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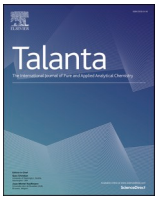

Evaluation of the effect of outer primer structure, and inner primer linker sequences, in the performance of Loop-mediated isothermal amplification

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ABSTRACT

Loop-mediated isothermal amplification, or LAMP, is nowadays the most popular isothermal nucleic acid amplification technique. This technique implements a minimum of four primers, named outer (F3/B3) and inner primers (FIP/BIP). The inner primers hybridize in two distinct regions, and some studies have reported that the usage of a linker, typically composed of four thymines, in the middle of these primers can improve assay performance. In addition to this, dual-priming oligonucleotides, DPO, have been reported to provide highly specific reducing non-specific amplifications. Considering the large number of primers implemented in LAMP assays, in the current study the suitability of DPO primers replacing regular outer primers; and their combination with different linker sequences in the inner primers were explored. The results demonstrated that replacing standard F3/B3 by DPO primers does not significantly affect that overall performance of the assay, and provides additional stability to temperature changes. This observations were consistent regardless the type of linker implemented in the inner primers, out of which in the current study a linker composed of thymines significantly outperformed the other options tested, most likely due to a combination of sequence and physical structure.

1. Introduction

Nowadays, molecular methods are widely accepted as diagnostic tools in many fields of application. Among them, those based on DNA amplification, such as PCR/qPCR, are the most popular ones [1,2]. More recently, isothermal techniques have attracted the interest of the scientific community due to the advantages they present over PCR/qPCR for applications, like the development of lab-on-chip devices or performing point-of-care tests. This is due to the fact that these techniques do not rely on expensive equipment, and in many occasions may be combined with different strategies for naked-eye results interpretation, thus making this step user-friendly [3].

Regarding isothermal techniques, Loop-mediated isothermal amplification (LAMP) originally described in 2000, is the most commonly used highlighted by the number of research articles published in the last few years. This technique implements two outer primers typically called F3 and B3, and two inner primers named FIP and BIP, which hybridize in four regions of the target; in addition to these, two extra primers may be added to accelerate the reaction known as loop primers (LF and LB), these are graphically represented in Fig. 1A [4,5]. In the original study, Notomi et al. included a linker sequence between the two oligonucleotides that form both, the FIP and BIP, F1c-F2 and B2–B1c respectively. This sequence was originally composed of four thymines to provide flexibility for intermolecular interaction [6] still, other nucleotides has not been assessed. Nowadays, there is no consensus on the need to implement, or not, such linker sequence in the primers designed, however when included, these are always composed of the same four thymines. Torres et al. have already highlighted that this configuration does not necessarily need to be the best one attending to their *in silico* predictions [7].

Even though LAMP has been reported as a highly sensitive, and reproducible technique, the high number of primers, significantly increases the complexity of novel assays design, and along with the concentrations used, may be behind the appearance of unspecific, spurious amplifications which may lead to false positive results [8–11] being these major limitations of existing LAMP-based methods. As a result,

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several studies have been published reporting improved performance of the technique by implementing different types of supplements such as betaine [4], dimethyl sulfoxide [12], guanidine hydrochloride [13], tetramethylammonium chloride [14], pullulan [15] or even the usage of enzymes such as helicases [16] among others. This is a simpler approach than others such as the design of different types of probes which, even though present several advantages, add more oligonucleotides to the reaction mixture, and complexity to the assays by needing more sophisticated equipment for the readout [9,17–22].

Unspecific amplification is not an exclusive problem of LAMP. This phenomenon has also been reported in PCR. In 2007 Chun et al. described dual-priming oligonucleotides (DPO) to block non-specific priming. Briefly, these DPO primers consist of a relatively long primer divided in two uneven regions bond by a poly(I) linker, this already creates certain instability so that only three mismatches are needed in either part of the primer sequence to completely avoid its extension, thus leading to more specific assays, this structure is depicted in Fig. 1B [23]. DPO primers have been used for multiplex assays targeting a wide variety of microbial pathogens [24–27] and have actually been implemented in certain commercially available kits [28]. Primer sequence and structure is not only relevant for nucleic acid amplification techniques, but also for other molecular biology-based methods such as those implementing aptamers [29–31] or those for specific capture/hybridization [32,33].

Considering what has been exposed, the goal of this study was to evaluate the effect of different primer structures on the performance of LAMP assays, taking a previously published assay targeting *Listeria monocytogenes* as a model [34]. To this end, different types of linkers in FIP/BIP primers were evaluated, and for the first time DPO primers were included replacing standard F3/B3 primers.

2. Materials & methods

2.1. Strains and culture media

L. monocytogenes WDCM 00021, purchased from the Spanish Type Culture Collection, was used as the reference microorganism since it is indicated as model strain for microbiological testing attending to ISO 11133 [35,36]. Fresh cultures were prepared by resuspending one single colony in 4 mL of Nutrient Broth (NB, Biokar Diagnostics S.A., France) and incubated overnight at 37 °C. This culture was one-hundred fold serially diluted and plated on Tryptic Soy Yeast Extract Agar (TSYEA, Biokar Diagnostics S.A., France) and the plates were incubated at 37 °C overnight to determine initial bacterial concentration of the fresh culture.

