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Alonso Molina, JL.; Amoros, I.; Cañigral Cárcel, I. (2011). Development and evaluation of a real-time PCR assay for quantification of Giardia and Cryptosporidium in sewage samples. Applied Microbiology and Biotechnology. 89(4):1203-1211. https://doi.org/10.1007/s00253-010-2984-6

The final publication is available at

https://doi.org/10.1007/s00253-010-2984-6

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Additional Information

1 **Development and evaluation of a real-time PCR assay for quantification of *Giardia* and**

2 ***Cryptosporidium* in sewage samples**

3

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19

Abstract

Cryptosporidium and *Giardia* are major causes of diarrhoeal disease in humans, worldwide and are major causes of protozoan waterborne diseases. Two DNA TaqMan PCR-based *Giardia* and *Cryptosporidium* methods targeting a 74-bp sequence of the ß-giardin *Giardia* gene and a 151-bp sequence of the COWP *Cryptosporidium* gene, respectively, were used as models to compare two different LNA/DNA TaqMan probes, to improve the detection limit in a real-time PCR assay. The LNA probes were the most sensitive resulting in 0.96 to 1.57 lower Ct values than a DNA *Giardia* TaqMan probe, and 0.56 to 2.21 lower than a DNA *Cryptosporidium* TaqMan probe. Evaluation of TaqMan *Giardia* and *Cryptosporidium* oligoprobes with LNA-substitutions resulted in real-time PCR curves with an earlier Ct values than conventional DNA TaqMan oligoprobes. In conclusion, the LNA probes could be useful for more sensitive detection limits.

Keywords: *Cryptosporidium*, *Giardia*, LightCycler, LNA probe, qPCR, sewage, TaqMan

1 **Introduction**

2 The protozoan parasites *Cryptosporidium* and *Giardia* are major causes of diarrhoeal 3 disease in humans, worldwide and have also been recognised as the predominant causes of 4 protozoan waterborne diseases (Karanis et al., 2007).

5 The human ID_{50} of 30 oocysts and 10 cysts reported for *C. parvum* (Dupont et al. 6 1995) and *G. lamblia* (Rendtorff 1954), respectively, requires a very sensitive technique for 7 their detection. A rapid detection of these pathogens is therefore of interest for public health 8 control. In environmental samples with low amounts of target pathogens, there is a need for 9 more sensitive probe technologies in order to detect very few target DNAs in the presence of 10 large back-ground flora in often PCR inhibitory sample matrices (Malorny and Hoorfar 11 2005).

12 The real-time PCR (qPCR) using the TaqMan fluorogenic detection system is 13 particularly adapted for the quantification of target sequences. This system use a molecular 14 fluorescent probe specific to the PCR product to generate a fluorescent signal proportional to 15 the initial amount of template DNA (Heid et al. 1996). The fractional cycle number at which 16 the real-time fluorescence signal mirrors progression of the amplification reaction above the 17 background noise level is used as an indicator of successful target amplification (Wilhelm et 18 al. 2001). Most commonly, this is called the threshold cycle (Ct) but the same value is 19 described for use with the LightCycler™ (Roche Applied Science) where the fractional cycle 20 is called the crossing point (Cp) (MacKay et al. 2007). Only a few studies have described a 21 TaqMan quantitative PCR specific to *Cryptosporidium* (Higgins et al. 2001; McDonald et al. 22 2002; Guy et al. 2003; Limor et al. 2002; Fontaine and Guillot 2002; Fontaine and Guillot 23 2003; Keegan et al. 2003; Giovanni and LeChevallier 2005) and *Giardia* (Guy et al. 2003, 24 Verweij et al. 2004; Bertrand et al. 2004).

1 The locked nucleic acid probes (LNAs) are modified nucleic acids in which the sugar
2 has been conformationally locked, imparting unprecedented hybridization affinity towards
3 DNA and RNA (Kumar et al. 1998). The introduction of LNA residues in oligonucleotides
4 increases the thermal stability of the oligonucleotide (Koshkin et al. 1998; Obika et al. 1997).
5 Introduction of one single LNA base into an oligonucleotide leads to an increase in T_M of 2 to
6 6ºC (Nitsche 2007). Locked nucleic acids obey the Watson-Crick pairing rules, but have an
7 increased specificity and high affinity to complementary DNA (Sanjay et al. 1998; Vester and
8 Wengel 2004; Koshkin et al. 1998; Nolan et al. 2006). Due to such advantages, LNA or
9 LNA/DNA mixmers (hereinafter called LNA) have been used for real-time PCR (Reynisson
10 et al. 2006; Alonso et al. 2010) and fluorescent *in situ* hybridization (Silahtaroglu et al. 2003;
11 Kubota et al. 2006). Thus, the purpose of the present study is the evaluation of the
12 performance of LNA probes in a real-time PCR assay for quantification of *Giardia* and
13 *Cryptosporidium* in sewage.

