



# NCBI Primer-BLAST

An online tool for designing target-specific PCR primer pairs (with internal probes)

<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>

National Center for Biotechnology Information • National Library of Medicine • National Institutes of Health • Department of Health and Human Services

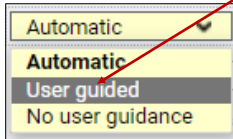
## Scope and Access

Primer-BLAST [1] is a PCR primer design and specificity checking tool from NCBI. It picks primers using the Primer3 algorithm [2] and then uses BLAST [3] to screen for primers specific to the input template. Similar to other BLAST searches, you can limit a Primer-BLAST search to specific taxa or a custom set of sequences specified by Entrez queries. It presents candidate primers along with their alignment to targets. Primer-BLAST is a web only application accessible through the “Specialized BLAST” section of the BLAST homepage (<https://blast.ncbi.nlm.nih.gov/>) or directly at <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>.



## Accepted Inputs

The Primer-BLAST search page (right) defaults to single template input form. This contains multiple sections. The top one (A) takes your input and allows the adjustment to a basic set of parameters. Given a template alone (B), Primer-BLAST will find a set of primer pairs optimal for PCR amplification. Primer-BLAST also accepts existing primers (C) and supports other combinations of input: 1) a primer pair with its template, 2) a template with a single primer, and 3) a pair of primers alone. In the case 1), Primer-BLAST validates the primer pair for the template sequence and performs a specificity check if this option is selected. In the case 2), Primer-BLAST finds candidate primers that work with the input primer and reports their target-specificity. In case 3) with primer pairs alone, Primer-BLAST finds the amplification target and provides primer template alignments.



With a RefSeq mRNA accession as a template, Primer-BLAST can take exon junctions into consideration through options given in the “Exon/intron selection” section (D). There you can set Primer-BLAST to have candidate primers span or not span splicing junctions, or ignore those junctions (E). You can also activate intron inclusion using the checkbox (F).

In the Primer Specificity Checking Parameters section (G), you can select different databases using the pull-down menu (H), restrict the search to a different organism by selecting from the suggested list upon typing (I), adjust the stringency of the specificity checking through parameters listed below the database (J), and check the box (K) to generates primer pairs that amplify all known transcript variants for the same gene. You can also adjust the search mode (L) to increase the chance of finding specific primers when the input template is highly similar to other targets in the database, and use the “Custom” database (M) option to upload a custom set of sequences (accessions or FASTA) for use as the specificity checking database.

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

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Primer sequences should be entered here in the 5' to 3' orientation.

Clicking the question mark icon next to a parameter to see the help information.

For details on “Primers common for a group of sequences, read our blogpost [5].

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Automatic  
Automatic  
User guided  
No user guidance

Refseq mRNA  
Refseq representative genomes  
Genomes for selected organisms (primary reference assembly only)  
nr  
Refseq RNA (refseq\_rna)  
Custom

barley  
barley (taxid:112509)  
domesticated barley (taxid:112509)  
two-rowed barley (taxid:112509)  
barleys (taxid:4512)

## Advanced Parameters for Primer-BLAST

Clicking the “Advanced Parameters” link (A) toggles open the section with infrequently adjusted parameters. The first section (B) contains parameters for BLAST that specify the exhaustiveness of specificity checking. The second section (C) contains parameters specific to the selected primers and their PCR products (D): such as, the T<sub>m</sub> of the PCR product, the primer length, the primer GC content, and GC clamps at the 3'-end of the primer. It also contains settings on PCR buffer conditions (E) since they can greatly affect the primer T<sub>m</sub> calculation. Note that, in favor of search speed, Primer-BLAST does not use thermodynamic alignment features by default (F). This section also allows you to instruct Primer-BLAST to take SNPs mapped to template into consideration during primer picking (Human RefSeq accession required) by checking the checkbox (G).

The screenshot shows the 'Advanced parameters' section of the Primer-BLAST web interface. It is divided into several sub-sections:

- Primer Parameters (C):** Contains fields for PCR Product T<sub>m</sub> (D), Primer Size (Min: 15, Opt: 20, Max: 25), Primer GC content (%), GC clamp, Max Poly-X, Max 3' Stability, Max GC in primer 3' end, Secondary Structure Alignment Methods, and various TH (Thermodynamic Hybridization) values for template mispriming, self-complementarity, and pair complementarity. It also includes fields for 5' and 3' side overlaps and concentration of monovalent/divalent cations and dNTPs (E).
- Primer Pair Specificity Checking Parameters (B):** Includes Max number of Blast target sequences (50000), Blast expect (E) value (30000), Blast word size (7), Max primer pairs to screen (500), Max targets to show for designing new primers (20), Max targets to show for pre-designed primers (1000), and Max targets per sequence (100).
- Internal hybridization oligo parameters (H):** Includes Hybridization oligo (with a checkbox to pick internal hybridization oligo), Hyb Oligo Size (Min: 18, Opt: 20, Max: 27), Hyb Oligo tm (Min: 57.0, Opt: 60.0, Max: 63.0), and Hyb Oligo GC% (Min: 20.0, Opt: 50, Max: 80.0).
- Other parameters (F, G, I, J):** Includes checkboxes for 'Use Thermodynamic Oligo Alignment' and 'Use Thermodynamic Template Alignment' (F), 'Show results in a new window' and 'Use new graphic view' (I), and a 'Get Primers' button (J). There is also a checkbox for 'Primer binding site may not contain known SNP' (G).
- Progress bar (K, L):** Shows the job status as 'Running' with a progress bar, current time (23 June 2014, 16:10:54), and time since submission (42 sec). It includes 'Check' and 'Cancel' links.

