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Providing a framework for reviewing PCR primer design guidelines

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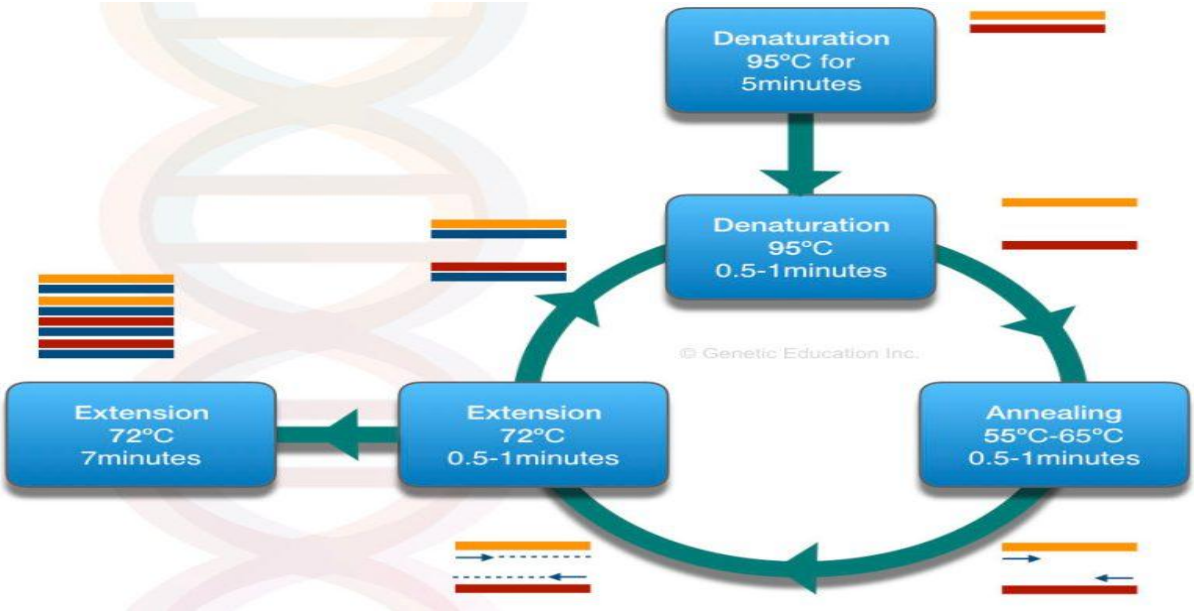
Abstract
Polymerase Chain Reaction is widely held as one of the most important inventions of the 20th century in molecular biology. Small amounts of the genetic material can now be amplified to be able to a identify, manipulate DNA, detect infectious organisms, including the viruses that cause AIDS, hepatitis, tuberculosis, detect genetic variations, including mutations, in human genes and numerous other tasks. Designing oligonucleotides and making sure that you have the right parameters for your oligo is an important step in securing results, especially in PCR Primer Design. In order to achieve successful DNA amplification, it's important to start off with the right primer. An appropriate primer design is one of the most important factors for good PCR. A poorly designed primer can result in little or no target product, due to non-specific amplification or primer dimer formation leading to reaction failure, even when all the other parameters are properly optimized. There are number of criteria such as primer length, Tm, GC contents, 3'-end sequence, 5'-end sequence, primer-dimer formation, hairpin-loop structure formation that need to be established in the design of primers to have efficient primers for PCR reaction. This article provides general guidelines for PCR primer design. In this article, we are discussing the role of PCR primer and their properties along with the PCR primer design guidelines as well.

Keywords: Polymerase Chain Reaction (PCR), Primer, DNA, PCR reaction.

