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Quantification of low-expressed mRNA using 5′ LNA-containing real-time PCR primers

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Abstract

Real-time RT-PCR is the most sensitive and accurate method for mRNA quantification. Using specific recombinant DNA as a template, real-time PCR allows accurate quantification within a 7-log range and increased sensitivity below 10 copies. However, when using RT-PCR to quantify mRNA in biological samples, a stochastic off-targeted amplification can occur. Classical adjustments of assay parameters have minimal effects on such amplification. This undesirable amplification appears mostly to be dependent on specific to non-specific target ratio rather than on the absolute quantity of the specific target. This drawback, which decreases assay reliability, mostly appears when quantifying low-expressed transcript in a whole organ. An original primer design using properties of LNA allows to block off-target amplification. 5′-LNA substitution strengthens 5′-hybridization. Consequently on-target hybridization is stabilized and the probability for the off-target to lead to amplification is decreased.

Keywords: Locked nucleic acid (LNA); Polymerase chain reaction (PCR); Interleukin-6 (IL6); Hybridization; LNA primer design; Low expressed mRNA

Real-time reverse transcription PCR (real-time RT-PCR) is to date the most sensitive and accurate method for mRNA quantification [1]. In particular, using specific recombinant DNA as a template, real-time PCR allows DNA quantification with an accurate dynamic range of 6–7 log orders of magnitude and improved sensitivity below 10 copies [2]. Indeed, under these conditions, the DNA template is limited to the specific target and the primers themselves. Therefore amplification should only lead to specific product, with exception of a primer-dimer formation, which is commonly avoided with an appropriate primer design. However, reverse-transcription (RT) products also contain numerous off-targeted primer-bound sequences. Thus, when quantifying very low-copy transcripts, an off-targeted amplification can frequently occur, but is only detected by using DNA binding dye (such as SYBRGreen I). Most of these non-specific amplifications can be avoided by using a correct primer design (primer-dimer) or checking for sequence homology (nucleotide–nucleotide BLAST). However, non-specific template can appear through an unforeseeable mechanism similar to jumping (bridging) PCR [3]. In this case, the primer-bound sequences are originally on separate template molecules. Bridging can occur if the templates contain a region of sequence similarity, leading to a template with specific primer-bound sequences at both ends. Such a drawback appears mostly to be dependent on specific to non-specific target ratio rather than on the absolute quantity of specific target. Indeed, non-specific product is most frequently observed for relatively low-expressed transcript quantification in a whole organ [4] than quantifying normally expressed target in single cell [5]. This undesirable amplification entails either an undervaluation of the mRNA quantities or a lack of detection. A possible way to bypass this

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problem is to use properties of locked nucleic acid nucleotides (LNA) to decrease non-specific amplification.

LNA nucleotides are defined as nucleotide analogues containing an extra 2′O,4′-C methylene bridge added to the ribose ring [6,7]. This bridge results in a 3′-endo conformation reducing the conformational flexibility. LNA base oligomers can be introduced into DNA and RNA sequences by linking to the phosphate-backbone. These LNA-containing oligonucleotides show exceptional stability when hybridized to complementary nucleic acids [6–9]. LNA-substitutions are increasingly used for PCR assays in order to increase maximum annealing temperature (T_{max}) or to improve hybridization specificity. Short intron-specific LNA-probes have been proposed to block amplification of contaminating DNA in real-time RT-PCR [10]. However, LNA-substitutions are mostly used in primers or probes for genotyping based on allele specific hybridization [11–13]. 3′-LNA residues improve the specificity of allele-specific primers compared to native DNA primers [11]. Latorra and colleagues have studied the effects on primer T_{Max} of one to four LNA-substitutions in different positional patterns [14], and suggested that too much LNA substitution in primer sequences was detrimental, and that positioning was an important consideration. More recently, Levin used LNA to enhance affinity of PCR primers in AT-rich sequences in three positional designs: near the 5′ end, near the 3′ end and distributed throughout. Only 5′-substituted primers were comparable to high yielding conventional primers [15].

The goal of this work was to bypass off-targeted amplification when quantifying low copy cDNA in a whole organ. Specially, the aims of this study were (1) to progress in the understanding of off-targeted amplification in biological samples, (2) to test an original design of LNA-containing primers to avoid off-targeted amplification.

