Restriction Endonucleases, (Cutting DNA)
(Ligation)
Ligase & Phosphatase

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Restriction enzyme = Restriction Endonuclease

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

Idea:

These enzymes are found normaly in bacteria to 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.

***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.**

Restriction enzyme = Restriction Endonuclease

In the 1960s, it was shown in work done in the laboratories of Werner Arber and Matthew Meselson and they defined 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:

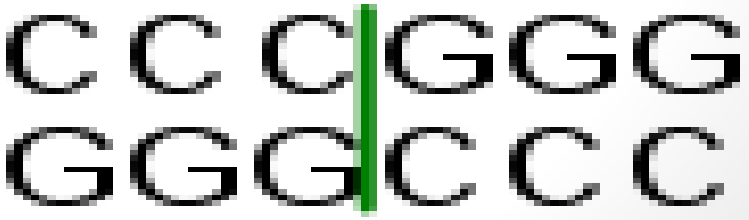
There are two types of palindromic sequences that can be possible in DNA:

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.**

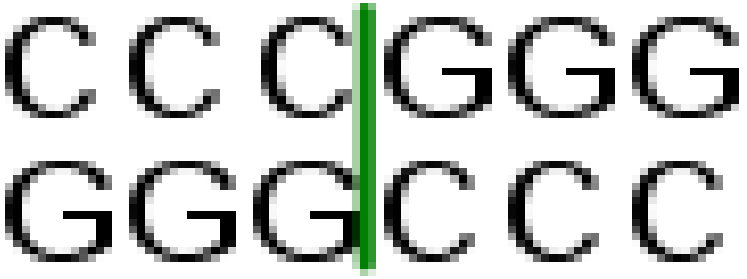
Inverted repeat palindromes are more common and have greater biological importance than mirror-like palindromes.



Restriction site:

- EcoRI digestion produces "sticky" ends:

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



Restriction endonucleases (restriction enzymes)

sticky ends

blunt ends

Nomenclature

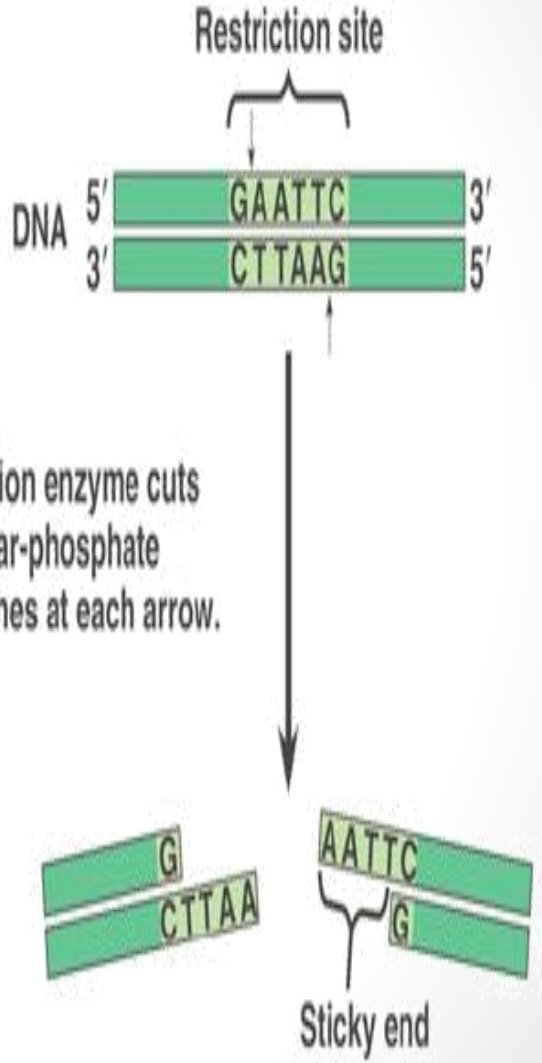
*Eco*RI

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.

