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SOME ASPECTS OF PRIMER DESIGN FOR REAL TIME PCR WITH SYBR® Green AS A DYE

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Abstract. This work considers the sequence of steps for designing primers for real time qPCR analysis with SYBR® Green as fluorescent dye. For primer design, we used Primer-Blast software. As an example, we design primers to *fum6* gene, a member of fumonisin biosynthetic gene cluster. The importance of a balance between specificity and efficiency of primers obtained with the help of Primer-Blast software is discussed. Challenges of obtaining specific primers are considered, possible solutions are suggested. BLAST analysis of the primers is discussed, potential approaches to choosing an optimal primer pair are considered. The testing results of the designed primers to *Fusarium verticillioides fum6* gene in real-time PCR reaction are shown. We used the designed primer pair in a qPCR reaction and analyzed the amplification curve and melting curve of the amplicon. The shape of the amplification curve reflects the kinetics of PCR reaction and gives some approximation about primer efficiency. Melt curve analysis gives the information on primer specificity, with a single melt peak corresponding to a single PCR amplicon.

Keywords: *Primer-BLAST, Primer3, qPCR, BLAST, Fusarium.*

Introduction

Primer design is one of the most important factors determining the success and quality of quantitative real-time PCR analysis (qPCR). Precise and reliable quantification of the analyzed DNA depends to a large extent on the efficiency of the primers used. In general, PCR primers must meet a number of requirements, important for their performance in PCR reaction. It is especially true for the primers designed for qPCR analysis. Basically, qPCR principle is based on monitoring the amplification of the target DNA in the end of each amplification cycle by measuring the fluorescence of the reporter dye. So, when designing primers for qPCR, one should consider the reporter dye to be used in the reaction. The most commonly used fluorescent dyes for qPCR analysis are: Molecular Beacon (Public Research Properties, Inc., USA), Scorpions® (Sigma-Aldrich, USA), SYBR® Green (Invitrogen, USA) and TaqMan® (Applied Biosystems, USA). One of the most commonly used reporter dyes is SYBR® Green. It is very easy to use and cost-effective. So, in the present work we focused on primer design for SYBR® Green – based qPCR analysis. SYBR® Green is an

intercalating dye which binds non-specifically to double-stranded DNA, and the resulting complex emits fluorescence which can be measured in the end of each amplification cycle. However, since the DNA-binding of this dye is non-specific, it will also bind to non-specific PCR products and primer dimers, and it will affect the quantification of the specific amplicon. So, the crucial requirements for primers used in qPCR reaction is their high specificity and low ability to form primer dimers. However, there is no need for expensive primer design software for SYBR® Green – based qPCR primer design, since there are several free instruments found in World Wide Web (www), which can help to design high quality primers[1 - 9].

Primer design

For primer design, we use the program Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), found on NCBI (National Center of Biotechnology Information) site. It is easily available, free of charge, uses Primer3 program for primer search. Besides, there is BLAST program necessary for analysis of primer specificity with an access to a comprehensive database of sequenced DNA and RNA sequences available on this site. Besides, this program uses BLAST analysis simultaneously with primer design, which makes it possible to choose the most suitable primers already at the stage of primer search. More details on the advantages of Primer-BLAST software are given elsewhere[4, 10].

The success of a regular PCR depends largely on primers, which should be balanced between two parameters: specificity and efficiency. Specificity is important because, if primers are poorly designed, an undesirable non-specific amplification of the sequences present in the analyzed DNA can occur. Efficiency is also important for primer design. An efficient primer pair leads to doubling the amount of the amplicon after each PCR cycle. Most programs for primer design have the default conditions set for traditional PCR. This will help to choose primer pairs with an acceptable balance between specificity and maximal efficiency in traditional PCR, but they will not necessarily be the best choice for qPCR. For SYBR® Green –based qPCR the specificity is of major importance, since the dye binds to any DNA, including non-specific fragments, and primer dimers. So, during qPCR primer design, some changes to default parameters suggested by Primer-BLAST software should be made, since the default parameters were optimized for traditional PCR.

In this work we describe primer design to *fum6* gene (a member of fumonisin biosynthetic gene cluster) of *Fusarium verticillioides* as an example. At <https://www.ncbi.nlm.nih.gov/> site we choose nucleotide data base in front of the search line, and in the search line type *Fusarium verticillioides fum6 gene* and do the search. Then we find *fum6* in search results, with NCBI registration number, for example, KF889190.1. We open this entry and save the sequence in FASTA format. At this point we follow with primer design using Primer-BLAST software. We open the software and either paste the nucleotide sequence, or open the saved file with the nucleotide sequence, or enter the reference number KF889190.1 in the field *PCR Template* (figure 1). Then we edit *PCR product size*. By default, there is 70-1000 nucleotides in this field. This number should be changed for 80-150 nucleotides. The optimal amplicon would have a size of ~120 b.p. As a rule, the amplicons of 80–200 b.p. are acceptable; however longer amplicons result in less efficient results of qPCR.

