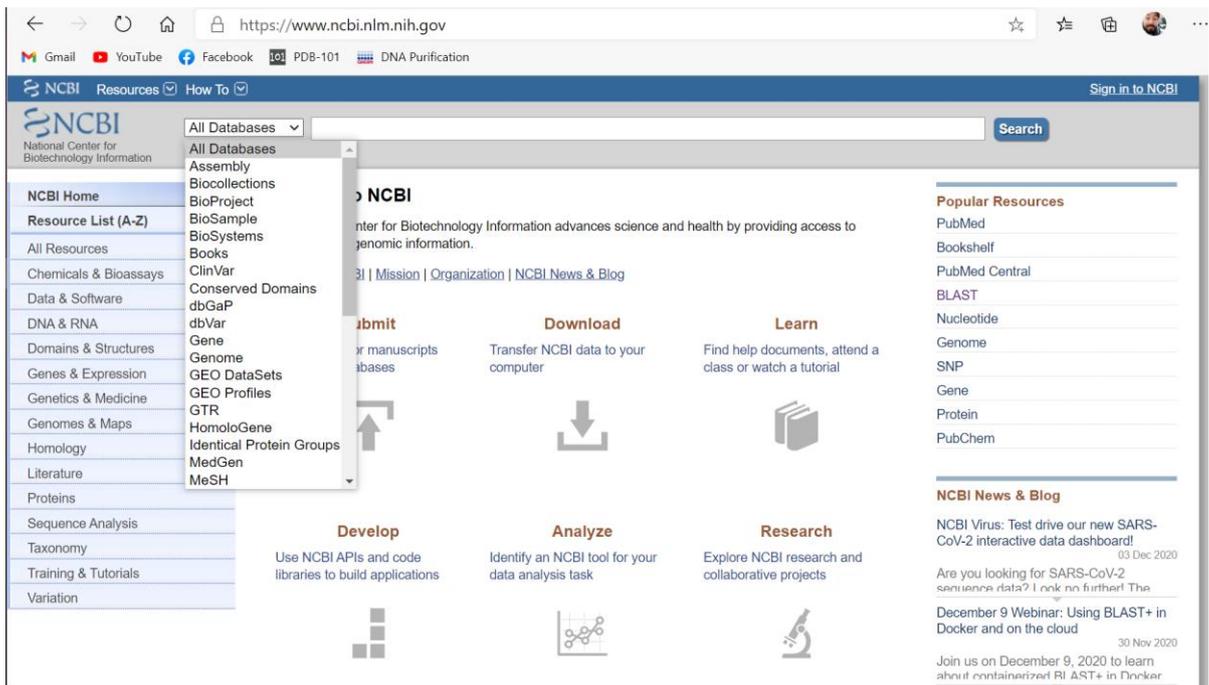


Primer Design

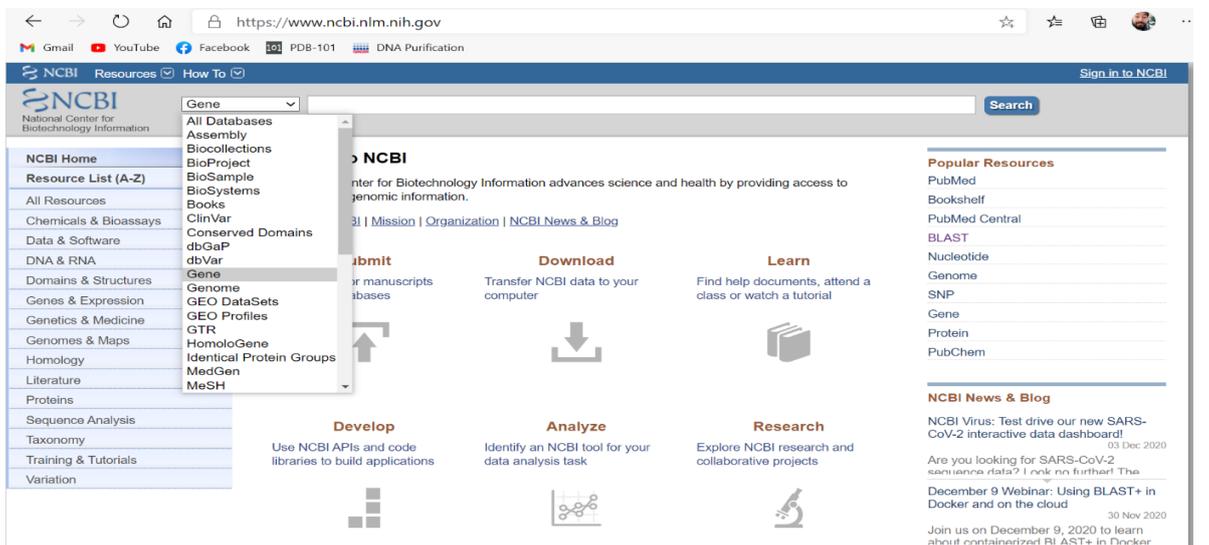
1. Go to NCBI (National Center for Biotechnology Information) website ([National Center for Biotechnology Information \(nih.gov\)](https://www.ncbi.nlm.nih.gov))
2. On the upper part of the page you will find:



3. Click on All Databases



4. Select 'Gene'



5. Type the name of your gene of interest in the query box (GFAP for example)

The screenshot shows the NCBI homepage with the search bar containing 'GFAP'. The navigation menu on the left includes categories like 'All Resources', 'Chemicals & Bioassays', 'Data & Software', 'DNA & RNA', 'Domains & Structures', 'Genes & Expression', 'Genetics & Medicine', 'Genomes & Maps', 'Homology', 'Literature', 'Proteins', 'Sequence Analysis', 'Taxonomy', 'Training & Tutorials', and 'Variation'. The central section is titled 'Welcome to NCBI' and provides information about the center's mission. It features six main action buttons: 'Submit' (Deposit data or manuscripts into NCBI databases), 'Download' (Transfer NCBI data to your computer), 'Learn' (Find help documents, attend a class or watch a tutorial), 'Develop' (Use NCBI APIs and code libraries to build applications), 'Analyze' (Identify an NCBI tool for your data analysis task), and 'Research' (Explore NCBI research and collaborative projects). On the right, there is a 'Popular Resources' list including PubMed, Bookshelf, PubMed Central, BLAST, Nucleotide, Genome, SNP, Gene, Protein, and PubChem. Below that, there is a 'NCBI News & Blog' section with recent news items.

