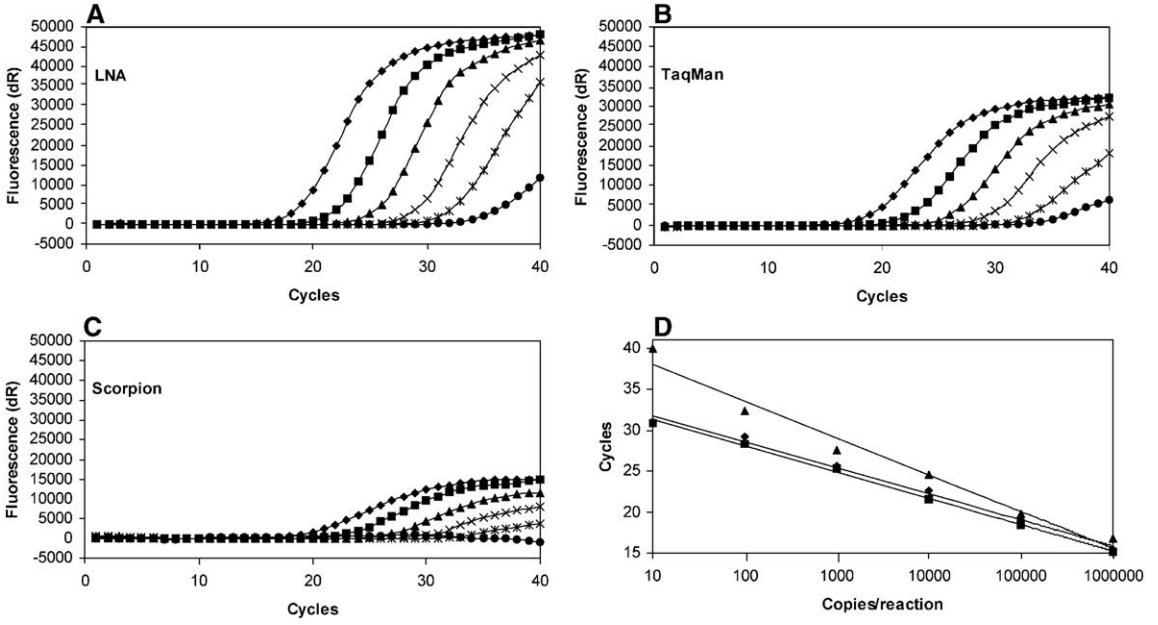

Fig. 3. The results of Experiment 3. Comparison on real-time PCR amplification plots on a 10-fold dilution concentrations of *Salmonella* DNA between LNA probe (A), a *Taq*Man (FAM–BHQ1) (B) and Scorpion system (C) and the corresponding standard curve from graphs A to C (D). (■) LNA probe, (♦) *Taq*Man probe and (▲) Scorpion. The assay was carried out in Mx3005p.

Table 3
Results of Experiment 2

Copies/reaction	*Taq*Man		Locked nucleic acids		Scorpion	
	Ct[a]	dR[b]	Ct[a]	dR[b]	Ct[a]	dR[b]
10^6	15.92 ± 0.23	32464	15.37 ± 0.25	47877	17.38 ± 0.60	14943
10^5	19.26 ± 0.19	32161	18.82 ± 0.16	48066	20.68 ± 0.68	14747
10^4	23.17 ± 0.43	30490	22.03 ± 0.14	46670	24.96 ± 0.14	11620
10^3	26.11 ± 0.09	27321	25.59 ± 0.10	42913	28.13 ± 0.01	7901
10^2	29.91 ± 0.33	18178	28.63 ± 0.18	36075	32.63 ± 0.49	3650
10^1	32.53 ± 1.94	6515	31.7 ± 0.73	11845	No Ct	–
Efficiency (%)	99.9		103.4		83.9	

Comparison of three probe chemistries, all labeled with FAM–BHQ1 in a real-time PCR assay in Mx3005p on a serially diluted DNA from *Salmonella typhimurium*.

[a] Ct: cycle threshold.

[b] dR: the fluorescence intensity in the last cycle of the PCR run using adaptive baseline normalization. Otherwise, see keys in Tables 1 and 2.

Man and the signal intensity was twice as high at low concentrations (Table 3). The PCR efficiency was satisfactory with both LNA and *Taq*Man but not with the Scorpion. The Scorpion showed least competitive results and failed to detect 10 gDNA copies per reaction. In an attempt to optimize the signal strength of the Scorpion probe, the PCR annealing temperature was reduced from 65 °C to 60 °C with no improvement (data not shown). However, a 3-step temperature profile including an extension step of 72 °C in combination-with higher $MgCl_2$ concentration may improve the Scorpion chemistry.

