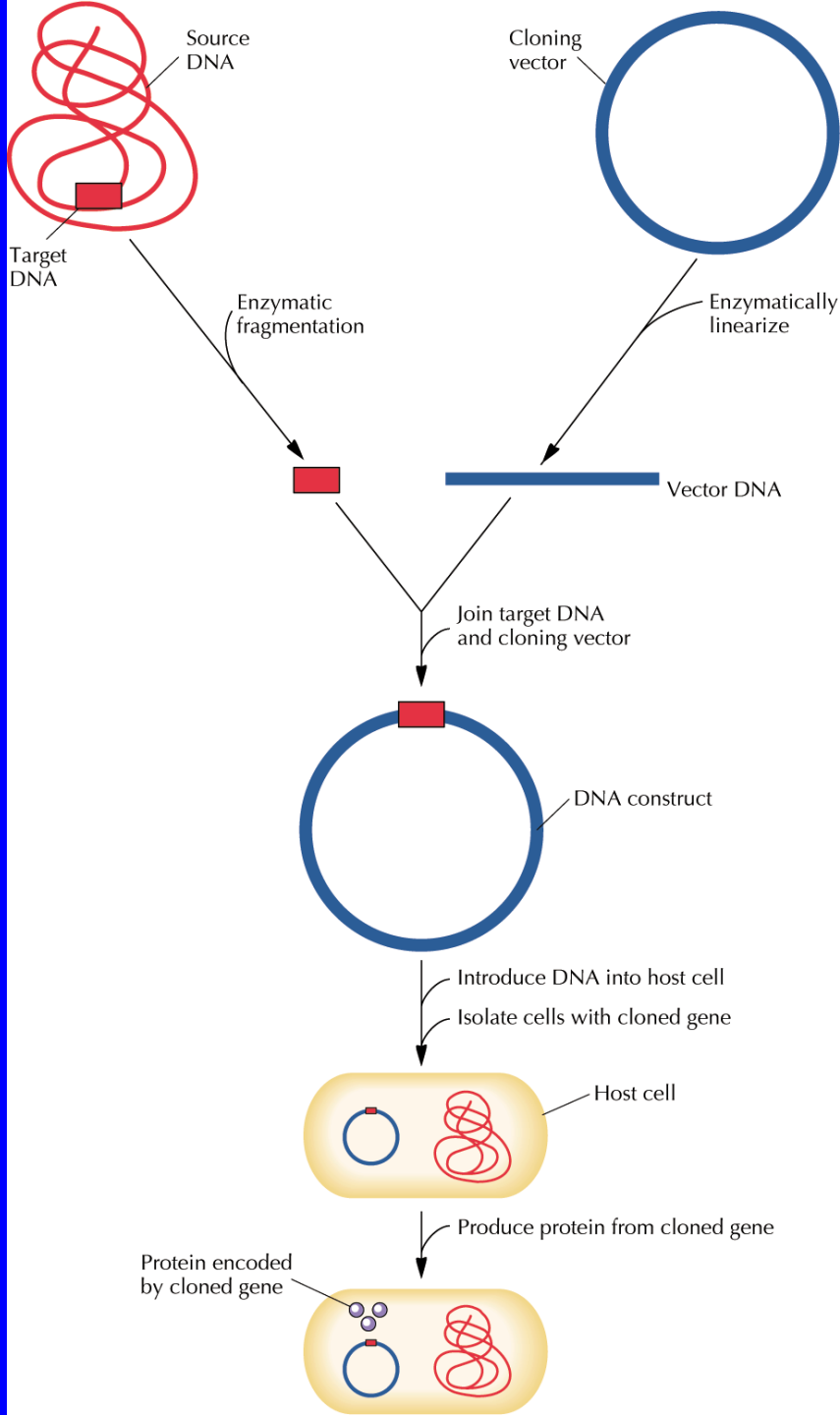
Recombinant DNA Technology

DNA Cloning

Cloning


Source DNA
Target DNA
Cloning vector
Enzymatic fragmentation
Enzymatically linearize
Vector DNA
Join target DNA and cloning vector
DNA construct
Introduce DNA into host cell
Isolate cells with cloned gene
Host cell
Produce protein from cloned gene
Protein encoded by cloned gene

