End is not the end, in fact E.N.D is "Effort Never Dies" and if you get NO in an answer, then remember NO is "Next Opportunity". Always be positive.

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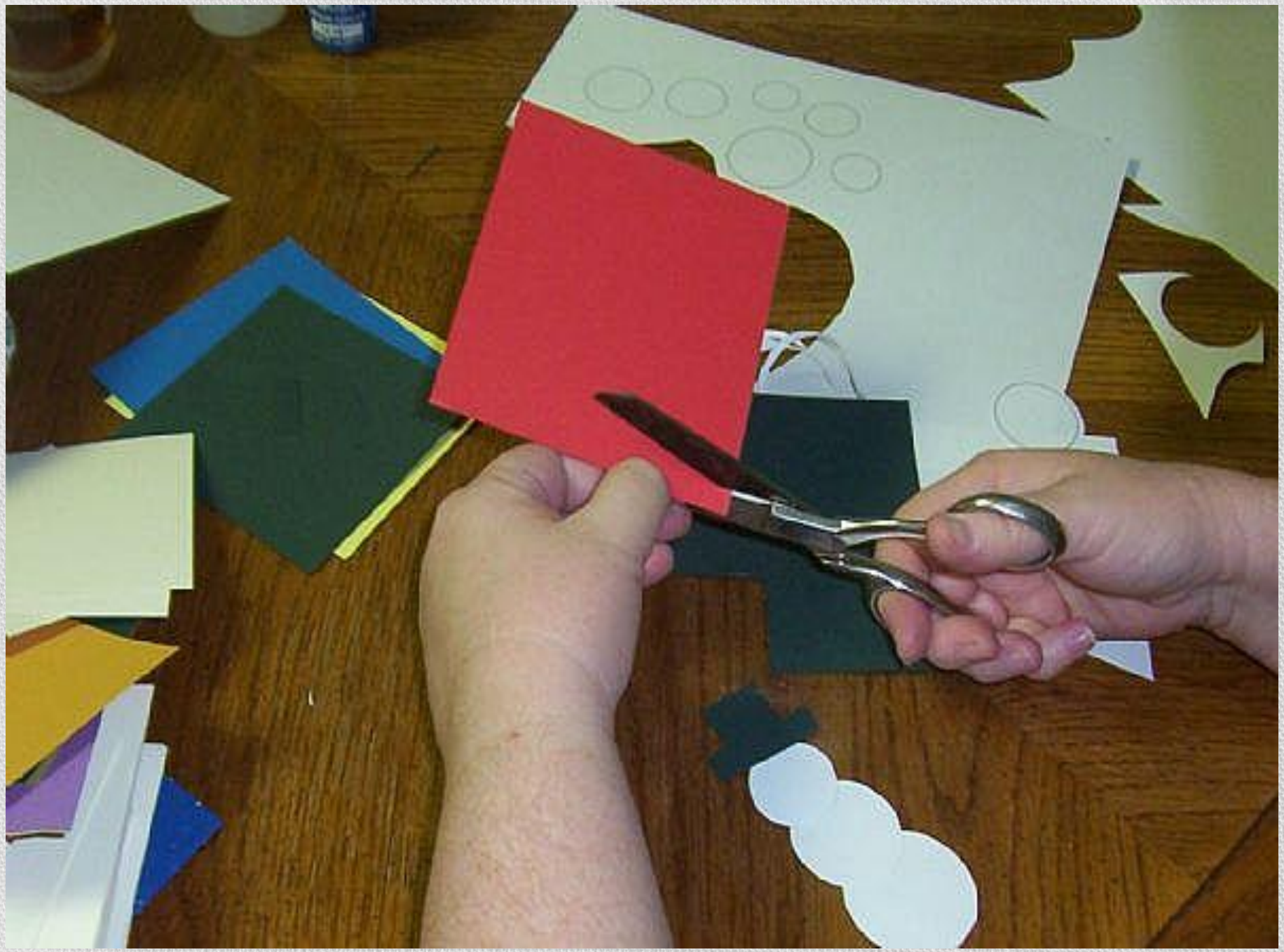
Faculty of Veterinary Medicine
Assiut University-Egypt



RESTRICTION ENDONUCLEASES, (CUTTING DNA)

(LIGATION)
LIGASES & PHOSPHATASES

Restriction enzyme = Restriction Endonuclease

Is an enzyme that cuts DNA at or near specific recognition nucleotides sequences known as Restriction site.

These enzymes are found in bacteria and provide a defense mechanism against invading viruses.

To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.

Restriction enzyme = Restriction Endonuclease

The phenomenon was first identified in work done in the laboratories of Salvador Luria and Giuseppe Bertani in early 1950s. It was found that *a bacteriophage λ* that can grow well in one strain of *Escherichia coli*, for example *E. coli C*, when grown in another strain, for example *E. coli* K, its yields can drop significantly, by as much as 3-5 orders of magnitude. *The E. coli K* host cell, known as the restricting host, appears to have the ability to reduce the biological activity of the phage λ. If a phage becomes established in one strain, the ability of that phage to grow also become restricted in other strains. In the 1960s, it was shown in work done in the laboratories of Werner Arber and Matthew Meselson that *the restriction is caused by an enzymatic cleavage of the phage DNA, and the enzyme involved was therefore termed a restriction enzyme*.

Restriction enzyme = Restriction Endonuclease

Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially.

Restriction site:

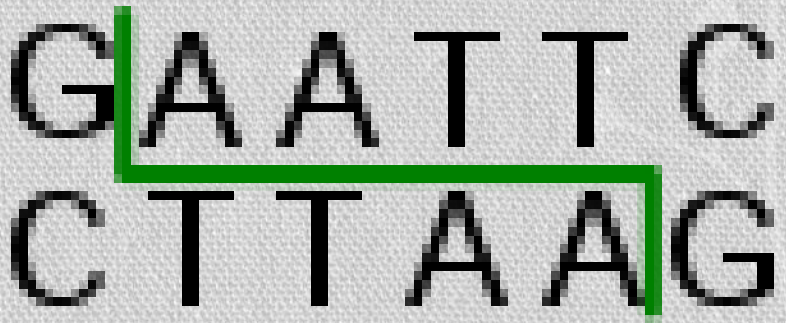
Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA.

The recognition sequences usually vary between 4 and 8 nucleotides, and many of them are **palindromic,** meaning the base sequence reads the same backwards and forwards

Restriction site:

In theory, there are two types of palindromic sequences that can be possible in DNA.

