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ORIGINAL PAPER

Development of plasmid calibrators for absolute quantification of the β-parvalbumin gene in *Lophius piscatorius*

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Abstract

The real-time quantitative PCR (qPCR) calibration curves are highly reproducible and allow the generation of specific, sensitive, and reproducible data that can be used for gene quantification. However, it is important to rigorously validate the external calibration curve model in qPCR since absolute quantification is dependent on the standards used. We present a method for standardising qPCR-based quantification of the β-parvalbumin (β-*pvalb*) gene of *Lophius piscatorius*, a major fish allergen, using a plasmid DNA (pDNA) calibrator. In parallel experiments, standard curves were generated and compared from the genomic DNA (gDNA) isolated from *L. piscatorius* and pDNA carrying the target, *pvalb*. The commutability of pDNA and gDNA calibrators for the quantification of β-*pvalb* was assessed by employing a TaqMan qPCR, targeting the second intron of the *pvalb* gene of *L. piscatorius*. Higher PCR efficiencies, good linearity, and lower standard deviation (S.D.) values were observed with pDNA instead of gDNA calibrants. pDNA calibrants exhibited a lower bias in terms of closeness to the expected value of unknown samples than their genomic counterparts. The assay was specific and sensitive, where the limit of detection (LOD) and limit of quantification (LOQ) were five copies and ten copies per reaction. The short-term stability study of the pDNA calibrants indicated its stability for 60 days at − 20 °C and 30 days at 4 °C. The efficient results indicated a plasmid calibrator as a potential tool for absolute quantification of the *pvalb* gene and an alternative to conventional gDNA standards.

Keywords Plasmid calibrator · Genetic marker · *Pvalb* · *Lophius* · DNA quantification

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Introduction

Quantitative PCR is currently the gold standard for nucleic acid quantification. There are various PCR-based quantification methods like qPCR, competitive PCR, and digital PCR. qPCR is the most precise and widely used method for gene quantification [1]. It is used for identification, quantification, and recombinant DNA (plasmid DNA) analysis, covering many medicine and food safety applications. In qPCR, the basic principle is the computation of DNA amplification through the level of fluorescence emitted during the synthesis of amplified double-stranded DNA (dsDNA). Although simple, there are some specific issues in qPCR, like standard selection, to achieve reliable quantification [2]. In this study, we constructed a plasmid DNA (pDNA)-based calibrator for absolute quantification of the β-*pvalb* gene in anglerfish *(Lophius piscatorius),* an important commercial fish.

Parvalbumins (Pvalbs) are heat-stable proteins from the calmodulin family, primarily found in the muscular tissues of vertebrates. A small acidic protein with high Ca^{2+} binding affinity comprising 108–109 amino acid residues with a molecular mass of 12 kDa is a major fish allergy-eliciting protein in humans [3, 4]. Prevention of adulteration is the commonly accepted reason for the development of detection tools that bypass the lack of morphological traits after processing fish meat. Less perceived, but not less important, is the factor associated with the wholesomeness of food, as in some consumers, consuming fish meat can trigger an allergic reaction. The scale of this reaction can start from mild symptoms, but it can reach, in extreme cases, life-threatening, severe conditions. Despite the general reactivity of such predisposed consumers to fish per se [5–8], in some individuals, their reactivity is limited to certain fish species [7, 9–11]. Especially for this reason, proper qualitative and quantitative determination of processed fish meat plays a role in public health.

Absolute quantification qPCR using a standard curve method is the most widely used technique in environmental and food microbiology [12–14]. The standard curve can be constructed by amplifying serial dilutions of known concentrations of standard templates, followed by linear regression of the logarithm of the DNA concentrations of the standard templates versus the threshold cycle (Ct). This method assumes that the sample's efficiency and standard are the same [2, 14, 15]. In practice, efficiency may not be similar, affecting accuracy. To overcome the efficiency fluctuations, both the standard dilutions and the unknown samples are amplified via the same protocol using the same amplification properties [16]. Therefore, the reliability and validity of the standard curve are of importance [17]. It also depends on the design and manufacture of the standard control, measurement of the exact concentration and long shelf life [18, 19].

The standard control or calibrator can be constructed using purified PCR amplicons, plasmid, genomic, cDNA or synthetic oligonucleotides. Recombinant pDNA and genomic DNA (gDNA) cloned together produce reproducible standard curves due to their high stability [19–21]. As various studies support the use of plasmid standard curves for absolute qPCR quantification, we also compare pDNA and gDNA standards. The advantages of plasmid calibrants are that they are relatively easy to construct, stable for extended periods when frozen, and can be manufactured, scaled up, and purified in large quantities over a broader working range [21].