14

15 **Materials and methods**

16 Specificity testing

17 To determine the specificity of qPCR for *Giardia intestinalis* and *Cryptosporidium*
18 *parvum*, nucleic acids were recovered from the following organisms: genomic DNAs (gDNA)
19 from *C. felis*, *C. hominis* and *C. meleagridis*, were obtained from the *Cryptosporidium*
20 *Reference Unit* (Chalmers R., National Public Health Service Microbiology, Swansea, UK),
21 gDNA from *G. intestinalis* ATCC 30888D (Portland 1 strain) and gDNA from
22 *Cryptosporidium parvum* ATCC PRA-670 (Iowa strain) were obtained from the American
23 Type Culture Collection, *C. parvum* oocysts (bovine, Iowa isolate) and *G. lamblia* (human,
24 H3 isolate) cysts were purchased from BTF (North Ryde, Australia), *G. muris* (Roberts-

1 Thompson isolate) was obtained from Waterborne (New Orleans, USA). DNA was isolated
2 from *Giardia* and *Cryptosporidium* strains by the method below described. Specificity of the
3 assay was also tested on DNAs obtained from three different bacterial cultures: *Escherichia*
4 *coli* CECT 515, *Pseudomonas aeruginosa* ATCC 10145 and *Vibrio parahaemolyticus* CECT
5 511. DNA was recovered and purified from bacteria by the Realpure genomic DNA
6 extraction kit (Durviz, Valencia, Spain). The genomic DNAs were quantified using the
7 Quant-iT^{TM} dsDNA HS and BR Assay kits (Invitrogen), and fluorescence was determined
8 using the Qubit Fluorometer (Invitrogen).

9

10 DNA extraction

11 The DNA was extracted from (oo)cysts using the QIAamp DNA minikit (Qiagen,
12 Hilden, Germany). A freeze-thaw protocol (Nichols et al. 2003), which maximizes DNA
13 extraction from oocysts and cysts was followed. Briefly, the (oo)cysts were suspended in 180
14 µl of ATL lysis buffer (Qiagen), and subjected to 15 freeze-thaw cycles (1 min in liquid
15 nitrogen and 1 min at 65ºC per cycle), and 20 µl of proteinase K was added per tube. The
16 tubes were incubated overnight at 56ºC. The manufacturer's protocol was followed for
17 purification of DNA through the column and the DNA was eluted from the columns in TE
18 buffer.

19

20 TaqMan PCR procedure

21 Primers and probes for detection of *Giardia* (P241 forward, reverse and DNA
22 TaqMan probe) and *Cryptosporidium* (P702 forward, reverse and DNA TaqMan probe) were
23 adopted from a previously reported qPCR method (Guy et al. 2003), targeting a 74-bp

sequence of the β-giardin gene and a 151-bp region of the COWP gene, respectively. The sequence of DNA β-giardin probe (P241) was AAGTCCGCCGACAACATGTACCTAACGA and the sequence of DNA COWP probe (P702) was TGCCATACATTGTTGTCCTGACAAATTGAAT (Guy et al. 2003). Additionally, the sequences of LNA β-giardin probe (P241: CGccGACaaCATGTACcTA and LNA COWP probe (P702: TGccATAcATTGTTGTCcTGACAA) were designed by Sigma-Aldrich (St. Louis, USA) from the DNA TaqMan *Giardia* and *Cryptosporidium* above probe sequences, respectively. The lower letters in the LNA sequences represented the locked nucleotides. The DNA and LNA *Giardia* and *Cryptosporidium* TaqMan probes were oligonucleotides with a 5'-end reporter dye (FAM-6-carboxyfluorescein) and a 3'-end quencher dye (TAMRA-6-carboxy-*N*,*N*,*N*',*N*'-tetramethylrhodamine). The effectiveness of BSA for the relief of PCR inhibitors during qPCR amplification was evaluated with the inclusion of nonacetylated BSA (fraction V; Sigma, St. Louis, Mo.) at different concentrations (400, 200, 50 and 20 ng/µl) in qPCR mixtures. To evaluate the efficiency of LNA *Taq*Man probes with the inclusion of BSA in the qPCR mixtures, three replicates were seeded with gDNA of *Giardia* and *Cryptosporidium* corresponding to 3.1×10^5 and 3.3×10^6 fg, respectively. Thus, we tested the effect of BSA on the amplification efficiency and reproducibility of the real-time PCR. Optimization experiments were performed to determine the most suitable reaction conditions. Optimization of the probe concentrations in the qPCR reaction were done using 50 nmol/L, 100 nmol/L, 150 nmol/L and 200 nmol/L final probe concentrations. Three replicates for each concentration in one qPCR run were used. In the probe comparison trial, the same thermal settings and PCR set up as previously described were used. The DNA and LNA TaqMan probes at 50 nmol/L, 100 nmol/L, 150 nmol/L and 200 nmol/L were run parallel at the same concentrations of *Giardia* and *Cryptosporodium* gDNA corresponding to 3.3×10^4 fg and 3.1×10^5 fg, respectively. Three replicates for each