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

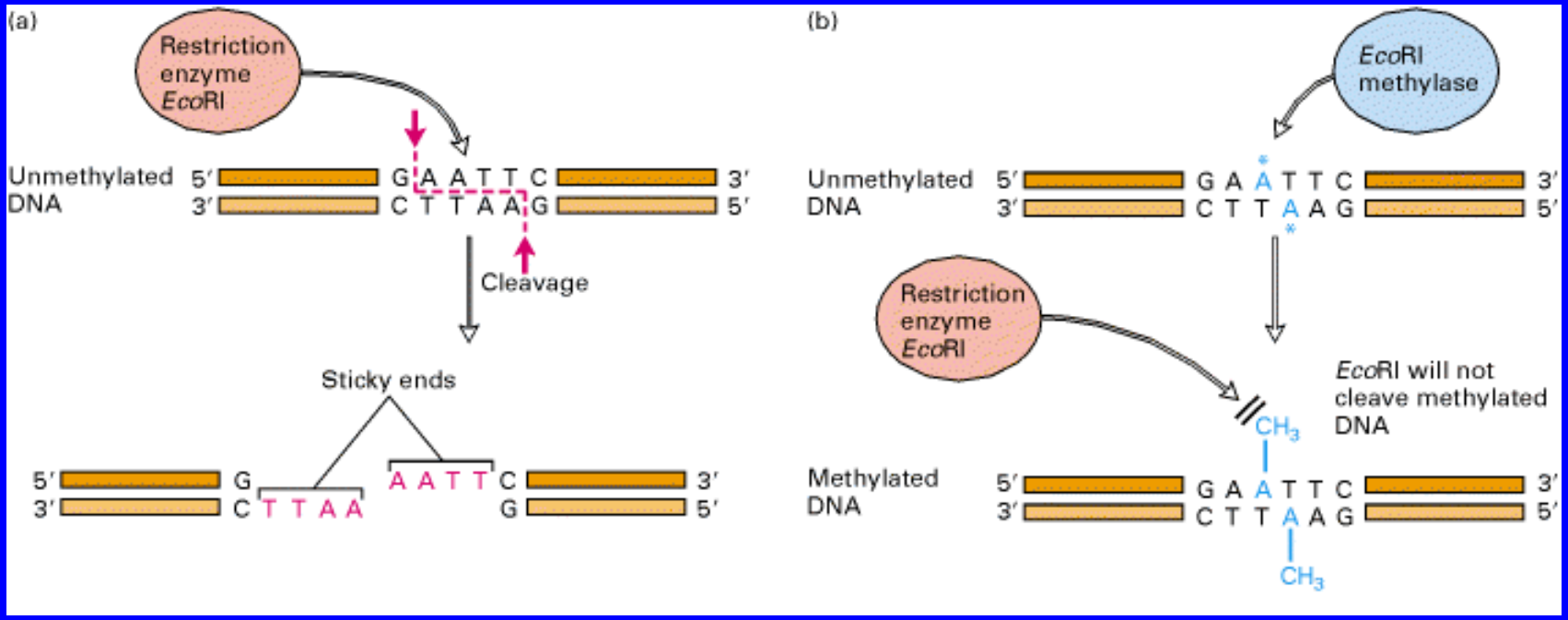
Availability

- 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


(a)
Restriction enzyme EcoRI
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)
EcoRI methylase
Unmethylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
Restriction enzyme EcoRI
EcoRI will not cleave methylated DNA
CH3
Methylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
CH3

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

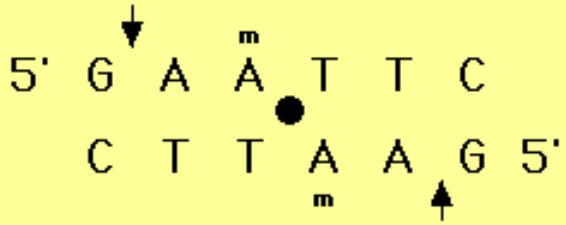
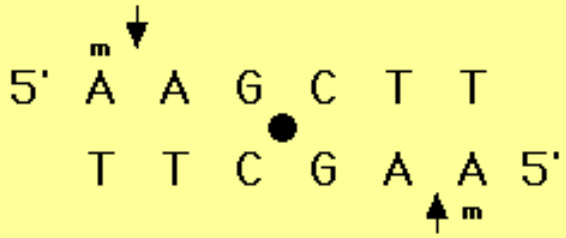
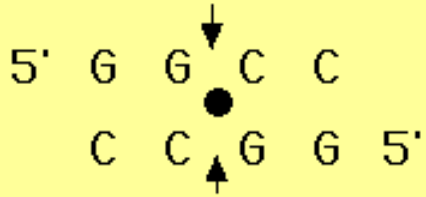
- Recognition sites
  - Generally 4, 6, or 8 bp in length
  - Most sites are palindromic
    - OTTO / HANNAH / REGAL LAGER
    - A MAN A PLAN A CANAL PANAMA
  - For REases - sequence reads the same in a 5'--->3' direction on each strand