An issue with DNA-based calibration curves is that they only undergo the PCR step, unlike the unknown mRNA samples in transcriptions studies, which must first be reverse transcribed. This increases the potential for variability in qPCR results, and the amplification results may not accurately compare to the results from the unknown samples [17, 18]. Substitution of the gDNA extracts by plasmid one fully addresses such technical challenges [22–24]. A stretch of DNA, like an amplicon originating from endpoint PCR, can be cloned into bacterial vectors. By action of enzymes ligases, an initial linear molecule of plasmid vector is circularised by adoption and a covalent bond connection with this DNA stretch [25]. The resulting clones are mainly selected by antibiotic resistance and screened for only those carrying inserted fragments utilising, e.g. the blue-white selection approach [26].

To find an alternative to the standard curve made from gDNA in quantitative PCR, an experiment using both genomic and cloned plasmid DNA needs to be performed [27]. The aim of this study was to confirm that the recombinant calibrator can be used in future plasmid assessments of the concentration or fraction of certain fish species in complex samples, reducing dependence on gDNA from specific fish species and standardising quantification abilities worldwide.

This article describes the successful cloning of *L. piscatorius* region of the second intron of the protein-coding part of the *pvalb* gene into a bacterial plasmid vector, pGEM-T Easy *pvalb*, and it is employed as a standard for quantification of *pvalb.* The resulting constructed pDNA was compared with the gDNA of *L. piscatorius* as standards for absolute qPCR assay. We also investigated the short-term stability of the plasmid standards against the gDNA standards, providing a tool in qPCR where the plasmid calibrator can substitute gDNA, which is the traditionally used assay.

Materials and methods

Sample collections and preparation

Fish samples of *L. piscatorius* (Lophiiformes, Lophiidae) were obtained from local fisheries and food stores in Prague, Czech Republic. Taxonomical identification was made from the label and was confirmed by ichthyologists from the Faculty of Science, Charles University. Fish tissue was stored in 50 ml tubes at − 20 °C for further downstream applications.

Isolation and quantification of DNA

The fish tissue was thawed and homogenised using a mortar before the extraction. gDNA extraction was performed using the NucleoSpin® Tissue kit (MACHEREY–NAGEL GmbH & Co. KG, Düren, Germany). The extractions were carried out according to the user manual procedures provided by the kit supplier. The reference samples from the panel of fishes were also extracted with the same kit. gDNA was eluted in 100 µl of elution buffer E. pDNA was isolated by PureYield™ Plasmid Miniprep System kit according to the manufacturer's instructions (Promega, Wisconsin, USA). After the chemical lysis of protein structures of bacteria, pDNA was eluted using the principle of silica membrane minicolumn. Samples were quantified spectrophotometrically using a NanoDrop (München, Germany). The A260/280 absorption ratio assessed the purity of the isolated DNA, wherein a value of 1.8 was considered pure.

Oligonucleotide primers

The primer pair targeting the second intron of β-*pvalb* of *L. piscatorius* was used as defined by Mukherjee et al. [28]. The primer pair was used to amplify the target region. Positive amplicons were purified and sequenced (SeqMe, Dobříš, Czech Republic). Obtained sequences were visualised and processed in BioEdit Sequence Alignment Editor, version 7.0.9.0 (California, USA) and used to design the probe (software Primer3Plus, Boston, USA) (Fig. 1) [29]. Primers (DAS F and DAS R) and probe (DAS_Probe1) used in the Taqman qPCR assay were as reported in Table 1. Primers and probe (Table 1) were synthesised by Generi Biotech (Hradec Kralove, Czech Republic).

Construction of target plasmid vector

Primers (DAS F and DAS R) were designed to clone into bacterial plasmid vector pGEM-T Easy Vector System, 3.2 kb (Promega, Wisconsin, USA) carrying the targeted region of the second intron of *pvalb* gene (196 bp) specific to *L. piscatorius.*

