

# Quantitative detection of a marine fish iridovirus isolated from large yellow croaker, *Pseudosciaena crocea*, using a molecular beacon

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## Abstract

A real-time polymerase chain reaction (PCR) assay utilizing a molecular beacon for the quantitative detection of a marine fish iridovirus isolated from large yellow croaker, *Pseudosciaena crocea* (LYCIV), was developed, which involved the amplification of a 122 bp DNA fragment from a conserved region of LYCIV ATPase gene. The specific probe consisting of two short arm and a central loop sequences complementary to the target amplicon was characterized with respect to its efficiency of quenching ( $E_{ff}$ ), and signal to background ratio by spectrofluorometric analysis of its hybridization with the complementary oligonucleotide target. The positive control plasmid pFHT-ATPase containing the target sequence was quantified to make the standard curve for sample detection after serial 10-fold dilution. Linear coefficient correlations between cycle threshold ( $C_T$ ) value and logarithmic positive plasmid concentration were close to one ( $r^2 = 0.998$ ) and the detection limit of the assay was 70 copies of positive plasmid/assay. The specificity of this real-time PCR was also demonstrated by using the genomic DNA templates from the healthy fish, white spot syndrome baculovirus (WSSV), and epizootic hematopoietic necrosis virus (EHNV), respectively. The coefficient of variation (CV) of the assay ranged from 1.16 to 4.42%, depending on the concentration of the positive plasmid. The quantitative detection of different tissues from LYCIV-infected fish showed that the spleen and kidney contained the largest number of viral particles ( $6.86 \times 10^6$  and  $4.62 \times 10^6$  viral genome copies/mg tissue, respectively) while no viral DNA was detected in the muscular tissue. These results suggested that the real-time PCR assay reported here could be used for rapid, sensitive, and quantitative detection of LYCIV infection.

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**Keywords:** Large yellow croaker, *Pseudosciaena crocea*; Iridovirus; Real-time PCR; Molecular beacon; Quantitation

## 1. Introduction

Iridoviruses recognized as causative agents of serious systemic diseases have been identified from more than 20 fish species in the recent years (Hyatt et al., 2000). Iridoviruses are icosahedral with a large double-stranded DNA genome, 120–300 nm in diameter and consist of a spherical deoxyribonucleoprotein core surrounded by a lipid membrane (Williams, 1996). At present, the Iridoviridae family is subdivided into four genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, and *Lymphocystivirus*. Most fish iridoviruses are members either of the genera *Lymphocystivirus* or of the genera *Ranavirus*. Iridoviruses in genera *Lymphocystivirus* cause the development of cluster of extremely hypertrophied fibroblasts or osteoblasts called lym-

phocystis cells, while viruses in genera *Ranavirus* may lead to systemic disease in infected animals and are associated with high morbidity and mortality (Hedrick and McDowell, 1995; Hedrick et al., 1992; Pozet et al., 1992). Recently, outbreaks of iridoviral disease with high mortality have been increasingly reported in some seawater fish and freshwater fish in Southeast Asian regions (Chou et al., 1998; He et al., 2000; Sudthongkong et al., 2001; Chen et al., 2003a). These iridovirus isolates are very similar in morphology, associated histopathological lesions, and nucleotide sequences of the conserved genes such as ATPase and major capsid protein genes to each other, but they are genetically distant from those viruses in genera *Ranavirus* and *Lymphocystivirus*, suggesting that these iridovirus isolates from different fish species in Southeast Asia should belong to a new group of the Iridoviridae family (He et al., 2001; Hyatt et al., 2000; Chen et al., 2003a).

