International Journal on Emerging Technologies **16(2): 36-41(2025)**

ISSN No. (Print): 0975-8364
ISSN No. (Online): 2249-3255

qRT-PCR's Significance and Efficacy in Molecular Biology Research

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(Received 10 April 2025, Revised 21 May 2025, Accepted 09 June 2025)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Quantitative Real-Time PCR (qRT-PCR) is one of the most *in vitro* innovative methods in molecular biology research for analyzing nucleic acids (RNA and DNA). It uses fluorescent reporter molecular techniques to track production of amplified products and analyze expression of specific genes (decipher the functional genes) through creation of cDNA from RNA as *in vivo* (DNA Replication) depending upon exponential amplification of specific sequence between target and reference genes producing millions of copies for various analysis such as standard curve method, CT and 2−ΔΔCq methods to analyze gene expression during linear ground phase, exponential phase, log-linear phase and plateau phase of reaction cycles. Therefore, this review paper discusses the important and effectiveness of qRT-PCR in molecular biology researches. Thus, the essential prerequisites for accurate gene expression quantification are RNA extraction, RNA purification, cDNA synthesis, housekeeping genes as an internal control and qRT-PCR analysis to ascertain the amount of gene expression to investigate expression profiles of multigene families and endogenous genes by choosing appropriate reference genes as standard for normalizing target gene expression data to detect chromosomal translocation and single nucleotide polymorphism of relatively large number of samples in a short period of time. Hence, qRT-PCR use to understand basic cellular mechanisms and identifying changes in gene expression levels in respond to particular biological stimuli.

Keywords: qRt-PCR reaction, housekeeping genes, Normalization, CT method, 2−ΔΔCq method.

INTRODUCTION

Since the invention of southern and northern blots for detection DNA and RNA, respectively, gene expression profiling considered in molecular biology labs (Greene *et al.*, 2024; Reddy *et al.*, 2025). But these methods require comparatively large amounts of nucleic acids from tissues based on band intensity for quantification (Mehta and Amit 2017). Therefore, one of the most efficient and sensitive approaches for analyzing gene expression profile to pursue functional genomic in recent molecular biology researches is quantitative real-time PCR (qRT-PCR) instead of blot techniques (Liang *et al.*, 2014; Shobha and Keshamma 2023). In order to monitor template amplification with more sensitivity and specificity expression in terms of accuracy and speed, qRT-PCR reaction cycles combine with fluorescent reporter chemistry, to quantify expression level of particular genes and to detect alternations at particular genomic regions (Ma *et al.*, 2016). As a result, qRT-PCR calculate initial amount of nucleic acid template in reaction without necessary post amplification process for quantitative data in accurate dynamic range (Nestorov *et al.*, 2013).

One of the most challenges aspects in qRT-PCR amplification efficiency is normalizing variance which caused by some errors in nucleic acid extraction and purification, efficiency in cDNA synthesis and stability of employed reference genes (Jian *et al.*, 2016). Consequently, in order to normalize relative expression of target genes, reliable reference genes must be chosen (Kong *et al.*, 2014; Taylor *et al.*, 2019; Katholnig, 2021). A number of genes have been employed as reference genes or housekeeping genes or internal control genes since their expression levels in different tissues or organs in primary metabolism and basic cellular processes remained largely consistent across

many experimental conditions such as actin (ACT), ubiquitin (UBQ), beta-tubulin-4 (TUB4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 16S RNA, 18S RNA and elongation factor 1 alpha (EF1α) (Yuan *et al.,* 2014; Wang *et al.,* 2020). However, recent researches revealed that some of these genes don't exhibit consistent expression across range of pant species, plant tissues and plant developmental stages (Liang *et al.,* 2014; Sandercock *et al.,* 2017). Therefore, choosing appropriate reference genes is critical for normalizing qRT-PCR analysis for appropriate interpretation of gene expression data. As a result, more and more researches focus on identifying and assessing putative reference genes in different plant species under varied experimental conditions (Hazem *et al.,* 2024; Gebril and Elsayed 2025; Saleh *et al.,* 2025).

Sum of all, attention in experimental design, sample and assay quality control and select appropriate reference genes for normalization significantly success qRT-PCR analysis that be important and effectiveness of qRT-PCR in molecular biology labs.

