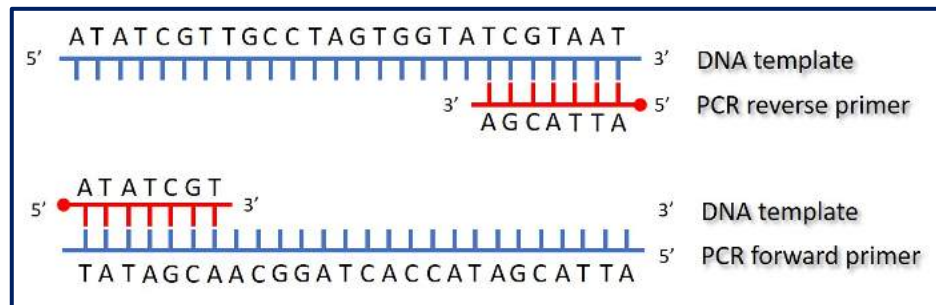


Designing PCR Primers using Primer3, UCSC in-Silico PCR and primer-BLAST

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 (T_m) of the primers between 50°C and 65°C, and the maximum difference between the T_m of two primers should be within 2- 5°C of each other.** Because the T_m is dependent on the length, it's important to keep primers on the shorter end. The bases also impact the T_m, G and C result in higher melting temperatures than A and T. If the T_m 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 T_m → can tend to bind to any similar sequence decreasing its specificity.*

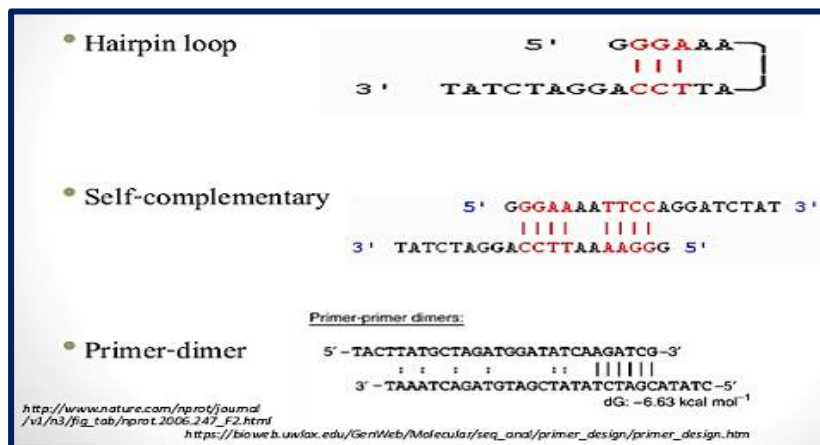
✓ *Higher T_m → can tend to form loose bonds making it difficult to bind during PCR cycles.*

⇒ **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.