Methods

Primer design and melting temperature prediction. Oligonucleotide and LNA-containing primers were synthesized at Eurogentec (Saraing, Belgium). Primer design and optimization regarding to primer-dimer, self-priming formation was done with MacVector software (Accelrys, San Diego, USA) as described previously [16]. Selected forward (FW) and backward (BW) primers are shown in Table 1.

For LNA-modified primers, four-spaced LNA were substituted in the 5′-region without modifying the 3′-region and chosen in order to maximize predicted T_m increase regarding the analogous DNA-primers.

DNA primer melting temperatures (T_m) were assumed as described previously [15]. Briefly, the unified $\Delta H°$ and $\Delta S°$ nearest-neighbour parameters of SantaLucia [17] were used associated to a standard salt correction formula [18] under PCR conditions, i.e., 32 mM monovalent cations, 4 mM $MgCl_2$, 1.2 mM dNTPs, and 400 nM primer concentration. For LNA–DNA primers, $\Delta H°$ and $\Delta S°$ were corrected using the LNA sequence dependent $\Delta\Delta H°$ and $\Delta\Delta S°$ parameters from McTigue [19].

Nucleic acid isolation and reverse transcription. Genomic DNA was isolated from 10^5 frozen baboon Bone Marrow cells using a QIAamps DNA Blood Midi Kit (Qiagen) according to the manufacturer's instructions.

Rat spleens mRNAs were isolated from 7 mg tissue samples with a MagnaPure LC mRNA Isolation Kit II (Roche Applied Science, Mannheim, Germany) in a MagnaPure LC instrument per the manufacturer's instructions. mRNAs were eluted in a 50 μL final volume. Reverse transcription was carried out in a 10 μL final volume from 3 μL of mRNA solution using the Reverse Transcriptase Core Kit (Eurogentec, Saraing, Belgium) with 50 μM oligo(dT)15 primer and RNase inhibitor (2 UI), according to the manufacturer's instructions.

All samples were collected from experimental studies in accordance with ethical French guidelines and approved by the Ethical Committee of the French Army Medical Research Center [16,20].

Real-time qPCR and qRT-PCR. Real-time PCRs were carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Diagnostics), using 4 mM/l $MgCl_2$ and 0.4 μM/L for each primer (final concentration). Quantitative PCR was performed using Lightcycler (Roche Diagnostics) for 45 cycles at 95 °C for 20 s, 55 °C (ACTB) 58 °C (IL6-1, IL6-2) or 60 °C (IL6-LNA) for 5 s, and 72 °C for 10 s. All assays were carried out in duplicate. Quantification was achieved using Cycle threshold (C_T) measured with the second derivative maximum method (LightCycler software v3.5, Roche Diagnostics). Specificities of the PCR amplification were documented with LightCycler melting curve analysis. Melting peaks obtained either from RT-product or from specific recombinant DNA were identical.

Table 1
PCR primer pairs

Primer set	5′–3′ Primer sequence	Position[b]	Predicted T_m[c] (°C)
ACTB			
FW	AGT GTG ACA TGG TGC ATC TCT GC	2064–2086	67.5
BW	ATA GCA CAG CCT GGA TAG CAA CG	2151–2129	67.2
IL6-1			
FW	GCG ATG ATG CAC TGT CAG AAA AC	300–322	65.1
BW	AAC GGA ACT CCA GAA GAC CAG AG	428–406	66.2
IL6-2			
FW	AGG AAC GAA AGT CAA CTC CAT CTG	25–48	64.9
BW	CAG TGG CTG TCA ACA ACA TCA GTC	134–111	66.6
LNA[a]			
FW	GCG ATG ATG CAC TGT CAG AAA AC	300–322	72.5
BW	AAC GGA ACT CCA GAA GAC CAG AG	428–406	72.4

[a] LNA substituted bases are highlighted and underlined.
[b] Positions are from human ACTB gene (E00829) and rat IL6 mRNA (NM_012589).
[c] Predicted using the $\Delta H°$ and $\Delta S°$ nearest-neighbour parameters under PCR conditions (32 mM monovalent cations, 4 mM $MgCl_2$, 1.2 mM dNTPs and 400 nM primer concentration).

