

Primer Design

Ameer Effat M. Elfarash

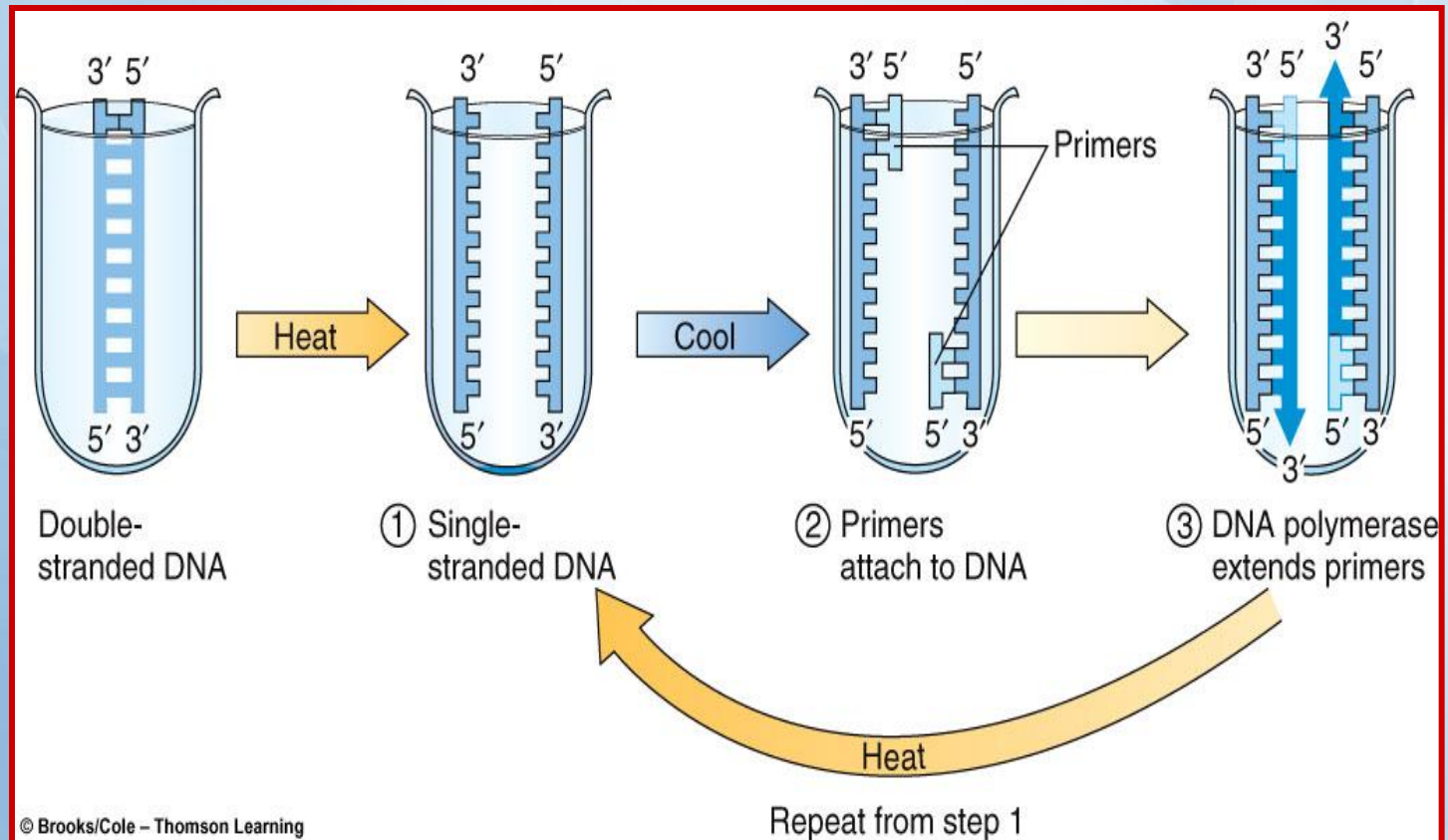
Dept. of Genetics
Fac. of Agriculture, Assiut Univ.
amir_effat@yahoo.com

PCR Reagents

- PCR buffer
- dNTP Mix
- Taq DNA polymerase
- **Primers**
- Template
- DDW

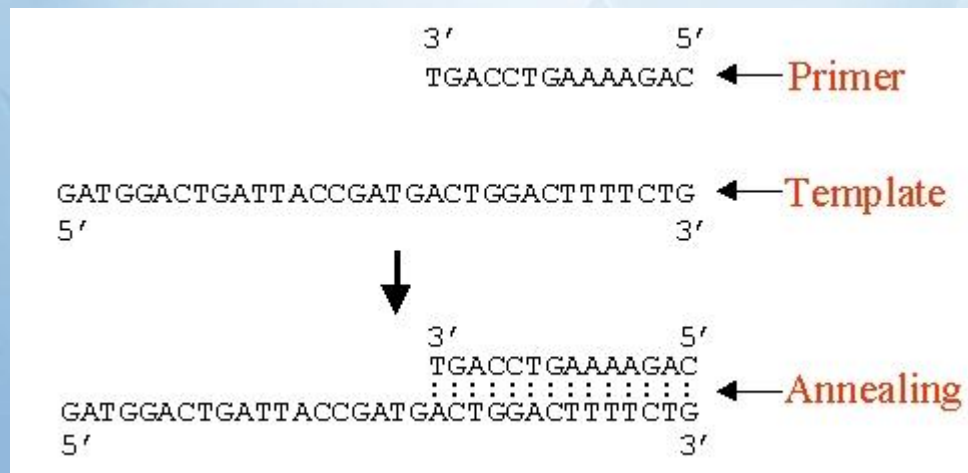
Very-Brief PCR Reminder

- 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...TCAACTTAGCATGATCGGGTAGTAGCTTGACTGTACAACCTCA

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 GTGGATGTGGTGTCTGATGGC 3



Max 3' end stability

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

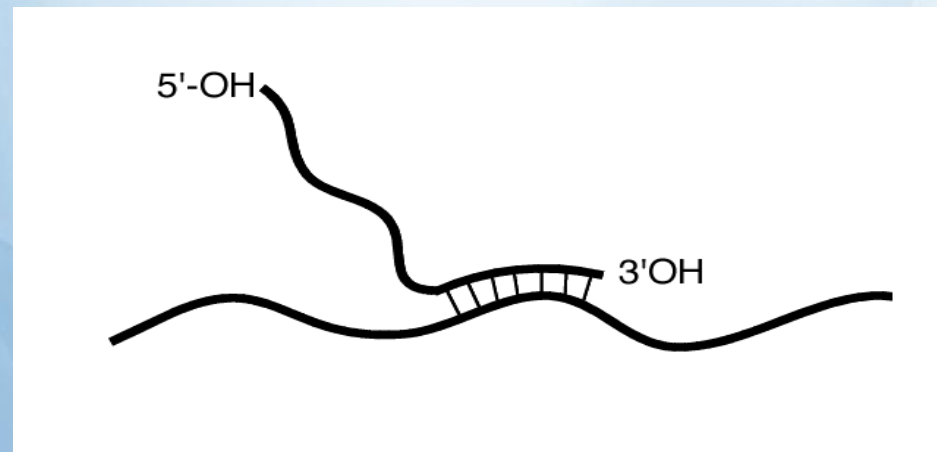
5 GTGGATGTGGTGTCTGATGGC 3



Primer melting temperature (T_m):

- ✓ The melting temperature (T_m) is the most important factor in determining the optimal PCR annealing temperature (T_a).