Large yellow croaker iridovirus (LYCIV) was first isolated from large yellow croaker, *Pseudosciaena crocea*, an

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economically important fish species of mariculture in China, in 2001. LYCIV infection had caused mass mortalities in cultured large yellow croaker juveniles during the last few years, resulting in great economic losses. The histopathological, morphological, and molecular biological characteristics of LYCIV have been investigated (Chen et al., 2003a). A polymerase chain reaction (PCR)-based method has been developed for the early, rapid detection of LYCIV infection, which involves the specific amplification of a 294 bp DNA fragment of the LYCIV ATPase gene (Chen et al., 2003b). Recently, real-time PCR has attracted a wider concern of the PCR due to its improved rapidity, sensitivity, reproducibility, and the reduced risk of carry-over contamination (Wittwer et al., 1997; Mackay et al., 2002). Real-time PCR using either hybridization probes such as TaqMan, adjacent probes and molecular beacons, or double-stranded DNA binding dyes have been widely used for the routine diagnosis of the medically important viruses and general virological studies (van Elden et al., 2001; Mackay et al., 2002; Gottlieb et al., 2002; Ruelle et al., 2004). The use of real-time TaqMan quantitative PCR was reported for the investigation of strain variation in an emerging iridovirus of warm-water fishes (Tony et al., 2003). Here we report the development of real-time PCR using a molecular beacon for the quantitative detection of a marine fish iridovirus isolated from large yellow croaker, *Pseudosciaena crocea*.

## 2. Materials and methods

### 2.1. Fish and virus

The moribund large yellow croakers were collected from mariculture farms in Ningde and Luoyuan areas of Fujian province, China, during summers of 1999–2001. The diseased fish (average length 8–12 cm) were characterized as having iridovirus infection by clinical symptoms, histopathology, electron microscopical observation, and polymerase chain reaction analysis. The genomic DNA of epizootic hematopoietic necrosis virus (EHNV) was a generous gift from Dr. Alex Hyatt (Australian Animal Health Laboratory), and white spot syndrome baculovirus (WSSV) DNA was stored in our laboratory.

### 2.2. Isolation of template DNA

For template DNA isolation, about 100 mg of spleens excised from the naturally infected large yellow croaker or healthy large yellow croaker were homogenized in 1 ml of sterile redistilled water on ice. After centrifugation at  $1500 \times g$  for 10 min at  $4^\circ\text{C}$ , the supernatants were mixed with an equal volume of lysis buffer (10 mM Tris-HCl pH 8.0, 100 mM EDTA,  $20 \mu\text{g ml}^{-1}$  RNase, 0.5% (w/v) SDS), meanwhile proteinase K was added to a final concentration of  $100 \mu\text{g ml}^{-1}$ . The mixtures were incubated for 3 h at  $55^\circ\text{C}$ , and then extracted twice with phenol-chloroform. DNA was precipitated with ethanol for 30 min at  $-20^\circ\text{C}$ , then redissolved in  $100 \mu\text{l}$  TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at  $-80^\circ\text{C}$  for further use.

Table 1

Nucleotide sequences of primer sets	Expected size of PCR products (bp)
F1: GACGCTGACATTATCACACG R1: ATCAACATGAGCCACGAGTG	83
F2: CGTCACTGGAATGTTCTGGT R2: TTGTCCACACATGTCTGGCT	122
F3: AGGAGGCCAACCACTTCTAC R3: CCACATTCTTTAGTGCCAGC	114

### 2.3. Positive control plasmid

The positive control plasmid pFHT-ATPase containing an insert of LYCIV ATPase gene was constructed previously (Wang and Chen, 2004). The positive plasmids were subsequently transformed into *Escherichia coli*, and individual clones were isolated. The plasmid DNA was purified, sequenced, and finally quantitated by spectrophotometry (ULTrospec2100 pro, Pharmacia). The resulting positive control plasmid concentration was determined to be  $7.0 \times 10^9$  copies/ $\mu\text{l}$ .

### 2.4. Primer design

The design of primers was based on a conserved region of LYCIV ATPase gene (GenBank accession number: AY165049). The nucleotide sequences of the three primer sets designed and the expected size of their PCR products are shown in Table 1. Primers first were tested for their ability to generate no signal in negative controls by dimer formation, and then their efficiency in the PCR reaction is evaluated by the slope of the regression curve obtained with several dilutions of the positive plasmid template. One primer set, with the highest amplification efficiency, was selected for real-time PCR assay.

