Article: RT1092

Biotica Research Today
Vol 4:9
2022
635
637

How to cite this article?
Pratapa *et al.*, 2022. Real-Time PCR: A Sensitive, Specific and Rapid Diagnostics Assay of Fish and Shellfish Pathogens. Biotica Research Today 4(9):635-637.

Real-Time PCR: A Sensitive, Specific and Rapid Diagnostics Assay of Fish and Shellfish Pathogens

Pratapa M.G.[1], David Waikhom[1*] and Laishram Soniya Devi[2]

[1]ICAR-Central Institute of Fisheries Education (CIFE), Mumbai, Maharashtra (400 061), India
[2]College of Fisheries, Central Agricultural University (Imphal), Lembucherra, Tripura (799 210), India

Open Access

Corresponding Author
David Waikhom
e-mail: davidwaikhom3@gmail.com

Keywords
Diagnostics, Pathogens, Real-time PCR, SYBR green

Abstract

The real-time PCR (polymerase chain reaction) assay is one of the most commonly used techniques in disease diagnostics. This advanced form of PCR has both pros and cons. Real-time PCR has advantages over conventional PCR in terms of specificity, rapidity, and sensitivity to identify a pathogen at the genomic level. It has many forms of chemistry, such as SYBR green-based, probe-based, *etc*., based on its working principle. Many researchers have reported that real-time PCR could accurately detect and identify various fish pathogens. However, it has some drawbacks, such as the high cost and required a skilled person to operate this assay.

Introduction

The intensification of aquaculture and the transboundary movement of aquatic animals has resulted in new emerging diseases that pose a high threat to the fisheries sector, especially aquaculture. To mitigate the spread of these diseases, pre- and post-transport screening are required for diagnosis. In the present scenario, molecular diagnostic techniques play a significant role in diagnostics, especially real-time PCR, even though conventional PCR is high enough to detect pathogens. Still, there have some limitations to conventional PCR, like its semi-quantitative nature and the need for post-amplification procedures; it may lead to cross-contamination resulting in false positive or false negative results. Moreover, sensitivity is very low as compared to real-time PCR. To overcome the limitations of conventional PCR, real-time PCR has been developed for many fish pathogens and is playing a significant role in screening pathogens. It detects as low as one viral copy. Apart from virus quantification, it is also used in gene expression studies (Valasek *et al.*, 2005). Further, it has many advantages over immunodiagnostic assays and culture-based techniques in terms of specificity, sensitivity, and rapidity.

Real-Time PCR Assay

It is a molecular technique that evolved in the 1990s and is the most advanced form of PCR. The name itself indicates that it monitors the target amplification in real time. Like conventional PCR, amplification happens exponentially, but the basic difference is that real-time PCR target detection is automatic and quantitative. There is no need for post-amplification procedures like agarose gel electrophoresis over real-time PCR with high diagnostic sensitivity and specificity. And its efficiency is also found to be very high and can be calculated with the help of the slope of the standard curve by using a mathematical equation as efficiency = $10^{-1/slope}$-1. Further, we can run this PCR within an hour, depending on the

Article History
Received on: 08th September 2022
Revised on: 11th September 2022
Accepted on: 12th September 2022

E-mail: *bioticapublications@gmail.com*

real-time PCR instrument such as Roche, Applied Biosystems, *etc*. However, it is pretty expensive compared to conventional PCR.

To understand real-time PCR more precisely, one should know the basic scientific terms used in real-time PCR as shown in table 1.

Table 1: Basic scientific terms used in real-time PCR

Terminology	Description
Baseline	The initial number of cycles before actual amplification in which there is little change in fluorescent signal.
Threshold	It is also called the initial level of detection and can be defined as a point at which the reactions show the fluorescent signal above the baseline.
Ct or Cq	It is also called a cycle threshold and is defined as the cycle number at which the sample amplification curve crosses the threshold line. CT value plays a significant role in data analysis.

Principles of RT-PCR

A) Based on the working principle, the real-time PCR may be broadly classified into two types:

1. SYBR green-based real-time PCR

2. Probe-based real-time PCR

1. SYBR Green-based Real-Time PCR

SYBR green is a dsDNA binding dye that binds to the minor grove of dsDNA, and in each PCR amplification cycle, new dsDNA of the target is formed. SYBR green will go and attach to these and emits fluorescence. This fluorescence is detected by connected computer software, and the fluorescence emission is directly proportional to the amplification. The main advantage of this PCR is that it is less expensive compared to probe-based real-time PCR. However, there are limitations in real-time PCR, such as non-specificity as SYBR green binds all dsDNA even to primer-dimers; hence, melt curve analysis is essential to know the specificity (Arya *et al.*, 2005).

Melt Curve

It is also called the dissociation curve. After the amplification program in SYBR green, the melt curve program needs to be set up to determine its specificity. As temperature rises, the dsDNA get denatures, SYBR green is released, and fluorescence intensity decreases gradually. These changes are recorded in the software and analyzed and generate melt curve. The melt curve should yield a single peak in specific amplification. In contrast, it produces more than one peak in the case of any non-specific amplification.