NCBI National Center for Biotechnology Information

Nucleotide NG_007073

Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RefSeqGene on chromosome 12

NCBI Reference Sequence: NG_007073.2

GenBank Graphics

>NG_007073.2 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RefSeqGene on chromosome 12

```

TCCCTCATCTCCCTTCCCTGACAGCAGCTCCCTCTCTACATCACCAAGGACAAGCAGACAGGAGCCTGGT
GGAAAAGCTGTGTGACGCGTTCCGCACATCCGGTATGTCGCCCTCCCTGAGGGTCTTTGTGCTGAGCG
GGGCCCTGCAGGGGAGAAAAGGCCATCCCTCACCCCTCAATGCCCCACCTGGATCCCTGGGACTGG
GGAGGCTGATGGGGAGGTTGAGCCTTTACTAGCTGGATCTCCAGTTCCTCACAAAGCCCTTCTATCT
GCAGACTGAGCGGCAGCAGCGAGACCTGGCTACTGTGTGTGCACAGTGCCTCACAGAGCGAGGCTT
CCGTAAGATGCTTGACAATTTGACTGTTTGGAGACAAACTGTGAGTGGTCCATCTTCAAGTCTTCA
TTGTCAGTTGTAGGCAAGCTGCGACTGGGGCCAAGCCCTGAGGCAAGGTGAGCAGCACAGGACATTTCA
ATGCCGTGTGGGTTCTGGGCTGGTAAAGCATCTGGCGGCCCTGGGCAAGATAGCGGCTCTTGCAGTCAAC
TTCCCGCTCTCTTATCTCCAGCTGGGTTGCAACCAAAATGCGAGAGTGACCTAAGACAGATCTTTGTC
TCCAGTCTTTTTTATTACTCCAAAACACCAACCAAGCAGCATCTCATCAATCTTGTGTTGTTGTTGTT
TTTTAATAGTTTTTATTTTTCAGAGCAGTTTTAGGTTCAAAGCAAAATGAGCAGAAAAGTACAGGGAGTCT
CCTTCTACCCCTTGCCTACACATCACAGCCTTCCCACTTCAACATCTGTCACAGGGGTGGCAGCATT
TGTTAACAGCTGAACCTACACTTACACATCATCTCTAAAGTCATGGTTTACCTTGGAGTTCACTGCACGT
AATGACATGTACCCACCATGTCAGTATCATACAGAGAGTTTCACTGCTTACAAATCCCTGCACTCCA
CCTATTATCCCTCTCTCCCAACCCCTGATCTTTTACTGTTGCCATCACTTTGTCTTTCCAGAAT
GTATCATTGGAAATGATCGGTATGGAGCCTCTCACCTTGGCTCTTAAATGTTGCGTTTAAAGGCTCC
ATGCTTCCATGGCTTGTCTTTTAAATCAGAAGTAACTGTTTTCAAGGCTGCTCTGAATCTCCTTTT
CTCCCTCAGGCTATAAATAGATGAAATTTGAGCAGAAGCTTGGGCTGTGTCATACAGAGGTTTGGATGGA
ATCAAGGAGCTTGAGATTGGCCAAGCAGGTAGCCAGAGAGCGCCATCAGCAGAAAACCATCCACTGGTA
CGTAAGGCAGCCTGTGCGGGCAGACAGACTGGGCCCTCCCTCTGTCAGTGAATTTGTTCTCTCTT
TTTTAAATCACGTTTTCTGCTTTTCTAGGTTCTAGGTACCAGCCTCTGGCTTCTACAGCCTCAGACAA
TGAATTTGTACACACAGAGCCCGCCGTAACCCGTCGGCATCCAAACACCCAGCAGCAGGCTTCCAAA
AAGAAACCCAAAGTTGTCTTCAAGTGATGAGTCCAGTGAGGAGGATGATGCTCCCGCTGTTCCCG
GCCGAGAAAGGCACACAGCTAGGGTGACAGGGCTGGTTCCATAGGACTGTGCTGCGGGGGCTGAGTGA
GATGCTGCTGCCCACTGCGCAGAAAGGCTCTCTGTACAGCTTGGATTTTATTTCTCTGTGCGGTGT
GGGATGTCTCACTTGTCTCTGATATCTATTTTTTCACTCTTTGTGACTCAGCTTTTCTTATTCTCT
TTAATTTCTGATAGATCTTTCAGCAGAGATGACAGAAGCAGAGACCCCAAGAAAACCACTCCATTCT
TCAGAGCATCGGCTCGAGGCAGATCTAGGAAGTCTGTTCTGCTCTCCCTGTGCGAGGATCTCTGT
AGGGTGACCTGGAATTCGAATCTGTTCCCTGTGAAAATTTTGTCTGCTCTTTTTTAAAAA
    
```

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.

Primer3Plus
pick primers from a DNA sequence

[Download Primer3](#) [Source Code](#)

[Molbi](#) [Wily](#)

Run Primer3Plus 1

This is the latest version of Primer3Plus with all the new features.

[Run Primer3Web](#)

The latest version of Primer3Web.

