Primer Design

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PCR Reagents

- PCR buffer
- dNTP Mix
- Taq DNA polymerase
- Primers
- Template
- DDW
Very-Brief PCR Reminder

PCR is a method to amplify large quantities of a DNA covering a specific sequence.
What is a primer?

A primer is a short synthetic oligonucleotide which is used in many molecular techniques from **PCR** to **DNA sequencing**. These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal.
Why Are Primers Important?

- Primers are what gives PCR its SPECIFICITY!!!
- Good primer design: PCR works great.
- Bad primer design: PCR works terrible.
Good Primer’s Characteristic

Primer length

5..TCAACTTTAGCATGATCGGGTAGTAGCTTGACTGTACAACTCAGCAA.. 3'

18-24 bp for general applications
General rules for primer design

- Primer length determines the specificity and significantly affect its annealing to the template
  - Too short -- low specificity, resulting in non-specific amplification
  - Too long -- decrease the template-binding efficiency at normal annealing temperature due to the higher probability of forming secondary structures such as hairpins.

Too short---less specific
Too long---wasting money
Base Composition

- Usually, average (G+C) content around 50-60% will give us the right melting/annealing temperature for ordinary PCR reactions, and will give appropriate hybridization stability.

5 GTGGATGTGGTGTCGATGCGATGAGC 3
Max 3’end stability

It’s critical that the stability at 3’ end be high

5 GTGGATGTGGTGTCGATGGC 3
Primer melting temperature (Tm):

- The melting temperature (Tm) is the most important factor in determining the optimal PCR annealing temperature (Ta).

Melting Tm between 50-70 °C are preferred
Tm Calculation

Wallace rule:

\[ Tm = 4 \times (G + C) + 2 \times (A + T) \]

Bolton and McCarthy:

\[ Tm = 81.5 + 16.6 \times \log [I] + 0.41 \times (%GC) - 600/L \]

The nearest neighbor method (Santalucia et.al, 1998):

\[ Tm_{NN} = \frac{\Delta H}{\Delta S + R \cdot \ln \left( \frac{c}{4} \right)} - 273.15 + 16.6 \cdot \log (K^+) \]
Annealing temperatures

37 – 60°C gradient
Primer Pair Matching

Primers work in pairs – forward primer and reverse primer. Since they are used in the same PCR reaction, it shall be ensured that the PCR condition is suitable for both of them.

One critical feature is their annealing temperatures, which shall be compatible with each other. The maximum difference allowed is 3 °C. The closer their $T_{\text{anneal}}$ are, the better.

<table>
<thead>
<tr>
<th>5 CTGATCAAGTCGATGGCTTG 3</th>
<th>Fw</th>
<th>59 C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 GATGGAGAGGCTTTGACTGC 3</td>
<td>Rv</td>
<td>58 C</td>
</tr>
</tbody>
</table>
A. Avoid hairpin and stem-loop formation

Hairpin: $^2\text{G} = -0.7 \text{ kcal/mol}$, Loop = 8 nt, Tm = 41°

5' CCAGTCGTT

3' ACAGTCAAAACA
Avoid complementary at 3` end of primers
when is a “primer” a primer?
PCR primers are designed to:

- For Cloning a special sequence
  - Full length

- For Detection
  - Gene of interest
  - Gene expression (mRNA)
  - Microbial agents detection
  - Mutation Detection
  - Quantification
  - Allelic discrimination
  - Disease

- Random
  - ??
Random primers
Example: RAPD-PCR
RAPD = Random Amplified Polymorphic DNA

5`-TCG GCG GTT C-3`
Primer 5:6-d

Fs-7
Fm-13
Fm-20
Fo-28
Fo-15
Fs-19
Universal Primers

Primers can be designed to amplify only one product.
Primers can also be designed to amplify multiple products. We call such primers “universal primers”. For example, design primers to amplify all HPV genes.