6. Find your species of interest. Click ctrl+F and type in the name of the species (in our case this is *Macaca mulatta* (Rhesus monkey))

<input type="checkbox"/>	GFAP ID: 419969	glial fibrillary acidic protein [<i>Gallus gallus</i> (chicken)]	Chromosome 27, NC_006114.5 (3697628..3702163, complement)	
<input type="checkbox"/>	GFAP ID: 396562	glial fibrillary acidic protein [<i>Sus scrofa</i> (pig)]	Chromosome 12, NC_010454.4 (18456268..18466594)	
<input type="checkbox"/>	GFAP ID: 712941	glial fibrillary acidic protein [<i>Macaca mulatta</i> (Rhesus monkey)]	Chromosome 16, NC_041769.1 (56169389..56180984, complement)	
<input type="checkbox"/>	GFAP ID: 454741	glial fibrillary acidic protein [<i>Pan troglodytes</i> (chimpanzee)]	Chromosome 17, NC_036896.1 (10323057..10333051)	CK820_G0052909
<input type="checkbox"/>	GFAP ID: 100033970	glial fibrillary acidic protein [<i>Equus caballus</i> (horse)]	Chromosome 11, NC_009154.3 (18815101..18824260)	
<input type="checkbox"/>	GFAP ID: 101081938	glial fibrillary acidic protein [<i>Felis catus</i> (domestic cat)]	Chromosome E1, NC_018736.3 (44868468..44877976, complement)	
<input type="checkbox"/>	GFAP ID: 101017460	glial fibrillary acidic protein [<i>Papio anubis</i> (olive baboon)]	Chromosome 17, NC_044992.1 (51723079..51734689, complement)	
<input type="checkbox"/>	GFAP ID: 101339778	glial fibrillary acidic protein [<i>Tursiops truncatus</i> (common bottlenose dolphin)]	Chromosome 20, NC_047053.1 (41007824..41018040, complement)	
<input type="checkbox"/>	Gfap ID: 100758767	glial fibrillary acidic protein [<i>Cricetulus</i>]		I79_011607

