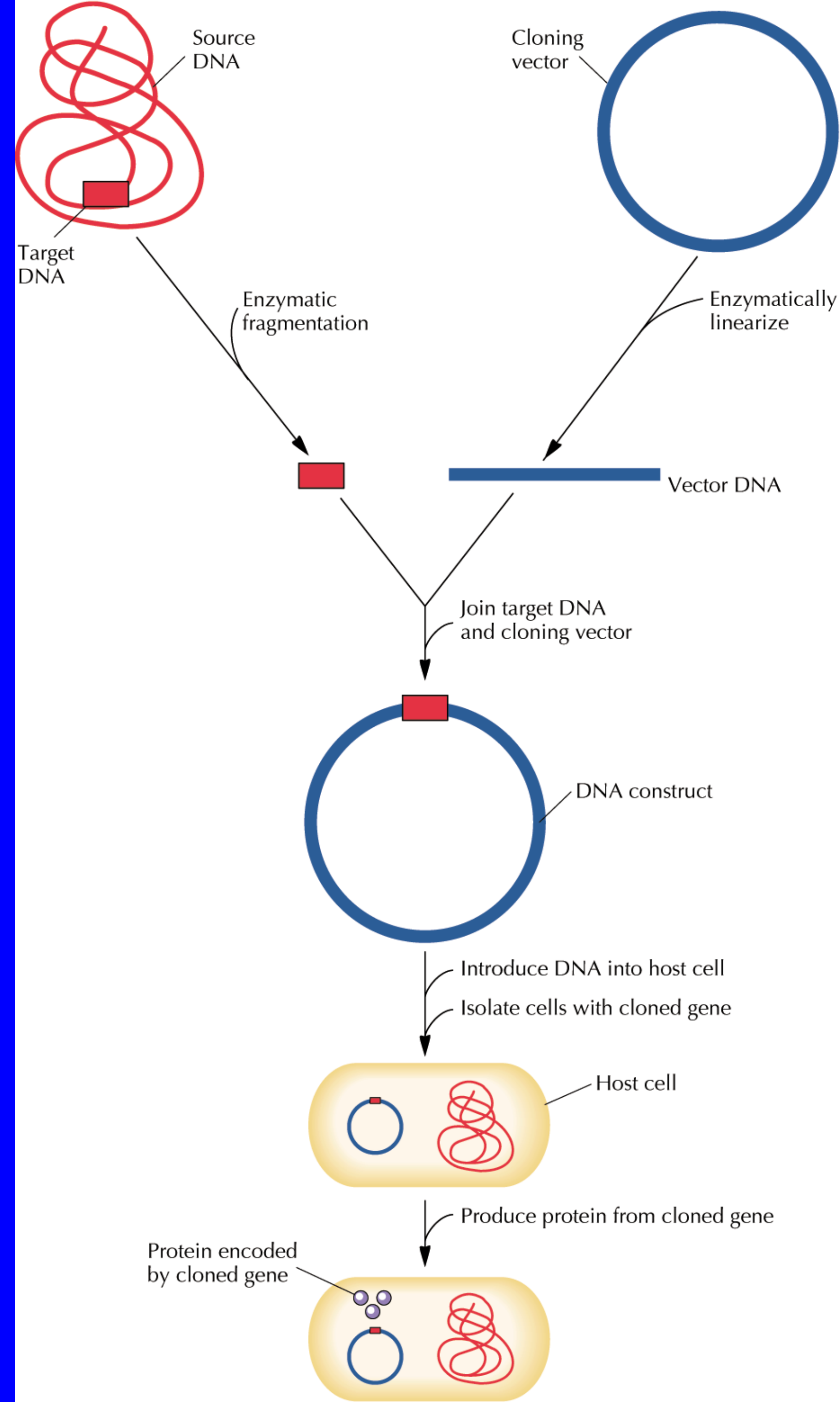
Recombinant DNA Technology

DNA Cloning

Cloning



rDNA Technology

- ***Restriction Enzymes and DNA Ligase***

Origin and function

- Bacterial origin = enzymes that cleave foreign DNA
- Named after the organism from which they were derived
  - EcoRI from *Escherichia coli*
  - BamHI from *Bacillus amyloliquefaciens*
- Protect bacteria from bacteriophage infection
  - Restricts viral replication
- Bacterium protects it’s own DNA by methylating those specific sequence motifs

Availability

- Several hundreds enzymes identified, many available commercially from biotechnology companies

Classes

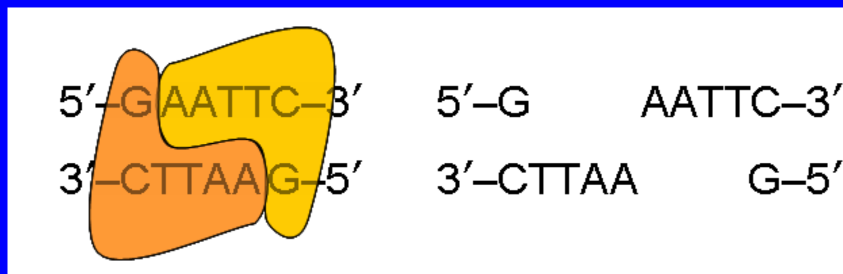
- ***Type I-III***
  - Cuts the DNA on both strands but at a non-specific location at varying distances (>1000 or 24-26bp) from the particular sequence that is recognized by the restriction enzyme
  - Therefore random/imprecise cuts
  - Not very useful for rDNA applications

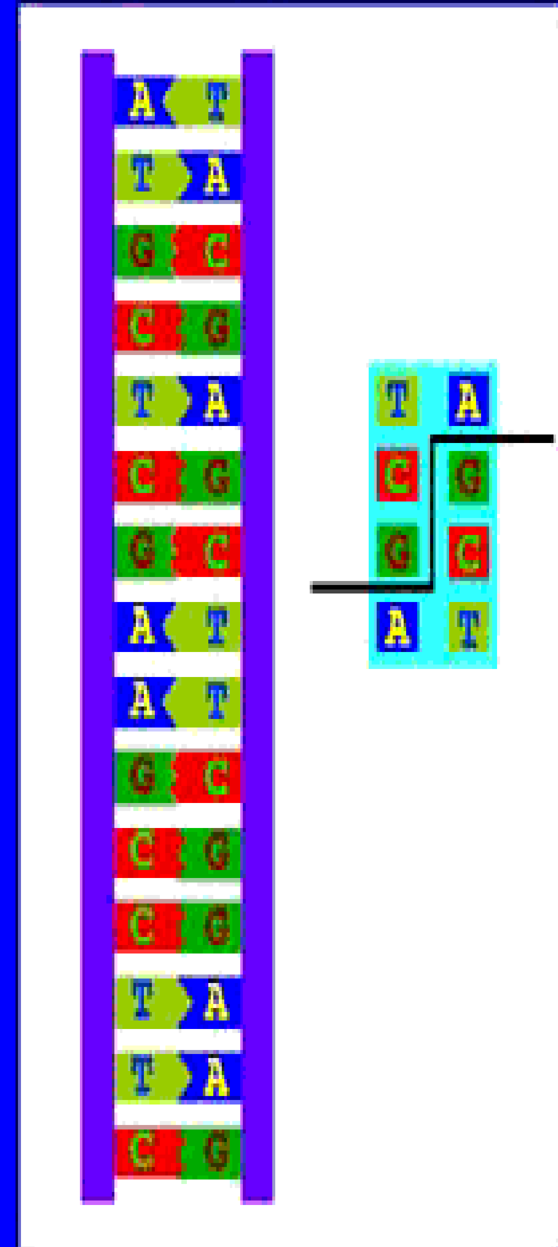
- ***Type II***
  - Cuts both strands of DNA within the particular sequence recognized by the restriction enzyme
  - Used widely for molecular biology procedures
  - DNA sequence = symmetrical
    - Reads the same in the 5'→ 3' direction on both strands = Palindromic Sequence
    - Some enzymes generate "blunt ends" (cut in middle)
    - Others generate "sticky ends" (staggered cuts)
      - H-bonding possible with complementary tails
      - DNA ligase covalently links the two fragments together by forming phosphodiester bonds of the phosphate-sugar backbones

Gli enzimi più utilizzati per la manipolazione del DNA sono

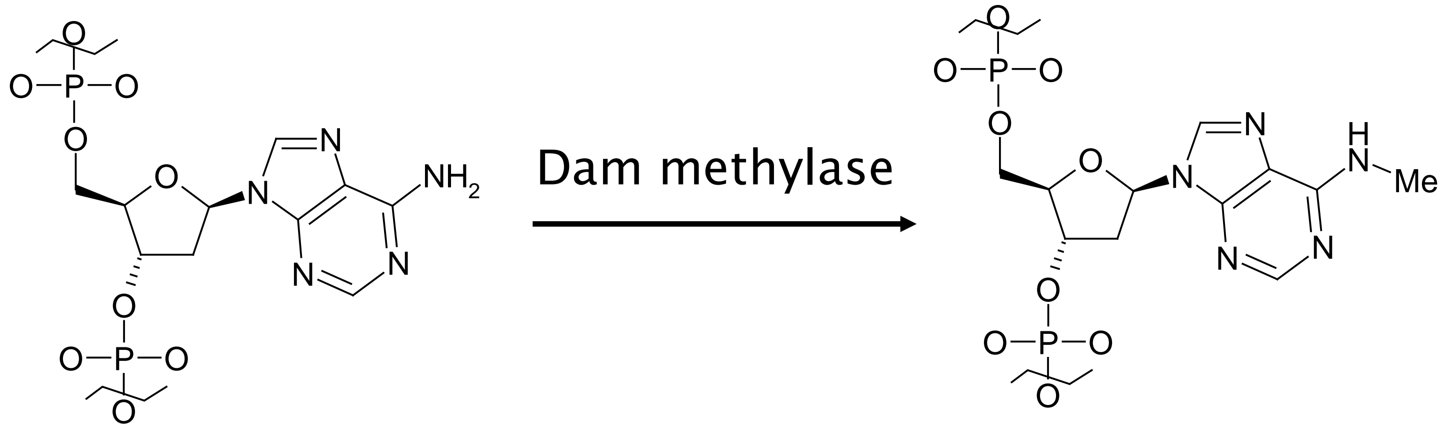
<u>Enzimi di restrizione di *tipo II*</u>:

- tagliano il Dna in corrispondenza del sito di riconoscimento, in posizione variabile su entrambi i filamenti
- necessitano di Mg^{++}
- sono in forma mono-dimerica
- non hanno attività metil-transferasica (presente nel batterio in un enzima separato)





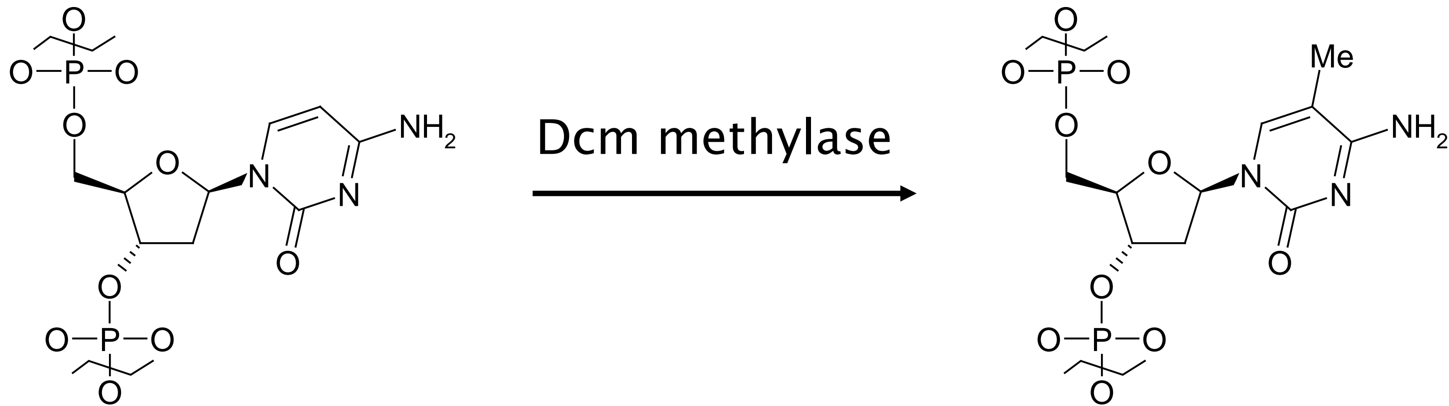
dam Methylation



- Dam methylase puts a methyl group on the nitrogen of 6th position of adenosine at the site: GATC
- All of the E. Coli that we use generate DNA with dam methylation
- Some enzymes ***only*** cut dam methylated DNA: eg DpnI
- Some enzymes ***do not*** cut dam methylated DNA: eg XbaI