Principles of qRT-PCR: Amplification efficiency of qRT-PCR depending upon nucleic acid extraction and purification, cDNA synthesis and stability of employed reference genes (Harshitha and Arunraj 2021). Detection and quantification of qRT-PCR depend upon fluorescent reporter under rapid change in temperatures during reaction cycles (Ching *et al.,* 2022). Fluorescent chemistries use as binding dyes and fluorescently labeled sequence specific primers or probes because it monitors target sequence amplification during reaction cycles (Özcan *et al.,* 2022). Fluorescent reporters in qRT-PCR involve double-strand DNA binding dyes or dyes molecules attach to primers or probes for hybridization with PCR product during reaction cycles to reflect the amount of amplified product in each reaction cycle (Kashani and Malau-Aduli 2014). Therefore, qRT-PCR integrates quantification and amplification into single process including nucleic acid template, two oligonucleotide primers as a binding complementary sequency for specificity, dNTPs, Taq DNA polymerase to catalyze synthesis of new strand from primed single strand template.

SYBR Green dye consider a common DNA binding dye in qRT-PCR, because it exhibits 1000 folds more fluorescence than ethidium bromide after binding to double strand DNA as compare to unbound DNA to increase target amplified as mentioned in Fig. 1.

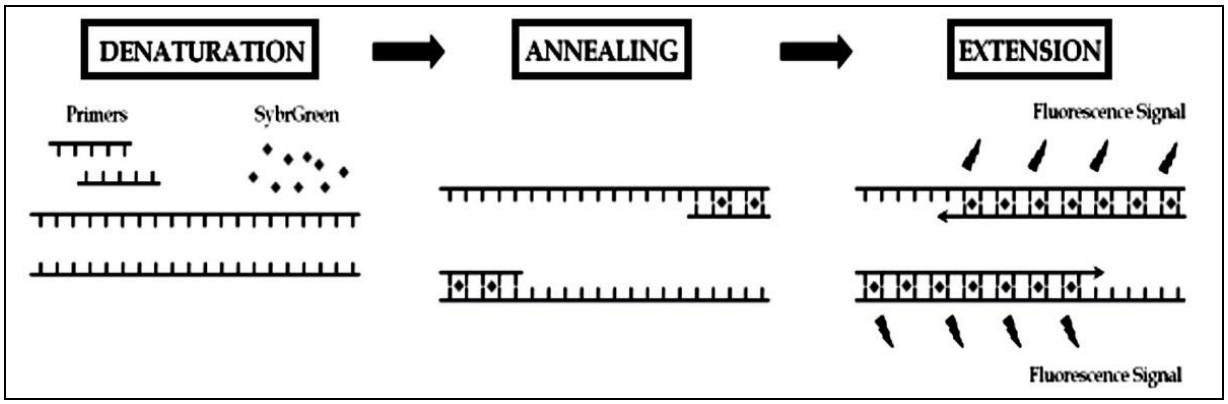

Fig. 1. Principle of SYBER Green day detection during qRT-PCR reaction.

Phases of qRT-PCR reaction cycles: In order to show product accumulation, qRT-PCR plots fluorescence reaction cycles in four phases (linear ground phase, exponential phase, log-linear phase and plateau phase) (Fig. 2). At linear phase (usually first 10 to 15 cycles), qRT-PCR initiate and emits fluorescence at each cycle without rising over background level (Threshold Line) in order to calculate baseline fluorescence. At exponential phase, the amount of fluorescence reach threshold point which is greater than background level known as threshold cycle (Ct) which represent the amount of target gene in sample. In log-linear phase, the florescence signal increase with each cycle with linear efficiency. At plateau phase, reactants reduce and exponential product accumulation don't occur anymore and fluorescence intensity no longer relevant for calculating data. Therefore, it is crucial to understand that the quantity of PCR products in exponential phase corresponds to quantity of initial template under optimum conditions since the reaction effectively amplified DNA up to specific amount prior plateau effect. Consequently, log transformed data from exponential phase can be plotted with slope of regression line indicating amplification efficiency of samples as referred in Fig. (3). Hence, gene expression profile examines for up-down regulation in one or more experimental samples across qRT-PCR to perform quantification.

