

Primer design using Primer-BLAST and OligoAnalyzer

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PCR

- Amplification of target DNA sequences

- Steps:

1. Initial denaturation

2. Denaturation

3. Annealing

4. Extension

5. Final Extension

} 30 cycles

- Reagents:

- DNA template

- Buffer (containing $MgCl_2$)

- Primers

- dNTPs

- Taq polymerase



Primer design

1. Primer length: 18-22 bases
2. Primer melting temperature: 52–58°C
3. Primer annealing temperature
4. GC content: 40–60%
5. GC clamp: GC at 3'
6. Primer secondary structures: try to avoid these
7. Repeats: maximum 4 repeats (e.g., ATATATAT)
8. Runs: maximum 4 bases (e.g., AAAA)
9. End stability: less stability at 3', less false priming
10. Avoid template secondary structure
11. Avoid cross homology

Primer-BLAST

- Available through NCBI
(URL: <http://www.ncbi.nlm.nih.gov/tools/primer-blast>)
- Used to construct primers
 - Paste a sequence or specify an NCBI accession number for a gene
- Adjustable parameters include:
PCR product size, melting temperature, GC content, GC clamp, salt concentrations, etc.
- Can also be used to check specificity of primer pairs
 - Databases: Refseq mRNA, Genome, Refseq RNA, nr
 - Narrow search to organisms from certain taxonomic groups

Primer-BLAST

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

Enter accession, gi, or FASTA sequence (A refseq record is preferred) [Clear](#)

Or, upload FASTA file No file chosen

Range

Forward primer From To [Clear](#)

Reverse primer

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

of primers to return

Primer melting temperatures (T_m) Min Opt Max Max T_m difference

Exon/intron selection

A refseq mRNA sequence as PCR template input is required for options in the section [Clear](#)

Exon junction span [Clear](#)

Exon junction match Exon at 5' side Exon at 3' side

Intron inclusion Primer pair must be separated by at least one intron on the corresponding genomic DNA [Clear](#)

Intron length range Min Max

Primer Pair Specificity Checking Parameters

Specificity check Enable search for primer pairs specific to the intended PCR template [Clear](#)

Database [Clear](#)

Organism [Clear](#)

Enter an organism name, taxonomy id or select from the suggestion list as you type. [Add more organisms](#)

Exclusion (optional) Exclude predicted Refseq transcripts (accession with XM, XR prefix) Exclude uncultured/environmental sample sequences [Clear](#)

Entrez query (optional) [Clear](#)

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. [Clear](#)

Ignore targets that have or more mismatches to the primer. [Clear](#)

Misprimed product size deviation [Clear](#)

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

Show results in a new window Use new graphic view [Clear](#)



OligoAnalyzer

- Available through IDT
(URL: <http://www.idtdna.com/analyzer/applications/oligoanalyzer>)
- User can input desired concentrations of primer, Na⁺, Mg²⁺ and dNTPs
 - Affect melting temperature
- Given a primer sequence, calculates:
 - Melting temperature
 - GC content
 - Hairpin formation
 - Self-dimer/hetero-dimer possibilities
- Useful for visualizing possible primer dimers

OligoAnalyzer

[Instructions](#) | [Definitions](#) | [Feedback](#)

Sequence # Bases 20
5'-GGT ACT GGT AAG TCC TTT GG
-3'

Target Type
Oligo Conc μM
Na⁺ Conc mM
Mg⁺⁺ Conc mM
dNTPs Conc mM

Clear Sequence

Add To Order

Default Settings

Analyze

Hairpin

Self-Dimer

Hetero-Dimer

NCBI Blast

TM Mismatch

Results | 5' mods | Internal Mods | 3' mods | Mixed Bases

HETERO-DIMER ANALYSIS ?

Primary Sequence

5'- GGTACTGGTAAGTCCTTGG -3'

Secondary Sequence

5'- TCCGTGGGICCATATTCGTC -3'

Maximum Delta G -39.53 kcal/mole

Delta G -5.02 kcal/mole
Base Pairs 3

```
5'   GGTACTGGTAAGTCCTTGG
      :   |||   :
3'  CIGCITATACCTGGGIGTCCT
```

Delta G -5.02 kcal/mole
Base Pairs 3

```
5' GGTACTGGTAAGTCCTTGG
      :   : |||
3'           CIGCITATACCTGGGIGTCCT
```

Primer construction

- Primers made using both Primer-BLAST and OligoAnalyzer
 - Primer-BLAST → picking primer pairs, specificity check
 - OligoAnalyzer → primer dimer analysis



Assignment

- Design a pair of primers to amplify the *CAT* (*chloramphenicol transacetylase*) gene of pCA24N
- Submit your candidate primers to Avenue to Learn.