Primer Design

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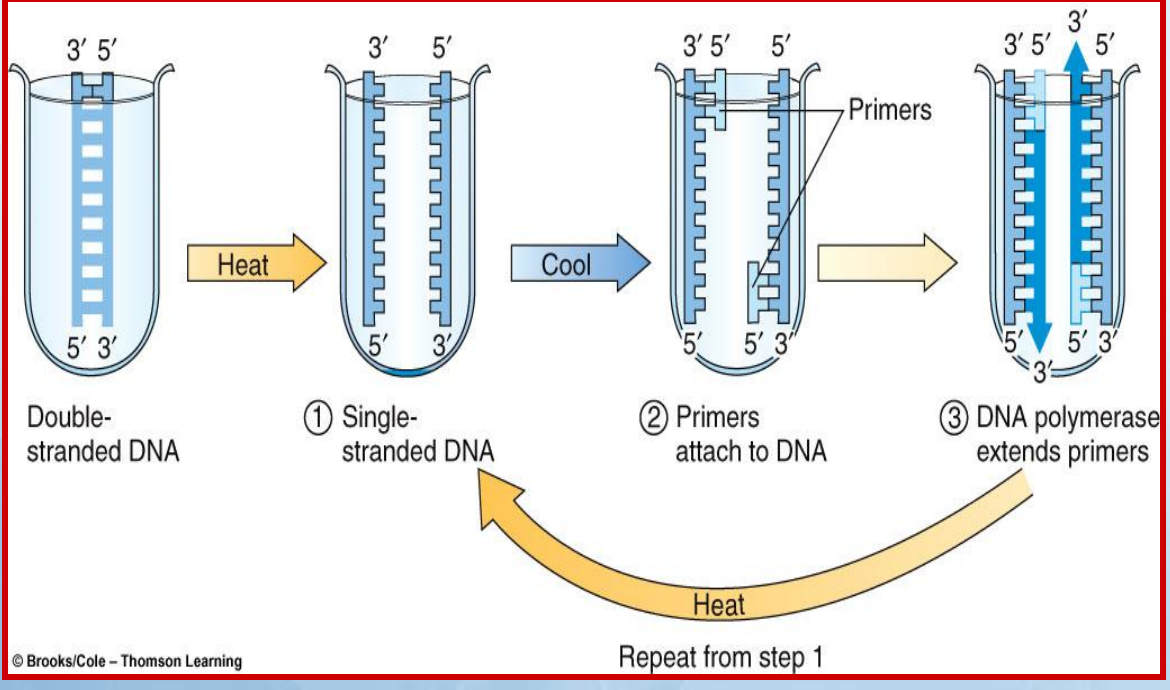
PCR Cycle

- Each cycle (Round) of PCR contains 3 steps:
  - 1- Denaturation
  - 2- Primer annealing
  - 3- Primer extension
- The cycle usually repeated for 25 – 40 times.



PROCEDURE …..

PCR

Agarose gel electrophoresis

3-4 hours

Reliable PCR from Every Sample

The final product

UV visualisation

Why Are Primers Important?

PCR Reagents

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

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

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.

Good Primer's Characteristic

Primer length

5..TCAACTTAGCATGATCGGGTAGTAGCTTGACTGTACAACTCAGCAA..3'

18-24 bp for general applications

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 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.

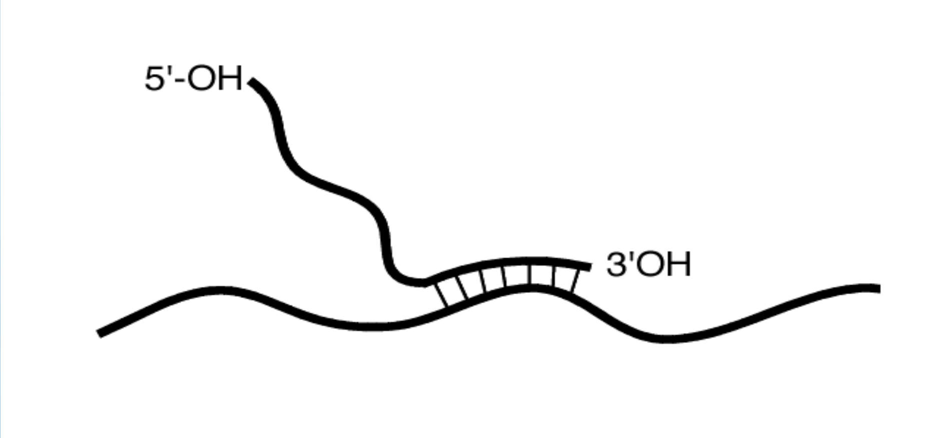
One critical feature is their annealing temperatures, which shall be compatible with each other. The maximum difference allowed is 3 °C. The closer their T_{anneal} are, the better.