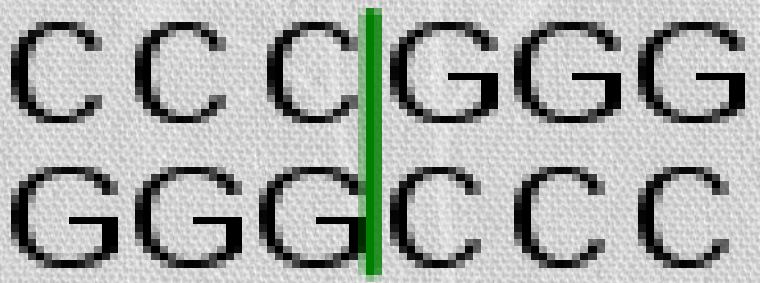
The *mirror-like* palindrome is similar to those found in ordinary text, in which a sequence reads the same forward and backwards on a single strand of DNA strand.



Restriction site:

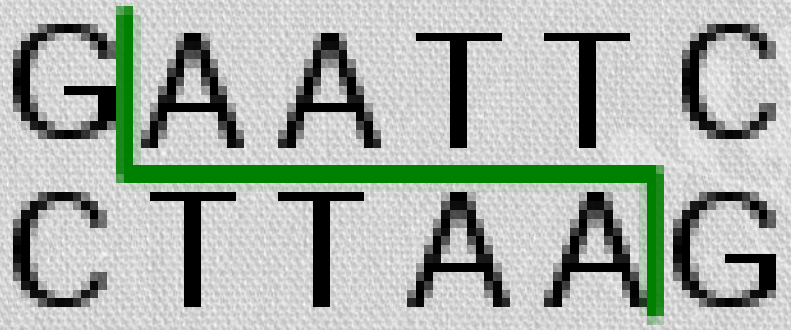
The inverted repeat palindrome is also a sequence that reads the same forward and backwards, but the forward and backward sequences are found in complementary DNA strands.

Inverted repeat palindromes are more common and have greater biological importance than mirror-like palindromes.

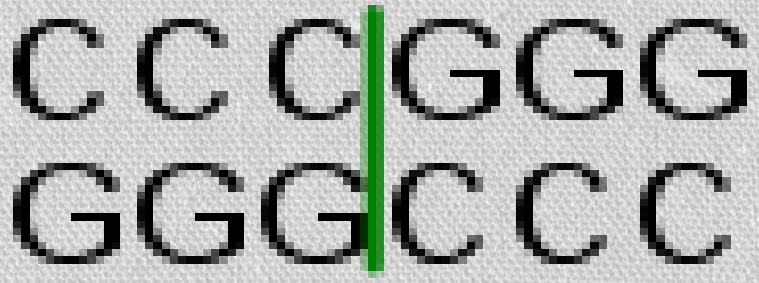


Restriction site:

- EcoRI digestion produces "sticky" ends:



- whereas SmaI restriction enzyme cleavage produces "blunt" ends:



Restriction endonucleases (restriction enzymes)

sticky ends

blunt ends

Nomenclature

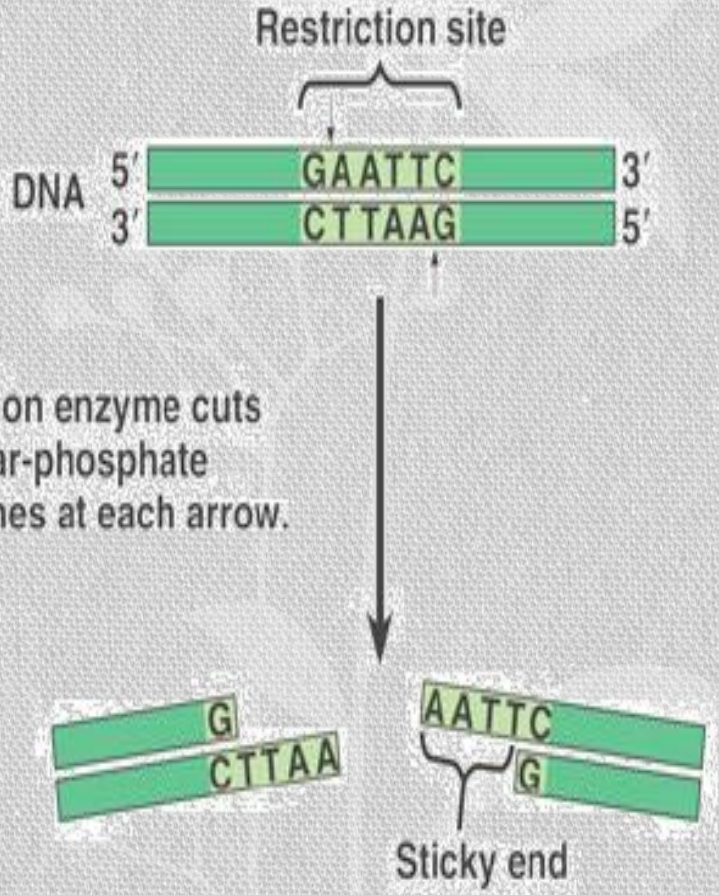
*Eco*RI

E = genus (*Escherichia*)

co = species (*coli*)

R = strain

I = # of enzyme



Restriction Endonucleases:

Enzyme	Source	Recognition Sequence	Cut
EcoRI	Escherichia coli	5'GAATTC 3'CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'
EcoRII	Escherichia coli	5'CCWGG 3'GGWCC	5'--- CCWGG---3' 3'---GGWCC ---5'
BamHI	Bacillus amyloliquefaciens	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'
HindIII	Haemophilus influenzae	5'AAGCTT 3'TTCGAA	5'---A AGCTT---3' 3'---TTCGA A---5'
TaqI	Thermus aquaticus	5'TCGA 3'AGCT	5'---T CGA---3' 3'---AGC T---5'
SmaI*	Serratia marcescens	5'CCCGGG 3'GGGCCC	5'---CCC GGG---3' 3'---GGG CCC---5'