Main General Settings Advanced Settings Internal Oligo Penalty Weights Advanced Sequence

Sequence Id:

Paste template sequence below Or upload sequence file: no file selected

```

TTGACTAAGGAGGAGAGAGAGAGACTATAATGCTCCCTCCGATTTAAGGAGGAGGCTAATTC
TGCATTAAGCCTTACTTCMAATTTGATGACCAAGGCTCTGAGCCTCAGCACTCAAAATTTGTA
ATGAGAGACCTCTGGCTTCTCCATGAGACACCCCTTAGAAGGCAACCAATGGGAATCTGCTGA
GGACTCTGTTATTTGGTCTCTCTGAGGAGAGAGGAGCTCTGGACCCATAAATCTGGAGCACAG
TTCTTTTGGCCATGGGCTCAAAAGATGATGATGATGATGATGATGATGATGATGATGATGATG
AGCTGTGGGCTCTTAAAGCTCATAGAGGCAATGCTCTGAGAGGAGGAGGAGGAGGAGGAGGAG
GCATTTTCAGTTCCTCAATAGAGATCATATGATGCTTCAAAATTAATGATGATGATGATGATG
TAAMACATGCTGGCTGATGATGAGAGATTAAGATGAGATCTTCCCAAGATGAGAGGAGGAG
CTAATAAAGGAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
TTTTATATCTAGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
TACTTTCCTAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
CTTACATCTTAACTGAGAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATG
    
```

2

Main General Settings Advanced Settings Internal Oligo Penalty Weights Advanced Sequence

Product Size Ranges:

Primer Size	Min: <input type="text" value="18"/>	Opt: <input type="text" value="20"/>	Max: <input type="text" value="26"/>	
Primer Tm	Min: <input type="text" value="50"/>	Opt: <input type="text" value="60"/>	Max: <input type="text" value="60"/>	Max Tm Difference: <input type="text" value="5"/>
Primer GC%	Min: <input type="text" value="40"/>	Opt: <input type="text" value="50.0"/>	Max: <input type="text" value="60"/>	

Concentration of monovalent cations: Annealing Oligo Concentration:

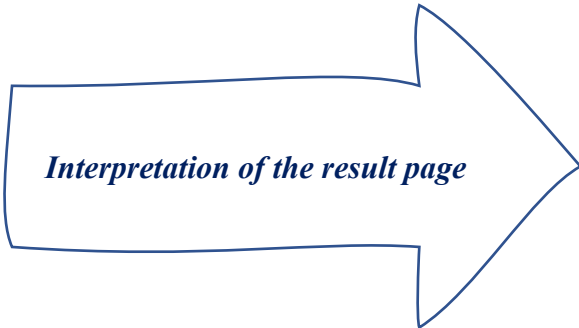
Concentration of divalent cations: Concentration of dNTPs:

3

Pair 4: Primer_3

Left Primer 4:	CCCTAATCCTCCGCACTAG
Start:	23656 Length: 20 bp Tm: 60.0 C GC: 60.0 % Any: 0.0 End: 0.0 TB: 9.0 HE: 0.0 3' Stab: 2.6 Penalty: 0.034
Right Primer 4:	ATGAGGGCAAGGAAACCTG
Start:	23970 Length: 20 bp Tm: 60.0 C GC: 55.0 % Any: 0.0 End: 0.0 TB: 10.0 HE: 33.9 3' Stab: 4.0 Penalty: 0.039
Pair:	Product Size: 315 bp Any: 0.0 End: 0.0 TB: 18.0 Penalty: 0.073

4



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

Primer-BLAST
A tool for finding specific primers
Finding primers specific to your PCR template (using Primer3 and BLAST).

Primer Parameters

Use my own forward primer (5'→3' on plus strand) Clear

Use my own reverse primer (5'→3' on minus strand) Clear

PCR product size
of primers to return: 10

Primer melting temperatures (T_m)
Min: 50, Opt: 60.0, Max: 60, Max T_m difference: 5

Exon/intron selection

Exon junction span: No preference

Exon junction match: Min 5' match: 7, Min 3' match: 4, Max 3' match: 8

Intron inclusion: Primer pair must be separated by at least one intron on the corresponding genomic DNA

Intron length range: Min: , Max:

Primer Pair Specificity Checking Parameters

Specificity check: Enable search for primer pairs specific to the intended PCR template

Search mode: Automatic

Database: nr

Exclusion: Exclude predicted Refseq transcripts (accession with XM, XR prefix) Exclude uncultured/environmental sample sequences

Organism: Homo sapiens

Results

Your PCR template is highly similar to the following sequence(s) from the search database. To increase the chance of finding specific primers, please review the list below and select all sequences (within the given sequence ranges) that are intended or allowed targets.