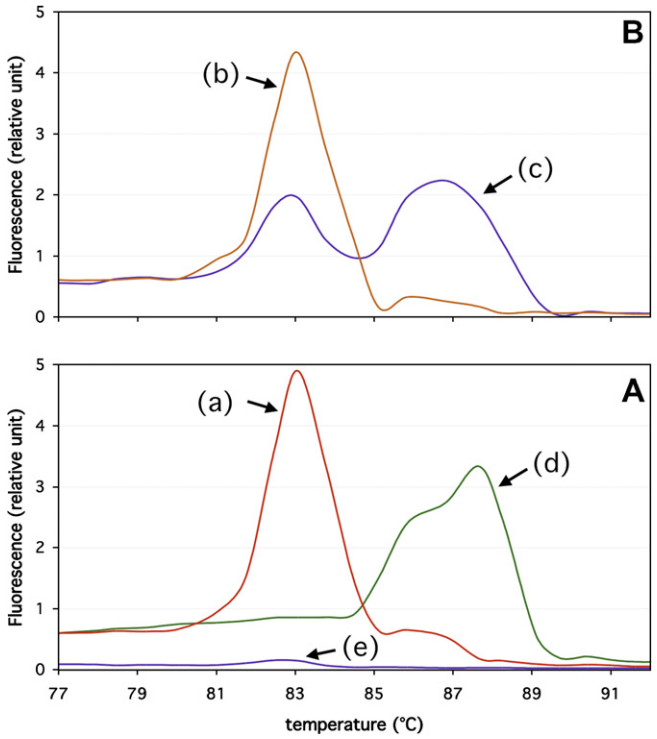

Fig. 1. Melting curve characterization of PCR products obtained quantifying human ACTB using decreasing quantities of genomic DNA in the presence of 25 ng baboon gDNA. Human to baboon gDNA ratio: (a) 50%, (b) 5%, (c) 1%, (d) baboon gDNA, and (e) control without DNA.

Results and discussion

Non-specific amplification in proportion to relative target concentration

On-target to off-target ratio is a key factor for non-specific product amplification. This phenomenon can be illustrated by varying the specific cDNA concentration in the presence of a constant amount of off-targeted template. Recently, we have conducted a nested quantitative PCR to assess stable engraftment of human mesenchymal stem cells in a baboon model of cell therapy [20]. The inner primer set was specific of human ACTB genomic DNA. However, it also amplified an off-targeted product among baboon gDNA. The difference in crossing threshold (C_T) for the same amount of both genomic DNA was a 4–5 range (human versus baboon). This off-targeted amplification was not predictable using nucleotide–nucleotide BLAST among baboon sequences. Decreasing quantities of human gDNA were added to the same amount of baboon gDNA to obtain a 50–0% human to baboon gDNA range. From 50% to 5% human to baboon ratio, only the specific human on-targeted amplification was observed (Fig. 1). An off-targeted amplification appeared from a 1% human to baboon ratio while isolated amplification of the same amount of human gDNA (0.25 ng) led to the human specific product, without primer-dimer formation and off-targeted amplification (data not shown). This non-specific amplification predominated using human to baboon ratio lower than 1%. Thus, off-targeted amplification was not linked to the absolute non-specific target amount that remained constant through out the experiment, but to the specific to non-specific target ratio. Similar results were observed using constant (baboon and human) DNA amount (data not shown).

Non-specific amplification in proportion to absolute target concentration

On-target to off-target ratio is not the only parameter involved in PCR non-targeted amplification. Such amplifications are also dependent on the absolute specific target

