Journal of Animal Science and Products (JASP)
International peer-reviewed journal
7 (2): 134-144, 2024

Bioinformatic Comparisons of Some Web-based PCR Primer Design Programs

Hasan KOYUN [1]*, M. Furkan ÜSTÜN [1]

[1]Van-Yüzüncü Yıl University, Agricultural Faculty, Department of Animal Science, Biometry-Genetics Unit, 65100 Van, Türkiye

Hasan KOYUN, ORCID No: 0000-0001-9424-6850, M. Furkan ÜSTÜN, ORCID No: 0009-0008-4219-070X

ARTICLE INFO

Research Article

This article was presented as an oral presentation at the 17th National Student Congress on Animal Science, May 17-19, 2024, and was awarded first place in the categories of best presentation and research project at the congress.

Received : 05.12.2024
Accepted : 17.12.2024

Keywords

Bioinformatics analysis
Primer design
Web-based PCR primers design programs

*** Corresponding Author**

hkoyun@yyu.edu.tr

ABSTRACT

Bioinformatics has become an indispensable tool for both basic and applied research in biotechnology in the life sciences. The polymerase chain reaction (PCR) is a laboratory method that can be used to quickly amplify a large number of identical copies of a specific DNA segment. In PCR, short synthetic DNA fragments known as primers are used to selectively amplify a specific section of the genome. For PCR to be as efficient and specific as possible, it is important to choose an effective primer sequence and use the correct concentration of primers. If the primer is not designed carefully, non-specific amplification and/or primer dimer formation may occur, which may prevent product formation. Currently, a number of different design tools are available on the internet to assist molecular geneticists in designing PCR primers under optimal conditions. In this study, out of 39 web-based PCR primer design programs, 7 accessible, freely available and widely used web-based PCR primer design programs (NCBI, Primer3, Biserach, Genscript and Primer3plus; Stitcher 2.0; and PrimerQest Tool) were compared using bioinformatics applications for genomic sequences. The advantages and disadvantages of the web-based PCR programs are discussed on the basis of the comparison results.

Web Tabanlı Bazı PCR Primer Tasarım Programlarının Biyoinformatik Karşılaştırılması

MAKALE BİLGİSİ

Araştırma Makalesi

Bu makale, 17-19 Mayıs 2024 tarihlerinde 17. Ulusal Zootekni Öğrenci Kongresi'nde sözlü bildiri olarak sunulmuş, kongrede en iyi sunum ve araştırma projesi dallarında 1.cilik ile ödüllendirilmiştir.

Geliş: 05.12.2024
Kabul: 17.12.2024

ÖZ

Biyoinformatik, yaşam bilimleri biyoteknolojisinde hem temel hem de uygulamalı araştırmalar için önemli bir araç haline gelmiştir. Polimeraz zincir reaksiyonu (PCR), DNA'nın belirli bir bölümünün çok sayıda özdeş kopyasını hızla çoğaltmak için kullanılan bir laboratuvar yöntemidir. PCR, genomun belirli bir bölümünü seçici olarak çoğaltmak için primer adı verilen kısa sentetik DNA parçalarını kullanır. PCR'nin mümkün olduğu kadar verimli ve spesifik olması için etkili bir primer dizisinin seçilmesi ve doğru primer konsantrasyonunun kullanılması önemlidir. Primer dikkatli bir şekilde tasarlanmadığı sürece spesifik olmayan amplifikasyon ve/veya primer dimer oluşumu meydana gelebilir ve bu durum hedeflenen PCR ürününün oluşumunu engelleyebilir. Günümüzde moleküler genetikçiler için optimum koşullarda PCR primerleri oluşturmasına yardımcı olacak çeşitli tasarım araçlarına internette kolaylıkla erişilebilir. Bu çalışmada

Anahtar Kelimeler

Biyoinformatik analiz
Primer tasarımı
Web tabanlı PCR primer tasarım programları

*** Sorumlu Yazar**

hkoyun@yyu.edu.tr

erişilebilir, kullanıma açık ve yagın olarak kullanılan 39 adet web tabanlı PCR primer tasarım programlarından, 7 adet web tabanlı PCR primer tasarım programları (NCBI, Primer3, Biserach, Genscript, Primer3plus, Stitcher 2.0, PrimerQest Tool) biyoinformatik tabanlı genomik dizi uygulamaları ile karşılaştırılmıştır. Karşılaştırma sonuçlarına göre web tabanlı PCR programlarının kullanım üstünlükleri ile olumsuz yanları tartışılmıştır.