### 2.5. Molecular beacon design

The specific molecular beacon was designed based on a modified design strategy of molecular beacon (Li et al., 2000). The sequence of the molecular beacon was as follows: 5'-FAM-GCCAGCCGTCTATACAACACCTCAGGctggc-DABCYL-3' with a 17 nucleotide loop sequence (not underlined) and 26 nucleotide sequence (capital letters) complementary to an internal sequence of the target amplicon. The seven nucleotide sequences (underlined) formed the stem structure. 5'-Carboxyfluorescein (FAM) and DABCYL were used as the fluorophore and quencher and were coupled to the 5' and 3' ends of the stem structure, respectively. A stock solution of the probe was prepared in 10 mM Tris pH 8.0, 2 mM  $\text{MgCl}_2$ , and stored at  $-70^\circ\text{C}$ .

A fluorescence spectrometer F-4010 (HITACHI) was used to detect the fluorescence emission of the molecular beacon. A fixed excitation wavelength of 491 nm was used and the fluorescence emission was measured at 518 nm. The signal to background ratio was determined by the following steps:  $100 \mu\text{l}$  of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM  $\text{MgCl}_2$ ) was placed in a 1-cm quartz cell and the flu-

orescence ( $F_{\text{buffer}}$ ) was measured. The molecular beacon was added to the buffer to a final concentration of 50 nM and the resulting fluorescence ( $F_{\text{close}}$ ) was noted. Subsequently, a 20-fold molar excess of oligonucleotide target was added to the solution above and fluorescence ( $F_{\text{open}}$ ) was measured 5 min after addition of the target. The signal to background ratio was calculated as:  $(F_{\text{open}} - F_{\text{buffer}})/(F_{\text{close}} - F_{\text{buffer}})$ . The efficiency of quenching ( $E_{\text{ff}}$ ) of light emitted from the fluorophore by the quencher was calculated as:  $E_{\text{ff}} = \{1 - (F_{\text{close}} - F_{\text{buffer}})/(F_{\text{open}} - F_{\text{buffer}})\} \times 100$ .

### 2.6. Real-time PCR amplification

Reaction mixture for real-time PCR was optimized and consisted of 1 × PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 2 mM MgCl<sub>2</sub>, 0.3 mM dNTPs, 0.25 μM each of sense and antisense primers, 0.5 μM molecular beacon probe, 1 μl plasmid DNA ( $7.0 \times 10^9$  copies) and 2.5 U Taq DNA polymerase in a total volume of 25 μl. PCR conditions in Rotor-Gene 3000 Fluorescence Cycler (Corbett Research) consisted of 1 cycle of initial denaturation of 3 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 45 s at 54 °C, and 45 s at 72 °C, and a final incubation for 10 min at 72 °C. Fluorescent data were acquired during each annealing phase.

### 2.7. Sensitivity, specificity and reproducibility of real-time PCR

The sensitivity of the real-time PCR was determined by using 10-fold serial dilutions of the quantified positive plasmid DNA as templates. Standard curve was generated using cycle threshold values calculated from assays over a range of logarithmic positive plasmid concentrations. The  $C_T$  value, which is defined as the fractional cycle number at which the fluorescence reaches 10 times the standard deviation (S.D.) of the mean baseline calculated for cycles 3–15, was determined by Roter-Gene Real-Time Analysis Software (Corbett Research).

The specificity of PCR amplification was investigated by detecting genomic DNAs from the healthy fish, EHNV, and WSSV. Meanwhile, two unrelated primer sets complementary to major capsid protein and DNA polymerase genes of LYCIV were used to evaluate the specificity of the molecular beacon hybridization with the target sequence.

To evaluate the reproducibility of this real-time PCR assay, the inter-run coefficient of variation (CV) was calculated on  $C_T$  values collected from five separate runs with two standard dilutions of positive plasmid at  $7.0 \times 10^7$  and  $7.0 \times 10^3$  copies, and the intra-run coefficient of variation was determined on three replicates of nine dilutions of positive plasmids ranging from  $7.0 \times 10^1$  to  $7.0 \times 10^9$ . The coefficient of variation was the ratio between the standard deviation and the mean of replicate measurements.