The thermal cycling conditions for PCR amplification were 95 °C, 5 min followed by 40 cycles of 94 °C, 30 s; 60 °C, 40 s; 72 °C, 60 s and a final extension of 72 °C, 7 min in Veriti™ Thermal cycler (Applied Biosystems, California, USA). The PCR products were analysed by agarose gel electrophoresis and purified using a PCR product purification kit (Qiagen GmbH, Hilden, Germany). The PCR amplicon concentration was assessed by NanoDrop (München, Germany). The PCR amplicons were directionally cloned into bacterial plasmid vector pGEM-T Easy Vector System and resulting product was designated as pGEM-T Easy *pvalb* (3.2 kb) (Promega, Wisconsin, USA). T4 DNA Ligase was used to create covalent bonds. Insert: vector molar ratio was kept 1:1. The plasmid was incorporated into *E. coli* JM109 High-Efficiency Competent Cells by heat shock according to the manufacturer's instructions (Promega, Wisconsin, USA). Selection of transformants was done on L.B. agar plates (SERVA Electrophoresis GmbH, Heidelberg, Germany) containing antibiotics, ampicillin with a final concentration of 100 µg/ml, with the addition of 0.5 mM IPTG (Carl Roth GmbH + Co.,

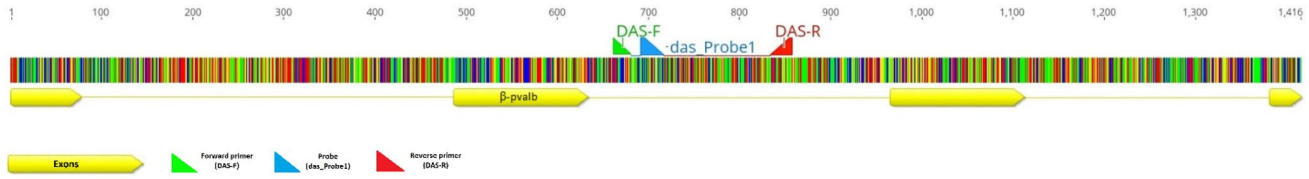

Fig. 1 Location of primer and probe with respect to β-*pvalb* gene of *L. piscatorius* [30]

Table 1 Primers and probes used in this study

Oligo name	Sequence (5′–3′ direction)	References
DAS F	ACAACTTTCCCCGAGAAGC	Mukherjee et al. [28]
DAS R	ACAACATCACAGTTTAAGTTTTGC	
DAS Probe1	FAM-TGCTCTCTATGACAGCTGTCTCGTC-BHQ1	This study

Karlsruhe, Germany) and 80 µg/ml X-gal (Carl Roth GmbH + Co., Karlsruhe, Germany), cultivated at 37 °C. White colonies carrying insert were picked up by sterile toothpick and inoculated in 2 ml L.B. medium containing antibiotic ampicillin (SERVA Electrophoresis GmbH, Heidelberg, Germany) and was incubated at 37 °C to OD A600 of 2.0–4.0. [27, 31] (Fig. 2). Verification of inserted product was confirmed by Sanger's dideoxy sequencing (Generi Biotech, Hradec Kralove, Czech Republic).

Preparation of genomic DNA and plasmid standards

Genomic DNA extracted from *L. piscatorius* tissue was used to prepare a five-step serial dilution as standard with a copy number of 10^5–10^1. No carrier DNA was used for the preparation of gDNA standards.

Linearised plasmid with cloned intron segment, pGEM-T Easy *pvalb* (196 bp, Fig. 2) was used to prepare a five-step serial dilution as standard with copy numbers ranging from 10^5 to 10^1.

The copy number was calculated based on the genome size of *L. piscatorius* gDNA of 747.36 Mb and pDNA of 3.2 kb, with the formula described below (1) as proposed by Lee et al.[13]

$$\text{DNA copies} = \frac{6.022 \times 10^{23} \left(\frac{\text{copy}}{\text{mol}}\right) \times \text{DNA amount (ng)}}{\text{DNA length (bp)} \times 1 \times 10^{9} \times 660 \left(\frac{\text{g}}{\text{mol}}\right)}. \quad (1)$$

The standards were used to construct standard curves and compare gDNA and pDNA PCR efficiency, linearity and regression coefficient. The results were used to derive and validate a plasmid-based calibrator for absolute quantification of *L. piscatorius* by qPCR.

qPCR

A StepOne plus™ qPCR system (Applied Biosystems, California, USA) was used to quantify the DNA by TaqMan assay (Applied Biosystems, California, USA) using primers and probes reported in Table 1. StepOne software V2.3 (Applied Biosystems, California, USA) was used to design the experiment and analyse the experimental data.