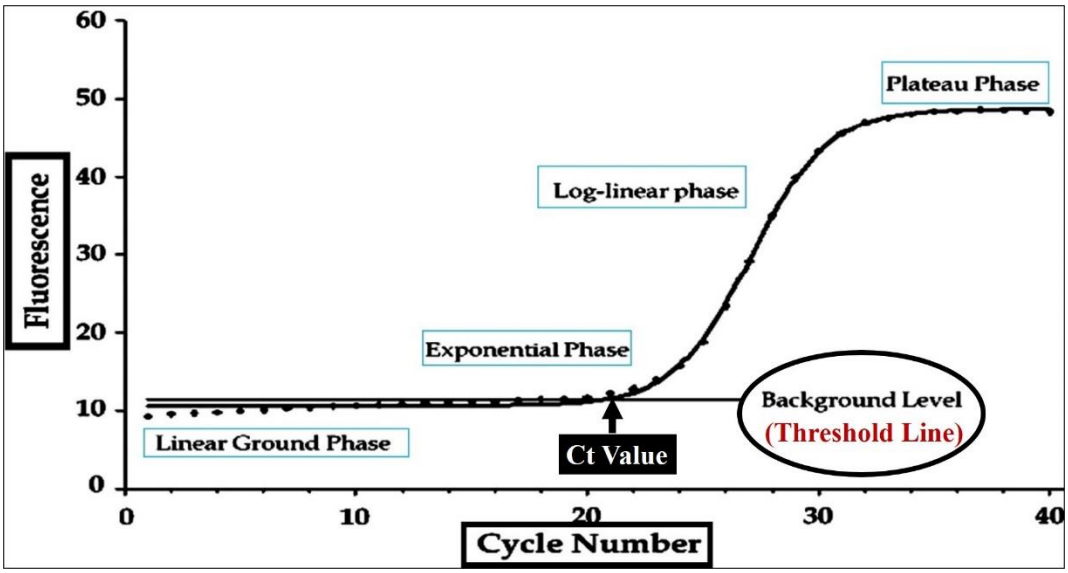

Fig. 2. Phases of qRT-PCR in a plot of fluorescence signal versus cycle.

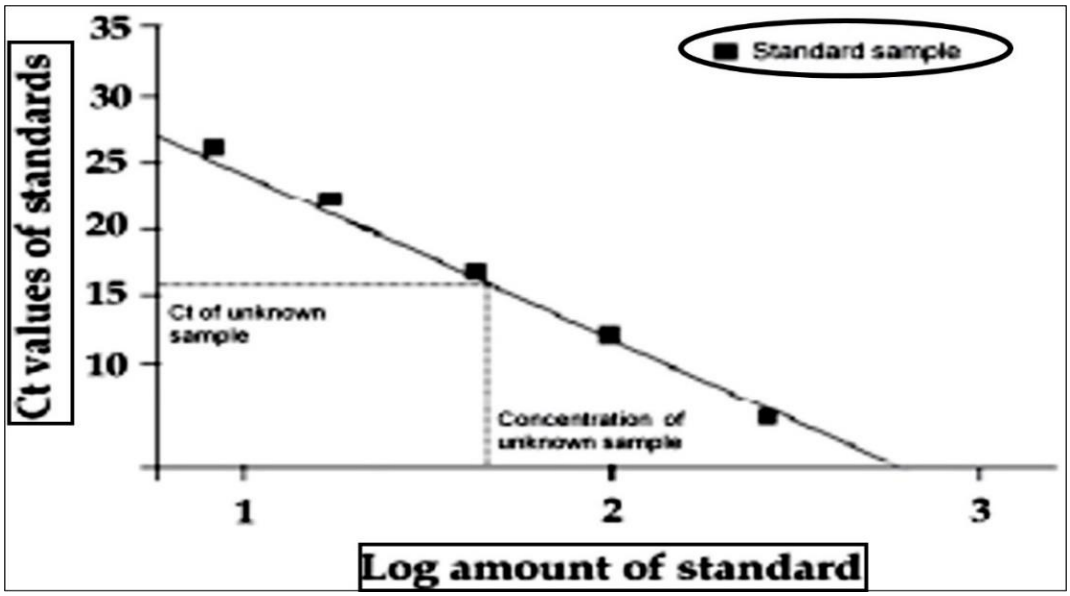

Fig. 3. Determination concentration of unknown sample in qRT-PCR by standard curve.

Primer Designing for qRT-PCR: To avoid non-specific PCR products, optimal primers are necessary by designing primers without similarities with other sequences containing nucleotides (16-28 bp) length and GC content (35-65%) by using BLAST (Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information). In addition, Primer melting temperatures I should be (60–65) °C, to give product size of 100-200 bp.

Chemistries of qRT-PCR:

1. Sequence non-specific detection: Employ DNA binding fluorogenic dyes which are unaffected to any variation in target sequence, not require design fluorogenic oligoprobes and easy to use.

2. Sequence specific detection: The advancement of fluorescent probe technique makes it possible to perform sensitive and specific detection of reaction. There are different kinds of probes that employed in response to various molecular structures and dyes attached, these probes are:

A. Hybridization probes: Bind to target sequence closely to each other in a head to tail arrangement (Fig. 4).

B. Hydrolysis probes: often refer to TaqMan probes or 5’ nuclease assay have a quencher dye at 3’ end and contain fluorescent reporter dye at 5’ end (Fig. 5).