Introduction:

Polymerase chain reaction (PCR) is a common laboratory technique used to make many copies (millions or billions!) of a particular region of DNA. This DNA region can be anything the experimenter is interested in. For example, it might be a gene whose function a researcher wants to understand, or a genetic marker used by forensic scientists to match crime scene DNA with suspects. Typically, the goal of PCR is to make enough of the target DNA region that it can be analyzed or used in some other way [1]. For instance, DNA amplified by PCR may be sent for sequencing, visualized by gel electrophoresis, or cloned into a plasmid for further experiments. PCR is used in many areas of biology and medicine, including molecular biology research, medical diagnostics, and even some branches of ecology [2]. PCR primers are short single-stranded DNA sequences which help in the amplification of DNA during PCR reaction." PCR technique is one of the most anticipated technique is genetic science, as it facilitates replication of DNA in vitro, Each and every component of PCR reaction are equally important. PCR reaction completes in three steps (denaturation, annealing and extension). In denaturation, the double-stranded DNA becomes single-stranded (DNA denatured), in the annealing step, the primer binds with its complementary sequence and in elongation step, with the help of dNTPs and Taq DNA polymerase the growing DNA strand expands.

The figure below,



Applications of PCR primers:

PCR makes it possible to obtain, by in vitro replication, multiple copies of a DNA fragment from an extract. Matrix DNA can be genomic DNA as well as complementary DNA obtained by RT-PCR from a messenger RNA extract (poly-A RNA), or even mitochondrial DNA. It is a technique for obtaining large amounts of a specific DNA sequence from a DNA sample [3]. This amplification is based on the replication of a double-stranded DNA template. It is broken down into three phases: a denaturation phase, a hybridization phase with primers, and an

elongation phase. The products of each synthesis step serve as a template for the following steps, thus exponential amplification is achieved [4]. Generally, PCR primers are DNA primers. As we all know that in replication short RNA primers are involved instead of DNA primer while in PCR we are using DNA primer. The polymerase chain reaction is carried out in a reaction mixture which comprises the DNA extract (template DNA), Taq polymerase, the primers, and the four deoxyribonucleoside triphosphates (dNTPs) in excess in a buffer solution. The tubes containing the mixture reaction are subjected to repetitive temperature cycles several tens of times in the heating block of a thermal cycler (apparatus which has an enclosure where the sample tubes are deposited and in which the temperature can vary, very quickly and precisely, from 0 to 100°C by Peltier effect). The apparatus allows the programming of the duration and the succession of the cycles of temperature steps. Each cycle includes three periods of a few tens of seconds [5]. There are several assumptions that favour the use of DNA primer in PCR:

1. DNA primers are more temperature stable than RNA primers.
2. The process of DNA polymerization in PCR is unidirectional so there is no chance of removal of short RNA primer after the polymerization is completed.
3. Additionally, DNA polymerase I help in removing of short RNA primer in replication *in vivo* which is not present in PCR.

Melting temperature is a temperature at which the half on the DNA (template DNA) is broken opens. The melting temperature of DNA depends on the GC and AT content of DNA. We know why, right. The primer binds to the DNA has the same melting temperature as its template. Otherwise, if the temperature is not appropriate then it binds other than its target site.

The annealing temperature is a temperature which required to anneal or bind primer to its complementary strand. The annealing temperature varies from primer to primer.

PCR product detection and analysis:

Like other DNA polymerases, Taq polymerase can only make DNA if it's given a primer, a short sequence of nucleotides that provides a starting point for DNA synthesis. In a PCR reaction, the experimenter determines the region of DNA that will be copied, or amplified, by the primers she or he chooses [6]. PCR primers are short pieces of single-stranded DNA, usually around 20202020 nucleotides in length. Two primers are used in each PCR reaction,

and they are designed so that they flank the target region (region that should be copied). That is, they are given sequences that will make them bind to opposite strands of the template DNA, just at the edges of the region to be copied [7]. The product of a PCR consists of one or more DNA fragments (the sequence or sequences of interest). The detection and analysis of the products can be very quickly carried out by agarose gel electrophoresis (or acrylamide). The DNA is revealed by ethidium bromide staining [2, 3, 5]. Thus, the products are instantly visible by ultraviolet transillumination (280–320 nm). Very small products are often visible very close to the migration front in the form of more or less diffuse bands. They correspond to primer dimers and sometimes to the primers themselves. Depending on the reaction conditions, nonspecific DNA fragments may be amplified to a greater or lesser extent, forming net bands or "smear" [6, 7, 8, 9]. On automated systems, a fragment analyzer is now used. This apparatus uses the principle of capillary electrophoresis. Fragment detection is performed by a laser diode. This is only possible if the PCR is performed with primers coupled to fluorochromes.

