Primer Design

Ameer Effat M. Elfarash

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

Why Are Primers Important?

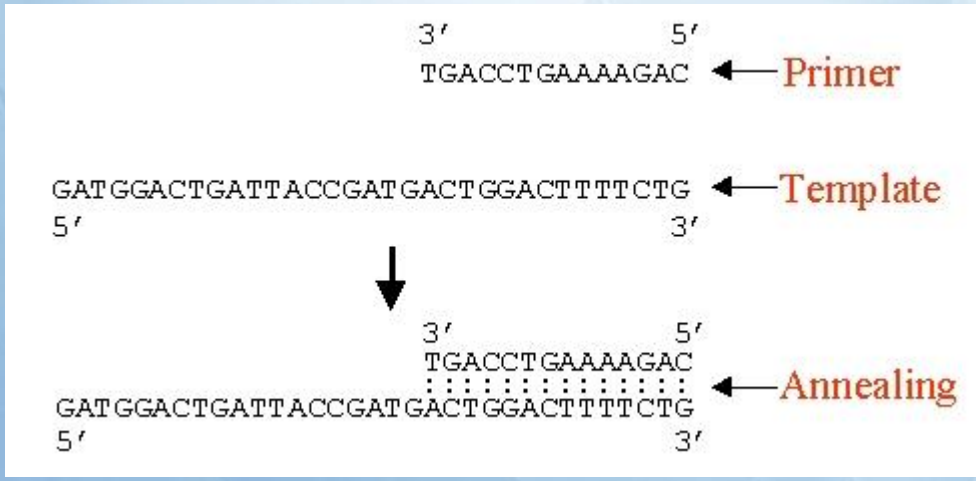
- Primers are what gives PCR its SPECIFICITY!!!
- Good primer design: PCR works great.
- Bad primer design: PCR works terrible.

PCR Reagents

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

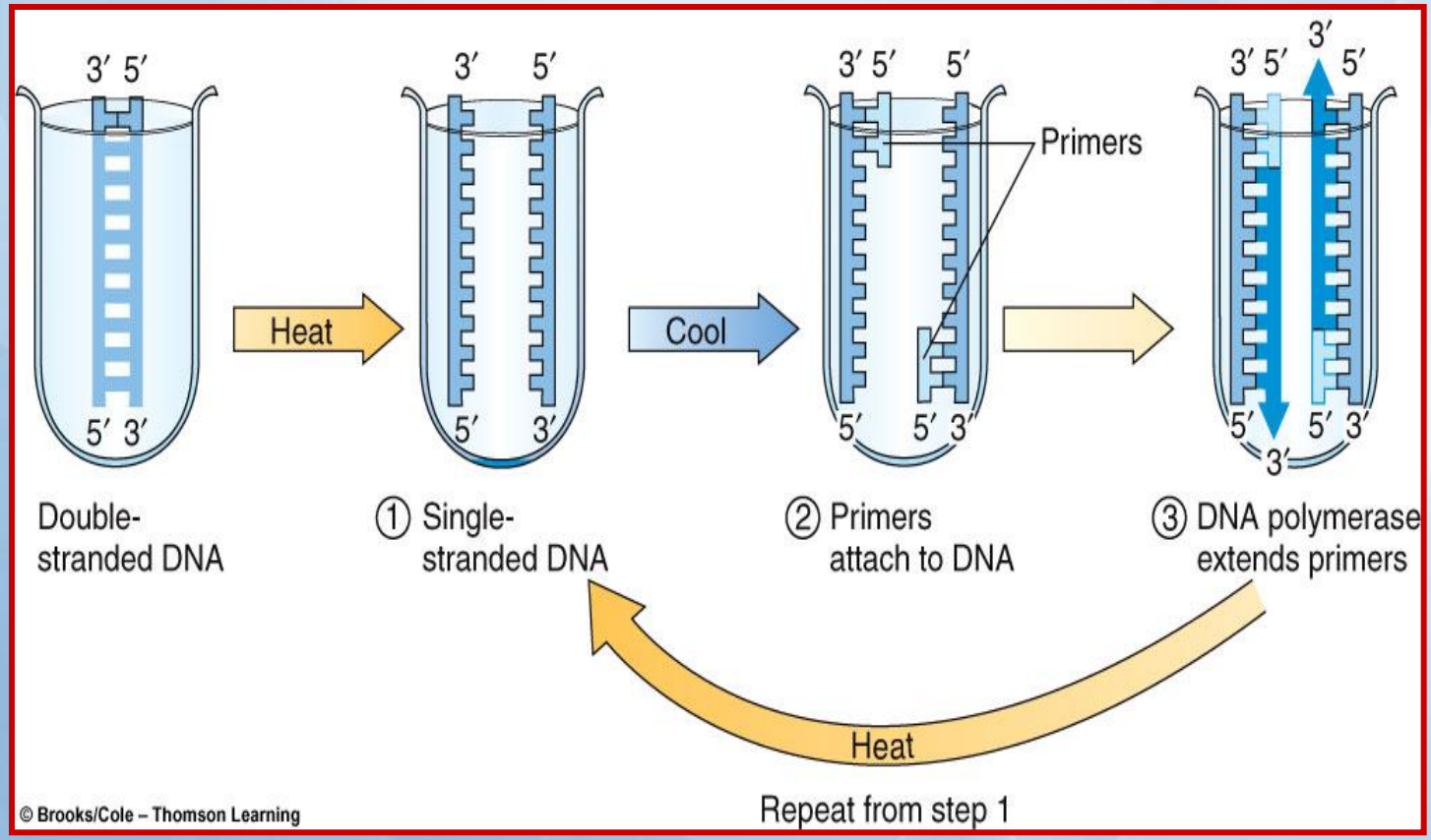
What is a primer?

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.



Very-Brief PCR Reminder

- PCR is a method to amplify large quantities of a DNA covering a specific sequence.



Good Primer's Characteristic

Primer length

5..TCAACTTAGCATGATCGGGTAGTAGCTTGACTGTACAACTCA

18-24 bp for general applications

General rules for primer design

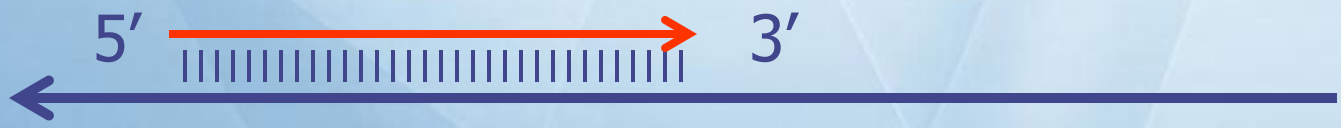
- **Primer length determines the specificity and significantly affect its annealing to the template**
  - **Too short -- low specificity, resulting in non-specific amplification**
  - **Too long -- decrease the template-binding efficiency at normal annealing temperature due to the higher probability 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 GTGGATGTGGTGTCGATGGC 3



Max 3'end stability

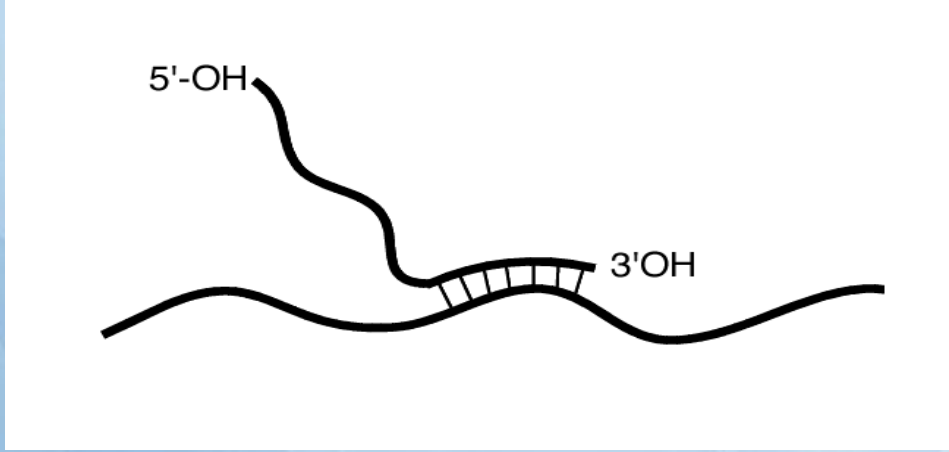
It's critical that the stability at 3' end be high