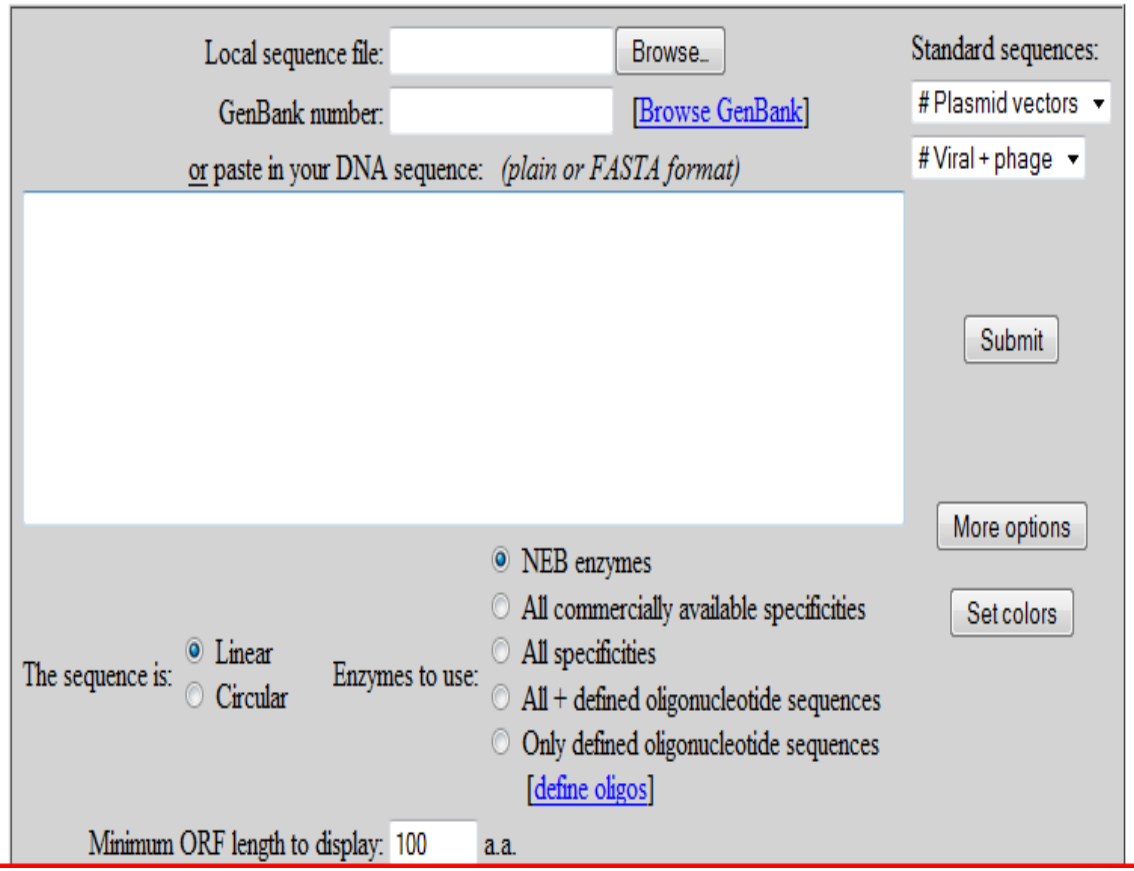
[NEB homepage]