The steps of PCR:

The key ingredients of a PCR reaction are Taq polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube, along with cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized [10]. The basic steps are [11-14]:

1. Denaturation (96°C96 °\text C96°C96, °, start text, C, end text): Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.
2. Annealing (55555555 - 65656565°C°\text C°C°, start text, C, end text): Cool the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.
3. Extension (72°C72 °\text C72°C72, °, start text, C, end text): Raise the reaction temperatures so Taq polymerase extends the primers, synthesizing new strands of DNA.

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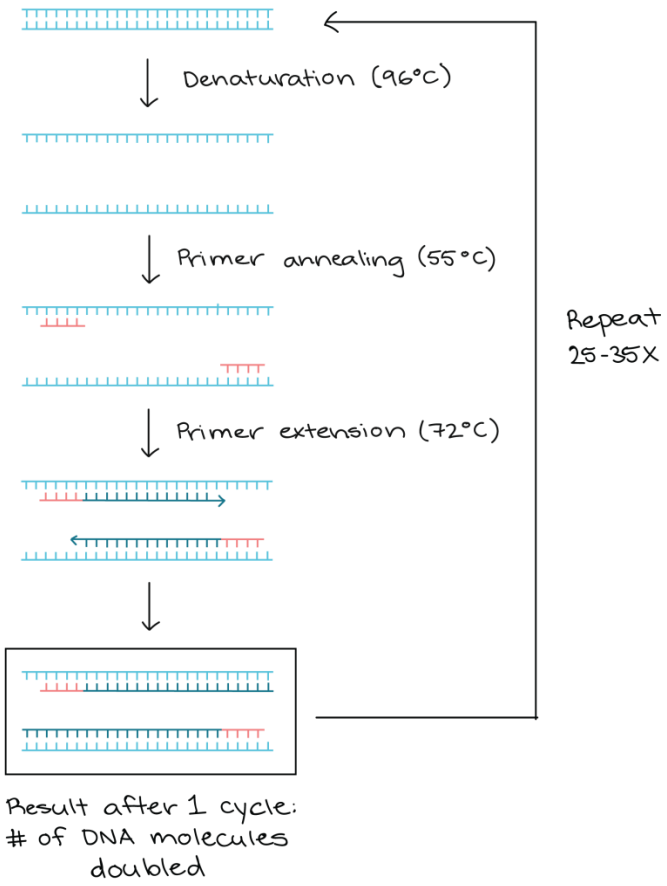
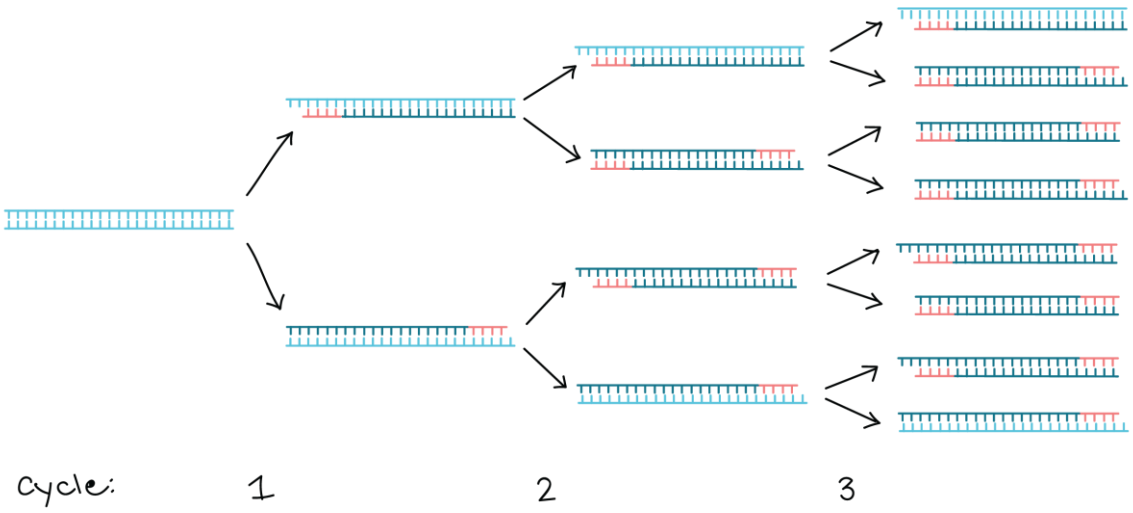