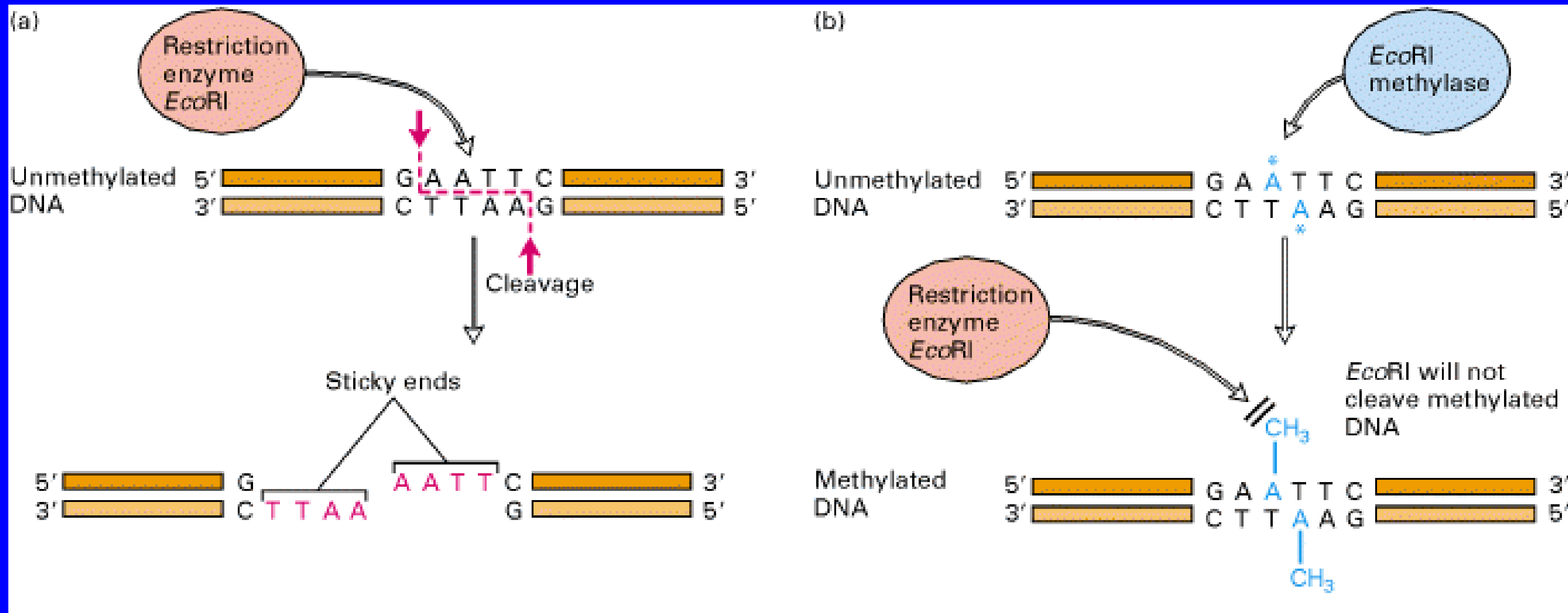
http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/dam_dcm_methylases_of_ecoli.asp

dcm Methylation



- Dcm methylase puts a methyl group on the carbon of 5th position of cytidine at the site: CCAGG and CCTGG
- The enzyme we use most that can be affected by dcm methylation is SfiI
- XL1-Blues and BL21s are both Dcm^+

http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/dam_dcm_methylases_of_ecoli.asp


(a)
Restriction enzyme *Eco*RI
Unmethylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
Cleavage
Sticky ends
5′ G
3′ C T T A A
A A T T C 3′
G 5′
(b)
*Eco*RI methylase
Unmethylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
Restriction enzyme *Eco*RI
*Eco*RI will not cleave methylated DNA
CH_3
Methylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
CH_3

Restriction Enzymes

- Nomenclature
  - EcoRI
    - E = Escherichia genus name
    - co = coli species name
    - R = strain RY12 strain or serotype
    - I = Roman numeral one = first enzyme
  - HinDIII
    - Haemophilus influenza serotype d
      3rd enzyme

Restriction Enzymes

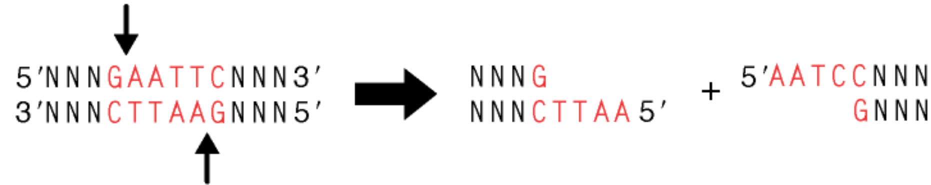
- Recognition sites
  - Generally 4, 6, or 8 bp in length
  - Most sites are palindromic
    - OTTO / HANNAH / REGAL LAGER
    - A MAN A PLAN A CANAL PANAMA
  - For REases - sequence reads the same in a 5'--->3' direction on each strand

Table 4.1 Recognition sequences of some restriction endonucleases

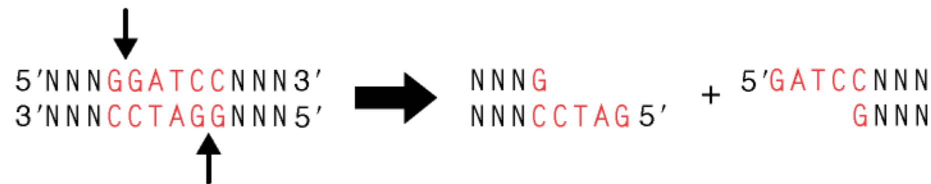
Enzyme	Recognition site	Type of cut end
*Eco*RI	G ↓ A—A—T—T—C C—T—T—A—A ↑ G	5′-phosphate extension
*Bam*HI	G ↓ G—A—T—C—C C—C—T—A—G ↑ G	5′-phosphate extension
*Pst*I	C—T—G—C—A ↓ G G ↑ A—C—G—T—C	3′-hydroxyl extension
*Sau*3AI	↓ G—A—T—C C—T—A—G ↑	5′-phosphate extension
*Pvu*II	C—A—G ↓ C—T—G G—T—C ↑ G—A—C	Blunt end
*Hpa*I	G—T—T ↓ A—A—C C—A—A ↑ T—T—G	Blunt end
*Hae*III	G—G ↓ C—C C—C ↑ G—G	Blunt end
*Not*I	G ↓ C—G—G—C—C—G—C C—G—C—C—G—G—C ↑ G	5′-phosphate extension

Gli enzimi di restrizione generano estremità specifiche nei loro siti di taglio

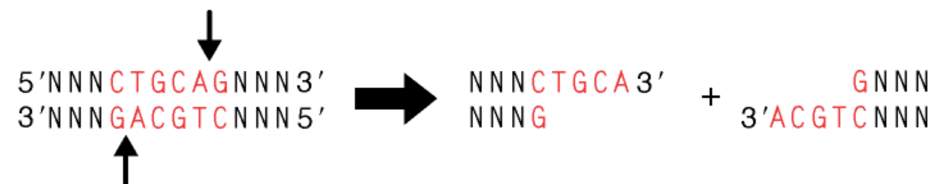
EcoR1 genera sporgenze 5′ di 4 basi in un bersaglio di 6 bp



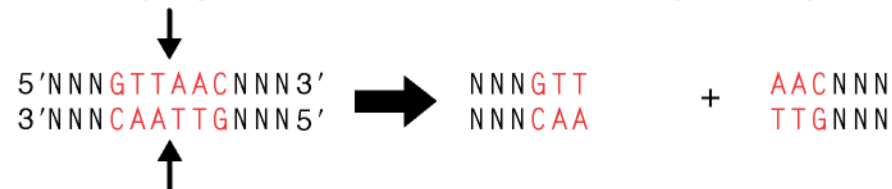
BamH1 genera sporgenze 5′ di 4 basi in un bersaglio di 6 bp



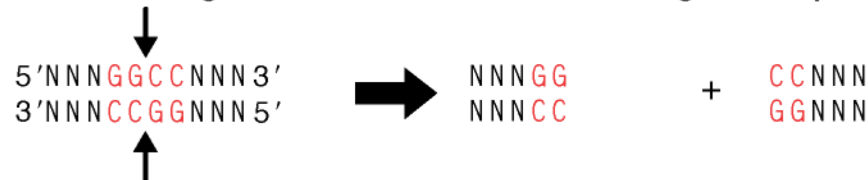
Pst1 genera sporgenze 3′ di 4 basi in un bersaglio di 6 bp



HpaI genera estremità nette in un bersaglio di 6 bp



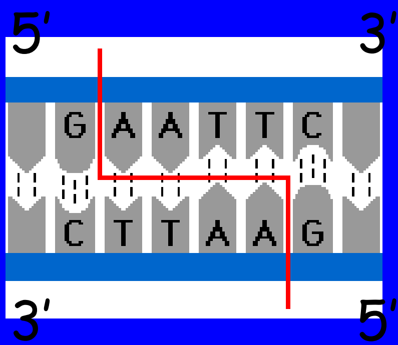
HaeIII genera estremità nette in un bersaglio di 4 bp



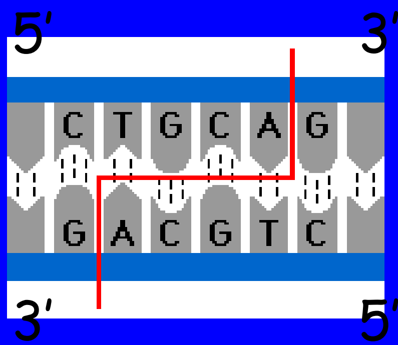
Lewin, IL GENE VIII, Zanichelli editore S.p.A. Copyright © 2006

Restriction Enzymes

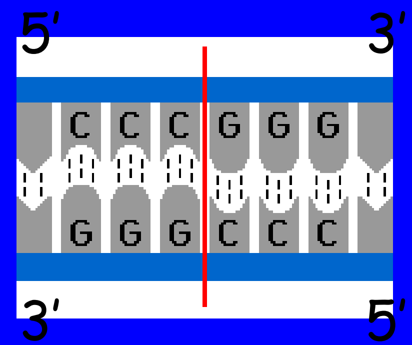
- Cleave DNA to generate different “ends”
  - Staggered cut
    - 5’ extension
    - 3’ extension
  - Blunt end



