GMR
The Original

VALIDATION OF QRT-PCR PRIMER AMPLIFICATION EFFICIENCY FOR REFERENCE AND FUNCTIONAL GENES IN THE BRINJAL SHOOT AND FRUIT BORER, *LEUCINODES ORBONALIS*

Nikhil Reddy K S[1,2], Pradeep S[2], Venkatesan Thiruvengadam[1*], Gandhi Gracy Ramasamy[1], Hanumanthaswamy B C[2], Prashantha C[3], Satish K M[4], Nagaraju M S[5]

[1]Division of Genomic Resources, ICAR-National Bureau of Agricultural Insect Resources, Bengaluru - 560024, India
[2]Department of Entomology, College of Agriculture, Shivamogga, Keladi Shivappa Nayaka University of Agricultural and Horticultural Sciences, Shivamogga – 577204
[3]Department of Entomology, AHRS, Ponnampet, Kodagu – 571216
[4]Department of Plant Biotechnology, College of Agriculture, Shivamogga, Keladi Shivappa Nayaka University of Agricultural and Horticultural Sciences, Shivamogga – 577204
[5]Department of Soil Science and Agricultural Chemistry, College of Agriculture, Shivamogga, Keladi Shivappa Nayaka University of Agricultural and Horticultural Sciences, Shivamogga – 577204
[*]Corresponding author Email I.D.: tvenkat12@gmail.com

ABSTRACT
The quantitative reverse transcription PCR (qRT-PCR) is widely used to quantify gene expression, but reliable measurements require validated primer amplification efficiency. This study evaluated the amplification efficiencies of five primer sets targeting *28S*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), bystin (*bysl*), ecdysone receptor (*EcR*) and ultraspiracle (*USP*) in the brinjal shoot and fruit borer, *Leucinodes orbonalis*. Standard curves were generated by plotting quantification cycle (Cq) values against the logarithm of the starting template quantity. The five-fold serial dilution studies exhibited amplification efficiencies ranging from 96.1% to 105%, with coefficients of determination (R^2) between 0.988 and 0.997. The corresponding slopes ranged from −3.420 to −3.208. *GAPDH* exhibited the lowest amplification efficiency (96.1%), whereas *28S* showed the highest (105%). All assays demonstrated strong linear relationships across the tested template concentrations. These results indicate that the evaluated primer sets of the candidate reference genes exhibit suitable amplification characteristics for subsequent qRT-PCR analyses. Nevertheless, primer efficiency alone does not establish the stability of reference genes under different experimental conditions. Further validation of reference-gene expression stability, amplicon specificity and assay reproducibility is recommended to ensure reliable relative quantification in *L. orbonalis*.

KEYWORDS: *Leucinodes orbonalis*, qRT-PCR, amplification efficiency, standard curves, gene expression.

1. Introduction

The quantitative reverse transcription PCR (qRT-PCR) is a sensitive and widely adopted technique for quantifying transcript abundance and gene expression profiling. Its accuracy depends on several experimental parameters, including RNA integrity, reverse-transcription performance, primer specificity, amplification efficiency and appropriate normalization of expression data. Inappropriate validation of these parameters can introduce systematic errors and compromise the interpretation of differential gene expression (Bustin et al., 2009; Taylor et al., 2019).

Amplification efficiency is a critical prerequisite of quantitative accuracy because it describes the increase in target DNA during each PCR cycle. It is commonly estimated from the slope of a standard curve constructed by plotting Cq values against the logarithm of serially diluted template concentrations. An amplification efficiency of 100% corresponds to an approximately twofold increase in product per cycle and a theoretical slope of −3.32. Assays with efficiencies near 100% and strong linearity are generally considered suitable for quantitative analysis, although acceptable performance must be assessed alongside specificity, precision and the assay's working range (Pfaffl, 2001; Bustin et al., 2009).

In *L. orbonalis*, molecular characterization of genes involved in development, reproduction and endocrine regulation can provide insights into pest biology and support the evaluation of RNA interference (RNAi)-based management strategies. Bystin (*bysl*), ecdysone receptor (*EcR*) and ultraspiracle (*USP*) are relevant functional targets, whereas *28S* and *GAPDH* may serve as candidate reference genes. However, the suitability of reference genes for normalization cannot be assumed solely from their conventional use; their expression stability must be evaluated under the specific experimental conditions (Vandesompele et al., 2002; Huggett et al., 2005). Therefore, the present study evaluated the standard-curve characteristics,

amplification efficiencies and linearity of five qRT-PCR primer pairs targeting *28S, GAPDH, bysl, EcR* and *USP* in *L. orbonalis*. This assessment provides a preliminary technical basis for their use in subsequent gene-expression studies.