Which enzymes I should use

PubMed

http://www.ncbi.nlm.nih.gov

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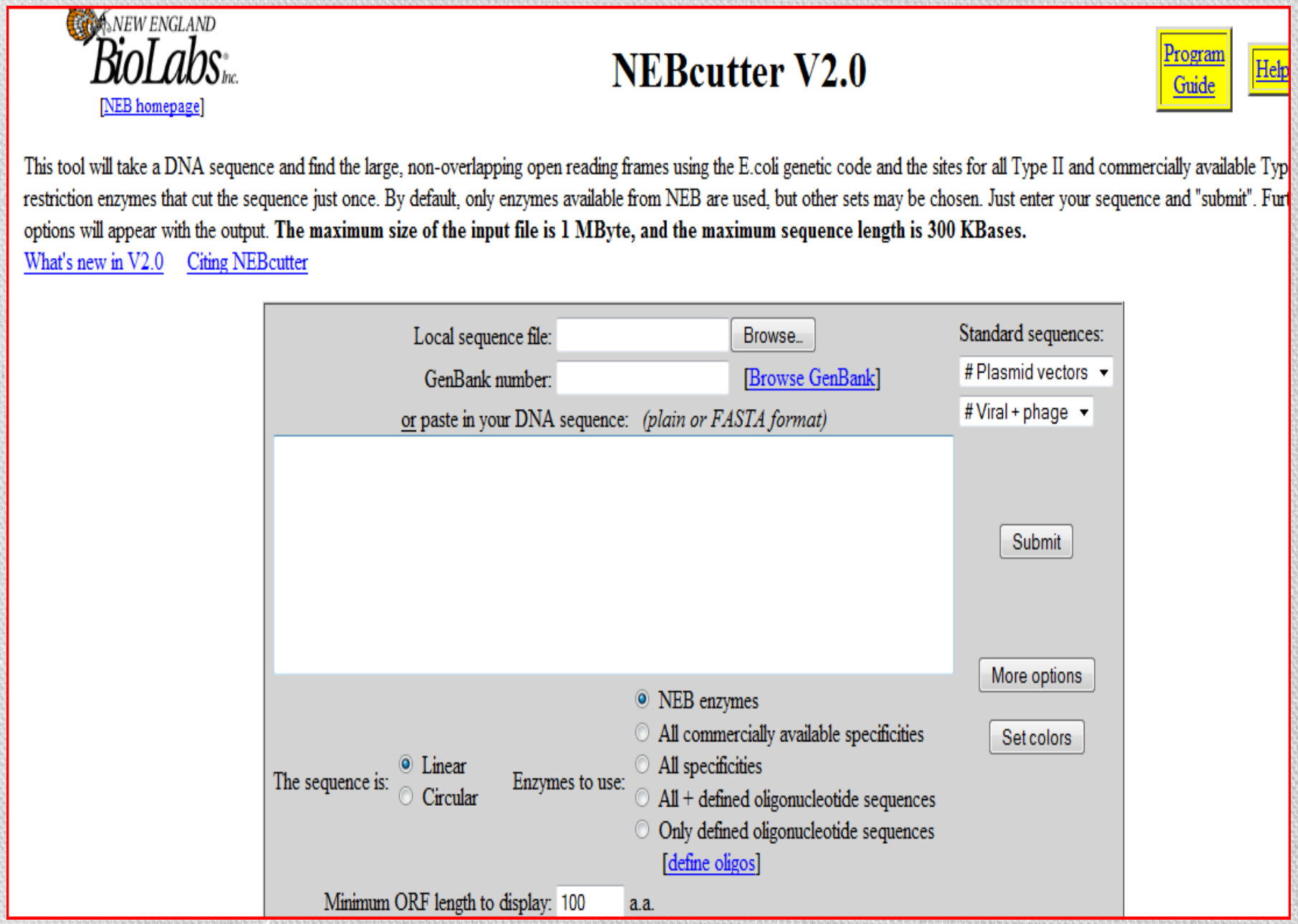
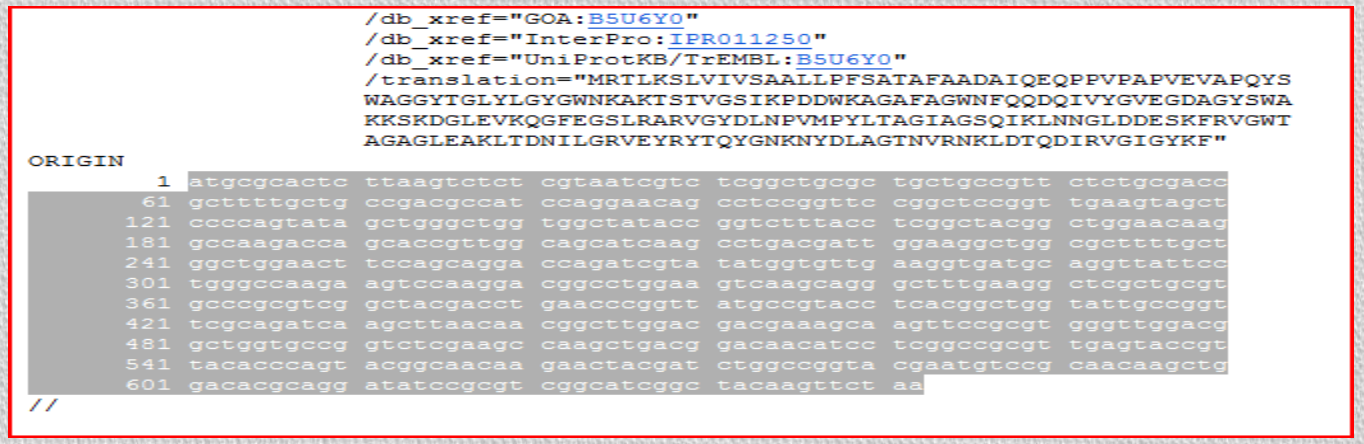

NEW ENGLAND
BioLabs Inc.
[NEB homepage]
NEBcutter V2.0
Program Guide
Help
This tool will take a DNA sequence and find the large, non-overlapping open reading frames using the E.coli genetic code and the sites for all Type II and commercially available Typ
restriction enzymes that cut the sequence just once. By default, only enzymes available from NEB are used, but other sets may be chosen. Just enter your sequence and "submit". Furt
options will appear with the output. The maximum size of the input file is 1 MByte, and the maximum sequence length is 300 KBases.
What's new in V2.0
Citing NEBcutter
Local sequence file:
Browse...
GenBank number:
[Browse GenBank]
or paste in your DNA sequence: (plain or FASTA format)
Standard sequences:
Plasmid vectors
Viral + phage
Submit
More options
Set colors
The sequence is:
Linear
Circular
Enzymes to use:
NEB enzymes
All commercially available specificities
All specificities
All + defined oligonucleotide sequences
Only defined oligonucleotide sequences
[define oligos]
Minimum ORF length to display: 100 a.a.