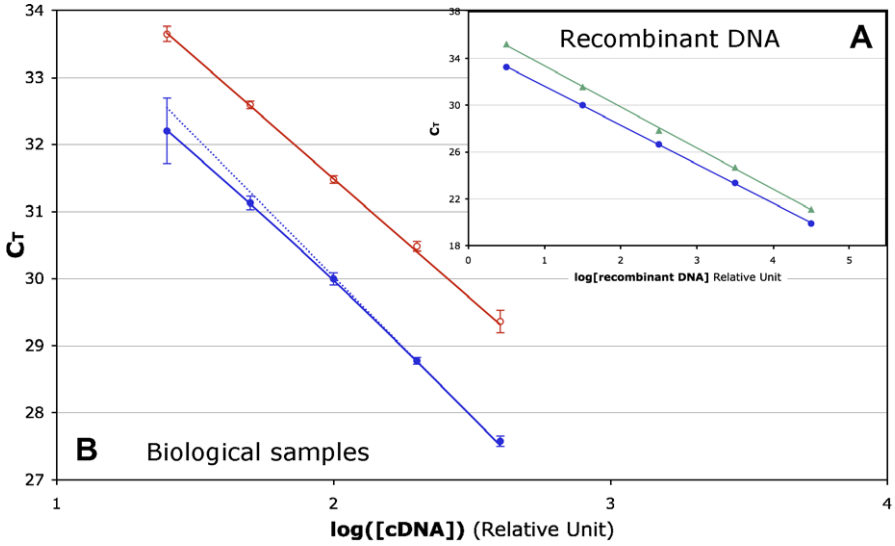

Fig. 2. Rat IL6 mRNA quantification qPCR standard curve using recombinant DNA or low copy biological samples. (A) Four-log range recombinant DNA template: (●) IL6-1, C_T: 19.8–33.2, $E_{PCR} = 99\%$, $r = 0.9999$; (▲) IL6-2: C_T: 21.1–35.2, $E_{PCR} = 93\%$, $r = 0.9996$. (B) Sixteen-fold dilution of biological samples from 40 to 2.5 μg rat spleen samples: (●) IL6-1: C_T: 27.5–32.2, $E_{PCR} = 82\%$, $r = 0.9863$; (○) IL6-LNA C_T: 29.2–33.7, $E_{PCR} = 92\%$, $r = 0.9999$.

concentration while specific to non-specific target ratio remains constant.

IL6-mRNA is expressed at very low level in spleen after thermal injury in rat [16]. New primer sets have been designed to improve IL6-mRNA quantification in such pathological conditions. IL6-1 and IL6-2, have been selected based on their high PCR efficiencies (99% and 93%) (Fig. 2A). Moreover, both IL6-1 and IL6-2 allowed accurate quantification over a 4-log range with a high linearity (Pearson's r 0.9999 and 0.9996) using recombinant DNA as a template (external standard). Crossing points range were, respectively, 19.8–33.2 and 21.1–35.2 for set-1 and set-2. Similar assays were then achieved with both sets, using as a template 16-fold serial dilutions of pooled biological samples namely reverse-transcription products obtained from 40 to 2.5 μg of tissue with low IL6-mRNA levels. Whereas the 27.5–32.2 C_T range on biological samples was included in the external standard range, the set displayed a significant decrease in PCR-linearity for the lowest template-concentrations (Fig. 2). The loss of reliability of the PCR assays was the result of amplification of an off-targeted product from the biological sample, while decreasing the absolute template concentration (Fig. 3). Adjustment of assay parameters, i.e., stringency and primer concentration did not improve PCR-efficiency (data not shown). Similar results were observed for IL6-2 (data not shown). As shown in Fig. 4 using the IL6-2 set, this non-specific amplification appeared stochastically close to the quantification threshold decreasing assay reliability: duplicate samples in the same qPCR run led to different on-targeted to off-targeted amplification ratios with fluctuating C_T. These ratio variations were most likely due to an exacerbation of the Monte Carlo effect in proportion to the off-targeted template. This occurrence is an inherent limitation of PCR amplification from small amounts of any template due to small and random differences in amplification efficiency between individual DNA templates [21].

In summary, the decrease of the absolute specific target concentration led to a stochastic off-target amplification although the on-target to off-target ratio remained constant in the template.

Model of amplification of off-targeted product

The rate of appearance of the on-target hybridized primers ($Hybridized_S$) at any time (t) is proportional to