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 * (G + C) + 2 * (A + T)*

- Bolton and McCarthy:

*Tm = 81.5 + 16.6 * Log [I] + 0.41 * (%GC) – 600/L*

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

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

Melting Temperature

Oligo sequence

CGCACTTCCAACAACCCTTC

Length	GC%	MW(kD)
20	55.0	5.98

Melting Temperature (°C):

Thermo	Hybrid	GC+AT
59.4	52.5	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	GC%	MW(kD)
50	54.0	14.94

Melting Temperature (°C):

Thermo	Hybrid	GC+AT
80.8	70.0	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

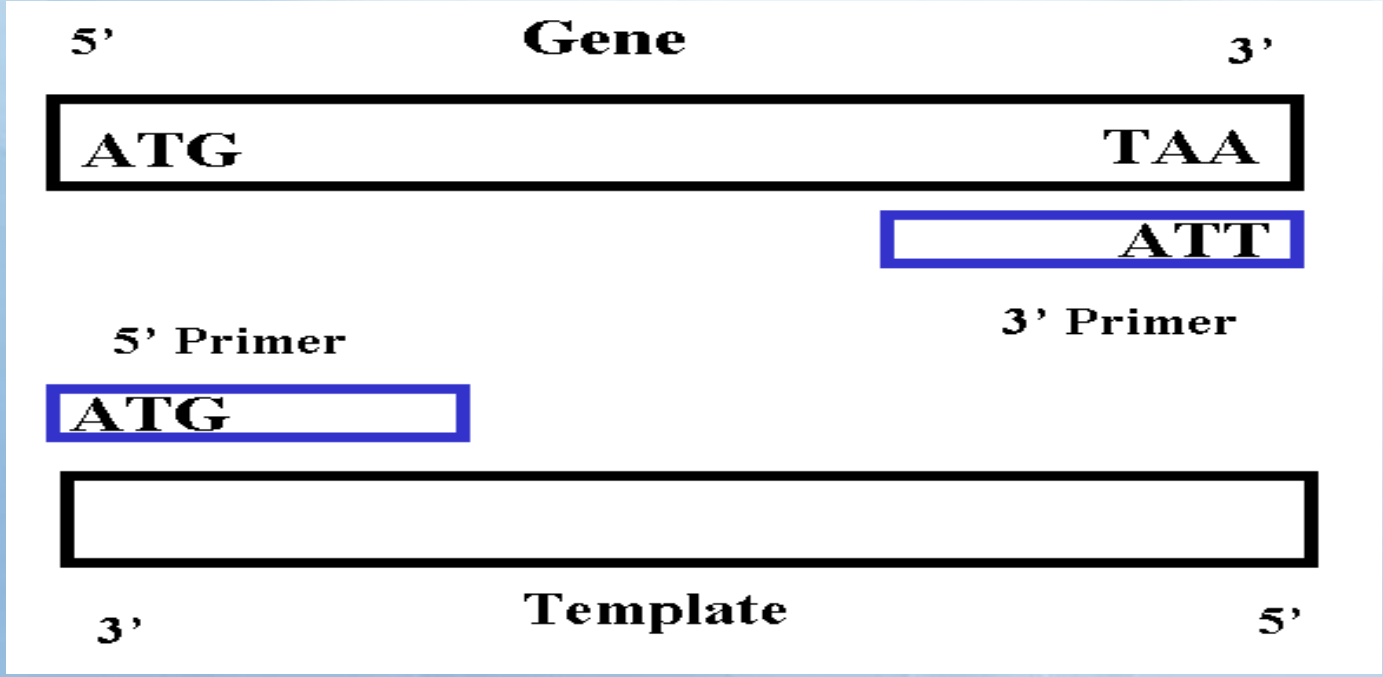
Primer Pair Matching

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

ATGTCCAATGACAACGAAGTACCTGGTTCCATGGTTATTGTCGCACAAGGTCCAGACGATCAATACGCATACGAGGTTCCCCCTATCGATAGCGCGGCCG
TTGCCGGGAATATGTTTGGCGACTTAATTCAAAGAGAAATATATCTACAGAAAAACATTTATTATCCAGTCCGATCTATTTTTGAACAAGGAACAAAAGA
AAAGAAGGAGATCAACAAGAAAGTATCTGATCAAGTCGATGGCTTGCTAAAGCAGATCACTCAAGGAAAAAGGGAGGCCACAAGGCAAGAGCGAGTCGAT
GTCATGTCGGCAGTCCTGCACAAGATGGAATCTGATCTTGAAGGATACAAAAAGACCTTTACCAAAGGCCCATTCATTGACTACGAAAAGCAGTCAAGCC
TCTCCATCTATGAGGCCTGGGTCAAGATCTGGGAGAAGAACTCTTGGGAAGAAAGAAAGAAGTACCCTTTTCAGCAGCTTGTTAGAGATGAACTGGAGCG
GGCGGTTGCCTACTACAAACAAGATTCACTCTCTGAAGCGGTAAAAGTGCTAAGACAGGAGCTCAACAAGCAAAAAGCGCTAAAGGAAAAAGAGGACCTC
TCTCAACTGGAGCGGGACTACAGAACCCGAAAGGCGAATCTCGAGATGAAAGTACAATCCGAGCTTGATCAAGCGGGAAGTGCTTTGCCTCCATTGGTCA
GTCCAACGCCAGAGCAATGGCTTGAACGTGCCACAAGACTGGTTACGCAAGCAATTGCTGATAAAAAGCAGCTGCAGACCACAAACAATACTCTTATCAA
GAATTCCCCAACCCCTCTAGAAAAGCAGAAAGCCATCTACAATGGTGAGCTACTTGTGGATGAGATAGCCAGTCTACAGGCCCGCTTAGTTAAGCTGAAC
GCCGAAACGACACGACGCAGGACAGAAGCAGAACGCAAGGCGGCCGAGGAACAAGCGTTGCAAGATGCTATTAAATTTACTGCCGACTTTTATAAGGAAG
TAACTGAGAAATTTGGCGCACGAACATCGGAGATGGCGCGCCAACTGGCCGAAGGCGCCAGGGGGAAAAATATCAGGAGTTCGGCGGAAGCAATCAAGTC
GTTTGAAAAGCACAAGGATGCGTTAAATAAAAAACTTAGCCTTAAAGATAGGCAAGCCATTGCCAAAGCCTTTGATTCTCTAGACAAGCAGATGATGGCG
AAGAGCCTTGAGAAATTTAGCAAAGGCTTTGGAGTTGTAGGCAAAGCTATTGACGCCGCCAGCCTGTACCAAGAGTTCAAGATATCTACGGAAACCGGGG
ACTGGAAACCATTCTTTGTAAAAATTGAAACACTAGCTGCTGGTGCGGCCGCCAGTTGGCTTGTGGGTATTGCATTTGCCACGGCAACAGCCACTCCTAT
AGGCATTCTGGGGTTCGCACTGGTAATGGCAGTTACCGGGGCGATGATTGACGAAGACCTTCTAGAAAAAGCAAACAATCTTGTAATATCCATTTAA



- **Determine the start and end sites of the DNA fragment you want to clone.**

3`GACTAAGGACAGTTGCACAGTTACC . . CTCAGGACTTCAGGAACGTAGCAC5`
5`CTGATTCCTGTCAACGTGTCAATGG . . GAGTCCTGAAGTCCTTGCATCGTG3`

- **Choose the first 17 -24 bases in the 5` ends as forward and reverse primers.**