Introduction

Due to the remarkable advancements in biological sciences during the last century, a substantial volume of genetic data has been amassed. Simultaneously, the emergence of the computer era has facilitated the processing of genetic data in a manner congruent with contemporary standards. Consequently, bioinformatics is an interdisciplinary field that integrates computational programs with established scientific disciplines such as mathematics, statistics, molecular genetics, physics, chemistry, biochemistry, and biology, facilitating the storage and analysis of extensive genetic data to elucidate the convergence of biology and computer science.

As an approach, Bioinformatics, was initially formulated by Pauling and Corey in the 1950s to examine the secondary structures of proteins, while the field of bioinformatics commenced with the publication of the inaugural article on molecular graphics created using computer programs in Scientific American in 1966. Today, the term bioinformatics is supported by many software programs and is widely used. The National Center for Biotechnology Information (NCBI) is one of the few methods established in 1988 for the analysis and interpretation of complex data. In October 1990, the "Human Genome Project" (HGP), one of the most important projects in this field, made significant contributions to the advancement of bioinformatics with its emergence. At the same time, bioinformatics science provides ease of analysis to the user with various informatics, mathematical, statistical models such as artificial intelligence models, internet-based uses, and different programming strategies. For this reason, while the development of genetics science is progressing rapidly, it will also increase and expand its intersections and common work areas with different branches of science in the near future, thus this natural intersection will develop an interdisciplinary approach and have an important share in its progress. This shows that bioinformatics science is one of the keys to science in the near future (Andrade and Sander, 1997; Collins et al., 2003).

Bioinformatics is primarily used to analyze the structures and functions of RNA, DNA, and amino acids or protein sequences. This includes areas such as pharmacology, the treatment of genetic diseases, genome analysis, and the development of vaccines. Bioinformatics uses a variety of techniques to carry out all associated research work. In relation to genetics and genomics, bioinformatics refers to the use of computer technology to capture, store, analyze and disseminate biological data. To improve our understanding of health and disease, and in some cases to provide medical care or improve the traits of farm animals, scientists use databases to organize and index biological information. (Sahu et al., 2024)

The most important of these instruments are the databases. These databases can independently store considerable amounts of genetic information and at the same time make

this data available to the user for analysis. The use of biological databases such as sequence databases and portals such as GenBank, the UCSC Genome Browser and Ensembl plays a crucial role in bioinformatics. This allows scientists to access a wide range of biologically relevant data, including genomic sequences of an increasingly broad range of organisms. In addition, there are databases for model organisms such as WormBase, Arabidopsis Information Resource (TAIR) and Mouse Genome Informatics (MGI) as well as databases that do not focus on sequences, e.g. Online Mendelian Inheritance in Man (OMIM), Protein Data Bank (PDB), MetaCyc and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Baxevanis and Bateman, 2015).

PCR is a widely used biotechnological technique for the enzymatic replication or amplification of a specific region between two known DNA segments. This technical approach was first discovered by Kjell Klape. Later, in 1985, Kary Mullis introduced polymerase chain reaction technology using the heat-resistant TAQ polymerase. PCR, which involves more than one method, has many advantages, including easy amplification of DNA, examination of regions coding for amino acids, quantitative yield (QTL and diseases, etc.), analysis and interpretation of loci. It is currently used in various fields such as molecular genetics, medical and pharmaceutical sciences, the detection of hereditary diseases, animal sciences, and archeology. (Rose, 1991; Desquesnes et al., 2002; Al-Samarai and Al-Kazaz 2015; Kaunitz, 2015).