### 2.8. Samples detection

This molecular beacon real-time PCR assay was used to measure viral load in the various tissues of LYCIV-infected

fish. Five diseased fish previously diagnosed as LYCIV-infected by histopathology, electronic microscopy, and PCR from a natural outbreak of LYCIV at farms of Ninde in Fujian province were selected for the real-time PCR assay. Viral DNA was extracted from the various tissues of infected fish, including spleen, kidney, liver, gills, and muscle. Viral loads were expressed as log<sub>10</sub> viral genomes per milligram of tissue, averaged over the three quantitative PCR replicates.

## 3. Results

### 3.1. Molecular beacon design and characterization

Three pairs of primers were designed for the testing of their amplifying efficiency in PCR (Table 1). The primer set of F2R2 with the highest amplifying efficiency was selected for the real-time PCR assay after the amplification efficiency of these three pairs of primers was evaluated by comparing their slope values (data not shown). This primer set was designed to amplify a 122 bp target sequence within the LYCIV ATPase gene (Table 1). The molecular beacon used here was 31 bp in length and consisted of a central 17 bp loop sequence and two complementary 7 bp arm sequences. The design of this molecular beacon was different from the conventional design, the sequence complementary to the target DNA was 26 bp in length, which included both loop and arm part sequences (Fig. 1). Sequence complementarity between the beacon probe and primer set F2R2 revealed that no probe–primer interactions existed. The melting temperature of the beacon probe–target hybrid, without the adjacent arm sequences at the 3' end of the probe, was predicted to be 61 °C, which was typically 7–10 °C higher than the PCR annealing temperature (54 °C in this case), allowing the probe to hybridize with the target during the annealing stage.

Using a fixed excitation wavelength of 491 nm and measuring the fluorescence emission at 518 nm, the signal to background ratio was calculated to be 19.9:1 following hybridization of a 20-fold molar excess of oligonucleotide target to the probe over 5 min at room temperature. The efficiency of quenching was found to be 95.0% by triplicate detection (data not shown). The results showed that the molecular beacon designed was of good quality and quenching efficiency.

### 3.2. Real-time PCR and molecular beacon assay

The optimization of the real-time PCR amplification was achieved by varying reaction components and conditions. The molecular beacon at a final concentration of 0.5 μM was sufficient to produce satisfactory accumulation of fluorescent signal, and a final MgCl<sub>2</sub> concentration of 2.0 mM and an annealing temperature of 54 °C were shown to be optimal for signal accumulation in this assay (data not shown). The optimized reaction mixture and condition were as described in Section 2.

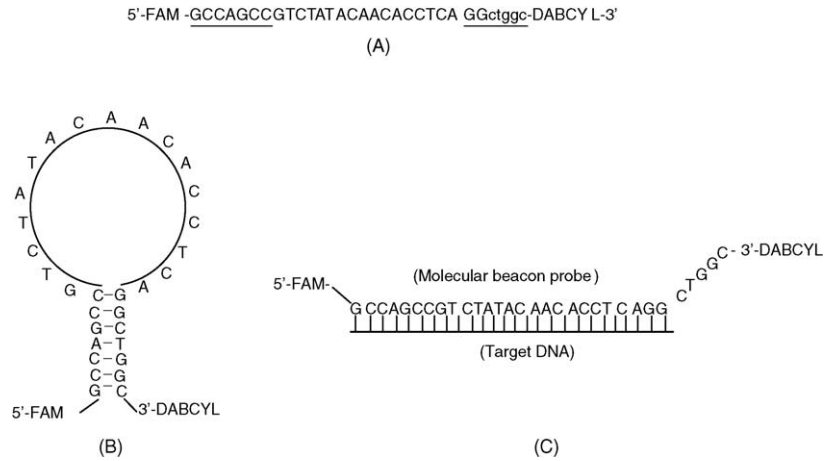


Fig. 1. Primary nucleotide sequence and predicted secondary structure of molecular beacon. (A) Primary nucleotide sequence of the molecular beacon with a 17 nucleotide loop sequence (not underlined) and 26 nucleotide sequence (capital letters) complementary to an internal sequence of the target amplicon. Two 7 nucleotide arm sequences (underlined) form the stem structure. FAM and DABCYL were coupled to the 5' and 3' ends of the stem structure, respectively. (B) Predicted secondary structure of the molecular beacon. (C) Molecular beacon-target DNA hybrid.

