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Original article

Design of Specific Primer for Methallothionein Gene of Tor Fish (*Tor tambra*)

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Abstract

To identify and integrate heavy metals at the molecular level, heavy metal biomarkers are required. Metallothionein gene is a gene that is expressed in fish organs that have been heavily polluted. The aim of the study was to find specific primers for Metallothionein of Tor fish. A primer in the form of a short chain DNA sequence is necessary as a particular target DNA identifier for the detection of the expression of the metallothionein gene in PCR instruments. The primer design was performed in silico using the NCBI site and multiple-aligned using Geneious Prime bioinformatic software. Primers were designed according to the conserved region of these genes. The primers specificity was checked using Primer BLAST tools in NCBI. The results showed that the forward primer 1 (5'- GAT TGC GCC AAG ACT GGA ACT –3') and reverse primer 1 (5' – ATC ACG TTG ACC TCC TCA CTG -3') qualified as good primers with an amplicon size 186 bp.

INTRODUCTION

Tor fish (*Tor tambra* Valenciennes 1842) is a fish with high economic value. Tor fish is one of 10 species of fish that found in the River of Batang Gadis. This species has the highest abundance and dominance in the four observation stations in Batang Gadis (Atifah & Lubis, 2017). Many community activities are carried out on this River, one of them is gold mining. Gold mining directly dumps its waste into the river in the form of oil and sediment from the excavation and causes the water quality in the Batang Gadis river to decline. According to a study, the Batang Gadis River has levels of Hg< 0.0008, Cd< 0.003 and Pb< 0.005. Metal concentrations discovered that were still below the cutoff value (Atifah & Harahap, 2019).

Decreased water quality can affect the life of the biota in the river. A water pollutant agent detection system is necessary as an early indicator of pollution due to the significant risk of water contamination in the Batang Gadis river. The presence of up to a quantity of water-polluting chemicals can be demonstrated by a number of detection technologies. To detect and monitor the presence of heavy metals at the molecular level, heavy metal biomarkers are required (Friedrich *et al*. 1996).

Metallothionein is a protein that is very sensitive and accurate as an indicator of pollution. This is based on a natural process where metals can be bound in the bodily tissues of organisms, which is made possible by the existence of these proteins. Every living thing's tissues contain metallothionein, a metal-binding protein that performs and participates in the process of binding metals. When organisms are polluted with metallic elements, its concentration in tissues (liver, gills, and digestive glands) rises. (Engel & Brouwer, 1991; Engel &

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Brouwer, 1993; Le Gal, 1988; Bebianno & Langston, 1995).

The biomarker metallothionein (MT) can be used to monitor metal exposure and toxicity in water. Since MT is currently the only biological substance in the body that naturally interacts with metals, its application as a bioindicator or biomarker is very appropriate. DNA amplification is required to identify metallothionein in an individual.

Many DNA-based molecular methods have been developed, one of them is the Polymerase Chain Reaction (PCR) method. PCR has several advantages, including: fast in analyzing results (1 day), has high sensitivity and specificity values (>90%). PCR can be used to detect the presence of gene expression that plays a role in the metal binding process in the tissues of every living thing. PCR is based on using the ability of DNA polymerase to synthesize a new DNA strand that is complementary to the offered template strand. Since DNA polymerase can only add nucleotides at the pre-existing 3'-OH group, primers are required that can add the first nucleotide. This requirement makes it possible to delineate a specific region of the template sequence that the researcher wishes to amplify. At the end of the PCR reaction, the specific sequence will accumulate in billions of copies. PCR aims to amplify DNA and consists of three stages. The initial stage of the amplification process is the denaturation of the DNA strand, then the primer pair attaches to the target DNA fragment (annealing), and the last stage is the elongation of the DNA sequence by DNA polymerase (Kadri, 2020).

In the PCR, a very important component needed is oligonucleotide primers. A good primer must have specific properties that are expected to amplify specific regions in the genome (Praja, 2021; Sint *et al*. 2012). Along with the development of the NCBI GenBank database, primer design can be done using in silico approach. In silico studies were conducted using a computational approach. In general, in silico studies utilize databases that already available to be used as research objects (Saraswati *et al*. 2019). This study was aimed to design and analyze oligonucleotide primers in silico to amplify the metallothionein gene.