7. Click on the gene name. In the following page you should find *mRNA and Protein(s) section*

Genomic

1. **NC_041769.1 Reference Mmul_10 Primary Assembly**

Range: 56169389..56180984 complement
Download: [GenBank](#), [FASTA](#), [Sequence Viewer \(Graphics\)](#)

mRNA and Protein(s)

1. **XM_015119892.2 → XP_014975378.1 glial fibrillary acidic protein isoform X2**

Related: [ENSMMUP00000049963.2](#), [ENSMMUT00000076046.2](#)

Conserved Domains (2) [summary](#)

pfam00038	Filament, Intermediate filament protein
Location:138 → 446	
pfam04732	Filament_head, Intermediate filament head (DNA binding) region
Location:73 → 136	

2. **XM_028836604.1 → XP_028692437.1 glial fibrillary acidic protein isoform X1**

Related: [ENSMMUP00000046183.2](#), [ENSMMUT00000077212.2](#)

Conserved Domains (2) [summary](#)

pfam04732	Filament_head, Intermediate filament head (DNA binding) region
Location:71 → 136	
pfam00038	Filament, Intermediate filament protein
Location:138 → 446	

Related sequences

Nucleotide	Protein
Heading	Annotation and History

8. Click **XM_015119892.2** which is accession number of the mRNA sequence
N.B. **XP_014975378.1** is the protein sequence ;
prefix XM_ (mRNA), XR_ (non-coding RNA), and XP_ (protein)
9. The following page will show you the all the information about the mRNA of the gene that you are searching.
10. Next click FASTA to obtain the full sequence of the mRNA of your gene of interest.

NCBI Resources How To Sign in to NCBI

Nucleotide Nucleotide Search Help

Advanced

GenBank Send to: Change region shown

PREDICTED: Macaca mulatta glial fibrillary acidic protein (GFAP), transcript variant X2, mRNA

NCBI Reference Sequence: XM_015119892.2

[FASTA](#) [Graphics](#)

[GenBank](#)

LOCUS XM_015119892 4016 bp mRNA linear PRI 26-APR-2019

DEFINITION PREDICTED: Macaca mulatta glial fibrillary acidic protein (GFAP), transcript variant X2, mRNA.

ACCESSION XM_015119892

VERSION XM_015119892.2

DBLINK BioProject: [PRJNA528504](#)

KEYWORDS RefSeq.

SOURCE Macaca mulatta (Rhesus monkey)

ORGANISM Macaca mulatta

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorhini; Catarrhini; Cercopithecoidea; Cercopithecoidea; Macaca.