Melting T_m between 50-70 °C are preferred



T_m Calculation

✦ Wallace rule:

$$T_m = 4 * (G + C) + 2 * (A + T)$$

✦ Bolton and McCarthy:

$$T_m = 81.5 + 16.6 * \log [I] + 0.41 * (\%GC) - 600/L$$

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

$$T_{m_{NN}} = \frac{\Delta H}{\Delta S + R \cdot \ln\left(\frac{c}{4}\right)} - 273.15 + 16.6 \cdot \log(K^+)$$

Melting Temperature



Oligo sequence

CGCACTTCCAACAACCCTTC

Length 20 GC% 55.0 MW(kD) 5.98

Melting Temperature (°C):

Thermo 59.4 Hybrid 52.5 GC+AT 62.0

[DNA] (nM) 50

DNA/RNA D:D

[Na+](mM) 50

Formamide(%) 0

Mismatch(bp) 0

Show Tm

Report

Cancel

Melting Temperature



Oligo sequence

AACAACCCTTCCGCACTTCCAACAACCCTTCACAACCCTTC

Length 50 GC% 54.0 MW(kD) 14.94

Melting Temperature (°C):

Thermo 80.8 Hybrid 70.0 GC+AT 154.0

[DNA] (nM) 50

DNA/RNA D:D

[Na+](mM) 50

Formamide(%) 0

Mismatch(bp) 0

Show Tm

Report

Cancel

Annealing temperatures

37 – 60°C
gradient





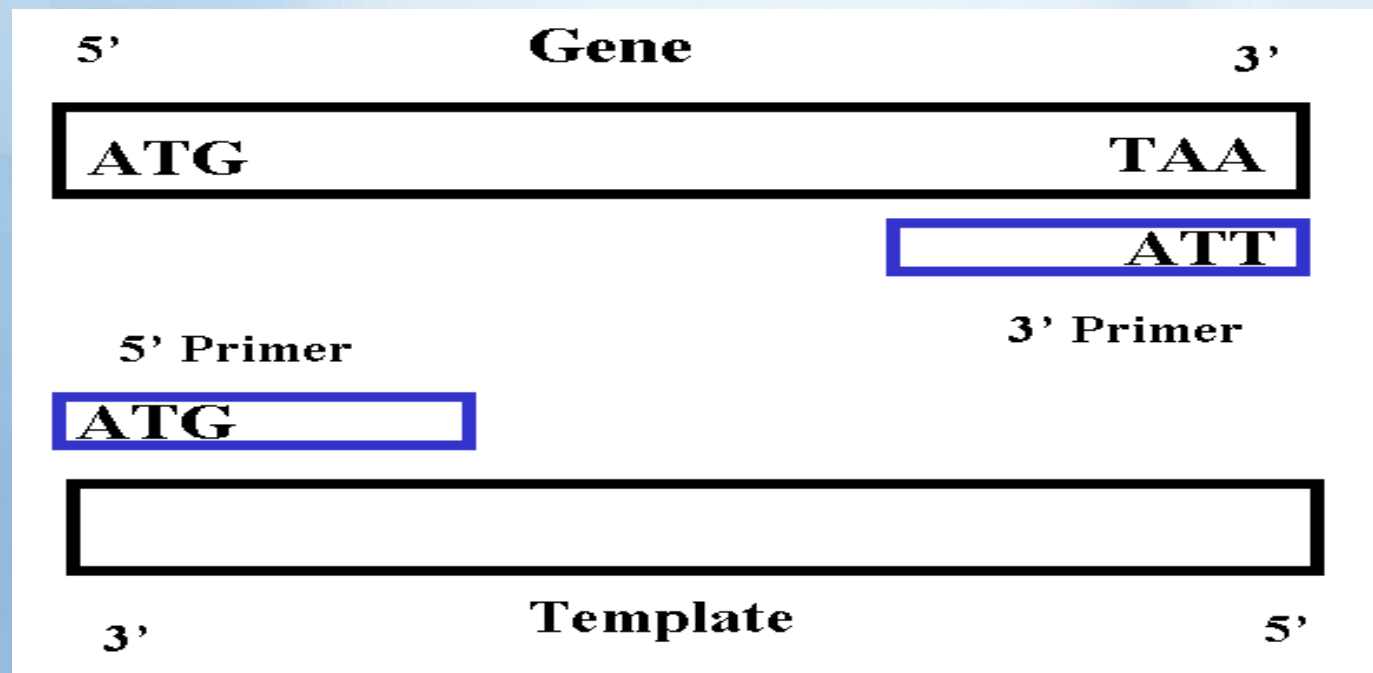
Primer Pair Matching

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_{anneal} are, the better.