Copy and paste sequence

copy

paste



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      121 ccccagtata gctgggctgg tggctatacc ggtctttacc tcggctacgg ctggaacaag
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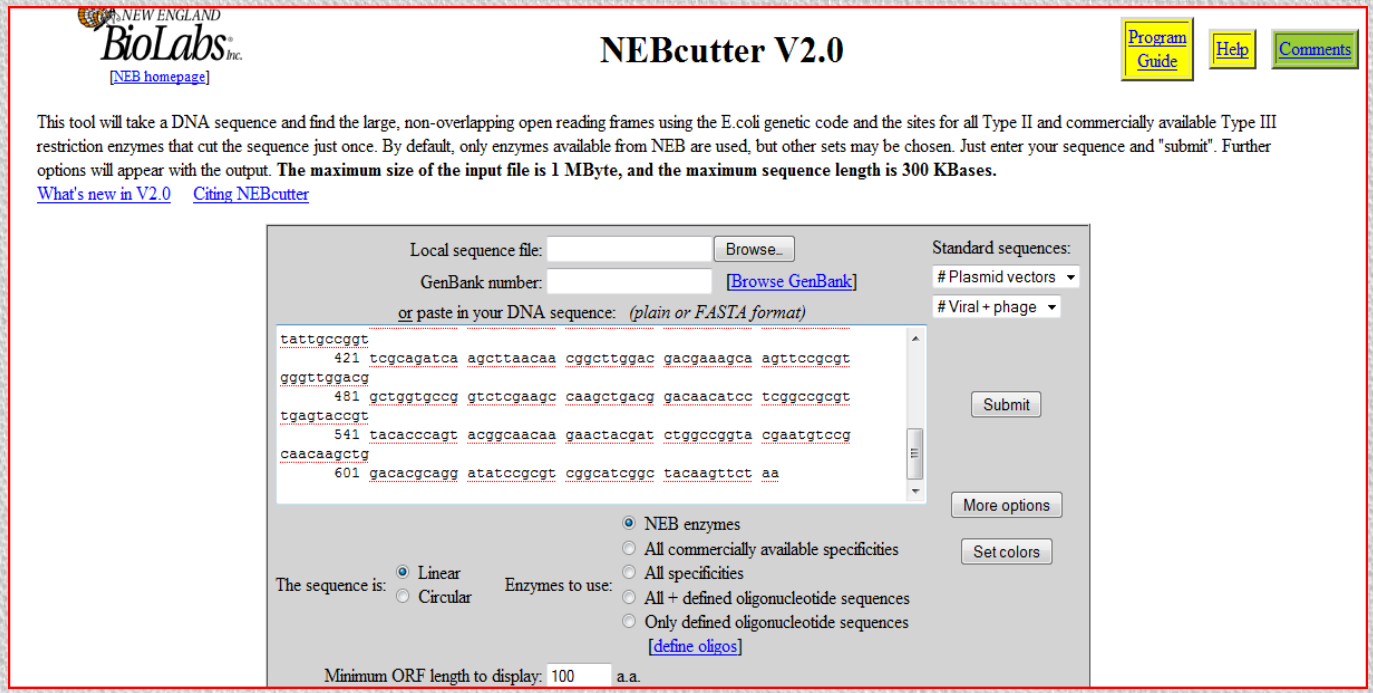


NEW ENGLAND BioLabs Inc.

[NEB homepage]

NEBcutter V2.0

Program Guide | Help | Comments

This tool will take a DNA sequence and find the large, non-overlapping open reading frames using the E.coli genetic code and the sites for all Type II and commercially available Type III restriction enzymes that cut the sequence just once. By default, only enzymes available from NEB are used, but other sets may be chosen. Just enter your sequence and "submit". Further options will appear with the output. **The maximum size of the input file is 1 MByte, and the maximum sequence length is 300 KBases.**

What's new in V2.0 Citing NEBcutter

Local sequence file: Browse..

GenBank number: [Browse GenBank]

or paste in your DNA sequence: *(plain or FASTA format)*

```
tattgccggt
      421 tcgcagatca agcttaacaa cggcttggac gacgaaagca agttccgcgt
gggttggacg
      481 gctggtgccg gtctcgaagc caagctgacg gacaacatcc tcggccgcgt
tgagtaccgt
      541 tacacccagt acggcaacaa gaactacgat ctggccggta cgaatgtccg
caacaagctg
      601 gacacgcagg atatccgcgt cggcatcggc tacaagttct aa
```

Standard sequences:

Plasmid vectors

Viral + phage

Submit

More options

Set colors

The sequence is: Linear / Circular

Enzymes to use:

- NEB enzymes
- All commercially available specificities
- All specificities
- All + defined oligonucleotide sequences
- Only defined oligonucleotide sequences

[define oligos]

Minimum ORF length to display: 100 a.a.

Restriction Map

Noncutters: AarI, AatII, AbsI, AccI, AclI, AflIII, AjuI, AlfI, AloI, AlwNI, ApaI, ApaLI, ApoI, ArsI, AscI, AsuII, AvaI, AvrII, BalI, BamHI, BarI, BbvCI, BcgI, BciVI, BclI, BdaI, BglI, BglII, BplI, Bpu10I, BsaAI, BsaBI, BsaXI, BseMII, BsePI, BseRI, BsgI, BsmI, Bsp1407I, BspHI, BsrBI, BsrDI, BstEII, BstXI, BtgZI, BtrI, BtsI, ClaI, CspCI, DraII, DraIII, DrdI, Eam1105I, EciI, Eco47III, Eco57I, Eco57MI, EcoNI, EcoRI, FseI, GsuI, HaeIV, Hin4I, HindII, HpaI, KpnI, MauBI, MboII, MfeI, MluI, MslI, NaeI, NarI, NcoI, NdeI, NheI, NotI, NruI, NspI, OliI, PacI, PasI, PflMI, PleI, PmaCI, PmeI, PpiI, PpuMI, PshAI, PsiI, PI-PspI, PstI, PvuI, PvuII, RsrII, SacI, SacII, SalI, SanDI, SapI, ScaI, PI-SceI, SexAI, SfiI, SgfI, SgrAI, SgrDI, SmaI, SnaBI, SpeI, SphI, SrfI, Sse8387I, SspI, StuI, SwaI, TaqII, TatI, TfiI, Tsp45I, TspDTI, TspRI, Tth111I, VspI, XbaI, XhoI, XhoII, XmnI