In addition, the efficiency and sensitivity of PCR tests depend largely on the correct design of the primers. These depend on several important parameters. These are: a) %GC ratio and primer melting temperature (Tm); b) primer length, which are the most critical points for PCR success. As a result of incorrect or incomplete adaptations, a poorly designed primer can be so dominant that it prevents product formation. For this reason, errors in the primers in question can lead to a PCR reaction that does not work. Non-specific primer formation can lead to low or no product yield in PCR, which has various consequences. Therefore, it is important to consider the above primer phases throughout the design process. Further detailed information on this topic can be found in the relevant publications (Yang et al., 2004).

The G/C ratio indicates how high the proportion of G/C is in the DNA. For a strong primer design, an ideal G/C ratio should therefore be present in the DNA. For efficient annealing, the G/C ratio should be kept at 40–55 %. The correct matching of the Tm values with the G/C ratio determines the specificity of the primer pairs. This is because under conditions that depend on a high Tm temperature, a low primer content can lead to no or incorrect results. Conversely, no specific result can be achieved at a low Tm temperature. For this reason, the correct matching of Tm temperature and G/C content is directly proportional to the specificity of the product and is very important. Therefore, the Tm temperature of primers with an ideal length of 15-25 nucleotides can be calculated using the formula “2*(nA+nT) + 4*(nG+nC)”, also known as Wallace's rule, and the Tm temperature corresponding to the G/C content can be determined (Dieffenbach et al., 1993; Benita et al., 2003; Ruijter et al., 2009; Svec et al., 2015).

Besides, annealing specificity and specific primer content are not completely effective, but depend on primer length. Therefore, for a successful PCR, an appropriate primer length should be selected. Primers with a length of 18-24 nucleotides (nt) can show good working

properties during PCR in terms of specificity and cause the least problems. The reason for this is that primers shorter than 18 nt may not bind completely to the DNA and/or in some cases bind to the wrong loci due to their short length. However, if the primers are longer than 24 nt, the primer may bind to its own sequence instead of the DNA sequence due to the length of the designed sequence. As the temperature required for binding increases at the same time, PCR efficiency may decrease. For this reason, this can prevent the formation of sufficient product during PCR and reduce the success rate of the PCR. Therefore, primers with a minimum length of 18 base pairs should be selected for the proper operation of PCR and optimization of the appropriate Tm temperature (Dieffenbach et al., 1993 and Obradovic et al., 2013).

As far as web-based PCR applications are concerned, they occupy a very important place in bioinformatics science as they store, process, and interpret large amounts of genetic data. In particular, web-based software allows researchers to perform analyzes outside the laboratory as they are accessible via internet browsers from any computer or device. At the same time, they help researchers to use the programs without complex programming knowledge by providing different and easy-to-use interfaces. These software programs are constantly updated so that researchers have access to the latest functions. Smilarly, web-based platforms can also store data in the cloud and make it available for sharing so that multiple users can access the same data. Cloud-based web services offer fast and powerful computing capabilities, often for large amounts of data, and bring significant benefits in analyzing large amounts of stored data sets and applying complex algorithms and interpreting their results. Finally, web-based programs can be easily integrated with other databases, tools, and services, making it easier to merge data from different sources. Since these programs usually support multiple users, it is possible to continuously develop the data and programs and offer new interfaces based on user feedback. As a result, there are numerous web-based software programs in the field of bioinformatics that have become an important tool for researchers to analyze genetic data, interpret the results, and publish their work (Ryan et al., 2008 and You et al., 2008).

Considering all these factors mentioned above, the aim of this study was to identify the system requirements of web-based bioinformatics programs for the design of PCR primers that are commonly used today. Another aim was to provide guidance to users by discussing the advantages and disadvantages of the web-based PCR programs based on the results of the bioinformatics comparisons.

Materials and Methods

Program running conditions

Two different customization methods were applied to the operation of the systems. Since the settings of the programs are unique for each system, we first used the internal settings of the program, i.e. the default settings, and then loaded the parameters (modified) we had previously defined into the program to optimize it for the system.