### 3.3. Sensitivity, specificity and reproducibility of real-time PCR

Serial 10-fold dilutions of the positive control plasmid with known concentration (copy number/ $\mu\text{l}$ ) were prepared ( $7.0 \times 10^9$  to  $7.0 \times 10^1$  copies/ $\mu\text{l}$ ). One microliter of positive plasmid from each dilution was used for the molecular beacon real-time PCR to determine the detection limit of the assay and to establish a standard curve for the quantification of LYCIV genomic DNA. A typical amplification plot of relative fluorescence versus cycle number is shown in Fig. 2. PCR amplification plots demonstrated characteristic exponential fluorescence accumulation (showing product formation) over time, and a 10-fold increase in the copy number of positive plasmid DNA resulted in a mean decrease in  $C_T$  of 2.3 (range 1.90–2.64). The detection limit of the assay was consistently showing 70 copies/assay (mean  $C_T = 26.45 \pm 0.58$ ).

Standard curve generated from the mean data of experiments carried out in triplicate demonstrated a good linear relationship between  $C_T$  values and logarithmic plasmid concentrations from  $7 \times 10^9$  to  $7 \times 10^1$  copies per reaction (Fig. 3). Linear coefficient correlations were shown reproducibly to be close to

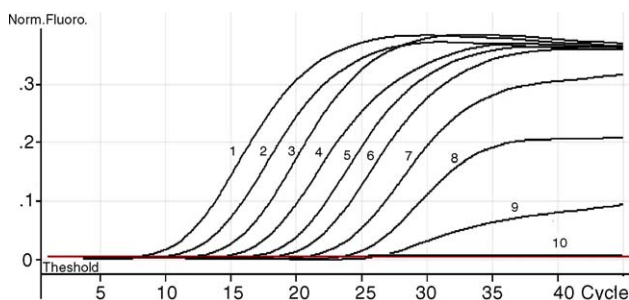


Fig. 2. Amplification plot of molecular beacon real-time PCR of the positive plasmids with different copy number. 1–10 represented the copy number of positive plasmid per reaction: (1)  $7 \times 10^9$ , (2)  $7 \times 10^8$ , (3)  $7 \times 10^7$ , (4)  $7 \times 10^6$ , (5)  $7 \times 10^5$ , (6)  $7 \times 10^4$ , (7)  $7 \times 10^3$ , (8)  $7 \times 10^2$ , (9)  $7 \times 10^1$ , and (10) no template DNA.

one ( $r^2 = 0.998$ ). The relation between the  $C_T$  values and corresponding concentrations of the template could be denoted as:  $C_T = -2.26 \times \log(\text{copies/reaction}) + 29.77$ .

To assess the specificity of the assay, the DNA templates extracted from the healthy fish, EHNV, and WSSV were analyzed by this real-time PCR, respectively. No accumulations of fluorescent signals were detected in these reactions. In addition, the specificity of molecular beacon hybridization with the target DNA was evaluated by amplifying the LYCIV DNA template using two primer sets complementary to major capsid protein and DNA polymerase genes of LYCIV, respectively, and no fluorescence was observed in their amplified products (data not shown).

To evaluate the reproducibility of this assay, the inter-run coefficient of variation was calculated on mean  $C_T$  values collected from five separate runs with two standard dilutions of positive plasmid at  $7.0 \times 10^7$  and  $7.0 \times 10^3$  copies. For reactions with positive plasmid copy number of  $7.0 \times 10^7$  and  $7.0 \times 10^3$ ,