1 probe concentration in one qPCR run were used. The TaqMan qPCR assays were performed
2 in the LightCycler 2.0 real-time PCR system (Roche Diagnostics Ltd., Rotkreuz, Switzerland)
3 and data were analysed with the LightCycler software version 4.1. The *Giardia* ß-giardin and
4 *Cryptosporidium* COWP sequences were amplied in a 20-µl reaction mixture containing 4 µl
5 of LightCycler *Taq*Man master (Roche Diagnostics). The concentrations of primers and
6 fluorescent probes, after optimization of the PCR conditions, were 600 nmol/L and 200
7 nmol/L (DNA or LNA TaqMan probe), respectively. BSA was used in *Giardia* and
8 *Cryptosporidium* qPCRs to final concentrations of 20 ng/µl and 200 ng/µl, respectively. The
9 concentration of BSA included in the Roche LightCycler *Taq*Man master mixture is
10 unknown. The *Giardia* qPCR cycling conditions consisted of 10 min of incubation at 95ºC
11 followed by 50 cycles of alternating temperatures of 95ºC for 10 s, 58ºC for 8 s and 72ºC for
12 3 s. The *Cryptosporidium* qPCR cycling conditions consisted of 10 min of incubation at 95ºC
13 followed by 50 cycles of alternating temperatures of 95ºC for 10 s, 66ºC for 8 s and 72ºC for
14 6 s. A no-template control was included in every assay, and no cycle threshold (Ct) values
15 were consistently obtained after 50 cycles of PCR. The standard curves were constructed from
16 gDNA from *G. intestinalis*. DNA concentrations serially diluted from 6.6 ng to 66 fg and
17 from gDNA from *C. parvum* DNA concentrations serially diluted from 0.62 ng to 62 fg,
18 respectively. From the slope of the standard curve, the amplification efficiency (E) was
19 estimated by the formula $E=(10^{-1/slope})-1$. A reaction with 100% efficiency generated a slope
20 of -3.32. (Ibekwe and Grieve, 2003). One PCR with triplicates was used for this experiment.

21

22 Field evaluation of LNA TaqMan probes for the detection of Giardia and Cryptosporidium

23 A total of 14 raw sewage samples were obtained from three wastewater treatment
24 plants (WWTPs), WWTP1 (capacity: 290,000 inhabitant equivalents), WWTP2 (capacity:

1,277,900 inhabitant equivalents) and WWTP3 (capacity: 249,000 inhabitant equivalents) between October 2007 and March 2008. Duplicate samples (100 ml) were concentrated by centrifugation at 2,000 g for 10 min at room temperature. *Giardia* cysts and *Cryptosporidium* oocysts in pellets were isolated by immunomagnetic separation (IMS), using the Dynal GC-Combo system (Dynal, A.S., Oslo, Norway) according to the manufacturer´s instructions. The duplicate concentrates were used for detection of *Giardia* and *Cryptosporidium* using immunofluorescence microscopy (IF) and qPCR, respectively.

The identification and enumeration of (oo)cysts was carried out by immunofluorescence assay using the commercial kit Crypto/Giardia IF test (Cellabs, Brookvale, Australia), according to the manufacturer's instructions. The slides were systematically examined by using epifluorescence microscopy (Olympus BX50; Olympus, Tokyo, Japan) at 600X magnification. *Giardia* cysts and *Cryptosporidium* oocysts were identified by fluorescence characteristics, size, and shape and then enumerated. *Cryptosporidium* oocysts and *Giardia* cysts were used directly in DNA extraction without oocyst and cyst detachment by adding 180 µl of the ATL buffer from the QIAamp DNA minikit (Qiagen GmbH, Hilden, Germany), as recommended Jiang et al. (2005). The DNA was extracted with the QIAamp DNA minikit after oocyst and cyst isolation by IMS. The suspension was subjected to 15 freeze-thaw cycles (1 min in liquid nitrogen and 1 min at 65ºC per cycle). DNA was extracted from the oocysts and cysts with the QIAamp DNA minikit and the manufacturer-recommended procedures. Successful qPCR amplification and contamination of qPCR were monitored by use of one positive DNA control (DNA of *Cryptosporidium parvum* for LNA/DNA COWP P702 probes and DNA of *Giardia intestinalis* for LNA/DNA β-giardin P241 probes) and one negative DNA control.

1 **Results**

2 The specificity of LNA TaqMan probes was investigated using the newly designed 3 LNA TaqMan probes. A BLAST search showed that the sequences of LNA P241 TaqMan 4 probe matched exactly and only with β-giardin gene sequences of *G. intestinalis* assemblage 5 A. Furthermore, it was found that the LNA P241 probe had one mistmatch with *G. intestinalis* 6 assemblages F and C, and two mismatches with *G. intestinalis* assemblages B, H and E, and 7 *G. muris*. In the second step, the specificity of the primers and probes was tested against *G.* 8 *intestinalis* genotypes A and B, *G. muris*, *Cryptosporidium* species and a set of two strains of 9 bacteria which are common water-borne organisms. The gDNA from *Cryptosporidium* and 10 bacteria species was not amplified in the *Giardia* TaqMan LNA qPCR. The P241 primer 11 LNA probe set detected *G. intestinalis* and *G. muris* (Table 1).