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



Copy and paste sequence

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ORIGIN
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       61 gcttttgctg ccgacgccat ccaggaacag cctccggttc cggctccggt tgaagtagct
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//
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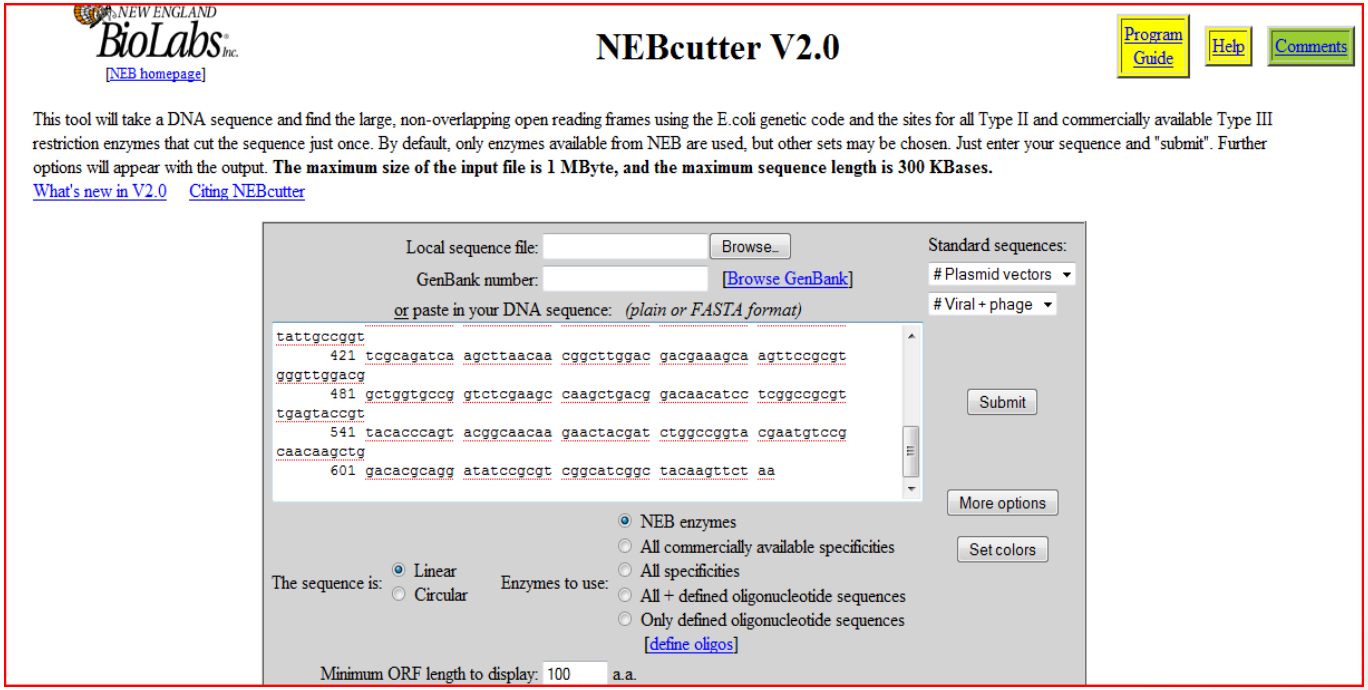
paste



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

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.

Ligation (Ligases & Phosphatases)