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
EcoRV	GATATC	6	blunt	1	612
FspAI	RTGCGCAY	8	blunt	1	4
AcyI	GRCGYC	6	five_prime	1	74
AflII	CTTAAG	6	five_prime	1	10
AgeI	ACCGGT	6	five_prime	1	148
AvaII	GGWCC	5	five_prime	1	258
BccI	CCATC	5	five_prime	1	85
BseYI	CCCAGC	6	five_prime	1	131
BspMI	ACCTGC	6	five_prime	1	280
Eco31I	GGTCTC	6	five_prime	1	496
Esp3I	CGTCTC	6	five_prime	1	33
FauI	CCCGC	5	five_prime	1	370

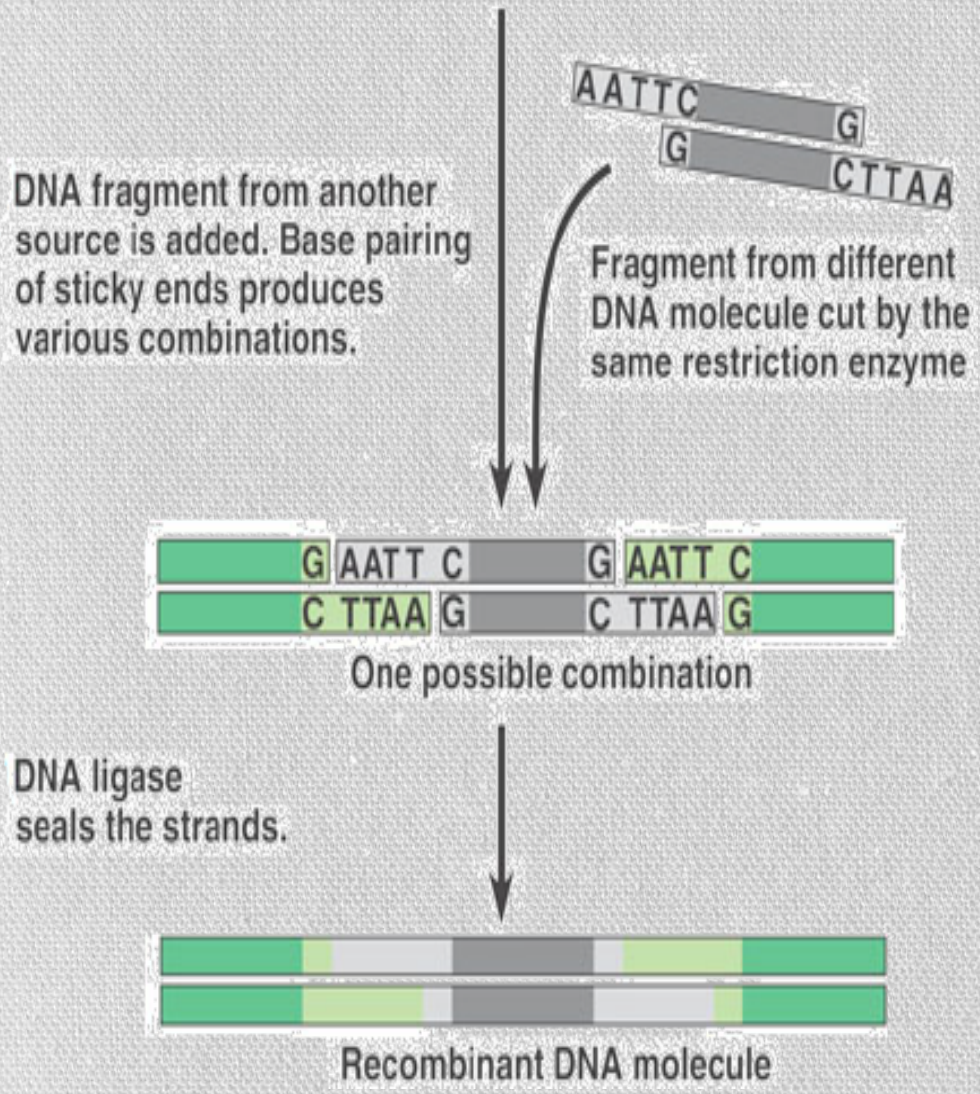
Ligation (Ligases & Phosphatases)

Ligation in molecular biology is the joining of two nucleic acid fragments through the action of an enzyme.