12 A BLAST search showed that the sequences of LNA P702 TaqMan probe matched 13 exactly with COWP gene sequences of *Cryptosporidium parvum*, *C. hominis*. *C. wrairi* and 14 *C. meleagridis*. Furthermore, it was found that the LNA P702 probe had six mismatches with 15 *C. felis*. The specificity was confirmed after the TaqMan LNA qPCR test was applied on 16 DNA from various *Cryptosporidium* species. The test detected *C. parvum* , *C. hominis* and *C.* 17 *meleagridis* but did not cross-react with *C. felis* (Table 1).

18 When comparing the LNA and DNA TaqMan probes, all PCRs were run in parallel 19 and with the probes at the same gDNA concentrations. The primers concentration was 600 20 nmol l^{-1}. Optimal concentrations of TaqMan probes were determined by comparison of the Ct 21 values for several concentrations (50 nmol/L, 100 nmol/L, 150 nmol/L and 200 nmol/L). 22 Optimal probe concentrations were determined in triplicate (Table 2). The LNA β-giardin 23 P241 and the LNA β-COWP P702 probes (FAM-Tamra) were the most sensitive for each 24 probe concentration, resulting in 0.39 (50 nmol/L) to 1.37 (200 nmol/L) lower Ct values than

1 a DNA β-giardin P241 probe, and 0.63 (200 nmol/L) to 1.37 (50 nmol/L) lower than a DNA
2 COWP P702 probe (Table 2). The Ct values observed for P241 LNA and P702 LNA probes
3 concentration 200 nmol/L were 29.52 $\pm$ 0.04 and 26.69 $\pm$ 0.04, respectively. We thus selected
4 the 200 nmol/L concentration for each probe used in this study. In the second step, we
5 compared the Ct values observed for five *G. intestinalis* gDNA concentrations ranging from
6 6.6 ng to 66 fg and six *C. parvum* gDNA concentrations ranging from 0.62 ng to 62 fg,
7 respectively. The fluorescence plateau from the LNA probes was higher that with the DNA
8 probes (data not shown). Likewise, the LNA probes showed a lower Ct value than DNA
9 probes at all gDNA concentrations tested (Table 3). The LNA probes were the most sensitive
10 resulting in 0.96 to 1.57 lower Ct values than a P241 DNA *Giardia* probe, and 0.56 to 2.21
11 lower than a P702 DNA *Cryptosporidium* probe. The PCR efficiency was satisfactory with
12 both LNA and DNA probes.

13 Tenfold standard dilutions of *Giardia* and *Cryptosporidium* gDNA were prepared
14 corresponding to 6.2 x 10^5 to 62 fg and 6.6 x 10^6 to 66 fg, respectively. Standard curves were
15 generated by plotting Ct values as a function of the logarithm of known *Giardia intestinalis*
16 *a*nd *Cryptosporidium parvum* gDNA concentrations. The Ct values of each dilution amplified
17 in triplicate by TaqMan PCR were plotted as a function of the logarithm of the starting
18 quantity of gDNA. Application of the real time PCR assays with LNA β-giardin probe P241
19 and LNA COWP probe P702 yielded a linear relationship between the cycle threshold and the
20 log of the starting concentration. All data points recorded are within the dynamic range
21 defined by the standard curve. All replicates were within 0.5 Ct of each other. The Ct values
22 of the real time PCR assays with LNA β-giardin probe P241 ranged from 25.53 (3,200 cysts)
23 to 39.22 (0.32 cyst). The results showed that the LNA probe was able to detect down to 1 cyst
24 per reaction. When no cyst was added to the reaction tube, no Ct value was achieved. The
25 slope of the curve was -3.46 with a squared correlation coefficient (r^2) of 0.989 (Fig. 1). PCR

1 amplification efficiency was then estimated to be 96.2 %. Thus, a detection limit of 0.32 *G. lamblia* cyst per reaction corresponding to 3.2 cysts in 100 µl purified suspension was reached. The Ct values of the real time PCR assays with LNA COWP probe P702 ranged from 21.66 (165,000 oocysts) to 40.31 (1,65 cyst). When no oocyst was added to the reaction tube, no Ct value was achieved. The slope of the curve was -3.81 with a squared correlation coefficient (r^2) of 0.995 (Fig. 2). PCR amplification efficiency was then estimated to be 83.3%. Thus, a detection limit of 1.65 *C. parvum* oocyst per reaction corresponding to 16.5 oocysts in 100 µl purified suspension was reached.

9 To assess the repeatability of TaqMan PCR assay alone, tenfold serial dilutions of *G. lamblia* cysts and *C. parvum* oocysts were amplified on three separate occasions under identical conditions. For each concentration, the mean values were associated with a coefficient of variation. The coefficients of variation of the real time PCR assays with LNA β-giardin probe P241 obtained for the study of repeatability of TaqMan PCR ranged from 0.58 (1,600 cysts) to a maximum of 1.56 (1.59 cysts). The coefficients of variation of the real time PCR assays with LNA COWP probe P702 obtained for the study of repeatability of TaqMan PCR ranged from 0.59 (82,500 oocysts) to a maximum of 3.01 (8.25 oocysts).