Strategy:
1. Align groups of sequences you want to amplify.
2. Find the most conservative regions at 5’ end and at 3’ end.
3. Design forward primer at the 5’ conservative region.
4. Design reverse primer at the 3’ conservative regions.
5. Matching forward and reverse primers to find the best pair.
6. Ensure uniqueness in all template sequences.
7. Ensure uniqueness in possible contaminant sources.
CLUSTAL multiple sequence alignment

WDV
ACCCCGCGTGGTGCCGCCGACGCACCTCGGCTTTTTCGTAGTGCACGGGACGCTTTTG
WDV-TAI
ACCCCGCGTGGTGCCGCCGACGCACCTCGGCTTTTTCGTAGTGCACGGGACGCTTTTG
WDV-F
ACCCCGCGTGGTGCCGCCGACGCACCTCGGCTTTTTCGTAGTGCACGGGACGCTTTTG
WDV-B
ACCCCGCGTGGTGCCGCCGACGCACCTCGGCTTTTTCGTAGTGCACGGGACGCTTTTG
WDV-SWE
ACCCCGCGTGGTGCCGCCGACGCACCTCGGCTTTTTCGTAGTGCACGGGACGCTTTTG
WDV-ENK
ACCCCGCGTGGTGCCGCCGACGCACCTCGGCTTTTTCGTAGTGCACGGGACGCTTTTG
WDV-BAR
ACCCCGCGTGGTGCCGCCGACGCACCTCGGCTTTTTCGTAGTGCACGGGACGCTTTTG

**** ******* ******* ***************** ***************** ********

WDV
ACCGCATCTTCTTCTG-ATCACTTTTCTGTGGAAGATGTTGATTTATACACACTTTTGACTTTG
WDV-TAI
ACCGCATCTTCTTCTG-ATCACTTTTCTGTGGAAGATGTTGATTTATACACACTTTTGACTTTG
WDV-F
ACCGCATCTTCTTCTG-ATCACTTTTCTGTGGAAGATGTTGATTTATACACACTTTTGACTTTG
WDV-B
ACCGCATCTTCTTCTG-ATCACTTTTCTGTGGAAGATGTTGATTTATACACACTTTTGACTTTG
WDV-SWE
ACCGCATCTTCTTCTG-ATCACTTTTCTGTGGAAGATGTTGATTTATACACACTTTTGACTTTG
WDV-ENK
ACCGCATCTTCTTCTG-ATCACTTTTCTGTGGAAGATGTTGATTTATACACACTTTTGACTTTG
WDV-BAR
ACCGCATCTTCTTCTG-ATCACTTTTCTGTGGAAGATGTTGATTTATACACACTTTTGACTTTG

**** ******* ******* ******** ******* ******* ***** ******

WDV
AAATCTGTGCCATGGCTTAGCTTATAAGGAGATGCGGAGCTCACCCTCGATGAGCACG
WDV-TAI
AAATCTGTGCCATGGCTTAGCTTATAAGGAGATGCGGAGCTCACCCTCGATGAGCACG
WDV-F
AAATCTGTGCCATGGCTTAGCTTATAAGGAGATGCGGAGCTCACCCTCGATGAGCACG
WDV-B
AAATCTGTGCCATGGCTTAGCTTATAAGGAGATGCGGAGCTCACCCTCGATGAGCACG
WDV-SWE
AAATCTGTGCCATGGCTTAGCTTATAAGGAGATGCGGAGCTCACCCTCGATGAGCACG
WDV-ENK
AAATCTGTGCCATGGCTTAGCTTATAAGGAGATGCGGAGCTCACCCTCGATGAGCACG
WDV-BAR
AAATCTGTGCCATGGCTTAGCTTATAAGGAGATGCGGAGCTCACCCTCGATGAGCACG

** ******* ******* ***************** ** *******
Cloning Overview

Four main steps in cloning:
- Insert synthesis
- Restriction enzyme digest
- Ligation
- Transformation
5’ \rightarrow 3’