Eco RI



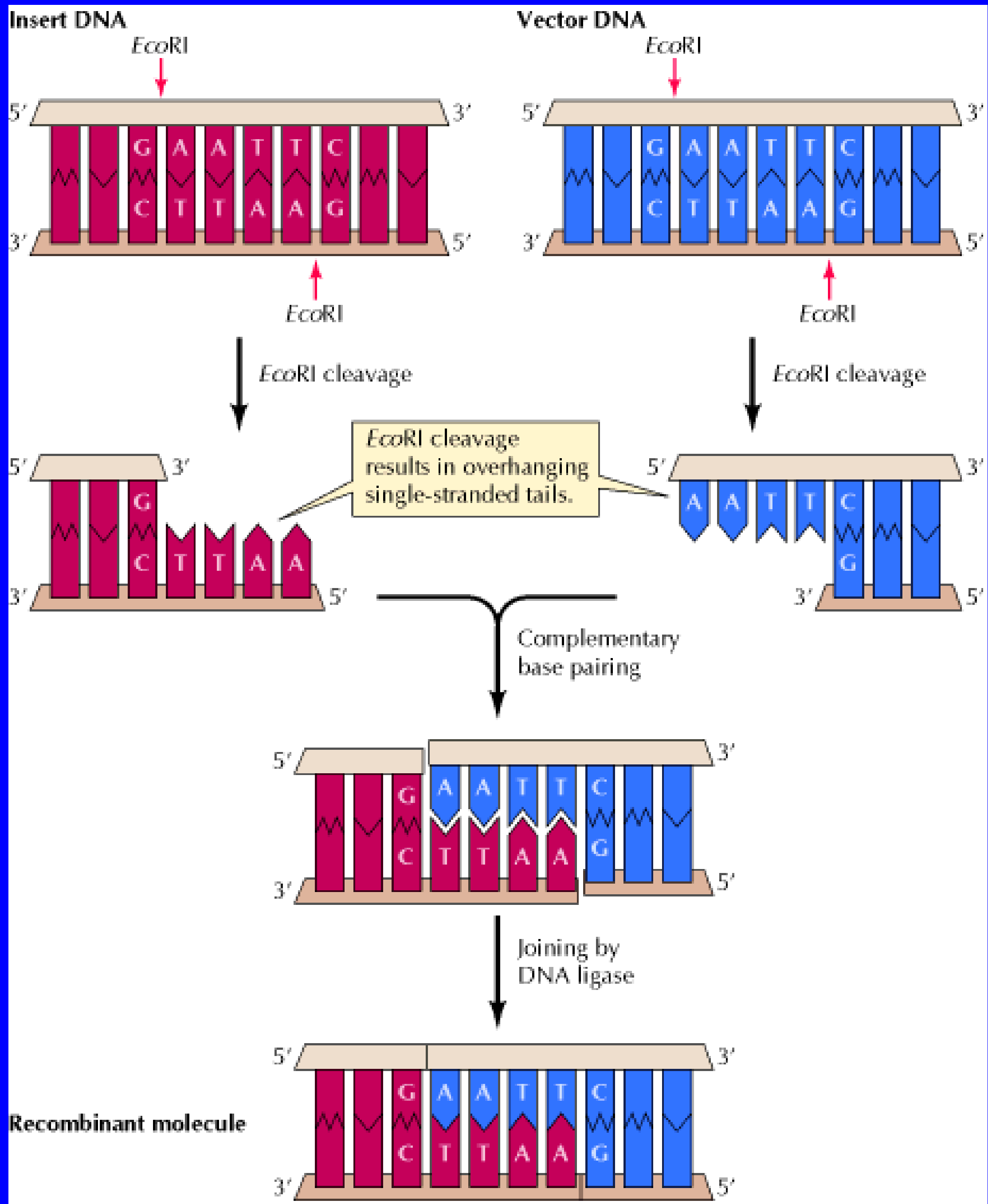
PstI



SmaI

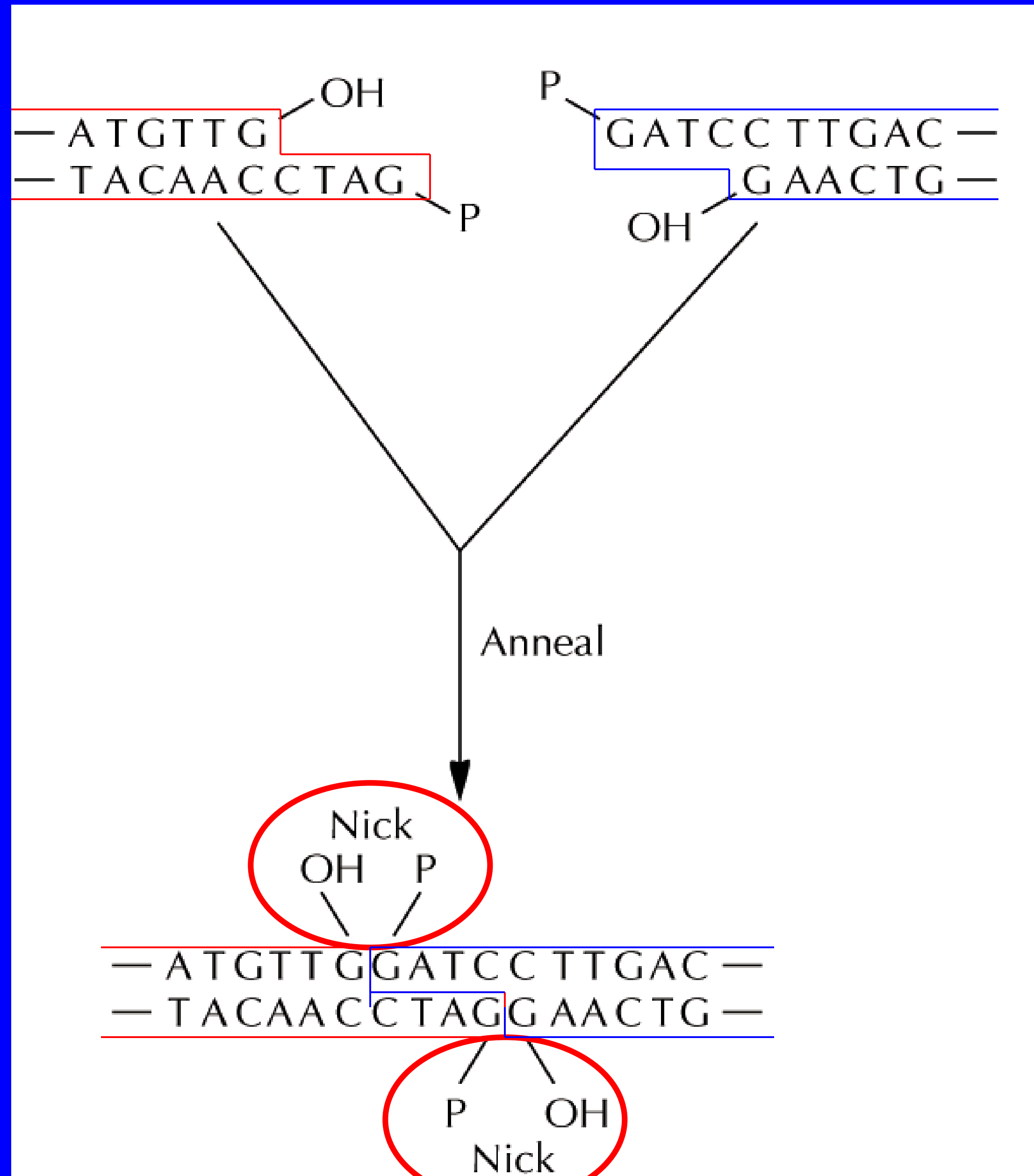
Restriction Enzymes in DNA Cloning

- How are REases used ?
  - Ends are “sticky”
  - Complementary
  - Any two DNAs cut with same enzyme can stick together through complementary base pairing



Insert DNA
Vector DNA
EcoRI
5′
3′
G A A T T C
C T T A A G
EcoRI cleavage
EcoRI cleavage results in overhanging single-stranded tails.
Complementary base pairing
Joining by DNA ligase
Recombinant molecule

Annealing Sticky ends



DNA strands held together only by basepairing
Nicks in strands need to be repaired

Restriction fragment length polymorphism

- RFLP is a polymorphic allele identified by the presence or absence of a specific restriction endonuclease recognition site:
  - GAATTC versus GATTTC
- RFLP is usually identified by digestion of genomic DNA with specific restriction enzymes followed by Southern blotting
- Regions of DNA with polymorphisms:
  - Introns
  - Flanking sequences
  - Exons

Diagnosi

Digestione

DNA allele 1 →

5' GCGC GCGC 3'

3' CGCG CGCG 5'

383 pb 80 pb 149 pb

PCR (612 pb)

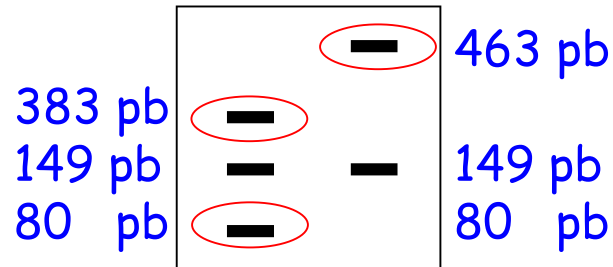
DNA allele 2 →

5' GCAC GCGC 3'

3' CGTG CGCG 5'