2. MATERIAL AND METHODS

2.1. Insect culture, RNA extraction and cDNA synthesis

The *L. orbonalis* culture was maintained under laboratory conditions at 27 ± 1°C and 70–80% relative humidity, and biological material was collected for molecular analysis. Total RNA was isolated using 1000 µL of RNAiso Plus (Takara, Japan) following the manufacturer's protocol. The quality and integrity of the isolated RNA were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA) and 1.2% (w/v) agarose gel electrophoresis, respectively. The complementary DNA (cDNA) was synthesized from the isolated RNA using PrimeScript First Strand cDNA Synthesis Kit with a gDNA Eraser (Takara, Japan).

2.2. Primer designing

The qPCR primers for checking amplification efficiencies were designed using the PrimerQuest Tool (Integrated DNA Technologies). The designed primers were further evaluated for key quality parameters, including melting temperature, GC content, and the potential for secondary structure formation, using OligoEvaluator and SMS Primer Stats tool. Primer sequences and corresponding amplicon sizes are presented in Table 1.

2.3. Standard-curve construction and amplification efficiency

The amplification efficiency of five primer pairs was evaluated using a serial dilution of cDNA. The undiluted cDNA sample was used as the initial template, followed by successive five-fold serial dilutions to obtain relative template concentrations of 1, 1:5, 1:25, 1:125 and 1:625. Each successive dilution was prepared by mixing one volume of the preceding cDNA dilution with four volumes of nuclease-free water. The resulting dilution series was used as a template for qPCR amplification with each gene-specific primer pair. Each dilution was analysed in separate qPCR reactions using the respective gene-specific primers. Technical triplicates were included for each dilution, along with a no-template control (NTC) to monitor potential contamination and nonspecific amplification.

Standard curves were generated by plotting Cq values against the logarithm of the starting template quantity. Linear regression was used to determine the slope, y-intercept and coefficient of determination (R^2) for each primer pair. Amplification efficiency was calculated using the equation:

E (%) = [10^(−1/S) − 1] × 100

where S represents the slope of the standard curve. An efficiency of 100% corresponds to a slope of approximately −3.32. The slope, y-intercept, amplification efficiency and R^2 values were transcribed from the supplied standard-curve output. Because the dilution scheme and replicate-level data were not provided, the number of dilution steps, technical replicates and statistical uncertainty could not be independently verified. Assay performance was interpreted in line with established qPCR validation principles (Pfaffl, 2001; Bustin et al., 2009).

3. RESULTS

3.1. Biological relevance of the selected genes

The five genes represent distinct biological functions, ranging from ribosomal structure and cellular metabolism to ribosome biogenesis and hormonal regulation of insect development.

28S rRNA: The 28S rRNA is a major structural and functional component of the large 60S ribosomal subunit in eukaryotes. Together with ribosomal proteins and other rRNAs, it contributes to ribosome architecture and protein synthesis. Because of its abundance, 28S rRNA is frequently employed as a candidate gene for mRNA normalization. Nevertheless, its suitability for qRT-PCR normalization must be verified under the relevant experimental conditions rather than assumed from its structural role alone (Alberts et al., 2002; Bustin et al., 2009).

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): *GAPDH* encodes a conserved glycolytic enzyme that catalyses the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, coupled with the reduction of NAD^+ to NADH. This reaction contributes to cellular energy metabolism. Although *GAPDH* is commonly used as a candidate reference gene in expression studies, its transcript abundance may vary with developmental state and experimental conditions. Therefore, therefore the suitability of a candidate reference gene for accurate quantification needs to be experimentally verified (Nicholls et al., 2012; Bustin et al., 2009).

