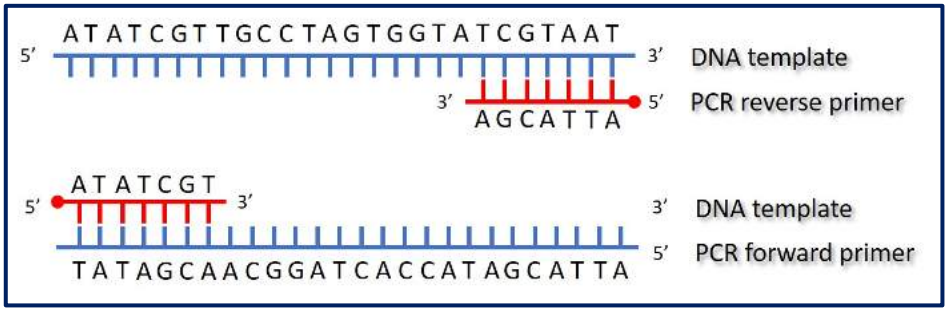
Designing PCR Primers using Primer3, UCSC in-Silico PCR and primer-BLAST

Primers are short sequences of single stranded DNA that mark both ends of the target sequence. Two primers are utilized, one for each of the complementary single strands of DNA released during denaturation. **The forward primer (LEFT)** attaches to the start codon of the template DNA (the anti-sense strand), while **the reverse primer (RIGHT)** attaches to the stop codon of the complementary strand of DNA (the sense strand). The 5' ends of both primers bind to the 3' end of each DNA strand.



Designing a primer and making sure that you have the right parameters for it is an important step in securing results. In order to achieve successful DNA amplification, it's important to start off with the right primer.

Here are some guidelines for designing your PCR primers:

⇒ **Aim for the GC content to be between 40 and 60% with the 3' of a primer ending in G or C to promote binding.** This is known as a GC Clamp.

- ✓ The G and C bases have stronger hydrogen bonding and ***help with the stability of the primer***.
- ✓ Be mindful not to have too many repeating G or C bases, as this can ***cause primer-dimer formation.***

⇒ **A good length for PCR primers is generally around 18-25 bases**. Specificity usually is dependent on length and annealing temperature. ***The shorter the primers are, the more efficiently they will bind or anneal to the target.***

- ✓ ***Short primers → can tend to find a similar sequence decreasing its specificity.***
- ✓ ***Long primers → can tend to form more hydrogen bonds making it difficult to denature and anneal during PCR cycles.***

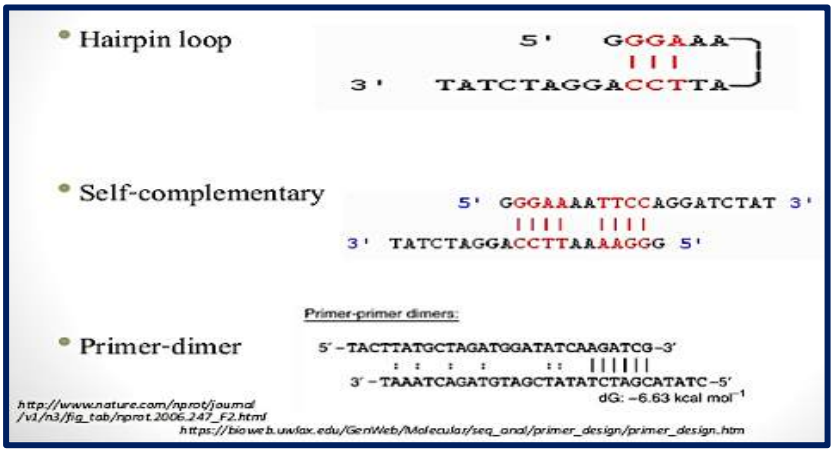
⇒ **Try to make the melting temperature (Tm) of the primers between 50°C and 65°C, and the maximum difference between the Tm of two primers should be with in 2- 5°C of each other**. Because the Tm is dependent on the length, it's important to keep primers on the shorter end. The bases also impact the Tm, G and C result in higher melting temperatures than A and T. If the Tm of your primer is very low, try to find a sequence with more GC content, or extend the length of the primer a little.