This cycle repeats 25252525 - 35353535 times in a typical PCR reaction, which generally takes 2222 - 4444 hours, depending on the length of the DNA region being copied. If the reaction is efficient (works well), the target region can go from just one or a few copies to billions. That's because it's not just the original DNA that's used as a template each time. Instead, the new DNA that's made in one round can serve as a template in the next round of DNA synthesis. There are many copies of the primers and many molecules of Taq polymerase floating around in the reaction, so the number of DNA molecules can roughly double in each round of cycling. This pattern of exponential growth is shown in the image below.



Annealing temperature:

A temperature at which primer can bind to its complementary sequence is called an annealing temperature. The annealing temperature is a very important parameter in designing a DNA primer. Annealing temperature should be 5ºC lower than the melting temperature. Melting temperature of the primer is calculated using the formula below,

$$\mathbf{Tm= 4\ (G + C) + 2\ (A + T)}$$

An ideal annealing temperature of the primer is ranging between 56ºC to 65ºC. Variation in this range hinders PCR amplification. If the annealing temperature is too low, the primer can bind to any of the complementary sequences and gives a non-specific result. The primer cannot bind if the annealing temperature is too high.

Length of the primers:

Primers are short sequences, generally, 18 to 23 nucleotide long primer always give the best result in PCR. Shorter primers (>18bp) do not have the affinity to amplify properly in each cycle. If the primer is too short, annealing temperature becomes lower and it reduces amplification capacity. Long primers are also not recommended because the annealing temperature of the long primer is too high, it leads to non-specific binding. However, a long primer is highly specific in long-range PCR.

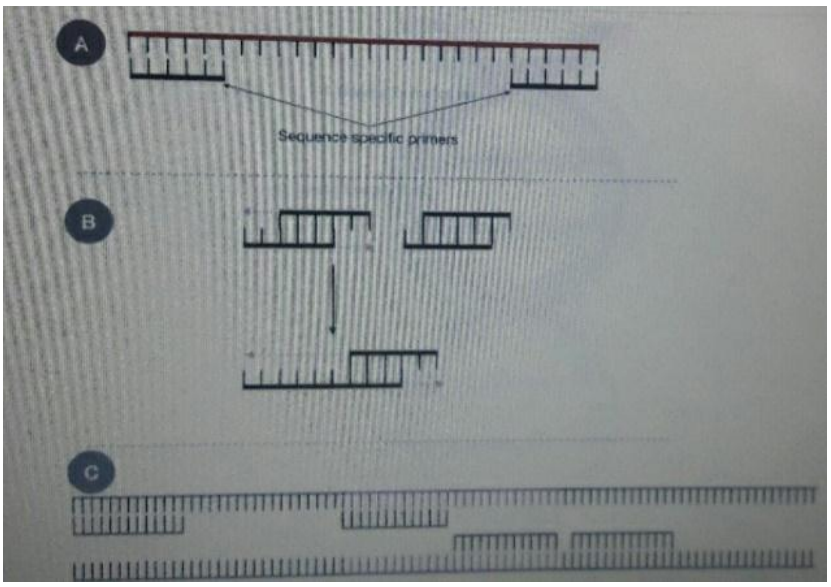
GC content of primers:

Another important factor in designing the primer is GC content. GC content between 40% to 60% is acceptable. The annealing temperature of the primer between 55ºC to 65ºC with 50% GC perform better. If GC content is too high, at given annealing temperature primer will mismatch with other sequences. Additionally, GC reach sequences are highly non-specific. The chance of non-specific amplification in GC rich region is very high as compared to the AT-rich region. It is critical to understand that if 8 to 10 bases of primer will match with

other sequences and the annealing temperature is too low, it definitely amplifies the DNA but gives the false result.

Complementation in forward and reverse primers:

While designing primer, keep in mind that both forward and reverse primer do not match with each other or are not complementary with each other. Otherwise, instead of binding with target sequences, both primer will bind with each other and creates a dimer.