2.2. DNA extraction

DNA was extracted from fresh pure cultures by centrifuging 1 mL at 16000×*g* for 2 min. The supernatant was discarded and the pellet was resuspended in 1 mL of Tris-EDTA 1× (TE 1×, Tris-HCl 10 mM, EDTA 1 mM, Sigma-Aldrich, St. Louis, USA) and centrifuged again under the same conditions. Once more, the supernatant was discarded, the pellet was resuspended in 200 μL of TE 1× and heated at 99 °C for 10 min at 1400 rpm in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany). After the heat treatment, the samples were centrifuged as detailed above at 4 °C. The supernatant, with the DNA, was transferred to a clean tube and stored at −20 °C until needed.

2.3. Primers

The assay developed by Garrido-Maestu et al. targeting the *plcA* gene of *L. monocytogenes* was used as model LAMP assay [34]. Two different primer structures/modifications were evaluated. On one hand replacement of the outer primers, F3/B3, for DPO primers, and on the other,

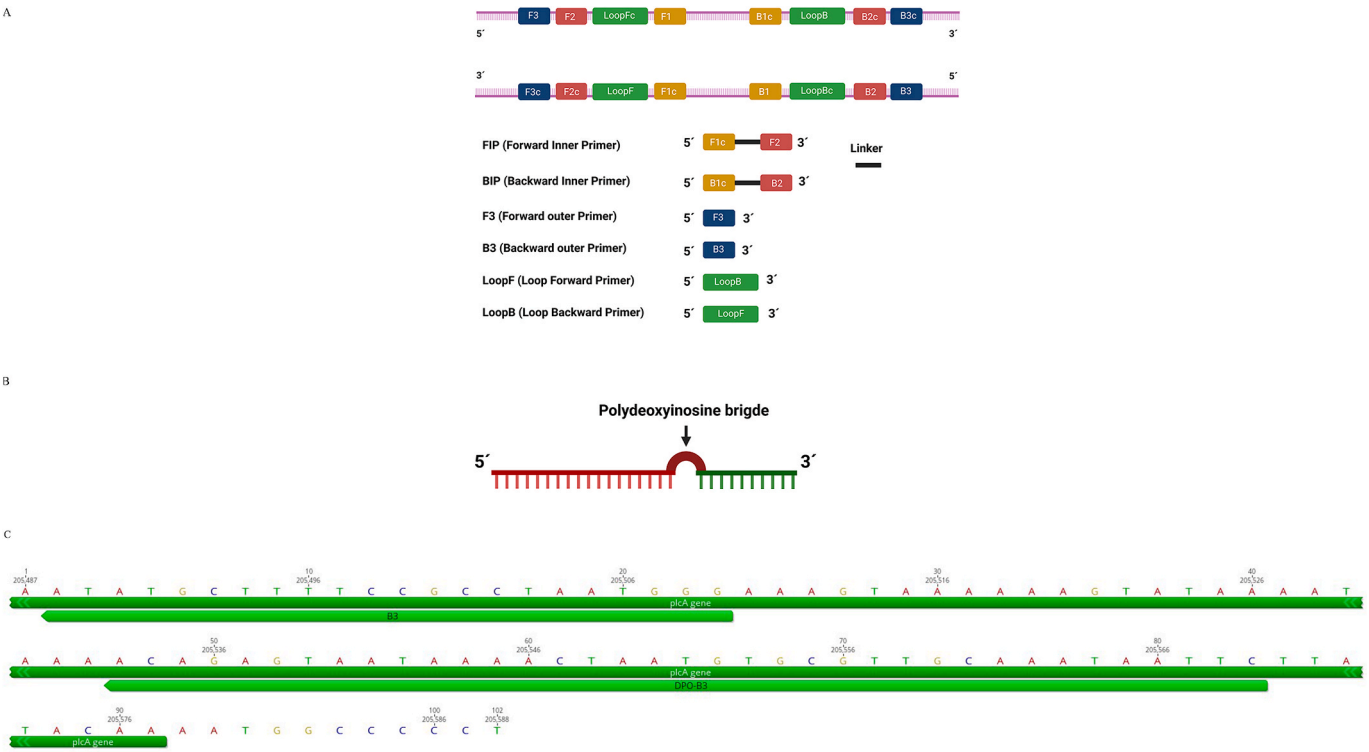

Fig. 1. A) Graphical summary of the structure and position of the primers used in LAMP assays. B) Representation of a DPO primer. C) Region of the *plcA* gene of *L. monocytogenes* NC_003210 depicting the position of the original B3 primer and the newly designed DPO-B3. Within each column different letters denote statiscally significant differences. Fig. 1A and B were created with www.biorender.com and 1C with Geneious Prime® version 2023.0.1.

implementing different linker sequences in the inner primers FIP/BIP. All the primers were purchased from Merck Life Science (UK Limited, Gillingham, UK) and were HPLC purified (see Table 1 for primer sequence details).