- ✓ ***Lower Tm → can tend to bind to any similar sequence decreasing its specificity.***
- ✓ ***Higher Tm → can tend to form loose bonds making it difficult to bind during PCR cycles.***

⇒ **Try to avoid regions of secondary structure and have a balanced distribution of GC-rich and AT-rich domains.**

- ✓ Avoid intra-primer homology (more than 3 bases that complement within the primer) or inter-primer homology (forward and reverse primers having complementary sequences).
- ✓ Avoid runs of 4 or more of one base, or dinucleotide repeats (for example, ACCCC or ATATATAT).

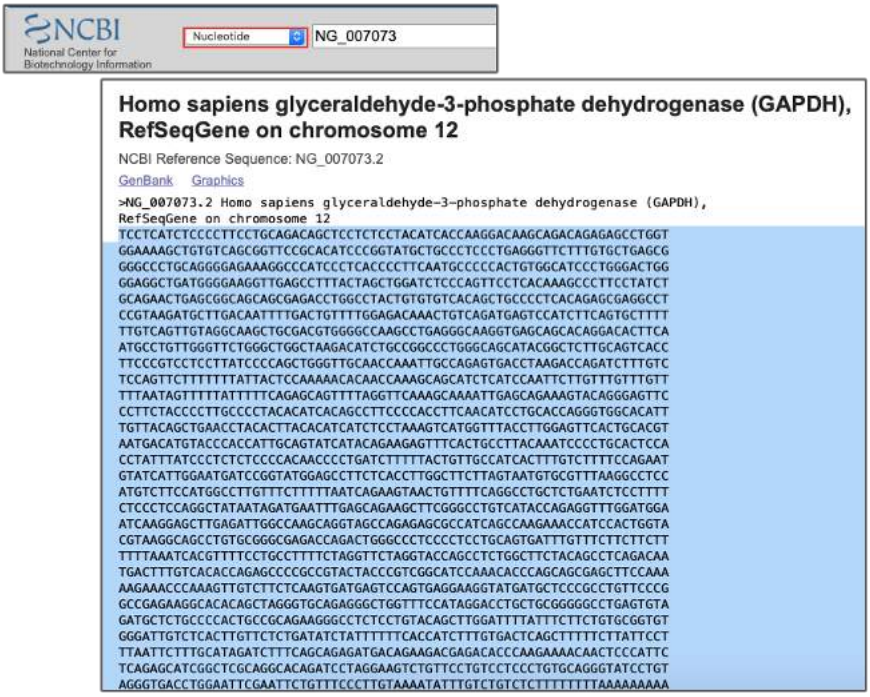
→ ***These circumstances can lead to self-dimers or primer-dimers instead of annealing to the desired DNA sequences.***



Designing primers using Primer3Plus:

In this lab exercise you are asked to design a set of PCR primers that specifically anneal to human **tumor protein p53 gene** (**accession number NG_017013.2**), To do so,

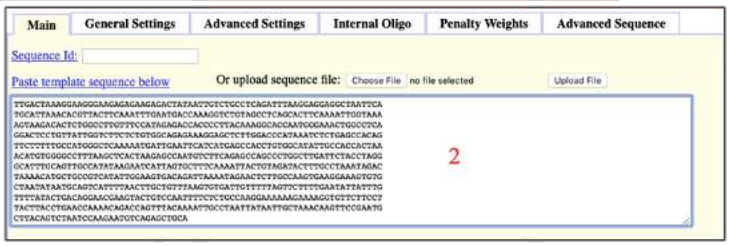
First, retrieve the DNA sequence of the human p53 gene and copy the full sequence from the GenBank record.

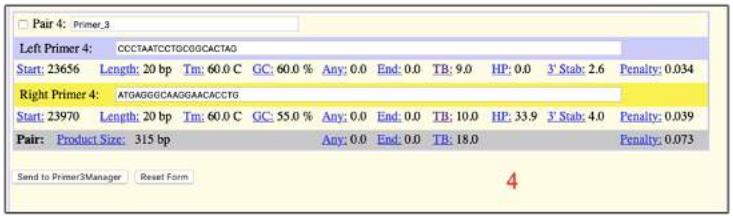


Then open **Primer3Plus (** **http://primer3plus.com/cgi-bin/dev/primer3plus.cgi)** and follow the steps below:

1. Paste the desired DNA sequence in the sequence box.
2. Set the range of the expected PCR product length, go to **Product Size Ranges** and type **300-500.**
3. Adjust the primer size, primer Tm and primer GC% ***according to the guidelines detailed in this manual.***
4. Then finally, click **Pick Primers.**

→ The results will appear to you as the program nominates different pairs of primers.