Primer length, the GC content and Melting temperature (Tm)

In this study, the primer length was chosen in the range of 15-20-25 nt to achieve optimal results for primer design programs and to obtain successful results in PCR. In addition, the ideal G/C ratio for a given primer design in the range of "20-35-50%" and the Tm temperature in the

range of "52-55-58 °C" were selected as appropriate for the G/C content according to Wallace's rule and the programs were run with these settings.

PCR Yield

The ideal working range for sequences in primer design programs is usually between 300 and 500 bp, but since the goal is to systematically challenge the programs, a 150-200 bp long amino acid coding (CDS) locus sequence of a preselected gene to be used in the study is loaded into all programs to be used in the study.

Gene (Leptin)

In view of the required parameters, the leptin gene was considered suitable for the primer design in the study carried out with reference to the NCBI "National Center for Biotechnology Information," both with regard to primer quantitative properties and because it contains the corresponding locus range.

The leptin gene is a potential protein and candidate gene that is secreted from the adipose region of the skin and plays a role in the growth and metabolism of cattle, affecting immune system functionality and muscle fattening, carcass characteristics, meat quality, milk quantity, and composition. Leptin is a gene with a total length of 16,751 base pairs, 2 exons, and 3 introns and the NCBI reference code: NC_037331.1. The selected locus region was chosen from the first exon region, which is 12,104-12,247, with a length of 143 base pairs (bp), and the analyzes were performed according to the settings contained in these parameters (Fitzsimmons et al., 1998; Leifers et al., 2005; Wylie, 2011).

Sequence analysis

The sequences loaded into the system for the primer design were then executed with the default settings of the programs. The data obtained were saved to limit the range of settings of the system, to force the programs, and to compare the results obtained with variable parameters. To systematically test the primer design programs, the previously determined system settings were adjusted to the programs, and the primer design program was run again, comparing the variable parameters obtained with the recorded data. Finally, using the comparative data obtained from the analysis results in both ways, web programs that are systematically suitable for primer design were determined.

Results

Parameters given above were operated by narrowing the range of settings and adjusting the subjective settings to the systems so that the systems could make more original designs. The default settings of the programs vary from system to system, and these parameters are available in the address links of the programs given in the table, and the subjective settings were loaded the gene sequences along with modifying parameters equally into each program.

Moreover, each program uses the web working order, and there are different and more than one programming language in the programs used. For this reason, it is thought that the working range of the systems is affected depending on the density of these programming languages in the results obtained. It was found that 5 of the programs that execute functions

work with JavaScript and 2 of them work with the programming languages PHP (Hypertext Preprocessor) and ASP (Active Server Pages Developer) (Table 1).

Based on the program running parameters (G/C ratio: 20-35-50; primer melting temperature (Tm): 52-55-58 °C; primer length: 15-20-25 nt), only 7 of the 39 previously selected web-based PCR primer design programs were able to obtain primers in different time units using the parameters determined in this study (primer length, GC content and melting temperature (Tm) and PCR yields).

Table 1 shows the results corresponding to the leptin gene sequences entered into the web-based PCR primer design programs.

Table 1. Results from liner design programs
Tablo 1. Primer tasarım programlarından elde edilen sonuçlar

Web-based PCR Primer Designing Programs	Program Language	Primer Tm (°C)	GC Content (%)	Product Size (bp)	Time (min-sec)
NCBI (Anonymous, 2024a)	php,asp	59.40/59.48	55.00/43.48	95	4m30s
	javascript	55.76/55.62	47.62/45.00	71	3m04s
Primer3 (Anonymous, 2024b)	php, java	58.59/59.78	55.00/45.45	144	1s
		55.53/54.83	45.00/45.00	118	1s
Bisearch (Anonymous, 2024c)	javascript	60.30/60.80	50.00/47.60	69	1.1s
		53.2/53.4	43.8/41.2	84	1.2s
Genscript (Anonymous, 2024ç)	javascript	59.59/59.21	-	100	1.5s
		55.17/55.02	-	119	1.6s
Primer3 Plus (Anonymous, 2024d)	javascript	59.90/59.60	63.20/45.50	101	0.6s
		55.50/55.00	45.00/45.00	109	0.7s
Stitcher 2.0 (Anonymous, 2024e)	Javascript,	55.27/57.86	42.11/45.00	-	1.92s
		56.91/57.51	42.11/42.11	-	0.90s
PrimerQest Tool (Anonymous, 2024f)	php, asp.net	60.00/60.00	47.60/42.90	108	1.91s
		55.00/55.00	44.40/44.40	101	3.60s

The data shown in red include the results of designs made with subjective settings, while the data shown in blue show the results of designs made with default settings belonging to the systems (minute (m), second (s)).