MATERIALS AND METHODS

Primer design

The gen methallothionein sequence was obtained using the "nucleotide" search menu provided by the NCBI GenBank (https://www.ncbi.nlm.nih.gov/-genbank/) and designed using Geneious Prime bioinformatic software (https://www.geneious.com) (Achyar *et al*. 2021). The metallothionein gene sequence used in this study was from Cyprinus carpio mRNA with accession number XM019095650.2|:1-619 because the database at NCBI is not yet available with the methallothionein gene sequence for Tor tambra. *Cyprinus carpio* was chosen because it is still at the same taxonomic level as T. tambra, namely the Cyprinidae family. The metallothionein gene sequences obtained were stored in FASTA format for further use in the in silico primer design process using Geneious Prime. After the primer pair candidates were obtained, the primers were selected based on ideal primer criteria (Shen *et al*. 2010; Hung & Weng, 2016). The specificity of candidate primer pairs generated by Geneious Prime were checked using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools-/primerblast/). The selected primer pair was ordered to synthesize at IDT, Singapore.

Gradient PCR

The synthesized primer pair was optimized using gradient PCR to obtain the right annealing temperature. The gradient temperature was set up according to the melting temperature (Tm) of each primer. The composition of the gradient PCR reaction used with a total volume of 10 µl consist of 5 µl 2x MyTaq HS Red Mix (Bioline), 1 µl cDNA of T. tambra (prepared from total RNA of liver tissue which was extracted using Ribozol and cDNA was synthesized using Sensifast cDNA kit, Bioline), and 0.5 µM forward primer and 0.5 µM reverse primer. The PCR reaction was made up to 10 µl with the addition of nuclease-free water. PCR was carried out under the following conditions (according to manual instruction of MyTaq HS Red Mix, Bioline): initial denaturation at 95°C for 1 minutes, followed by 35 cycles consisting of denaturation at 95°C for 15 seconds, annealing at gradient temperature (50-59.5°C) for 15 seconds, and elongation at 72°C for 10 seconds. The PCR process ended with an elongation step at 72°C for 5 minutes. The PCR products were analysed by electrophoresis using 2% agarose gel, 100 V for 27 minutes.

RESULTS AND DISCUSSION

Primer design

The Geneious Prime software generated two pairs of candidate primers to amplify the

metallothionein gene in the 135-502 region of the complete mRNA sequence of the fish Cyprinus carpio (Figure 1). The two pairs of primers designed by Primer-BLAST showed variability in terms of PCR product size, primer length, Tm, and GC percentage (Table 1). The shortest and longest products were produced by primer pair 1 with 186 bp and by primer pair 2 with 296 bp, respectively (Table 1). Furthermore, the melting temperature (Tm) of each primer pair has a difference not exceeding 50°C with temperatures ranging from 57.9 to 61.2ºC.

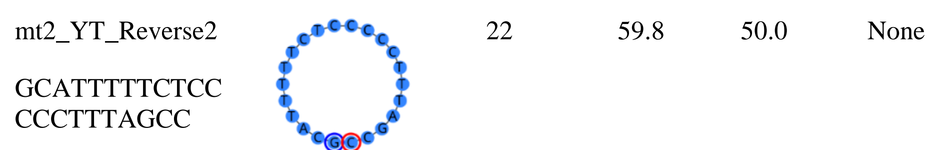
The oligonucleotide primer sequences obtained from Primer-BLAST were then further analyzed to identify the most efficient primer. Analysis to identify self dimer, hairpin, repeat, and run was performed using Geneious Prime. All primers analyzeshowed varying results in terms of self dimer, hairpin, repeat and run (Table 1).

The first step in the PCR test process is primer design and this will determine the amplification performance of the PCR test (Praja, 2021). The following factors, including primer length, melting temperature, GC percentage, and other criteria, including the minimal amount of self-dimer, hairpin, repeat, and run, must be taken into consideration while creating the primer. (Saraswati *et al*. 2019; Pradnyaniti *et al*. 2013).