Interpretation of the result page

Primer seq.: LEFT and RIGHT primers are highlighted in purple and yellow colors, respectively.
Start: the position of the 5′ base of the primer. For left primers it is the position of the leftmost base and for right primers it is the rightmost base.
Length: length of the primer.
TM: melting temperature of the primer.
GC: the percent of G and C bases in the primer.
ANY: self-complementary score of the primer.
SELF: 3′ self-complementary of the primer.

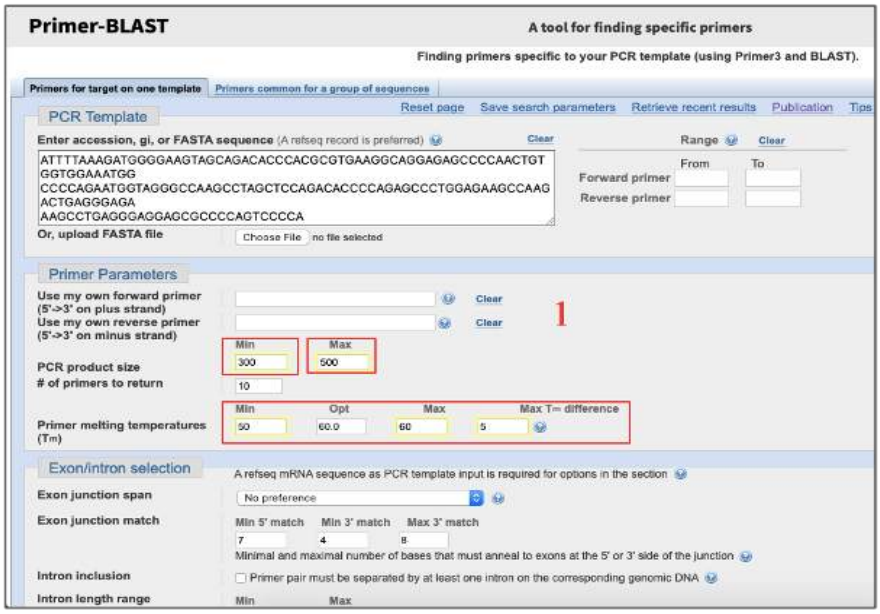
Should be zero or as low as possible

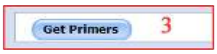
The best primer pair to choose is the one that matches the criteria you had once entered into your search.

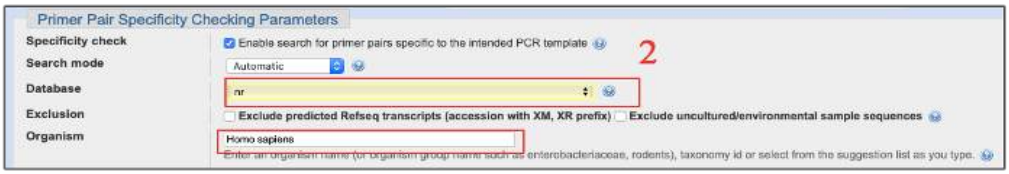
Primer-BLAST:

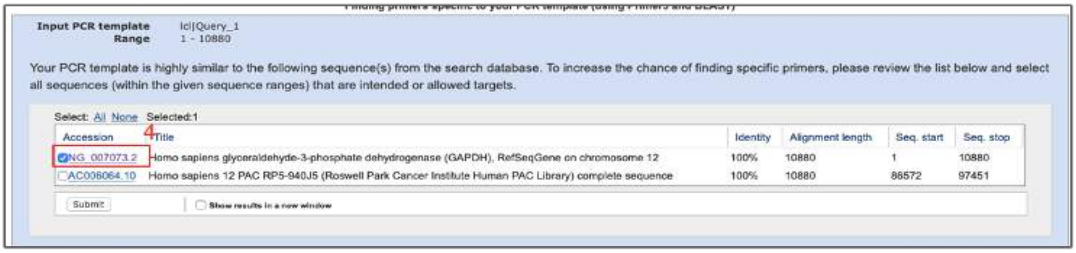
There are two primary function for primer-BLAST: Designing new primers and checking the specificity of the designed primers.