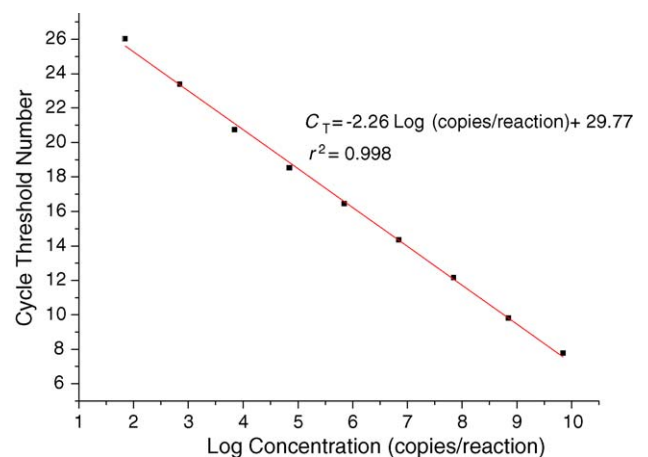


Fig. 3. Standard curve showing a linear relationship between positive plasmid concentration and cycle threshold number. Plots are  $C_T$  values vs. log positive plasmid concentrations (copies/reaction) generated from mean data of experiments performed in triplicate.

Table 2  
Inter-run and intra-run coefficient of variation

Copy number	Mean $C_T$	S.D.	CV (%)
Inter-run coefficient of variation ( $n = 5$ )			
7.00E+07	12.24	0.165	1.35
7.00E+03	20.85	0.617	2.96
Intra-run coefficient of variation ( $n = 3$ )			
7.00E+09	7.81	0.103	1.32
7.00E+08	9.92	0.115	1.16
7.00E+07	12.37	0.174	1.41
7.00E+06	13.66	0.171	1.25
7.00E+05	16.59	0.227	1.36
7.00E+04	18.71	0.288	1.54
7.00E+03	20.96	0.514	2.45
7.00E+02	23.62	0.734	3.11
70	26.45	1.170	4.42

S.D.: standard deviation; CV: coefficient of variation; inter-run coefficient of variation was calculated on values collected from five separate runs with two standard dilutions of positive plasmid at  $7.0 \times 10^7$  and  $7.0 \times 10^3$  copies; intra-run CV was determined on three replicates of nine dilutions of positive plasmids ranging from  $7.0 \times 10^1$  to  $7.0 \times 10^9$  copies.

Table 3  
Viral load in various tissues of LYCIV-infected fish

Tissues	Spleen	Kidney	Liver	Gills
Average $C_T$ value	14.31	14.72	18.90	21.47
Viral load <sup>a</sup>	6.84	6.66	4.81	3.67
Number of viral particle <sup>b</sup>	$6.86 \times 10^6$	$4.62 \times 10^6$	$6.47 \times 10^4$	$4.63 \times 10^3$

<sup>a</sup>  $\log_{10}$  (viral genome copies/mg tissue).

<sup>b</sup> Viral genome copies/mg tissue.

the inter-run CV was 1.35 and 2.96%, respectively. The intra-run CV was determined on three replicates of nine dilutions of positive plasmids ranging from  $7.0 \times 10^1$  to  $7.0 \times 10^9$  copies. Inter-run and intra-run coefficients of variation are presented in Table 2. The results suggest that the good reproducibility is more important for samples where the amounts of virus are low.

### 3.4. Samples detection

To determine viral load in the various tissues, viral DNAs extracted from the spleen, liver, kidney, gills, and muscle of LYCIV-infected fish were amplified by this real-time PCR. The result showed obvious differences in the viral load in different tissues (Table 3). The spleen and kidney contained the largest number of viral particles ( $6.86 \times 10^6$  and  $4.62 \times 10^6$  genome copies/mg tissue, respectively) while no viral DNA was detected in the muscle, indicating that the spleen and kidney may be the most sensitive target tissues for LYCIV infection.

## 4. Discussion

Iridoviruses have been identified to be the causative agents of serious systemic diseases in fish. In recent years, outbreaks of iridoviral disease with high mortality have been reported increasingly in different species of cultured fish in Southeast Asian

regions, resulting in great economic losses (Chou et al., 1998; He et al., 2000; Sudthongkong et al., 2001; Chen et al., 2003a). The early and rapid detection of iridovirus infection has been considered important for providing appropriate measures to control the transmission of diseases. Several PCR-based techniques have been used for the rapid diagnosis of iridovirus infection in fish (Oshima et al., 1998; Grizzle et al., 2003; Chen et al., 2003b). However, these PCR assays are only able to detect the presence of iridovirus infection, and not able to determine the viral load in infected fish, which may be a useful indicator of disease outbreak. Recently, a real-time TaqMan PCR has been developed to apply to the investigation of strain variation in an emerging iridovirus of warm-water fishes (Tony et al., 2003). Here we first report the development of a molecular beacon real-time PCR assay for the quantitative detection of LYCIV infection.