More than 4 complementary bases and lower annealing temperature induces dimer formation. When primers are bind with each other instead of binding with the target sequence, it creates a dimer. Dimers can easily be amplified in PCR because it is shorter sequences of up to 50 to 60 bp.

Repeat bases in primers:

Repeated bases can bind within the primer and makes primer non-active. If some of the dinucleotide or dinucleotide is repeated in primer, it binds within the primer and creates a hairpin loop. More specifically, if repeat bases are present on the terminal end of 3' end it will create a serious problem in PCR.

How to design PCR primers?

During my research works my topics are majorly PCR centred. I want to share my experience on how we can successfully design a primer. Firstly, identify your template sequence. It is very important to identify which gene or DNA fragment we want to amplify. Identify that sequence and obtain it from NCBI. Now go to the primer 3 software which is open access and freely available primer designing tool and it is widely accepted. However, each primer designing companies have their own primer design software. You can go to primer 3 from here: http://bioinfo.ut.ee/primer3-0.4.0/ Actually, I think you should try it side by side in another tab. I will Give you one sequence

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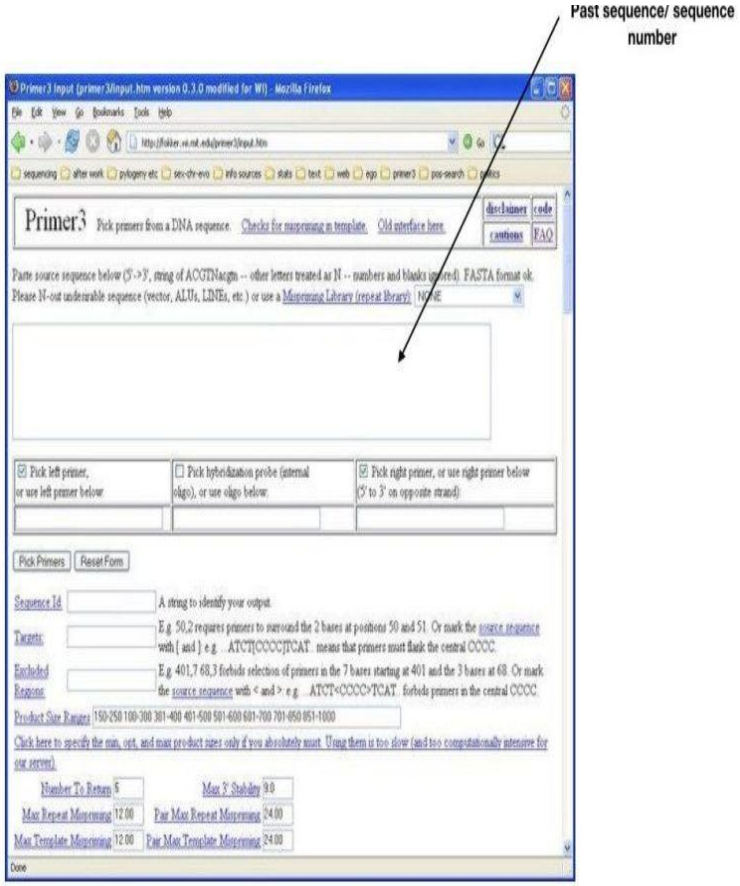
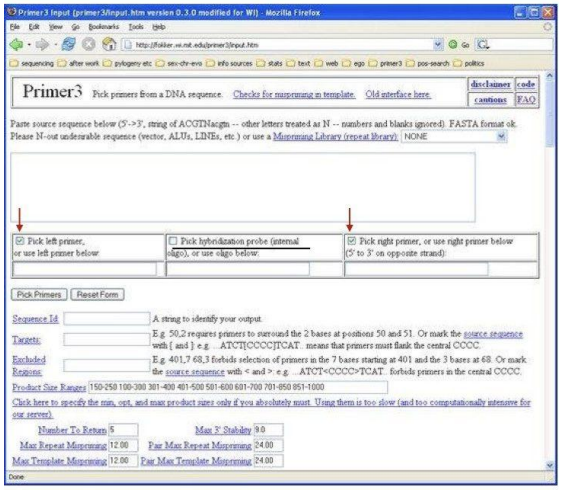