The reaction mix contained 10 µl of 2 × TaqMan™ Universal Master Mix 2 with UNG (Uracil-N-glycosylase) (Life Technologies, California, USA), which contained ROX Reference Dye (glycine conjugate of 5-carboxy-X rhodamine, succinimidyl ester) as a passive reference dye, primers (Table 1) at a final concentration of 0.4 mmol/l. 0.1 mmol/l probes (Table 2), 2 µl of template DNA (gDNA and pDNA) and nuclease-free water, the total reaction volume being

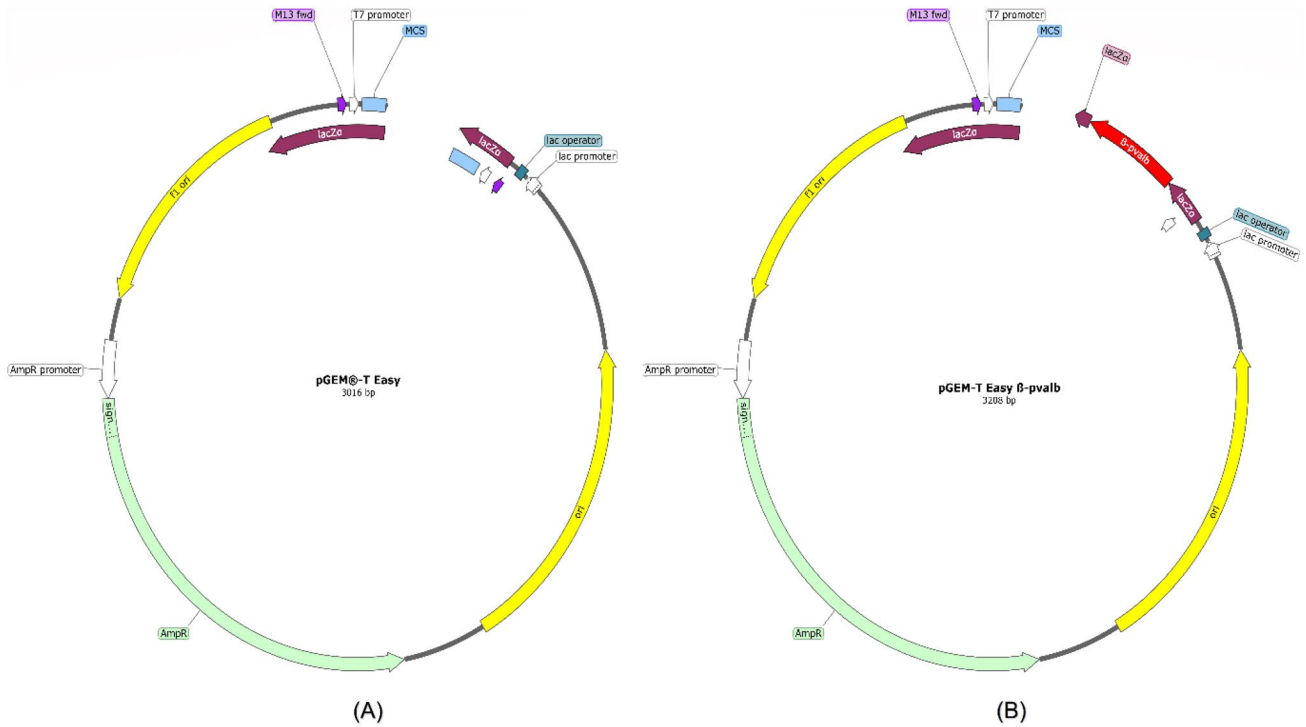

Fig. 2 Genetic map of **A** pGEM®-T Easy plasmid transformed into **B** pGEM®-T Easy β-*pvalb* (3.2 kb) carrying the *pvalb* target gene of *L. piscatorius*

Table 2 Comparison of qPCR efficiencies generated using pDNA and *L. piscatorius* gDNA as standards

Standards	pGEM-T Easy *pvalb*	*Lophius* gDNA
Mean PCR efficiency	100.64	104.18
Mean SD (±)	0.24	1.07
Mean coefficient of determination (R^2)	0.996	0.992
Single-factor ANOVA (*p* value)	0.51^{NS}	0.17^{NS}

Mean ± SD ($N = 3$, $n = 3$),

Ns non-significant

20 µl. The following thermal cycle profile was used: initial holding step at 50 °C for 2 min, initial denaturation and polymerase activation at 95 °C for 10 min, 35 cycles with denaturation at 95 °C for 15 s and annealing with polymerisation at 60 °C for 1 min. All PCR reactions were carried out in triplicates in three parallels.

Determination of limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ are the most important parameters of a qPCR assay, referring to the lowest quantity of the target that can be reliably detected and quantified with a probability of ≥ 95% [32, 33]. Both LOD and LOQ are essential parameters in analytical chemistry and are used to determine the sensitivity and precision of analytical methods and instruments. The absolute limit is the lowest number of initial template copies that can be detected and quantified. The absolute LOD and LOQ of the TaqMan qPCR assay were determined by serially diluted pDNA and gDNA. All PCR runs were evaluated as cycle parameters described above.