463 pb 149 pb

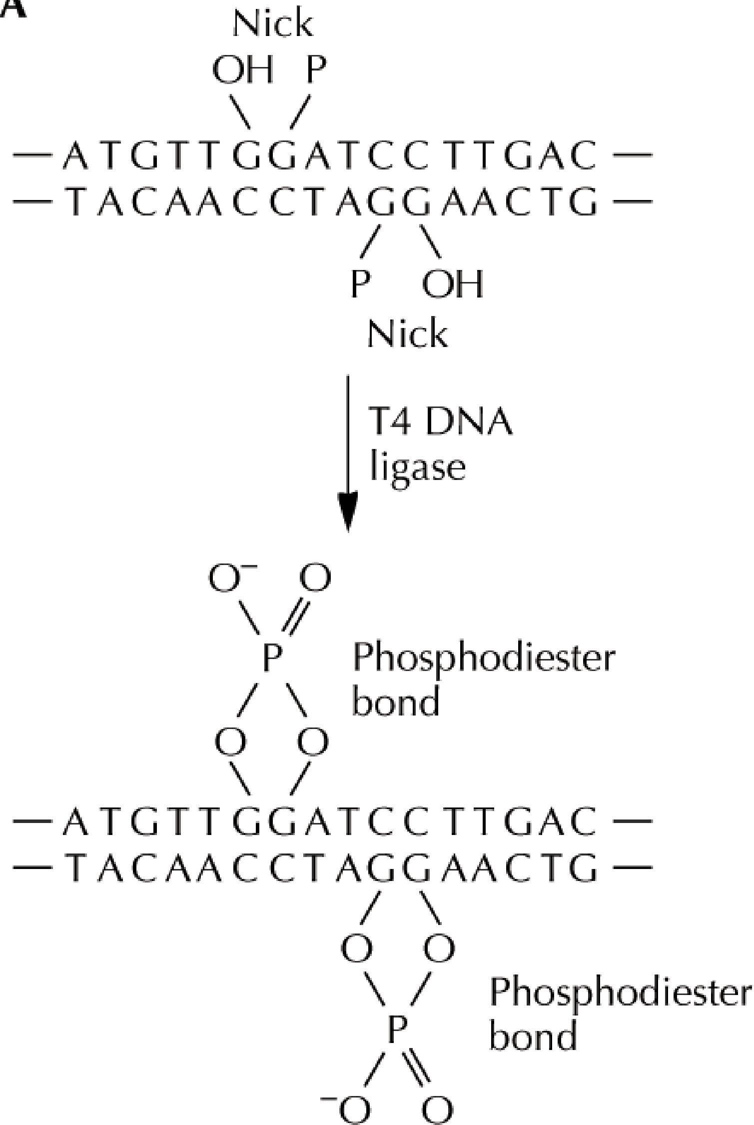
Separazione dei Frammeti su gel

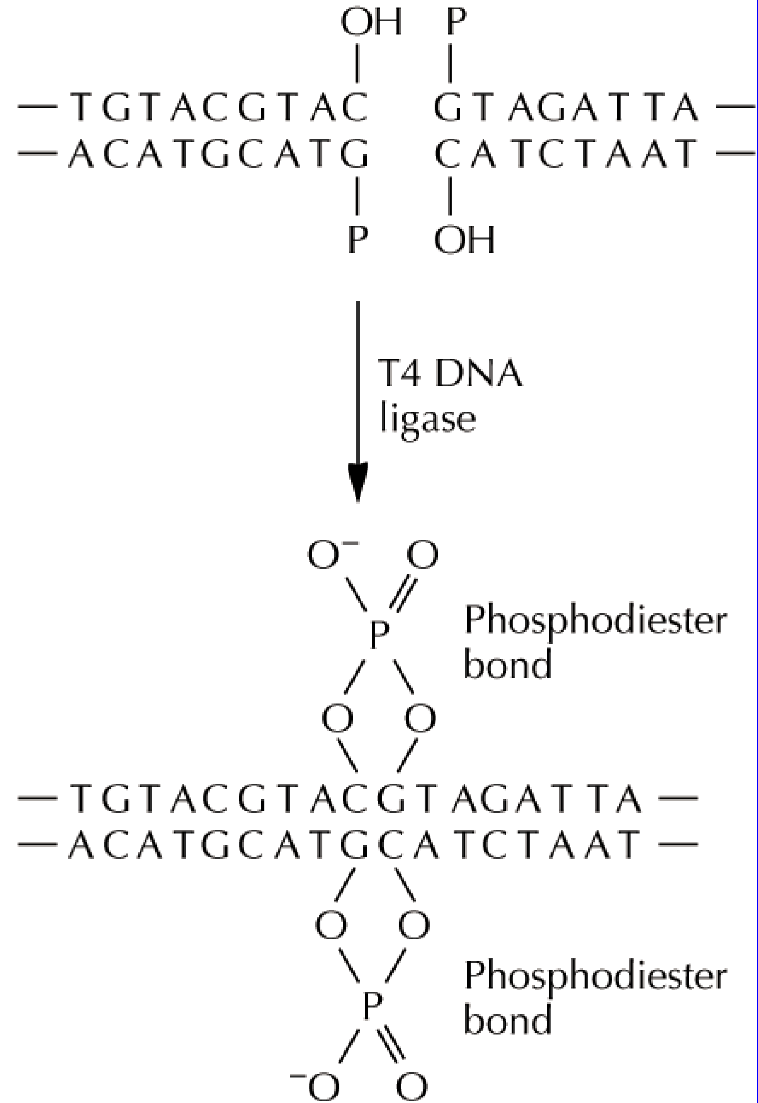


Linking Restriction Fragments

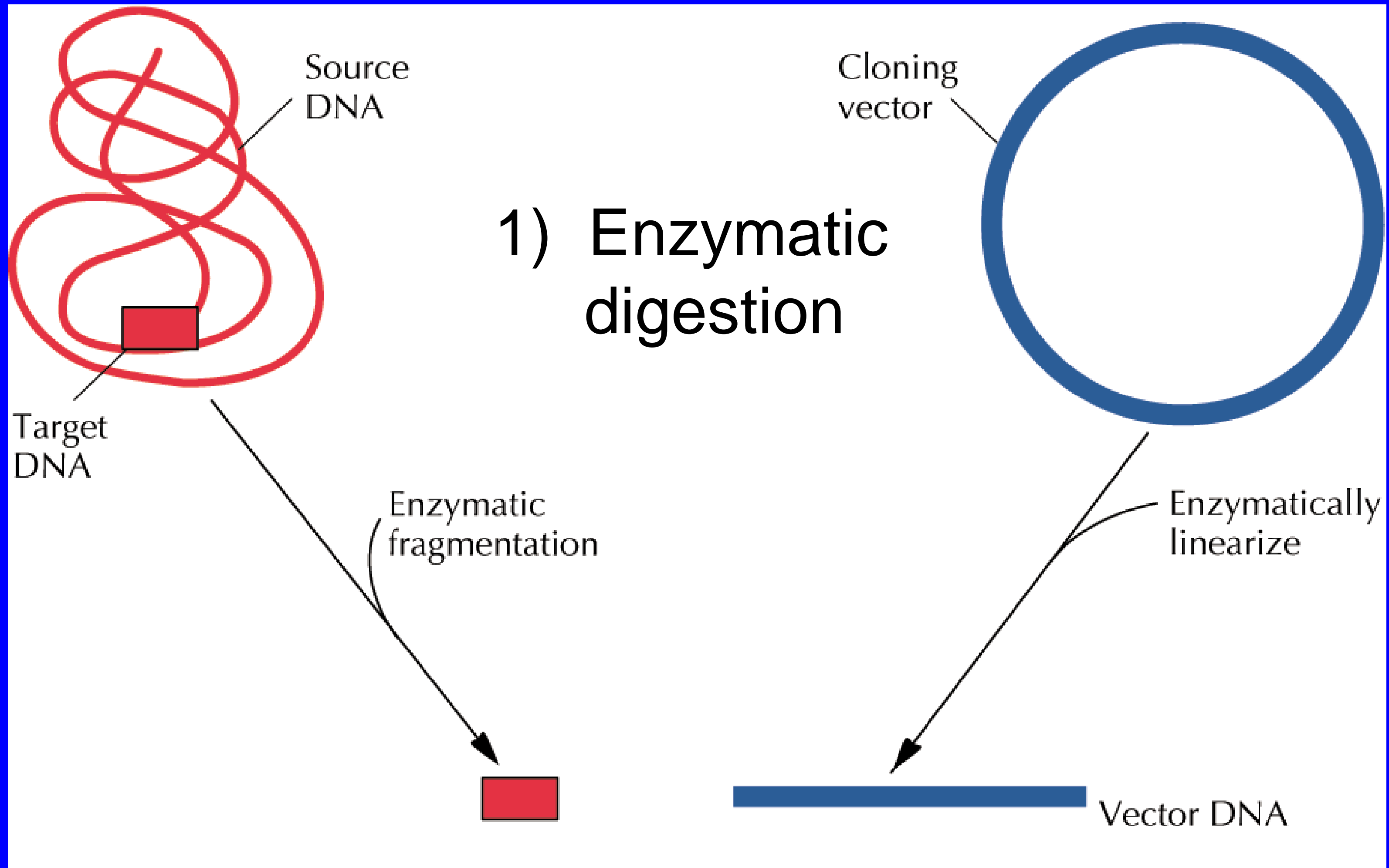
- T4 DNA Ligase
  - repairs nicks in DNA strands (reforms phosphodiester bond)
  - uses energy from ATP
  - works on blunt or sticky ends

T4 DNA Ligase Mode of Action

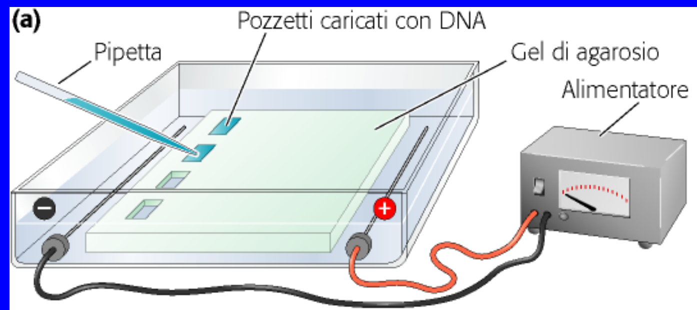


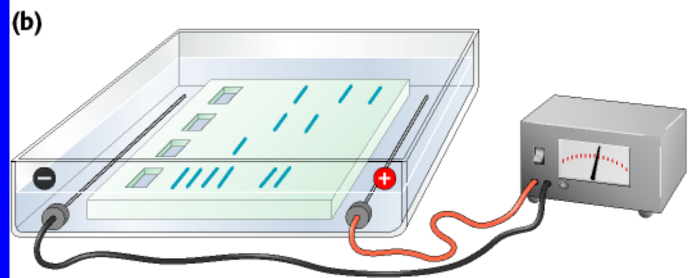


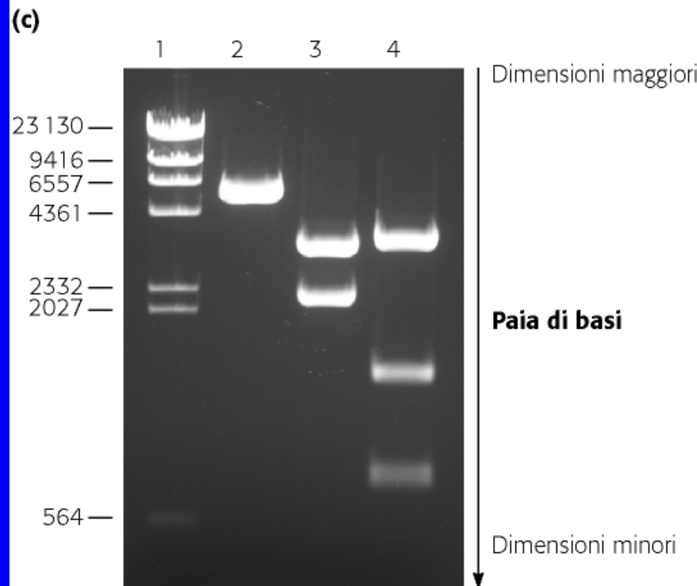
Recombinant DNA Cloning Procedure



Separation of DNA fragments by gel electrophoresis

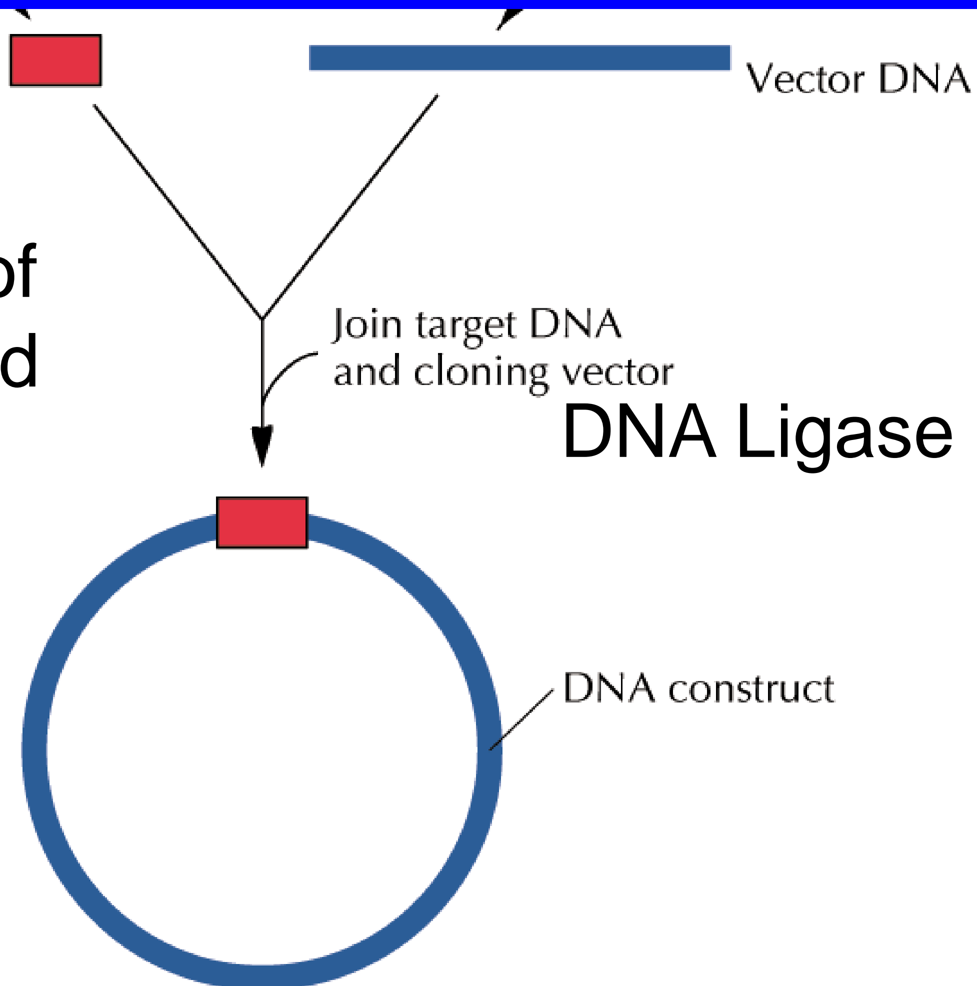






Recombinant DNA Cloning Procedure

2) Ligation of Target and vector



And in the case we don't have the possibility of using the same restriction enzyme for the vector and the insert?
How can we overcome this problem?