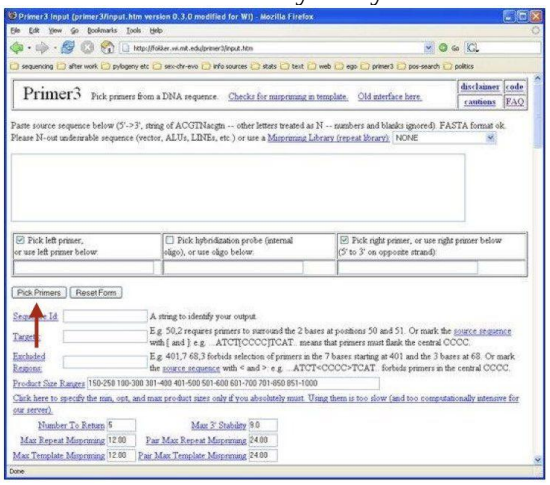
This is a beta-globin gene sequence (copy and paste it in a box of primer 3)

ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGT
GCATCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAAC
GTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGAC
AGGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGG
GTTTCTGATAGGCACTGACTCTCTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCT
GCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCA
CTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCT
CGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCC
ACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGG
TGAGTCTATGGGACGCTTGATGTTTTCTTTCCCCTTCTTTTCTATGGTTAAGTTCAT
GTCATAGGAAGGGGATAAGTAACAGGGTACAGTTTAGAATGGGAAACAGACGAA
TGATTGCATCAGTGTGGAAGTCTCAGGATCGTTTTAGTTTCTTTTATTTGCTGTTC
ATAACAATTGTTTTCTTTTGTTTAATTCTTGCTTTCTTTTTTTTTCTTCTCCGCAATT
TTTACTATTATACTTAATGCCTTAACATTGTGTATAACAAAAGGAAATATCTCTGA
GATACATTAAGTAACTTAAAAAAAAACTTTACACAGTCTGCCTAGTACATTACTA
TTTGGAATATATGTGTGCTTATTTGCATATTCATAATCTCCCTACTTTATTTTCTTT
TATTTTTAATTGATACATAATCATTATACATATTTATGGGTTAAAGTGTAATGTTT
TAATATGTGTACACATATTGACCAAATCAGGGTAATTTTGCATTTGTAATTTTAAA
AAATGCTTTCTTCTTTTAATATACTTTTTTGTTTATCTTATTTCTAATACTTTCCCTA
ATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTCTTTGCACCATTC
TAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAATATCTCTGCATAT
AAATATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCTAATAGC
AGCTACAATCCAGCTACCATTCTGCTTTTATTTTATGGTTGGGATAAGGCTGGATT

ATTCTGAGTCCAAGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTC
CCACAGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAG
AATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAA
TGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAA
AGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCC
TTGAGCATCTGGATTCTGCCTAATAAAAAACATTTATTTTCATTGC



Now select the options for forward primer and reverse primer shown as red arrows. Never select the option given in the middle (labelled as black) because we want to run the simple PCR hence we do not need a probe. In the next step just for understanding read the information given on primer-3 page, read the specification but do not click on any of the boxes because all the information is automatically or by default set by the software.



In the next step as shown in the figure, click on the "Pick primer" button and wait for the result.

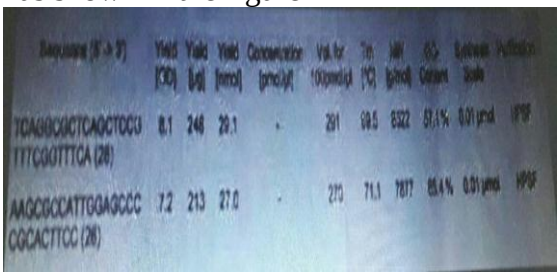
Primer3 Output

```
PRIMER PICKING RESULTS FOR

Template masking not selected
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len       tm      gc%  any_th  3'_th hairpin seq
LEFT PRIMER        183   20    58.93    50.00    0.00   0.00    0.00 AGAAACTGGGCATGTGGAGA
RIGHT PRIMER       413   20    59.01    50.00    8.22   0.00    0.00 TGAGCCAGGCCATCACTAAA
SEQUENCE SIZE: 1606
INCLUDED REGION SIZE: 1606

PRODUCT SIZE: 231, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

     1 ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCATC

    61 TGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAG

   121 TTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCA

   181 ATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCACTGACTCT
         >>>>>>>>>>>>>>>>>>>

   241 CTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGGTCTACCCTTGGACCCAG

   301 AGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAG

   361 GTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGAC
                                     <<<<<<<<<<<<<<<<<<<

   421 AACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGAT
```