3`GACTAAGGACAGTTGCACAGTTACC . . CTCAGGACTTCAGGAACGTAGCAC5`
5`CTGATTCCTGTCAACGT3`---→
Forward primer

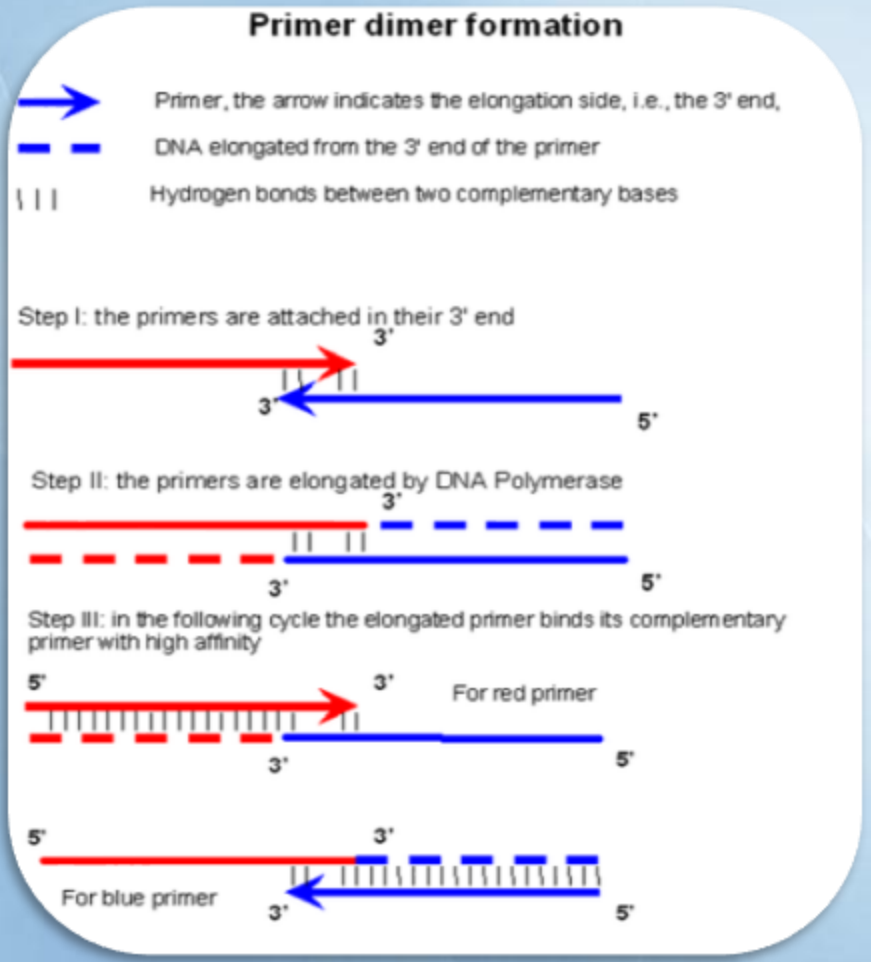
Reverse primer
←-----3`CTTCAGGAACGTAGCAC5`
5`CTGATTCCTGTCAACGTGTCAATGG . . GAGTCCTGAAGTCCTTGCATCGTG3`

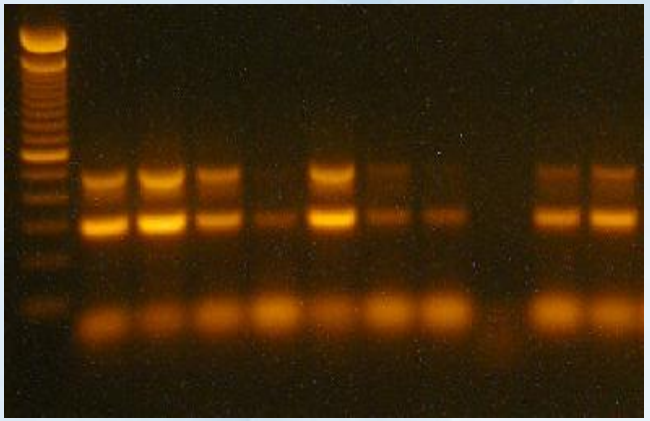
Avoid

A. Avoid hairpin and stem-loop formation

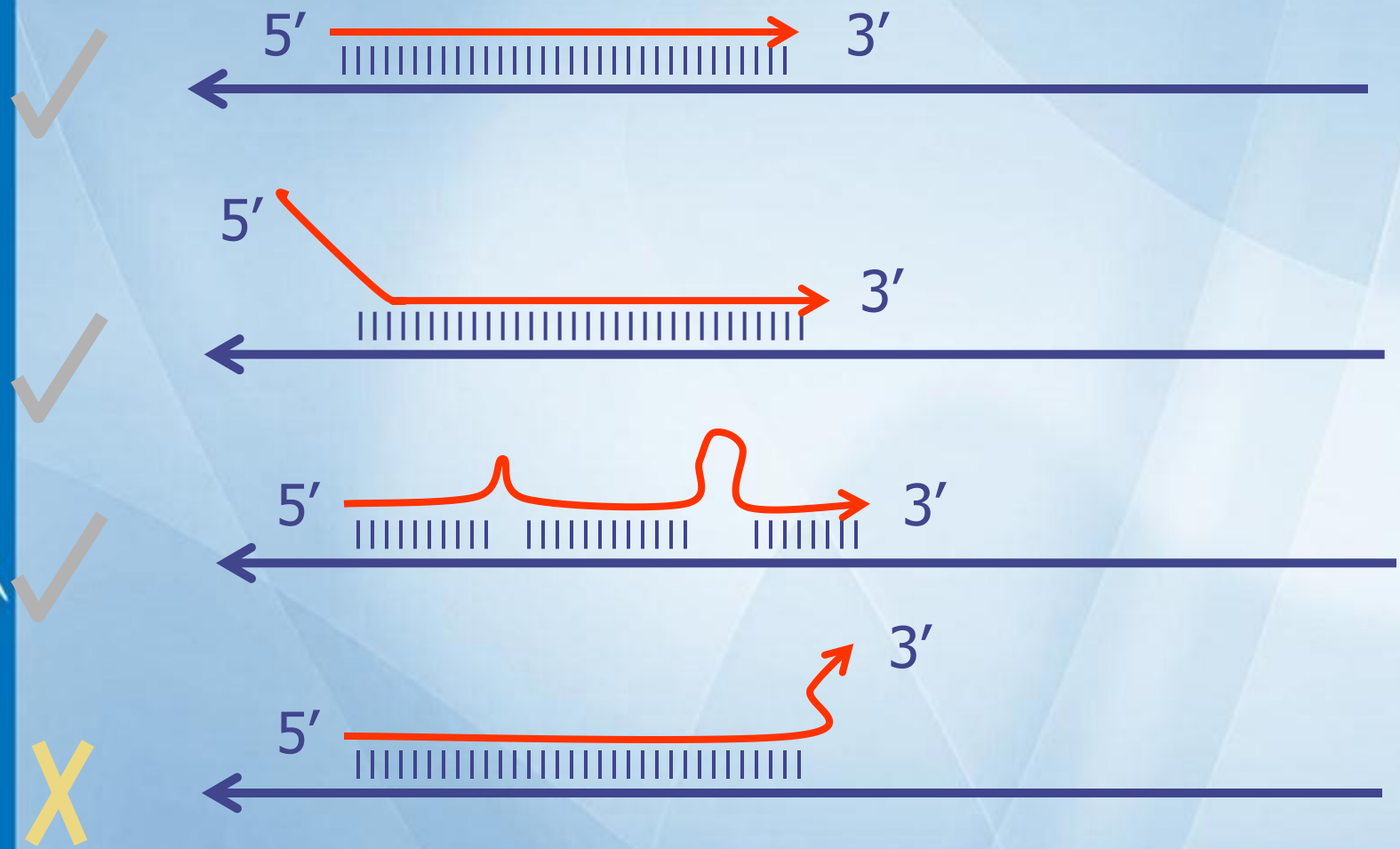
```
Hairpin: ²G = -0.7 kcal/mol, Loop = 8 nt, Tm = 41°
    5'  CCAGTCGTT┐
          ||||    A
  3'  ACAGTCAAACA┘
```

- Avoid complementary at 3` end of primers





when is a “primer” a primer?