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

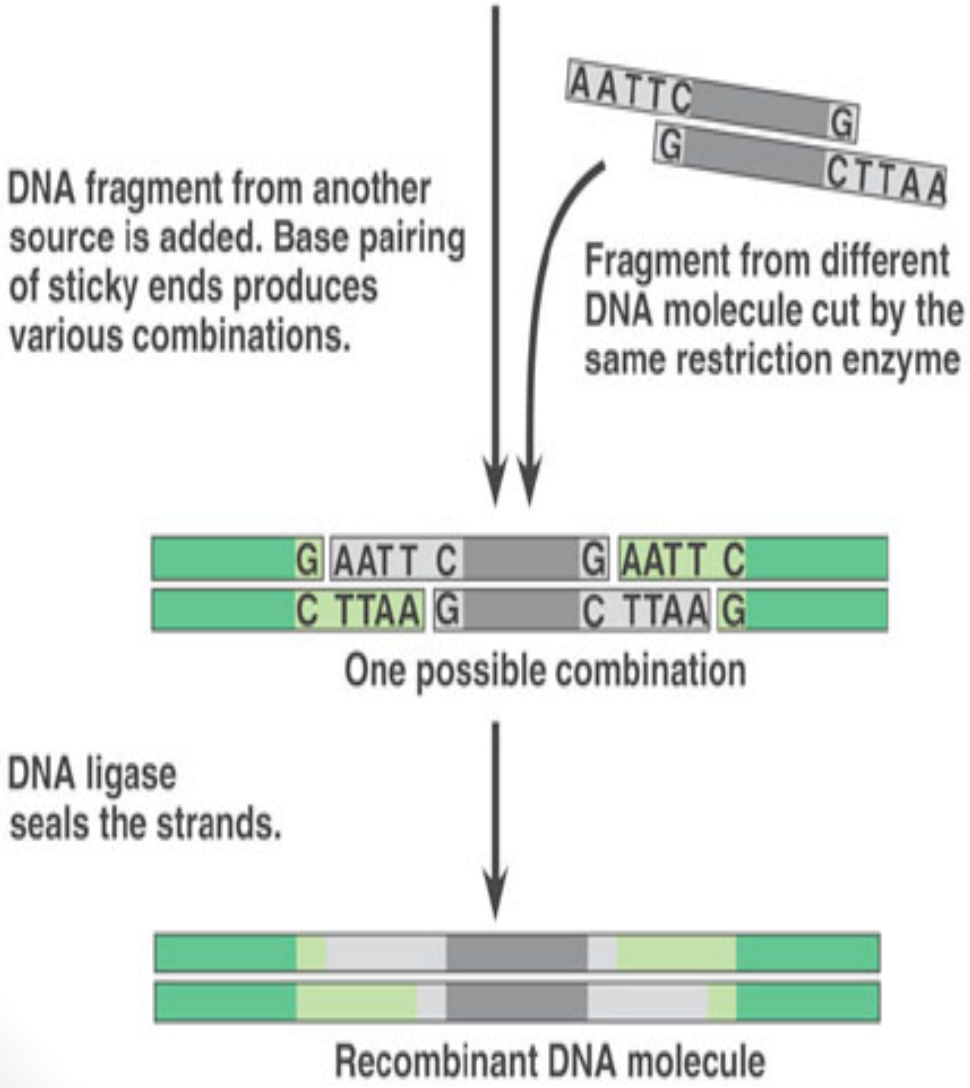
It is an essential laboratory procedure in the **molecular cloning** by which DNA fragments are joined together to **create recombinant DNA molecules.**

Ligation (Ligases & Phosphatases)

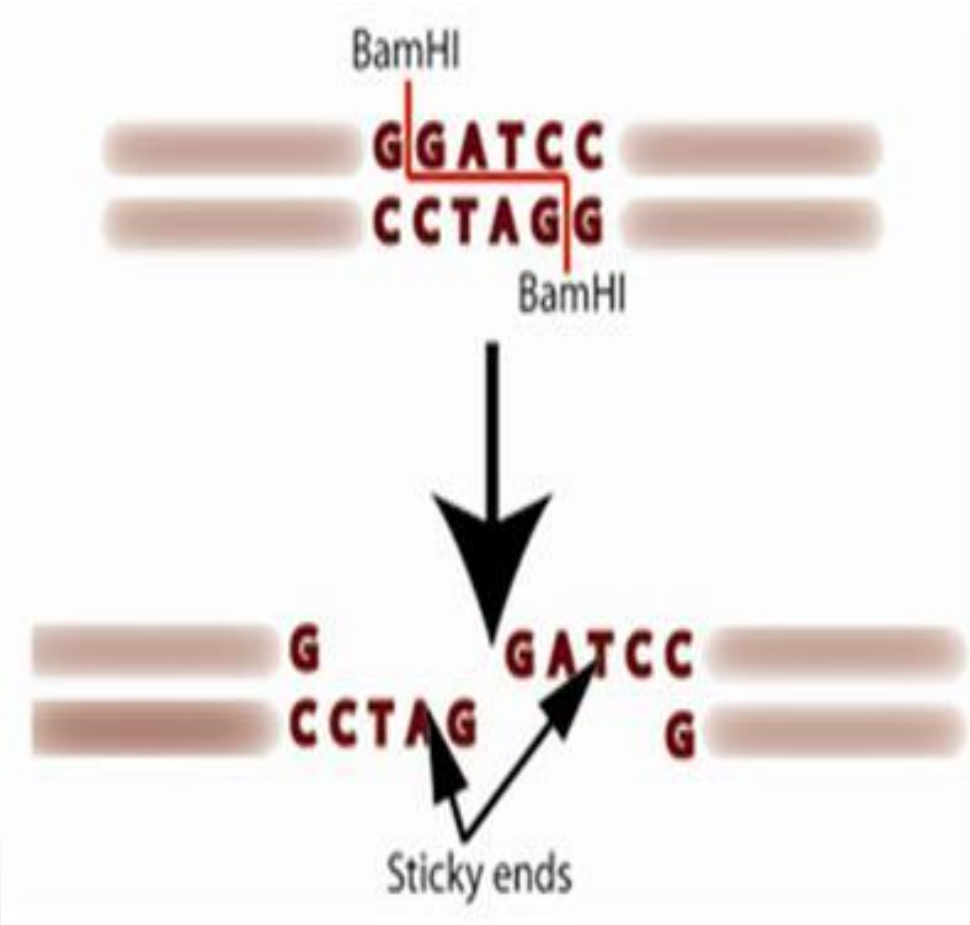
when a foreign DNA fragment is inserted into a plasmid.