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

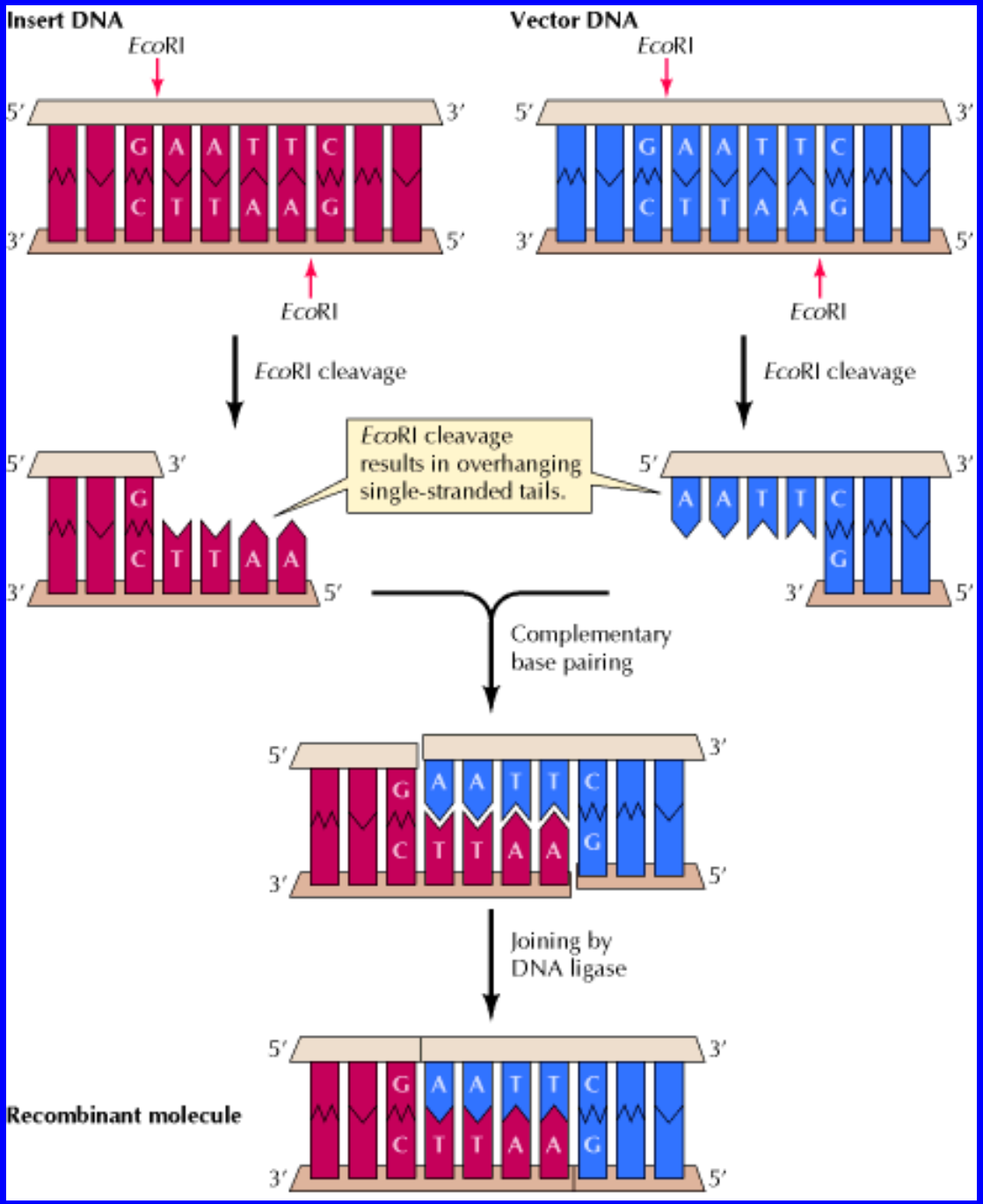
Restriction Enzymes

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

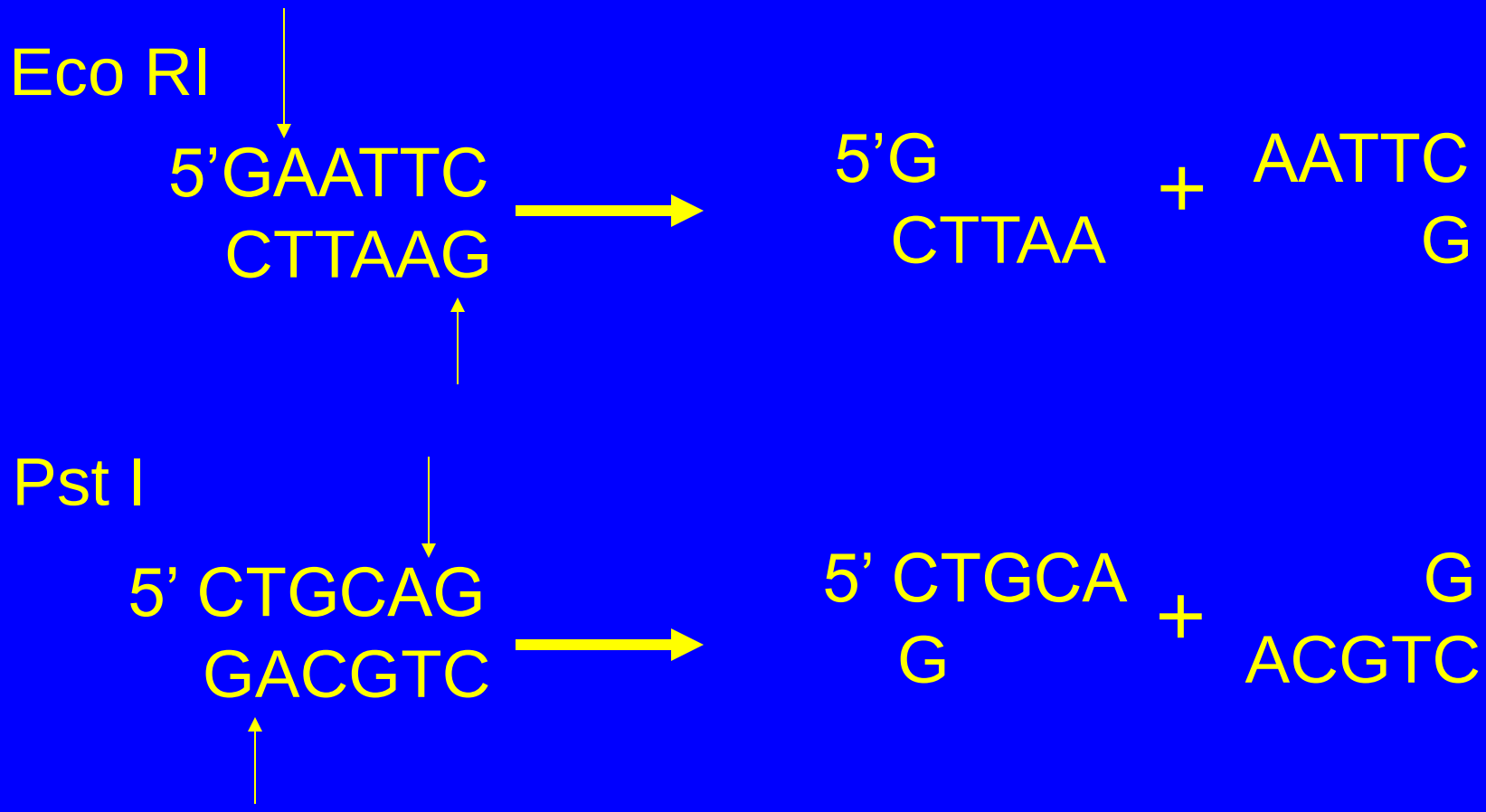
enzyme	restriction site
EcoRI	
HindIII	
HaeIII	