Short-term stability study

The experimental design for the stability study of *L. piscatorius* gDNA and pGEM-T Easy *pvalb* pDNA was based on previously reported stability studies for plasmid calibrators [26]. Three tubes of each serial dilution of linearised pGEM-T Easy *pvalb* pDNA and gDNA standards were stored at 4 °C and − 20 °C. Aliquots of the gDNA and pDNA standards in triplicate were withdrawn after 0, 15, 30 and 60 days and evaluated as described above. PCR efficiency, regression coefficient and three parallel tests were performed in triplicates.

Statistical analysis

The PCR efficiency for the target-specific qPCR method and the linearity of corresponding regression lines calculated based on serial dilutions of the pDNA and gDNA calibrant were compared and statistically analysed using single-factor ANOVA. Statistical tests were performed using Microsoft Excel software (Analysis ToolPack for Microsoft Excel 2020).

Results and discussion

In the present work, we demonstrate the successful use of a plasmid-based (pGEM-T Easy *pvalb*) calibrator for absolute quantification of the β-*pvalb* gene targeting the second intron of *pvalb* of *L. piscatorius*. We used it as a model organism. DNA from *L. piscatorius* tissue was extracted using the NucleoSpin® Tissue kit. Nanodrop quantification was reported to be 412 ± 6 µg/µl. The primer set (DAS F and DAS R) is designed for gDNA amplification amplified *Lophius* samples.

Construction of pGEM-T Easy *pvalb* plasmid

The process of constructing the plasmid pGEM-T Easy *pvalb* involved inserting a 196 bp fragment of the second intron of the β-*pvalb* gene from *L. piscatorius* into the pGEM-T Easy vector (Fig. 2). This resulted in the formation of the plasmid pGEM-T Easy *pvalb*, as shown in Fig. 2B. The DNA sequence of pGEM-T Easy *pvalb* was then confirmed to contain the 196 bp *pvalb* sequence.

qPCR

A TaqMan qPCR assay targeting the second intron of the *pvalb* gene in the plasmid DNA forms the foundation of the present study. The TaqMan assay was used to detect both gDNA and pDNA independently. The standard curve was generated with a fivefold dilution ranging from 10^5 to 10^1 copies of gDNA and pDNA per reaction. The C_T value was plotted against the log of copy number for both calibrants (Fig. 3). Simple linear regression (least squares analysis) was applied to each data set comprising the calibration curve to give the best fitting straight line accommodating all the data points. A multi-parametric approach was adopted wherein the correlation coefficient (R^2) and PCR efficiencies of the pDNA and gDNA system calibration curves were compared.

Comparison of PCR efficiency of the calibrants

PCR efficiency, a measure of how closely the majority of the PCR cycles in a given run approximate a perfect duplication of product, is one of the most important parameters in determining the reliability and performance of an assay [2]. An efficient qPCR has an efficiency close to 100%; however, if the efficiency is low, it can lead to inaccurate quantification, as the relationship between the starting template and

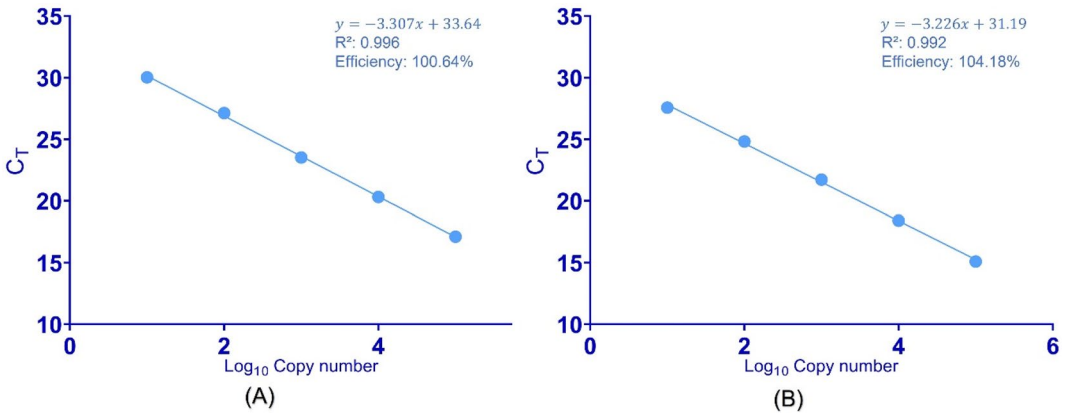

Fig. 3 Standard curves for qPCR of *L. piscatorius* targeting the second intron of β-*pvalb* gene. **A** Standard curve obtained from qPCR of fivefold dilution series of extracted pDNA (pGEM-T Easy *pvalb*); **B** standard curve obtained from qPCR of serial dilutions of the gDNA