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

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:

$$Tm = 3 * (G + C) + 2 * (A + T)$$

CTGATCAAGTCGATGGCTTG

Melting Temperature

Oligo sequence

CGCACTTCCAACAACCCTTC

Length 20 GC% 55.0 MW(kD) 5.98

Melting Temperature (°C):

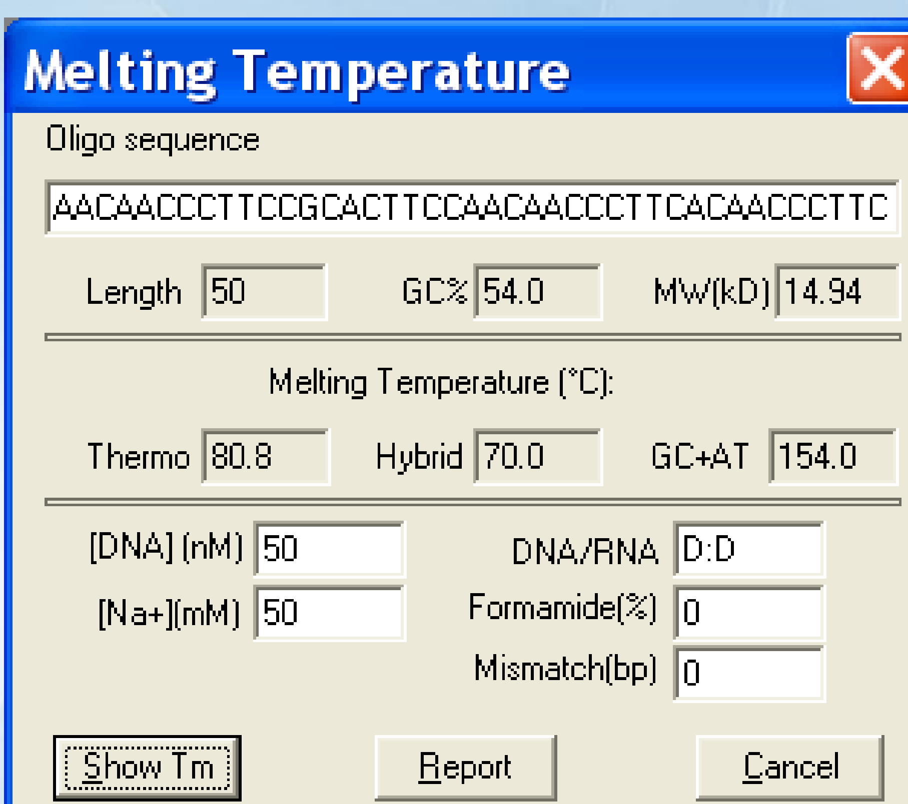
Thermo 59.4 Hybrid 52.5 GC+AT 62.0

[DNA] (nM) 50 DNA/RNA D:D

[Na+](mM) 50 Formamide(%) 0

Mismatch(bp) 0

Show Tm Report Cancel



ATGTCCAATGACAACGAAGTACCTGGTTCCATGGTTAT
TGTCGCACAAGGTCCAGACGATCAATACGCATACGAGG
TTCCCCCTGCCAGTTGGCTTGTGGGTATTGCATTTGCC
ACGGCAACAGCCACTCCTATAGGCATTCTGGGGTTCGC
ACTGGTAATGGCAGTTACCGGGGCGATGATTGACGACC
TTCTAGAAAAAGCAAACAATCTTGTAATATCCATTTAA

5' Gene 3'

ATG TAA

ATT

3' Primer

5' Primer

ATG

3' Template 5'

