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 (shown below) 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), in FASTA or Accession/GI format, 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, such as a primer pair with its template, a template with a single primer, and a pair of primers alone. In the first case, Primer-BLAST validates the primer pair for the template sequence and performs a specificity check if this option is selected. In the second case, Primer-BLAST finds candidate primers that work with the input primer and reports their target-specificity. With primer pairs alone, Primer-BLAST finds the amplification target and provides primer template alignments.

With a RefSeq mRNA accession (or GI) as a template, Primer-BLAST can take exon junctions into consideration when finding optimal primer pairs (to differentiate mRNA from genomic target), through options given in the “Exon/intron selection” section (D). You can also set it to have candidate primers span or not span splicing junctions, or ignore the junctions altogether (E). You can also activate intron inclusion using the checkbox (F). Check the box (G) to generate primer pairs that amplify all known transcript variants for the same gene.

Parameters in the last open section (H) govern the specificity checking. Here, you can select different databases using the pull-down menu (I) and limit the search to a different organism by selecting from the suggested list upon typing (J), and adjust the stringency of the specificity checking through parameters listed below the database (K). 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.
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, such as the Tm of the PCR product (D), the primer length (E), the primer GC content (F), GC clamps at the 3’-end of the primer (G), and the PCR buffer conditions (H). Settings in buffer condition can greatly affect the primer Tm calculation. Note that, in favor of search speed, Primer-BLAST does not use thermodynamic alignment features (I) by default. You can instruct Primer-BLAST to take SNPs mapped to template into consideration during primer picking (RefSeq accession/GI required) by checking the checkbox (J).

You can pick internal probe for real-time PCR by activating and adjusting options given in the third section (K). An option of “Use new graphic view” (L), 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 (M) to submit the search. The browser tracks the progress of the submitted job via an intermediate polling page (N) and displays the result when it becomes available. You can manually check it by using the “Check” link (O).
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.

For the template sequence provided 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 (D)
- The candidate primer pairs with their predicted products (E)
- The properties of a specific primer pair, viewable in the popup (F) activated upon hovering
- 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, such as SNPs mapped to the template, through the “Configure page” dialog box (H) activated by clicking the “Tracks” button
Primer-BLAST Results: Primer Pairs and Their Alignment to Targets

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

In the example to the left, two pairs of primers for human HFE gene transcript variant 1 (NM_000410) also amplify variants 12 and 6 (F). Alignments (with mismatches) to a truly unintended target FOXK1 is also shown (G).

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

The “User guided” (H) mode allows primer-BLAST to distinguish between the intended template and other targets that are highly similar to it upon job submission (I).

Selecting custom database (J) allows you to provide custom 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.

References


Technical Assistance

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

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