2.3.1. DPO

The effect of DPO was evaluated by replacing the standard F3/B3 primers for DPO alternatives which were designed extending the original oligonucleotides in their 5' ends, and implementing the polyI bridge, 5 nucleotides long, as described by Chun et al., 2007 [23]. The new F3 primer, DPO-F3, ended being 35 nucleotides long. Regarding the B3, it was redesigned due to the presence of repetitive sequences in the surroundings of the original primer, see Fig. 1C, and so, the newly designed DPO-B3, was 37 nucleotides long.

2.3.2. Linkers for FIP/BIP

Five different linkers between the F2–F1c and B2–B1c, from FIP and BIP primers respectively, were tested. They consisted on a four nucleotide long polyA, polyT, polyG, polyC and polyI structure. Additionally, the same primers without any linker were purchased for comparison.

2.4. LAMP assay

LAMP reactions were performed under the optimized conditions described by Roumani et al. [37]. These were: a final reaction volume of 20 μL with 12 μL of GspSSD2.0 Isothermal Master Mix (OptiGene Ltd., Horsham, UK), 0.04 μL of ROX as a passive reference dye (Invitrogen, Carlsbad, CA, USA), 800 nM FIP/BIP, 200 nM F3/B3, 400 nM LB and 3 μL of template DNA, the remaining volume was filled with sterile milliQ water. The reactions were run at 65 °C for 30 min with fluorescence acquisition every 30 s (60 cycles) in a QuantStudio™ 5 System and analyzed with QuantStudio™ Design & Analysis Software v1.5.1 (Applied Biosystems™, Foster City, CA, USA). Results confirmation was accomplished performing a melt curve analysis, which consisted in heating at 95 °C for 1 s, 80 °C for 20 s and heating again up to 95 °C with temperature increments of 0.015 °C and fluorescence acquisition during the process.

Table 1
LAMP primers targeting *plcA* gene of *L. monocytogenes*.

Primer	Sequence 5 → 3′
plcA-F3	TGT GTT TGA GCT AGT GGT TTG G
plcA-B3	CCC ATT AGG CGG AAA AGC ATA T
DPO-plcA-F3	CAA TGA CAT CGT TTG TGT TTG*iiiii*GTG GTT TGG
DPO-plcA-B3	GAA TTA TTT GCA ACG CAC ATT AG*iiiii*GTA CTC TGT
plcA-FIPnl	GCA GCG CTC TCT ATA CCA GGT ACA-AAT GTC CAT GTT ATG TCT CCG TTA
plcA-BIPnl	AGG TTT GTT GTG TCA GGT AGA GCG-CGC TTA ATA ACT GGA ATA AGC CAA
plcA-FIPa	GCA GCG CTC TCT ATA CCA GGT ACA*aaaaa*AAT GTC CAT GTT ATG TCT CCG TTA
plcA-BIPa	AGG TTT GTT GTG TCA GGT AGA GCG*aaaaa*CGC TTA ATA ACT GGA ATA AGC CAA
plcA-FIPt	GCA GCG CTC TCT ATA CCA GGT ACA*ttttt*AAT GTC CAT GTT ATG TCT CCG TTA
plcA-BIPt	AGG TTT GTT GTG TCA GGT AGA GCG*ttttt*CGC TTA ATA ACT GGA ATA AGC CAA
plcA-FIPg	GCA GCG CTC TCT ATA CCA GGT ACA*ggggg*AAT GTC CAT GTT ATG TCT CCG TTA
plcA-BIPg	AGG TTT GTT GTG TCA GGT AGA GCG*ggggg*CGC TTA ATA ACT GGA ATA AGC CAA
plcA-FIPc	GCA GCG CTC TCT ATA CCA GGT ACA*ccccc*AAT GTC CAT GTT ATG TCT CCG TTA
plcA-BIPc	AGG TTT GTT GTG TCA GGT AGA GCG*ccccc*CGC TTA ATA ACT GGA ATA AGC CAA
plcA-FIPi	GCA GCG CTC TCT ATA CCA GGT ACA*iiiii*AAT GTC CAT GTT ATG TCT CCG TTA
plcA-BIPi	AGG TTT GTT GTG TCA GGT AGA GCG*iiiii*CGC TTA ATA ACT GGA ATA AGC CAA
plcA-LB	CAT CCA TTG TTT TGT AGT TAC AGA G

Lower case, italic letters denote the different linkers tested for FIP and BIP, and the Inosine (i) "bridge" of the DPO primers. The "-" denote the FIP/BIP primers without linker used as control. The underlined section of the DPO-plcA-F3 primer identifies the region in common with the original plcA-F3 primer.

2.5. Evaluation and statistical analysis

The effect of the different F3/B3 primers, regular and DPO, in combination with FIP/BIP with the different linkers was performed in sequential steps based on the cycle of quantification (Cq) data obtained (initial comparison performed at 65 °C). The linkers providing the best results were compared against the same primers but without linkers, then, those with the best results were combined to evaluate the effect of differential linker composition in the inner primers, and finally, the effect in an amplification temperature gradient from 62 to 67 °C was also assessed.