The primer 3 output is shown in the figure (above). Analyze first the result window. You can see that the parameters like the length of the primer, GC content, annealing temperature and hairpin formation all are under the standard criteria. Now take a look at the red line. The primer gives you 231bp fragment so when you run the PCR based on the criteria of this primer, your product should be 231. The arrows (>>>>>> and <<<<<<<) shows the annealing site of primer to your sequence. Additionally, the software gives you other pairs of possible primers in the bottom as shown in the figure (Below)

```
KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                 start  len       tm      gc%  any_th  3'_th hairpin seq

 1 LEFT PRIMER        52   20    59.10    55.00    0.00   0.00    0.00 TGGTGCATCTGACTCCTGAG
   RIGHT PRIMER      202   20    58.93    50.00    0.00   0.00    0.00 TCTCCACATGCCCAGTTTCT
   PRODUCT SIZE: 151, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 8.77

 2 LEFT PRIMER       110   20    59.13    55.00    0.00   0.00    0.00 CGTGGATGAAGTTGGTGGTG
   RIGHT PRIMER      345   20    58.94    50.00    0.00   0.00    0.00 AACAGCATCAGGAGTGGACA
   PRODUCT SIZE: 236, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

 3 LEFT PRIMER       114   20    58.83    55.00    0.00   0.00    0.00 GATGAAGTTGGTGGTGAGGC
   RIGHT PRIMER      288   20    59.10    55.00    0.00   0.00    0.00 GTAGACCACCAGCAGCCTAA
   PRODUCT SIZE: 175, PAIR ANY_TH COMPL: 23.48, PAIR 3'_TH COMPL: 12.69

 4 LEFT PRIMER      1289   20    58.92    50.00    0.00   0.00    0.00 AGTCCAAGCTAGGCCCTTTT
   RIGHT PRIMER     1441   20    59.23    50.00    0.00   0.00    0.00 ACCAGCCACCACTTTCTGAT
   PRODUCT SIZE: 153, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

Statistics
        con   too    in    in   not          no    tm    tm   high  high  high        high
        sid  many   tar  excl    ok   bad    GC   too   too any_th 3'_th hair-  poly   end
       ered    Ns   get   reg   reg   GC% clamp   low  high  compl compl   pin     X  stab    ok
Left    8957     0     0     0     0  2354     0  3428  1142      0     1    72    23     0  1937
Right   8864     0     0     0     0  2442     0  3425  1096      0     0    19    49     0  1833
Pair Stats:
considered 8637, unacceptable product size 8630, primer in pair overlaps a primer in a better pair 370, ok 7
liborimer3 release 2.4.0
```

Your primer is ready for the order. In the next step find out the company which gives service in your area. Send them the detail or fill the online form of primer detail. While filling the detail, keep backcrossing your sequence. If you made a mistake in a single base, you will not get the PCR result or false result. You will receive the primers in precipitated form with one primer specification paper as shown in the figure



The primer specification report. The report is from our standard protocol and just for your understanding. The specification paper has all the information regarding the primer. It

contains the yield at 260nm OD, a sequence of primer, the yield of primer in microgram, the yield of primer in nano mol and other specification as shown in the figure (above). Our primer is in the form of the solid precipitate. We have to revive it for further use. Recall the PCR procedure, we need an approximately 10pmol primer for our PCR reaction.