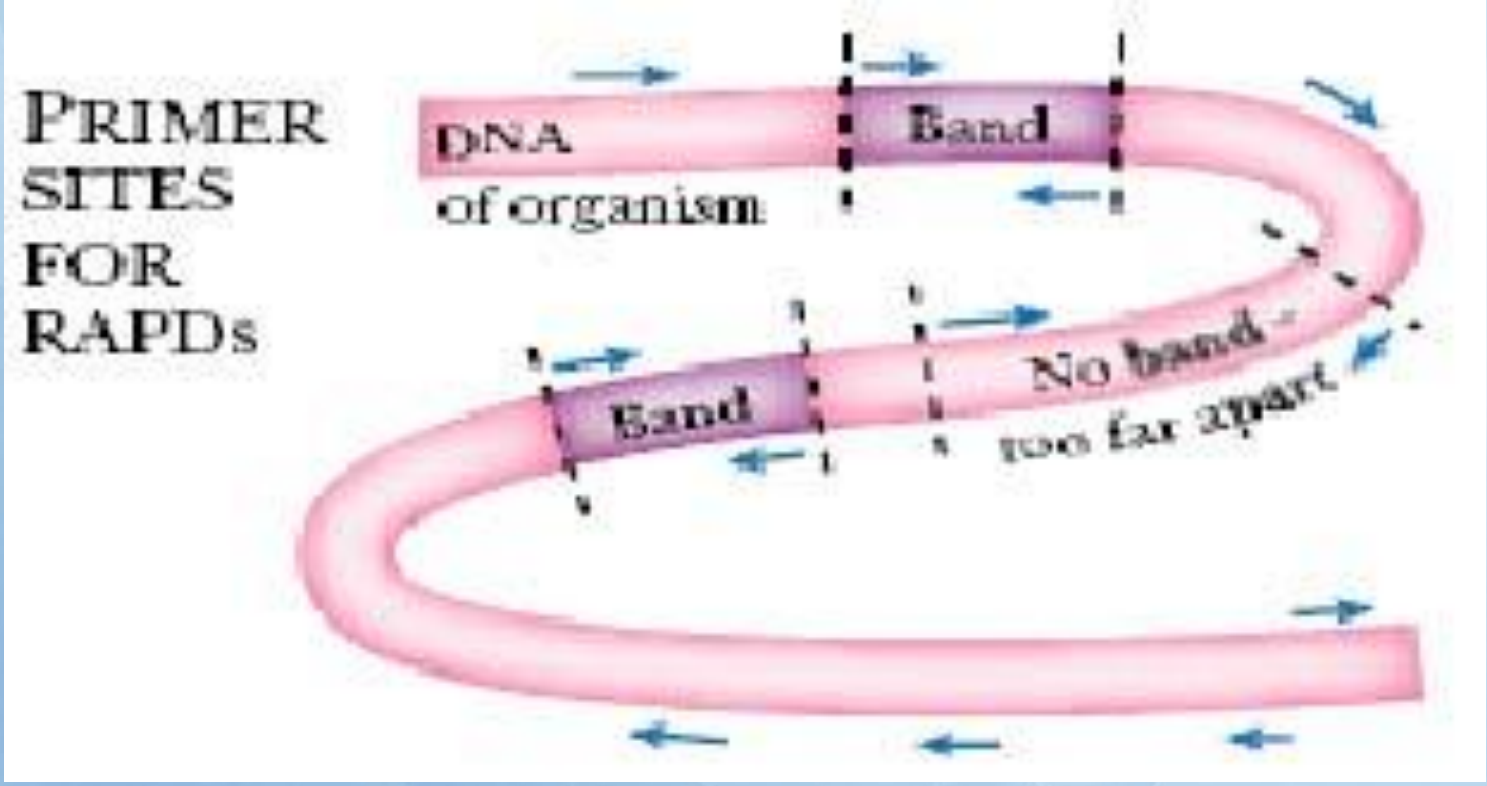


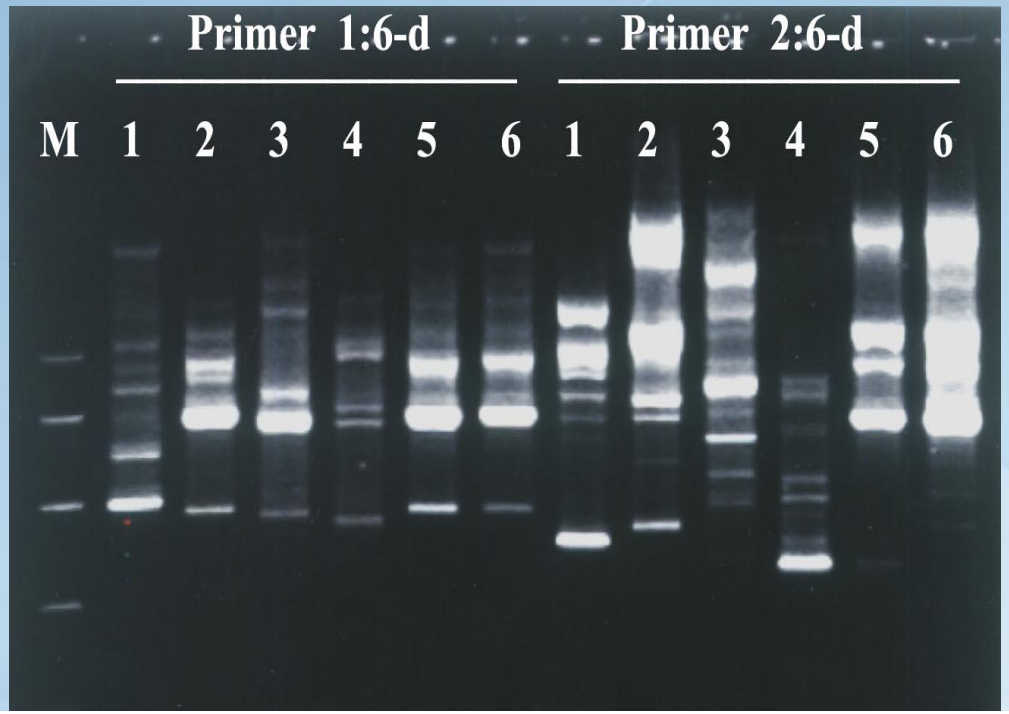
PCR primers are designed to:

- For Cloning a special sequence
  - Full length
- For Detection
  - 100 - 500 bp
  - Gene of interest
  - Gene expression (mRNA)
  - Microbial agents detection
  - Mutation Detection
  - Quantification
  - Allelic discrimination
  - Disease
  - ...
- Random
  - ??

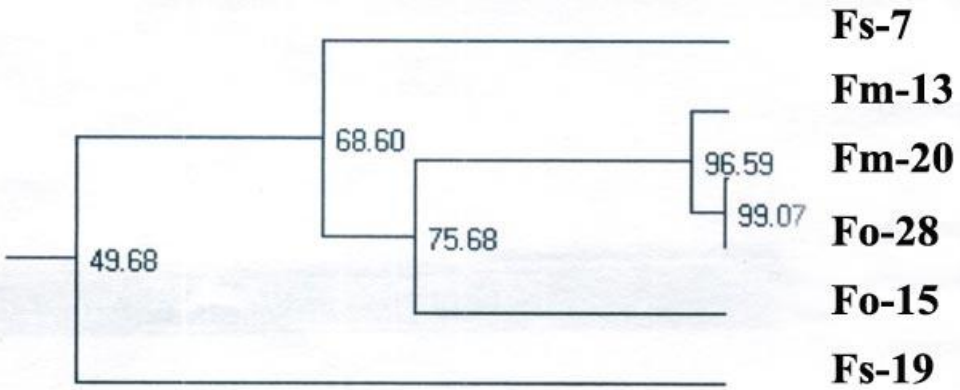
Random primers
Example: RAPD-PCR
RAPD = Random Amplified Polymorphic DNA