Bystin (*bysl*): *Bysl* gene is associated with ribosome biogenesis and the processing of precursor rRNA. Functional annotation studies in *Drosophila melanogaster* indicate that the insect bystin homolog participates in ribosomal RNA processing and the formation of ribosomal subunits (Fukuda et al., 2008). *Drosophila* studies have also associated bystin with cellular growth and cell–cell interactions, suggesting broader developmental relevance. These conserved functions provide a biological rationale for examining *bysl* in *L. orbonalis*, although its specific physiological roles in this pest require direct experimental confirmation (Adams et al., 2008; Anon, 2026).

Ecdysone receptor (*EcR*): *EcR* gene encodes a ligand-dependent nuclear receptor that mediates the biological effects of the insect steroid hormone 20-hydroxyecdysone (20E). Upon hormone binding, *EcR* participates in transcriptional regulation of ecdysteroid-responsive genes, coordinating developmental transitions, moulting and metamorphosis. Consequently, *EcR*

represents an important molecular target for investigating endocrine regulation and developmental processes in insects (Nakagawa and Henrich, 2009; Hill et al., 2013).

Ultraspiracle (*USP*): *USP* encodes an insect nuclear receptor homologous to vertebrate retinoid X receptor (RXR). It commonly forms a heterodimer with *EcR*, enabling the receptor complex to interact with ecdysone-response elements and regulate hormone-responsive gene transcription. Genetic studies in *Drosophila melanogaster* have demonstrated that *USP* is required for appropriate developmental and transcriptional responses to ecdysone during metamorphosis. The functional association between *EcR* and *USP* provides a rationale for evaluating both genes in studies of insect development and molecular pest management (Yao et al., 1993; Ghbeish et al., 2001; Nakagawa and Henrich, 2009)

3.2. Polymerase Chain Reaction (PCR) based amplification check

All the genes were amplified with the cDNA which is synthesized earlier (in the above section) in Bio-Rad C1000 Touch Thermal Cycler Thermocycler Dual 48 Well Fast, low usage from Bio-Rad. As soon as the primers were synthesized, gradient PCR was kept for a standard 25 µL reaction to check the temperature at which the primers were amplified which can be used for further studies. PCR amplification was performed with an initial denaturation step at 95 °C for 5 minutes. This was followed by 39 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute. A final extension step was carried out at 72 °C for 5 minutes. After confirming the amplification temperature, the PCR product was loaded on 1.2% gel, confirming the band amplified at their respective amplicon length. The amplicon lengths of *28S, GAPDH, bysl, EcR* and *USP* were confirmed as 152, 156, 165, 160 and 147 bp, respectively (Figure 1).

3.3. Standard-curve characteristics and amplification efficiency

All five primer sets exhibited negatively sloped standard curves, indicating that Cq values decreased with increasing starting template quantity (Figure 2). Primer amplification experiments yielded values ranging from 96.1% to 105%, while R^2 values ranged from 0.988 to 0.997. The standard-curve parameters are summarized in Table 2.

Among the evaluated assays, *28S* showed the highest amplification efficiency (105%), followed by *bysl* (104.4%), *USP* (101.9%), *EcR* (98.4%) and *GAPDH* (96.1%). The *EcR* and *USP* assays showed the highest coefficients of determination ($R^2 = 0.997$), followed by *GAPDH* (0.996), *28S* (0.991) and *bysl* (0.988) (Table 2). These results indicate generally strong linearity, although the *bysl* assay showed marginally greater deviation from its fitted regression line than the other assays.

3.4 Melt-peak analysis of qRT-PCR primers

The specificity of the five candidate genes in *L. orbonalis* was evaluated through post-amplification melt-peak analysis (Figure 3). All five genes exhibited a prominent and relatively sharp dominant melting peak, indicating the amplification of a predominant PCR product. The *28S* gene exhibited a major melting peak at approximately 81–82°C, while the *GAPDH* gene showed a distinct peak around 82–83°C. Similarly, the *bysl* and *EcR* genes displayed prominent melting peaks at approximately 83–84°C and 82–83°C, respectively. The *USP* gene also showed a distinct dominant melting peak at approximately 83–84°C. The melting profiles were relatively consistent across the replicate reactions for each gene.

Although minor fluorescence-derived signals were observed at lower temperatures (approximately 73–77°C) in some primer sets, these signals were substantially lower than the corresponding major melting peaks. The dominant peaks were clearly distinguishable from these minor signals, suggesting that the predominant amplification products had consistent melting characteristics.