Additional text on the right side of the interface states: "You can pick internal probe for real-time PCR by activating and adjusting options given in the third section (H). An option of “Use new graphic view” (I), checked by default, allows Primer-BLAST to create a visually informative and interactive graphical summary of the result using the embedded Graphical Sequence Viewer [4]."

## Submitting a Search

Click the “Get Primers” button (J) to submit the search. The browser tracks the progress of the submitted job via an intermediate polling page (K) and displays the result when it becomes available. You can manually check it by using the “Check” link (L).

## Primer-BLAST Results: the Graphical Summary

The Primer-BLAST displays results by breaking them into several sections: the search summary, the graphical overview, and a tabular list of primer pairs with their properties plus alignments to the annealing sites on different targets. The summary section (A) reiterates the template, an informational message with additional details on the primers returned, and a “Search Summary” link (B) with detailed search statistics.

Primer-BLAST >> Job **A** yxwVUC3TR9qIVckWkRzFIBfYIQNTHK5DA

Primer-BLAST Results

Input PCR template: NM\_000410.4 Homo sapiens homeostatic iron regulator (HFE), transcript variant 1, mRNA  
 Range: 1 - 5176  
 Specificity of primers: Primers may not be specific to the input PCR template as targets were found in selected database: RefSeq mRNA (Organism limited to Homo sapiens)...help on specificity primers  
 Other reports: Search Summary **B**

**Graphical view of primer pairs**

Template: NM\_000410.4 | Find: | Tools | Tracks

Genes: HFE **D**

Primer pairs for job LyxwVUC3TR9qIVckWkRzFIBfYIQNTHK5DA

Primer 1  
 Primer 2  
 Primer 3  
 Primer 4  
 Primer 5  
 Primer 8  
 Primer 9 **E**

**Primer 1 Details**  
 Forward: 195..214 length 20 Tm 59.90 GC 55.00% Seq TGATCATGAGAGTCCCGTG  
 Reverse: 808..827 length 20 Tm 60.03 GC 55.00% Seq ACAGCCAAGGTATCCAGCC  
 PCR product length: 633  
 Links & Tools  
 BLAST nr: NM\_000410.4 (195..827)  
 BLAST to Genome: NM\_000410.4 (195..827)  
 FASTA record: NM\_000410.4 (195..827)  
 GenBank record: NM\_000410.4 (195..827)

Select a Range  
 Set New Marker At Position  
 Set Sequence Origin At Position  
 Set Sequence Origin At Feature  
 Flip Sequence Strands  
 Zoom In  
 Zoom Out  
 Zoom To Sequence  
 Zoom On Range  
 BLAST and Primer Search  
 Printer-Friendly (PDF/SVG)  
 Configure tracks **F**

Configure Page **H**

Available Tracks: Find Tracks Custom Data

Active Tracks: Active Track name

Variation: ExAC Release 1 Frequency, dbSNP b155 v2

Sequence: Frameshift Variations, dbSNP b155 v2  
 In-frame Any, dbSNP b155 v2

Genes/Products: Live RefSNPs, dbSNP b155 v2

Features: Missense Variations, dbSNP b155 v2  
 Rare Variations (MAF < 0.01), dbSNP b155 v2  
 Stop Gain or Loss Variations, dbSNP b155 v2

Uploaded Data: Synonymous Variations, dbSNP b155 v2  
 UK10K ALSPAC Frequency, dbSNP b155 v2  
 UK10K TWINSUK Frequency, dbSNP b155 v2  
 Unfiltered RefSNPs, dbSNP b155 v2

Track Settings: Cited Variations, dbSNP b155 v2 Track legend  
 dbSNP 2.0 Build 155 v2 all data based on Homo sapiens  
 Rendering options: Show variants for 50 or less

Remove track(s) Configure Reset tracks Cancel

Sequence: A G G G C T G G A T A A C C T T G G C T G T A C C C  
 T C C C G A C C T A T T G G A A C C G A C A T G G G  
 Variation: rs1762824283  
 Variation ID: rs1762824283  
 Variation Type: SNV, length 1  
 Alleles: G/A  
 [Genomic locations]  
 GCF\_000001405.39: NC\_000006.12 @ 26092868  
 GCF\_000001405.25: NC\_000006.11 @ 26093096  
 [Links & Tools]  
 SNP summary: rs1762824283 **I**

For the template sequence submitted in RefSeq accession format, NM\_000410 in this case, the Graphical Sequence Viewer provides much more information. Specifically, it displays:

- A clear overview of the results in the context of the target sequence, by showing the exon boundaries of the template plus its annotated protein product, pulled out from the feature table of this record (D),
- The candidate primer pairs, their predicted products, and exact locations on the template (E),
- The properties of a specific primer pair, viewable in the hovering activated popup (F),
- The sequence-level details of the annealing site through the “Zoom to Sequence” option (G) in the right-click menu
- The highlighted relationship of suggested primers with other features through the “Configure page” dialog box (H) activated by clicking the “Tracks” button, with the example shown being one of the known SNPs (I) mapped to one of the primer’s annealing site on this human mRNA template.