C. Hairpin probes: also refer to stem-loop DNA probes, these probes have higher specificity of target recognition such as molecular beacons, scorpions, Lux fluorogenic primers and sunrise primers (Fig. 6).

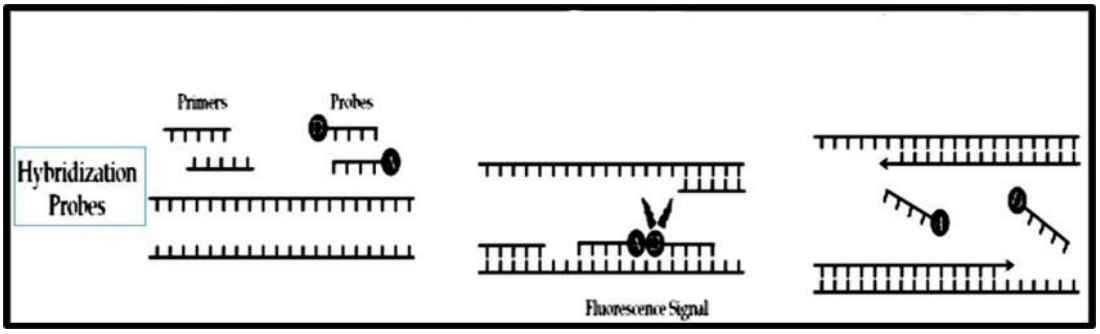

Fig. 4. Detection by hybridization probes.

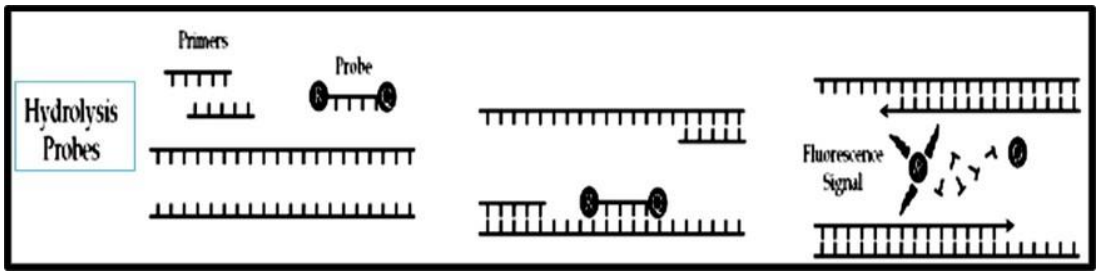

Fig. 5. Detection by hydrolysis probes.

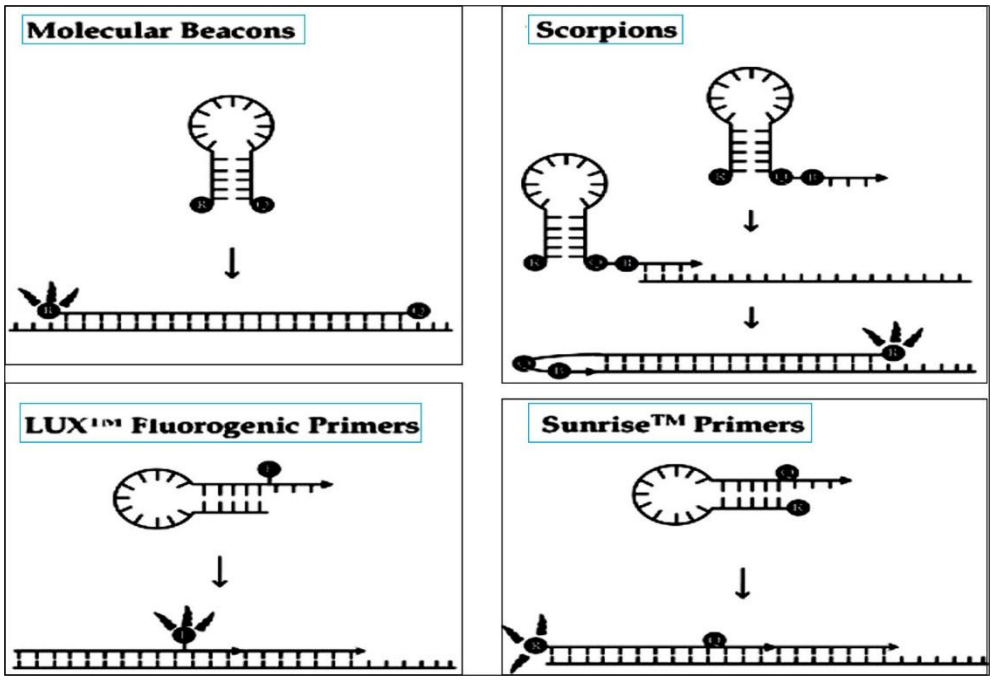

Fig. 6. Detection by Hairpin probes.