Although Primer3 Plus (0.6s / 0.7s) and Primer3 (1s/ 1s) had the fastest response time for primer design for both default and modified parameters, NCBI (4m30s / 3.04s) was the most time-consuming program (Table 1). It was also found that 32 of the 39 programs did not work or were not functional. Table 2 shows the other thirty-two web-based PCR primer design programs that did not executed and the explanations for their failure.

Discussion

In this study, of 7 web-based PCR primer design programs, Primer3 Plus (0.6s / 0.7s) and Primer3 (1s / 1s) achieved the fastest response time, while NCBI (4m30s / 3.04s) was the most time-consuming program. for the other programs, the order of speed was found to be Bisearch (1.1s / 1.2s), Genscript (1.5s / 1.6s), Stitcher 2.0 (1.92s / 0.9s) and PrimerQuest Tool (1.91s / 3.6s) (Table 1.).

Primer3 and Primer3Plus are programs with simple interfaces and very fast primer design. In addition, it is one of the fastest programs in terms of time (min) and at the same time

shows very stable and design-compliant data for the precursor parameters. At the same time, the Primer3Plus program allows the user to instruct the NCBI program to perform BLAST (Basic Local Alignment Search Tool) at the end of the primer design. Besides, The NCBI program enables the design of primers and also provides detailed information on genes at three levels (GenBank, Fasta, Graphics). In addition, it has a very large data set and enables the comparison of nucleic acid and protein-based sequences with BLAST. At the end of the study, the Tm temperature and G/C ratio in the precursor parameters were found to be ideal and suitable for PCR. The reason why the time (min) required is higher than other programs is probably due to the intensive use of the software languages used, the inclusion of more than one application and the wide distribution.

Although the Biserach program offers a simple interface and ease of use, it has been found to contain less data in terms of parametrics than other programs. In addition, there are differences between the primers and the precursor parameters at the end of the design. In addition, the programs Genscript and Stitcher 2.0 have a new interface and deliver fast results. However, they provide missing precursor parameters at the end of the design. In Genscript this is the G/C content. This parameter is a very important precursor for the PCR, so its absence can negatively influence the success of the PCR. In the Stitcher 2.0 program, the product size is not indicated at the end of the design. This gives us incomplete information about the quantity of the product obtained, which seems to have a negative impact on the chances of success. The PrimerQest Tool program has a new and improved interface. It is also a program that can provide quite adequate and ideal results in terms of preliminary parameters and can be quite fast in terms of time (min).

Furthermore, for the primer design, 39 primer design programs were selected, but it was found that 32 of the programs did not work during the design. Anonymous (2024ö) and Abd-Elsalam (2003) provided the web addresses of the 32 programs in question, and commented on whether they functioned optimally. However, in this study, after 21 years, 32 programs were found to be either not currently working or not being operated. For this reason, the reasons for the non-functioning of the programs and/or their shortcomings are listed in Table 2.

Conclusion

Genetics, which has made incredibly rapid progress over the last century, has generated a huge amount of data. For this reason, bioinformatics occupies a very important place in the processing, storage, interpretation, and presentation of genetic data to the users.

For primer designs to give effective results in PCR, the necessary criteria must always be considered. These variable criteria play an important role in obtaining unique primers for primer design and PCR to achieve effective results in bioinformatics analysis programs. To achieve an optimal primer design, 39 web-based PCR software programs from the Internet were used in this study to determine the strategies and accuracy of the programs. Based on the results, it can be said that the NCBI program, which initially provides the user with all current information on the genes and displays the homology rates at the end of the design, requires more time than other programs (min). However, the NCBI program is one of the programs suitable for primer design because it can perform BLAST and provides the user with up-to-date information about the candidate gene. Moreover, The Primer3Plus and PrimerQuest Tool

programs are suitable programs for primer design and are recommended to other users as they provide fast design results, have a simple and ergonomic user interface and also provide ideal parametric data for PCR success, more conveniently than other programs. In addition, 32 programs seem to work on the Internet, but it was found in this study that they do not work for various reasons. The reasons are explained in detail in Table 2. Thus, with the results obtained, this study also provides the current information on whether these programs work or not.