17 The β-giardin and COWP qPCR assays greatly depended on the BSA concentration (Table 4). Addition of 20 ng/µl of BSA to the qPCR LNA β-giardin mixture enhances lightly the amplification of *Giardia* gDNA (Ct 25.83). However, the use of BSA at the final concentration of 400 ng/µl was detrimental to the reaction (Ct 27.53). We observed that the addition of BSA to the TaqMan LNA COWP qPCR mixture, decreased Ct values from 23.40 (20 ng/µl of BSA) to 21.15 (400 ng/µl of BSA) (Table 4).

23 The sensitivity of the DNA and LNA probes were compared with sewage samples. To this end the influent from wastewater treatment plants were examined in parallel with

1 *Taq*Man DNA and LNA probes, and the real-time PCR assay. Fourteen influent samples were
2 *Giardia* positive using the primer-LNA probe set P241 and the primer-DNA probe set P241.
3 The P241 LNA probe showed a lower Ct value than the P241 DNA probe at all influent
4 samples analysed (table 5). Except for one sample the Cts for *Giardia* positive samples were
5 above cycle 35 (table 4). The fourteen sewage samples analyzed with the IFA procedure were
6 positive for *Giardia* and contained between 38 and 145 cysts 100 ml^{-1} (Table 5).

7 No *Cryptosporidium* was detected in the analysed samples as determined by qPCR. Of
8 the fourteen sewage samples analyzed for the presence of *Cryptosporidium* oocysts by IFA, 7
9 (50%) were positive.

10 To detect the presence of inhibitors, four and three sewage samples were spiked with
11 60,000 cysts of *G. lamblia* and 40,000 oocysts of *C. parvum*, respectively. *G. lamblia* DNA
12 was detected using DNA and LNA P241 probes (table 6), and *C. parvum* DNA and LNA
13 P702 probes (Table 7) in the qPCR assays. The Ct values of LNA probes were compared to
14 those of DNA probes obtained from the same sample. We observed that LNA probes showed
15 a lower Ct value than the DNA probes at all influent samples analysed (tables 6 and 7).

16

17 **Discussion**

18 The specificity testing showed that qPCR amplification of a region of the giardin gene
19 distinguished *Giardia* spp. from other microorganisms. The fluorescence plateau from the
20 gDNA of *G. lamblia* was higher that the gDNA of *G. muris* (data not shown).

21 It was previously shown that the ß-giardin primer-probe LNA P241 sets do not detect
22 DNA from several bacterial isolates as well as from two isolates of *Cryptosporidium parvum*
23 (Guy et al., 2003). Currently, eight *Cryptosporidium* spp. have been reported in humans: *C.*

1 *hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, *C. suis*, and *Cryptosporidium*
2 cervine genotype (Xiao and Cama 2006). Of these species/genotypes found in humans, the
3 probe LNA P702 hybridized with *C. hominis*, *C. parvum* and *C. meleagridis* but did not
4 cross-react with *C. felis*. Sequence mismatches reduce the efficiency of DNA amplification in
5 the 5' exonuclease assay by reducing the efficiency of extension of the mismatched base
6 pair(s) by the Taq enzyme (Guy et al. 2004). Smith et al. (2002) concluded that mismatches in
7 the probe region have the greatest effect on real-time PCR and that an increased number of
8 mistmatches led to lowered real-time PCR efficiency. However, the real-time PCR-based
9 assays developed in our study were not totally specific for *G. lamblia* and, *Cryptosporidium*
10 *hominis* and *C. parvum* species, respectively.

11 To our knowledge, this the first comparison of LNA versus standard DNA Taqman
12 probes for protozoan detection. Real-time PCR reactions are characterized by an increase in
13 fluorescence emission due to probe degradation by DNA polymerase in each elongation step
14 during PCR cycling. The higher the starting copy number of the nucleic acid target, the earlier
15 the fluorescence will reach the predetermined threshold and the smaller will be Ct (Fontaine
16 and Guillot 2002). Evaluation of TaqMan Giardia and *Cryptosporidium* oligoprobes with
17 LNA-substitutions resulted in real-time PCR curves with an earlier Ct values than
18 conventional DNA TaqMan oligoprobes. This may have been caused by their slightly
19 decreased length, resulting in an enhanced level of quenching due to the changed proximity of
20 reporter and quencher and thus an improved signal to noise ratio (Mackay et al. 2007). The
21 superiority of LNA over the DNA TaqMan probe could also be explained by its higher T_m but
22 at the same time it emphasizes the possibilities which shorter probe sequences with increased
23 T_m can offer, such as LNA, minor groove binders (Fontaine and Guillot 2003) and peptide
24 nucleic acid (Reynisson et al. 2006). This is an advantage since long probes perform relatively
25 poorly compared to short probes (Fontaine and Guillot 2003).