The screenshot displays the Primer-BLAST web interface. At the top, it identifies the tool as 'Primer-BLAST: A tool for finding specific primers' and notes it uses 'Primer3 and BLAST'. The main section is titled 'Primer Parameters' and includes fields for 'PCR Template' (accession number KF889196.1), 'PCR product size' (80-150), '# of primers to return' (10), and 'Primer melting temperatures (Tm)' (60.0-63.0, Max Tm difference 2). There are also options for 'Exon/intron selection' and 'Intron inclusion'.

Figure 1. The screen of Primer-BLAST.

We usually change *Primer melting temperature* (T_m) to 60°C – Min, 61°C – Opt, 63°C – Max. *Primer melting temperature* (T_m) – is a temperature at which hybridize 50% of primer and its complementary template. Usually, primers with melting temperatures between 60°C and 67°C, and optimal melting temperatures T_m from 62°C to 64°C. The difference between T_m of forward and reverse primers should be no more than 1-2 °C. Theoretically, it is possible to make a larger temperature range for primers, for example, 60-68°C, but we prefer to have all primers with similar melting temperature. In this case, different primers, specific to different genes, can be used in a single PCR run, which makes the analysis easier and faster.

At this page, one can additionally indicate the region on the template for *Forward* and *Reverse* primers, in case it is necessary to specify where to look for the primers. It is possible to enter own nucleotide sequence of previously designed primer in the field *my own primer*, if it is necessary to use the existing primer and find a pair for it. It can be useful to tick the box *Show results in a new window*. All other parameters on this page can remain with default settings. Then, we open the tab *Advanced parameters*. In this tab, in the field *Max GC in primer 3' end*, the value is changed from 5 to 2. The increase of this parameter will result in a faster and stronger binding of the primer with the template and increase primer efficiency. However, such an increase in binding efficiency in primer 3' end will decrease primer specificity, and this parameter is of crucial importance in case of using SYBR® Green dye. So, it is better to start primer search from the value 2, and increase this value only in case the program cannot find any primers satisfying this condition.

Max Self Complementarity. Max Pair Complementarity. *Primer Self Complementarity* or *primer pair Complementarity*, especially at 3' end of the nucleotide are not recommended to exceed the values 2 - 3. *Primer ends* should not complement each other, since it will result in primer dimers. Sometimes it is inevitable, when Primer3 does not suggest primer list. In this case, its recommended gradually, with a step on 1 nucleotide, to increase this value. We also change the value 8 to 5 in the sections *Self Complementarity* and *Pair Complementarity* in other fields (*Any*) primers.

Generally, the above changes are necessary for qPCR primer search. Naturally, Primer3 software is capable of selecting primer search conditions for a lot more parameters for primer optimization, this information is given in more details elsewhere[11, 12]. After selecting the conditions for primer design, we push the button Get Primers and wait for the program to suggest primers. In the end of the process, *Graphical view of primer pairs* and *Detailed primer reports* (figure2) appear on the screen. They give the diagram of the position of primer pair on the template and the detailed information of the suggested primers for *Fusarium verticillioides fum6* gene.