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

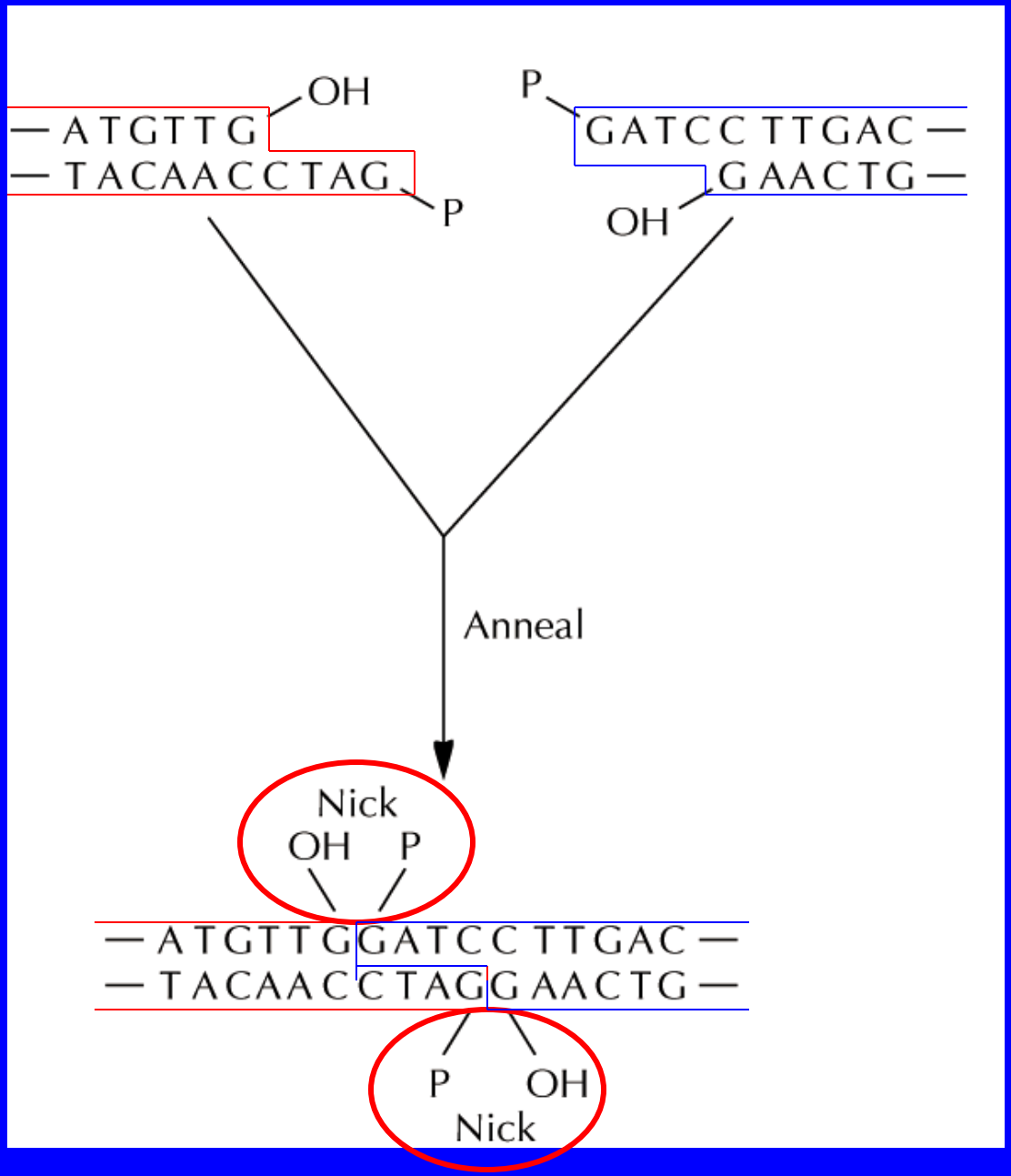

Insert DNA
Vector DNA
EcoRI
EcoRI
EcoRI
EcoRI
EcoRI cleavage
EcoRI cleavage
EcoRI cleavage results in overhanging single-stranded tails.
Complementary base pairing
Joining by DNA ligase
Recombinant molecule
5′
3′
G A A T T C
C T T A A G

Staggered Cut / 5’ or 3’ Extension



Annealing Sticky ends

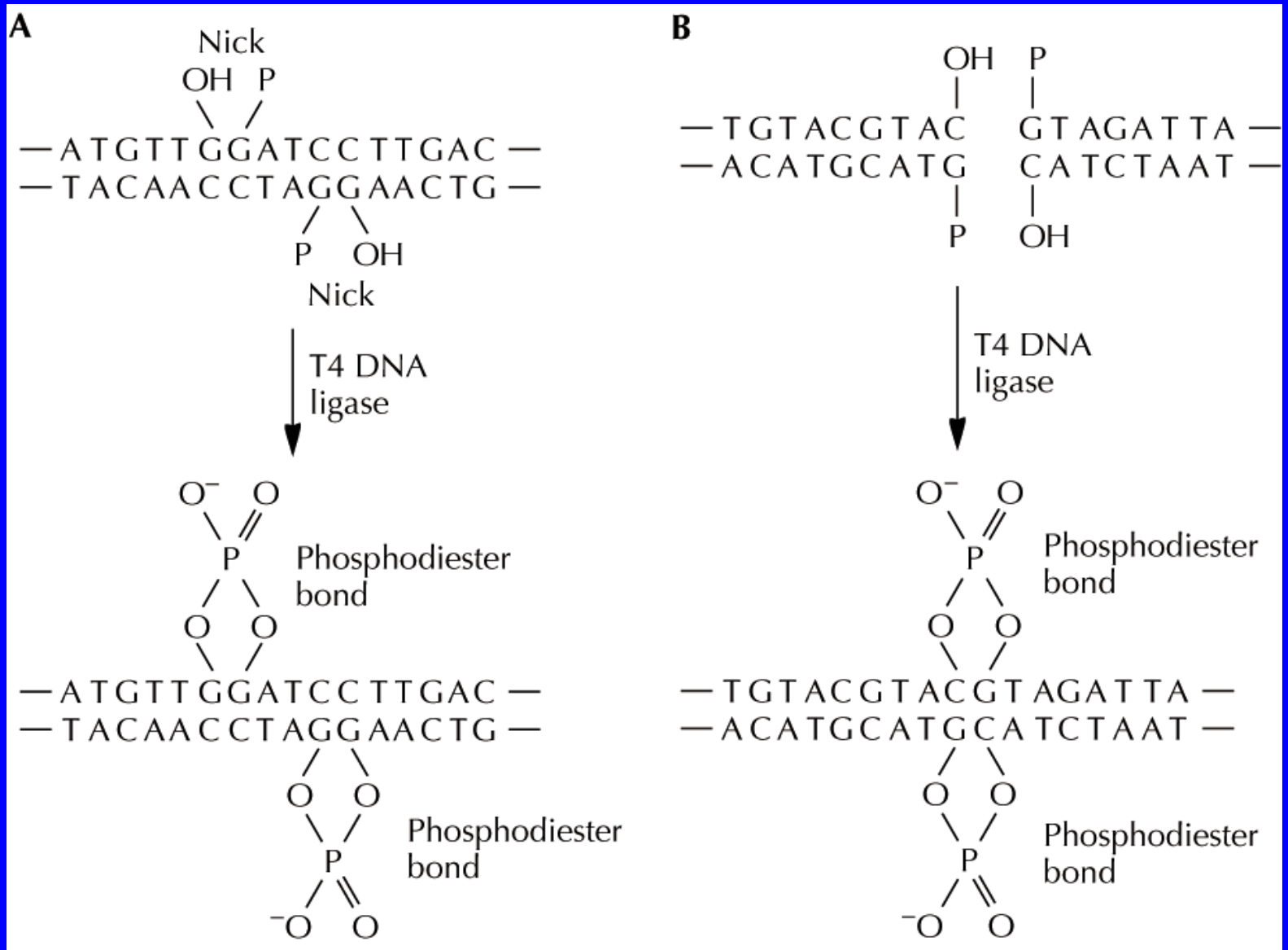
DNA strands held together only by basepairing
Nicks in strands need to be repaired