5 CTGATCAAGTCGATGGCTTG 3	Fw	59 C
5 GATGGAGAGGCTTGACTGC 3	Rv	58 C

ATGTCCAATGACAACGAAGTACCTGGTTCCATGGTTATTGTCGCACAAGGTCCAGACGATCAATACGCATACGAGGTTCCCCCTATCGATAGCGGGCCG
 TTGCCGGGAATATGTTTGGCGACTTAATTCAAAAGAGAAATATATCTACAGAAAAACATTTATTATCCAGTCCGATCTATTTTTGAACAAGGAACAAAAGA
 AAAGAAGGAGATCAACAAGAAAGTATCTGATCAAGTCGATGGCTTGCTAAAGCAGATCACTCAAGGAAAAAGGGAGGCCACAAGGCAAGAGCGAGTCGAT
 GTCATGTCGGCAGTCCTGCACAAGATGGAATCTGATCTTGAAGGATACAAAAAGACCTTTACCAAAGGCCCATTCATTGACTACGAAAAGCAGTCAAGCC
 TCTCCATCTATGAGGCCTGGGTCAAGATCTGGGAGAAGAACTCTTGGGAAGAAAGAAAGAGTACCCTTTTCAGCAGCTTGTTAGAGATGAACTGGAGCG
 GGCGGTTGCCTACTACAAACAAGATTCACCTCTCTGAAGCGGTAAAAGTGCTAAGACAGGAGCTCAACAAGCAAAAAGCGCTAAAGGAAAAAGAGGACCTC
 TCTCAACTGGAGCGGGACTACAGAACCCGAAAGGCCAATCTCGAGATGAAAGTACAATCCGAGCTTGATCAAGCGGGAAGTGCTTTGCCTCCATTGGTCA
 GTCCAACGCCAGAGCAATGGCTTGAACGTGCCACAAGACTGGTTACGCAAGCAATTGCTGATAAAAAGCAGCTGCAGACCACAAACAATACTCTTATCAA
 GAATTCCCCAACCCCTCTAGAAAAGCAGAAAGCCATCTACAATGGTGAGCTACTTGTGGATGAGATAGCCAGTCTACAGGCCCGCTTAGTTAAGCTGAAC
 GCCGAAACGACACGACGACGAGGACAGAAGCAGAACGCAAGGCGGCCGAGGAACAAGCGTTGCAAGATGCTATTAAATTTACTGCCGACTTTTATAAGGAAG
 TAACTGAGAAATTTGGCGCACGAACATCGGAGATGGCGCGCCAACTGGCCGAAGGCGCCAGGGGGAAAAATATCAGGAGTTCGGCGGAAGCAATCAAGTC
 GTTTGAAAAGCACAAGGATGCGTTAAATAAAAAAAGCTTAGCCTTAAAGATAGGCAAGCCATTGCCAAAGCCTTTGATTCTCTAGACAAGCAGATGATGGCG
 AAGAGCCTTGAGAAATTTAGCAAAGGCTTTGGAGTTGTAGGCAAAGCTATTGACGCCGCCAGCCTGTACCAAGAGTTCAAGATATCTACGGAACCGGGG
 ACTGGAAACCATTCTTTGTAAAAATTGAAACACTAGCTGCTGGTGCGGCCGCCAGTTGGCTTGTGGGTATTGCATTTGCCACGGCAACAGCCACTCCTAT
 AGGCATTCTGGGGTTCGCACTGGTAATGGCAGTTACCGGGGCGATGATTGACGAAGACCTTCTAGAAAAAGCAAACAATCTTGTAAATATCCATTTAA



Avoid

A. Avoid hairpin and stem-loop formation

Hairpin: $\Delta G = -0.7$ kcal/mol, Loop = 8 nt, $T_m = 41^\circ$

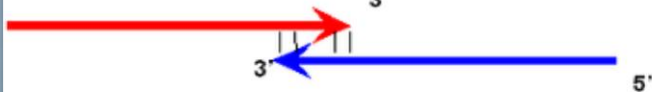


● Avoid complementary at 3' end of primers

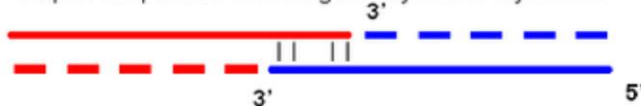
Primer dimer formation

-  Primer, the arrow indicates the elongation side, i.e., the 3' end,
-  DNA elongated from the 3' end of the primer
-  Hydrogen bonds between two complementary bases

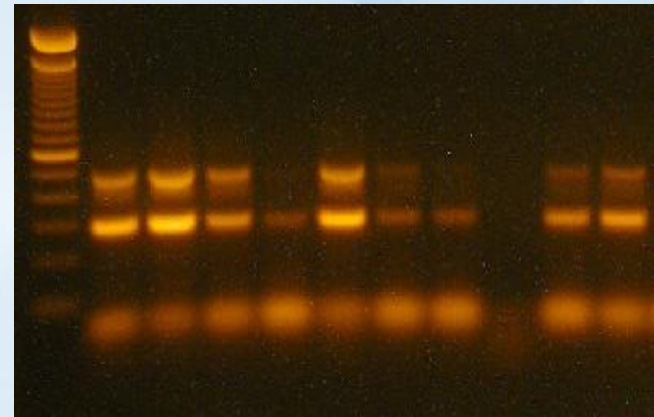
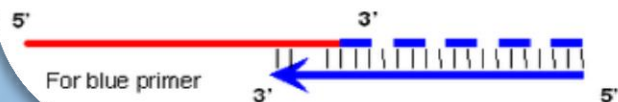
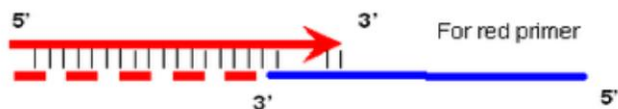
Step I: the primers are attached in their 3' end



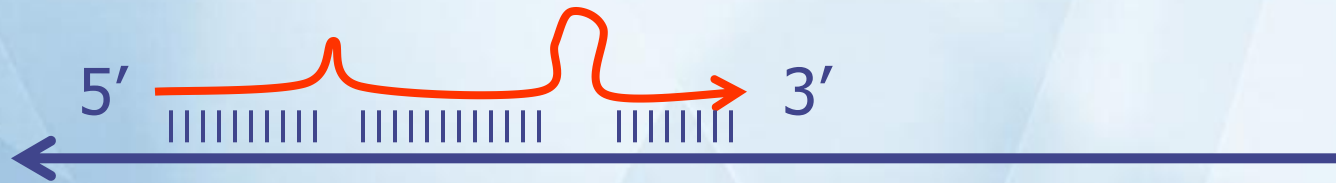
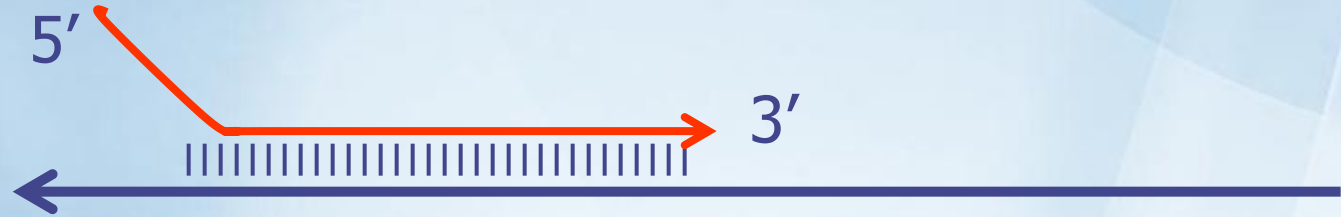
Step II: the primers are elongated by DNA Polymerase



Step III: in the following cycle the elongated primer binds its complementary primer with high affinity



when is a "primer" a primer?



PCR primers are designed to:

For Cloning a special sequence

Full length

For Detection

100 - 500 bp

Random

??