The data were represented with GraphPad Prism version 8.0.1 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The comparison of the Cq values obtained was performed by one-way ANOVA with a Tukey post-hoc and Mann-Whitney – *U* test was the chosen one for direct average analyses. Additionally, the analysis of the effect of temperature increase on the different outer primer structures and linker sequences was performed by two-way ANOVA with Tukey or Dunnett post-hoc.

3. Results

3.1. Initial linker comparison

LAMP amplification was successful regardless the type of F3/B3 primer structure, regular or DPO. Consistently, regardless the type of outer primer structure, LAMP assays implementing FIP/BIP with linkers consisting on "C", "G" or "T" provided significantly lower Cq values compared to those of linkers composed by "A" or "I" as it can be observed in Fig. 2A and B. Even though there were no statistically significant differences, the assays where "T" was the linker of choice seemed to provide lower Cq values. Additionally, no statistically significant differences were observed among the Cq values obtained with the different outer primer structures when the same type of linker was selected for the inner primers as denoted in Fig. 2C.

3.2. Comparison against no linker

Based on the results mentioned above, FIP/BIP primers implementing linkers consisting on "C", "G" or "T" were selected for comparison against no linker (NL). Consistently with the previous observation, LAMP amplification was also possible with both F3/B3 primer structures when NL was included in the FIP/BIP primers. The elimination of the linker in the inner primers significantly increased the Cq value obtained with regular, and DPO, outer primers, see Fig. 3A and B. In this set of experiments, the assays implementing the pyrimidine linkers, namely "T" and "C", obtained significantly lower Cq values, see Fig. 3C. Once more, no significant differences were observed regardless the type of outer primer structure selected.

3.3. Temperature effect

An increase in the Cq values obtained was observed with the increase in the amplification temperature from 62 to 67 °C. This phenomenon was consistent with both sets of outer primers, regardless the linker used in the inner primers, "T", "G", "C" or NL.

When implementing regular outer primers with a "T" linker, significantly lower Cq values were obtained at 62, 63 and 64 °C compared to 65, 66 and 67 °C, without differences among the data for the lowest