4. DISCUSSION

The five qRT-PCR assays exhibited amplification efficiencies between 96.1% and 105% and R^2 values between 0.988 and 0.997. These results demonstrate satisfactory amplification efficiency and strong linearity across the tested cDNA dilution series. All five primer pairs displayed efficiencies within the commonly accepted range of 90–110%, indicating that the assays are suitable for reliable amplification and subsequent quantitative gene-expression analysis. The high R^2 values further demonstrate a strong linear relationship between Cq values and the logarithm of relative starting template quantity. Amplification efficiency and linearity are important analytical parameters for evaluating the performance of qRT-PCR assays (Bustin et al., 2009; Taylor et al., 2010; Bustin et al., 2025).

Melt-curve assessment is an important component of qPCR quality control because it helps evaluate amplification specificity and identify potential nonspecific products or primer-dimer formation. The melt-curve analysis in the present study further supported the specificity of the five primer assays, with distinct dominant melting peaks observed for *28S, GAPDH, bysl, EcR* and *USP*. The presence of predominant single melting peaks indicates amplification of major products with consistent melting characteristics, an important criterion for assessing qRT-PCR assay specificity (Bustin et al., 2009; Taylor et al., 2019).

A distinction must be made between primer efficiency and reference-gene stability. Although *28S* and *GAPDH* are frequently considered for normalization, their suitability in *L. orbonalis* cannot be confirmed from standard curves alone. Though earlier studies on these reference genes were evaluated (Kariyanna et al., 2020), these must be thoroughly studies across all the developmental studies in *L. orbonalis* using all possible web tools to analyze its stability. The use of multiple validated reference genes can improve the reliability of normalized expression estimates (Vandesompele et al., 2002; Andersen et al., 2004).

The standard-curve performance of *bysl, EcR and USP* supports their further use in functional gene-expression studies, including investigations of insect development and RNAi-mediated gene silencing. Where assay efficiencies differ, efficiency-adjusted relative-quantification methods may be preferable to the conventional $2^{-\Delta\Delta}$ Cq method (Pfaffl, 2001). Overall, these findings provide preliminary technical validation of the five primer pairs, while further specificity, precision and reference-gene stability assessments are needed for comprehensive validation.

The successful standardisation of the five primer assays provides an important molecular resource for investigating gene expression in *L. orbonalis*. Reliable primer performance is fundamental to generating reproducible transcript-expression data and enables the application of qRT-PCR in studies involving insect development, reproduction and RNAi mediated gene silencing. The inclusion of both reference gene candidates (*28S* and *GAPDH*) and functionally relevant genes (*bysl, EcR* and *USP*) further increases the research value of the validated assays. In particular, the availability of validated primers for *EcR* and *USP* facilitates investigations into ecdysone mediated developmental regulation, while the *bysl* assay provides an opportunity to examine the molecular mechanisms associated with ribosome biogenesis and cellular functions. Thus, the present work establishes a useful foundation for future functional genomics research and the development of RNAi based molecular pest management strategies against *L. orbonalis* (Pfaffl, 2001; Bustin et al., 2009; Taylor et al., 2010).

5. CONCLUSION

The five qRT-PCR primer pairs targeting *28S, GAPDH, bysl, EcR* and *USP* exhibited amplification efficiencies of 96.1–105% and R^2 values of 0.988–0.997. These results indicate generally satisfactory amplification performance and strong standard-curve linearity, providing a useful foundation for subsequent gene-expression studies in *L. orbonalis*. Validation of primer specificity, experimental reproducibility, and reference-gene stability is essential before establishing comprehensive assay validation. Furthermore, accurate qPCR quantification requires an appropriate normalization strategy, as inaccurate data normalization can compromise the reliability of experimental result.

Acknowledgments

The authors gratefully acknowledge College of Agriculture, Navile, Keladi Shivappa Nayaka University of Agricultural and Horticultural Sciences, Shivamogga for providing this opportunity for the doctoral programme in ICAR-NBAIR, Bengaluru.

Conflict of interest

The authors declare that they have no conflict of interest related to this work.