The design of primers must take into account a number of factors, including primer length, melting temperature (TM), GC base proportion, and Self 3' complementarity. In this study, all primers that had been designed showed lengths ranging from 21-23 bases (Table 1). Theoretically, a primer with a length of 18-30 bases is an ideal primer length (Sasmito *et al*. 2014). Short PCR primer lengths will be susceptible to mispriming (pasting errors) while primers that are too long can undergo hybridization so that it will inhibit the DNA polymerization process (Anika *et al*. 2019). Hung & Weng (2016) also stated that a good primer length is 18-24 nucleotides, because primers are too short will be less specific. All primers that have been successfully designed in this study have GC percentages ranging from 39.1 to 52.4% (Table 1). GC percentage is an important factor in designing PCR primers. GC percentage is the number of guanine and cytosine bases in a primer that related to the melting temperature. In addition, the percentage of GC is also related to the binding between DNA strands. To break the hydrogen bonds of GC, it takes a higher energy and temperature compared to AT. The high content of GC will make it difficult to break the double-threaded chain in primers and molds. Furthermore, the low GC content in the primers causes the primers to be unable to attach and will have an impact on decreasing PCR efficiency. Ideally, the percentage of GC content is in the range of 40 – 60% (Sasmito *et al*. 2014; Maitriani *et al*. 2015) 45-55% (Shen *et al*. 2010).

Table 1. Primer design results from Primer-BLAST

No	Sequence (5'--> 3')	DNAFold	Length (nt)	Tm (^{0}C)	GC (%)	Self 3' Complem-entarity	Amplicon Size (bp)
1	mt2_YT_Forward GATTGCGCCAAG ACTGGAACT		21	61.2	52.4	4.2	186
	mt2_YT_Reverse ATCACGTTGACC TCCTCACTG		21	59.7	52.4	None	
2	mt2_YT_Forward2 TGTAAAACATGC AAGAAGAGCTG		23	57.9	39.1	None	296

mt2_YT_Reverse2 GCATTTTTCTCC CCCTTTAGCC		22	59.8	50.0	None

A suitable melting temperature (Tm) is in the temperature range of 57-63ºC (Shen *et al*. 2010). The self 3' complementarity should be as tiny as possible. The maximum self 3' complementarity is 3 (Shen *et al*. 2010) and the maximum repetition of the same base sequentially is 4 bases (Lorenz, T 2012). Other things that need to be considered in designing primers are the structure of the dimers in the primer, the number and position of G and C bases in the selected primer sequence (Saraswati *et al*. 2019). The presence of G or C bases will help for specific binding because the bonds between G and C bases are stronger than the bonds between A and T bases (Borah, 2011). However, the number of G and C bases that are more than three in the last five bases at the 3' end of the primer should be avoided (Borah, 2011).

Based on the primer design results in Table 1, primer 1 shows the characteristics of a good primer for DNA amplification. The main reason for choosing primer 1 is the amplicon size for real time PCR should be in the range of 100-250 bp, and primer 1 has a size of 186 bp while primer 2 has 296 bp. Primer 1 does have a reasonably large Tm, but even if there is a chance for self-dimerization, the Tm for self-dimerization is only 4 degrees, meaning that during PCR analysis, the temperature utilized is much higher than that (95°C for denaturation, 50-60 °C for annealing, and elongation 72°C).

Figure 1. PCR Simulation of two pairs of designed primer candidates on the mRNA sequence Cyprinus carpio metallothionein 2 (mt2) (NCBI Accession Number: ref|XM_019095650.2|:1-619)

The optimal melting temperature (Tm) is in the temperature range of 57–63°C (Shen *et al*. 2010). The smaller the self 3' complementarity, the better. The maximum self 3' complementarity is 3 (Shen *et al*. 2010) and the maximum repetition of the same base sequentially is 4 bases (Lorenz, 2012). Other things that need to be considered in designing primers are the structure of the dimers in the primer, the number and position of G and C bases in the selected primer sequence (Saraswati *et al*. 2019). The presence of G or C bases will help for specific binding because the bonds between G and C bases are stronger than the bonds between A and T bases (Borah, 2011). However, the number of G and C bases that are more than three in the last five bases at the 3' end of the primer should be avoided (Borah, 2011).

Based on the primer design results in Table 1, primer 1 shows the characteristics of a good primer for DNA amplification. The main reason for choosing primer 1 is because the amplicon size for real time PCR should be in the range of 100–250 bp, and primer 1 has a size of 186 bp while primer 2 has 296 bp. Primer 1 does have a fairly large Tm, but even though there is potential for self-dimer, the Tm self-dimer is only 4 degrees, which means the self-dimer will open at a temperature above that because during PCR analysis, the temperature used is far above that (95°C for denaturation, 50–60°C for annealing, and elongation 72°C).

