ACEMFS FUT Minna Bioinformatics Workshop

Primer Design

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Primer Design

DNA & PCR Essentials

- **DNA Structure Review:**
  - DNA is a double helix composed of two complementary strands.
  - Bases: Adenine (A) pairs with Thymine (T); Guanine (G) pairs with Cytosine (C).
  - DNA is antiparallel; primers bind 3'→5' on template, extend 5'→3'
- **What is a Primer?**
  - A short, synthetic, single-stranded DNA sequence (typically 18-25 bases long).
  - They act as starting points for DNA polymerase.
  - Designing primers for PCR is an essential wet-lab skill with a bioinformatics foundation.

DNA & PCR Essentials

- **PCR Basics:**
  - **P**olymerase **C**hain **R**eaction: An in vitro method to amplify specific DNA segments.
  - **Three Steps:** Denaturation, Annealing, and Extension.
  - Specificity = primer sequence + annealing temp + template uniqueness
  - Amplicon length:
    - Conventional PCR: 100–1000 bp (sweet spot 300–800 bp)
    - qPCR: 70–200 bp (for efficiency)

Primer Design Rules

- **Length:** 18-25 nucleotides (20–22 nt typical).
- **Melting Temperature (Tm):**
  - The temperature at which half of the DNA duplex separates into single strands.
  - **Rule:** Primers should have a Tm between 55-65°C. For a primer pair, the Tm should be within a 5°C range of each other.
  - **Calculation:** Varies by formula (e.g., Wallace Rule: $Tm=2(A+T)+4(G+C)$). Tools calculate this for you.
- **GC Content:**
  - The percentage of Guanine and Cytosine bases.
  - **Rule:** Aim for 40-60%. High GC content can increase Tm and lead to stronger binding.

Primer Design Rules

- **A-T/G-C Clamps:** A G or C at the 3' end strengthens binding. Avoid an A or T.
- **Avoid:**
  - 3 G/Cs at 3' end in a row (non-specific priming)
  - Runs of ≥4 identical bases
  - Self-complementarity (hairpins) and cross-dimerization (ΔG too negative)
- **Amplicon:** pick region without long repeats, extreme GC, or low complexity
- **Check:** secondary structures, off-targets, SNPs, indels

Melting Temperature (Tm) Basics

- **Rule-of-thumb (for short primers):** Tm ≈ 2°C×(A+T) + 4°C×(G+C)
- **Preferred:** nearest-neighbor model with salt/oligo corrections (tool-computed)
- Aim for **annealing ≈ Tm–3 to –5 °C**; optimize empirically

Primer Secondary Structures & Specificity

- **Secondary Structures:**
  - **Hairpins:** A primer folding back on itself.
  - **Self-dimers:** Two identical primers binding to each other.
  - **Cross-dimers:** Two different primers binding to each other.
  - **Rule:** Avoid all of these! They compete with the target DNA and reduce PCR efficiency.
- **Specificity is Key:**
  - Primers must bind only to the intended target sequence.
  - **Primer-BLAST:** The essential tool to check for off-target binding in a genome.

Mycotoxin Pathways & Gene Targets

Aflatoxins (*Aspergillus flavus/parasificus*)

- Clustered genes; key regulator **aflR**; PKS **aflC/pksA**; structural genes (**aflD**, **aflM**, …)
- Targets: **aflR** (regulation), **aflD/aflM** (biosynthesis), **pksA** (polyketide step)

Trichothecenes (*Fusarium graminearum*, etc.)