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.


BamHI
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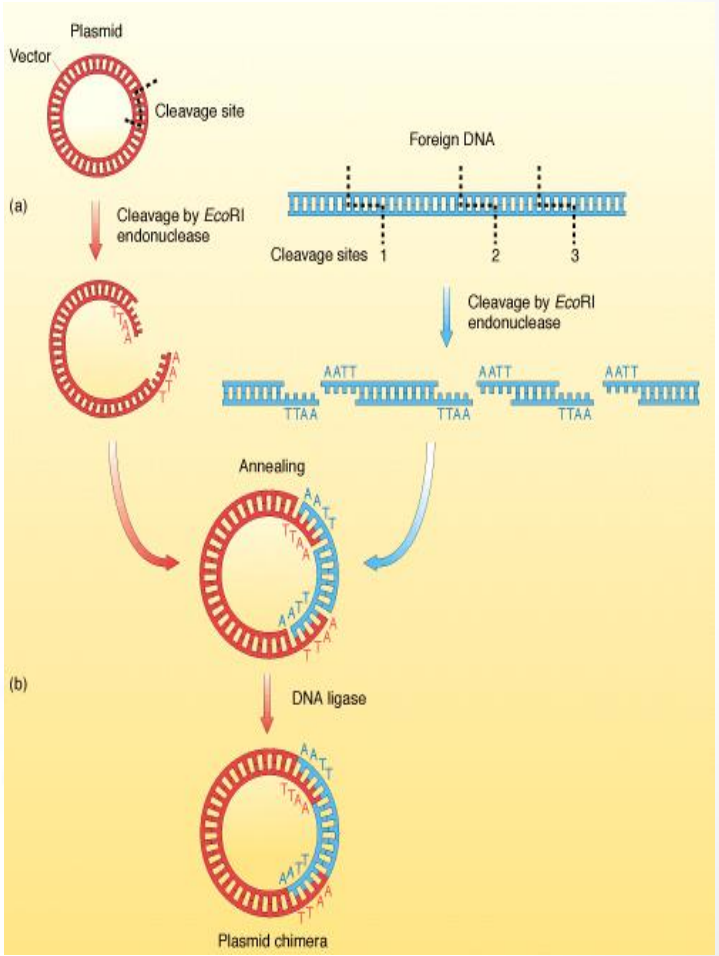
CCTAGG
BamHI
G
GATCC
CCTAG
G
Sticky ends


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

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 (cohesive end) ligation.

Sticky-end ligation:

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.

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.