REFERENCES

1. Anonymous. Flybase Database on bystin gene in *Drosophila melanogaster*. Accessed from FlyBase Gene Report: Dmel\bys. Accessed on 29-09-2026
2. Alberts B. Molecular biology of the cell. Garland science; 2017 Aug 7.
3. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer research. 2004 Aug 1;64(15):5245-50.
4. Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., ... & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.
5. Bustin, S. A., Ruijter, J. M., Van Den Hoff, M. J., Kubista, M., Pfaffl, M. W., Shipley, G. L., ... & Wittwer, C. T. (2025). MIQE 2.0: revision of the minimum information for publication of quantitative real-time PCR experiments guidelines. *Clinical Chemistry*, *71*(6), 634-651.
6. Fukuda MN, Miyoshi M, Nadano D. The role of bystin in embryo implantation and in ribosomal biogenesis. Cellular and molecular life sciences. 2008 Jan;65(1):92-9.
7. Ghbeish N, Tsai CC, Schubiger M, Zhou JY, Evans RM, McKeown M. The dual role of ultraspiracle, the Drosophila retinoid X receptor, in the ecdysone response. Proceedings of the National Academy of Sciences. 2001 Mar 27;98(7):3867-72.
8. Hill RJ, Billas IM, Bonneton F, Graham LD, Lawrence MC. Ecdysone receptors: from the Ashburner model to structural biology. Annual Review of Entomology. 2013 Jan 7;58:251-71.
9. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. Genes & Immunity. 2005 Jun;6(4):279-84.
10. Kariyanna, B., Prabhuraj, A., Asokan, R., Babu, P., Jalali, S. K., Venkatesan, T., ... & Mohan, M. (2020). Identification of suitable reference genes for normalization of RT-qPCR data in eggplant fruit and shoot borer (Leucinodes orbonalis Guenée). *Biologia*, *75*(2), 289-297.
11. Nakagawa Y, Henrich VC. Arthropod nuclear receptors and their role in molting. The FEBS journal. 2009 Nov;276(21):6128-57.
12. Nicholls C, Li H, Liu JP. GAPDH: a common enzyme with uncommon functions. Clinical and Experimental Pharmacology and Physiology. 2012 Aug;39(8):674-9.

13. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic acids research. 2001 May 1;29(9):e45-.
14. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. Trends in biotechnology. 2019 Jul 1;37(7):761-74.
15. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology. 2002 Jun 18;3(7):research0034-1.
16. Yao TP, Forman BM, Jiang Z, Cherbas L, Chen JD, McKeown M, Cherbas P, Evans RM. Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. Nature. 1993 Dec 2;366(6454):476-9.

List of tables

Table 1. Primer sequences and expected amplicon lengths for the five qRT-PCR assays

SLNO.	Gene	Primer name	Primer sequence (5′–3′)	Amplicon size (bp)
1	*28S*	RT-Lo28S-F	AGGAATATTCAAACCCCAGCC	152
	28S	RT-Lo28S-R	AACTTTCCCTTGCTCTGTCC	
2	*GAPDH*	RT-LoGAPDH-F	GCGAAGGTCATTTCTAATGCG	156
	GAPDH	RT-LoGAPDH-R	GTCTACGGTCTTTGGGTGG	
3	*bysl*	RT-Lobysl-F	TCTAAACCTGAACGGACAAG	165
	bysl	RT-Lobysl-R	TTGAAGGACATCTCTGACAC	
4	*EcR*	RT-LoEcR-F	AAACGGATGCAGTACAGATG	160
	EcR	RT-LoEcR-R	AACACTCCGTCTGAAGAAAC	
5	*USP*	RT-LoUSP-F	GAGCGAGGAGTGTCAATTC	147
	USP	RT-LoUSP-R	TGCCCGAAATGTGGAATG	

Table 2. Standard-curve parameters and amplification efficiencies of the five qRT-PCR assays

Gene	Slope	Y-intercept	Efficiency (%)	R^2
28S	−3.208	42.843	105	0.991
GAPDH	−3.420	42.489	96.1	0.996
bysl	−3.220	43.292	104.4	0.988
EcR	−3.360	42.989	98.4	0.997
USP	−3.277	39.114	101.9	0.997