Gene of interest

Gene expression (mRNA)

Microbial agents detection

Mutation Detection

Quantification

Allelic discrimination

Disease

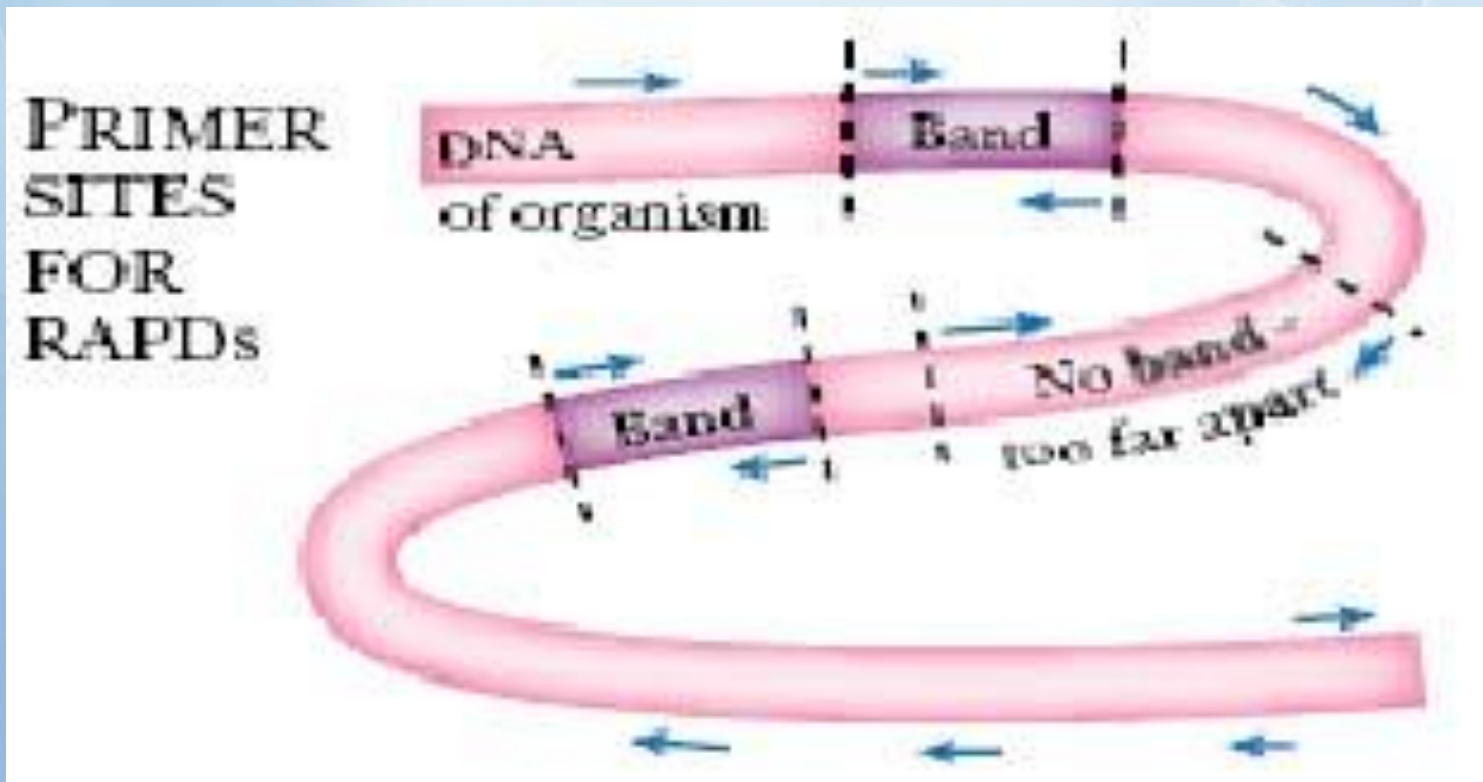
...

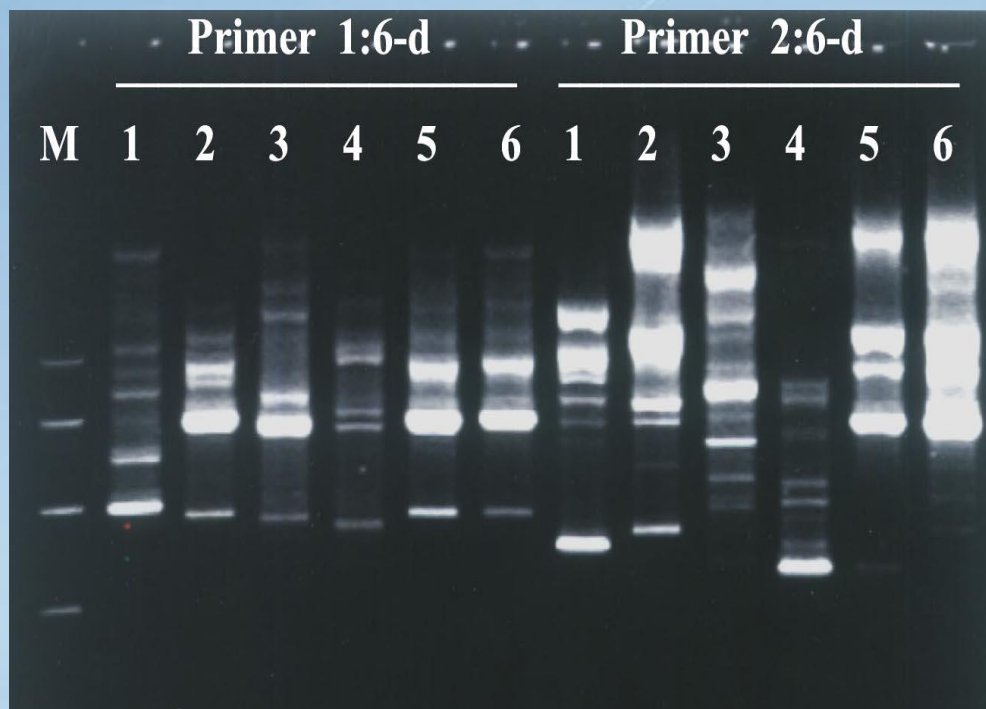
Random primers

Example: RAPD-PCR

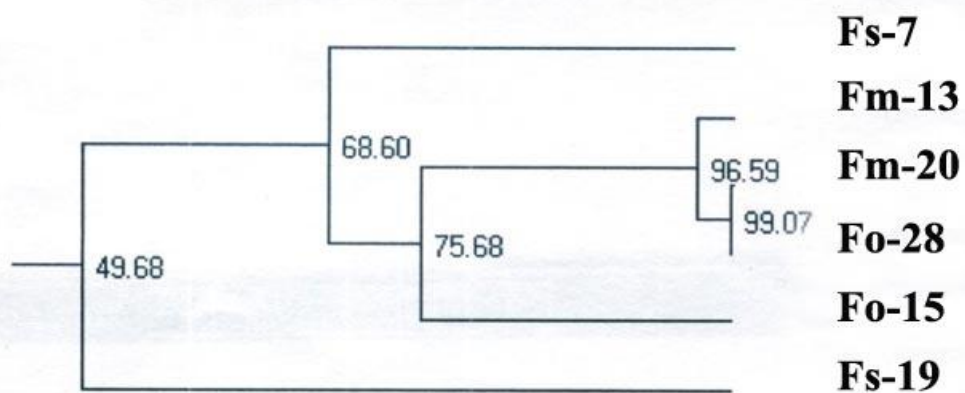
RAPD = Random Amplified Polymorphic DNA

5'-TCG GCG GTT C-3'