## Primer-BLAST Results: Primer Pairs and Their Alignment to Targets

**— Detailed primer reports** **A**

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers **Submit** **E**

**Primer pair 1** **B**

	Sequence (5'→3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGATCATGAGAGTCGCCGTG	Plus	20	195	214	59.90	55.00	6.00	1.00
Reverse primer	ACAGCCAAGGTTATCCAGCC	Minus	20	827	808	60.03	55.00	4.00	1.00
Product length	633								

**Products on intended targets** **C**

>NM\_000410.4 Homo sapiens homeostatic iron regulator (HFE), transcript variant 1, mRNA

product length = 633

Forward primer	1	TGATCATGAGAGTCGCCGTG	20
Template	195	.....	214
Reverse primer	1	ACAGCCAAGGTTATCCAGCC	20
Template	827	.....	808

**Products on potentially unintended templates** **D**

> NM\_001384164.1 Homo sapiens homeostatic iron regulator (HFE), transcript variant 13, mRNA

product length = 633

Forward primer	1	TGATCATGAGAGTCGCCGTG	20
Template	195	.....	214
Reverse primer	1	ACAGCCAAGGTTATCCAGCC	20
Template	827	.....	808

> NM\_139006.3 Homo sapiens homeostatic iron regulator (HFE), transcript variant 6, mRNA

product length = 591

Forward primer	1	TGATCATGAGAGTCGCCGTG	20
Template	195	.....	214
Reverse primer	1	ACAGCCAAGGTTATCCAGCC	20
Template	785	.....	766

The “Detailed Primer Reports” section (**A**) contains the details for returned primer pairs. Each primer pair is in its own subsection (**B**), with a summary of basic properties along with alignments to their intended target (**C**) and to potentially unintended targets (**D**).

In the example pair of primers for human HFE gene transcript variant 1 (NM\_000410) also amplify variants 13 and 6 (under **D**). Alignments, which are considered unintended in automatic mode. Checking them will mark them as intended targets in re-search through Submit button (**E**).

**Primer Pair Specificity Checking Parameters**

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

**Search mode** **User guided** **F**

**Database** **User guided**

**Organism**

Enter an organism name (or organism group name such as enterobacteriaceae, rodents), taxonomy id or select from the suggestion list as you type.

### More on “User guided” Mode and “Custom” Database

The “User guided” (**F**) search mode allows you to instruct Primer-BLAST whether certain targets that are highly similar to the input template should be considered as intended target upon job submission (**G**).

The Custom database option (**H**) allows you to provide your own input dataset for specificity checking. System constraints limit the size of sequence files to 300 MB. For sequences from the NCBI Nucleotide database, you can use their accessions or GI's to specify a larger custom dataset.

NCBI/ Primer-BLAST: Finding primers specific to your PCR template (using Primer3 and BLAST).

**Input PCR template** NM\_000249.3 Homo sapiens mutL homolog 1 (MLH1), transcript variant 1, mRNA

**Range** 1 - 2662

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.

Select: **All** None Selected:0

Accession	Title	Identity	Alignment length	Seq. start	Seq. stop
<input type="checkbox"/> XM_005265164.1	PREDICTED: Homo sapiens mutL homolog 1 (MLH1), transcript variant X3, mRNA	99.8%	2520	1	2515

**Submit**  Show results in a new window

**Primer Pair Specificity Checking Parameters**

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

**Search mode** **Automatic**

**Database** **Custom** **H**

**Organism**

**Exclusion (optional)**

**Entrez query (optional)** **Custom** **J**

### References

- Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 13:134.
- Rozen, S and Skaletsky, HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386.
- Altschul, SF, Madden, TL, Schäffer, AA, Zhang, J, Zhang, Z, Miller, W and Lipman, DJ (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Res*. 25:3389-3402.
- The Graphical Sequence Viewer Factsheet: [https://ftp.ncbi.nih.gov/pub/factsheets/Factsheet\\_Graphical\\_SV.pdf](https://ftp.ncbi.nih.gov/pub/factsheets/Factsheet_Graphical_SV.pdf).
- NCBI Insight Blogpost: Primer-BLAST now designs primers for a group of related sequences. <https://go.usa.gov/xUJcg>

### Technical Assistance

Please send you feedback, questions and bug reports to [blast-help@ncbi.nlm.nih.gov](mailto:blast-help@ncbi.nlm.nih.gov)