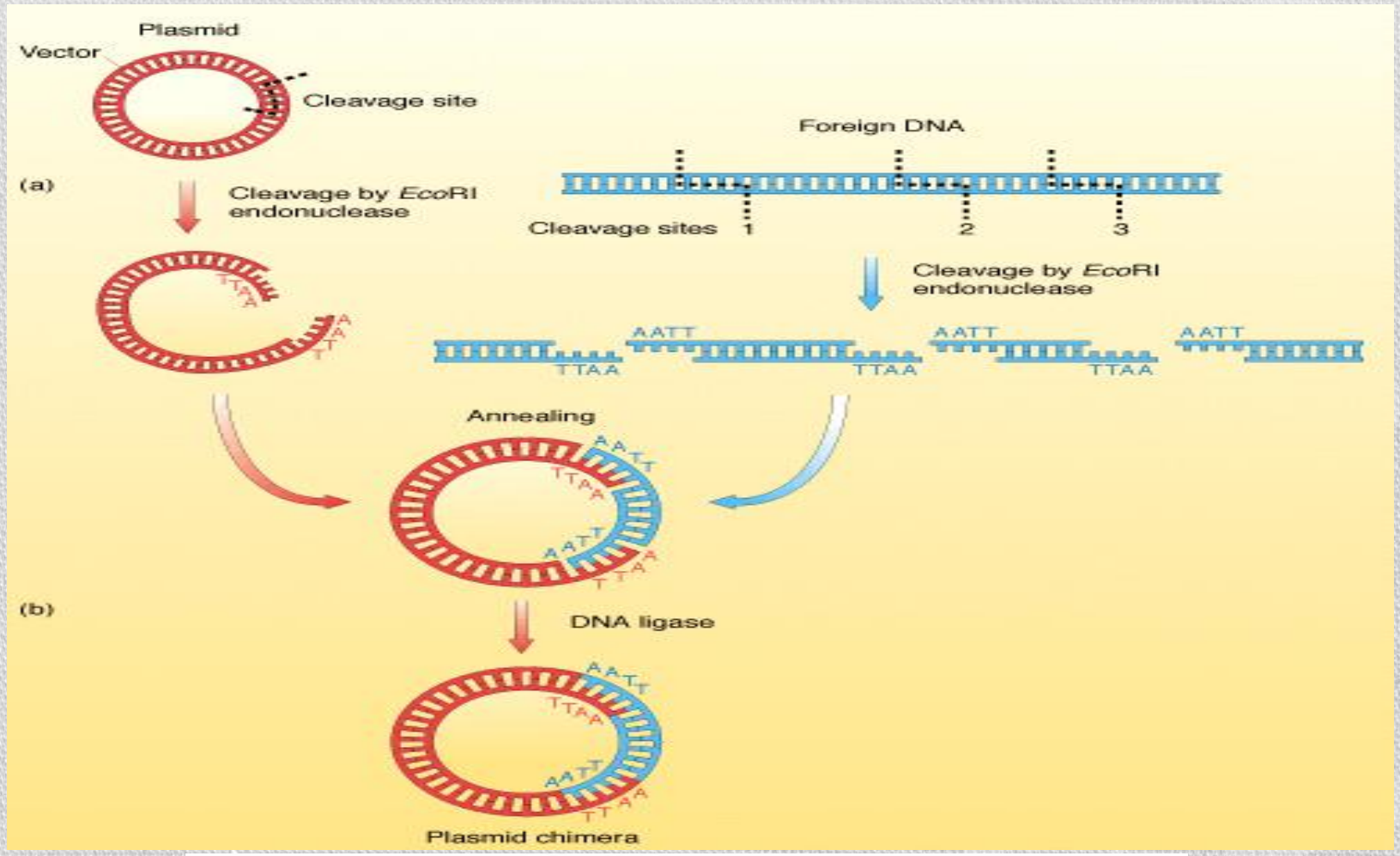
It is an essential laboratory procedure in the molecular cloning by which DNA fragments are joined together to create recombinant DNA molecules, such as when a foreign DNA fragment is inserted into a plasmid.

The ends of DNA fragments are joined together by the formation of phosphodiester bonds between the 3'-hydroxyl of one DNA termini with the 5'-phosphoryl of another.

Pasting DNA



- Complementary ends (sticky ends) H-bond
- Ligase forms phosphodiester bond to seal strands together.


Plasmid
Vector
Cleavage site
(a)
Cleavage by EcoRI endonuclease
Foreign DNA
Cleavage sites
1
2
3
Cleavage by EcoRI endonuclease
AATT
TTAA
Annealing
(b)
DNA ligase
Plasmid chimera


BamHI
G GATCC
CCTAG G
BamHI
G
GATCC
CCTAG
G
Sticky ends

Sticky-end ligation

In cloning experiments most commonly-used restriction enzymes generate a 4-base single-stranded overhang called the sticky or cohesive end.

These sticky ends can anneal to other compatible ends and become ligated in a sticky-end (or cohesive end) ligation.

EcoRI for example generates an AATT end, and since A and T have lower melting temperature than C and G, its melting temperature T_m is low at around 6˚C. For most restriction enzymes, the overhangs generated have a T_m that is around 15˚C. For practical purposes, sticky end ligations are performed at 12-16˚C, or at room temperature, or alternatively at 4˚C for a longer period.

Blunt-end ligation

Blunt end may be ligated to another blunt end, Blunt ends may be generated by restriction enzymes such as *Sma*I and *Eco*RV.

However a major advantage of blunt-end cloning is that the desired insert does not require any restriction sites in its sequence as blunt-ends are usually generated in a PCR, and the PCR generated blunt-ended DNA fragment may then be ligated into a blunt-ended vector generated from restriction digest.

Disadvantages of blunt-end ligation:

1- ligation is much less efficient than sticky end ligation, typically the reaction is 100X slower than sticky-end ligation.

2- The concentration of ligase used is higher than sticky end ligation (10x or more).

3- The concentration of DNA used in blunt-end ligation is also higher to increase the likelihood of collisions between ends.

4- Longer incubation time may also be used for blunt-end ligations.

