



**Primer-BLAST** is a combination of a program called **Primer3** that aids in the design of primers with specific properties and BLAST. Primer-BLAST allows for the construction of primers for qPCR where the user can specify the melting temperature, reduce the amount of self-priming and to span exon-exon junctions in order to avoid amplification of contaminating genomic DNA. After the design of primers, each primer pair is sent into BLAST to identify if similar products within the genome of the model organism will also be primed and amplified. This process ensures that the primers designed fall within your design parameters and most likely only amplify your gene of interest.

1. Enter the sequence OR the NCBI accession number for the gene of interest
2. Define the PCR product length
  1. limiting the product between 100-500 permits for good efficiency in qPCR
  2. longer products may not be efficiently replicated depending on your cycling protocol
3. Define the desired melting temperature ( $T_m$ ) of the primers (minimum, optimal, maximum, difference between the set)
  1. 60°C is fairly high and will aid in the enhanced specificity of the primer with the target during amplification to avoid false priming
  2. Try to have the  $T_m$  as close as possible so that they are annealing about equally
4. Choose the option “Primer must span an exon-exon junction”
  1. This aids in amplifying cDNA and not genomic DNA that may be contaminating
  2. do not select this if it a single exon gene as this will fail
5. Select Refseq mRNA as the database to search against.
  1. **Refseq** provides sequences to naturally occurring sequences.
  2. Things like plasmid sequences or vector constructs do not show up in Refseq
6. Select the organism you are BLASTing against
  1. there are options for model organisms as well as cell lines
  2. If you are using something like PC12 cells, you may use *Rattus norvegicus* or PC12 genome since that is also an option in the database
7. Evaluate the location of the primers and the other parameters. We generally choose primers at the 3' end of the RNA since RT reactions often have a 3' bias in eukaryotes by using oligo-dT priming in the reverse transcription



**Primer-BLAST** A tool for finding specific primers

• NCBI/ Primer-BLAST: Finding primers specific to your PCR template (using Primer3 and BLAST). [More...](#) [Tips for finding specific primers](#)

**PCR Template** [Reset page](#) [Save search parameters](#) [Retrieve recent results](#)

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

**Enter target**

NM\_001081212.1

Or, upload FASTA file  No file chosen

**Primer Parameters**

Use my own forward primer (5'->3' on plus strand)  [Clear](#)

Use my own reverse primer (5'->3' on minus strand)  [Clear](#)

PCR product size

Min	Max
100	500

**Define Product Length**

# of primers to return

Primer melting temperatures (T<sub>m</sub>)

Min	Opt	Max	Max T <sub>m</sub> difference
58	60.0	62	3

**Define T<sub>m</sub>**

**Exon/intron selection** A refseq mRNA sequence as PCR template input is required for options in the section

Exon junction span  [Clear](#) **Span exon-exon junctions**

Exon junction match

Exon at 3' side  Exon at 5' side

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

Intron inclusion  Primer pair must be separated by at least one intron on the corresponding genomic DNA

Intron length range

Min	Max
1000	1000000

**Primer Pair Specificity Checking Parameters**

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

Search mode

Database

Organism  Enter an organism name, taxonomy id or select from the suggestion list as you type

**BLAST for false products**

Exclusion (optional)  Exclude predicted Refseq transcripts (accession with XM\_ prefix)  Exclude predicted environmental sample sequences

Entrez query (optional)

Primer specificity stringency

Primer must have at least  total mismatches to unintended targets, including at least  mismatches within the last  bps at the 3' end.

Ignore targets that have  or more mismatches to the primer.

Misprimed product size deviation

Splice variant handling  Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)

**Get Primers**  Show results in a new window  Use new graphic view **Show results in new window**

[Advanced parameters](#) Note: Parameter values that differ from the default are highlighted in yellow