Compatible Coesive Ends

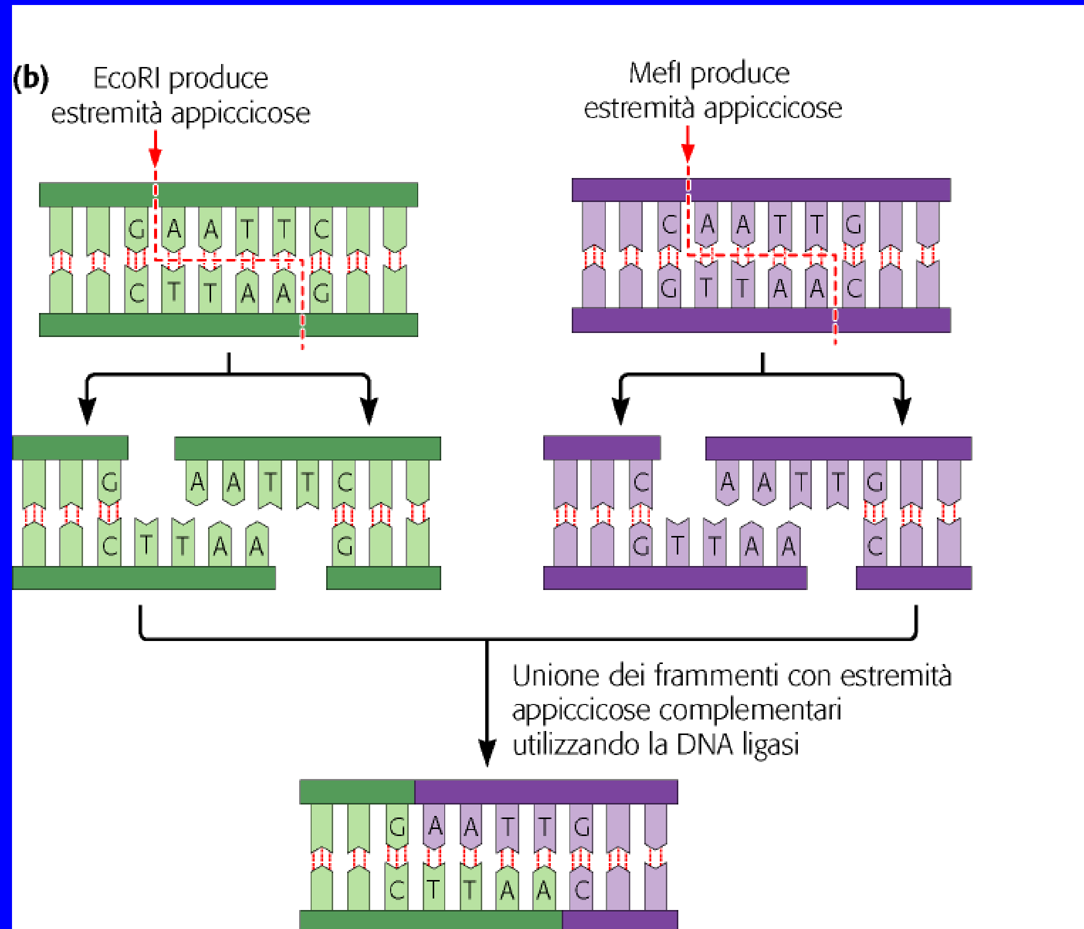


Table 1.19. Newly Generated Recognition Sequences Resulting from Ligation of Protruding Compatible DNA Ends.

First RE	Second RE	RE cleaving newly generated recognition sequence
AatII (GACGT↓C)	**TaiI** (ACGT↓)	MaeII, **TaiI**
Acc65I (G↓GTACC)	**BshNI*** (G↓GTACC)	**Acc65I**, **BshNI**, **BspLI**, **Csp6I**, **KpnI**, **RsaI**
	Bsp1407I (T↓GTACA), **Pfl23II** (C↓GTACG), **TatI*** (W↓GTACW)	**Csp6I**, **RsaI**
AciI* (C↓CGC)	**Bsp119I** (TT↓CGAA), **Bsu15I** (AT↓CGAT), **Hin1I*** (GR↓CGTC), MaeII (A↓CGT), **Psp1406I** (AA↓CGTT), **TaqI** (T↓CGA), **XmiI*** (GT↓CGAC)	
	Hin1I* (GR↓CGCC), **Hin6I** (G↓CGC), NarI (GG↓CGCC)	AciI
	HpaII (C↓CGG), **MspI** (C↓CGG)	**HpaII**, **MspI**
AciI* (G↓CGG)	**Bsp119I** (TT↓CGAA), **Bsu15I** (AT↓CGAT), MaeII (A↓CGT), **Psp1406I** (AA↓CGTT), **TaqI** (T↓CGA), **XmiI*** (GT↓CGAC)	
	Hin1I* (GR↓CGCC), **Hin6I** (G↓CGC), NarI (GG↓CGCC)	HhaI, **Hin6I**
	Hin1I* (GR↓CGTC)	HgaI
	HpaII (C↓CGG), **MspI** (C↓CGG)	AciI
AflIII* (A↓CGCGT)	AscI (GG↓CGCGCC), **PauI** (G↓CGCGC)	**Bsh1236I**, HhaI, **Hin6I**
	DsaI* (C↓CGCGG)	AciI, **Bsh1236I**
	MluI (A↓CGCGT)	AflIII, **Bsh1236I**, **MluI**
AflIII* (A↓CGTGT)	DsaI* (C↓CGTGG)	MaeII, **TaiI**
AflIII* (A↓CACGT)	DsaI* (C↓CACGG)	
AflIII* (A↓CATGT)	BspLU11I (A↓CATGT)	AflIII, BspLU11I, NlaIII, **XceI**
	DsaI* (C↓CATGG), **Eco130I*** (C↓CATGG), **NcoI** (C↓CATGG), **PagI** (T↓CATGA)	NlaIII
Alw21I* (GTGCT↓C)	**SduI*** (GTGCT↓C)	**Alw21I**, **SduI**
Alw21I* (GTGCA↓C)	**BseSI*** (GTGCA↓C), **SduI*** (GTGCA↓C)	**Alw21I**, **Alw44I**, **BseSI**, CviRI, **Hpy8I**, **SduI**
	Mph1103I (ATGCA↓T)	CviRI
	PstI (CTGCA↓G), **SdaI** (CCTGCA↓GG)	BsgI, CviRI
Alw21I* (GAGCT↓C)	**Eco24I*** (GAGCT↓C), **SacI** (GAGCT↓C), **SduI*** (GAGCT↓C)	**AluI**, **Alw21I**, CviJI, **Ecl136II**, **Eco24I**, **SacI**, **SduI**
Alw21I* (GAGCA↓C)	**SduI*** (GAGCA↓C)	**Alw21I**, **SduI**
Alw44I (G↓TGCAC)	**BfmI*** (C↓TGCAG)	BsgI, CviRI
ApaI (GGGCC↓C)	**BseSI*** (GGGCC↓C), **Eco24I*** (GGGCC↓C), **SduI*** (GGGCC↓C)	**ApaI**, **BseSI**, **Bsp120I**, **BspLI**, **BsuRI**, **Cfr13I**, CviJI, **Eco24I**, **SduI**
AscI (GG↓CGCGCC)	AflIII* (A↓CGCGT), **MluI** (A↓CGCGT)	**Bsh1236I**, HhaI, **Hin6I**
	DsaI* (C↓CGCGG)	AciI, **Bsh1236I**, HhaI, **Hin6I**
	PauI (G↓CGCGC)	**Bsh1236I**, Cac8I, HhaI, **Hin6I**, **PauI**
BamHI (G↓GATCC)	**BclI** (T↓GATCA), **Bsp143I** (↓GATC), **MboI** (↓GATC)	**Bsp143I**, **BspPI**, **MboI**
	BglII (A↓GATCT), **PsuI*** (R↓GATCT)	**Bsp143I**, **BspPI**, **MboI**, **PsuI**
	PsuI* (R↓GATCC)	**BamHI**, **Bsp143I**, **BspLI**, **BspPI**, **MboI**, **PsuI**
BbeI (GGCGC↓C)	**Bsp143II*** (RGCGC↓C)	BbeI, **BshNI**, **Bsp143II**, **BspLI**, **EheI**, HhaI, **Hin1I**, **Hin6I**, KasI, NarI
	Bsp143II* (RGCGC↓T)	**Bsp143II**, HhaI, **Hin6I**
BbvCI* (CC↓TCAGC)	**Bpu10I*** (CC↓TCAGC), **Bpu1102I*** (GC↓TCAGC)	BbvCI, **Bpu10I**, **BseMII**, BspCNI, DdeI, **MnlI**
	Bpu10I* (GC↓TCAGG), **Eco81I*** (CC↓TCAGG)	**BseMII**, BspCNI, DdeI, **Eco81I**, **MnlI**
	DdeI* (C↓TCAG)	**BseMII**, BspCNI, DdeI, **MnlI**
BbvCI* (GC↓TGAGG)	**Bpu10I*** (CC↓TGAGC), **Bpu1102I*** (GC↓TGAGC)	**Bpu1102I**, **BseMII**, BspCNI, DdeI
	Bpu10I* (GC↓TGAGG), **Eco81I*** (CC↓TGAGG)	BbvCI, **Bpu10I**, **BseMII**, BspCNI, DdeI, **MnlI**
	DdeI* (C↓TGAG)	**BseMII**, BspCNI, DdeI

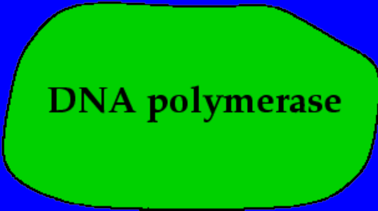
*Bam*HI (G↓GATCC)	*Bcl*I (T↓GATCA), *Bsp*143I (↓GATC), *Mbo*I (↓GATC)
	*Bgl*II (A↓GATCT), *Psu*I* (R↓GATCT)

Unfortunately, you have no luck!!!
How can we modify the ends to make them compatible??

Filling recessed 3’ ends

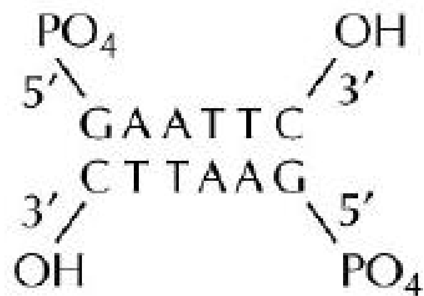
Overhengende ende

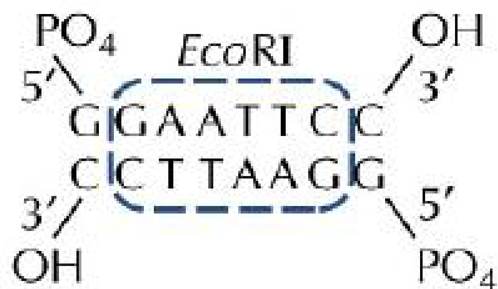
+

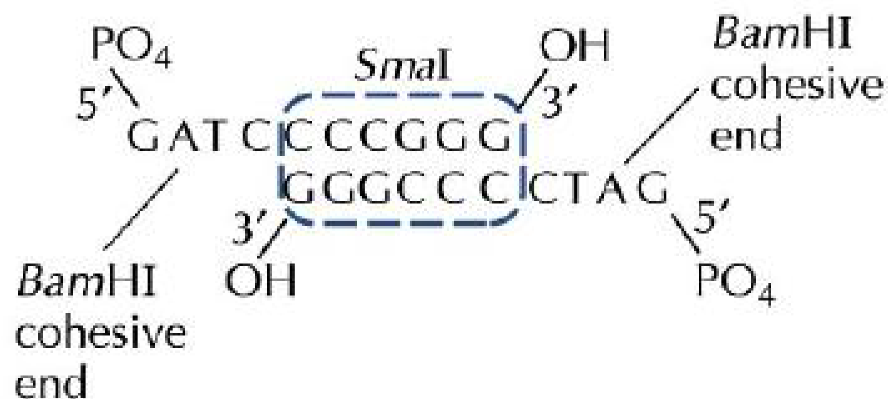


Polymerasen fyller ut

Linkers *Adapters*







TdT *(terminal deossinucleotidil transferase)*

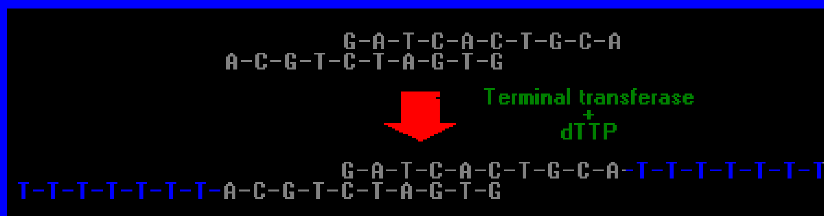
5′ 3′
3′ 5′
λ-esonucleasi
5′ 3′
3′ 5′
Terminal transferasi +dATP
5′ A(A)$_n$A 3′
3′A(A)$_n$A 5′

5′ 3′
3′ 5′
λ-esonucleasi
5′ 3′
3′ 5′
Terminal transferasi + TTP
5′ T(T)$_n$T 3′
3′T(T)$_n$T 5′