Method of Ligation

Add 1 µl of the PDrive vector of the ligation in 0.2 µl or 0.5 µl PCR tubes,

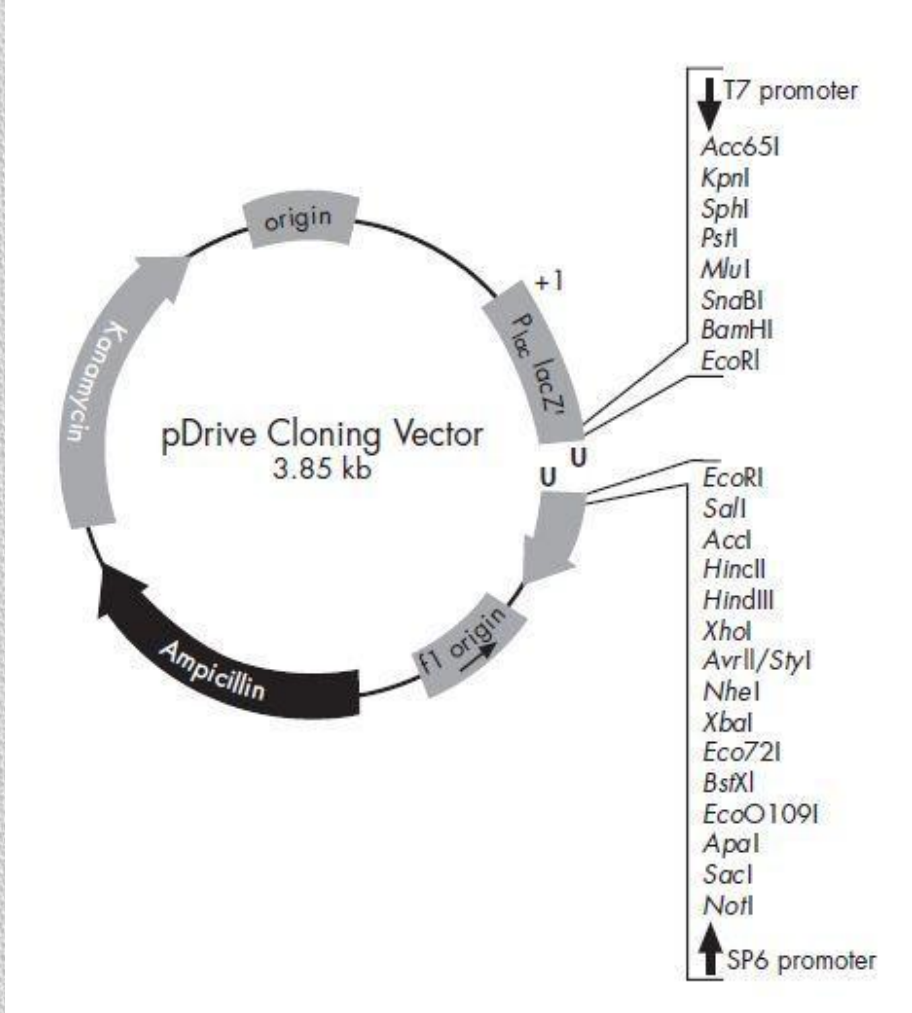
Add 1:4 µl of the purified PCR product,

Add 5 µl of ligation master mix &

Variable µl of water then put in the thermo cycler for four hours at 16°C.

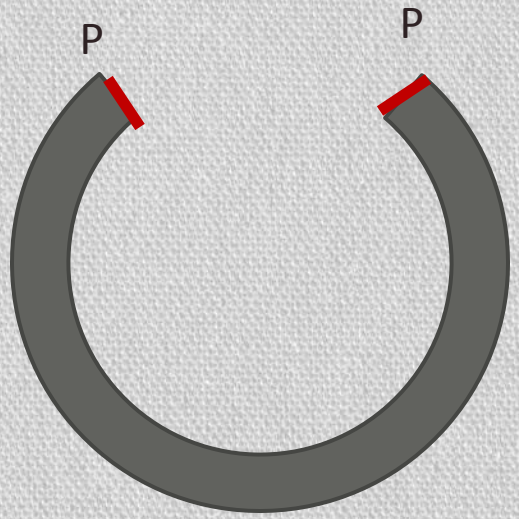
pDrive Cloning Vector

- Ampicillin and kanamycin selection
- Blue/white screening
- Unique restriction endonuclease recognition sites around the cloning site
- T7 and SP6 promoter on either side of the cloning site allowing *in vitro* transcription of cloned PCR products as well as sequence analysis using standard sequencing primers.
- Phage f1 origin to allow preparation of single-stranded DNA



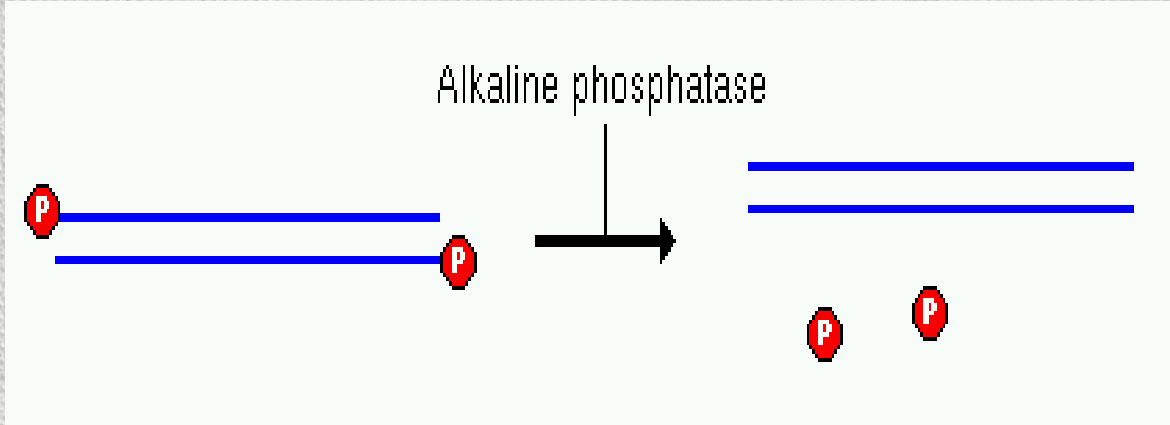
Alkaline phosphatase dephosphorylation

Removing phosphate group to prevent self ligation of the vector



Alkaline phosphatase:

Removes 5' phosphate groups from DNA and RNA. It will also remove phosphates from nucleotides and proteins. These enzymes are most active at alkaline pH - hence the name.



There are several sources of alkaline phosphatase that differ in how easily they can be inactivated:

Bacterial alkaline phosphatase (BAP) is the most active one, but also the most difficult to destroy at the end of the dephosphorylation reaction.

Calf intestinal alkaline phosphatase (CIP) is purified from bovine intestine. This is phosphatase most widely used in molecular biology labs because, although less active than BAP, it can be effectively destroyed by protease digestion or heat (75C for 10 minutes in the presence of 5 mM EDTA).

Shrimp alkaline phosphatase is derived from a cold-water shrimp and is promoted for being readily destroyed by heat (65C for 15 minutes).