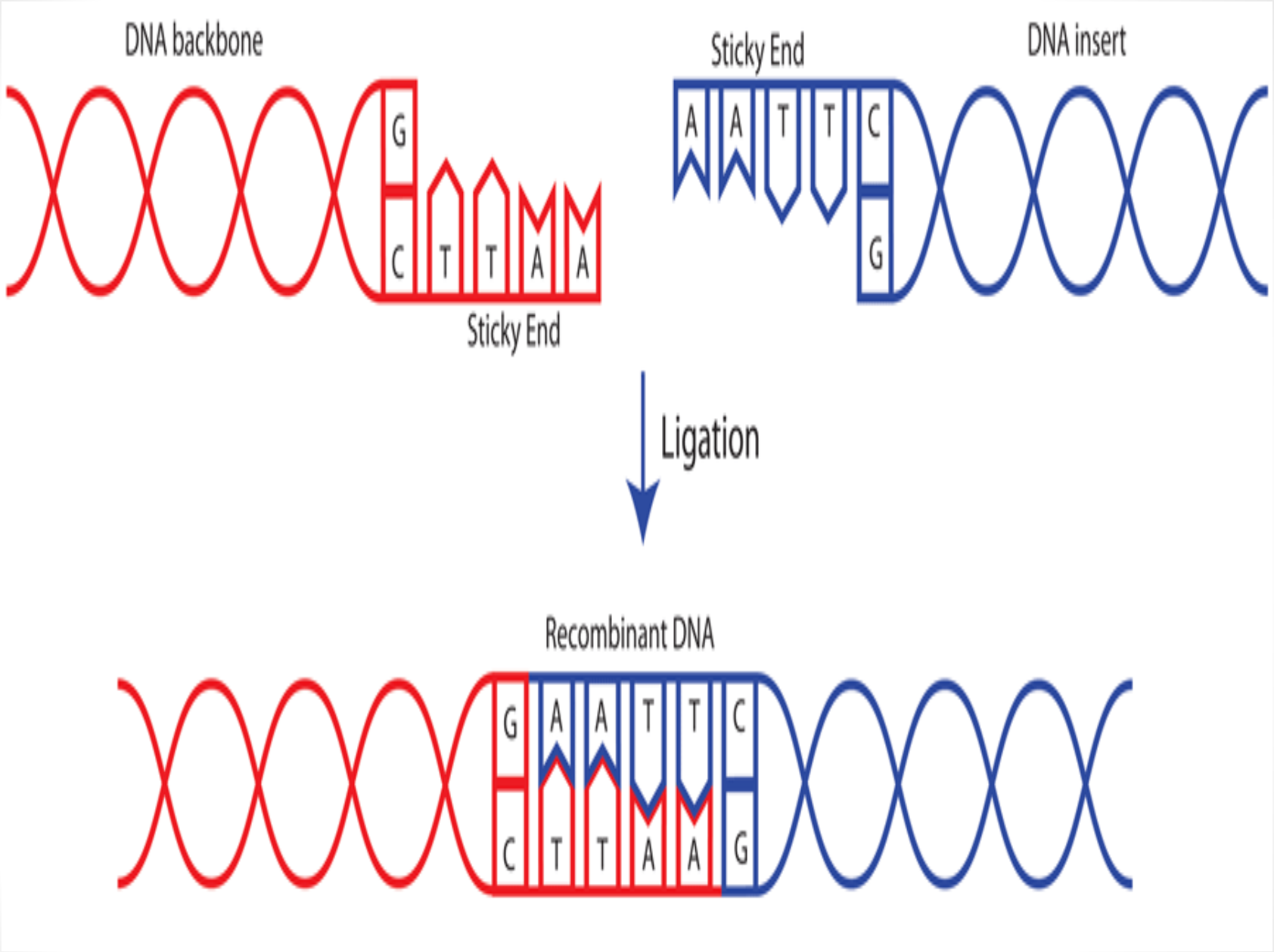
Method of Ligation:

Add 1 µl of the vector of the ligation in o.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^{o}C.


DNA backbone
G
C
T
T
A
A
Sticky End
Sticky End
DNA insert
A
A
T
T
C
G
Ligation
Recombinant DNA
G
A
A
T
T
C
C
T
T
A
A
G

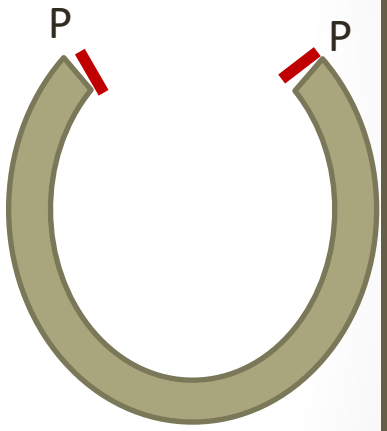
Alkaline phosphatase dephosphorylation

Digested DNA typically possesses a 5′ phosphate group that is required for ligation.