Primer 5:6-d



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.

```

Untitled
CLUSTAL multiple sequence alignment

WDV          ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-TAI      ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-F        ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-B        ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-SWE      ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-ENK      ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-BAR      ACCCTGCGTGGTGGGTCCCGAGGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
*****

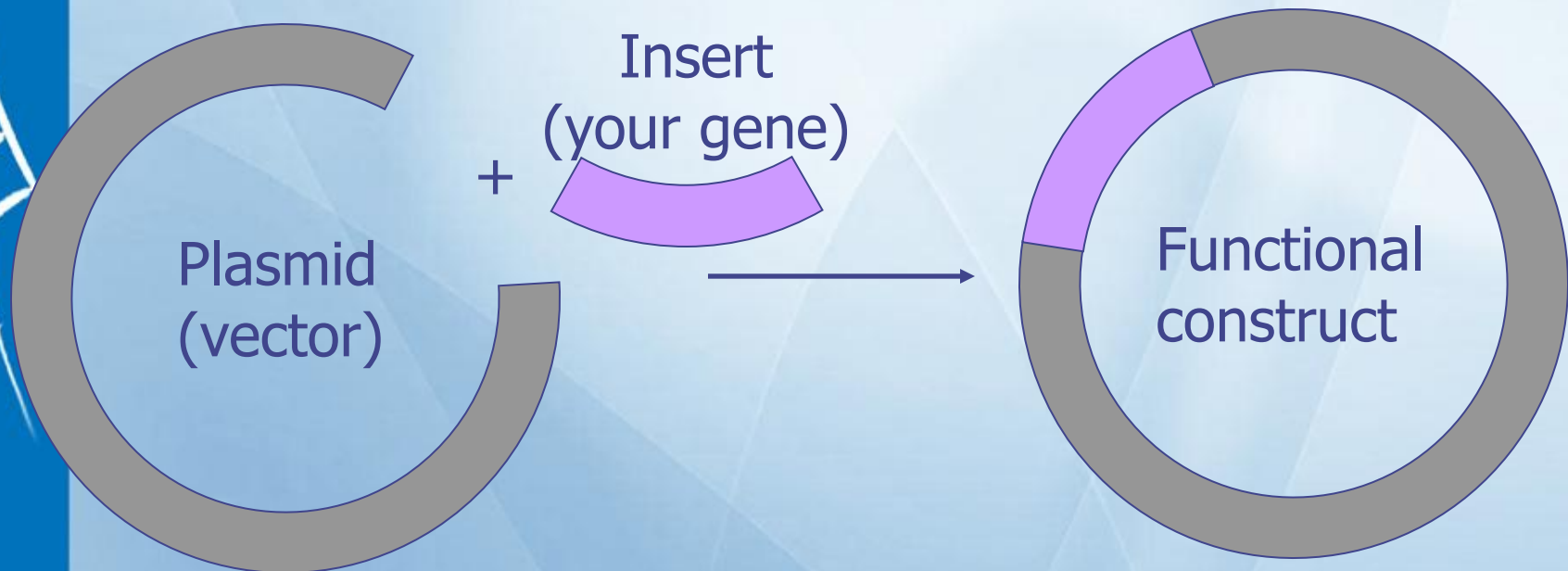
WDV          ACCACATCTTTTCTG-ATCACTTTCGTGGAAGATGTTGATTTATCACACTTTTGACTTGG
WDV-TAI      ACCACATCTTTTCTG-ATCACTTTCGTGGAAGATGGTGATTTATCACACTTTTGATGTGG
WDV-F        ACCACATCTTTTCTG-AGAACTTTCGTGGAAGATGGTGATTTATCACACTTTTGACGCGG
WDV-B        ACCACATCTTTTCTG-AGAACTTTCGTGGAAGATGGTGATTTATCACACTTTTGACGCGG
WDV-SWE      ACCACATCTTTTCTG-ATCACTTTCGTGGAAGATGTTGATTTATCACACTTTTGACGGGG
WDV-ENK      ACCACATCTTTTCTG-ATCACTTTCGTGGAAGATGTTGATTTATCACACTTTTGACTTGG
WDV-BAR      ACCACGTCTTTATGTTATCACTCCAATAATTAGAGGTGAGTCATCACTCTTTGCACCTGC
*****

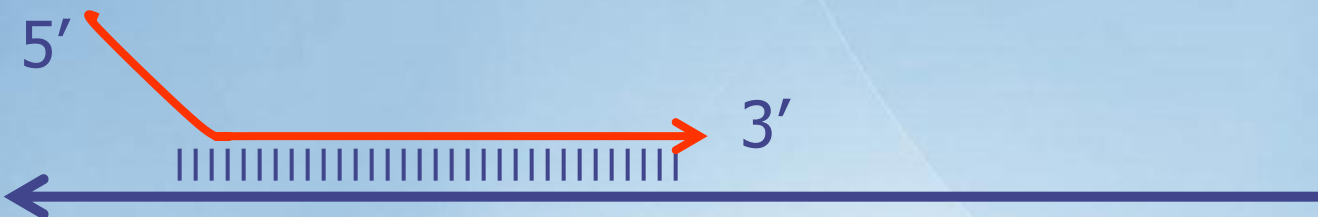
WDV          AAATCTGTGCCATGCCTTAGCTTATAAGGAAAGTGCGCGGTAGCCCATCTCGATGGAGCAG
WDV-TAI      AAATGTGTGCCATGCCTTAGCTTATAAGGAAAGTGCGTGGGAGCCCATCTCGATGGAGCAG
WDV-F        AAATGTGTGCCATGCCTTAGCTTATAAGGAAAGTGCGTGGTAGCCCATCTCGATGGAGCAG
WDV-B        AAATGTGTCCCATGCCTTAGCTTATAAGGAAAGTGCGTGGTAGCCCATCTCGATGGAGCAG
WDV-SWE      AAATCTGTGCCATGCCTTAGCTTATAAGGAAAGTGCGTGGTAGCCCATCTCGATGGAGCAG
WDV-ENK      AAATCTGTGCCATGCCTTAGCTTATAAGGAAAGTGCGCGGTAGCCCATCTCGATGGAGCAG
WDV-BAR      AATTATGTGGCATCGCTTAGCTTATAAGGAAAGTGTCGGGGAAGATATCTCGATGGATCAG
** * ***   ***   *****   ** *   *****   **
  