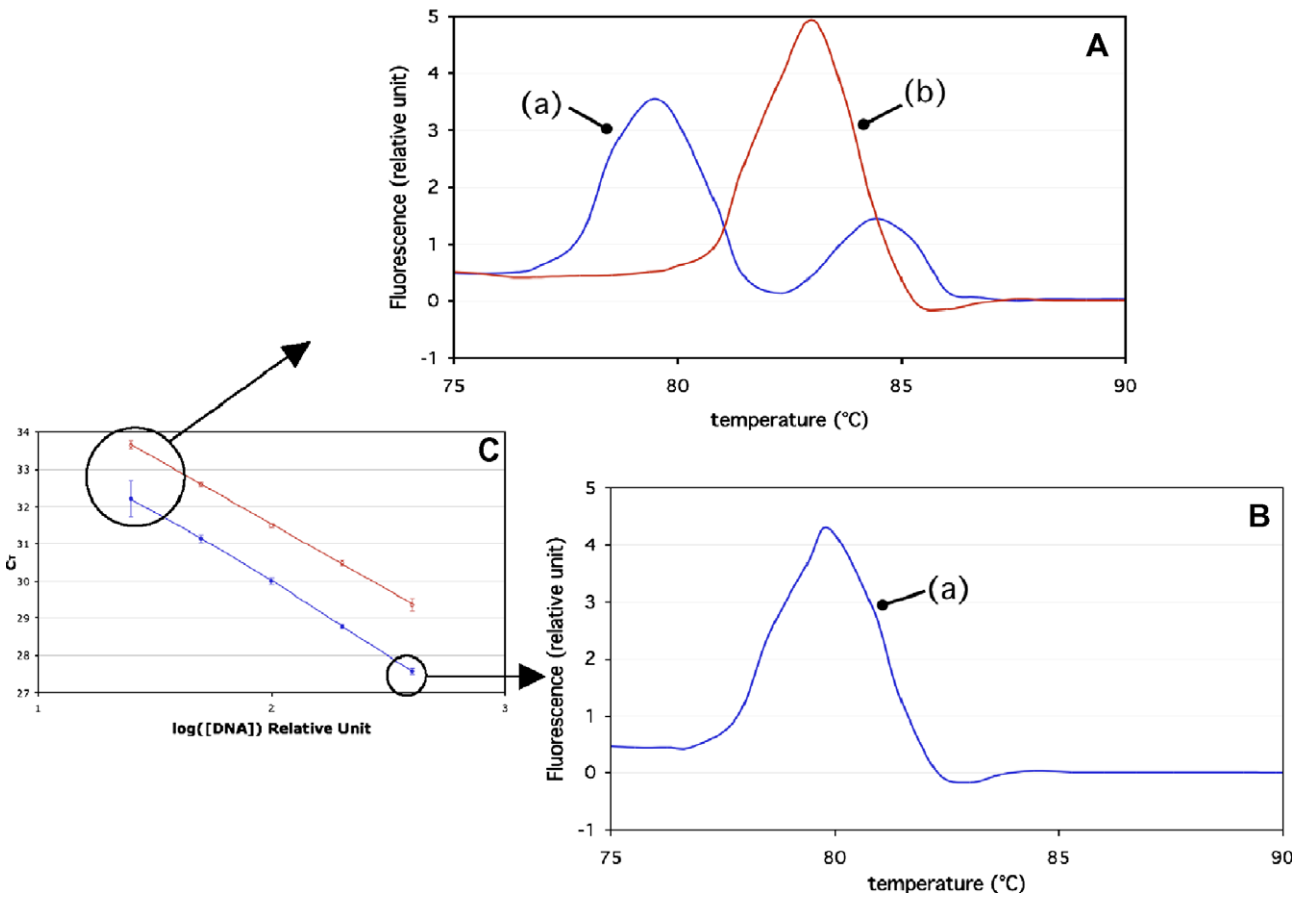

Fig. 3. Melting curve characterization of PCR products obtained quantifying low-expressed rat IL6 mRNA from 40 to 2.5 μg rat low copy biological samples: (A) 2.5 μg tissue sample: (a) IL6-1, (b) IL6-LNA, (B) 40 μg tissue sample: (a) IL6-1, (C) standard curves for IL6-1 (lower curve) and IL6-LNA (upper curve). This figure is identical to Fig. 2A.

