Current Trends in Biotechnology and Pharmacy
Vol. 17(2) 828 - 834, April 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2023.2.21

Simple and Rapid DNA Isolation from Shrimps by Using Single tube extraction buffer with Single step

MVSS Pavan Kumar[1], A Ranganadha Reddy[1]

[1]. Department of Biotechnology, VFSTR Deemed to be University, Guntur, India 522213.
*Corresponding author: rangaaluri@gmail.com

Abstract

Aquaculture and aquatic animals are important for national food security since they are a good source of animal meat and protein for mankind. Aquaculture is currently the most economically and financially viable option for farmers, so the majority of them are interested in it. In aquaculture, shrimp farming plays a major role and exists in either a freshwater or marine environment for human consumption. Although shrimps are very susceptible to diseases during breeding and farming, these diseases are identified by several methods, i.e., microbiology test, antigen-antibody testand molecular methods. For molecular screening and testing of shrimps, it is necessary to extract nucleic acid, i.e., DNA (deoxyribonucleic acid). So many traditional and commercial methods are available on the market for nucleic acid extraction. In our study, we developed a simple, rapid, efficient, and effective nucleic acid extraction method. DNA was isolated from shrimps and shrimp seeds using this method (A260/280 range: 70 ng/uL to 350 ng/uL) and tested using molecular (PCR-Polymerase Chain Reaction) methods, specifically the SYBR Green assay on Real Time PCR.

Key words: Aquaculture, Shrimp, PCR, SYBR Green assay

Introduction

Aquaculture is the managed procedure of cultivating aquatic organisms which includes fish, aquatic plants, Molluscs and especially shrimp for human consumption(1). Shrimp is one of the most commercialized seafood products and shrimp farming is growing on a global scale(2). As a result of increasing consumer demand, the shrimp industry has become a large-scale operation.Shrimp aquaculture provides high-quality food products, socioeconomic development and significant employment opportunities for skilled and unskilled workers (3). In worldwide, the shrimp aquaculture industry faces many challenges, most importantly various bacterial, fungal & viral diseases. Viruses are considered the major pathogenic agent affecting shrimp aquaculture (4). To date, over 20 viral diseases have affected shrimp, primarily white spot disease and hepatic microsporidiosis.White spot disease caused by the white spot syndrome virus (WSSV), a double-stranded DNA virus that can cause 100% mortality in 2 to 10 days.The major targets of WSSV infection are ectodermic and mesodermic tissues in shrimp. Recent studies estimate the total economic loss of WSSV at USD 8-15 billion and it continues to increase by 1 billion a year (5). *Enterocytozoonhepatopenaei*(EHP) *i*s an intra-

cellular microsporidian parasite that lyses epithelial cells in the liver and pancreas tubules in shrimp (6).Still, the life cycle of EHP & the way of eradication of this virus is completely obscure. So, the prevention and control of EHP is a challenge for the shrimp Industry (7).The key step in disease management requires that accurate and reliable diagnostic methods are available. To date, various diagnostic methods have been used for the detection of EHP and WSSV in shrimp, but it is crucial to choose the best methodology for the application. molecular detection of shrimp diseases via PCR and LAMP is the most reliable method(8-9).Nucleic acid extraction is the preliminary step for every PCR based molecular assay. In conventional DNA isolation method and other chemical method has some disadvantages, i.e.,phenol chloroformbased extractionis time consuming, laborious & hazardous (10). Meanwhile, other commercial DNA extraction kits are available, but they are labour-intensive and costly for a large number of animal samples.It is therefore very important to develop a method for the rapid, simple and cost-effective detection of EHP and WSSV infection to prevent disease epidemics and economic losses associated with growing shrimp (11). In this study, we demonstrate the single-tube extraction method for DNA isolation of shrimp tissue. Our protocol does not require any hazardous organic solvents and has been deemed suitable for a PCR method. Our method will help shrimp farming communities find a less expensive and user-friendly diagnostic tool for detecting shrimp DNA viral disease.

Materials and Methods

Sample collection

Accurate sampling is one of the most important steps for determining the shrimp diseases. Prior to sampling, take note of any changes in the colour, appearance, or lesions of the shrimp (12). In our study, adult shrimps were collected from a farm area and shrimp seed from a hatchery.Samples were collected in sterile container with 1X PBS saline buffer (130 mM sodium chloride, 2 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate mono basic and pH 7.4). After collection of samples, stored in a cold container maintain 2-8°C(13).

Nucleic acid extraction

Nucleic acid extractionis one of the major steps for molecular detection. Nucleic acids can extract from all living kingdoms like bacteria, virus, yeast, Plants, animals, human and also some dried samples.Several methods are used to extract the nucleic acids, i.e. chromatography based, alkaline based, silica based, salting out, CTAB, phenol-chloroform, magnetic bead based, filter based and paper based methods(14). So many commercial extraction method kits available in market like QIAamp, Roche MN kits etc., and different kinds protocols also available on web sites. Whatever, any extraction methods aim was to isolate good yield and purity of nucleic acid with minimal inhibitors. Nucleic Acid (either DNA or RNA) have to extract Based on the study & targeted organism. In our study, WSSV & EHP viruses has a DNA as a genetic material. So we developed single tube shrimp DNA extraction method with simple, reliable, fast & cost-effective(15-16).

Single Tube Shrimp DNA Extraction Protocol (17):

The shrimp seeds (10 -12nos) & specimen of adult shrimp was separately transferred to the 50 ml tube containing single step extraction Buffer solution as mentioned in Table 1. The tube was incubated at heating block at 65°C for 30 min. After Incubation the required volume of upper clear solution was taken for the further analysis.