Miscelazione e appaiamento

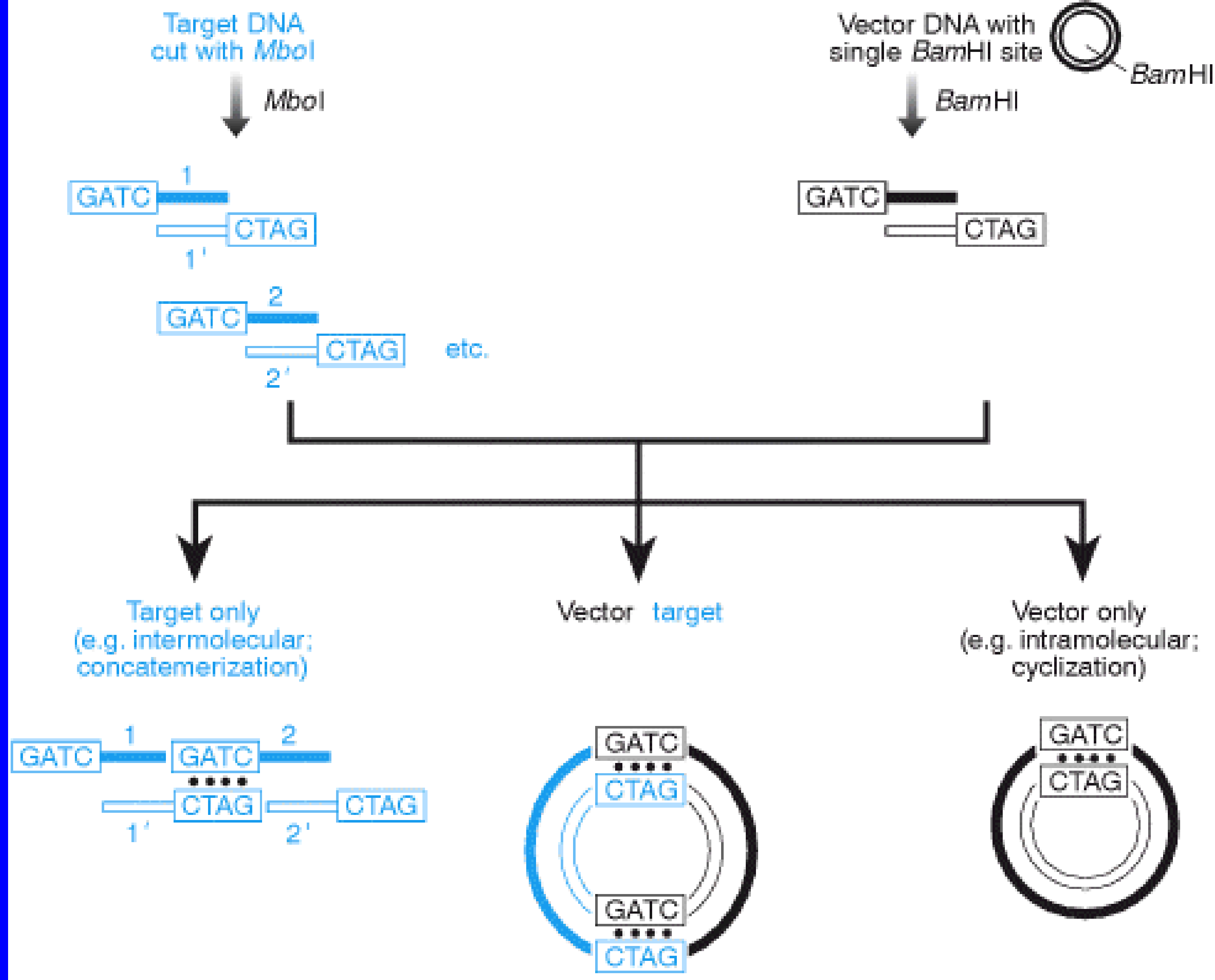
T(T)$_n$T
A(A)$_n$A

A(A)$_n$A
T(T)$_n$T



- ***What prevents plasmid DNA from reforming during ligation and transforming cells as do the recombinant molecules?***

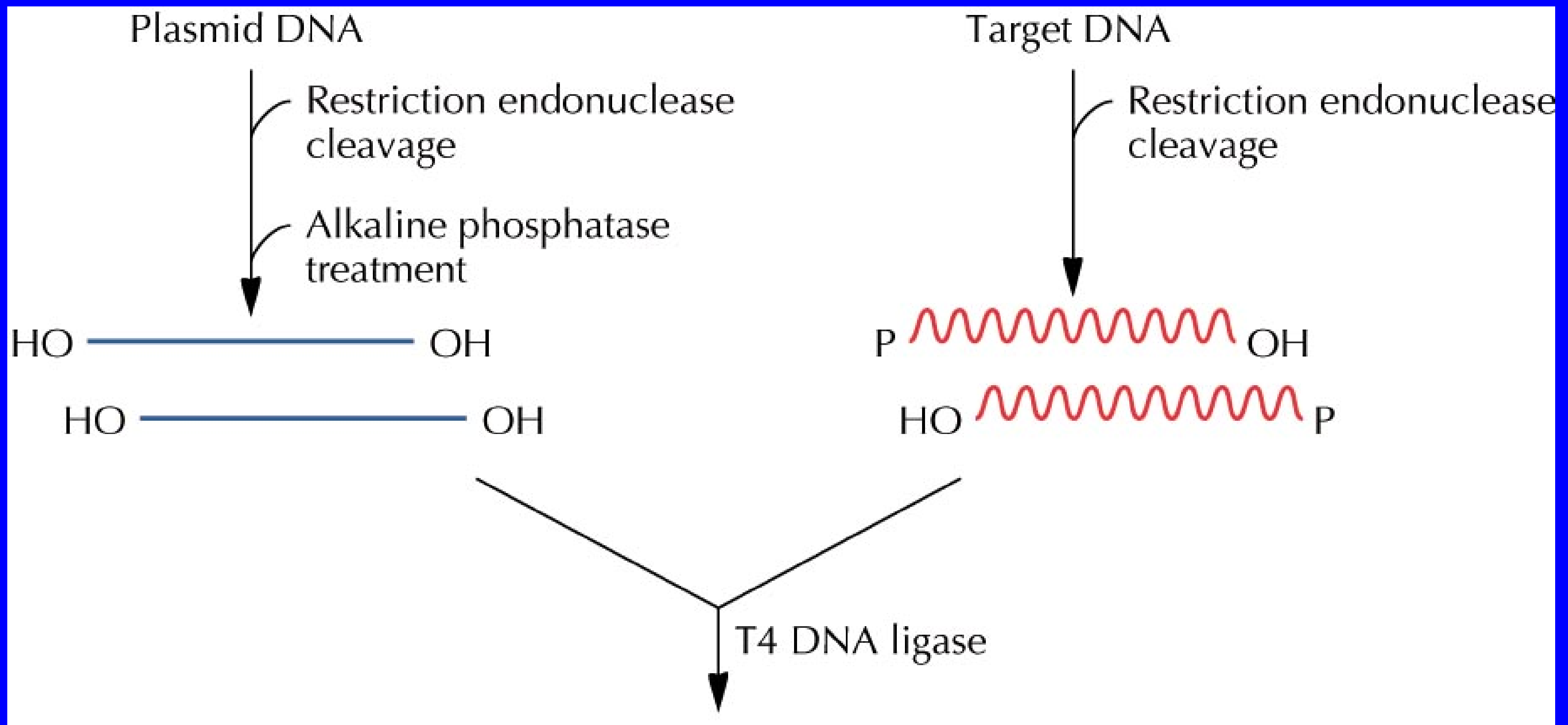
- ***Two ways to prevent***
  - ***Treat with Alkaline Phosphatase***
  - ***Directional Cloning***



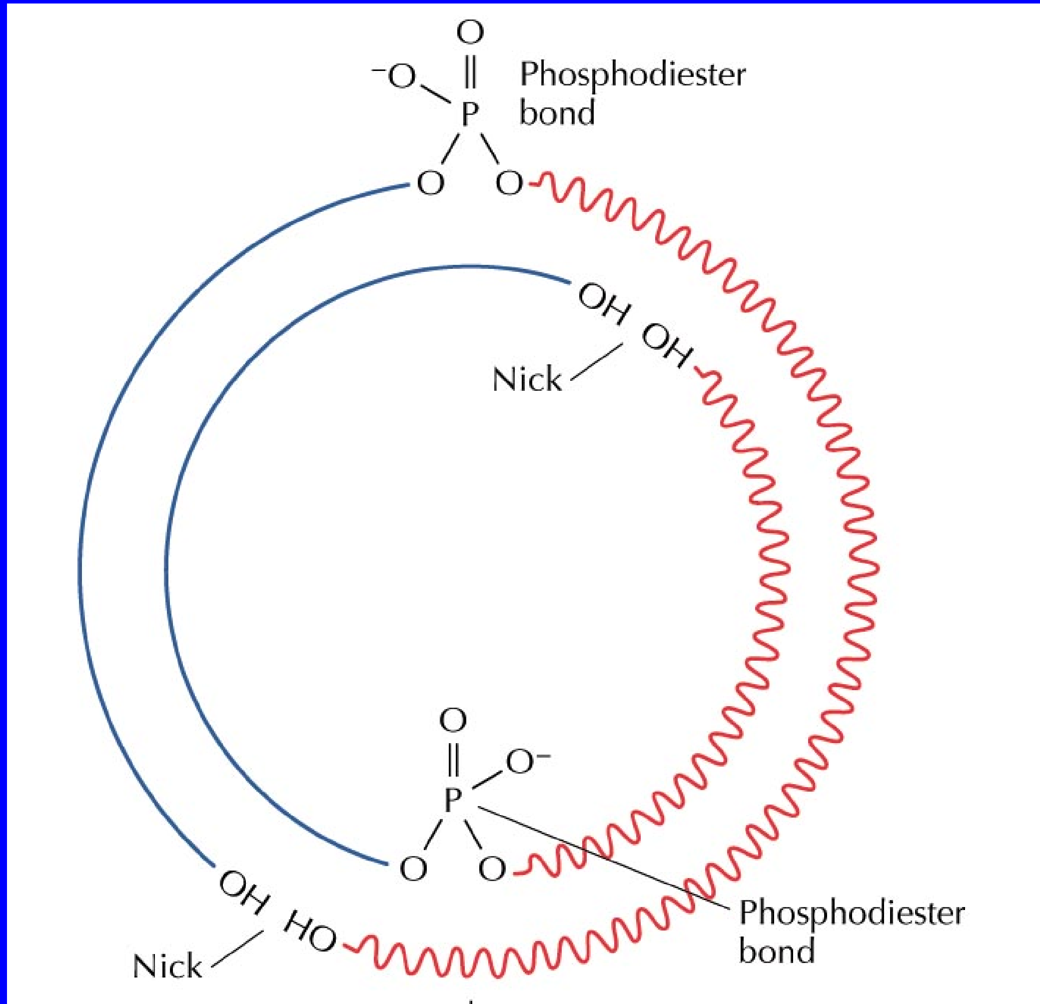
Cohesive termini can associate intramolecularly and intermolecularly. *Note* that only some of the possible outcomes are shown. For example, vector molecules may also form intermolecular concatemers, multimers can undergo cyclization and co-ligation events can involve two different target sequences being included with a vector molecule in the same recombinant DNA molecule. The tendency towards cyclization of individual molecules is more pronounced when the DNA is at low concentration and the chance of collision between different molecules with complementary sticky ends is reduced

- ***Alkaline Phosphatase***
  - removes 5’ PO_4 from end of DNA strand
  - prevents formation of new phosphodiester bond by DNA Ligase