amplified product becomes non-linear. The mean amplification efficiency of pDNA and gDNA was reported to be 100.64% and 104.18%, respectively. Three parallel tests were performed in triplicates, and a single-factor ANOVA indicated no significant statistical difference amongst individual calibrants (Table 2). Results were in the optimal efficiency acceptance range of 90–110%, according to MIQE guidelines. The correlation coefficient (R^2), demonstrating how well experimental data fit the regression line, was also observed in the acceptable average value of $R^2 \geq 0.98$. No significant statistical difference was observed between R^2 values between the standard curve obtained from gDNA ($R^2 = 0.992$) and pDNA ($R^2 = 0.996$). The results with reasonable efficiency, good linearity, sensitivity and accuracy were observed for both standard curves, making them suitable as calibrants for the *pvalb* quantification. However, as expected, pDNA PCR efficiency was significantly better than the gDNA calibrator efficiency ($p = 0.01$). *Pvalb* is a significant allergen identified in fish products making it an essential gene target for quantification. Owing to the exponential nature of PCR amplification, minor differences in PCR efficiencies can generate more significant differences in copy number estimation [34]. This can have serious implications for the quantitative determination of allergens present in the food. As the amount of amplified DNA is proportional to the amount of allergen present in the sample, lower efficiency can result in inaccurate quantification and, consequently, non-compliance with regulations regarding allergen labeling. Therefore, pDNA calibrant with an efficiency of 100.64% appears more suited for quantification than gDNA.

Repeatability and reproducibility of qPCR assay using pDNA and gDNA standards

The repeatability and reproducibility of the qPCR assay were tested using linearised pGEM-T Easy *pvalb* pDNA and *L. piscatorius* gDNA as calibrators. As shown in Table 3, the results indicate that the assay has good repeatability with SD and bias values below 25% and within the ENGL acceptable range [35]. The results indicated that the pDNA calibrant was better than the gDNA calibrant in calculating the experimental copy numbers of β-*pvalb*. Thus, the qPCR assay employing the pGEM-T Easy *pvalb* calibrator is reliable and suitable for quantifying *pvalb*, and its performance is comparable to a gDNA calibrant.

The accuracy of the quantitative results was evaluated as the bias between the observed copy number and achieved value. The precision of the assay was assessed in terms of RSD. The RSD and bias values (Fig. 4) using plasmid calibrants were within the acceptable range of < 25% [35]. Higher RSD and bias values were observed with gDNA calibrants. RSD and bias values obtained in this study are similar to those previously reported [36–38] and demonstrated that the choice of the calibrant impacts the reliability and accuracy of the analysis. Observations in this study indicate that the constructed pGEM-T Easy *pvalb* was reliable and credible for quantifying β-*pvalb*. Our results indicate that gDNA calibrators tend to underestimate the *pvalb* content. Underestimation of the samples using gDNA calibrants is probably due to the low PCR efficiency of the gDNA calibrants compared to pDNA calibrants (Table 2). pDNA calibrants displayed better performance characteristics in terms of closeness to the expected value than their genomic counterparts (Fig. 4). This agrees with the superior performance characteristics associated with single and multiple target plasmid calibrants reported for the quantification of food allergens [38].

Determination of limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ of qPCR assay are defined as the lowest quantity of the target gene that can be detected and quantified

Table 3 Repeatability and reproducibility of the $C_{T.}$ values using pGEM-T Easy *pvalb* and *L. piscatorius* gDNA

Actual copy number	Mean CT[a]	Mean SD[b]	RSD[c]	Mean experimental copy number	Bias %[d]
pDNA					
100,000	17.12	0.17	1.02	99,188.67	−0.81
10,000	20.44	0.23	1.15	9806.93	−1.93
1000	23.76	0.24	1.03	972.53	−2.75
100	27.00	0.40	1.50	101.52	1.52
10	30.21	0.22	0.73	10.59	5.94
gDNA					
100,000	15.09	0.14	0.93	97,881.49	−2.12
10,000	18.51	0.20	1.08	8522.44	−14.78
1000	21.62	0.34	1.57	926.25	−7.37
100	24.72	0.49	1.98	101.73	1.73
10	27.77	0.38	1.36	10.74	7.40

*Mean CT ($N=3$, $n=3$)