↓ PCR

RE
Ligation covalently attaches the vector and the insert via a phosphodiester bond (5’phosphate and 3’ hydroxyl of the next base)
### Restriction enzymes (NEB)

<table>
<thead>
<tr>
<th>Oligo sequence</th>
<th>% Cleavage 2h</th>
<th>% Cleavage 20h</th>
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<tbody>
<tr>
<td><strong>BamHI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGGAATCCGG</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>CGGGATCCCG</td>
<td>&gt;90</td>
<td>&gt;90</td>
</tr>
<tr>
<td>CGCGGAATCCGCG</td>
<td>&gt;90</td>
<td>&gt;90</td>
</tr>
<tr>
<td><strong>EcoRI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGAAATTCGG</td>
<td>&gt;90</td>
<td>&gt;90</td>
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<tr>
<td>CGGGAAATTCGG</td>
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<td>&gt;90</td>
</tr>
<tr>
<td>CCGGGAAATTCGGG</td>
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<td>&gt;90</td>
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<tr>
<td><strong>HindIII</strong></td>
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<td>CAAAGCTTTG</td>
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<td>0</td>
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<td>CCCCAAGCTTTGGG</td>
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<td><strong>NcoI</strong></td>
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<tr>
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<tr>
<td>CATGCCATGGCATG</td>
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<td><strong>NdeI</strong></td>
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<tr>
<td>GGGTTTCCATATGGAACCC</td>
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<td>0</td>
</tr>
<tr>
<td>GGAATTCCCATATGGAATTCC</td>
<td>75</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>
Site-directed mutagenesis

Step 1: Plasmid Preparation
- Amplification using mutagenic primers
- PCR

Step 2: Temperature Cycling
- Template
- Synthesized

Step 3: Digestion
- DpnI Digestion of Template DNA
- 5′...GATC...3′
- 3′...CTAG...5′

Step 4: Transformation
- Mutated plasmid (contains nicked circular strands)