5`-TCG GCG GTT C-3`




Primer 1:6-d
Primer 2:6-d
M 1 2 3 4 5 6 1 2 3 4 5 6

Primer 5:6-d


68.60
96.59
99.07
75.68
49.68
Fs-7
Fm-13
Fm-20
Fo-28
Fo-15
Fs-19

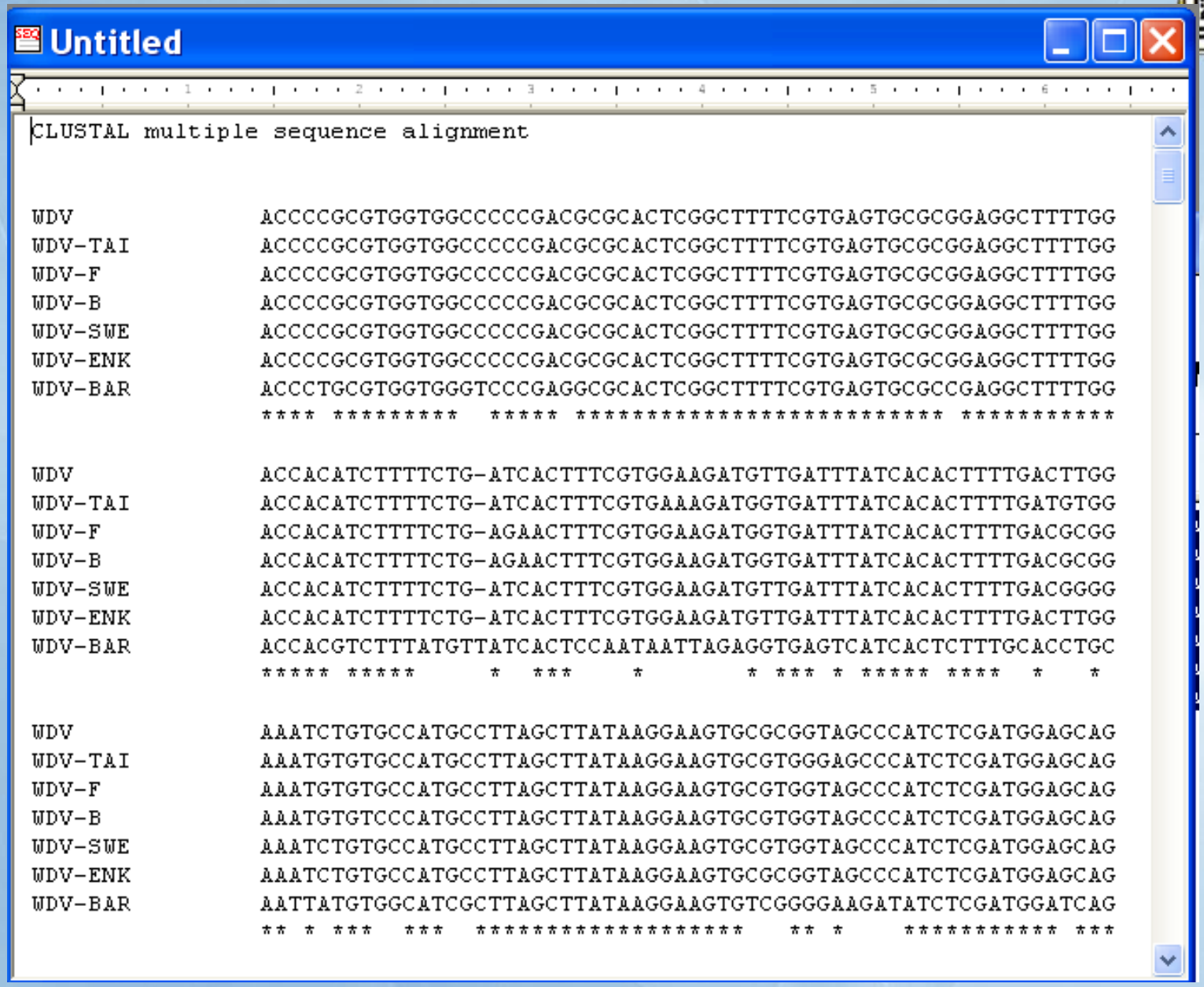
Universal Primers

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:

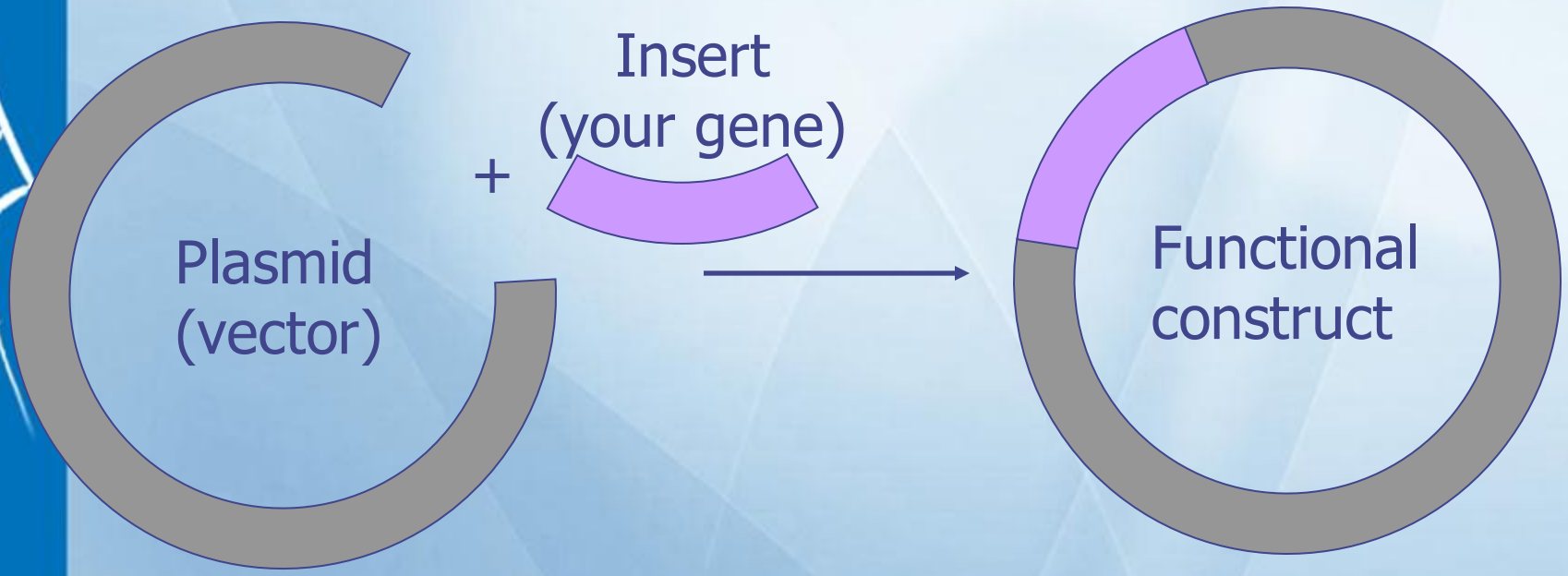
1. Align groups of sequences you want to amplify.
2. Find the most conservative regions at 5′ end and at 3′ end.
3. Design forward primer at the 5′ conservative region.
4. Design reverse primer at the 3′ conservative regions.
5. Matching forward and reverse primers to find the best pair.
6. Ensure uniqueness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


Untitled
CLUSTAL multiple sequence alignment
WDV ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-TAI ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-F ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-B ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-SWE ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-ENK ACCCCGCGTGGTGGCCCCCGACGCGCACTCGGCTTTTCGTGAGTGCGCGGAGGCTTTTGG
WDV-BAR ACCCTGCGTGGTGGGTCCCGAGGCGCACTCGGCTTTTCGTGAGTGCGCCGAGGCTTTTGG
**** ********* ***** ************************** **********
WDV ACCACATCTTTTCTG-ATCACTTTCGTGGAAGATGTTGATTTATCACACTTTTGACTTGG
WDV-TAI ACCACATCTTTTCTG-ATCACTTTCGTGAAAGATGGTGATTTATCACACTTTTGATGTGG
WDV-F ACCACATCTTTTCTG-AGAACTTTCGTGGAAGATGGTGATTTATCACACTTTTGACGCGG
WDV-B ACCACATCTTTTCTG-AGAACTTTCGTGGAAGATGGTGATTTATCACACTTTTGACGCGG
WDV-SWE ACCACATCTTTTCTG-ATCACTTTCGTGGAAGATGTTGATTTATCACACTTTTGACGGGG
WDV-ENK ACCACATCTTTTCTG-ATCACTTTCGTGGAAGATGTTGATTTATCACACTTTTGACTTGG
WDV-BAR ACCACGTCTTTATGTTATCACTCCAATAATTAGAGGTGAGTCATCACTCTTTGCACCTGC
***** ***** * *** * * *** * ***** **** * *
WDV AAATCTGTGCCATGCCTTAGCTTATAAGGAAGTGCGCGGTAGCCCATCTCGATGGAGCAG
WDV-TAI AAATGTGTGCCATGCCTTAGCTTATAAGGAAGTGCGTGGGAGCCCATCTCGATGGAGCAG
WDV-F AAATGTGTGCCATGCCTTAGCTTATAAGGAAGTGCGTGGTAGCCCATCTCGATGGAGCAG
WDV-B AAATGTGTCCCATGCCTTAGCTTATAAGGAAGTGCGTGGTAGCCCATCTCGATGGAGCAG
WDV-SWE AAATCTGTGCCATGCCTTAGCTTATAAGGAAGTGCGTGGTAGCCCATCTCGATGGAGCAG
WDV-ENK AAATCTGTGCCATGCCTTAGCTTATAAGGAAGTGCGCGGTAGCCCATCTCGATGGAGCAG
WDV-BAR AATTATGTGGCATCGCTTAGCTTATAAGGAAGTGTCGGGGAAGATATCTCGATGGATCAG
** * *** *** ********** ** * ********** ***