2. Probe-based Real-Time PCR

This real-time PCR takes advantage of the 5' exonuclease activity of Taq polymerase, which helps probe cleavage and fluorescence emission. A probe is a short oligonucleotide sequence consisting of a reporter at one end and a quencher at another end. When the probe is intact with a quencher, it inhibits the fluorescence emission by a reporter. When the probe is cleaved, the reporter is free from the quencher and emits fluorescence; target amplification is directly proportional to the fluorescence signal. The main advantage of this PCR over SYBR green PCR is high diagnostic specificity, and no need for melt curve analysis. Nevertheless, the disadvantage is that it is costly. Among probe-based real-time PCR, the Taqman probe is widely used. Several other reported probes are also used in real-time PCR, including molecular beacons, scorpion probe, dual hybridization probe, *etc*. (Arya *et al.*, 2005).

B) Based on the quantification, real-time PCR is divided into two types:

1. Absolute quantification

2. Relative quantification

1. Absolute Quantification

This is mainly used for quantifying the target copy number in an unknown sample, especially for detecting exact viral copies. Quantification is done based on the standard curve. The standard curve is generated by using a ten-fold serially diluted plasmid with known copy numbers and hence comparing the unknown target with known standards, and the quantification is determined.

2. Relative Quantification

The comparative threshold method is mainly used for gene expression studies. In this method, the expression of a target gene is measured by comparing it with control or housekeeping gene expression. The commonly used housekeeping genes in the relative quantification of real-time PCR include beta-actin, GAPDH, cytoskeletal protein, and ribosomal RNA.

There are three methods used to calculate the relative expression of the target gene, which include:

1. Livak method or $2^{-\Delta\Delta ct}$

2. $2^{-\Delta Ct}$ method

3. pfaffl method

Among these, the livak method is widely used.

Application of Real-Time PCR in Fisheries

Cell culture-based isolation and quantification are the gold standard for most viral diseases. Still, there are many limitations in these techniques like laborious, time-consuming, and challenging in developing different cell lines for different viruses. For crustaceans like shrimp and crabs, there are limited cell lines available. Real-time PCR has been developed to overcome these problems to detect many viral, bacterial, and parasite diseases (Durand *et al.*, 2002; Du *et al*., 2017; Sahoo *et al.*, 2019) as shown in table 2. Besides, many researchers have reported that real-time PCR is a sensitive, specific, and rapid assay for detecting many fish pathogens.

Table 2: Developed real-time PCR for the identification of various pathogens in fish and shrimp

Virus	Host
IPNV (Infectious Pancreatic Necrosis Virus)	Salmons
ISAV (Infectious Salmon Anaemia Virus)	Salmons
IHNV (Infectious Hematopoietic Necrosis Virus)	Salmons
VHSV (Viral Hemorrhagic Septicemia Virus)	Salmo salar, Oncorhynchus mykiss
IHHNV (Infectious Hypodermal and Haematopoietic Necrosis Virus)	Shrimp
TSV (Taura Syndrome Virus)	Shrimp
WSSV (White Spot Syndrome Virus)	Shrimp
YHV (Yellow Head Virus)	Shrimp
Bacteria	
Mycobacterium spp.	Zebrafish
Aeromonas hydrophila	Fishes
Aeromonas salmonicida	Fishes
Parasite	
Ciliates	Fishes
Argulus spp.	Fishes
Lernaea sp.	Fishes

Conclusion

Molecular diagnostic tools are boon for disease diagnostics. Among different techniques, PCR and its variants take a leading role. Real-time PCR is the most advanced form of PCR and has many advantages over conventional PCR, immunodiagnostics, and cell culture-based techniques. Real-time PCR has a wide dynamic range and low intra- and inter-assay variations. It can quantify even one viral copy due to its sensitivity. Therefore, this assay is widely used in fish disease diagnostics including confirmatory diagnostics of disease at the genomic level. The sensitivity, accuracy, rapidity, and specificity of real-time PCR have made this assay immensely popular in the field of disease diagnosis.

References

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., Patel, H.R., 2005. Basic principles of real-time quantitative PCR. *Expert Review of Molecular Diagnostics* 5(2), 209-219.

Du, Y., Liu, Y., Xiao, P., Meng, L., Liu, P., 2017. Development and application of a quantitative real-time polymerase chain reaction assay for the detection of *Aeromonas salmonicida*. *Journal of the World Aquaculture Society* 48(4), 574-582.

Durand, S.V., Lightner, D.V., 2002. Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. *Journal of Fish Diseases* 25(7), 381-389.

Sahoo, P.K., Parida, S., Mohapatra, A., Mohanty, J., 2019. Selection of candidate reference genes for RT-qPCR analysis in *Argulus siamensis* and their validation through screening of drugs and drug targets. *Scientific Reports* 9(1), 1-11.

Valasek, M.A., Repa, J.J., 2005. The power of real-time PCR. *Advances in Physiology Education* 29(3), 151-159.