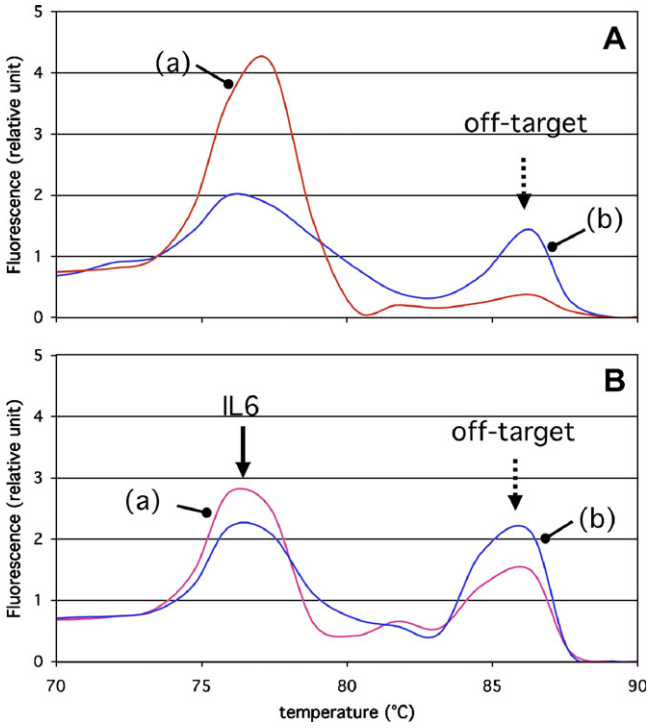

Fig. 4. Melting curve characterization of products obtained from duplicate samples (a and b) in the same qRT-PCR run, near the quantification threshold, using IL6-2 primer set. (A) Stochastic amplification of on-target (a) and off-target (b) products. (B) Reproducible mix of on-target and off-target products.

the concentration of targeted cDNA ([cDNA]) and of unbound primers ([Primer]) according to the following equation [22]:

$$\frac{d[\text{Hybridized}_S]}{dt} = k_S[\text{Primer}][\text{cDNA}_S] \quad (1)$$

Since at the start of the PCR the primer concentration is higher than the cDNA concentration, [Primer], does not decrease, thus can be substituted for the initial concentration, [Primer$_0$], consistent with the following equation:

$$\frac{d[\text{Hybridized}_S]}{dt} = k_S[\text{Primer}_0][\text{cDNA}_S] = K_S[\text{cDNA}_S] \quad (2)$$

By analogy, the rate of appearance of off-target hybridized primers (Hybridized$_{NS}$) at any time (t) is proportional to the concentration of non-specific template (Template$_{NS}$) according to the following equation:

$$\frac{d[\text{Hybridized}_{NS}]}{dt} = K_{NS}[\text{Template}_{NS}] \quad (3)$$

Since only 3′-hybridization leads to the primer extension, regarding to off-targeted amplification, the constant K_{NS} can be reduced to 3′-K_{NS} ($K_{NS} = 3'\text{-}K_{NS} + 5'\text{-}K_{NS}$).

The greatest affinity of the primers for the specific sequence (perfect matching) compared to off-targeted template (mismatches) results in a K_S coefficient higher than 3′-K_{NS}, favoring on-target hybridization. However, for very low-copy transcripts ([cDNA$_S$] ≪ [Template$_{NS}$]), 3′-off-targeted hybridization can coexist with the specific on-targeted hybridization, or can even dominate. Modifications of the assay stringency affect both on-target and 3′-off-target affinity constant in the same manner. The decrease in the reaction stringency can weakly affect the on-targeted to 3′-off-targeted hybridization ratio, however on the other hand, an increase in the reaction stringency, inhibits specific amplification.

However, off-target hybridization can only led to linearly amplified primer extension. PCR exponential amplification needs primer extension to reach homologous or reciprocal priming site. Paboo and colleagues have shown that primers can be extended on different templates provided that these templates contain a similar region [3]. Such bridging can occur between primer extension products, leading to a molecule containing primer sites at both ends and thus allowing an exponential amplification. Artifactual generation of these "shuffle clones" has been well described [23].

In summary, when using RT-PCR to quantify low-copy transcripts an off-targeted hybridization can occur. Variation of the assay stringency cannot alter on-target to off-target hybridization ratio. By analogy to bridging-PCR [3], off-targeted hybridization most likely leads to off-targeted exponential amplification after an initial lag phase, the length of which is proportional to the amount of off-targeted template.