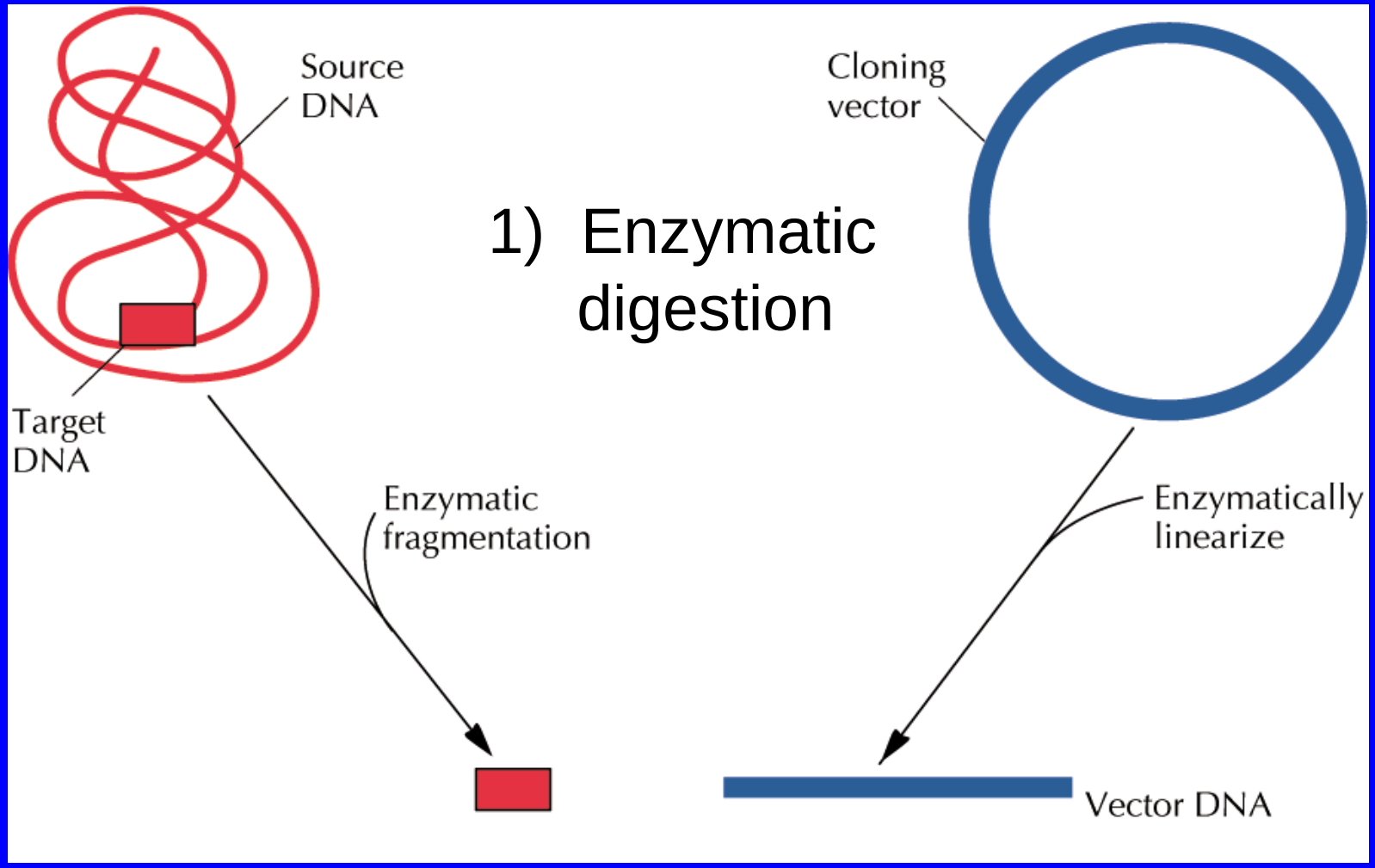
Linking Restriction Fragments

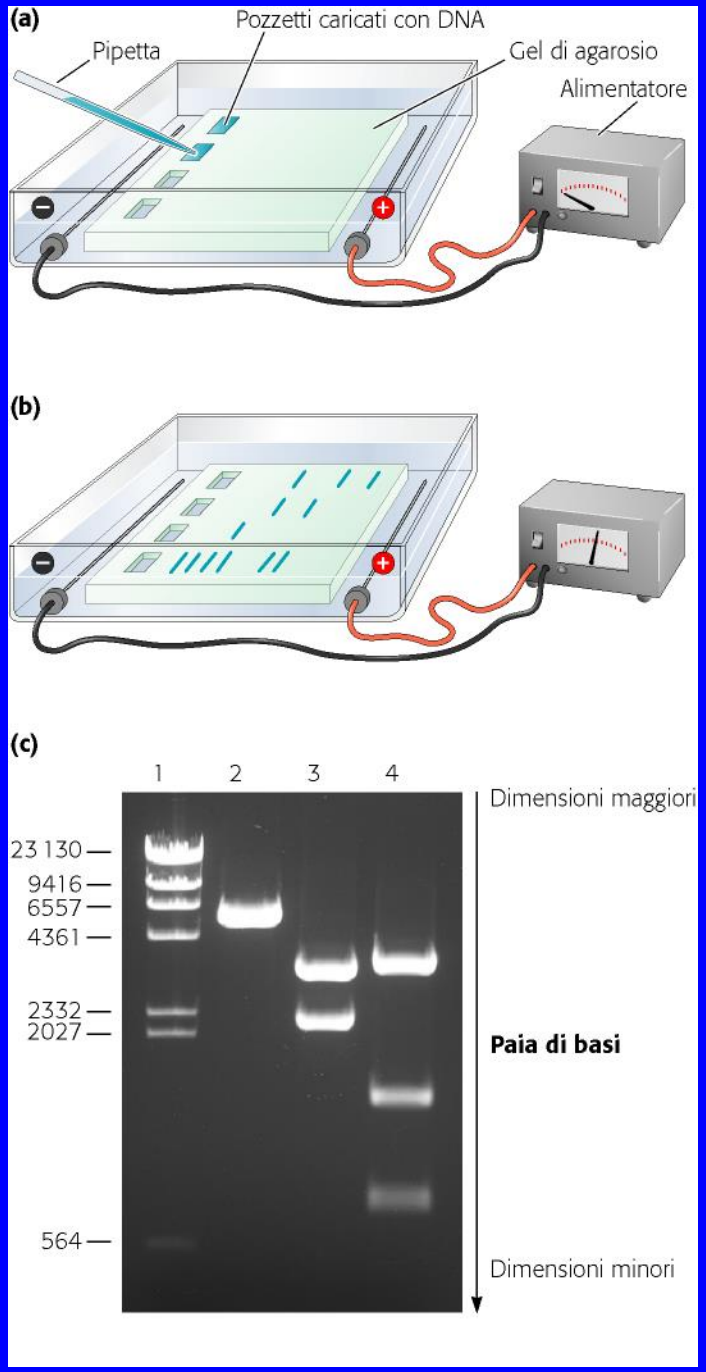
- T4 DNA Ligase
  - repairs nicks in DNA strands
    (reforms phosphodiester bond)
  - uses energy from ATP
  - works on blunt or sticky ends