Trouble-shooting

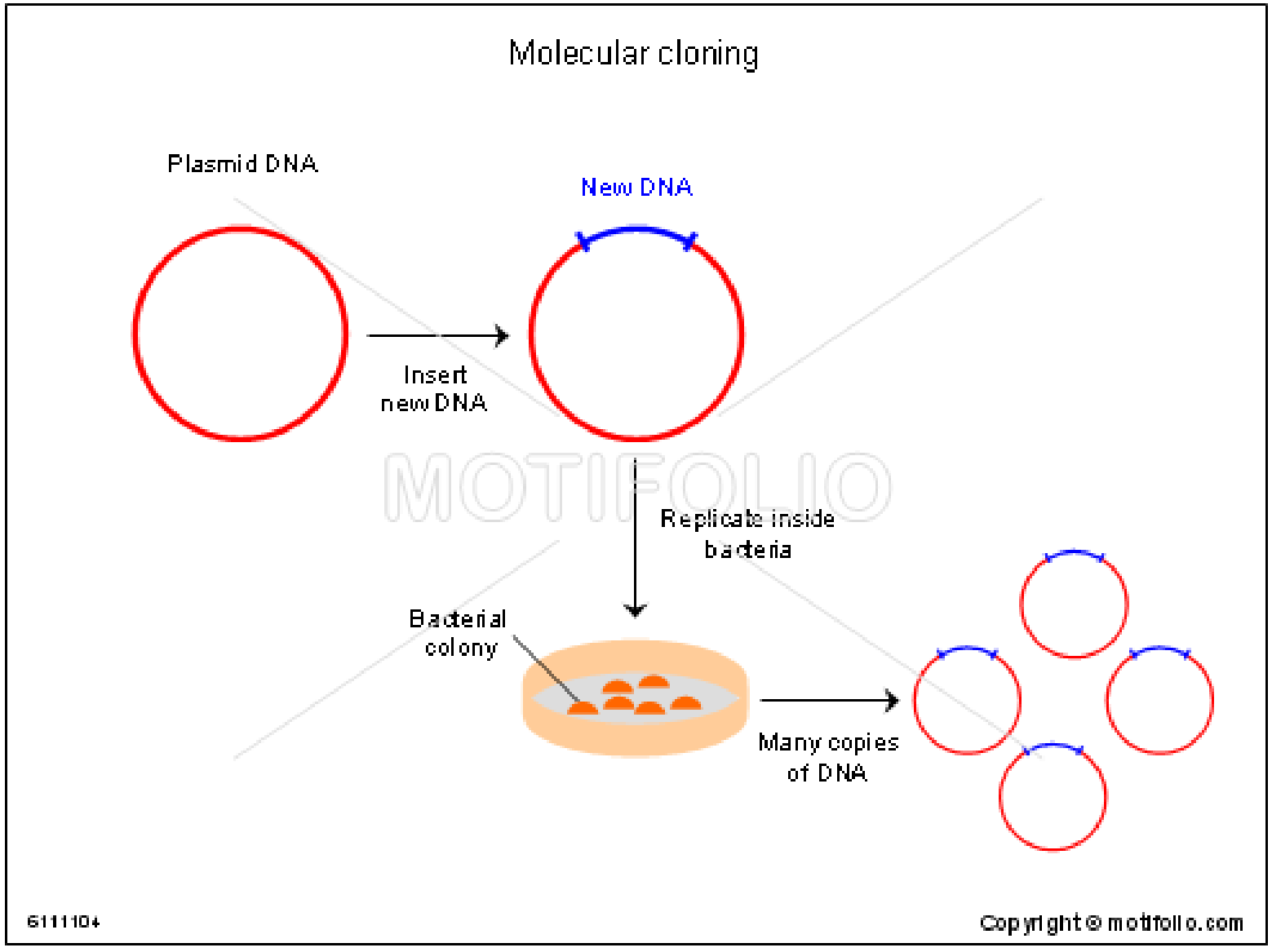
Sometimes ligation fail to produce the desired ligated products, and some of the possible reasons may be:

1- Damaged DNA - over-exposure to UV radiation during preparation of DNA for ligation can damage the DNA.

2- Excessive amount of DNA used.

3- Incomplete DNA digest, The vector DNA that is incompletely digested will give rise to a high background. Insert that is not completely digested will also not ligate properly and circularize.

4- Incomplete ligation. Blunt-ends DNA and some sticky-ends DNA that have low-melting temperature require more ligase and longer incubation time.


Molecular cloning
Plasmid DNA
New DNA
Insert
new DNA
Replicate inside
bacteria
Bacterial
colony
Many copies
of DNA
611104
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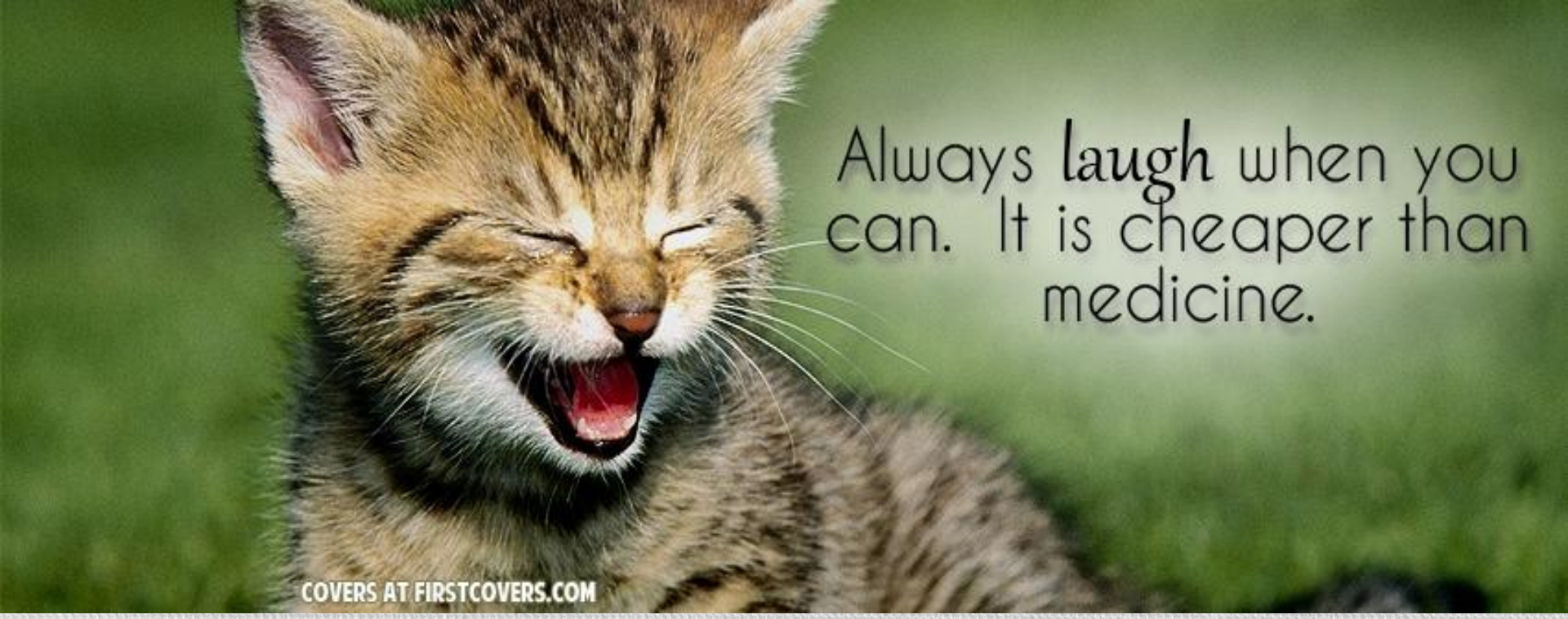

Thanks a lot

with my Best Regards and My Best wishes

Amira A. AL-Hosary
E-mail: Amiraelhosary @yahoo.com
Mob. (002) 01004477501