3.4. Experiment 3: comparison of platforms

The LNA probe was compared with the *Taq*Man probe labeled with FAM–BHQ1 on three instruments. The results showed that the assay using the LNA probe is robust since it was able to detect down to 10 copies per reactions in all instruments tested with optimal amplification efficiency (Table 4). The real-time PCR reaction with the *Taq*Man probe also proved to be successful to detect low levels of gDNA but with higher Ct values (Table 4). The RotorGene instrument showed better results when comparing the Ct values (Table 4).

3.5. Experiment 4: detection probability in feces

In order to statistically assess the performance of the final assay (Radstrom et al., 2003), the ability to detect *Salmonella* gDNA in three matrices was carried out by spiking each matrix with 2 to 2×10^6 copies/ml with the same standard dilution. The results showed the ability of the assay to detect *Salmonella* down to 2×10^3 copies/ml in all matrices (Table 5). However, in the feces matrix, one replicate of three, inoculated with 2×10^2 copies/ml, was detected but not in other matrices (Table 5). Therefore the detection probability was determined in the feces matrix.

Table 4
Results of Experiment 3

Copies/PCR reaction	Mx3005p		RotorGene		ABI 7700[a]
	*Taq*Man (Ct)	LNA (Ct)	*Taq*Man (Ct)	LNA (Ct)	LNA (Ct)
10^6	15.51 ± 0.17	14.46 ± 0.06	14.67 ± 0.73	13.37 ± 0.05	15.60 ± 0.29
10^5	18.80 ± 0.12	17.94 ± 0.16	18.18 ± 0.13	17.04 ± 0.12	18.49 ± 0.06
10^4	22.01 ± 0.16	21.25 ± 0.17	21.22 ± 0.20	20.27 ± 0.14	22.68 ± 1.12
10^3	25.36 ± 0.06	24.38 ± 0.18	24.99 ± 0.50	23.53 ± 0.17	25.45 ± 1.08
10^2	28.03 ± 0.07	27.68 ± 0.15	27.56 ± 0.55	26.88 ± 0.55	28.57 ± 0.49
10^1	31.37 ± 0.70	30.97 ± 0.97	30.92 ± 0.85	30.59 ± 1.65	33.56 ± 1.47
Efficiency	109.4	101.7	103	97	97.8
R^2	0.998	0.996	0.996	0.994	0.98
Slope	−3.116	−3.282	−3.25	−3.395	−3.51

See keys in Tables 1 and 2.

Comparison of three platforms in a real-time PCR with a locked nucleic acids (LNA) probe and a *Taq*Man probe (FAM–BHQ1) on serially diluted DNA from *Salmonella typhimurium*.

[a] No *Taq*Man experiment was made on ABI 7700.

Table 5
The results of Experiment 4

Inoculated gDNA (copies/ml)	DNA copies/reaction[a]	Fishmeal (Ct)	Pig feces (Ct)	Chicken neck-skin (Ct)
2×10^7	10^6	14.15 ± 0.16	15.04 ± 0.36	14.26 ± 0.16
2×10^6	10^5	18.60 ± 0.22	18.47 ± 0.28	16.81 ± 0.35
2×10^5	10^4	21.62 ± 0.48	21.87 ± 0.81	20.87 ± 0.02
2×10^4	10^3	25.40 ± 0.19	24.12 ± 0.52	24.98 ± 1.36
2×10^3	10^2	35.48 ± 3.98	29.15 ± 1.80	27.21[b]
2×10^2	10^1	–	31.81[b]	–
2×10^1	10^0	–	–	–

Detection sensitivity of the final real-time assay (FAM–BHQ1 labeled LNA probe) in RotorGene instrument in three sample types inoculated with a 10-fold dilution concentrations of genomic *Salmonella* DNA.
Values are means ± S.D.
[a] Theoretical value, assuming 100% yield in DNA purification.
[b] One replicate of three gave positive response.

The probability of detecting *S. enterica* serotype Typhimurium inoculated into the feces matrix after KingFisher DNA purification step was determined for serially tenfold-diluted gDNA. The detection probability of a gDNA at a concentration of 2×10^3 copies/ml was 80% and, at a concentration of 10^4 copies/ml, the detection probability was 100% (Fig. 4). As shown above, 10 copies/reaction of purified gDNA could be detected using both LNA and a *Taq*Man probe.