Evaluation of DNA Quantity

Nucleic acid quantification is crucial to obtaining precise and reliable data for many molecular biology tests, including PCR (polymerase chain reaction) and real-time PCR assays (18). Quantitative analysis carried out for 21 DNA

Table 1: Single tube extraction buffer composition:

Components	Concentration
KCl	100 mM
Tris-HCl	2 mM
MgCl2	10 mM
Glycerol	0.1%
NP 40	0.2%
Tween 80	0.1%
pH	7.0

samples which are extracted from shrimp seed and specimen of adult shrimp by usinga UV visible spectrophotometer (Eppendorf BioPhotometer D30). The concentration and purity of DNA were determined from the A260/A280 ratio. The concentrations of the sample were in the range of 80 ng/uL to 365 ng/uL and an absorbance quotient value of 1.8 < ratio consider to be good purified DNA.

Real Time PCR

However, Animal tissues can contain inhibitory molecules that can affect PCR. Therefore, we tested if DNA from single tube extraction can be amplified directly by Real Time PCR (19). Real Time PCR is the result of PCR's incredible sensitivity as well as real-time monitoring of its products (20). In Real Time PCR, SYBR Green & TaqMan based methods are more more popularity & sensitivity. In current study, we used the SYBR Green assay for real-time PCR because it is inexpensive and simple to use. SYBR Green assay was performed on BIO-RAD CFX95 Real time System with using specific primers (21). Primers for housekeeping gene β-actin was (5'CAACCGTGAGAAGATGACTC3') and (5' AGCATGAGGAAGAGCATAAC 3'). Reaction mixture containing 10 uL of 2X TB Green Mix (Cat No. 1234), 1 uL of forward primer (10uM), 1 uL of reverse primer (10uM) and 8uL of single tube DNA. The condition used for the amplification was followed: Initial Denaturation 95°C for 3 mins, followed by 40 cycles of 94°C for 15 sec and 60°C for 30 sec.At the end of each amplification cycle's extension phase, the fluorescence signal was acquired using 465 nm excitation and 510 nm emission wavelengths(22).

Evaluation of SYBR Green assay with melt curve

After the amplification, a melting curve analysis was done to confirm that only one gene-specific product had been synthesized. For each gene, a standard curve was generated to estimate amplification efficiency as shown in Figure 1.(23).

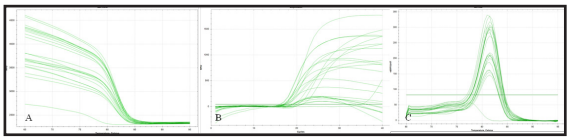

Fig 1: Amplification plot of shrimp DNA extracted by single tube extraction buffer. A. shows amplification plots on linear scale with florescence B. shows amplification plot on melt curve with linear scale C. shows determine melting temperature on melt curve analysis scale.

Results and Discussion

DNA Estimation

The results of this study showed that the single tube extracted shrimp DNA samples of 21 subjects ranged approximately 160 ng/uL(24). On purity assessment of shrimp samples we found that all 21 samples were within the optimal range of 1.5-2.4 as mentioned in Table 2.

Table 2: Quantitative results of Shrimp DNA samples extracted by single tube extraction buffer:

S.No.	Samples	Concentration (ng/uL)	A(260/280)
1	01	143	1.5
2	02	200	1.6
3	03	213	1.7
4	04	198	1.8
5	05	175	1.7
6	06	126	1.9
7	07	100	2.0
8	08	168	2.2

9	09	172	1.5
10	10	220	1.8
11	11	210	1.7
12	12	154	1.8
13	13	164	1.8
14	14	209	1.9
15	15	202	2.0
16	16	135	2.4
17	17	139	1.6
18	18	148	1.7
19	19	155	1.8
20	20	168	2.0
21	21	173	1.9

SYBR Green assay with melt curve

For set up of SYBR Green assay to detect beta actin gene from shrimp DNA by single tube extraction buffer. Satisfactory results were obtained as mentioned in Table 3.(25).

Conclusion

The performance of SYBR Green assay has been evaluated by amplification curves and melting temperatures. The melting curves of the SYBR Green assay with shrimp DNA isolated

Table 3: Melt curve temperatures of amplification of shrimp DNA samples

S.No.	Well	Fluorophore	Content	Sample	MeltTemp
1	A01	SYBR	Unkn	01	81.50
2	A02	SYBR	Unkn	09	81.50
3	A03	SYBR	Unkn	17	81.50
4	B01	SYBR	Unkn	02	81.00
5	B02	SYBR	Unkn	10	81.50
6	B03	SYBR	Unkn	18	81.50
7	C01	SYBR	Unkn	03	81.50
8	C02	SYBR	Unkn	11	81.50
9	C03	SYBR	Unkn	19	81.50
10	D01	SYBR	Unkn	04	81.50
11	D02	SYBR	Unkn	12	81.50
12	D03	SYBR	Unkn	20	81.50
13	E01	SYBR	Unkn	05	81.50
14	E02	SYBR	Unkn	13	81.50
15	E03	SYBR	Unkn	21	81.50
16	F01	SYBR	Unkn	06	81.50
17	F02	SYBR	Unkn	14	81.50
18	F03	SYBR	Unkn	22	81.50
19	G01	SYBR	Unkn	07	81.50
20	G02	SYBR	Unkn	15	81.00
21	G03	SYBR	Unkn	PC	81.50
22	H01	SYBR	Unkn	08	81.50
23	H02	SYBR	Unkn	16	81.50
24	H03	SYBR	NTC	NTC	None

by single tube extraction buffer were successfully evaluated. The evaluation of the SYBR Green assay on shrimp seed and tissue of adult shrimp samples already quantified by Biophometer method.

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