- Core **TRI** cluster: **TRI5** (trichodiene synthase), **TRI4**, **TRI6**, **TRI10**
- Targets: **TRI5** for presence/chemotype panels

Ochratoxin A (*Aspergillus/Penicillium*)

- **ota** cluster (e.g., **otaA/pks**, **otaB**, **otaC**)—species dependent

Use NCBI Nucleotide/Gene to fetch sequences (genomic or mRNA/cDNA)

Applications & Assay-Specific Considerations

- **End-point PCR (screening/ID):** 300–800 bp; robust primers; gel visualization
- **qPCR/RT-qPCR (quantification):** 80–150 bp; high efficiency (90–110%); MIQE compliance; melt curve or probe
- **Sanger sequencing:** product ~600–900 bp; avoid homopolymers; flank mutation site
- **Mutation/SNP detection:** allele-specific primers or HRM; position variant at/near 3' end for AS-PCR
- **Multiplexing:** balanced Tm/GC, minimal primer-primer interactions, staggered product sizes

Primer Design Tools

- **Primer3:**
  - The classic, open-source tool.
  - **Strengths:** Highly customizable, calculates various primer metrics, excellent for designing standard PCR primers.
- **NCBI Primer-BLAST:**
  - Combines primer design with a powerful specificity check against the NCBI database.
  - **Strengths:** Ensures primers are unique and won't amplify off-target sequences.
- **OligoAnalyzer (IDT):**
  - A tool for analyzing existing primers.
  - **Strengths:** Calculates Tm, checks for hairpins and dimers, provides a comprehensive report on primer quality.
- **Other Tools:**
  - **Geneious:** Integrated software with a user-friendly primer design module.
  - **SnapGene:** Another integrated tool for molecular biology.

Workflow

- Get target DNA/mRNA sequence (GenBank/RefSeq; confirm species/strain)
- Define region (avoid low complexity; include/exclude sites as needed)
- Design candidate pairs in Primer3 (set product size & Tm)
- Check specificity in Primer-BLAST (select organism DB; add host crops to “exclude”)
- QC in OligoAnalyzer (hairpins/dimers, ΔG thresholds)
- Pick final pair(s); document/export; order
- Bench validation: gradient Ta, efficiency, specificity (melt curve/gel)

Common Pitfalls & Troubleshooting

No PCR Product:

- **Potential Cause:** Primers aren't binding (wrong Tm), DNA quality is poor, or primers are forming dimers.
- **Troubleshoot:** Re-design primers, check DNA concentration/quality, run a gel to check for dimers.

Non-Specific Bands:

- **Potential Cause:** Primers are binding to unintended sites in the genome.
- **Troubleshoot:** Increase annealing temperature, re-design primers with higher specificity, use Primer-BLAST.

Low Yield:

- **Potential Cause:** Sub-optimal primer design, low primer concentration, or poor enzyme performance.
- **Troubleshoot:** Optimize annealing temperature, check primer quality, increase primer concentration.

Demo 1: Primer3 (Design on Aflatoxin aflR)

Goal: Design qPCR-grade primers (100–150 bp) for **aflR** (Aspergillus flavus)
Inputs: *aflR* coding sequence (mRNA or genomic region)
Constraints: Length 20–24 nt; Tm 60 ± 1 °C; GC 40–60%; product 100–150 bp
Deliverables: 2–3 candidate primer pairs
Steps:
Get sequence: In NCBI Gene, search "*aflR Aspergillus flavus*". Open Gene record → pick RefSeq mRNA or genomic sequence; click **FASTA**; copy sequence.
Open Primer3/Primer3Plus web: paste sequence. Set:

- **Product Size Ranges**: 90-160
- **Primer Size**: 20–24; **Tm**: 59–61 °C; **GC%**: 40–60
- (Optional) Mark **Targets/Included/Excluded Regions** (e.g., avoid low complexity)

Click **Pick Primers**. Review top 5 pairs: lengths, Tm, GC, penalty score.
Copy candidates to a scratch sheet/notepad for downstream checks.

