- Primers for target on one template
- Primers common for a group of sequences

Retrieve recent results Publication Tips for finding specific primers Reset page Save search parameters

PCR Template

Enter accession, gi, or FASTA sequence (A refseq record is preferred) Help Clear

Enter the PCR template here (multiple templates are currently not supported). It is highly recommended to use refseq accession or GI (rather than the raw DNA sequence) whenever possible as this allows Primer-BLAST to better identify the template and thus perform better primer specificity checking.

A template is not required if both forward and reverse primers are entered below.

The template length is limited to 50,000 bps. If your template is longer than that, you need to use primer range to limit the length (i.e., set forward primer "From" and reverse primer "To" fields but leave forward primer "To" and reverse primer "From" fields empty).

Or, upload FASTA file

Choose File No file chosen

Range Help Clear

	From	To
Forward primer		
Reverse primer		

Enter the position ranges if you want the primers to be located on the specific sites. The positions refer to the base numbers on the plus strand of your template (i.e., the "From" position should always be smaller than the "To" position for a given primer). Partial ranges are allowed. For example, if you want the PCR product to be located between position 100 and position 1000 on the template, you can set forward primer "From" to 100 and reverse primer "To" to 1000 (but leave the forward primer "To" and reverse primer "From" empty).

Note that the position range of forward primer may not overlap with that of reverse primer.

Primer Parameters

Use my own forward primer (5'->3' on plus strand)

Help

Optionally enter your pre-designed forward primer. Always use the actual primer sequence (i.e., 5'->3' on plus strand of the template). Please enter the primer sequence only (No any other characters are allowed).

Clear

Use my own reverse primer (5'->3' on minus strand)

Help

Optionally enter your pre-designed reverse primer. Always use the actual primer sequence (i.e., 5'->3' on minus strand of the template). Please enter the primer sequence only (No any other characters are allowed).

Clear

PCR product size

Min Max

100 200

# of primers to return

500

Primer melting temperatures (T_m)

Min Opt Max Max T_m difference

57.0 60.0 63.0 3 Help

The Tm calculation is controlled by Table of thermodynamic parameters and Salt correction formula (under advanced parameters). The default Table of thermodynamic parameters is "SantaLucia 1998" and the default Salt correction formula is "SantaLucia 1998" as recommended by primer3 program.

Exon/intron selection

Help A refseq mRNA sequence as PCR template input is required for options in the section

A refseq mRNA sequence (for example an entrez sequence record that has accession starting with NM_) allows the program to properly identify the corrsponding genomic DNA and thus find correct exon/intron boundaries.

Exon junction span

No preference Help

This controls whether the primer should span an exon junction on your mRNA template. The option "Primer must span an exon-exon junction" will direct the program to return at least one primer (within a given primer pair) that spans an exon-exon junction. This is useful for limiting the amplification only to mRNA. You can also exclude such primers if you want to amplify mRNA as well as the corresponding genomic DNA.

Exon junction match

Min 5' match Min 3' match Max 3' match

7 4 8

Minimal and maximal number of bases that must anneal to exons at the 5' or 3' side of the junction Help

This specifies the minimal number of bases that the primer must anneal to the template at 5' side (i.e., toward start of the primer) or 3' side (i.e., toward end of the primer) of the exon-exon junction. Annealing to both exons is necessary as this ensures annealing to the exon-exon junction region but not either exon alone. Note that this option is effective only if you select "Primer must span an exon-exon junction" for "Exon junction span" option.

Intron inclusion

☐ Primer pair must be separated by at least one intron on the corresponding genomic DNA Help

With this option on, the program will try to find primer pairs that are separated by at least one intron on the corresponding genomic DNA using mRNA-genomic DNA alignment from NCBI. This makes it easy to distinguish between amplification from mRNA and genomic DNA as the product from the latter is longer due to presence of an intron.

Intron length range

Min Max

1000 100000 Help

This specifies the range of total intron length on the corresponding genomic DNA that would separate the forward and revervse primers.

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

Primer Pair Specificity Checking Parameters

Specificity check

☑ Help Enable search for primer pairs specific to the intended PCR template

With this option on, the program will search the primers against the selected database and determine whether a primer pair can generate a PCR product on any targets in the database based on their matches to the targets and their orientations. The program will return, if possible, only primer pairs that do not generate a valid PCR product on unintended sequences and are therefore specific to the intended template. Note that the specificity is checked not only for the forward-reverse primer pair, but also for forward-forward as well as reverse-reverse primer pairs.