Acknowledgments

We would like to thank Dr. Hasan ÇELİKYÜREK of Van-Yuzuncu Yil University for giving us his valuable time for this study and sharing his interest and expertise with us.

Ethical approval

No ethical documentation was required in this study. As this study is a bioinformatic research, no living being (human, animal, etc.) was used as material.

Table 2. Non-operating liner design programs and their causes

Tablo 2. Çalışmayan primer tasarım programları ve nedenleri

Primer Programmes and web addresses	**Explanation**
MFEprimer	The site appears to be active. However, it takes 10-12 hours for the design to be finalized (Anonymous, 2024g).
dnaMATE	The site appears to be active, but the interface is not sufficient and up-to-date. Therefore, it has a complex structure for the user (Anonymous, 2024ğ)
TaxMan	The site is known as a free Primer design program. However, it does not design, it only gives BLAST results of the designed primers (Anonymous, 2024h).
Mongo Oligo Mass Calculator V2.06	The site appears to be free and active. However, it is an out of use and invalid page. The server is not visible (Anonymous, 2024ö).
Primer Desingner 4	The system appears to be active. However, it is not working and is an invalid page. The server is not visible (Abd-Elsalam, 2003).
Primaclade	The system appears to be active. However, it is out of use and not working (Anonymous, 2024ö).
AMUSER	The system is active. However, it does not meet the criteria required for liner design (Anonymous, 2024ı).
The PCR Suite	The system appears active but does not design a liner and gives a system error (Anonymous, 2024ö).
Overlapping Primers	The system appears active but does not design a liner and gives a system error (Anonymous, 2024ö).
Genomic Primers	The system appears active but does not design a liner and gives a system error (Anonymous, 2024ö).
SNP Primers	The system appears active but does not design a liner and gives a system error (Anonymous, 2024ö).
cDNA Primers	The system appears active but does not design a liner and gives a system error (Anonymous, 2024ö).
Protein to DNA reverse translation	The site is active but does not meet the necessary setting criteria for the liner design. Also, the interface is inadequate (Anonymous, 2024i).
Overlapping Primersets	The system appears active but does not design a liner and gives a system error (Anonymous, 2024ö).
Primerize	The system is active and working. However, it does not have the necessary setting parameters for the liner design. For this reason, the specificity of the design may be insufficient (Anonymous, 2024j).
OligoWalk	The system appears to be active. However, it is an invalid page and is out of use (Anonymous, 2024ö).
VIRsiRNApred	The system is actively working. However, it does not contain the necessary setting parameters for the liner design. For this reason, the specificity of the design may be insufficient (Anonymous, 2024k).
pssRNAit	The system is actively working. However, it does not contain the necessary setting parameters for the liner design. For this reason, the specificity of the design may be insufficient (Anonymous, 2024l).
PCR Primer Desing	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
siDRM	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Anonymous, 2024ö).
PCR Primer Design Tool	The site appears to be active and includes sufficient design criteria. However, the design takes 10-12 hours to complete (Anonymous, 2024m).
Web Primer	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
Primerx	The system is actively working. However, it does not contain the necessary setting parameters for liner design (Anonymous, 2024n).
PCR Desinger	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
DoPrimer	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
Primer Selection	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
The primer Genetor	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
OligoEvaluator	The site is active but does not contain the necessary setting criteria for the liner design. Therefore, it may negatively affect the specificity of the design (Anonymous, 2024o).
Array Desinger 2	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
GenomPRİDE 1.0	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
Primer Premier	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).
PrimerDesing	The system appears to be active. However, it is an out of use and invalid page. The server is not visible (Abd-Elsalam, 2003).

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