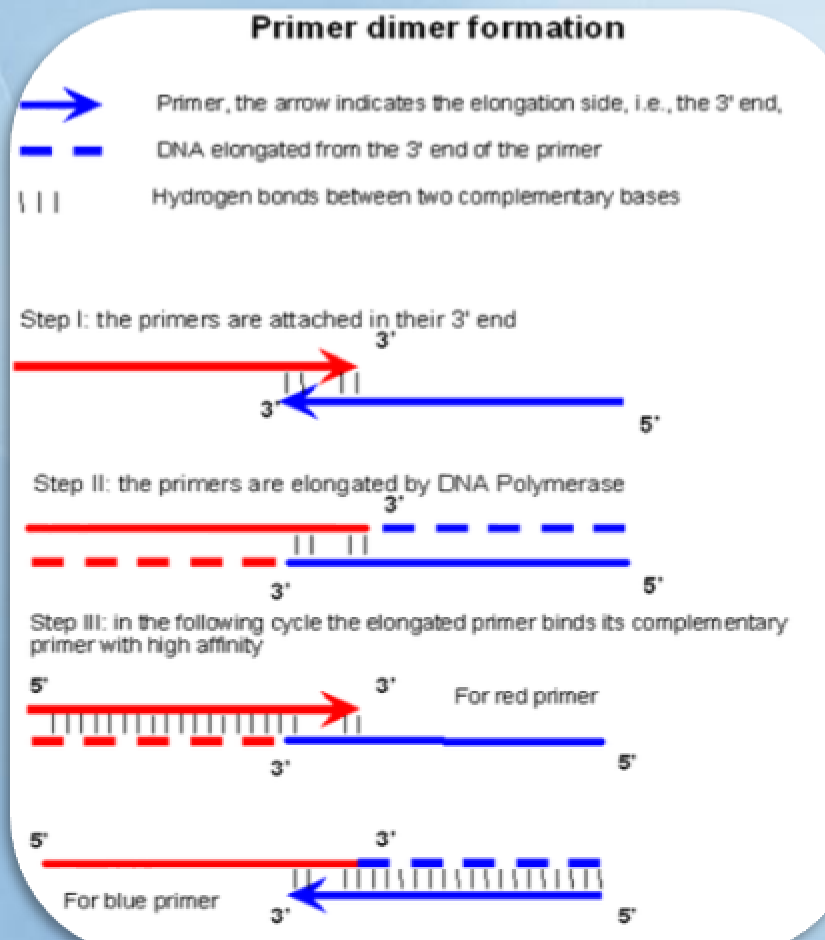
Avoid

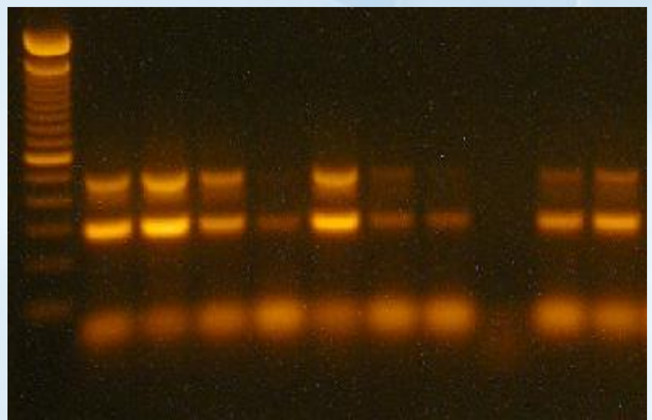
A. Avoid hairpin and stem-loop formation

Hairpin: ^{2}G = -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?

5′ 3′ ✓

5′ 3′ ✓

5′ 3′ ✓

3′
5′ ✗

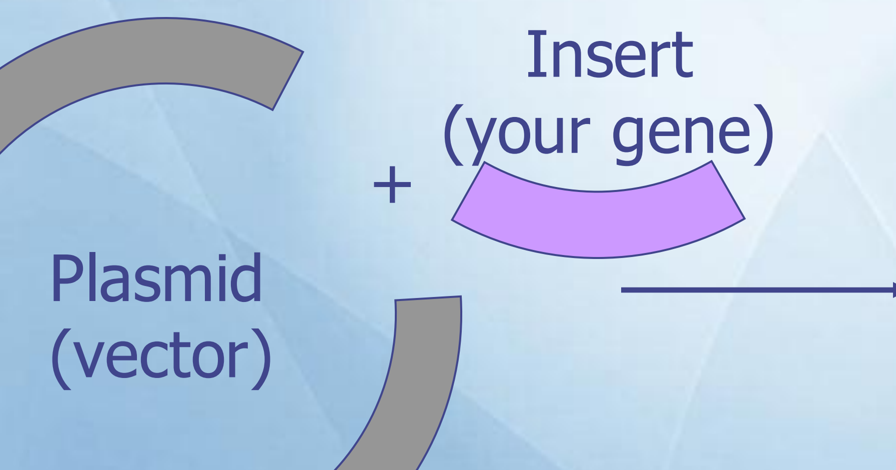
PCR primers are designed to:

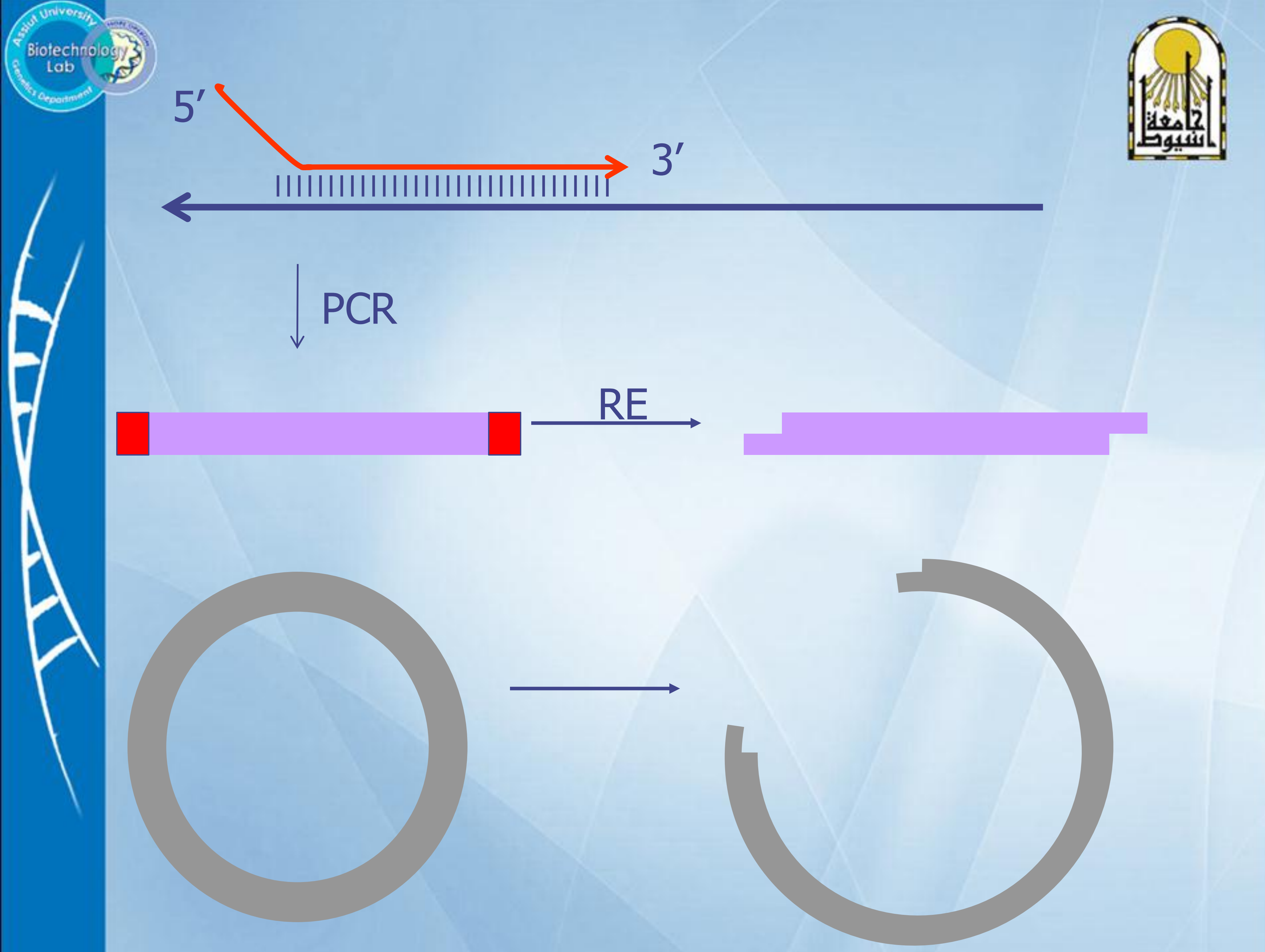
- For Cloning a special sequence
  - Full length
- For Detection
  - 100 - 500 bp
  - Gene of interest
  - Gene expression (mRNA)
  - Microbial agents detection
  - Mutation Detection
  - Quantification
  - Allelic discrimination
  - Disease
  - ...
- Random
  - ??

Cloning Overview

Four main steps in cloning:

- Insert synthesis
- Restriction enzyme digest
- Ligation
- Transformation





Assiut University
Biotechnology Lab
Genetics Department
5′
3′
PCR
RE

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)

Imperial College London

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

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	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): 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: maoa — 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
                                                       <<<<<<<<<<<

  541 CCATGAACATTATGAGTGCAAATACGTAATTAATGCGATCCCTCCGACCTTGACTGCCAA
      <<<<<<<<<

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

Assiut University
Biotechnology
Lab
Genetics Department
جامعة
اسيوط