Alkaline Phosphatase Action



Alkaline Phosphatase Action



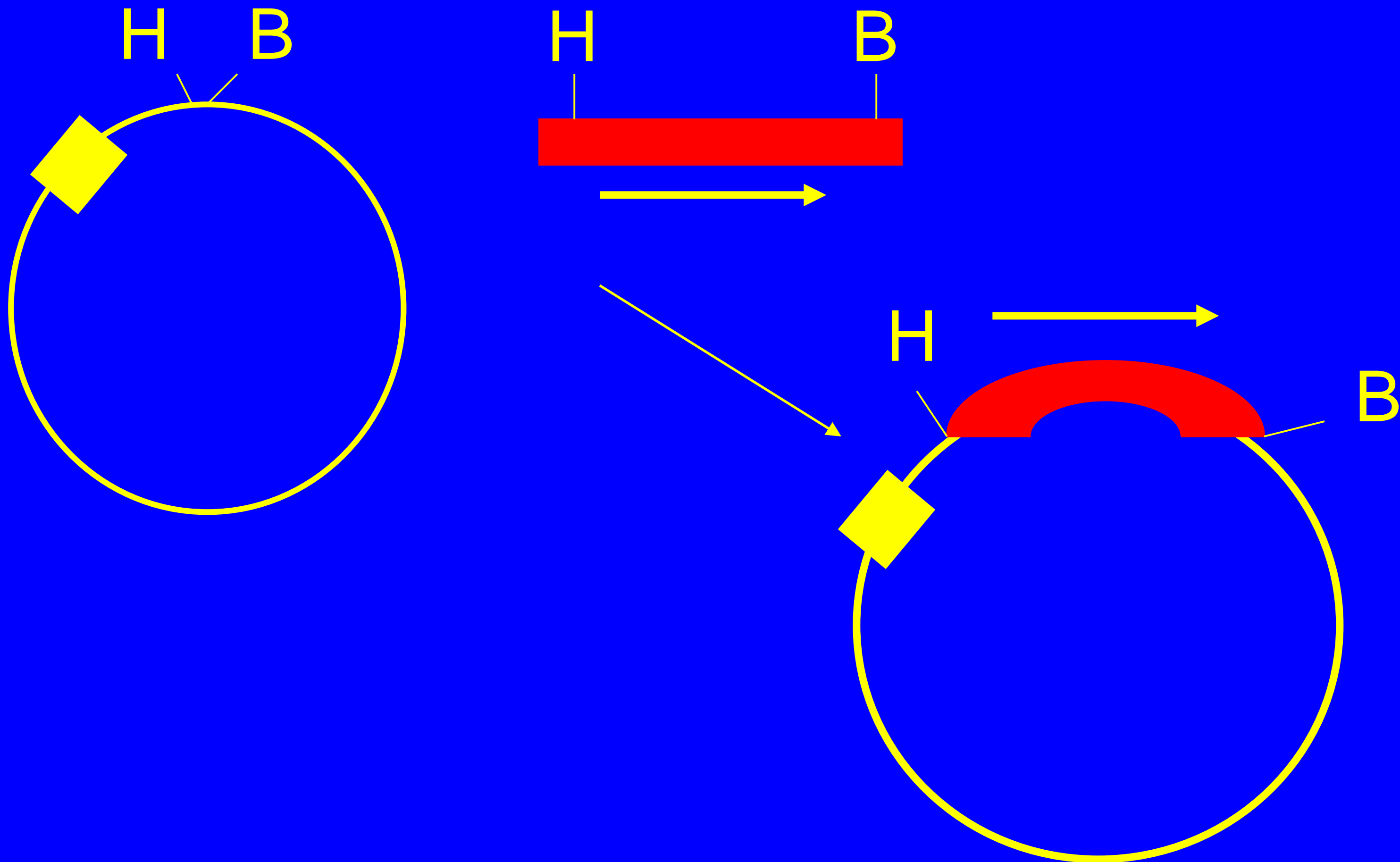
Two nicks remain

Will be repaired in bacterial cell following transformation

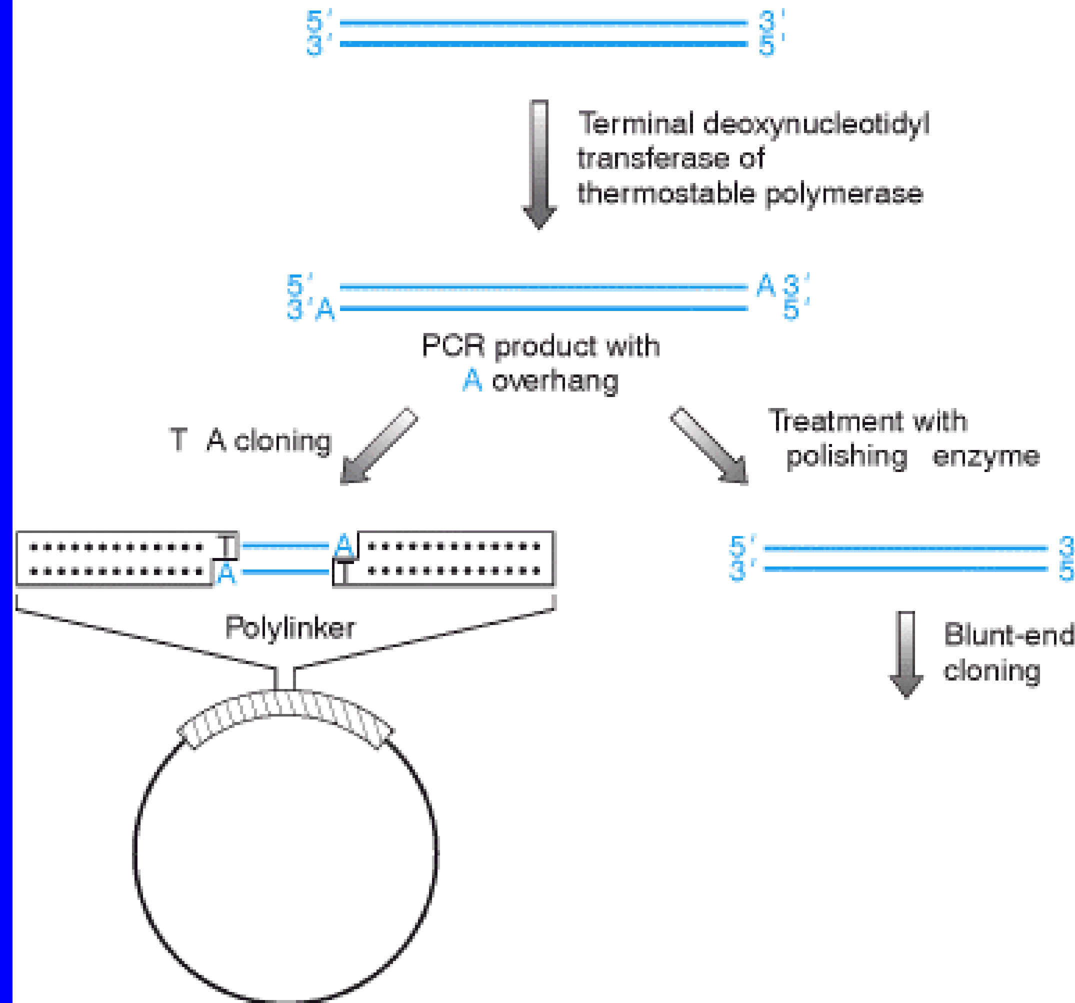
Directional Cloning

- Digest plasmid and target DNA with two different restriction enzymes
  - Hind III and BamHI
  - Ends are not compatible (can’t basepair)
  - Plasmid won’t re-circularize unless target DNA has inserted

Directional Cloning



Cloning of PCR fragments

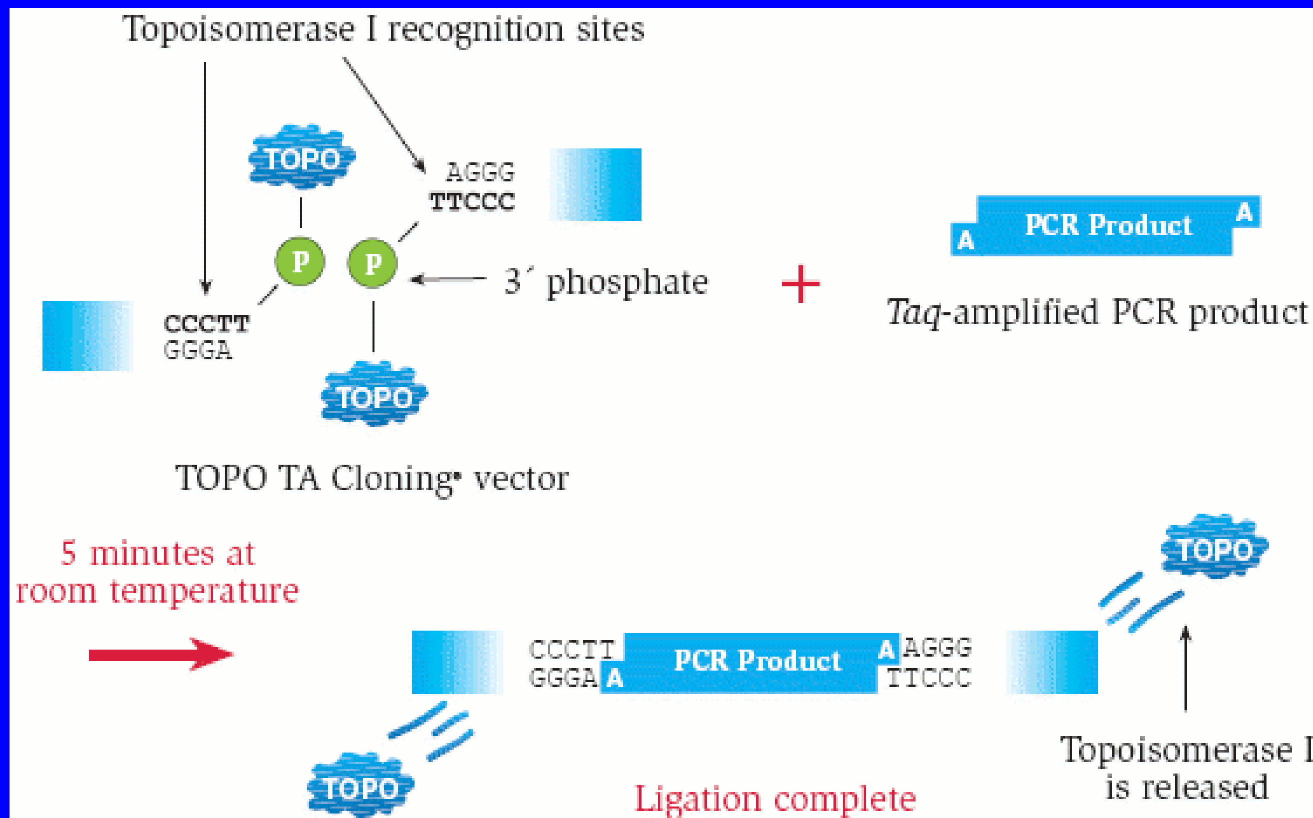


Cloning of PCR products

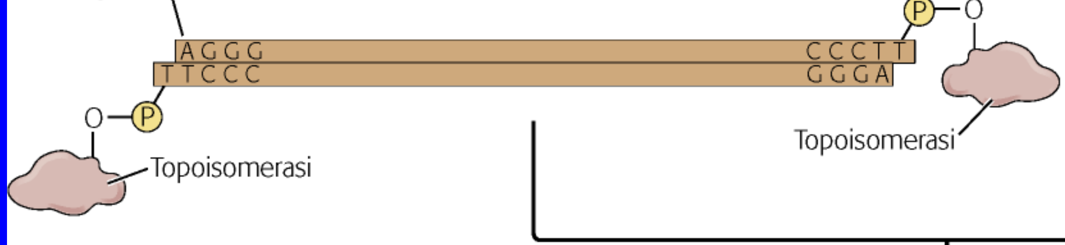
PCR products frequently have an overhanging adenosine at their 3 ends. The **T-A cloning** system has a polylinker system with complementary thymine overhangs to facilitate cloning.

Topo Cloning

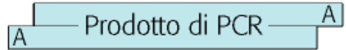
- DNA topoisomerase I from *Vaccinia* virus binds to double strand DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991).
- The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I.