Colonies With Mutated Gene
Excess of this primer → "Asymmetric" PCR

Use as a "megaprimer" for second PCR reaction

Gene with desired insertion
## Web-based Softwares

<table>
<thead>
<tr>
<th>Tool name</th>
<th>URL</th>
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<tbody>
<tr>
<td>CODEHOP</td>
<td><a href="http://blocks.fhcrc.org/codehop.html">http://blocks.fhcrc.org/codehop.html</a></td>
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<tr>
<td>Gene Fisher</td>
<td><a href="http://bibiserv.techfak.uni-bielefeld.de/genefisher/">http://bibiserv.techfak.uni-bielefeld.de/genefisher/</a></td>
</tr>
<tr>
<td>DoPrimer</td>
<td><a href="http://doprimer.interactiva.de/">http://doprimer.interactiva.de/</a></td>
</tr>
<tr>
<td>Primer3</td>
<td><a href="http://frodo.wi.mit.edu/primer3/">http://frodo.wi.mit.edu/primer3/</a></td>
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<tr>
<td>Primer Selection</td>
<td><a href="http://alces.med.umn.edu/rawprimer.html">http://alces.med.umn.edu/rawprimer.html</a></td>
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<td>Web Primer</td>
<td><a href="http://genome.www2.stanford.edu/cgi.bin/SGD/web.primer">http://genome.www2.stanford.edu/cgi.bin/SGD/web.primer</a></td>
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<td>PCR designer</td>
<td><a href="http://cedar.genetics.ston.ac.uk/public_html/primer.html">http://cedar.genetics.ston.ac.uk/public_html/primer.html</a></td>
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<td>Primo pro 3.4</td>
<td><a href="http://www.changbioscience.com/primo.html">http://www.changbioscience.com/primo.html</a></td>
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<td>Primo Degenerate 3.4</td>
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<td>The Primer Generator</td>
<td><a href="http://www.med.jhu.edu/medcenter/primer/primer.cgi">http://www.med.jhu.edu/medcenter/primer/primer.cgi</a></td>
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<td>EPRIMERS</td>
<td><a href="http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html">http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html</a></td>
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<td>PRIMO</td>
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<td>PrimerQuest</td>
<td><a href="http://www.idtdna.com/biotools/primer_quest/primer_quest.asp">http://www.idtdna.com/biotools/primer_quest/primer_quest.asp</a></td>
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<td>MethPrimer</td>
<td><a href="http://itsa.uscf/~uralab/methprimer/index1.html">http://itsa.uscf/~uralab/methprimer/index1.html</a></td>
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<td>Rawprimer</td>
<td><a href="http://alces.med.umn.edu/rawprimer.html">http://alces.med.umn.edu/rawprimer.html</a></td>
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<td>MEDUSA</td>
<td><a href="http://www.cgr.ki.se/cgr/MEDUSA/">http://www.cgr.ki.se/cgr/MEDUSA/</a></td>
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<tr>
<td>The Primer Prim’er Project</td>
<td><a href="http://www.nmr.cabm.rutgers.edu/bioinformatics/primer_primer_project/primer.html">http://www.nmr.cabm.rutgers.edu/bioinformatics/primer_primer_project/primer.html</a></td>
</tr>
<tr>
<td>GAP</td>
<td><a href="http://promoter.ics.uci.edu/primers/">http://promoter.ics.uci.edu/primers/</a></td>
</tr>
<tr>
<td>Software name</td>
<td>Description</td>
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<tr>
<td>Primerselect</td>
<td>Analyses a template DNA sequence and chooses primer pairs for PCR and primers for DNA sequencing</td>
</tr>
<tr>
<td>DANSIS Max</td>
<td>DANASIS Max is a fully integrated program that includes a wide range of standard sequence analysis features.</td>
</tr>
<tr>
<td>Primer Primer 5</td>
<td>Primer design for windows and power macintosh.</td>
</tr>
<tr>
<td>Primer Primer</td>
<td>Comprehensive primer design for windows and Power Macintosh.</td>
</tr>
<tr>
<td>NetPrimer</td>
<td>Comprehensive analysis of individual primers and primer pairs.</td>
</tr>
<tr>
<td>Array Designer 2</td>
<td>For fast, effective design of specific oligos or PCR primer pairs for microarrays.</td>
</tr>
<tr>
<td>AlleleID 7</td>
<td>Design molecular beacons and TaqMan probes for robust amplification and fluorescence in real time PCR.</td>
</tr>
<tr>
<td>GenomePRIDE 1.0</td>
<td>Primer design for DNA-arrays/chips.</td>
</tr>
<tr>
<td>Fast PCR</td>
<td>Software for Microsoft Windows has specific. Ready-to-use template for many PCR and sequencing applications; standard and long PCR inverse PCR. Degenerate PCR directly on amino acid sequence. Multiplex PCR.</td>
</tr>
<tr>
<td>OLIIGO 7</td>
<td>Primer Analysis Software for Mac and Windows.</td>
</tr>
<tr>
<td>Primer Designer 4</td>
<td>Will find optimal primers in target regions of DNA or protein molecules, amplify features in molecules, or create products of a specified length.</td>
</tr>
<tr>
<td>GPRIME</td>
<td>Software for primer design.</td>
</tr>
<tr>
<td>Sarani Gold</td>
<td>Genome Oligo Designer is a Software for automatic large scale design of optimal oligonucleotide probes for microarray experiments.</td>
</tr>
<tr>
<td>PCR Help</td>
<td>Primer and template design and analysis.</td>
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<tr>
<td>Genorama chip Design</td>
<td>Genorama Chip Design Software is a complete set of programs required for genotyping chip design. The programs can also be bought separately.</td>
</tr>
<tr>
<td>Primer Designer</td>
<td>The Primer Designer features a powerful, yet extremely simple, real-time interface to allow the rapid identification of theoretical ideal primers for your PCR reactions.</td>
</tr>
<tr>
<td>Primer Primer</td>
<td>Automatic design tools for PCR. Sequencing or hybridization probes, degenerate primer design, restriction, Nested/Multiplex primer design, restriction enzyme analysis and more.</td>
</tr>
<tr>
<td>PreimerDesign</td>
<td>DOS-program to choose primer for PCR or oligonucleotide probes.</td>
</tr>
</tbody>
</table>
Primer3
(v. 0.4.0) Pick primers from a DNA sequence.