4. Discussion

To our knowledge, this is the first independent study of the effect of different reporter and quencher dyes in a *Taq*Man real-time PCR and possibly also one of the first comparisons of LNA versus standard *Taq*Man probes. The results indicate that the reporter and quencher combinations can make a difference when quantifying DNA

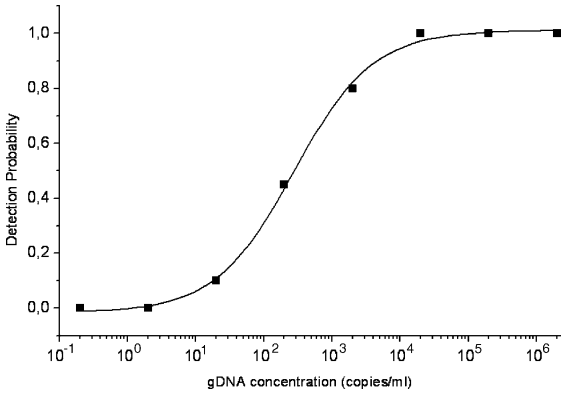

Fig. 4. The results of Experiment 4. Detection probability of the final real-time PCR method in RotorGene, using LNA probe. A pig fecal sample was enriched and inoculated with 10-fold dilution concentrations of genomic *Salmonella typhimurium* DNA. The graph displays a Sigmodial curve fitting generated from 20 samples at each concentration.

at low concentrations. Dye–quencher combinations were chosen with regard to minimal spectral overlap between the reporter fluorophores and the quenchers (Johansson et al., 2002). Since the Cy5 dye has a threefold extinction coefficient of FAM and Cy3 has two (table of fluorescent dyes at www.synthegen.com, accessed on 28/07/2005), it was expected that Cy5- and Cy3-labeled probes would give stronger signal than the FAM-labeled probes. Nevertheless, the probe labeled with Cy3 fluorophore reporter and BHQ2 quencher showed the lowest fluorescence signal of all dyes tested on the Mx3005p instruments which had specialized filter sets for all dyes in question. The dyes and quenchers tested have a small difference in their stabilizing effects on a duplex probe according to Moreira et al. (2005), where both Cy5 and Cy3 gave the probe slightly higher thermal stability (at 1.6 °C and 1.5 °C) than FAM. These small differences do not explain, however, the low signal generated by the Cy3 flourophore.

The three instruments used in the present study have different technical specifications, which have to be considered when interpreting the data. The Mx3005p uses quartz tungsten–halogen source lamp for excitation with 5 positions on the excitation and the detection filter wheels. The detector system is a single-well scanning photomultiplier tube and has a standard 96-well format. The excitation range is between 300 nm and 750 nm, while the emission range is between 300 nm and 700 nm. The RotorGene 3000 has a centrifugal format and uses a separate high-power light emitting diodes for the excitation. The excitation source is at 470 nm, 530 nm, 585 nm, and 625 nm, and the four detection filters can be chosen in the range from 510 to 610 nm. The ABI 7700 is made up of a 96-well thermal cycler attached to an array of 96 optical fibers. A laser light passes through these fibers to excite the fluorochromes in the samples. The emit-

ted fluorescence passes back through the optical fibers and is directed to a spectrograph attached to a Carbon-Charged Camera. Fluorescence detection between 500 and 660 nm is possible.

After the gain settings on the Mx3005p instrument had been optimized for each probe, the largest signal (dR) was observed with the FAM–BHQ1 probe. With gain setting at 8, the FAM–BHQ1 probe succeeded the Cy5–BHQ3 probe with gain setting at 2, but when raising the Cy5 gain setting to 8, it resulted in baseline fluorescence above the recommended limit and gave a higher Ct value as a consequence. The results also show that, before adopting new labeling on an existing *Taq*Man probe, or the development of a multiplex real-time PCR from a single reaction, it is essential to optimize both the probe concentration and the gain setting on the instrument to be used. Optimization of primer concentrations is also highly important, although it was kept constant in the present study for the comparison of different probe technologies.

The Gain Setting option on the instruments is usually used when the raw fluorescence is not in the optimal range. This can influence the PCR performance, when the raw baseline fluorescence lies over or under a specified limit. In the Mx3005p instrument, the producer recommends baseline fluorescence between 3000 R and 35 000 R. Above 35 000 R, the fluorescence is too close to the saturation level (65 536 R) and is therefore less accurate for quantification than in the optimal range (Mx3005p manual). The fluorescence scale on the RotorGene 3000 is from 0 to 100. The producer does not specify a certain range for the baseline fluorescence, but rather advises users to use the Automatic Gain Calibration function, which selects a gain to provide a pre-determined level of fluorescence for a given sample.