This model provides a solution to block off-target amplification by strengthening 5′ hybridization with LNA-substitution. This primer modification increases K_S (as 5′-K_{NS}) without modifying 3′-K_{NS}. This 5′-LNA substitution allows a significant increase of annealing temperature without decreasing on-targeted hybridization. Consequently, on-target hybridization would be stabilized and decreasing the probability of off-target amplification.

Use of 5′ region LNA-modified primers decrease non-specific amplification

IL6 rat primers set-1 and 5′ LNA-modified analogous primers (Table 1) were used to quantify low IL6-mRNA levels, using serial dilutions of biological sample as described previously. According to the predicted primer melting temperature, the LNA-substitution was supposed to allow a significant increase in hybridization temperature (up to 6 °C). However, LNA-substitution only enabled a 2 °C increase (60 °C versus 58 °C) in annealing temperature. This weak temperature gap could not be the consequence of the high annealing temperature of the DNA set (58 °C) because this gap could not be heightening using 2.5% or 5% DMSO in the qPCR mix (data not shown). DMSO increases the stringency of the PCR assay via a decrease of melting temperatures. However, this gap is consistent with previous report (T_{max} average value of 1.4°C) [14]. Moreover, the eight nucleotides-LNA substitution entailed only a weak 2 °C amplicon melting temperature increase (Fig. 3).

Table 2
PCR performances of IL6 primer sets using biological samples

Primer pair set	DMSO (%v/v)	Non-specific amplification[a]	C_T range
IL6-1	—	+	27.5–32.2
	2.5	+	28.3–33.4
	5	+	28.6–33.6
IL6-1/LNA	—	—	29.2–33.7
	2.5	—	29.1–33.4
	5	—	29.0–33.2
IL6-2	—	+	28.66–33.0

PCR performances of IL6 primer sets using a 16-fold dilution of pooled reverse-transcription products from 40 to 2.5 μg of rat lungs with low IL6 mRNA level. Annealing temperature: DNA sets 58 °C, LNA set 60 °C.
[a] Related to the lowest cDNA concentrations.

LNA-substitution in the 5′ part of the primer allowed enhanced PCR-linearity range (Fig. 2A) regarding to DNA primer, leading to a 4-fold ($\Delta C_T > 2$) decrease in quantification threshold. The improvement of accuracy of LNA-primer set can be attributed to the disappearance of unspecific amplification for the lower template-concentrations (Fig. 3).

These essential results are consistent with the recent study on the position-dependent effects of LNA on PCR primers [15]. Levin substituted 2 or 3 LNA near the 5′ end (LNA-5′), near the 3′ end (LNA-3′) or distributed throughout (LNA-even) in low T_m real-time PCR primers. LNA-5′ displayed higher PCR performance than LNA-3′ and LNA-even when using the C_T as a unique criterion of PCR sensibility. In our study, in spite of their increased performances LNA primer set displayed greater C_Ts ($\Delta C_T \approx 1.6$) than the homologous DNA primer set. The C_T ranges were, respectively, 29.2–33.7 and 27.5–32.2 (Fig. 2A and Table 2). However, contrary to DNA-primers ($\Delta C_T \approx 1.2$), LNA-primers hybridization was not altered by using 2.5% or 5% DMSO in the qPCR mix (Table 2). Furthermore, Levin and colleagues compared performance of short LNA-containing AT-rich primers to longer DNA-primers. The discrepancy between LNA and DNA primers set C_Ts, could be explained by a decreased dye fixation in LNA-containing DNA due to the 3′-endo conformation leading to a fluorescence detection delay. Nevertheless our LNA primer set allows for a 4-fold increase in quantification range toward the lower mRNA concentrations and fluorescence remains substantially above the detection threshold.

Conclusion

Real-time qRT-PCR is the gold standard to quantify mRNA. Meanwhile, non-specific amplification is the major restriction for accurate low-expressed mRNA quantification. Modifying the reaction parameters when quantifying low-copy transcripts has minimal effects on non-specific amplification. The data presented here demonstrate that LNA-5′ incorporation in primers enhances in specificity, quantifying low-expressed mRNA with real-time qRT-PCR. This improvement allows a significant shift of the quantification range toward the lower mRNA concentrations. Further improvement of LNA primer design, especially concerning the positional rules and the thermodynamic parameters, will probably allow new progress in quantification of low-expressed mRNAs.

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