Paste source sequence below (5'→3', string of ACGTNggtn -- 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

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  Reset Form

Sequence Id
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.

Excluded 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>TACAT... forbids primers in the central CCCC.

Product Size Ranges 150-260 100-300 301-400 401-500 501-600 601-700 701-850 851-1000

Number To Return: 6 Max 3 Stability 90

Max Repeat Mispriming 12.00 Pair Max Repeat Mispriming 24.00
Max Template Mispriming 12.00 Pair Max Template Mispriming 24.00

Pick Primers  Reset Form
### General Primer Picking Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Min</th>
<th>Opt</th>
<th>Max</th>
<th>Max Tm Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer Size</td>
<td>18</td>
<td>20</td>
<td>22</td>
<td>2</td>
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<tr>
<td>Primer Tm</td>
<td>55</td>
<td>58.0</td>
<td>61</td>
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<tr>
<td>Product Tm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer GC%</td>
<td>45</td>
<td></td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

- **Max Self Complementarity:** 4
- **Max 3' Self Complementarity:** 3.00
- **Max #N's:** 0
- **Max Poly-X:** 3
- **Inside Target Penalty:**
- **Outside Target Penalty:** 0
- **First Base Index:** 1
- **CG Clamp:** 0
- **Salt Concentration:** 50.0
- **Annealing Oligo Concentration:** 50.0

- **Liberal Base**
- **Show Debugging Info**
- **Do not treat ambiguity codes in libraries as consensus**

**Pick Primers**  **Reset Form**

*Set Inside Target Penalty to allow primers inside a target.*

*Not the concentration of oligos in the reaction mix but of those annealing to template.*
Primer3 Output

PRODUCT SIZE: 356, PAIR ANY COMPL: 7.00, PAIR 3' COMPL: 3.00

1  GTCTAAGGAGCTGCGCATAGGACCTAAAAGTTGATGATGCTGCTGTTCATATA

61  TGTCAAGGGAACATATCATATTCTGGGCGCCCTTTCCACCAGTGAGATCCCATGTC

121  ATATTGGATTACAATAATCTGTTGGAGGACATAGATAAACATGCGAGGAGATTCCAC

181  TGTGACACCCCTGGAGGCTCAACATGACTGACAAATTGGAACAAATGACCACATGAAAGAGCT

241  CATTGACAAAATCTGGTGGACAAAGACTGCTAGGGGGTTTGTATTCTTCTTTTTTTTGTGAATAT

301  CAATGTGACTCTCTGAGGCTCTACAGGATATTCTTCTGTCACCAATGGTGCCAGGAGAAGGTG

361  GTGCGGGGGCCACCACCTGCGATATTTCTTCTGTCACCAATGGTGCCAGGAGAAGGTG

421  AGGTGATCTGCTCAAGGTGAGCGAACGATAATGCGACCTCTCGGAGACCACAGTTAAGCT

481  GAACCATCTCTGTACCTACGTTGACCAGTCAAGTGACAACATCATCATAGAGACGCTGAA

54  CCATGAACATATGAGTGCAAAATACGTAATTATCGATACCTCCGACCTTTGACTGCCAA

 KEYS (in order of precedence):
 >>>>>> left primer
 <<<<<< right primer