Search mode

Automatic Help

Primer-blast tries to find target-specific primers by placing candidate primers on unique template regions that are not similar to other targets. However, in some cases, primer-blast cannot determine if a database sequence is an intended target or not, thus the user guidance might be helpful (For example, when your template is a polymorphic form or a partial region of an entry in the search database, or when the database such as the nr contains redundant entries of your template).
The "Automatic" option will ask for user guidance only when the program does not find sufficient unique template regions while the "User guided" option will always ask for user guidance if your template shows high similarity to any other database sequences.

Database

nr Help

Refseq mRNA:
This contains mRNA only from NCBI's Reference Sequence collection

Refseq representative genomes:
This database contains NCBI RefSeq Reference and Representative genomes across broad taxonomy groups including eukaryotes, bacteria, archaea, viruses and viroids. These genomes are among the best quality genomes available at NCBI. This database contains minimum redundancy in genome representation. For the eukaryotes, only one genome is included per species (However, alternate loci of eukaryotic genomes are included where applicable). For other species, genomes from diverse isolates of the same species may be included. Mitochondrion genomes are included where applicable.

Refseq RNA:
This contains all RNA entries from NCBI's Reference Sequence collection

Genomes for selected organisms (primary reference assembly only):
These are complete or nearly complete genome sequences from primary chromosome assemblies (i.e., no mitochondrion or alternate loci) for the following selected organisms:

apis mellifera
bos taurus
danio rerio
dog

drosophila melanogaster
gallus gallus
human
mouse
pan troglodytes
pig
rat

Although sequences in this database are completely covered by the Refseq representative genomes database, it does not contain the alternate loci and therefore has even less redundancy than the Refseq representative genomes database. This database is recommended if you are not concerned about missing alternate loci or mitochondrion sequences.

Custom:
You can use your own sequences (accession number, gi, or FASTA sequence) as a search database. The database size is limited to 300M.

Enter accession number, gi, or FASTA sequence Clear

Or, upload file: Choose File No file chosen

Exclusion

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

You can choose to exclude sequences in the selected database from specificity checking if you are not concerned about these. There are a large number of predicted Refseq transcripts in the Refseq mRNA, Refseq RNA and nr database. There are also many uncultured/environmental sample sequencesare in the nr database.

Organism

bacteria (taxid:2) Add organism

bacteria (taxid:2)

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

This will limit the primer specificity checking to the specified organism. It is strongly recommended that you always specify the organism if you are amplifying DNA from a specific organism (because searching all organisms will be much slower and off-target priming from other organisms is irrelevant). Click on "Add more organisms" label if you want to restrict to multiple organisms (enter only one organism in each input box).

Entrez query (optional)

Help

You can use a regular entrez query to limit the database search for primer specificity. For example, enter a GenBank accession number to limit search to that particular sequence only (Caution: this means the primer specificity will NOT be checked against any other sequences except the specified one).

Primer specificity stringency

Primer must have at least 5 total mismatches to unintended targets, including at least 2 mismatches within the last 3 bps at the 3' end. Help
This requires at least one primer (for a given primer pair) to have the specified number of mismatches to unintended targets. The larger the mismatches (especially those toward 3' end) are between primers and the unintended targets, the more specific the primer pair is to your template (i.e., it will be more difficult to anneal to unintended targets). However, specifying a larger mismatch value may make it more difficult to find such specific primers. Try to lower the mismatch value in such case.

Ignore targets that have 6 or more mismatches to the primer. Help
This is another parameter that can be used to adjust primer specificity stringecy. If the total number of mismatches between target and at least one primer (for a given primer pair) is equal to or more than the specified number (regardless of the mismatch locations), then any such targets will be ignored for primer specificity check. For examaple, if you are only interested in targets that perfectly match the primers, you can set the value to 1. You can also lower the E value (see advanced parameters) in such case to speed up the search as the high default E value is not necessary for detecting targets with few mismatches to primers.
Additionally this program has limit detecting targets that are too different from the primers...it will detect targets that have up to 35% mismatches to the primer sequences (i.e., a total of 7 mismatches for a 20-mer).
You may need to choose more sensitive blast parameters (under advance parameters) if you want to detect targets with a higher number of mismatches than default.

Max target amplicon size
4000 Help
This specifies the max amplicon size for a PCR target to be detected by Primer-BLAST. In general, the non-specific targets become less of a concern if their sizes are very large since PCR is much less efficient for larger amplicons.

Allow splice variants
☐ Help
Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)
If enabled, this program will NOT exclude the primer pairs that can amplify one or more mRNA splice variants from the same gene as your PCR template, thus making primers gene-specific rather than transcript-specific (Note that it is NOT intended to generate primers that will amplify all variants. It only means that the primers may amplify one or more other slice variants, in addition to the one you have specified). Enabling this option will make it much easier to find gene-specific primers since there is no need to distinguish between splice variants. This option requires you to enter a refseq mRNA accession or gi or fasta sequence as PCR template input because other type of input may not allow the program to properly interpret the result.

