



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 finds unique regions on the input template, picks primers from those regions using the Primer3 algorithm [2], and uses relaxed settings to BLAST [3] 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, or even your own custom sequences not present in the public databases (subject to size limit). 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 access 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) as input and supports the following combinations

- 1) a primer pair with its template
- 2) a template with a single primer
- 3) a pair of primers alone.

In case 1), Primer-BLAST validates the primer pair for the template sequence and performs a specificity check if this option is selected. In 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. You can also activate intron inclusion using the checkbox.

In the Primer Specificity Checking Parameters section (E), you can select different databases using the pull-down menu (F), restrict the search to a different organism by selecting from the suggested list upon typing (G), adjust the stringency of the specificity checking through parameters listed below the database (H), and check the box (I) to generates primer pairs that amplify all known transcript variants for the same gene. You can also adjust the search mode (J) 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 (K) option to upload a custom set of sequences (accessions or FASTA) for use as the specificity checking database through the file upload option. More on this at the end.

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

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.

Automatic

Automatic

User guided

No user guidance

Refseq mRNA

Refseq mRNA

Refseq representative genomes

Genomes for selected eukaryotic organisms (primary assembly only)

core_nt

Refseq RNA (refseq_rna)

Custom (use your own sequence accession, assembly accession, etc.)

nt

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_m 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_m 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).

A **Advanced parameters**

B **Primer Pair Specificity Checking Parameters**

C **Primer Parameters**

D **PCR Product Tm**

E **PCR buffer conditions**

F **Use Thermodynamic Oligo Alignment** **Use Thermodynamic Template Alignment (warning: search**

H **Internal hybridization oligo parameters**

I **Use new graphic view**

J **Get Primers**

K **Check**

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].

Primer Parameters

PCR Product Tm

Primer Size

Primer GC content (%)

GC clamp

Max Poly-X

Max 3' Stability

Max GC in primer 3' end

Secondary Structure Alignment Methods

TH: Max Template Mispriming

TH: Max Self Complementarity

TH: Max Pair Complementarity

TH: Max Primer Hairpin

Max Template Mispriming

Max Self Complementarity

Max Pair Complementarity

Excluded regions

Overlap junctions

Concentration of monovalent cations

Concentration of divalent cations

Concentration of dNTPs

Salt correction formula

Table of thermodynamic parameters

Annealing Oligo Concentration

SNP handling

Repeat filter

Low complexity filter

Primer Pair Specificity Checking Parameters

Max number of Blast target sequences

Blast expect (E) value

Blast word size

Max primer pairs to screen

Max targets to show (for designing new primers)

Max targets to show (for pre-designed primers)

Max targets per sequence

Use Thermodynamic Oligo Alignment Use Thermodynamic Template Alignment (warning: search

Primer Pair

Primer

Pair

Any

3'

Any

3'

Any

3'

Primer

Pair

Any

3'

Any

3'

5' side overlaps

3' side overlaps

Minimal number of nucleotides that the left or

50.0

1.5

0.6

SantaLucia 1998

SantaLucia 1998

50.0

Primer binding site may not contain known SNP

Automatic

Avoid repeat region for primer selection by filtering with repeat database

Avoid low complexity region for primer selection

Internal hybridization oligo parameters

Hybridization oligo

Pick internal hybridization oligo

Hyb Oligo Size

Hyb Oligo tm

Hyb Oligo GC%

Min

Opt

Max

Min

Opt

Max

Min

Opt

Max

Get Primers

Show results in a new window Use new graphic view

NCBI/ Primer-BLAST: Making primers specific to your PCR template. [more...](#)

Status

Running

Current time

23 June 2014, 16:10:54

Time since submission

42 sec

Progress Message

Check

Cancel

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, where you can manually check it by using the “Check” link (K). Browser will display the result when it becomes available.

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

Detailed primer reports

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

Download primer pairs

Primer pair 1

	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	ACAGCCAAGGTTATCCAAGCC	Minus	20	827	808	60.03	55.00	4.00	1.00
Product length	633								

Products on intended targets

>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 ACAGCCAAGGTTATCCAAGCC 20
Template 827 808

Products on potentially unintended templates

>NM_001300749.3 Homo sapiens homeostatic iron regulator (HFE), transcript variant 12, mRNA

product length = 633

Forward primer 1 TGATCATGAGAGTCGCCGTG 20
Template 195 214

Reverse primer 1 ACAGCCAAGGTTATCCAAGCC 20
Template 827 808

>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 ACAGCCAAGGTTATCCAAGCC 20
Template 827 808

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 12 and 13 (D), which are considered as unintended in automatic mode. Checking them will mark them as intended targets in re-run through the activated Submit button (E).

The result page also allows the saving of displayed results through the "Download primer pairs" button (F, new) in text or tabular format.

More on "User guided" Mode and "Custom" Database

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

The Custom database option (I) allows you to provide your own input dataset for specificity checking. System constraints limit the size of sequence files to 300 MB through file upload. For sequences from the NCBI Nucleotide database, use their accessions as input.

Primer Pair Specificity Checking Parameters

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

Search mode **User guided**

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.

Primer-BLAST

A tool for finding specific primers

Finding primers specific to your PCR template (using Primer3 and BLAST)

Input PCR template: F12345.1 HSC38H011 normalized infant brain cDNA Homo sapiens cDNA clone c-38H01, mRNA sequence
Range: 1 - 327

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"/> NM_002622.5	Homo sapiens prefolin subunit 1 (PFDN1), mRNA	97.21%	323	222	544

Submit Show results in a new window

Technical Assistance

Please send your feedback, questions and bug reports to blast-help@ncbi.nlm.nih.gov

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.nlm.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>

Note: Parameter values that differ from the default are highlighted in yellow

Primer Pair Specificity Checking Parameters

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

Search mode **Automatic**

Database **Refseq mRNA**

Exclusion **Refseq mRNA**

Organism **Refseq representative genomes**

Entrez query (optional) **Genomes for selected eukaryotic core_nt**

Primer specificity stringency **Custom (use your own sequence accession, assembly accession, etc.)**