**Mean standard deviation

[c]Relative standard deviation

[d]Bias = [(mean experimental copy number − actual copy number)/actual copy number] × 100

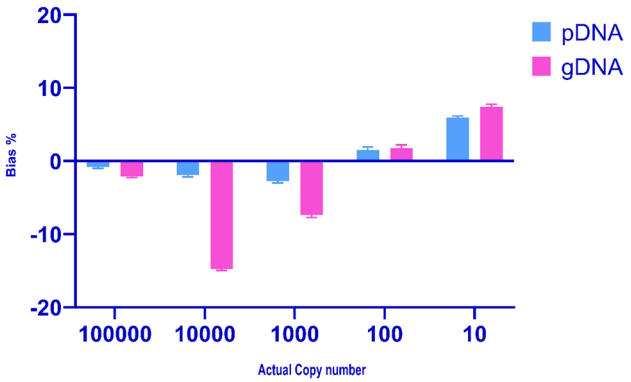

Fig. 4 Bias between the observed value and true/assigned value

with more than 95% probability. To test the LOD and LOQ of plasmid pGEM-T Easy *pvalb* in the qPCR, fivefold dilutions of concentrations (100, 50, 25, 10 and 5 copies/µL) were prepared. According to ENGL [35], the acceptance criterion of LOD and LOQ should be less than 25 copies and 50 copies, respectively. In the assays, the results determined that the LOD and LOQ were 5 copies and 10 copies per reaction for pGEM-T Easy *pvalb*, whilst the LOD and LOQ of *L. piscatorius* gDNA were 10 copies and 25 copies per reaction, respectively. Both pGEM-T Easy *pvalb* and gDNA could be detected seven times in nine repeated reactions. LOD and LOQ are critical for evaluating the performance of analytical methods and instruments and ensuring that data are reliable and accurate. In many fields, including environmental analysis, food safety, and pharmaceuticals, regulatory agencies set minimum LOD and LOQ requirements for analytical methods to ensure accurate and meaningful results [38]. The high sensitivity of the assay indicates its suitability to quantify allergenic proteins, even in small amounts, which is important for detecting trace amounts of allergens in processed foods and meeting current global labelling legislations.

Short-term stability study of pGEM-T Easy *pvalb*

The stability of pDNA and gDNA standards stored at 4 °C and − 20 °C was tested for 60 days, standard curve construction was performed on the 0th, 15th, 30th and 60th day and PCR efficiency was compared. Plasmid DNA, pGEM-T Easy *pvalb*, showed PCR efficiency better than that of gDNA calibrants over a period of 60 days (Fig. 5). PCR efficiency of pDNA was within the acceptable range for 60 days at − 20 °C whereas PCR efficiency was within the acceptable range for 30 days at 4 °C. Similarly, gDNA standards were also stable for 60 days at − 20 °C, however, with a lower efficiency than pDNA standards. Also, at 4 °C, the standards showed some partial degradation with more than 110% PCR efficiency.

pDNA can be conveniently stored at − 20 °C and transported around the world in cold packs or dry ice. Whilst DNA degradation is expected during storage and transportation, an ideal reference material should be resistant to loss and degradation. The correlation coefficient (R^2) showed a linear regression with values of ≥ 0.99 observed at − 20 °C for 60 days with no significant statistical variation, indicating a stable target with no dilution error. The results also suggest that the PCR assay could still detect and quantify a significant amount of *pvalb*, even after being stored for a prolonged period. This is important because it indicates

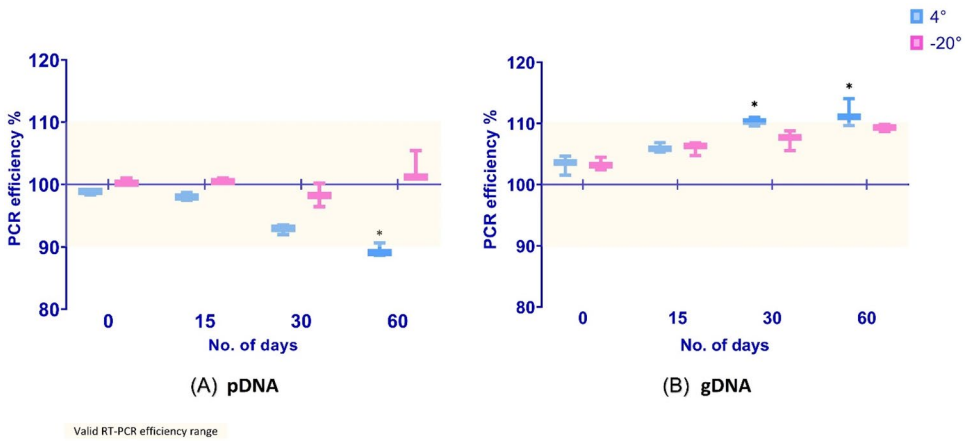

Fig. 5 PCR efficiency obtained for independent experiments of **A** plasmid DNA standards; and **B** genomic DNA standards after various storage times