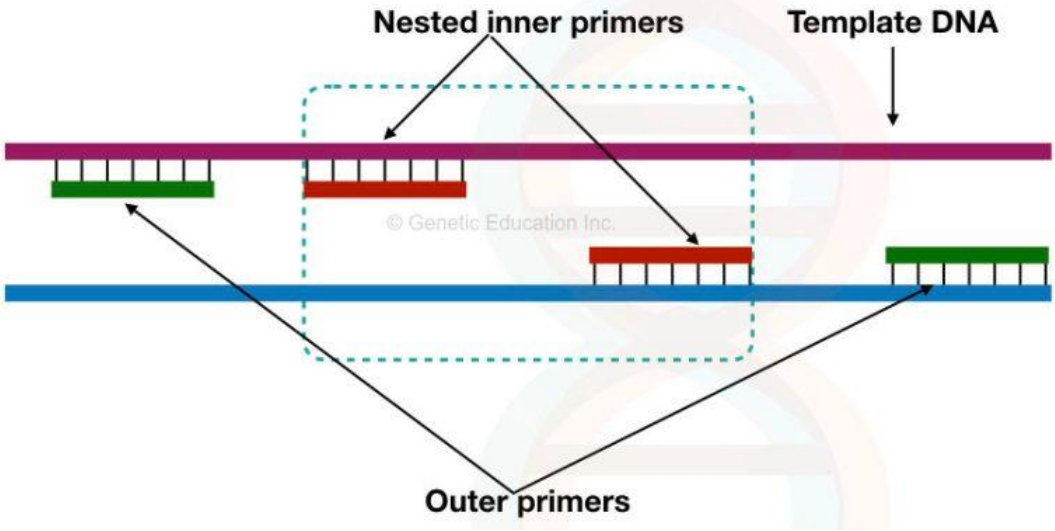
Suppose the given concentration of or primer is 29.1nM. When we add PCR grade water of 291µl to the primer tube, the final concentration of our tube become 100pM/µl. Do all the procedure in a sterile area now gently try to dissolve the primer in water. This concentration is our stock concentration of PCR primer. To achieve 10pmol final concentration for PCR reaction, take 1microliter from the stock primer and add 9µl of water (again PCR grade) to it. Now our primer with 10 pM/µl concentration is read. We can use 1µl from this working.

Degenerate primers:

For amplification and analysis of microbial samples, the degenerative primers are highly recommended. The degenerate primers have the same DNA sequences but are not exactly the same. By using this type of primers, the same gene in two different organisms can be amplified and variation among the species can be determined.

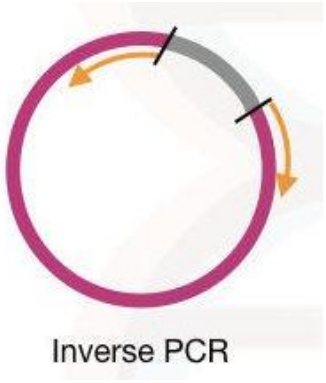
Nested primers:

Nested primers are a special type of primers used into the nested PCR reaction. Two sets of primers are used to amplify the gene in which one set of primer is nested. This nested set of primer binds to the amplified product on the first set of primer. The nested primer increases the chance of specific amplification by reducing the non-specific bindings.



Inverse primers:

Inverse primers are the primer having the 3′ end outside of the template DNA, therefore, it amplified DNA other than the target DNA. Inverse primers are used majorly into the site-directed mutagenesis and *in vitro* mutagenesis. Further, it is widely applicable in plasmid studies. See the figure below, how inverse primer amplify the DNA.



Conclusion:

The extension of genotyping approaches to all living organisms has made significant advances in the reconstruction of the history of life. At the population level, the distribution and frequency of known genetic polymorphisms in a species can highlight the evolving forces at play, reveal the effects of natural selection, and infer demographic change. Moreover, the comparison of the sequences of the same genes between different species and that of whole genomes is at the origin of the molecular phylogenies that currently prevail in the classification. They make it possible to trace the relationships between species on the basis of the divergence of their DNA sequences. As such, the PCR is a key stage at two levels. The first concerns the isolation of homologous genes in several species and their characterization. The second is the production of amplified total genomic DNA for genome sequencing and comparative analysis. But PCR is also used to identify the genetic heritage of missing organisms. The key to success in the PCR process is to design and build a suitable primer. It is important to know the basics when it comes to building a starter. Various parameters affect the quality of the primer, the most important of which are the length of the primer, melting temperature, GC rate, end 3 of the primer, end 5 of the primer, formation of the primer, ring formation and pin structures. After designing the starter sequence, with different software, these items can be manually set in the optimal mode.

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