COMMENT **MODEL RESEQ:** This record is predicted by automated computational analysis. This record is derived from a genomic sequence ([NC_041769.1](#)) annotated using gene prediction method: Gnomon, supported by mRNA and EST evidence.
Also see: [Documentation of NCBI's Annotation Process](#)

[Articles about the GFAP gene](#)
A new rhesus macaque assembly and annotation for next-generation sequencing [Biol Direct. 2014]
Age-related decreases in SYN levels associated with increases in MAP-2, apo [Age (Dordr). 2010]

[Pathways for the GFAP gene](#)

11. Copy the sequence and transfer it into a word file
12. Go to [Primer3 Input](#) website
13. Paste the sequence in the box

Primer3web version 4.1.0 - Pick primers from a DNA sequence. [disclaimer](#) [code](#)

[cautions](#)

Select the [Task](#) for primer selection

[Template masking](#) before primer design ([available species](#))

[Select species](#) [Nucleotides to mask in 5' direction](#)

[Primer failure rate cutoff](#) [Nucleotides to mask in 3' direction](#)

Paste source sequence below (5'→3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINES, etc.) or use a [Mispriming Library \(repeat library\)](#)

```
GGTAGTGCCATATTTTACAATTTGTAAAAACAAGCAGCAGCAAATGAAGACACTGGCTCATATTCCTGCA
GCCTGGAGGCCGGGTGCTCAGGGCTGACATGTCCACCCAGTGCACCCACTCTGCTTTTAACTGGGAG
ACTGGTAAGCAGACTGGTGGGATCTGTGCCAGAAATGGGACTGGGAGGGCCACTTCAGGGTTCTCCTC
TCCCTCTAAGGCTGAAGAAGGGTCTTCCCTCCCAAGACTTGGTGTCTTTCCCTCCACTCCTTCC
TGCCACTGCTGCTGCTGCTGCTAATCTTCAAGGGCACTGCTGCTCTTAGTGCCTGAGGAAAAAT
AAAGACACATGCTGCGCCCTTCCCA
```

<input checked="" type="checkbox"/> Pick left primer, or use left primer below	<input type="checkbox"/> Pick hybridization probe (internal oligo), or use oligo below	<input checked="" type="checkbox"/> Pick right primer, or use right primer below (5' to 3' on opposite strand)
<input type="text"/>	<input type="text"/>	<input type="text"/>

14. Next go down and find General Primer Picking Conditions. Using the information that we have provided in Primer Design Rules file, fill the spaces as indicated.

General Primer Picking Conditions

Upload the settings from a file No file chosen

Primer Size	Min <input type="text" value="19"/>	Opt <input type="text" value="20"/>	Max <input type="text" value="21"/>	
Primer Tm	Min <input type="text" value="58.0"/>	Opt <input type="text" value="59.0"/>	Max <input type="text" value="61"/>	Max Tm Difference <input type="text" value="5.0"/> Table of thermodynamic parameters <input type="text" value="SantaLucia 1998"/>
Product Tm	Min <input type="text" value="-1000000"/>	Opt <input type="text" value="0.0"/>	Max <input type="text" value="1000000"/>	
Primer GC%	Min <input type="text" value="45"/>	Opt <input type="text" value="50.0"/>	Max <input type="text" value="55"/>	
Product Size Ranges	<input type="text" value="700-800"/>			
Number To Return	<input type="text" value="5"/>		Max 3' Stability	<input type="text" value="9.0"/>
Max Library Mispriming	<input type="text" value="12.00"/>		Pair Max Library Mispriming	<input type="text" value="20.00"/>

N.B. Choose your product size range accordingly to the length of your mRNA sequence. Optimal range amplicon length product is between 600-1000bp. For genes with shorter than 600bp of length we usually design amplicon which ranges between 60-70% of the total length. Note that for those smaller gene there is no strict rule about the length of the amplicon.