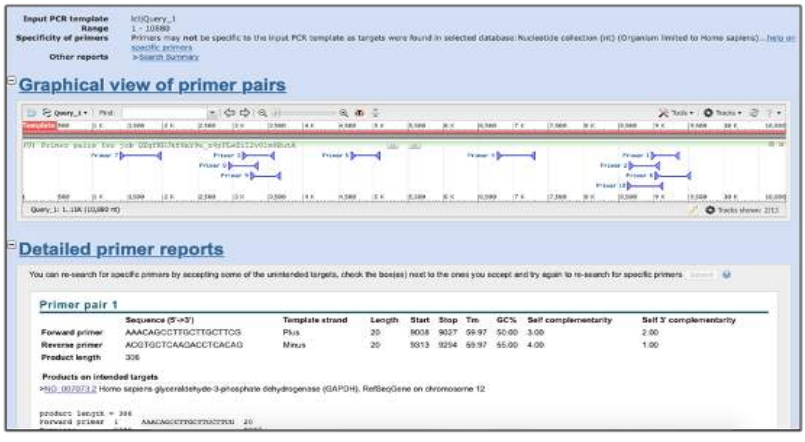
1) How to design primers using primer-BLAST





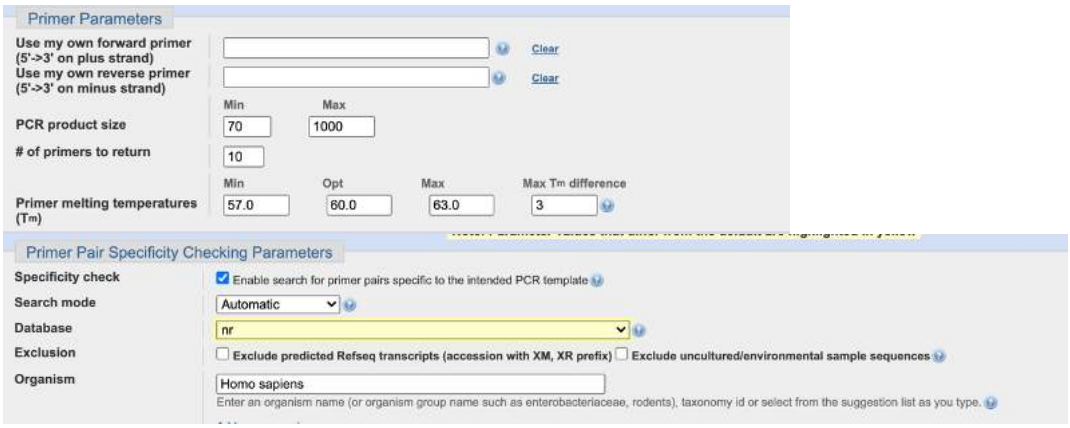




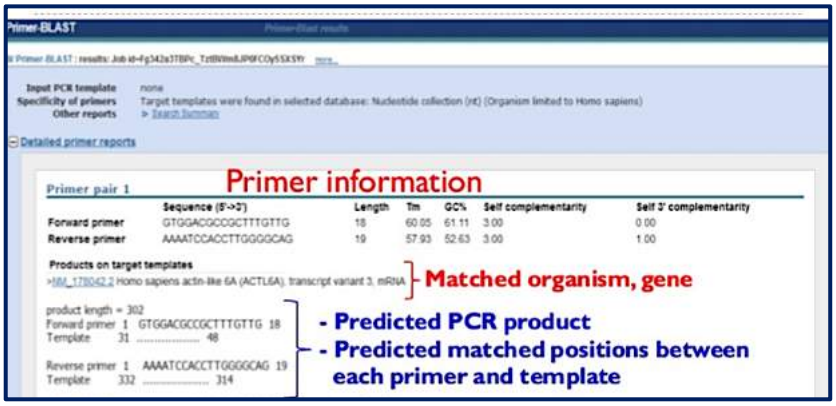


2) How to check primer specificity using primer-BLAST

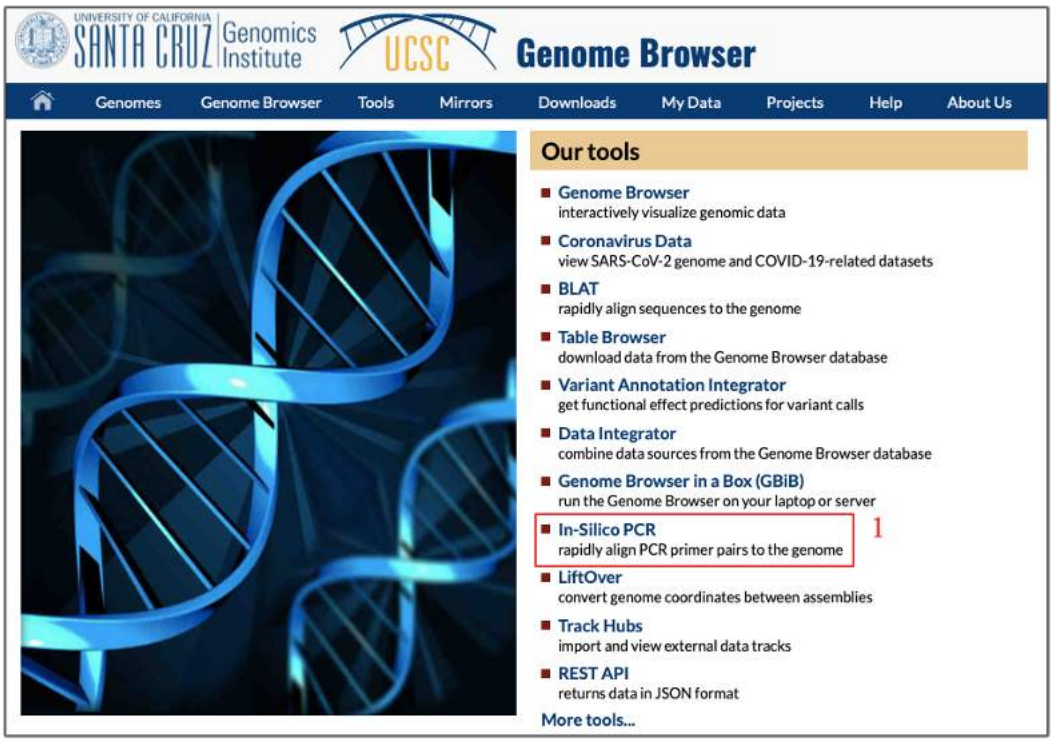
- Enter your designed primer sequences.
- Adjust the PCR product size and melting temperatures accordingly.
- Change the data base to (nr) → since your primers are DNA-specific
    - If it was mRNA specific then the chosen database would be (Refseq mRNA)
- Choose the organism of interest → Homo sapiens
- Finally, Get Primers

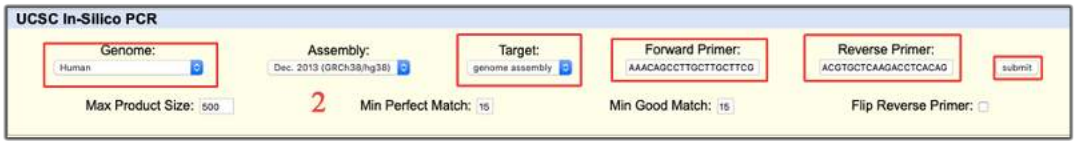