Normalization qRT-PCR by Housekeeping genes: Normalizing strategy by housekeeping genes consider simple and convenient method in qRT-PCR. These genes are expressed in a wide range of tissues and cell and show minimal change in expression levels between samples and experimental conditions. Therefore, these genes reduce errors that are generated from RNA extraction and purification, cDNA synthesis and samples variations. Typically, (3-5) genes are required for accurate normalization.

Amplification efficiency of qRT-PCR: Amplification efficiency consider an essential component of precise relative quantification. In an ideal reaction (100% efficient) every amplicon replicates to double the amount of product after each cycle to produce a plot of copy number versus cycle numbers (Rebouças *et al.*, 2013; Eldenary, 2015). While, if the reaction 90% efficient, the product doesn't double after each cycle and the slop of plot be less than plot of 100% efficiency (Sanders *et al.*, 2014; Sundgren, 2020). In addition, low efficiency between target and reference gene lead to inaccurate expression ration leading to difference in PCR efficiency (ΔE) between target gene and reference gene (Hongxia *et al.*, 2019). Therefore, amplification efficiency affects by sample impurities, amplicon length and efficiency of primer annealing which affect

primer binding and melting point of target sequence (Perini *et al.,* 2014).

Data Analysis of qRT-PCR: The mean normalized gene expression from relative quantitation assays can be computed by variety of mathematical models. Quantitative endpoint of qRT-PCR is threshold cycle (Ct) at which fluorescent signal of reporter dye cross arbitrarily threshold in exponential phase (Kuang *et al.*, 2018). Therefore, the amount of amplicon in reaction has an inverse relationship with Ct value (*i.e.*, the lower Ct the greater amount of amplicon). Therefore, Ct value mainly determine by the amount of template at start reaction (Hu *et al.*, 2016). Thus, if there are large amount of template at start reaction, relatively few amplification cycles require to accumulate enough product to provide fluorescent signal above background level (threshold line) and vice versa (Cameron Boyda, 2021). Hence, differences in cycle number (ΔΔCt) and threshold cycle (Ct) widely compares findings from experimental samples, e.g., gene of interest (GOI) in both test and control samples and the results are expressed as target/reference ratio after reaction. Consequently, the fold difference in gene expression calculate by incorporate results of ΔΔCt as follow:

ΔΔCt = ΔCt (target gene) – ΔCt (reference gene).

ΔCt (sample) = Ct (target gene) – Ct (reference gene).

ΔCt (control) = Ct (target gene) – Ct (reference gene).

Ratio $\Delta Ct = 2^{\Delta Ct}$ $(= 2^{[Ct\ sample\ -\ Ct\ control]})$.

Ratio $\Delta\Delta Ct = 2^{-\Delta\Delta Ct}$ $(= 2^{-\ [\Delta Ct\ sample\ -\ \Delta Ct\ control]})$.

Programs Used for Normalization in qRT-PCR: geNorm software program, estimate pairwise variation of reference gene with all other reference genes as standard deviation of logarithmically transformed expression ratios. In addition, BestKeeper combine descriptive statistics and regression analysis of Ct values to identify stable expression reference genes. Besides, NormFinder is algorithm find ideal reference genes of group of candidate genes by assigning stability value of each candidate gene with focus on variation between sample groups rather than expression on data set.

CONCLUSIONS

qRT-PCR consider one of the most sensitive, effective, quick and reproducible technique for measuring gene expression profiles. Therefore, it preferred by many researches worldwide to achieve biological results by choosing suitable reference genes to guarantee accuracy data across molecular biology researches.

FUTURE SCOPE

More studies by qRT-PCR should be done to understand basic cellular mechanisms and identifying changes in gene expression levels in respond to particular biological stimuli.

Acknowledgements. Authors want to thanks Genetic Dept., Faculty of Agric., Sohag Univ., Sohag, Egypt, for help and support.

Conflicts of Interest. None.

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How to cite this article: Ammar M. F. Mohammed, Fatma A. Ali, Shahd A. Mohammed, Shahd M. Omran, Marwa M. Dosokey, Sayed Gebril, Tarek H.M. Elsharouny, Ahmed E. Mahmoud, Ismail M.A. Bedawy, E. Ismail and Haitham M.A. Elsayed (2025). qRT-PCR's Significance and Efficacy in Molecular Biology Research. *International Journal on Emerging Technologies*, *16*(2): 36–41.