15. Scroll down in the page, find, and click the button PICK PRIMERS.

The screenshot shows the primer3.ut.ee web interface. It contains numerous input fields for configuring primer design parameters. Key fields include:

- Max GC in primer 3' end: 5
- 3' End Distance Between Left Primers: 3
- 3' End Distance Between Right Primers: 3
- 5 Prime Junction Overlap: 7
- 3 Prime Junction Overlap: 4
- Concentration of Monovalent Cations: 50.0
- Salt Correction Formula: SantaLucia 1998
- Concentration of Divalent Cations: 1.5
- Concentration of dNTPs: 0.6
- Annealing Oligo Concentration: 50.0
- Sequencing Spacing: 500
- Sequencing Interval: 250
- Sequencing Lead: 50
- Sequencing Accuracy: 20

 There are also checkboxes for 'Liberal Base', 'Lowercase masking', 'Pick anyway', 'Print Statistics', and 'Treat ambiguity codes in libraries as consensus'. A section titled 'Objective Function Penalty Weights for Primers' includes fields for Size, Tm, GC%, and various self-complementarity and hairpin penalties, all set to 0.0. Buttons for 'Pick Primers', 'Download Settings', and 'Reset Form' are visible at the bottom of the form.

16. The page that will open will show you the primes that the program picked

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	1775	20	59.14	55.00	3.32	0.00	0.00	GTTTGACACCTGACAGACG
RIGHT PRIMER	2512	20	59.05	55.00	0.00	0.00	0.00	GTTGGAGTTCTGGGTGCTG

SEQUENCE SIZE: 4016
 INCLUDED REGION SIZE: 4016

PRODUCT SIZE: 738, PAIR ANY_TH COMPL: 0.30, PAIR 3'_TH COMPL: 0.00

```

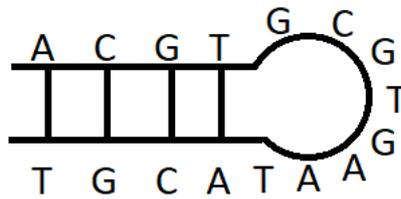
1 TGCTTTGTGGAGCTGTCAAGGCCCTGGGCTCTGGGAAAGAGGCACAGGGAGGCCAGGCAAG
61 GAAGGAGTGACCCGGAGGGACAGACCCAGGGGCTAAAGTCTGATAGGGCAAGAGAGTGC
121 CAGCCCCCTCTTGTCTATAAGGACCTCCACTGCCACATACAGGCCATGATTGATGCTTA
181 GACAAAGGGCTGGTGTCCAATCCAGCCCCAGCCCCAGAACTCCAGGGAAATGAATGGGC
241 AGAGAGCAGGAATATGGGACATCTGTGTTCGAGGGGAGGACTCCAGGAGTCTGGGAAATG
301 GGGCTAGTAGGAAATGATGATATAGGCACCCCTTGAGGGTACTGAACAGGTTTGTCTT
361 CGCCAAATCCAGCACCTTGCAAGTACTTACAGCTGAAGTAAAAGAGGCTGGGTATG
421 AAATCAAAAAGTTGAAAAGCAGGTCAGAGGTCATCTGGTACAGCCCTTCTCTTTTTT
    
```

You can see some information about the primers that the program picked.

1. In the **red box** (start) is the information about the position of the 5' base of the primer.
2. In the **green box** (len) is the information regarding the length of the primer.
3. In the **yellow box** (Tm) is the information regarding the melting temperature of the primer.
4. In the **violet box** (gc%) is the information about the GC% content of the primers or the percent of G or C bases in the primer.
5. In the **orange box** -any th (Self-Complementarity) and **brown box** -(hairpin) in the information about the tendency to primers to anneal to itself or to form secondary structure (hairpins). The lower the value the better.

Self-Complementarity

5' ACGTGCGTGAATACGT 3'



6. In the **violet box** (3`th)
The self-complementarity of the primer at the 3` ends (primer dimers formation with itself). The lower the value the better.



7. The final **Gray box** (seq) represent the primers sequence.
8. Copy and paste all the information from the **BLACK BOX** into your word file.
9. Add T7 promotor sequence to the forward primer-

T7 GCGTAATACGACTCACTATAGGG + Forward PRIMER

10. Add Sp6 promotor sequence to the reverse primer-

Sp6 GCGATTTAGGTGACACTATAG + Reverse PRIMER