Primer3web version 4.1.0 - Pick primers from a DNA sequence.

disclaimer | code | cautions

Select the Task for primer selection: generic

Template masking before primer design (available species)

Select species	Example: Mus musculus	Nucleotides to mask in 5' direction	1
Primer failure rate cutoff	< 0.1	Nucleotides to mask in 3' direction	0

Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a Mispriming Library (repeat library): NONE

```
TATCTTGCGGATGGGAAGCTGTGGGAGGTAGAGGGTGCCCGAGCTGAAGCATTGGCCGCGACAAACTCCG
GGGGCTCGATTTCTGGGGCCGCTATCGTGGTGTCCCAACATGACTCTGTCCTGCTCTCTCTGGAGGATGG
AAAATTCTCCCCACTGGAAATGGATCTTGGAGGGGAAGGAAACGCAGATTTGGTTGGTGCGGCACGTCTC
GAATGGAAACCAAACTTGGACTTTGCCGATATGCATAGCCTTGTTCGCCCAAGTCATGGATCTATGAACG
ACGGCCC
```

Pick left primer, or use left primer below	Pick hybridization probe (internal oligo), or use oligo below	Pick right primer, or use right primer below (5' to 3' on opposite strand)

Pick Primers | Download Settings | Reset Form

Sequence Id		A string to identify your output.
Targets		E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the source sequence with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.
Overlap Junction List		E.g. 27 requires one primer to overlap the junction between positions 27 and 28. Or mark the source sequence with -: e.g. ...ATCTAC-TGTCAT.. means that primers must overlap the junction between the C and T.

Primer3Plus

pick primers from a DNA sequence

More... | Source Code | Help | About

Load server settings: Default

Activate Settings

Task: generic

Select primer pairs to detect the given template sequence. Optionally targets and included/excluded regions can be specified.

Pick Primers | Reset Default

Main | General Settings | Advanced Settings | Internal Oligo | Penalties | Advanced Seq.

Sequence Id:

Paste template sequence below or upload sequence file: Choose File No file chosen Load Example

```
TATCTTGCGGATGGGAAGCTGTGGGAGGTAGAGGGTGCCCGAGCTGAAGCATTGGCCGCGACAAACTCCG
GGGGCTCGATTTCTGGGGCCGCTATCGTGGTGTCCCAACATGACTCTGTCCTGCTCTCTCTGGAGGATGG
AAAATTCTCCCCACTGGAAATGGATCTTGGAGGGGAAGGAAACGCAGATTTGGTTGGTGCGGCACGTCTC
GAATGGAAACCAAACTTGGACTTTGCCGATATGCATAGCCTTGTTCGCCCAAGTCATGGATCTATGAACG
ACGGCCC
```

Mark selected region: < Excluded > | [Target] | { Included } | Clear | Regions from Seq. | Save Sequence

Excluded Regions: < >

Demo 2: Primer-BLAST (Specificity Check)