Accession	Title	Identity	Alignment length	Seq. start	Seq. stop
U007073.2	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RefSeqGene on chromosome 12	100%	10880	1	10880
AC008064.10	Homo sapiens 12 PAC RPS-640J5 (Roswell Park Cancer Institute Human PAC Library) complete sequence	100%	10880	86572	97451

Graphical view of primer pairs

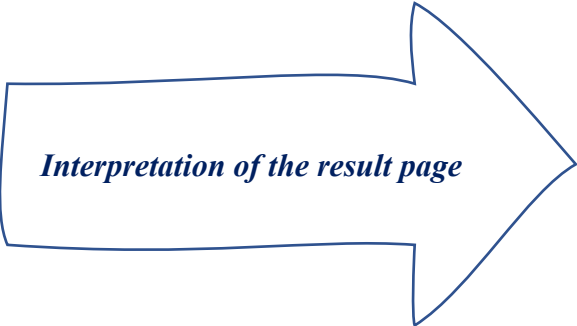
Detailed primer reports

Primer pair 1	Sequence (5'→3')	Template strand	Length	Start	Stop	T _m	GC%	Self complementarity	Self 3' complementarity
Forward primer	AAACAGCCTTGGCTTGCCTTOS	Plus	20	8038	8037	69.97	50.00	3.00	2.80
Reverse primer	ACGGTCTCAAGACCTGCACAG	Minus	20	8013	8294	69.97	55.00	4.00	1.90

Products on intended targets
U007073.2 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RefSeqGene on chromosome 12
Product length = 334
Forward primer 1 AAACAGCCTTGGCTTGCCTTOS 20

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**



Primer information

Primer pair 1	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GTGGACGGCGCTTTGTTG	18	60.05	61.11	3.00	0.00
Reverse primer	AAATCCACCTGGGGCAG	19	57.93	52.63	3.00	1.00

Products on target templates

>NM_173042.2 Homo sapiens actn-like 6A (ACTL6A); transcript variant 3, mRNA

product length = 302

Forward primer 1	GTGGACGGCGCTTTGTTG	18
Template	48
Reverse primer 1	AAATCCACCTGGGGCAG	19
Template	314

- Predicted PCR product
- Predicted matched positions between each primer and template

Checking primers specificity using UCSC in-Silico PCR

The screenshot shows the UCSC Genome Browser homepage. The navigation bar includes 'Genomes', 'Genome Browser', 'Tools', 'Mirrors', 'Downloads', 'My Data', 'Projects', 'Help', and 'About Us'. The 'Our tools' section lists various utilities: Genome Browser, Coronavirus Data, BLAT, Table Browser, Variant Annotation Integrator, Data Integrator, Genome Browser in a Box (GBiB), **In-Silico PCR** (circled in red with a red '1'), LiftOver, Track Hubs, and REST API. A 'More tools...' link is also present.

UCSC In-Silico PCR

Genome: Assembly: Target: Forward Primer: Reverse Primer:

Max Product Size: Min Perfect Match: Min Good Match: Flip Reverse Primer:

UCSC In-Silico PCR

```
>chr12:6538498-6538803 306bp AAACAGCCTTGCCTTGCCTCG ACGTCTCAAGACCTCACAG
AAACAGCCTTGCCTTGCCTCGagaaccatttgcttccogctcagagcttctt
gagtcctacaggaagctggcaccactctcagagaacaaggcttcttcc
tctctcctccagctcctagctatctgctgttggccaaacatggaagaa
getattctgtggcagccccaggagctgacaggtggaaggaagtcaggg
ctcgcactgggctctgacgctgactggttagtggagctcagcctggagct
gagctgcaagggcaattccagcttggcctccgcagCTGTGAGGCTCTGA
GCACGT
```

Primer Melting Temperatures

Forward: 62.8 C aaacagccttgccttgcctcg
Reverse: 59.0 C acgtgctcaagacctcacag
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temp comes from [Primer3](#).

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