List of figures

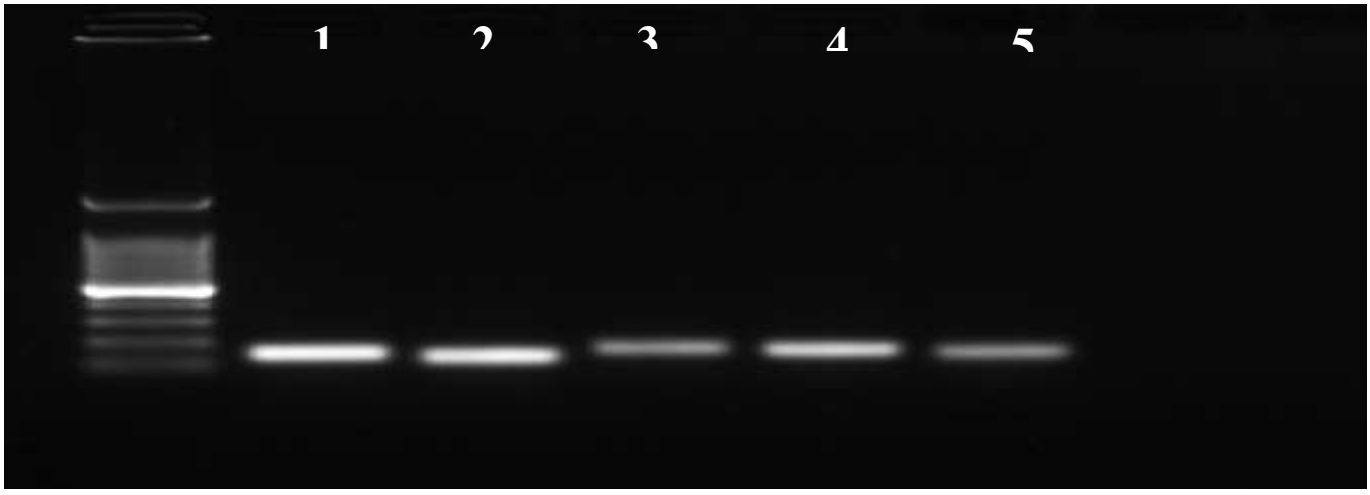


Figure 1. Agarose gel electrophoresis analysis of PCR-amplified genes. Ethidium bromide-stained 1.5% agarose gel showing PCR amplification products for *28S, GAPDH, bysl, EcR* and *USP* genes. Lane L: 100 bp DNA ladder; Lanes 1–5: PCR amplicons corresponding to *28S* (152 bp), *GAPDH* (156 bp), *bysl* (165 bp), *EcR* (160 bp), and *USP* (147 bp), confirming successful amplification and target size specificity.