1 Inhibitory components frequently found in biological samples can result in a
2 significant reduction in the sensitivity and kinetics of qPCR (Nolan et al. 2006). BSA has had
3 widespread use for relieving the effects of PCR inhibitors during the PCR step (Jiang et al.
4 2005; Guy et al. 2003; Kreader 1996). Jiang et al. (2005) demonstrated that the effect of PCR
5 inhibitors could be relieved significantly by the addition of 400 ng/µl of BSA to the PCR
6 mixture in the detection of *Cryptosporidium.* Guy et al. (2003) demonstrated that the addition
7 of BSA (final concentration, 20 ng/µl) to the *Giardia* PCR mixture removed the inhibitory
8 effect of substances with the potential to inhibit PCRs. Capillary based systems from Roche
9 (LightCycler range) require the addition of BSA into the reaction buffer to prevent binding of
10 reaction components and nucleic acid to the glass (Nolan et al. 2006). The β-giardin and
11 COWP qPCR assays greatly depended on the BSA concentration.

12 In our experiments, the total concentrations of cysts detected with IFA (from 3.8×10^2
13 to 1.4×10^3 cysts/L) agreed with the concentrations observed in published studies (Caccio et
14 al. 2003; Bertrand et al. 2004). For the fourteen sewage samples, all positive with the IFA
15 procedure and all produced amplification curves with the *Giardia* TaqMan PCR DNA and
16 LNA assays. Except for one sample the Cts for *Giardia* positive samples were above cycle 35
17 (table 4). Above cycle 35 the variability will be greater and quantification may be unreliable
18 (Nolan et al. 2006). Bertrand et al. (2004) observed *G. lamblia* cysts concentrations with the
19 IFA procedure always higher than the concentrations obtained with the TaqMan PCR assay.
20 No *Cryptosporidium* was detected in the analysed samples as determined by qPCR. Lack of
21 detection of *Cryptosporidium,* as determined by qPCR in sewage samples has been reported
22 by Guy et al. (2003), suggesting that oocysts were either absent or present at very low levels.
23 The difference was attributable to increased probe affinity for the target sequence. Although
24 we were unable to detect any DNA corresponding to *Cryptosporidium* in unspiked collected

1 samples, the method developed may be useful to assess the presence of these pathogenic
2 protozoa in water and wastewater.

3 In summary, the most appropriate primer TaqMan Probe combination is the one that
4 gives the lowest C_t and the highest normalized fluorescence. The results presented here
5 demonstrated that the introduction of LNA nucleotides into DNA TaqMan probes is useful for
6 improving the efficiency of hybridization with rRNA targets. Two probe LNA sequences,
7 LNA β-giardin and LNA COWP, evaluated in this study showed signal enhancements after
8 substituting four LNA bases for DNA bases.

9

10 **Acknowledgements**

11 This work was supported by the Spanish *Ministerio de Ciencia e Innovación* grants
12 AGL2005-07776-C03-03 and AGL2008-05275-C03-03/ALI). Part of this work was also
13 funded by the Fondo Europeo de Desarrollo Regional (Feder) grant POICV 2000-2006. We
14 thank staff at the wastewater treatment plants, and the Entidad de Saneamiento de Aguas for
15 assistance in sample collection.

16

17 **Conflict of interest** None

18

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1 **TTable 1.** Specificity of β-giardin and COWP LNA probes for detection of *Giardia* and *Cryptosporidium* in qPCR

DNA source	q PCR Result [a]	
	β-giardin P241	COWP P702
G. lamblia H3	+	-
G. lamblia (ATCC 30888 D)[b]	+	-
G. muris (Roberts-Thompson)	+	-
C. parvum (ATCCC PARA-67 D)[c]	-	+
C. parvum (Cp 3)	-	+
C. hominis (12599)	-	+
C. meleagridis (8716)	-	+
C. felis (13866)	-	-
E. coli (CECT 529)	-	-
P. aeruginosa (ATCC 10145)	-	-
V. parahaemolyticus (CECT 511)	-	-

[a]+, detected; -, not detected.

[b]Portland 1 isolate.

[c] Iowa isolate.

Table 2. Mean Ct values for different concentrations of β-giardin and COWP LNA probes for detection of *Giardia* and *Cryptosporidium* in qPCR

Probe concentration	β-giardin P241 probes[a]		COWP P702 probes[b]	
	LNA	DNA	LNA	DNA
50 nmol/L	32.88 ± 0.32	33.27 ± 0.32	28.70 ± 0.61	30.07 ± 0.01
100 nmol/L	30.67 ± 0.03	31.70 ± 0.32	27.69 ± 0.14	28.82 ± 0.26
150 nmol/L	29.80 ± 0.10	30.99 ± 0.29	27.53 ± 0.09	28.29 ± 0.11
200 nmol/L	29.52 ± 0.04	30.89 ± 0.23	26.69 ± 0.04	27.25 ± 0.37

[a]Mean + standard deviation of three replicates seeded with 3.1×10^4 fg of *G. lamblia* DNA.

[b]Mean + standard deviation of three replicates seeded with 3.3×10^5 fg of *C. parvum* DNA.