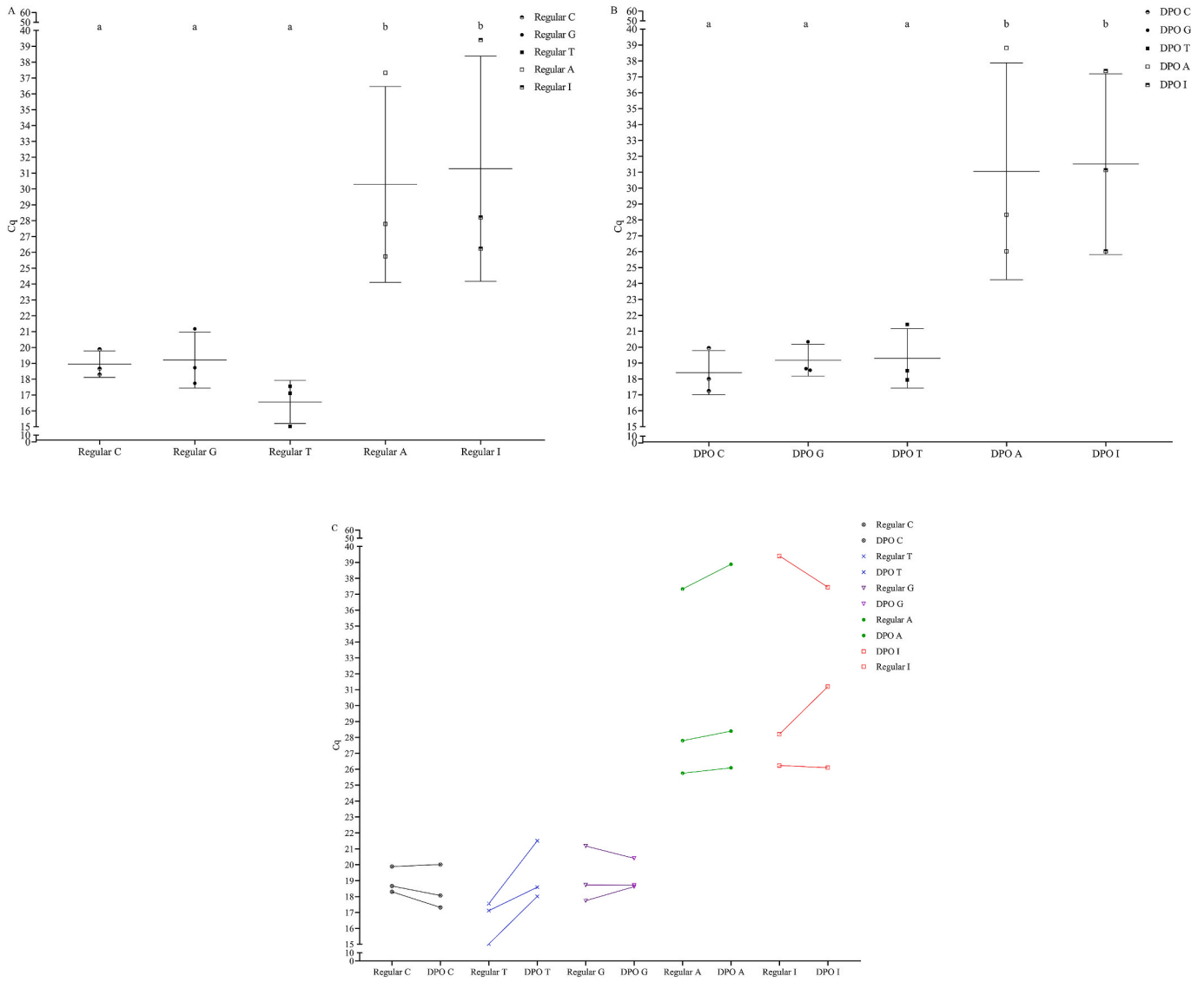

Fig. 2. Linker screening with regular outer primers A). Linker screening with DPO outer primers B). Comparison of the different linkers implementing regular or DPO outer primers C). Within each column different letters denote statiscally significant differences.

temperatures. After this, the other linker providing low Cq values, consistent with previous experiments, was "C" however, contrary to the observations from "T" significant differences were determined for all temperatures except among 65–67 °C and 66–67 °C. Within these two linkers only significant differences were observed at 66 °C however with a p value of 0.0476. Regarding the usage of "G" or NL, the Cq values obtained within each temperature tested were significantly higher than those implementing "T" and/or "C" where Cq obtained was around 18 at 62 °C but increased to 25–30 with NL and "G" respectively, being this trend consistent for all temperatures tested. All these results are shown in Fig. 5A.

When comparing the experiments performed with DPO, as mentioned above, a similar trend to that of regular outer primers was observed, however for all temperatures significantly lower Cq values were obtained with the "T" linker being only non-significant when compared against "C" at 62 and 63 °C. It is worth to note that at 67 °C no amplification was obtained with the "G" linker. These data can be observed in Fig. 5B.

In Fig. 5C the data obtained for all the different combinations are presented together. As it may be observed, the implementation of DPO primers seems to provide higher stability as the Cq variation among different temperatures with a given linker is smaller. This is in agreement with the results of comparing data among the lowest, 62 °C, and the highest, 67 °C, temperatures tested where for all the linkers, except for "G", and NL the smallest Cq variation was observed with DPO primers, e.g. the average Cq at 62 °C was 18.79 and 18.29 for regular and DPO outer primers respectively, and the value increased to 26.16 and 23.16 resulting in variation of 7.37 and 4.87 cycles.

3.4. Linker combinations

The linker combinations tested were FIP with "C" (FIPc) with BIP "T" (BIPt), FIP with "G" (FIPg) with BIP "T" (BIPt), FIP with "T" (FIPt) with BIP "C" (BIPc), FIP with "T" (FIPt) with BIP "G" (BIPg) and finally FIP with "T" (FIPt) with BIP "T" (BIPt) were used as controls due to the previous consistent observation that assays with "T" as linker provided lower Cq values.

As depicted in Fig. 4A and B, significantly lower Cq values were obtained with the control assay which implemented "T" in both inner primers, followed by FIPc-BIPt. This was consistent with both types of outer primer structures. However, contrary to the previous observations, the implementation of DPO primers obtained statistically lower Cq