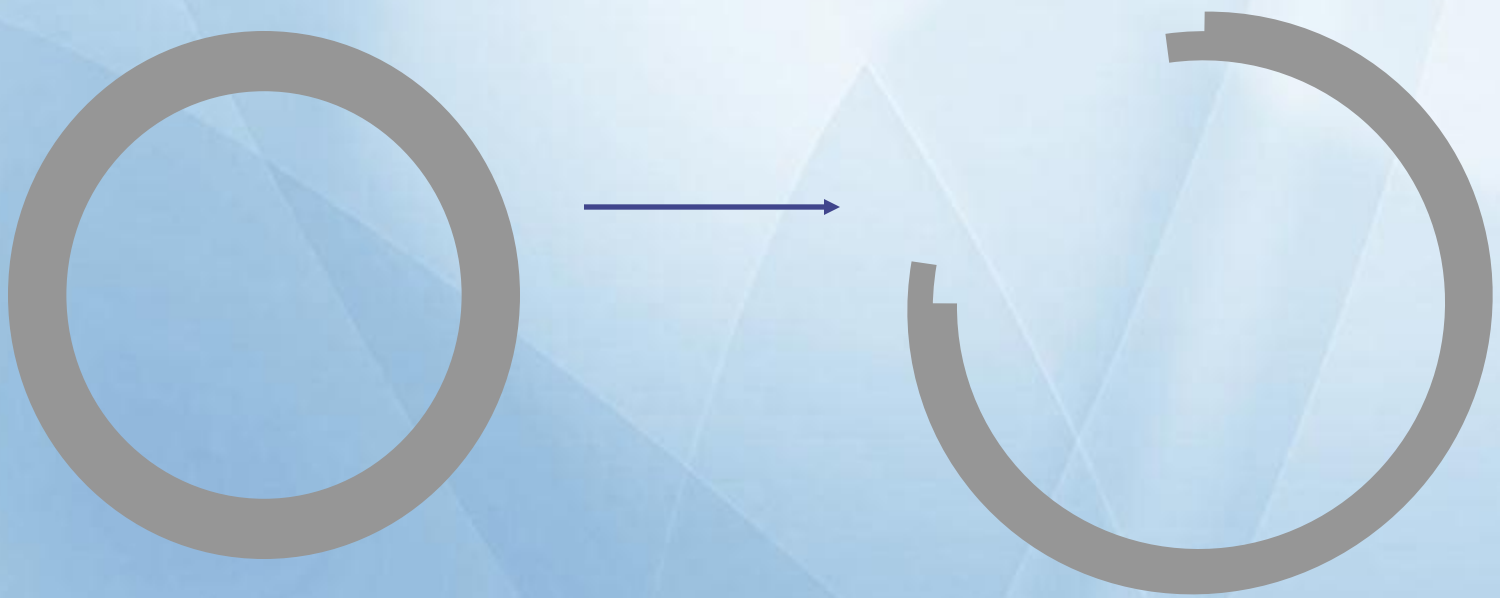
Cloning Overview

Four main steps in cloning:

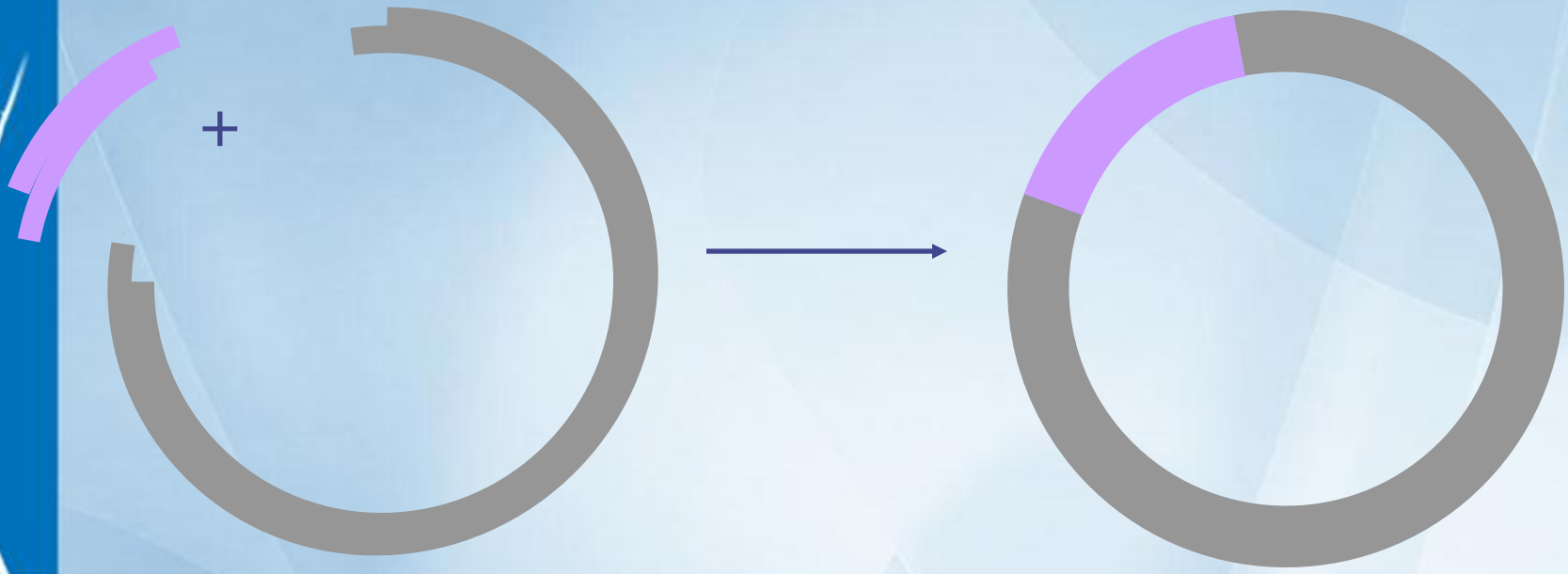
- Insert synthesis
- Restriction enzyme digest
- Ligation
- Transformation