```


Cloning Overview

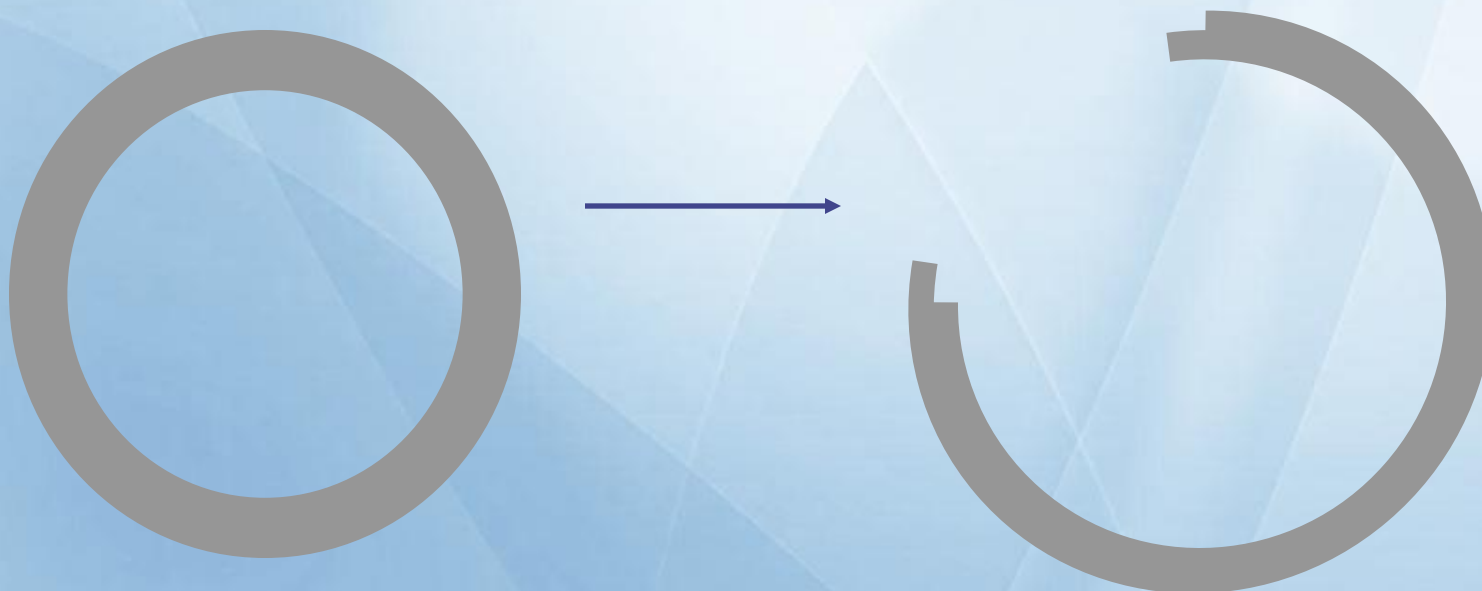
Four main steps in cloning:

- Insert synthesis
- Restriction enzyme digest
- Ligation
- Transformation

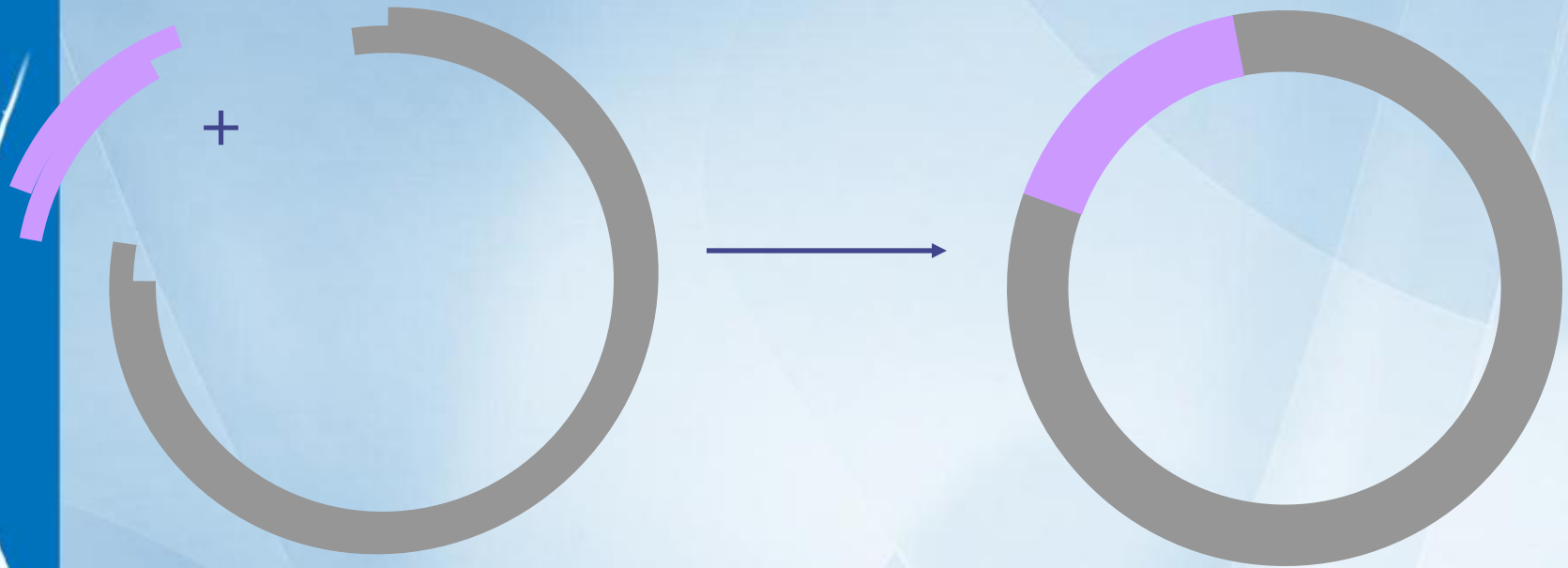




PCR



Ligation of the Insert into the Vector

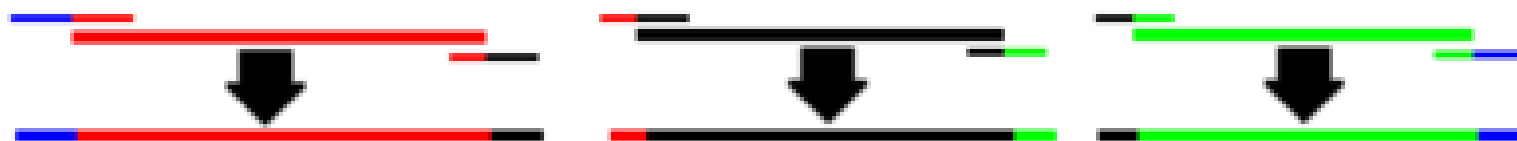


- Ligation covalently attaches the vector and the insert via a phosphodiester bond (5'phosphate and 3' hydroxyl of the next base)