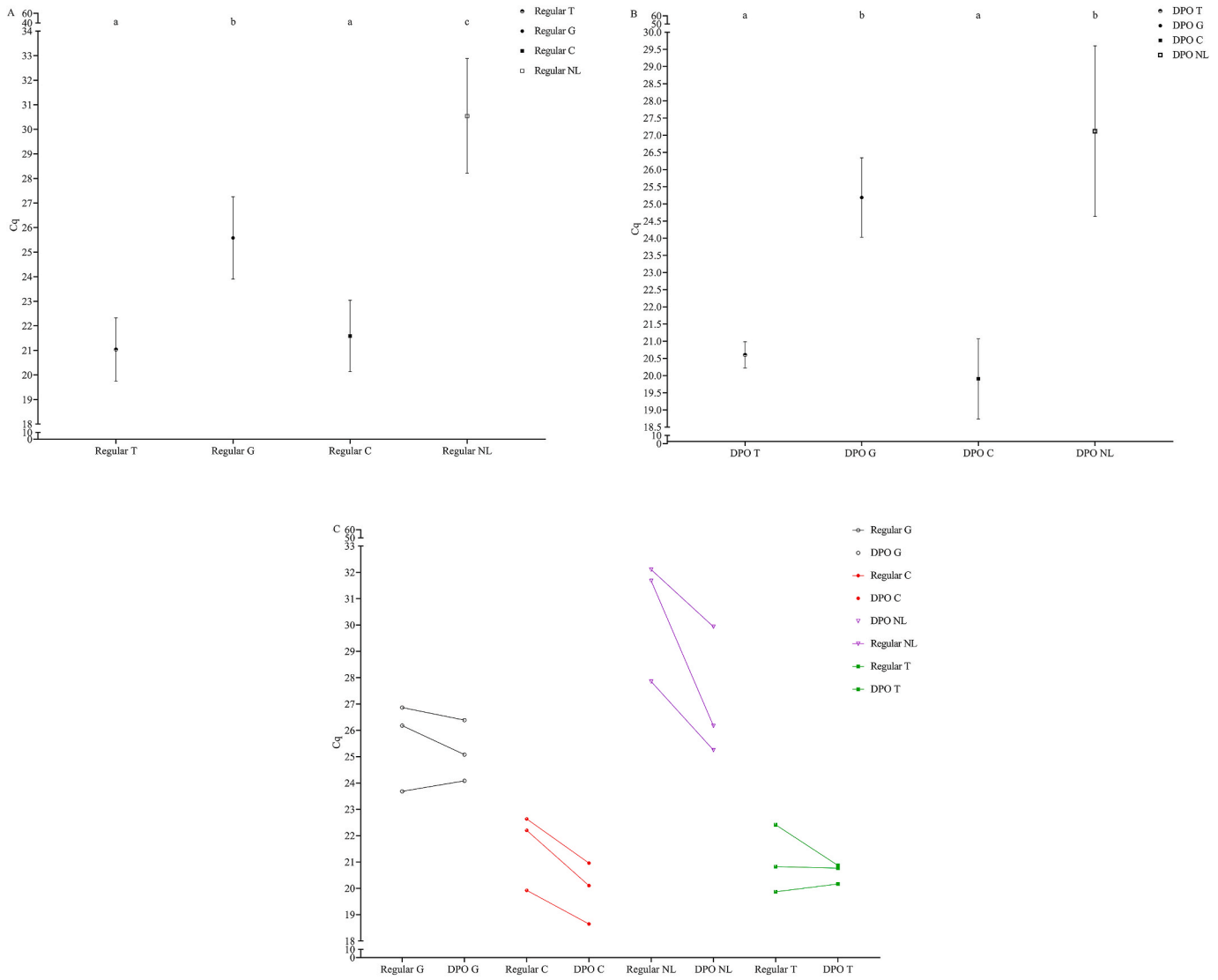

Fig. 3. Thymine "T", cytosine "C" and guanosine "G" linkers comparison against no linker "NL" with regular outer primers A). Thymine "T", cytosine "C" and guanosine "G" linkers comparison against no linker "NL" with DPO outer primers B). Comparison of the different linkers implementing regular or DPO outer primers C). Within each column different letters denote statiscally significant differences.

values compared to regular outer primers when the same linker combination was tested, see Fig. 4C.

4. Discussion

Over the last decades adoption of molecular methods for the detection of microbial pathogens, more specifically, those based on nucleic acid amplification, has revolutionized the development of rapid methodologies capable of providing an early response in situations of infection or outbreaks, and allowed to also improve microbiological quality and safeness of foods. In recent years isothermal amplification techniques have attracted the interest of the scientific community, and among the vast variety that exist, LAMP has become the most popular one [3]. Even though in the original description of this technique a tetra thymine linker was included in the inner primers [4], subsequent studies have demonstrated that this is not needed even though some researchers indicated improvements in the performance of specific assays [7,38], thus a lack of consensus exist on whether to use such linker or not. In addition to this, as commented above, the sequence of such linker is always composed of thymines. For this reason, the first objective of the current study was to evaluate the effect of the implementation of such linkers using a previously published LAMP assays targeting *L. monocytogenes* [34]. In addition to this, LAMP typically implements four primers, or up to six if the loop primers are also designed, thus final number of primers in a single reaction is relatively high. Furthermore, their final concentration is also high (800–1600 nM for inner primers, 200–400 nM for outer primers and 400–600 nM for loop primers) making the assay optimization critical in order to avoid, or reduce, unspecific amplifications. In 2007 the description of DPO primers by Chun et al. [23], which provided higher specificity and reduced non-specific amplification by PCR, opened the possibility of improving multiplex assays which have a large number of primers in the same reaction [39–41]. For this reason it was envisioned that the implementation of DPO primers replacing regular outer primers in LAMP assays may improve the overall performance, and so their evaluation was the second goal of the current study.

A preliminary linker screening performed, indicated that even though amplification occurs regardless the sequence of such linker, it has a direct impact in the result. As commented in the results section, the best results were obtained with linkers composed of "T", "C" and "G". Interestingly, the assays were "A" and "I" were used obtained significantly worse results. One possible explanation for these results may be