In order to prevent self-ligation, the 5′ phosphate can be removed prior to ligation.

Dephosphorylation of the 5′ end prohibits self-ligation, enabling us to manipulate the DNA as desired before re-ligating.

Removing phosphate group to prevent self ligation of the vector

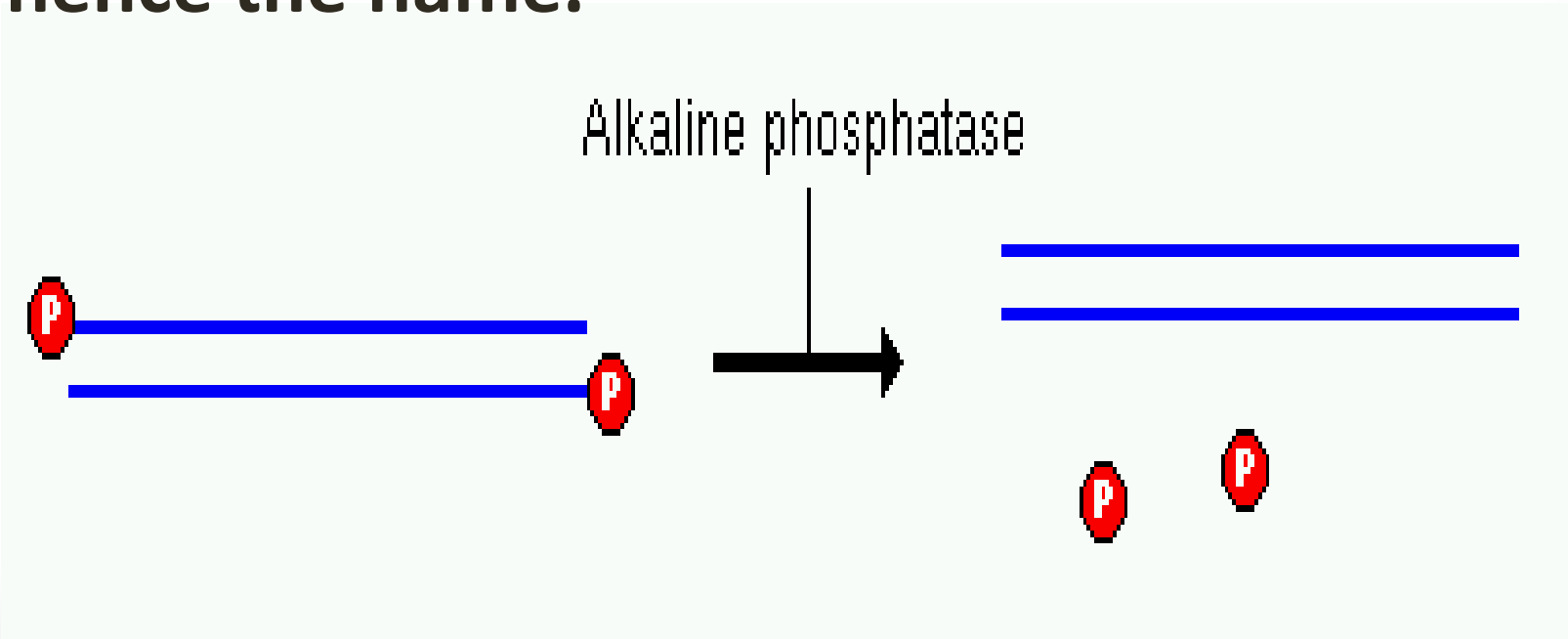


Alkaline phosphatase:

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:

1. **Bacterial alkaline phosphatase (BAP)** is the most active one, but also the most difficult to destroy at the end of the dephosphorylation reaction.
2. **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).
3. **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