Get Primers

☑ Show results in a new window ☑ Use new graphic view Help
This enables our new graphic display that offers enhanced overview for your template and primers.

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

Advanced parameters + −

Primer Pair Specificity Checking Parameters

Max number of sequences returned by Blast
50000 Help
Maximum number of database sequences (with unique sequence identifier) Blast finds for primer-blast to screen for primer pair specificities. Note that the actual number of similarity regions (or the number of hits) may be much larger than this (for example, there may be a large number of hits on a single target sequence such as a chromosome). Choose a higher value if you need to perform more stringent search.

Blast expect (E) value
30000 Help
Expected number of chance matches in a random model. A higher E value should be used if you want more stringent specificity checking (i.e., to identify targets that have more mismatches to the primers, in addition to the perfectly matched targets). On the other hand, a lower E value is recommended if you are only interested in perfect or nearly perfect matches as this will significatly shorten the search time.

Blast word size
7 Help
The minimal number of contiguous nucleotide base matches between the query sequence and the target sequence that is needed for BLAST to detect the targets. Set a lower value if you need to find target sequences with more mismatches to your primers. However this will increase the search time.

Max primer pairs to screen
500 Help
The maximum number of candidate primer pairs to screen in order to find specific primer pairs (The candidate primers are generated by primer3 program). Increasing this number can increase the chance of finding a specific primer pair but the process will take longer.

Max targets to show (for designing new primers)
20 Help
The maximum number of PCR targets (amplicons) to be shown when designing new primers.

Max targets to show (for pre-designed primers)
1000 Help
The maximum number of PCR targets (amplicons) to be shown when checking specificity for pre-designed primers.

Max targets per sequence
100 Help
The maximum number of PCR targets (amplicons) to be found on any single sequence in the search database.

Primer Parameters

PCR Product Tm
Min Opt Max

Primer Size
Min Opt Max

18 22 26
Primer GC content (%)
Min Max
40 60
GC clamp
0 Help
The number of consecutive Gs and Cs at the 3' end of both the left and right primer.

Max Poly-X
5 Help
The maximum allowable length of a mononucleotide repeat, for example AAAAAA.

Max 3' Stability
9 Help
The maximum stability for the last five 3' bases of a left or right primer. Bigger numbers mean more stable 3' ends.

Max GC in primer 3' end
5 Help
The maximum number of Gs or Cs allowed in the last five 3' bases of a left or right primer.

Secondary Structure Alignment Methods
☐ ☐ Help Use Thermodynamic Oligo Alignment
Use Thermodynamic Template Alignment (warning: search may be very slow with this option on)
The option "Use Thermodynamic Oligo Alignment" instructs Primer3 to use thermodynamic alignment models (instead of old traditional secondary structure alignment) for calculating the propensity of oligos to form hairpins and dimers while the option "Use Thermodynamic Template Alignment" instructs Primer3 to use thermodynamic alignment models (instead of old traditional secondary structure alignment) for calculating the propensity of oligos to anneal to undesired sites in the template sequence.

TH: Max Template Mispriming
Primer Pair
40.00 70.00 (For thermodynamic alignment model only)
TH: Max Self Complementarity
Any 3'
45.0 35.0 (For thermodynamic alignment model only)
TH: Max Pair Complementarity
Any 3'
45.0 35.0 (For thermodynamic alignment model only)
TH: Max Primer Hairpin
24.0 (For thermodynamic alignment model only)
Max Template Mispriming
Primer Pair
12.00 24.00 (For old secondary structure alignment model only)
Max Self Complementarity
Any 3'
8.00 3.00 (For old secondary structure alignment model only)
Max Pair Complementarity
Any 3'
8.00 3.00 (For old secondary structure alignment model only)

Excluded regions

Help

E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the source sequence with < and >: e.g. ...ATCT<CCCC>TCAT... forbids primers in the central CCCC.

Overlap junctions

Help

Enter a list of space separated nucleotide positions. This requires that the left or the right primers to span a junction that is just 3' of any such positions. For example, entering "50 100" would mean that the left or the right primers must span the junction between nucleotide position 50 and 51 or the junction between position 100 and 101 (counting from 5' to 3'). You can also specify in the fields below the minimal number of nucleotides that the left or the right primer must have on either side of the junctions. This option is useful if you want a primer to a span specific junction on the template. Note that this option cannot be used in association with the "Exon/intron selection" options above.

5' side overlaps 3' side overlaps

7 4

Minimal number of nucleotides that the left or the right primer must have at the 5' or 3' side of the junctions

Concentration of monovalent cations

50.0 Help

The millimolar concentration of salt (usually KCl) in the PCR. Primer3 uses this argument to calculate oligo melting temperatures.