Table 3. Comparison of LNA and DNA probe chemistries, all labelled with FAM-TAMRA in a real-time PCR assay in LigthCycler 2.0 on a serially diluted DNA from *Giardia duodenalis* and *Cryptosporidium parvum*

DNA (fg)[a]	P241 probes		DNA (fg)[b]	P702 probes	
	LNA(Ct[c])	DNA (Ct)		LNA (Ct)	DNA (Ct)
---	---	---	66×10^5	21.56 ± 0.06	23.77 ± 0.27
62×10^4	25.53 ± 0.07	27.04 ± 0.15	66×10^4	25.48 ± 0.08	27.26 ± 0.03
62×10^3	29.03 ± 0.08	20.24 ± 0.07	66×10^3	30.14 ± 0.17	31.05 ± 0.14
62×10^2	32.45 ± 0.03	33.41 ± 0.02	66×10^2	33.41 ± 0.09	33.97 ± 0.21
62 x 10	36.13 ± 0.56	37.11 ± 0.55	66 x 10	37.58 ± 0.24	38.61 ± 0.27
62	39.22 ± 1.14	40.79 ± 0.61	66	40.31 ± 0.12	41.94 ± 0.80
Efficiency (%)	96.2	93.1		83.3	88.7

[a]gDNA from *G. intestinalis*. DNA concentrations serially diluted from 6.6 ng to 66 fg.

[b]gDNA from *C. parvum* DNA concentrations serially diluted from 0.62 ng to 62 fg.

[c]Ct, Cycle threshold.

Table 4. Effect of BSA concentration on the β-giardin and COWP LNA qPCR assays

BSA concentration (ng/μl)	Mean Ct value ± SD	
	LNA P241[a]	LNA P702[b]
20	25.83 ± 0.26	23.40 ± 0.51
50	25.45 ± 0.10	22.17 ± 0.12
200	26.55 ± 0.28	21.87 ± 0.23
400	27.53 ± 0.08	21.15 ± 0.09

[a]Mean ± standard deviation of three replicates seeded with 3.1×10^5 fg of *G. lamblia* DNA.

[b]Mean ± standard deviation of three replicates seeded with 3.3×10^6 fg of *C. parvum* DNA.

1 **Table 5.** Comparison of LNA and DNA qPCR assays for detection of *Giardia* and

2 *Cryptosporidium* in 100-ml sewage samples

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Sample[a]	IF[b] cysts	P241 probes Ct[c]		IF oocysts	P702 probes Ct	
		LNA	DNA		LNA	DNA
C1	90	36.79	37.85	0	No Ct	No Ct
C2	151	35.31	37.31	2	No Ct	No Ct
C3	104	36.57	39.05	3	No Ct	No Ct
C4	109	35.04	37.57	0	No Ct	No Ct
C5	145	35.44	37.24	1	No Ct	No Ct
C6	133	34.87	36.59	0	No Ct	No Ct
C7	120	35.74	37.72	0	No Ct	No Ct
P1	78	35.35	37.42	5	No Ct	No Ct
P2	78	36.14	38.73	3	No Ct	No Ct
P3	38	38.69	41.01	0	No Ct	No Ct
P4	83	37.31	39.65	0	No Ct	No Ct
P5	128	35.31	37.02	0	No Ct	No Ct
P6	61	37.61	38.96	1	No Ct	No Ct
Q1	70	37.36	38.53	3	No Ct	No Ct

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23 [a]C, WWTP1; P, WWTP2; Q, WWTP3

24 [b]IF, Enumeration of cysts/oocysts was carried out by immunofluorescence microscopy

25 [c]Ct, Cycle threshold

1 **Table 6**. Mean Ct values for four WWTP samples (sample 1, sample 2, sample 3 and sample 4) seeded with 60,000 cysts of *G. lamblia* for

2 detection of *Giardia* with β-giardin P241 probes[a]

3

Sample	Sample 1		Sample 2		Sample 3		Sample 4	
	LNA	DNA	LNA	DNA	LNA	DNA	LNA	DNA
undiluted	27.92 ± 0.13	29.71 ± 0.01	27.48 ± 0.17	29.29 ± 0.12	29.71 ± 0.25	32.07 ± 0.10	28.66 ± 0.41	30.85 ± 0.45
dil 1:5	29.48 ± 0.10	32.51 ± 0.05	29.71 ± 0.00	32.24 ± 0.22	31.65 ± 0.49	33.99 ± 0.23	31.34 ± 0.12	33.65 ± 0.54
dil 1:10	30.01 ± 0.55	32.94 ± 0.66	30.12 ± 0.45	32.56 ± 0.66	32.31 ± 0.20	35.01 ± 0.23	31.65 ± 0.25	34.64 ± 0.42

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13 [a] Mean ± standard deviation of two replicates DNA undiluted and, dilutions 1:5 and 1:10.

Table 7. Mean Ct values for three WWTP samples (WWTP1, WWTP2 and WWTP3) seeded with 40,000 oocysts of *C. parvum* for detection of *Cryptosporidium* with COWP P702 probes*

Sample	Sample 1		Sample 2		Sample 3	
	LNA	DNA	LNA	DNA	LNA	DNA
undiluted	29.26 ± 0.29	31.87 ± 0.67	30.36 ± 0.21	32.44 ± 0.39	29.68 ± 0.35	32.48 ± 0.32
dil 1:5	32.56 ± 0.19	34.42 ± 0.52	32.22 ± 0.18	34.09 ± 0.54	31.75 ± 0.19	34.21 ± 0.18
dil 1:10	33.71 ± 0.34	35.07 ± 0.49	33.74 ± 0.03	35.43 ± 0.70	33.31 ± 0.24	35.18 ± 0.39

*Mean ± standard deviation of two replicates DNA undiluted and, dilutions 1:5 and 1:10.