Table 2 Primer specificity test generated by using Primer-BLAST

No	Target Template	Detect Target	Product Length	Accession Number
1	*Cyprinus carpio* metallothionein 2 (mt2), mRNA	Yes	186	XM_019095650.2
2	*Cyprinus carpio* class I metallothionein mRNA,	Yes	186	AF002162.1
3	*Cyprinus gibelio*	Yes	186	XM_052583010.1
4	*Cyprinus rohita*	Yes	186	XM_051135697.1
5	*Cyprinus auratus*	Yes	186	XR_003294303.1
6	*Clarias bathracus*	No		
7	*Oreochromis niloticus*	No		
8	*Puntius japonicus*	No		
9	*Oreochromis mossambicus*	No		
10	*Hemibagrus nemurus*	No		

Primer specificity

Primer specificity was checked in silico using NCBI primerBLAST. The primer pair BLAST results are summarized in Table 2. The primer pair was amplified in the Cyprinus genus, and was not detected in other species.

The most important criteria that primers must have is to be specific to the target (Ye *et al*. 2012). Ideally, primers will only attach to the desired target. If the primers can connect to undesirable targets during DNA amplification, the test results will not be able to specifically identify the species (Ye *et al*. 2012). From the Primer-BLAST results of the selected primer pairs, it was found that the primers could recognize the *Cyprinus carpio* genes contained in the NCBI database. In addition, the primers also recognize genes in *Cyprinus auratus*, *Cyprinus gibelio*, *Cyprinus rohita* and *Cyprinus auratus* indicating that the primers can still attach to the methallothionein gene of other cyprinidae family

The selected primers were tested using Geneious Prime software to determine the accuracy of the primer amplification on the target gene. The primer pairs amplify the metallothionein gene by attaching forwards to the 147th to 167th nucleotides and reversely attaching to the 332nd to 312th nucleotides.

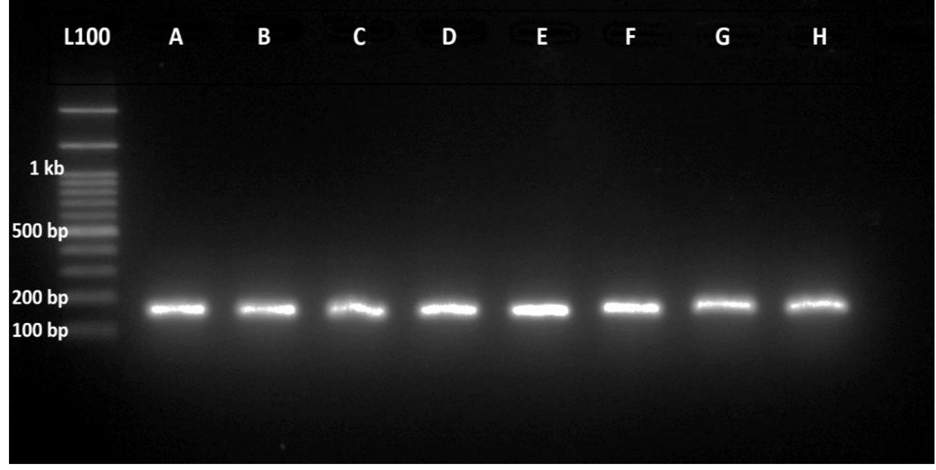


Figure 2. PCR Gradient Test Results using mt-YT-F & mt-YT-R primers A: 50ºC B: 51.3ºC C: 53.3ºC D: 55.6ºC E: 56.7ºC F 57.8ºC G: 58.7ºC H: 59.5ºC

Gradient PCR

The synthesized primer pair was optimized using gradient PCR to obtain the right annealing temperature. The gradient temperature was set up according to the melting temperature (Tm) of each primer. The results of the synthesis of primer 1 show that the Tm of the primer mt2_YT_Fwd is 58 °C, mt2_YT_Rev is 56.7 °C, the temperature gradient used in the temperature range of 50 °C - 59.5 °C. The results of gradient PCR showed the thickest and clearest DNA bands were at 56.7°C. Based on the results in Figure 2, the optimum annealing temperature for the mt-YT-Fwd & mt-YT-Rev sequences is 56.7 °C as shown in the letter E. This is based on the thickest band and the appropriate amplicon size. This demonstrates that the primer can attach to a region of the DNA template at a temperature of 56.7 °C that is complementary to the base sequence.

CONCLUSION

The primer with the best primer criteria for the MT2 gene is primer 1, which has an amplicon size of 186 bp and an annealing temperature of 56.7 °C. Its forward and reverse primer base sequences are 5'-GAT TGC GCC AAG ACT GGA ACT -3' and 5'-ATC ACG TTG ACC TCC TCA CTG -3', respectively.

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