Concentration of divalent cations

1.5 Help

The millimolar concentration of divalent salt cations (usually MgCl2+ in the PCR). Primer3 converts concentration of divalent cations to concentration of monovalent cations using formula suggested in the paper Ahsen et al., 2001. [Monovalent cations] = [Monovalent cations] + 120* (v([divalent cations] - [dNTP])). According to the formula concentration of desoxynucleotide triphosphate [dNTP] must be smaller than concentration of divalent cations. The concentration of dNTPs is included to the formula beacause of some magnesium is bound by the dNTP. Attained concentration of monovalent cations is used to calculate oligo/primer melting temperature. See Concentration of dNTPs to specify the concentration of dNTPs.

Concentration of dNTPs

0.6 Help

The millimolar concentration of deoxyribonucleotide triphosphate. This argument is considered only if Concentration of divalent cations is specified.

Salt correction formula

SantaLucia 1998 Help

Option for specifying the salt correction formula for the melting temperature calculation. There are three different options available:

1. Schildkraut and Lifson 1965, DOI:10.1002/bip.360030207 (this is used until the version 1.0.1 of Primer3).The default value of Primer3 version 1.1.0 (for backward compatibility)
2. SantaLucia 1998, DOI:10.1073/pnas.95.4.1460 This is the *recommended* value.
3. Owczarzy et al. 2004, DOI:10.1021/bi034621r

Table of thermodynamic parameters

SantaLucia 1998 Help

Option for the table of Nearest-Neighbor thermodynamic parameters and for the method of melting temperature calculation. Two different tables of thermodynamic parameters are available: Breslauer et al. 1986, DOI:10.1073/pnas.83.11.3746 In that case the formula for melting temperature calculation suggested by Rychlik et al. 1990 is used.
SantaLucia 1998, DOI:10.1073/pnas.95.4.1460 This is the *recommended* value.

Annealing Oligo Concentration

50.0 Help

The nanomolar concentration of annealing oligos in the PCR. Note that this is not the concentration of oligos in the reaction mix but of those annealing to template. Primer3 uses this argument to calculate oligo melting temperatures. The default (50nM) works well with the standard protocol used at the Whitehead/MIT Center for Genome Research--0.5 microliters of 20 micromolar concentration for each primer oligo in a 20 microliter reaction with 10 nanograms template, 0.025 units/microliter Taq polymerase in 0.1 mM each dNTP, 1.5mM MgCl2, 50mM KCl, 10mM Tris-HCL (pH 9.3) using 35 cycles with an annealing temperature of 56 degrees Celsius. This parameter corresponds to 'c' in Rychlik, Spencer and Rhoads' equation (ii) (Nucleic Acids Research, vol 18, num 21) where a suitable value (for a lower initial concentration of template) is "empirically determined". The value of this parameter is less than the actual concentration of oligos in the reaction because it is the concentration of annealing oligos, which in turn depends on the amount of template (including PCR product) in a given cycle. This concentration increases a great deal during a PCR; fortunately PCR seems quite robust for a variety of oligo melting temperatures.

SNP handling

☐ Help Primer binding site may not contain known SNP

With this option on, the program will automatically retrieve the SNP information contained in template (using GenBank accession or GI as template is required) and avoid choosing primers within the SNP regions.

Repeat filter

Automatic Help

If the default "Automatic" setting is selected, the program will automatically select the repeat database using the following rules.

1. If a repeat database is available from the same organism as specified in the "Organism" field by user (see above), then that repeat database will be used. For example, if "Human" is specified, then the human repeat database will be selected.

2. If a repeat database from the same organism is not available, the database from the closest parent of that organism in the taxonomy tree will be selected. For example, the rodent repeat database will be selected if "Mouse" is specified in "Organism" field. However, no repeat database will be selected if "Gallus gallus" is specified since a repeat database from its taxonomical parents is not available.

Avoid repeat region for primer selection by filtering with repeat database

Low complexity filter

☑ Help Avoid low complexity region for primer selection

Low complexity regions are some regions in a DNA sequence that have biased base compositions such as a stretch of ACACACACACACACACACA.

Internal hybridization oligo parameters

Hybridization oligo

Pick internal hybridization oligo

Hyb Oligo Size

Min	Opt	Max
18	20	27

Hyb Oligo tm

Min	Opt	Max
57.0	60.0	63.0

Hyb Oligo GC%

Min	Opt	Max
20.0	50	80.0

Get Primers

Show results in a new window Use new graphic view Help

This option enables our new graphic view which offers much more details for your template and primers. It will replace the current graphic view in the future.