T4 DNA Ligase Mode of Action



Recombinant DNA Cloning Procedure

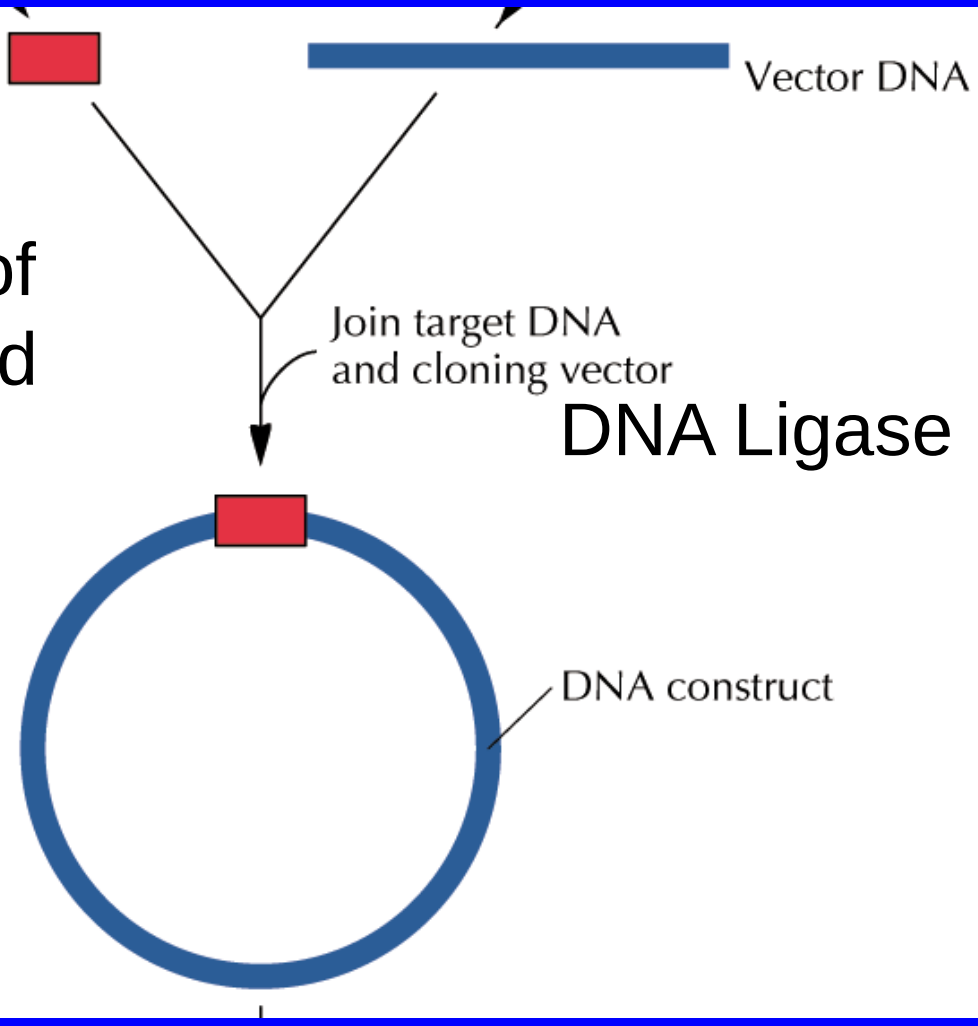




Separation of DNA fragments by gel electrophoresis

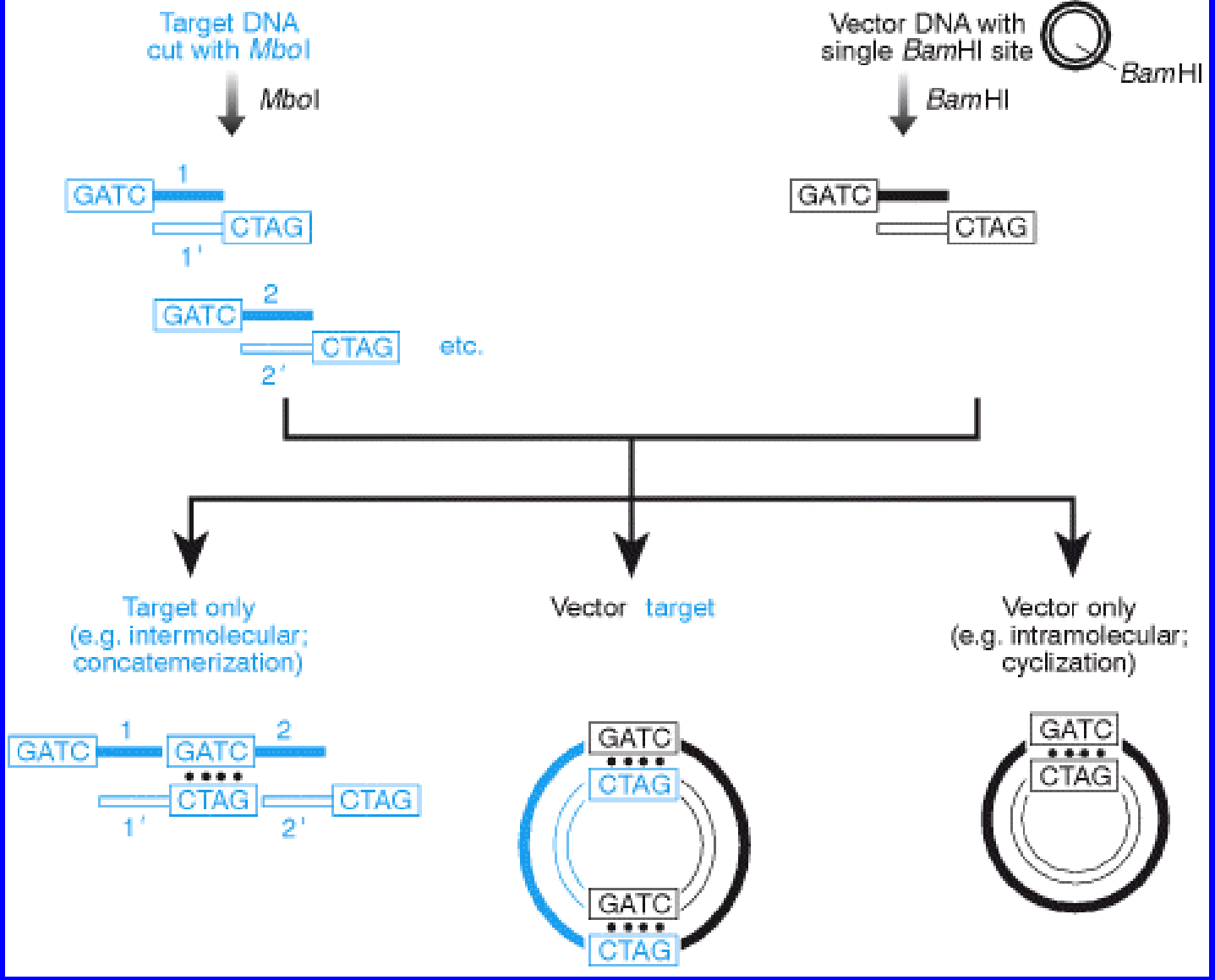
Recombinant DNA Cloning Procedure