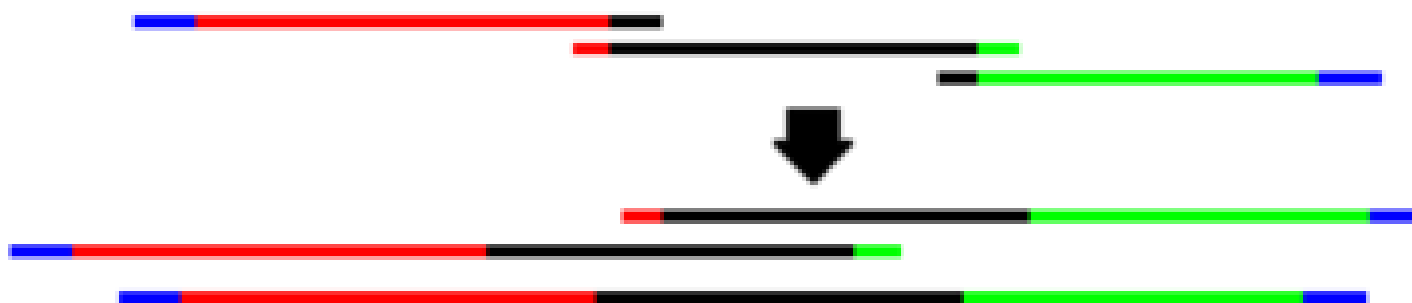
Restriction enzymes (NEB)

	oligo sequence	% cleavage	
		2h	20h
BamHI	<u>CGGATCCG</u>	10	25
	CGGGATCCCG	>90	>90
	CGCGGATCCGCG	>90	>90
EcoRI	<u>GGAATTCC</u>	>90	>90
	CGGAATTCCG	>90	>90
	CCGGAATTCCGG	>90	>90
HindIII	<u>CAAGCTTG</u>	0	0
	CCAAGCTTGG	0	0
	CCCAAGCTTGGG	10	75
NcoI	<u>CCCATGGG</u>	0	0
	CATGCCATGGCATG	50	75
NdeI	GGGTTT <u>CATATG</u> AAACCC	0	0
	GGAATTCC <u>CATATG</u> GGAATTCC	75	>90

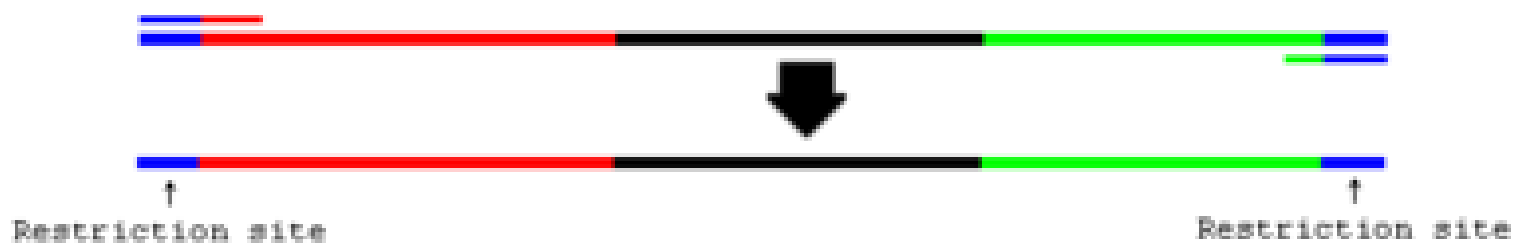
Extension PCR



Overlap PCR



Purification PCR



Site-directed mutagenesis

Step 1
Plasmid Preparation



Step 2
Temperature Cycling



Mutagenic primers

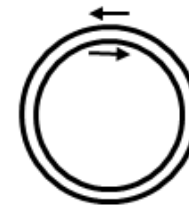


Step 3
Digestion



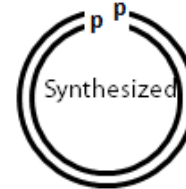
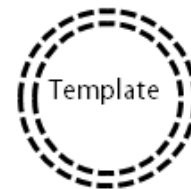
Mutated plasmid
(contains nicked circular strands)