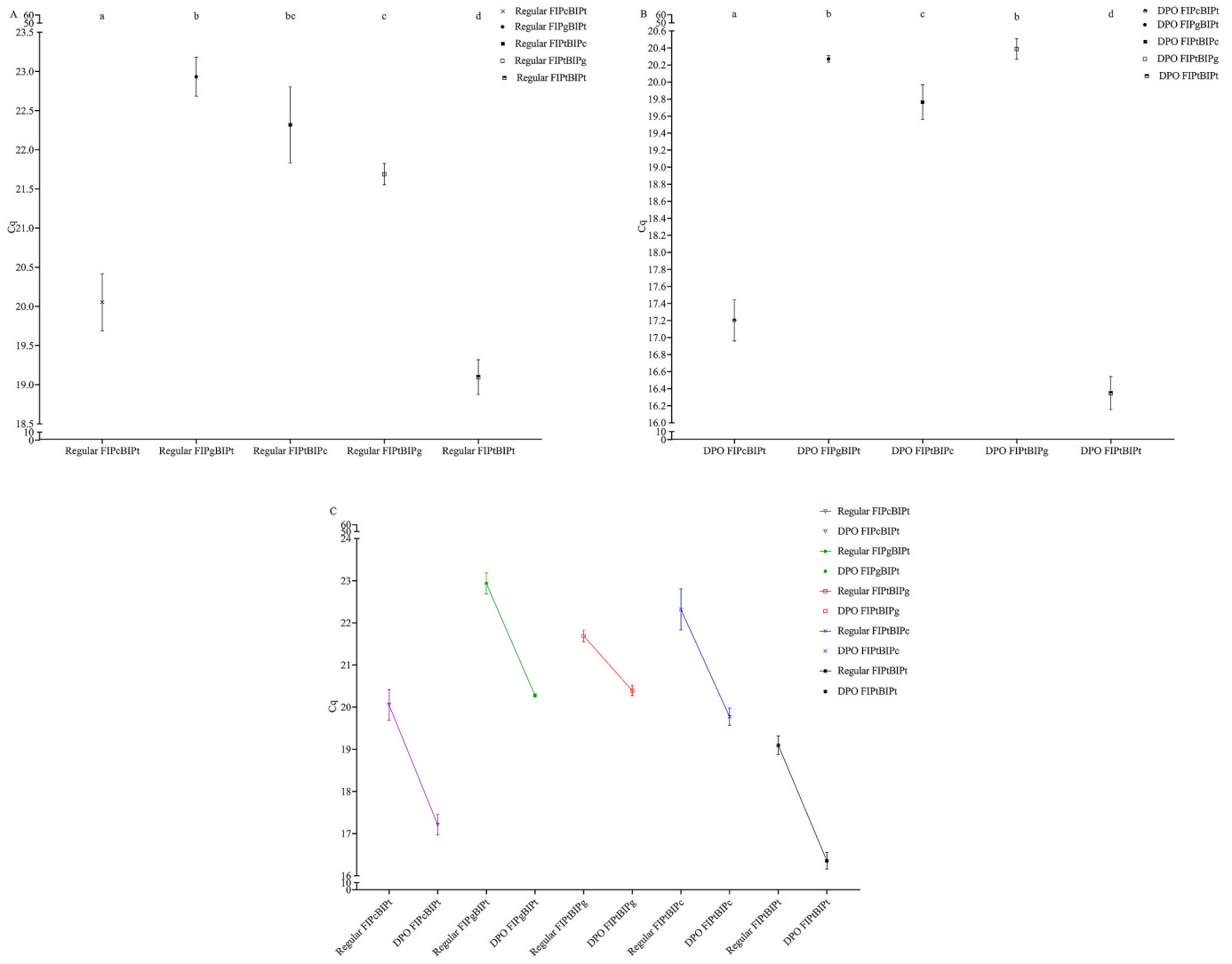

Fig. 4. Combination of FIP and BIP implementing different linkers with regular outer primers A) or DPO outer primers B) and comparison of both C). Within each column different letters denote statiscally significant differences.

associated to the fact that both are purines, and structurally larger than pyrimidines like "T" and "C", however it was surprising that the Cq value obtained with "G" was not statistically different from that of the pyrimidines. The same results were obtained when DPO primers were used instead of regular outer primers without significant differences thus demonstrating for the first time that DPO primers are suitable for LAMP assays.

Once confirmed the effect of the sequence, and the suitability of DPO, the best performing options were compared against NL. In this set of experiments, the beneficial effect the addition of the linker has, in terms of Cq reduction, compared to native FIP/BIP primers was confirmed. It is worth to note that in this second set of experiments the pyrimidine linkers obtained lower Cq values regardless the type of outer primer thus seeming to confirming our hypothesis. Most likely the lack of significance originally observed was due to the large SD obtained, and will support the original idea about the role of the linker in inner primers [6].

To further evaluate this hypothesis, linker combinations were tested and, even though the results were consistent regardless the outer primer structure used, the fact that placing the "C" linker in FIP or in BIP, along with a companion "T" in the other primer, generated significantly different results. This indicates that the results cannot be fully explained by a physical phenomenon, and so that the linker itself is exerting some interaction. It was noted that the sequence flanking FIP and BIP regions of *plcA* gene has "T" and "A" thus the "T" linker may potentially bind providing additional primer stability.

Finally, we sought to evaluate the effect temperature had on this LAMP assay. It was noted that, on a general basis, using DPO as outer primers provided lower Cq values even though at certain temperatures the differences were not statistically significant; however, this is in line with the results typically reported for PCR assays implementing DPO primers [41,42]. Focusing on the different linkers, consistently at all temperatures "T" obtained the best results while the worst were also consistently obtained with "G" and NL.