1 **Figure legends**

2

3 **Fig. 1.** Amplification plot and standard curve of β-giardin LNA TaqMan PCR assay with

4 tenfold dilutions of *G. intestinalis* DNA ranging from $6.2x10^5$ to 62 fg by using the

5 LightCycler 2.0 The number of PCR cycles is indicated on the *x* axis. Ct values determined

6 for individual standards were as follows: $6.2x10^5$ fg, 25.53; $6.2x10^4$ fg, 29.03; $6.2x10^3$ fg,

7 32.45; $6.2x10^2$ fg, 36.13; and 62 fg, 39.22. The horizontal red line indicates the threshold.

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9

10 **Fig. 2.** Amplification plot and standard curve of COWP LNA TaqMan PCR assay with

11 tenfold dilutions of *C. parvum* DNA ranging from $6.6x10^6$ to 66 fg by using the LightCycler

12 2.0 The number of PCR cycles is indicated on the *x* axis. Ct values determined for individual

13 standards were as follows: $6.6x10^6$ fg, 21.66; $6.6x10^5$ fg, 25.48; $6.6x10^4$ fg, 30.14; $6.6x10^3$ fg,

14 33.41; $6.6x10^2$ fg, 37.58; and 66 fg, 40.31. The horizontal red line (black?) indicates the

15 threshold.

1 **Fig. 1**

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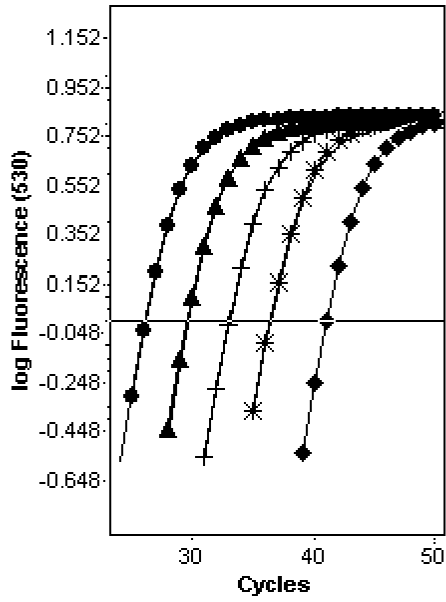

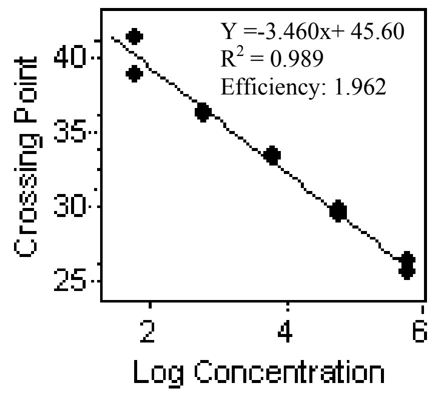

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1 **Fig. 2**
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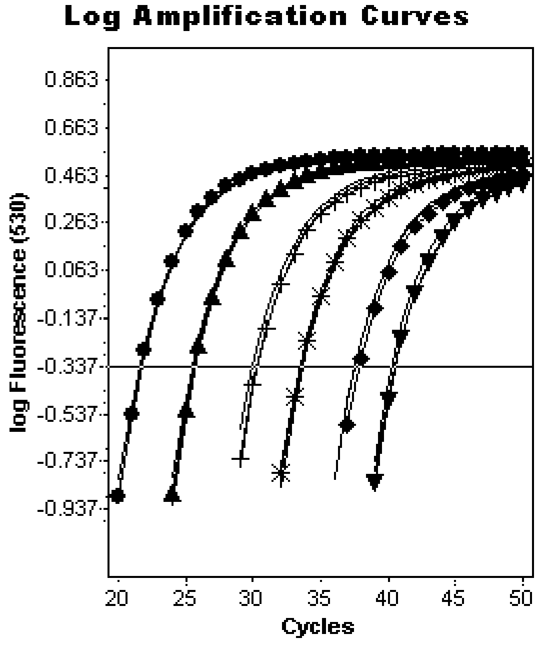

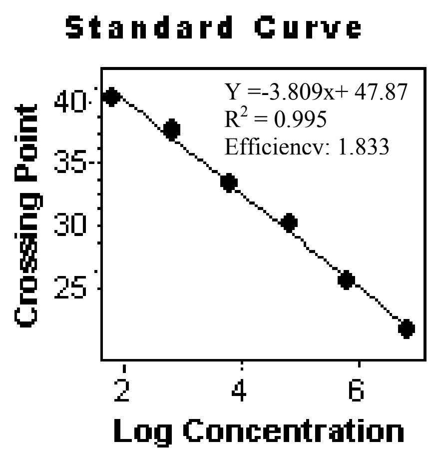

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