that the sample was preserved in a way that allowed for the PCR to produce reliable results even after a significant amount of time had passed. This can be particularly useful in situations where the samples need to be stored for long periods before analysis, as it shows that the sample can be reliably preserved and used for PCR analysis later. These results are also in agreement with previously reported studies [16]. The results confirm that plasmid reference material can be stored for 60 days, increasing present knowledge of plasmid calibrant stability. Further, whilst we used linearised pDNA, non-linearised plasmids can also be used for 14 days at − 20 °C due to their high stability [39].

The advent of real-time qPCR has greatly improved the use of molecular biomarkers to diagnose and monitor human diseases, quantitative determination of GMO ingredients, and analysis of microbial communities [14, 40, 41]. The use of qPCR protocols may offer an advantage in quantifying potential allergens, although further evaluation is necessary to draw definitive conclusions. Fish species often contain both α- and β-*pvalb*, with multiple β-*pvalb* isoforms expressed by most species, exhibiting significant variation in amino acid composition and sequence [37, 42–44]. Such heterogeneity adds complexity to the diagnosis of fish allergy and the detection of allergenic parvalbumin, as an individual may have a stronger reaction to one β-*pvalb* isoform than another [37]. Our assay, hence, targeted the β-*pvalb* gene due to allergenic nature.

qPCR has become the favoured method of widespread use due to its reliability, high sensitivity, good reproducibility, a large dynamic range of quantification with a small amount of starting material, relatively low costs, and short analysis time [16, 45].

Absolute and relative quantification are two quantification strategies, each with advantages and disadvantages. Whilst the absolute method quantifies the concentration of the target gene by relating the Ct value to a standard curve, the relative method uses a reference sample with reference genes for quantification [46]. One of the significant drawbacks of using reference samples is that they cannot be normalised worldwide, resulting in a differences between inter-laboratories results. Hence, there is an effort to establish a standardised biomolecule calibrator approach between laboratories. This is often the case in immunodiagnostics, where some protein molecule plays the role of the standard as the calibrator [47]. Even though DNA-based diagnostics and detections took place in the first attempts to bring some interlaboratory standards, the International WHO standard for establishing the Epstein–Barr virus was announced in 2016 [48] Despite using cloned fragments of the virus as one of the alternative calibrators within a particular laboratory, whole-genome DNA of the EBV was used as the international standard. Regarding the scale of variance between laboratories, the context from EBV viral load determination, more than two orders difference between laboratories seems to be common in these assays [48]. To circumvent the limitations, quantification using plasmid calibrators is used. Whilst plasmid construction can be time-consuming, it is straightforward to reproduce once constructed. As our data highlighted, plasmid DNA can generate highly reproducible standard curves with high stability up to 60 days of storage. Due to this, pDNA are easy to distribute worldwide, normalising reference material.

The presented assay standardises PCR-based detection of *L. piscatorius pvalb* by introducing a plasmid standard. It provides an inevitable aid in substituting a calibrator based on DNA isolate from a particular specimen with a plasmid one. The variation, SD and RSD of obtained value between individual experiments are within one order, which is satisfactory in the context of interexperimental variance in other DNA-based qualitative assays. In the present study, we demonstrated using a plasmid calibrator to quantify the *pvalb* gene of *L. piscatorius* by cloning the target into a plasmid (pGEM-T Easy *pvalb*). We also compared the standard curve of both genomic and plasmid and demonstrated the stability of plasmid standards over 60 days.

Conclusion

The current work aimed to check the reliability of a plasmid-based calibrator for detecting and quantifying the β-*pvalb* gene in *Lophius piscatorius*. The developed plasmid was successfully cloned with our target gene (second intron of *pvalb*; 196 bp) and showed high efficiency, repeatability and stability. The plasmid DNA calibrator performed better compared with the gDNA calibrator. The study contributes to the increasing knowledge of the profound use of plasmid calibrators for quantitative detection. The alternate option of using a plasmid instead of isolated gDNA can be a helpful tool and a step towards standardising fish identification amongst laboratories. We have used *L. piscatorius* as a model organism. However, the results can be implemented and transferred to other organisms, establishing an essential tool in fish identification, and creating a database for the *pvalb* content of different fish species.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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