5. Conclusions

The data obtained in the current study indicate that the sequence selected to serve as linker of the inner primers of LAMP assays, has a significant impact in the final outcome of the assay thus, even though such linker is expected to simply provide physical flexibility, sequence interactions cannot be fully ruled out and so, must be kept into consideration when designing these primers. In addition to this, it has been demonstrated that DPO primers are also suitable for LAMP assays, and that due to their ease of design they can be implemented after regular primers design providing any given assay with an additional

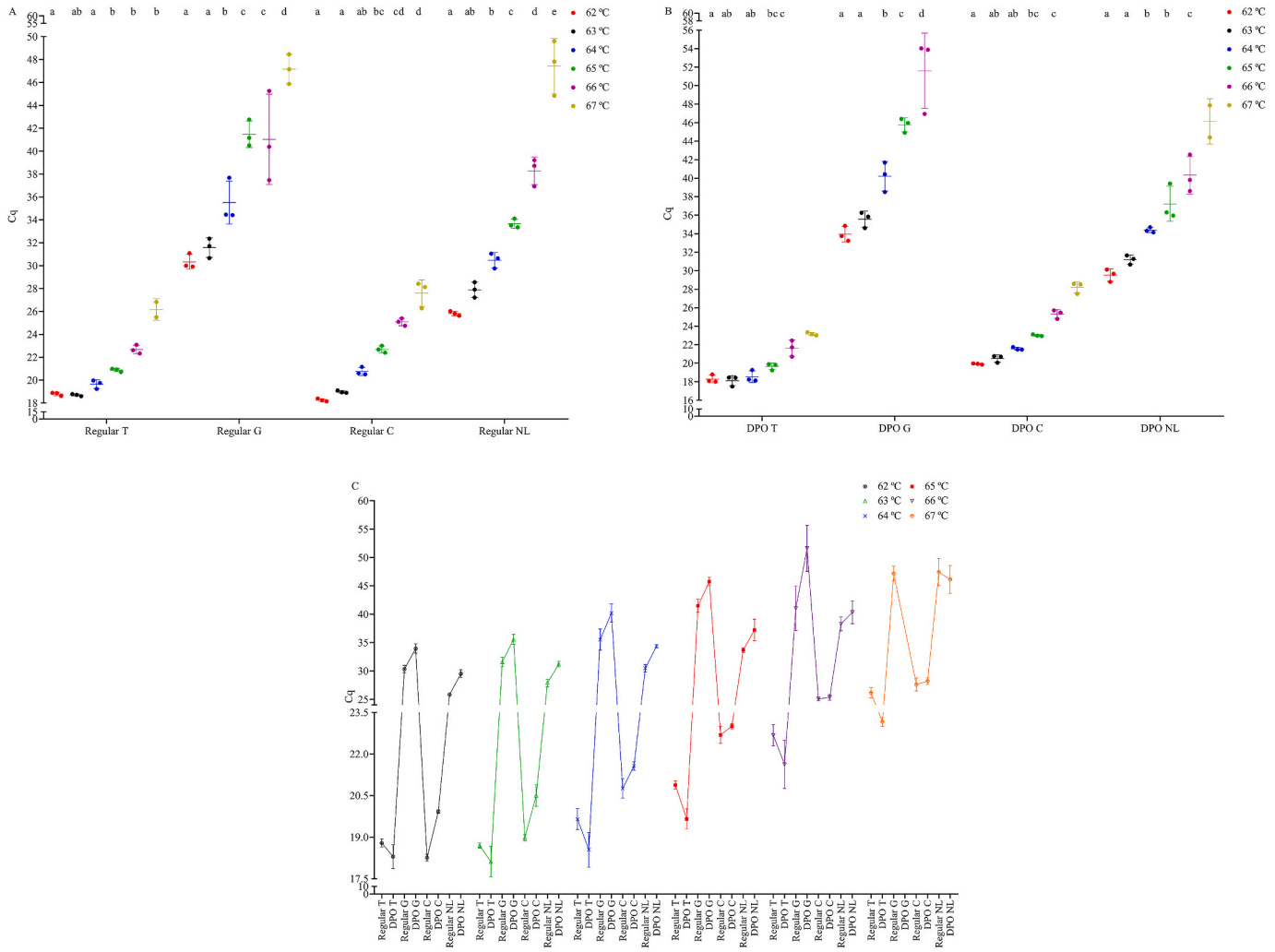

Fig. 5. Effect of the temperature with different linkers, and no linker "NL", with regular outer primers A). Effect of the temperature with different linkers, and no linker "NL", with DPO outer primers B). Effect of the temperature with different linkers, and no linker "NL", comparing the implementation of regular vs DPO outer primers C). Within each column different letters denote statiscally significant differences.

degree of flexibility in terms of temperature optimization, simplifying assay design and optimization and so, overcoming several of the limitations of LAMP-based methods.

Credit author statement

Alexandre Lamas: investigation, revision and editing of the original and revised manuscripts. Sarah Azinheiro: investigation and revision. Foteini Roumani: investigation and revision. Marta Prado: funding acquisition, revision and editing. Alejandro Garrido-Maestu: funding acquisition, conceptualization, methodology, supervision, validation, writing of the original draft and revised manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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