PCR



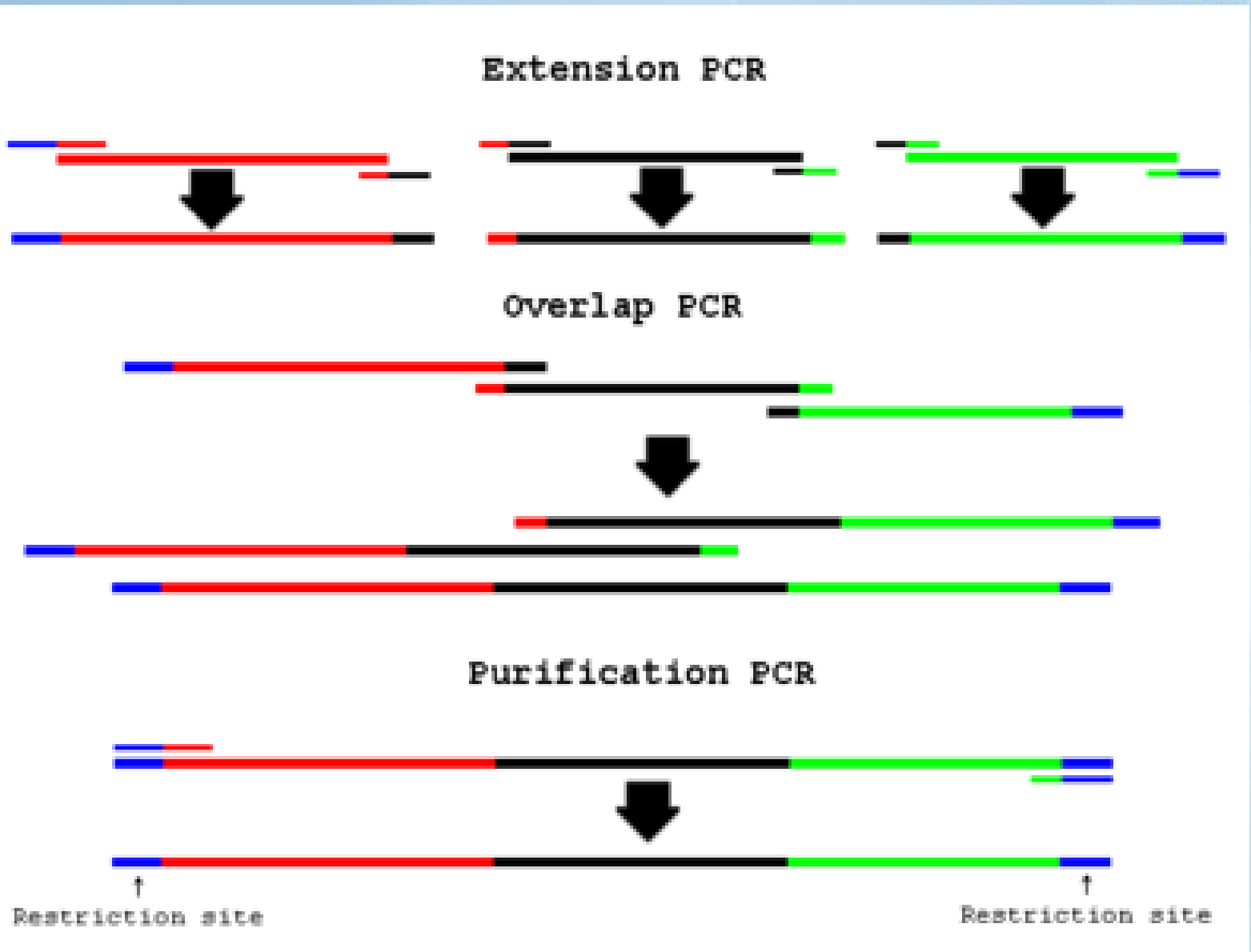
Ligation of the Insert into the Vector



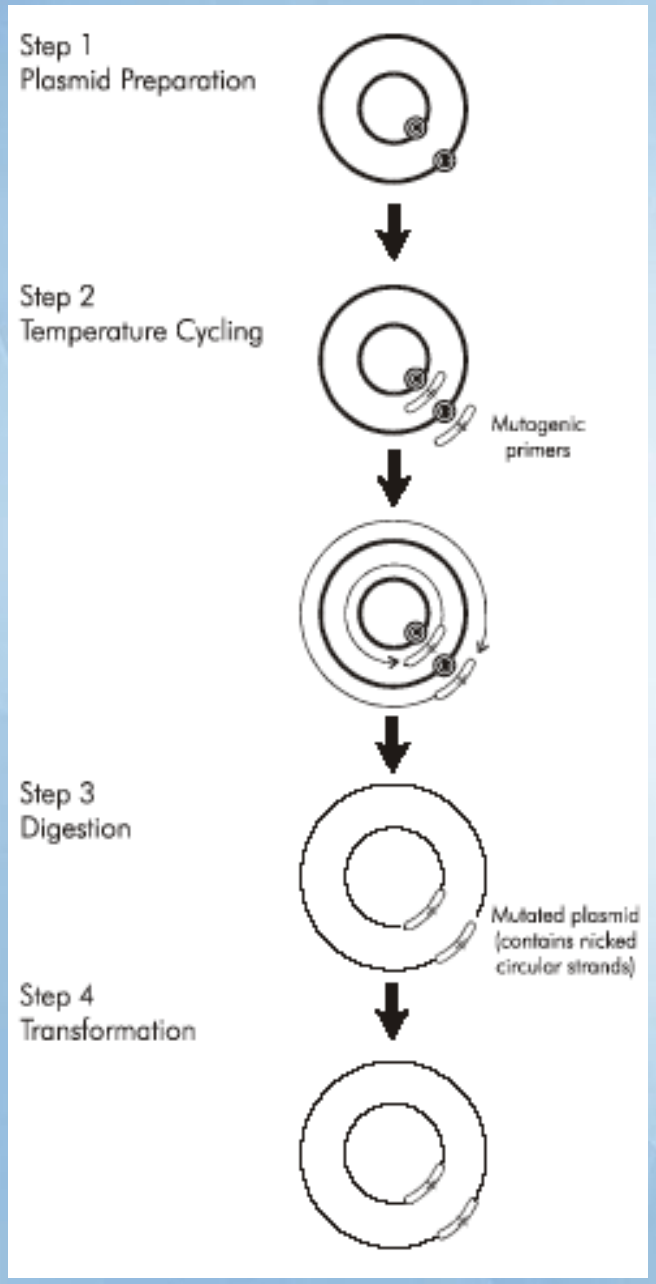
- Ligation covalently attaches the vector and the insert via a phosphodiester bond (5'phosphate and 3' hydroxyl of the next base)

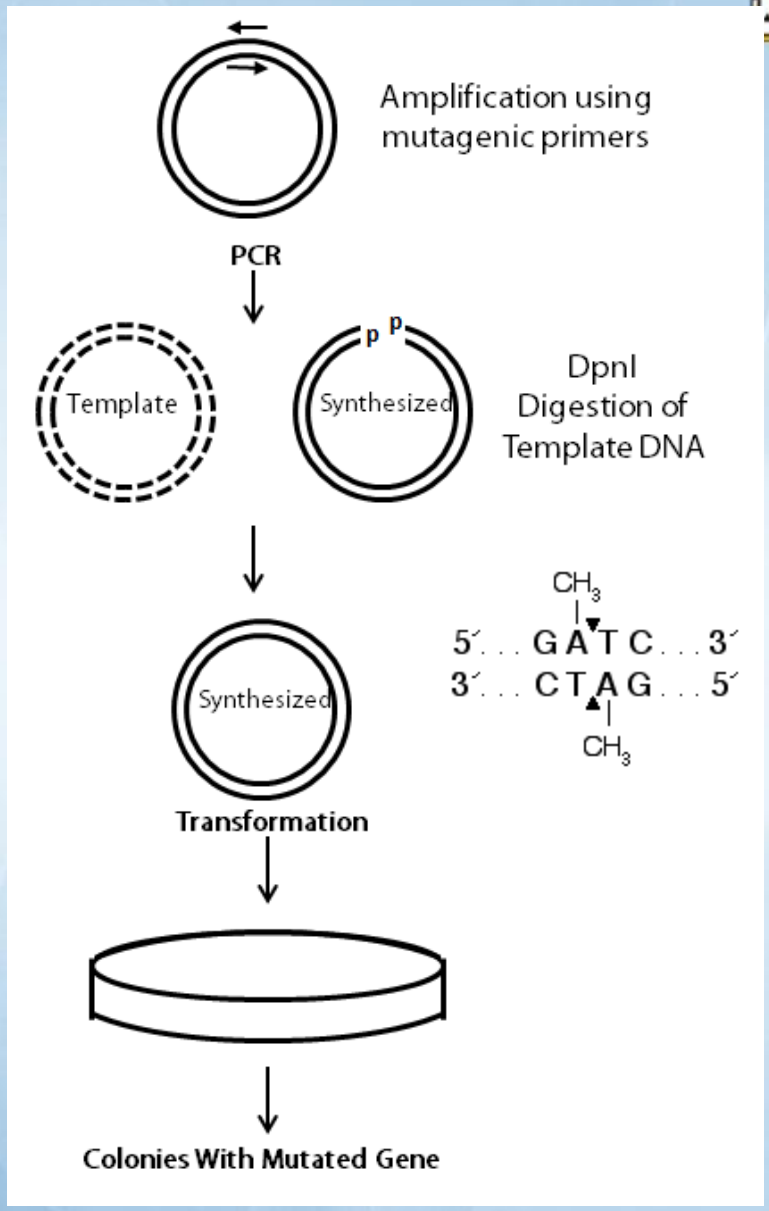
Restriction enzymes (NEB)