Alexa Fluor dye was used by Proudnikov et al. (2003) to replace FAM (quenched with TAMRA) with no improvement, although Alexa Fluor had been demonstrated to be more stable towards photo bleaching, temperature and pH. The same study also revealed that, when the quencher molecule was at an internal position in the probe, the fluorescence signal became stronger and Ct values lower (Proudnikov et al., 2003). This is in accordance with our results where the LNA probe, which is shorter and therefore has the quencher in closer proximity to the fluorophore, showed higher signal and lower Ct values than the corresponding *Taq*Man probe. This observation is suggesting that efficient quenching of the reporter is critical; presumably because this allows the use of a higher gain setting and therefore improved sensitivity, in addition to allowing greater differentiation between background and signal.

The Scorpion primer did not show comparable results to other probe technologies tested, although the concentration of the Scorpion probe was as high as 400 nM, compared to 240 nm for the *Taq*Man probes using either LNA or DNA backbone. The probing mechanism of a Scorpion primer is a unimolecular event, which should otherwise favor a strong signal (Whitecombe et al., 1999). When the annealing temperature was reduced from 65 °C to 60 °C, which favors the conformational change necessary for the Scorpion primer, it was inferior to the *Taq*Man and LNA probes used. It is noted, however, that the Scorpion primer tested here was a uni-probe, whereas duplex-probe (bi-probe) has been reported to show greater fluorescent signal due to the vastly increased separation between fluorophore and quencher in the active form (Solinas et al., 2001).

A recent publication (Thelwell et al., 2000) reported that the Scorpion primers showed better results when compared to *Taq*Man probes and Molecular beacons. In that study, the *Taq*Man probe was designed from the existing Scorpion primer where the stem sequence was removed, and reporter and quencher were added to the resulting probe sequence. It led to a *Taq*Man probe with fewer base pairs and a lower T_m than the corresponding primers used in the assay. This does not conform to the design guidelines for *Taq*Man probes, which recommends a T_m of 10 °C higher than the primers.

The RotorGene showed the best performance of the instruments tested. Mx3005p and ABI 7700 both have a 96-microwell format while the RotorGene instrument has a 36- or 72-well centrifuge rotor. Previous studies at our lab for the detection of another pathogen, namely the *Campylobacter*, showed a more sensitive detection limit by RotorGene compared to ABI 7700 (Josefsen et al., 2004). As for the Mx3005p, a similar real-time PCR but for *Campylobacter* also performed slightly better in the RotorGene than Mx3005p (M. H. Josefsen, unpublished data). Whether this could be due to more efficient temperature convection in the centrifugation format, a more accurate ramping, or even a better optical detection system needs to be further investigated.

The LNA probe showed the best performance when detecting and quantifying purified gDNA. The LNA probe has a T_m of 78 °C, which would enable stronger hybridization to the target molecule and thus increase the chance of a fluorophore cleavage, whereas the T_m of the *Taq*Man probe is 72 °C according to the producers.

The better performance of the LNA could well be due to more efficient quenching. However, a clarification needs an additional experiment, which could be done by using the same *Taq*Man probe but with the internal quencher linked to base pair no. 14. The superiority of LNA over the *Taq*Man probe could also be explained by its higher T_m but at the same time it emphasizes the possibilities which shorter probe sequences with increased T_m can offer, such as the LNA, minor groove binders and peptide nucleic acid (Fiandaca et al., 2001; Kumar et al., 1998; Kutyavin et al., 2000; Letertre et al., 2003), which would have been interesting to include in a comparison trial. Furthermore, LNA may be more robust to use in a multicenter ring trials. The results of Experiment 4 do not show improvements when compared to the original report from Malorny et al. (2004) who used cell suspension directly in the PCR reaction to determine the detection probability. In the present study, however, gDNA was inoculated into the matrices, which were then placed in the automated DNA purification unit. Our current efforts are focused on the use of LNA probes for the detection of pathogens in naturally contaminated foods, which could provide the final proof of the usefulness of this probe system.

In conclusion, the LNA probe showed better performance when detecting purified DNA and in the presence of matrix components from fishmeal, pig feces and chicken neck skin. The work is in progress to combine this PCR assay with an short enrichment for the sensitive detection of *S. enterica* in naturally contaminated samples and for validation in a multicenter collaborative trial.

Acknowledgement

Mr. Eyjólfur Reynisson was a visiting Ph.D. student at DFVF. The work was supported by a grant from the Icelandic R&D Fund for Added Value of Seafoods (AVS, grant no. S 006-05), Danish Agri Business (Innovation law, grant no. 314-04-01032), EU project Food-PCR 2, work package 10 of MedVetNet (FOOD-CT-2004-506122), CampyFood project from the Nordic Innovation Centre (grant no. 04008), Norfa (grant no. 040468) and a grant from BÍSN graduates Placement (Leonardo grant no. PL-ISL-05-411). The authors thank Mr. Stefan Jensen and Mrs. Christina Arnhart for their excellent technical assistance.

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