2) Ligation of Target and vector



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

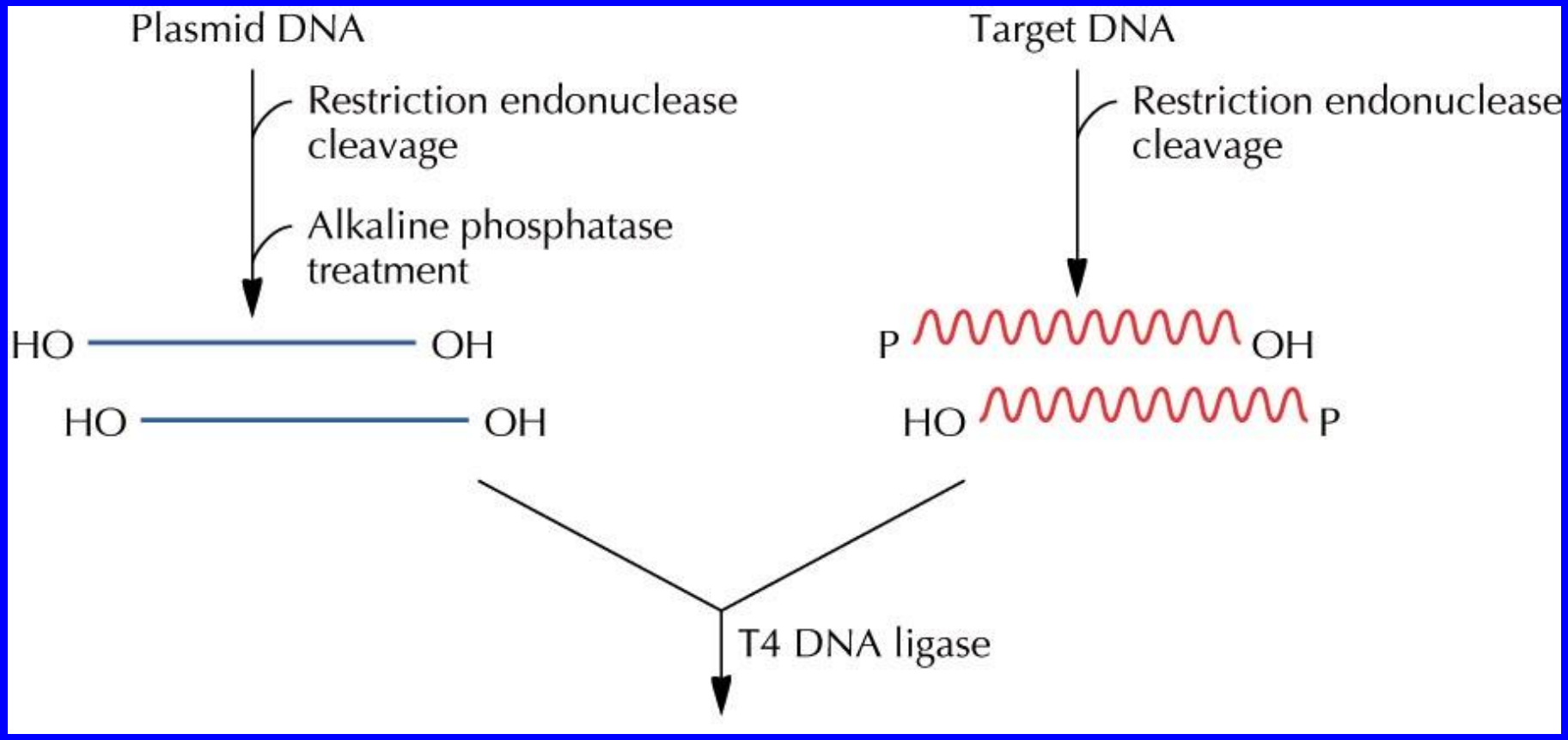
- ***Two ways to prevent***
  - ***Treat with Alkaline Phosphatase***
  - ***Directional Cloning***