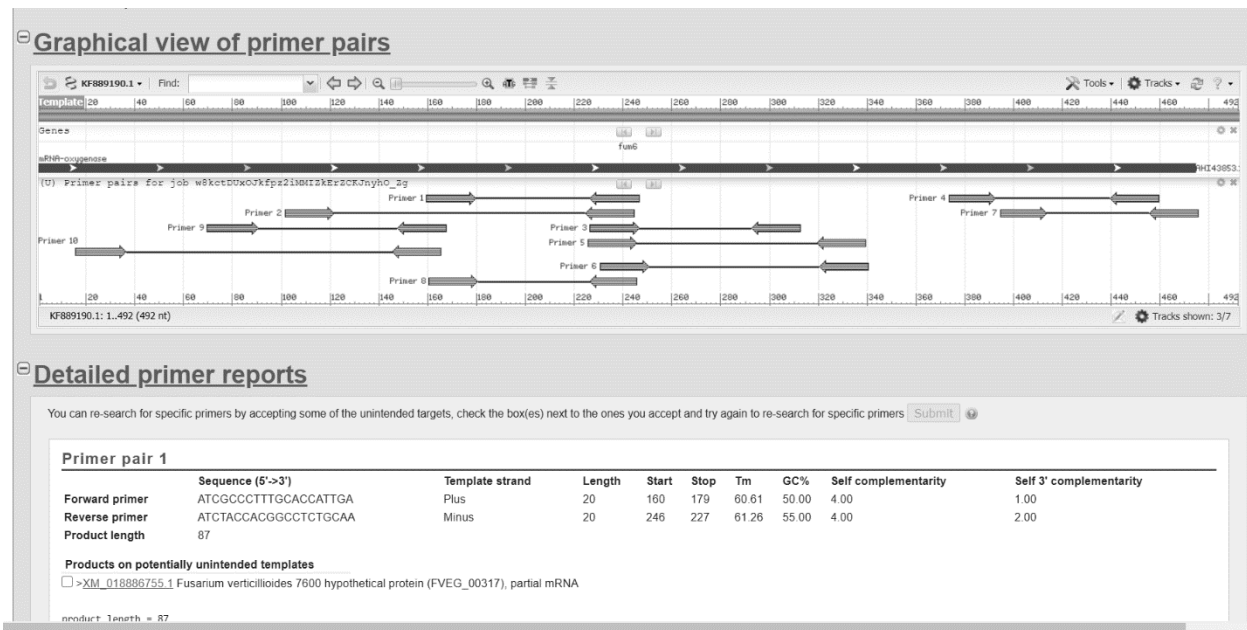


Figure 2. Graphical view of primer pairs and Detailed primer reports.

Next important stage of primer design is BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) of the primers, suggested by Primer-BLAST software. The goal of this analysis is the choice of the most appropriate primer pair both by primer characteristics and their specificity. Blast analysis makes it possible to choose the primer pairs recognizing the maximal number of sequenced sequences of a gene (inclusivity) on one hand, and minimal number of other unrelated sequences which these primers might recognize (specificity). For BLAST[13 - 15] analysis, in the field Enter accession number(s), gi(s), or FASTA sequence(s) we introduce the primer sequence and hit the BLAST button. It is useful to tick the box *Show results in a new window*. The value Max target sequences equals 100 by default. However, if the number of sequenced entries is close to 100 or more, it is better to increase the number of Max target sequences, by opening Algorithm parameters and choosing the appropriate number not exceeding *Number of Hits*, which indicate the number of sequenced entries with 100% homology. The value of the *Number of Hits* appears on the tab *Taxonomy*.

After primer design and their synthesis, an important step is in vitro testing of the primers. First step, is using the designed primer pair in a qPCR reaction and analyzing the amplification curve and melting curve of the amplicon. Figure 3 shows the results of the in vitro testing of the primer pair to *Fusarium verticillioides fum6* gene. Amplification graph (Figure 3a) reflects the kinetics of PCR reaction and shows the increase in the fluorescence after each PCR cycle. The shape of the curve and its slope gives some approximation about

primer efficiency. For precise determination of the efficiency of the primer pair, PCR of serial dilutions of the template DNA is necessary. Melt curve analysis (Figure 3b) gives the information on primer specificity, with a single melt peak corresponding to a single PCR amplicon.

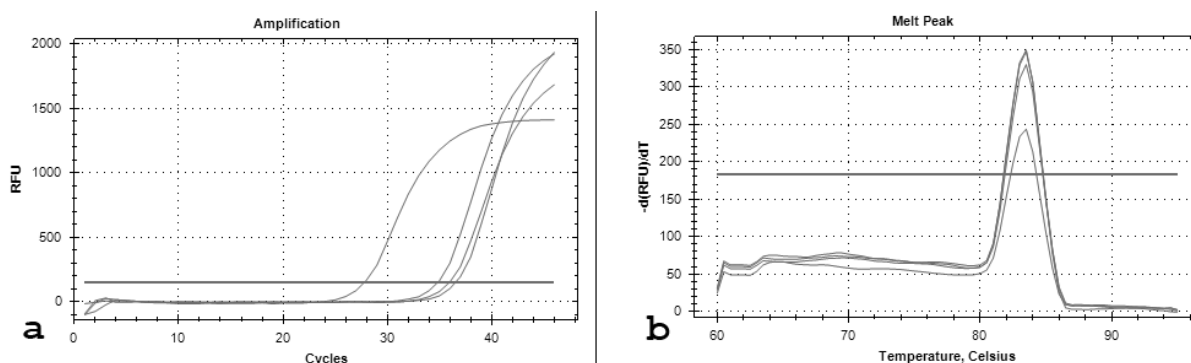


Figure 3. Amplification (Figure 3a) and Dissociation (Figure 3b) curves obtained with one of the described pairs of primers and DNA of wheat grain infected with Fusarium.

Conclusion

The above procedure of primer design for qPCR is at most simplified, however, it is effective for designing primers suitable for qPCR in SYBR® Green-based applications. An important problem for primer design issue is a choice of the gene suitable for obtaining efficient and specific primers. In this case, literature search and analysis of the genes commonly used for primer design can be helpful. Besides, the problems can appear in obtaining specific primers, Alignment of the sequenced sequences of the gene of choice, selection of specific gene regions and design of primers using these specific regions can facilitate the task of primer design.

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