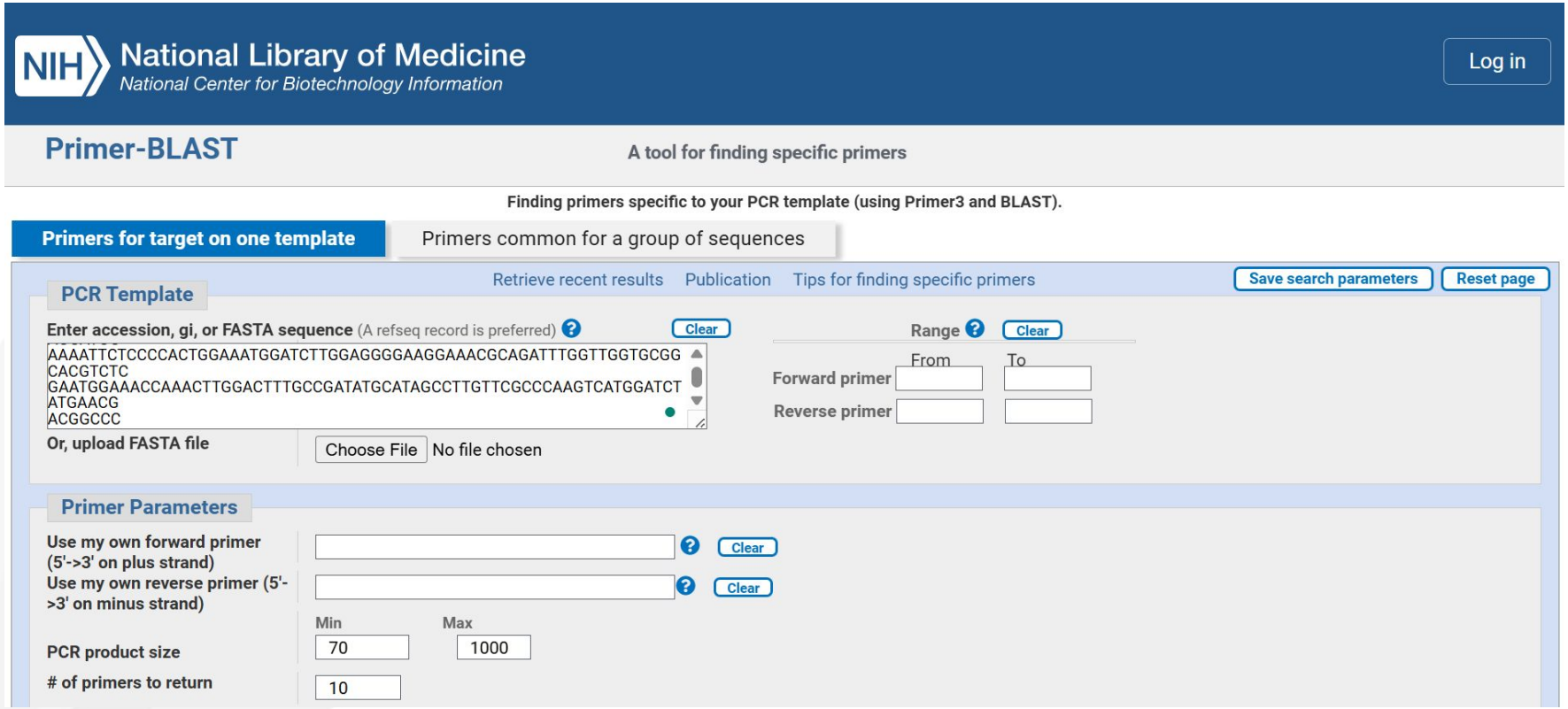
Goal: Verify **aflR** primer specificity
DB/Organisms:

- **Target include:** *Aspergillus flavus* (taxid 5059)
- **Also test against:** *Aspergillus* spp. (cross-reactivity), host crops (e.g., *Zea mays*, *Triticum aestivum*)

Settings: Default specificity **ON**; choose smallest relevant database (e.g., RefSeq genomic) for precision
Open **NCBI Primer-BLAST**.
Paste forward/reverse primers (from Primer3) **and** paste the template FASTA (improves alignment context).
Under **Primer Pair Specificity Checking**:

- **Organism**: *Aspergillus flavus*; database: **RefSeq genomic** (or **nr** for broad checks)
- Add additional organisms (maize/wheat/rice) to ensure no host hits

Get Primers / Submit → inspect **Amplification products** table, predicted sizes, off-target hits, and alignment details.
Export: "Send to → File" (or copy TSV). Keep Job ID for records.


National Library of Medicine
National Center for Biotechnology Information
Log in
Primer-BLAST
A tool for finding specific primers
Finding primers specific to your PCR template (using Primer3 and BLAST).
Primers for target on one template
Primers common for a group of sequences
Retrieve recent results
Publication
Tips for finding specific primers
Save search parameters
Reset page
PCR Template
Enter accession, gi, or FASTA sequence (A refseq record is preferred)
Clear
AAAATTCTCCCCACTGGAAATGGATCTTGGAGGGGAAGGAAACGCAGATTTGGTTGGTGCGG
CACGTCTC
GAATGGAAACCAAACTTGGACTTTGCCGATATGCATAGCCTTGTTCGCCCAAGTCATGGATCT
ATGAACG
ACGGCCC
Range
Clear
From
To
Forward primer
Reverse primer
Or, upload FASTA file
Choose 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
Min
Max
PCR product size
70
1000
# of primers to return
10