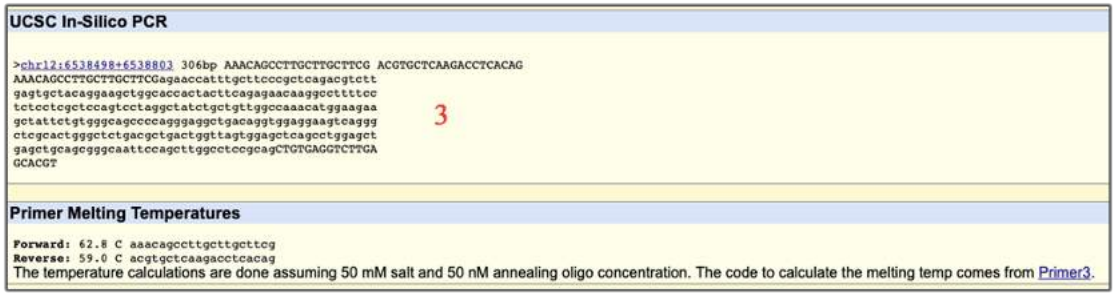
Interpretation of the result page



Checking primers specificity using UCSC in-Silico PCR







Note: The **reverse primer** is complementary to the the sense strand and pointing back toward the **forward primer** at the anti-sense strand. But if your reverse primer sequence is from the same strand, then ***Flip Reverse Primer checkbox***—must be checked this will reverse complement the sequence of your reverse primer.

→ Primers designed by primer3plus and primer-BLAST are already flipped, both forward and reverse primers are shown in 5' to 3' direction. Hence, no need to check the Flip Reverse Primer checkbox