Real-time PCR techniques involving the use of either hybridization probes or double-stranded DNA binding dyes have been increasingly applied to both pathogen detection and basic researches such as gene expression and regulation, and allelic discrimination (Das et al., 2000; Tyagi et al., 1998; Mackay et al., 2002). Fluorescence detection monitors the accumulation of PCR products in 'real-time' during the exponential phase of amplification, facilitating the rapid detection, and quantitative assessment of specific targets by using a standard curve. Molecular beacons are a novel type of single-stranded oligonucleotide probes capable of reporting the presence of target nucleic acids in homogenous solutions and in vivo, and can be used for real-time quantitative PCR analysis (Tyagi and Kramer, 1996; Poddar, 1999). Compared with the linear probes for amplicon detection during real-time PCR, molecular beacons have obvious advantages. The hairpin structure of molecular beacons provides a highly stable conformation. Therefore, molecular beacons have been shown to be more specific than linear probes such as TaqMan probes and adjacent probes (Mackay et al., 2002). The background emission of molecular beacons is also significantly less than that of linear probes because the fluorescence of non-hybridized molecular beacons is quenched with greater efficiency than the fluorescence of uncleaved non-hybridized linear probes (O'Shea and Cane, 2004). Here, the design of the molecular beacon was different from the conventional design, the beacon probe sequence complementary to the target DNA included both loop and arm part sequences (Fig. 1), this adjustment did not affect the function of the hairpin, furthermore, it would enhance the hybridization efficiency of molecular beacon and increase the distance between the fluorophore and quencher after hybridization, which is helpful for the restoration of fluorescence (Li et al., 2000).

In the present study, the signal to background ratio was 19.9:1 following hybridization of a 20-fold molar excess of oligonucleotide target to molecular beacon over 5 min at room temperature, the efficiency of quenching was up to 95.0%, showing molecular beacon designed here is suitable for the real-time PCR detection. The melting temperature of a molecular beacon used for real-time PCR (the loop part) is expected to be higher than the annealing temperature, so that the target-loop hybrid is stable during real-time measurement (during annealing). In this study, the melting temperature of the probe-target hybrid is

predicted to be 61 °C, which is typically 7–10 °C higher than the PCR annealing temperature (54 °C), thus providing high stringent primer annealing and increasing specificity of reaction.

A very strong inverse association between cycle threshold number and positive plasmid concentration was noted and the generating standard curve demonstrated linear correlation coefficients of 0.998 over several logarithmic plasmid concentrations. The detection limit of this real-time PCR assay was 70 copies of positive plasmid/assay, more sensitive than conventional PCR-based assays developed previously (Oshima et al., 1998; Chen et al., 2003b). The high degree of specificity of this assay was also demonstrated by detecting template DNAs from healthy fish, EHNV, and WSSV. The coefficient of variation on  $C_T$  values ranged from 1.16 to 4.42%, depending on the concentration of the positive plasmid. A coefficient of variability of 4.42% was reached only for lower copy numbers, which is acceptable and has no impact on the detection result. Finally, this real-time PCR is time efficient. Analysis of 32 samples, from DNA extraction to the completion of real-time PCR, could be finished within 4.5 h.

The viral loads in different tissues of LYCIV-infected large yellow croaker were measured by this molecular beacon real-time PCR, the result showed that most virions existed in the spleen and kidney, relatively fewer virions were detected in the liver and gills, and no viral DNA was detected in the muscle. This result corresponds well to those obtained by the electron microscopical observation and by the in situ hybridization-based method (Chen et al., 2003a; Huang et al., 2004). At present, the replication analysis of LYCIV in fish cells by this real-time PCR is underway.

In summary, the development of a rapid and sensitive real-time PCR assay using a molecular beacon for the quantitative detection of LYCIV is described in this paper and it will provide an ideal system for the routine detection of LYCIV and associated basic studies.

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