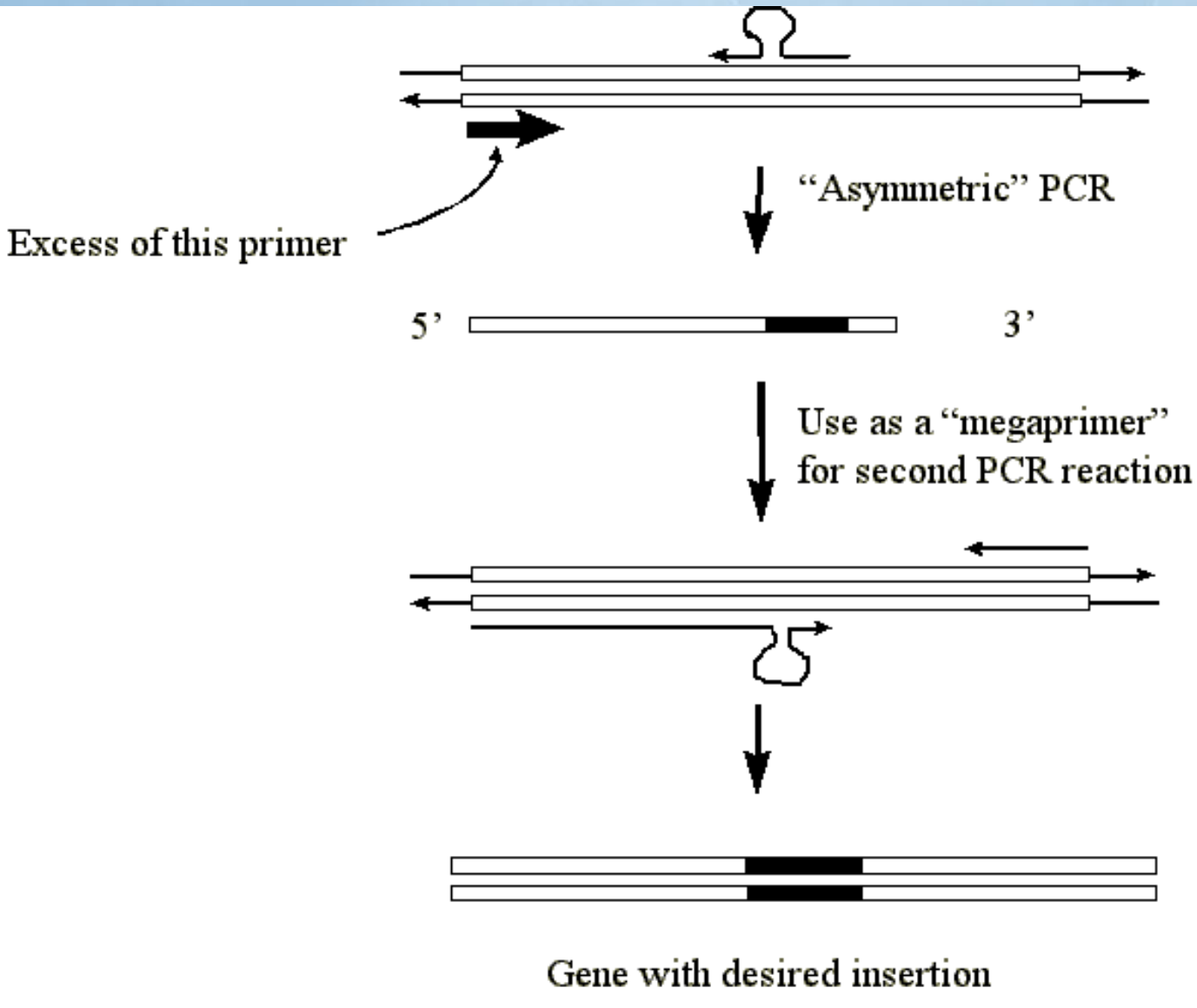
	oligo sequence	% cleavage 2h	% cleavage 20h
BamHI	CGGATCCG	10	25
	CGGGATCCCG	>90	>90
	CGCGGATCCGCG	>90	>90
EcoRI	GGAATTCC	>90	>90
	CGGAATTCCG	>90	>90
	CCGGAATTCCGG	>90	>90
HindIII	CAAGCTTG	0	0
	CCAAGCTTGG	0	0
	CCCAAGCTTGGG	10	75
NcoI	CCCATGGG	0	0
	CATGCCATGGCATG	50	75
NdeI	GGGTTTCATATGAAACCC	0	0
	GGAATTCCATATGGAATTCC	75	>90


Extension PCR
Overlap PCR
Purification PCR
Restriction site
Restriction site

Site-directed mutagenesis






Excess of this primer
"Asymmetric" PCR
5'
3'
Use as a "megaprimer"
for second PCR reaction
Gene with desired insertion

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 leatures 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 | Old (0.3.0) interface | disclaimer | cautions | Primer3 Home | 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): 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:		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: 150-250 100-300 301-400 401-500 501-600 601-700 701-850 851-1000

Number To Return	5	Max 3' Stability	9.0
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

GTCTAAGGAGCTGGGCATAGAGACTTACAAAGTGAATGTCAGTGAGCGTCTCGTTCAATATGTCAAGGGG
AAAACATATCCATTTCGGGGCGCCTTTCCACCAGTATGGAATCCCATTGCATATTTGGATTACAATAATC
TGTGGAGGACAATAGATAACATGGGGAAGGAGATTCCAACTGATGCACCCTGGGAGGCTCAACATGCTGA
CAAATGGGACAAAATGACCATGAAAGAGCTCATTGACAAAATCTGCTGGACAAAGACTGCTAGGCGGTTT
GCTTATCTTTTTGTGAATATCAATGTGACCTCTGAGCCTCACGAAGTGTCTGCCCTGTGGTTCTTGTGGT
ATGTGAAGCAGTGCGGGGGCACCACTCGGATATTCTCTGTCACCAATGGTGGCCAGGAACGGAAGTTTGT

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: maca — 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: 300-400

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

Primer Size	Min: 18	Opt: 20	Max: 22				
Primer Tm	Min: 55	Opt: 58.0	Max: 61	Max Tm Difference: 2			
Product Tm	Min:	Opt:	Max:				
Primer GC%	Min: 45	Opt:	Max: 60				
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	Set Inside Target Penalty to allow primers inside a target.			
First Base Index:	1	CG Clamp:	0				
Salt Concentration:	50.0	Annealing Oligo Concentration:	50.0	(Not the concentration of oligos in the reaction mix but of those annealing to template.)			

☑ Liberal Base ☐ Show Debuging Info ☑ Do not treat ambiguity codes in libraries as consensus

Pick Primers | Reset Form

Primer3 Output

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

     1 GTCTAAGGAGCTGGGCATAGAGACTTACAAAGTGAATGTCAGTGAGCGTCTCGTTCAATA

    61 TGTCAAGGGGAAAACATATCCATTTCGGGGCGCCTTTCCACCAGTATGGAATCCCATTGC

   121 ATATTTGGATTACAATAATCTGTGGAGGACAATAGATAACATGGGGAAGGAGATTCCAAC

   181 TGATGCACCCTGGGAGGCTCAACATGCTGACAAATGGGACAAAATGACCATGAAAGAGCT
                >>>>>>>>>>>>>>>>>>>>>

   241 CATTGACAAAATCTGCTGGACAAAGACTGCTAGGCGGTTTGCTTATCTTTTTGTGAATAT

   301 CAATGTGACCTCTGAGCCTCACGAAGTGTCTGCCCTGTGGTTCTTGTGGTATGTGAAGCA

   361 GTGCGGGGGCACCACTCGGATATTCTCTGTCACCAATGGTGGCCAGGAACGGAAGTTTGT

   421 AGGTGGATCTGGTCAAGTGAGCGAACGGATAATGGACCTCCTCGGAGACCAAGTGAAGCT

   481 GAACCATCCTGTCACTCACGTTGACCAGTCAAGTGACAACATCATCATAGAGACGCTGAA
                                                    <<<<<<<<<<<

    54 CCATGAACATTATGAGTGCAAATACGTAATTAATGCGATCCCTCCGACCTTGACTGCCAA
        <<<<<<<<<

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