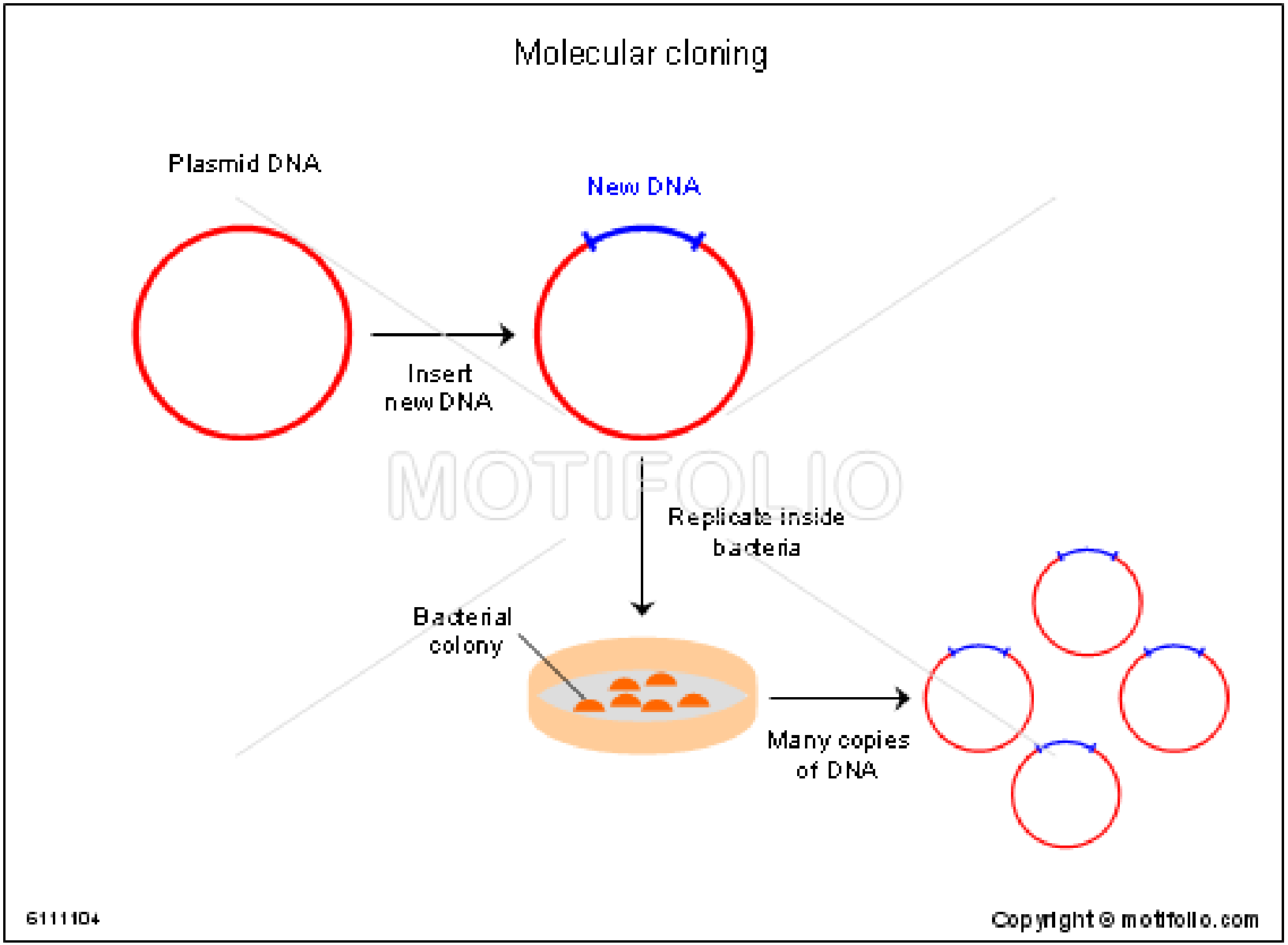
Sometimes ligation fail to produce the desired ligated products, and some of the possible reasons may be:

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
6111104
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Thanks a lot

with my Best Regards and My Best wishes

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