Step 4
Transformation



Amplification using
mutagenic primers

PCR



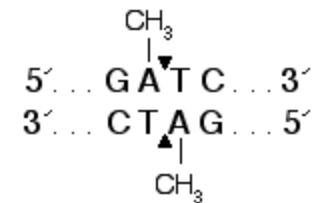
DpnI
Digestion of
Template DNA

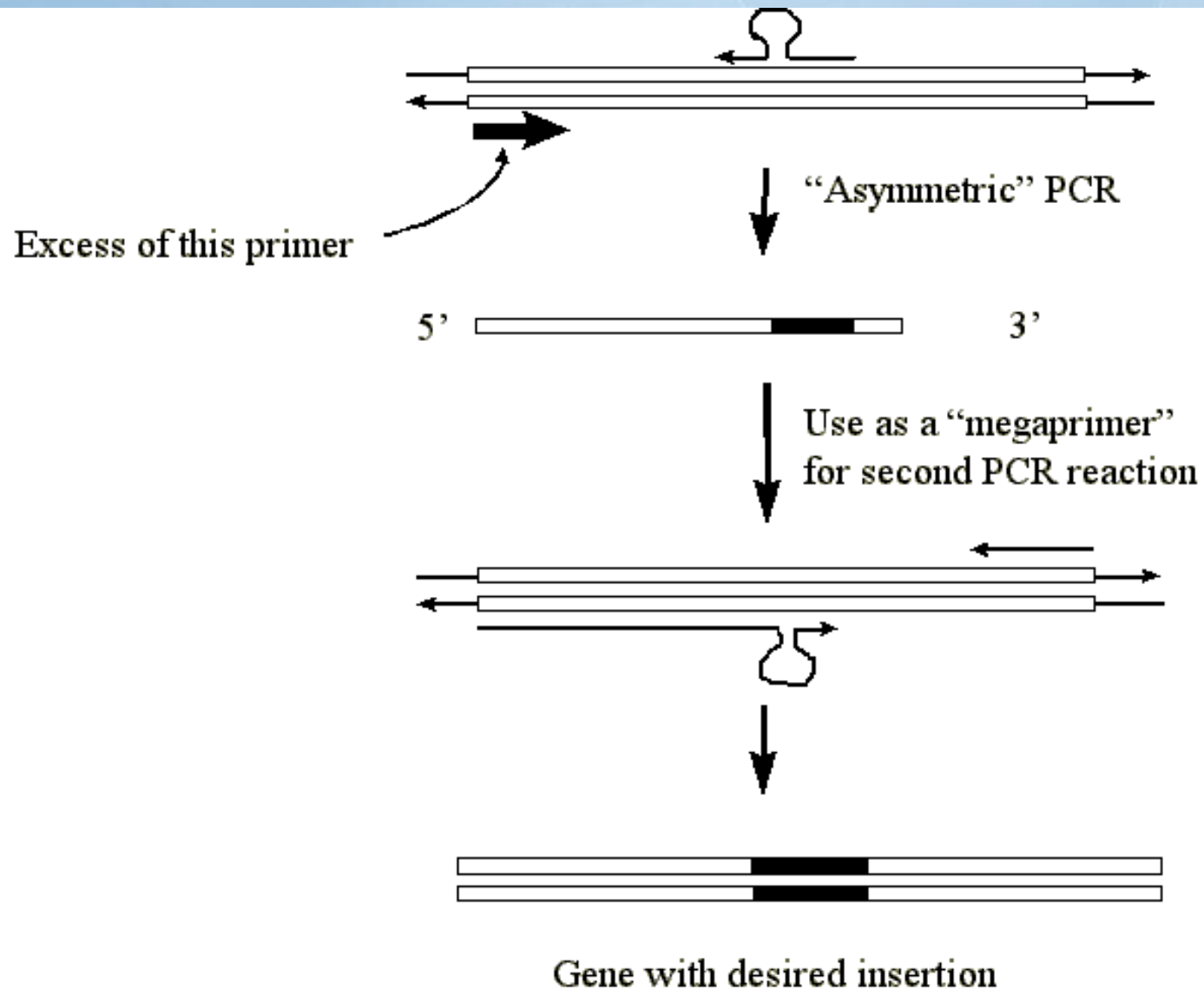


Transformation



Colonies With Mutated Gene





Web-based Softwares



Tool name	URL
CODEHOP	http://blocks.fhcrc.org/codehop.html
Gene Fisher	http://bibiserv.techfak.uni-bielefeld.de/genefisher/
DoPrimer	http://doprimer.interactiva.de/
Primer3	http://frodo.wi.mit.edu/primer3/
Primer Selection	Http://alces.med.umn.edu/rawprimer.html
Web Primer	http://genome.www2.stanford.edu/cgi.bin/SGD/web.primer
PCR designer	http://cedar.genetics.ston.ac.uk/public_html/primer.html
Primo pro 3.4	http://www.changbioscience.com/primo.html
Primo Degenerate 3.4	http://www.changbioscience.com/primo/primod.html
PCR Primer Design	http://pga.mgh.harvard.edu/serviet/org.mgh.proteome.primer
The Primer Generator	http://www.med.jhu.edu/medcenter/primer/primer.cgi
EPRIMERS	http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html
PRIMO	http://bioweb.pasteur.fr/seqanal/interfaces/eprimo.html3
PrimerQuest	http://www.idtdna.com/biotools/primer_quest/primer_quest.asp
MethPrimer	http://itsa.uscf/~uralab/methprimer/index1.html
Rawprimer	http://alces.med.umn.edu/rawprimer.html
MEDUSA	http://www.cgr.ki.se/cgr/MEDUSA/
The Primer Prim'er Project	http://www.nmr.cabm.rutgers.edu/bioinformatics/primer_primer_project/primer.html
GAP	http://promoter.ics.uci.edu/primers/

Installable Softwares

Software name	Description
Primerselect	Analyses a template DNA sequence and chooses primer pairs for PCR and primers for DNA sequencing
DANSIS Max	DANASIS Max is a fully integrated program that includes a wide range of standard sequence analysis features.
Primer Primer 5	Primer design for windows and power macintosh.
Primer Primer:	Comprehensive primer design for windows and Power Macintosh.
NetPrimer	Comprehensive analysis of individual primers and primer pairs.
Array Designer 2	For fast, effective design of specific oligos or PCR primer pairs for microarrays.
AlleleID 7	Design molecular beacons and TaqMan probes for robust amplification and fluorescence in real time PCR.
GenomePRIDE 1.0	Primer design for DNA-arrays/chips.
Fast PCR	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.
OLIGO 7	Primer Analysis Software for Mac and Windows.
Primer Designer 4	Will find optimal primers in target regions of DNA or protein molecules, amplify features in molecules, or create products of a specified length.
GPRIME	Software for primer design.
Sarani Gold	Genome Oligo Designer is a Software for automatic large scale design of optimal oligonucleotide probes for microarray experiments.
PCR Help	Primer and template design and analysis.
Genorama chip Design Software	Genorama Chip Design Software is a complete set of programs required for genotyping chip design. The programs can also be bought separately.
Primer Designer	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.
Primer Primer	Automatic design tools for PCR. Sequencing or hybridization probes, degenerate primer design, restriction, Nested/Multiplex primer design, restriction enzyme analysis and more.
PreimerDesign	DOS-program to choose primer for PCR or oligonucleotide probes.

Primer3

Primer3

(v. 0.4.0) Pick primers from a DNA sequence.

[Primer3plus interface](#) [More primer/oligo tools](#)

[disclaimer](#)

[Primer3 Home](#)

[Old \(0.3.0\) interface](#)

[cautions](#)

[FAQ/Wiki](#)

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 \(repeat library\)](#):

☒ 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:](#) 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.

[Excluded Regions:](#) 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.

[Product Size Ranges](#)

[Number To Return](#)

[Max 3' Stability](#)

[Max Repeat Mispriming](#)

[Pair Max Repeat Mispriming](#)

[Max Template Mispriming](#)

[Pair Max Template Mispriming](#)

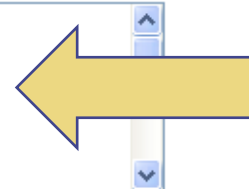
Pick Primers

Reset Form



and/or a sequence (vector, H2O, DNA, etc.) or use a [multiplexing library \(repeat library\)](#).

GTCTAAGGAGCTGGGCATAGAGACTTACAAAGTGAATGTCAGTGAGCGTCTCGTTCAATATGTCAAGGGG
AAAACATATCCATTTTCGGGGCGCCTTTCCACCAGTATGGAATCCCATTTGCATATTTGGATTACAATAATC
TGTGGAGGACAATAGATAACATGGGGAAGGAGATTCCAACTGATGCACCCTGGGAGGCTCAACATGCTGA
CAAATGGGACAAAATGACCATGAAAAGAGCTCATTGACAAAATCTGCTGGACAAAGACTGCTAGGCGGTTT
GCTTATCTTTTTGTGAATATCAATGTGACCTCTGAGCCTCACGAAGTGTCTGCCCTGTGGTTCTTGTGGT
ATGTGAAGCAGTGGGGGCACCACTCGGATATTCTCTGTCACCAATGGTGGCCAGGAACGGAAGTTTGT



☒	☐	☒
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'- >3' on opposite strand).

Pick Primers

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.

Excluded Regions: 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.

NEW Product Size Ranges

[Click here to specify the min, opt, and max product sizes only if you absolutely must. Using them is too slow \(and too computationally intensive for our server\).](#)

General Primer Picking Conditions

<u>Primer Size</u>	Min: 18	Opt: 20	Max: 22	
<u>Primer Tm</u>	Min: 55	Opt: 58.0	Max: 61	<u>Max Tm Difference:</u> 2
<u>Product Tm</u>	Min:	Opt:	Max:	
<u>Primer GC%</u>	Min: 45	Opt:	Max: 60	
<u>Max Self Complementarity:</u>	4	<u>Max 3' Self Complementarity:</u>	3.00	
<u>Max #N's:</u>	0	<u>Max Poly-X:</u>	3	
<u>Inside Target Penalty:</u>		<u>Outside Target Penalty:</u>	0	<u>Set Inside Target Penalty to allow primers inside a target.</u>
<u>First Base Index:</u>	1	<u>CG Clamp:</u>	0	
<u>Salt Concentration:</u>	50.0	<u>Annealing Oligo Concentration:</u>	50.0	<u>(Not the concentration of oligos in the reaction mix but of those annealing to template.)</u>

☒ Liberal Base
☐ Show Debugging Info
☒ Do not treat ambiguity codes in libraries as consensus

Pick Primers

Reset Form

```
<<<<< right primer
```