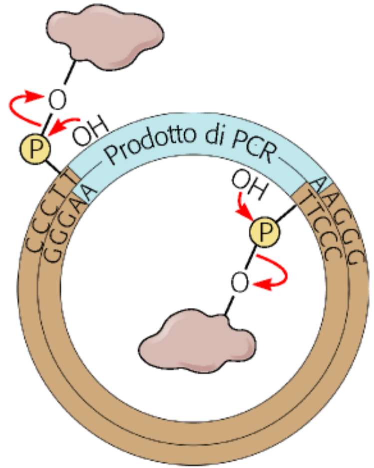
(a) Vettore con la topoisomerasi legata covalentemente



La *Taq* polimerasi lascia una A sporgente all'estremità 3′ dei prodotti di PCR, che può essere usata per il clonaggio

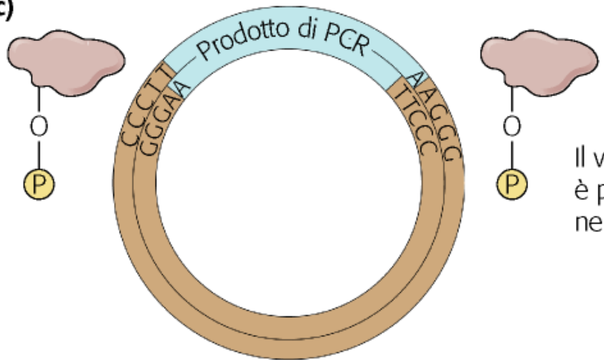


(b)



La topoisomerasi catalizza il legame covalente del vettore con il prodotto di PCR

(c)



Il vettore covalentemente chiuso è pronto per la trasformazione nei batteri

rDNA Technology

- ***Transformation of Bacteria***

CHOICE OF THE BACTERIAL HOST

(generally E.Coli)

res – → **mutants of the restriction system**

Rec A (-) → **mutants of the recombination system**

Transformation of Bacteria

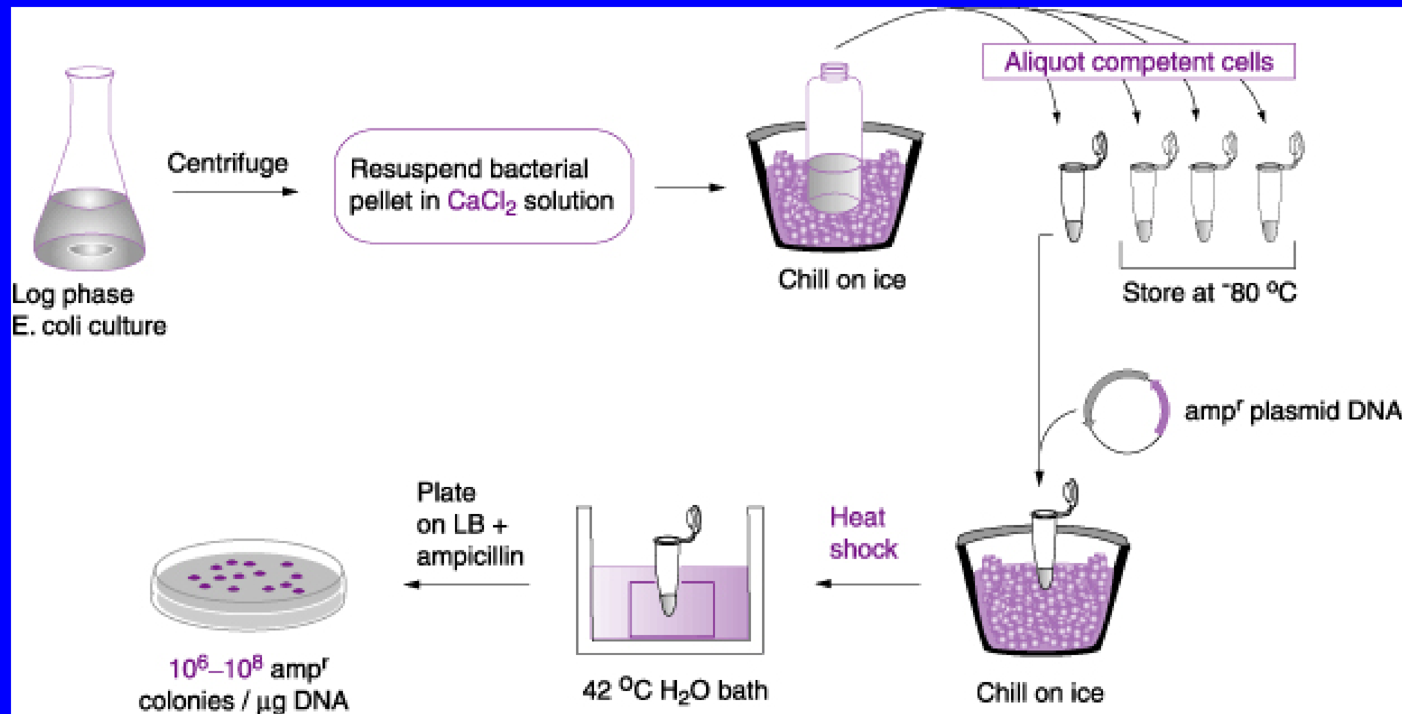
- rDNA constructed in the lab must be introduced into “host” cell
- Cells must be able to take up DNA - “COMPETENT”
- Growing bacteria will produce lots of copies of the DNA

Transformation of Bacteria

- Two basic methods to produce competent bacteria (able to take up added DNA)
  - Chemical competent
  - Electroporation

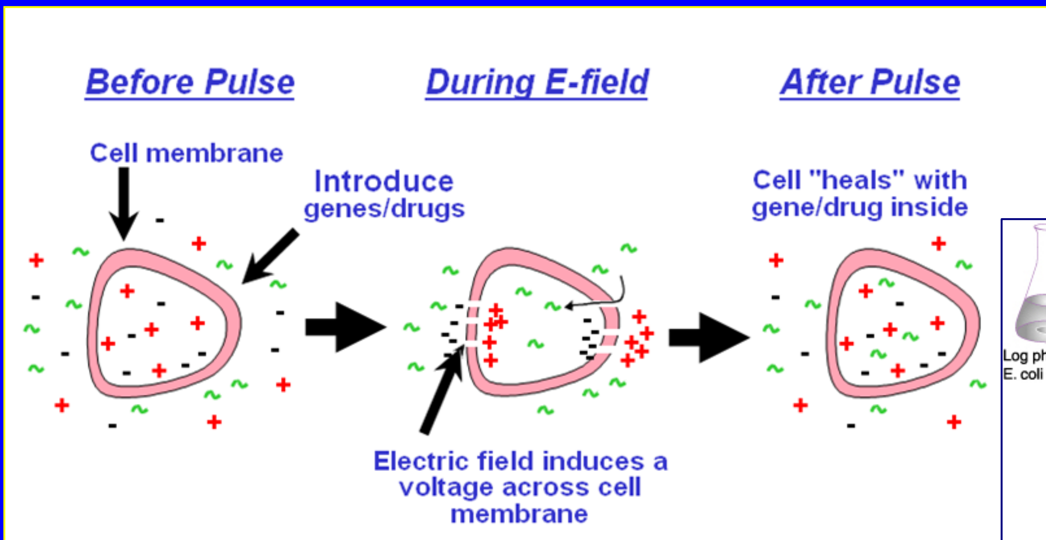
Transformation of Bacteria

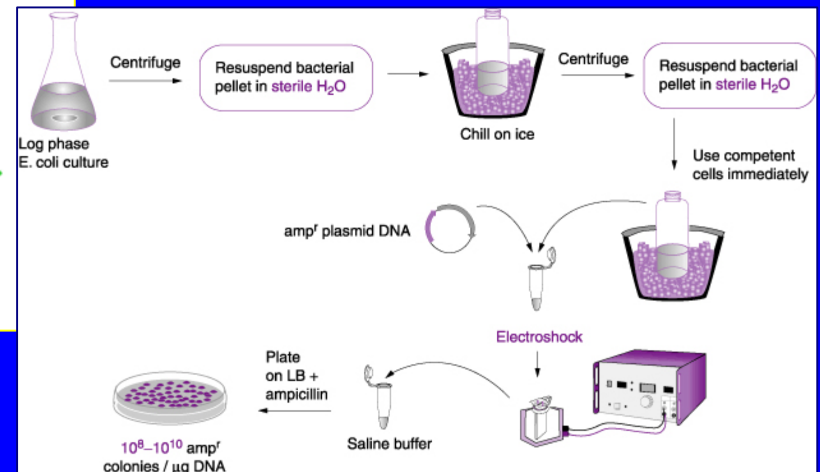
- Chemical competent
  - Divalent metal ion Ca^{++} , required
  - treat cells with ice-cold $CaCl_2$ solutions
  - Ca^{++} ions alter membrane so it is permeable to DNA



Transformation of Bacteria

- Electroporation
  - Cell/DNA mix given high voltage electric shock
  - 2.5kvolts, ~5msec
  - useful for high efficiency transformation
  - 10^9 transformants / µg of DNA

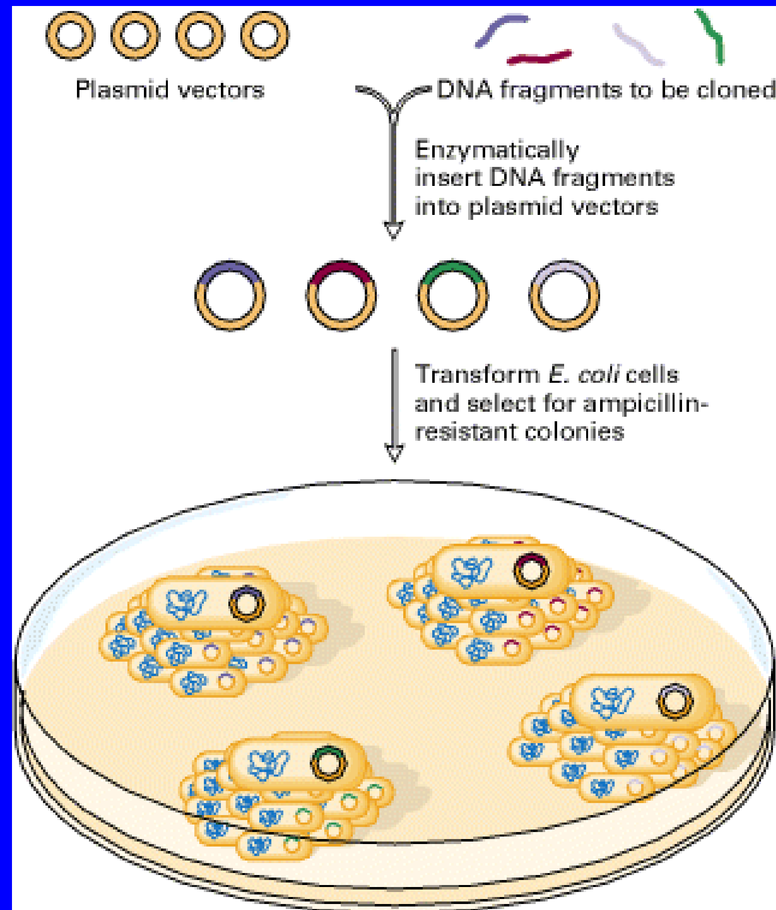


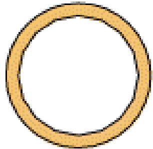


Transformation of Bacteria

- Both methods are very inefficient
  - only a few % of cells actually take up DNA
- How are the transformed cells selected?
  - antibiotic resistance gene on plasmid
  - ampicilin, tetracycline, chloramphenicol, etc.
  - transformed cells grow; non-transformed die

Selection of clones by antibiotic



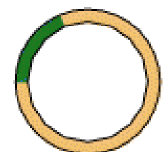


Plasmid vector

+

DNA fragment
to be cloned

Enzymatically insert
DNA into plasmid vector



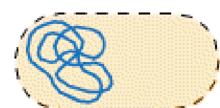
Recombinant plasmid

Mix *E. coli* cells with
plasmids in presence of
$CaCl_2$

Culture on nutrient agar
plates containing ampicillin

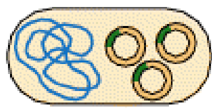
Bacterial
chromosome

Transformed
E. coli cell survives

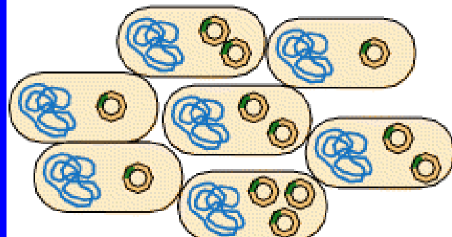


Cells that do not
take up plasmid die
on ampicillin plates

Independent
plasmid replication



Cell multiplication



Colony of cells each containing copies
of the same recombinant plasmid

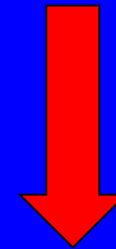
Chemically Competent E. coli

1×10^9 cfu/µg plasmid DNA

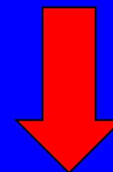
1 µg plasmid DNA

99,999% is cut

0,001% isn't cut



1ng plasmid DNA



1×10^6 cfu