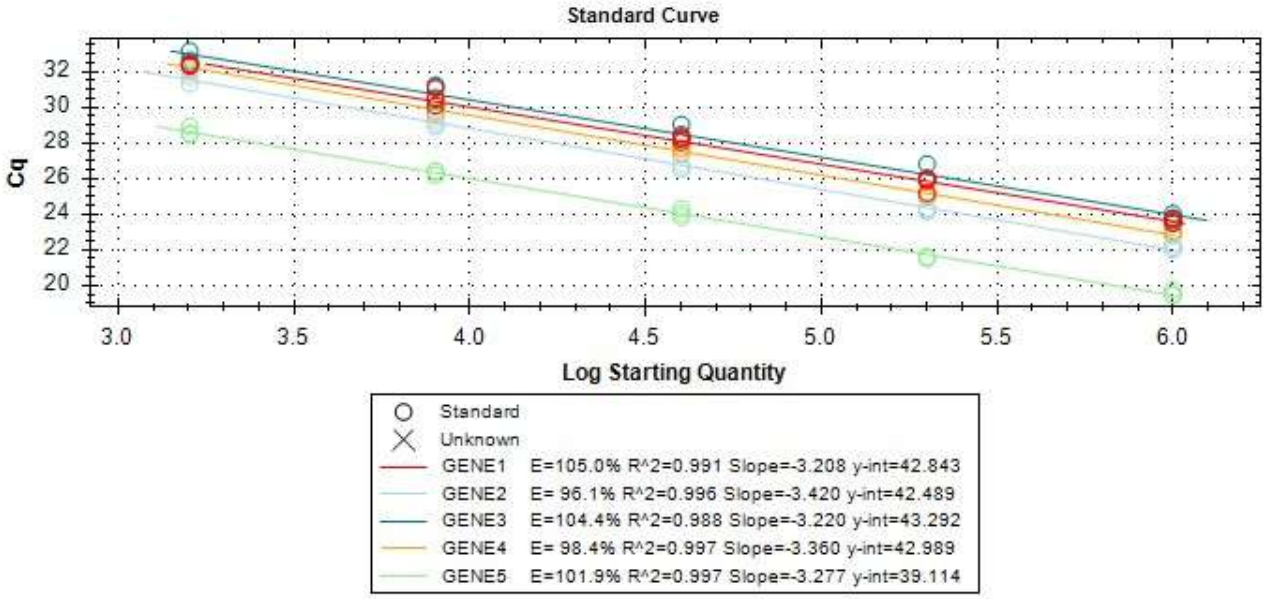


Figure 2. RT-qPCR standard curves and amplification efficiencies for RT-qPCR assays**.** Linear regression plots of quantification cycle values (Cq) plotted against the logarithmic starting template quantity across serial dilutions. Circular points represent standards evaluated in replicate. All five primer pairs demonstrated high linearity ($R^2 \geq 0.988$) and amplification efficiencies within the optimal range ($96.1\% \leq E \leq 105\%$)

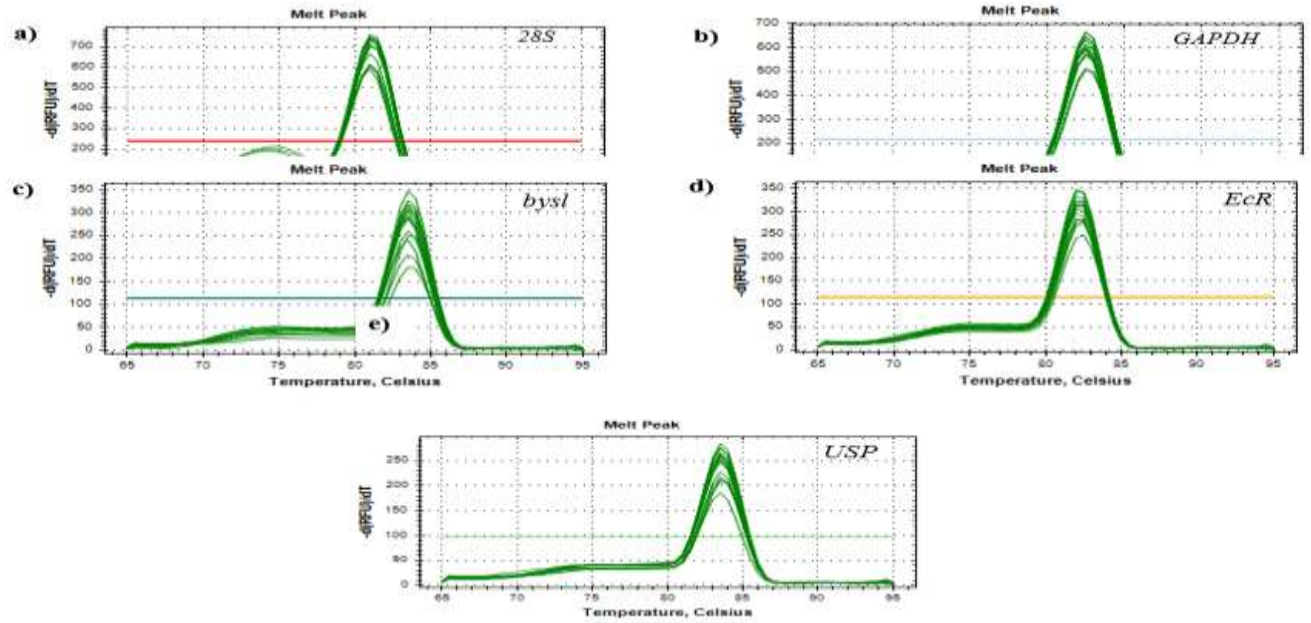


Figure 3. Melting curve profiles confirming primer specificity in RT-qPCR assays. Representative derivative melting curves ($-\mathrm{d(RFU)/d}T$ *vs*. temperature) for RT-qPCR assays: **(a)** *28S*, **(b)** *GAPDH*, **(c)** *bysl*, **(d)** *EcR*, and **(e)** *USP*. The horizontal-coloured lines indicate threshold cutoff baselines for peak detection.