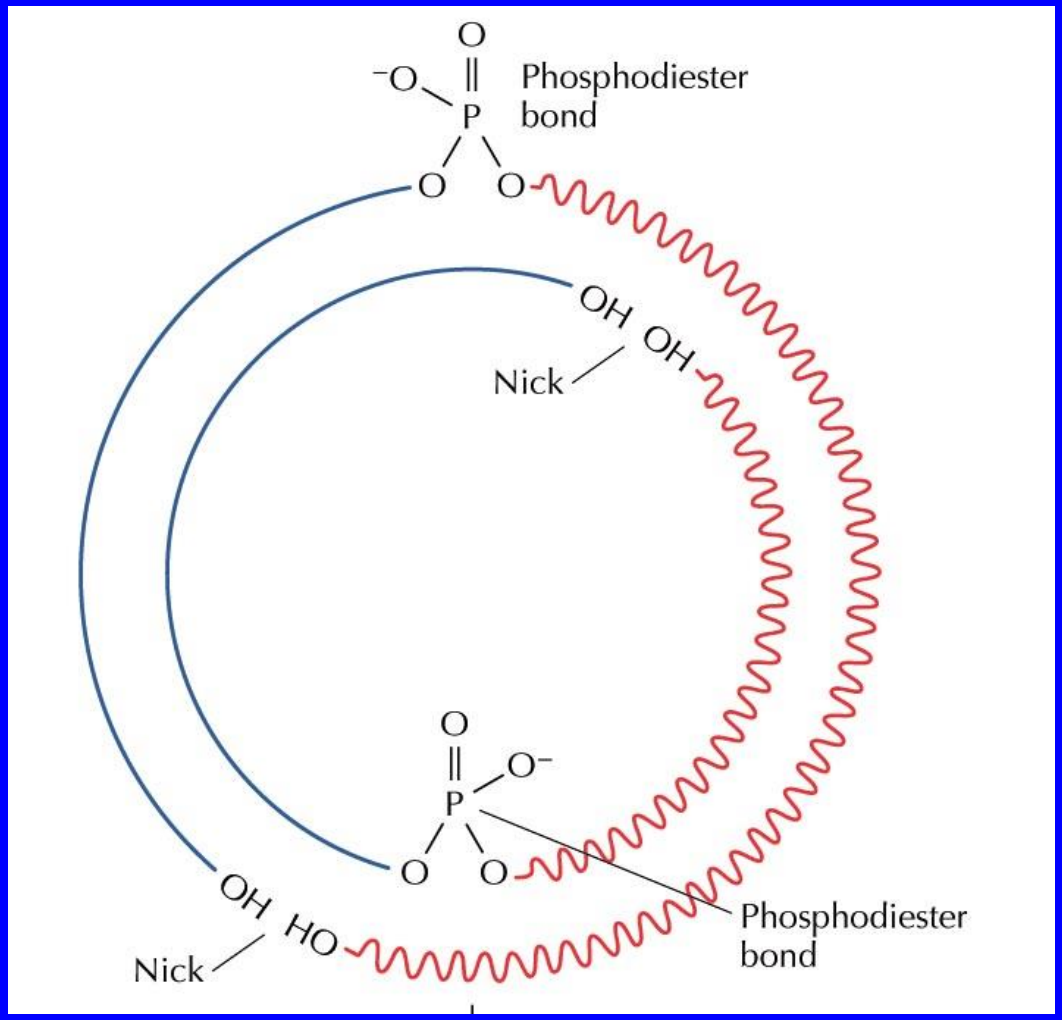
Cohesive termini can associate intramolecularly and intermolecularly. *Note* that only some of the possible outcomes are shown. For example, vector molecules may also form intermolecular concatemers, multimers can undergo cyclization and co-ligation events can involve two different target sequences being 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

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

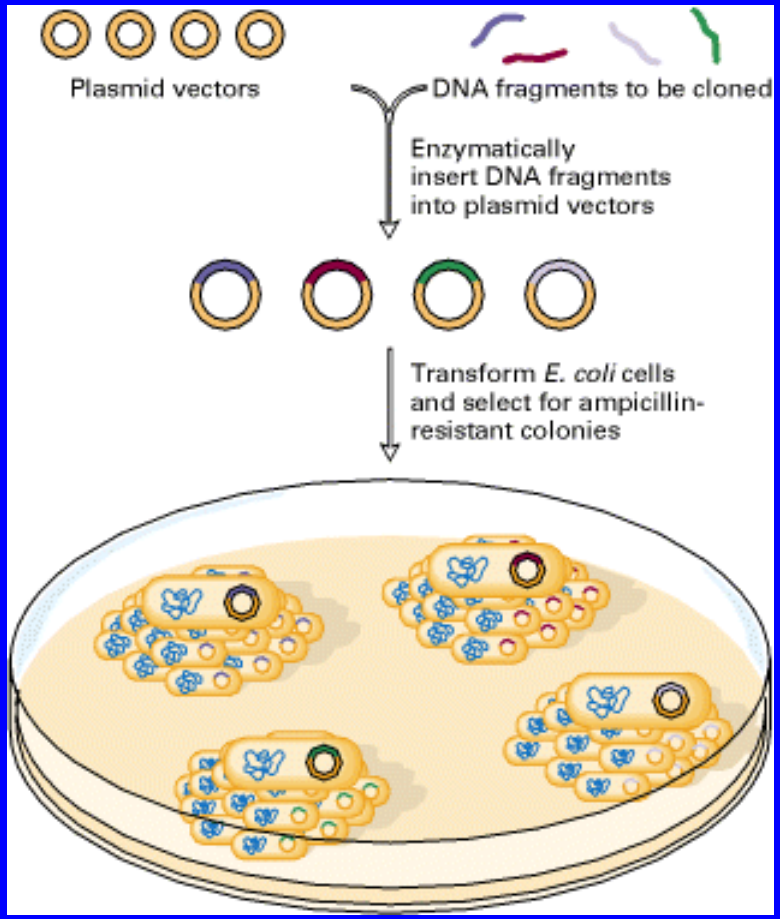
Transformation of Bacteria

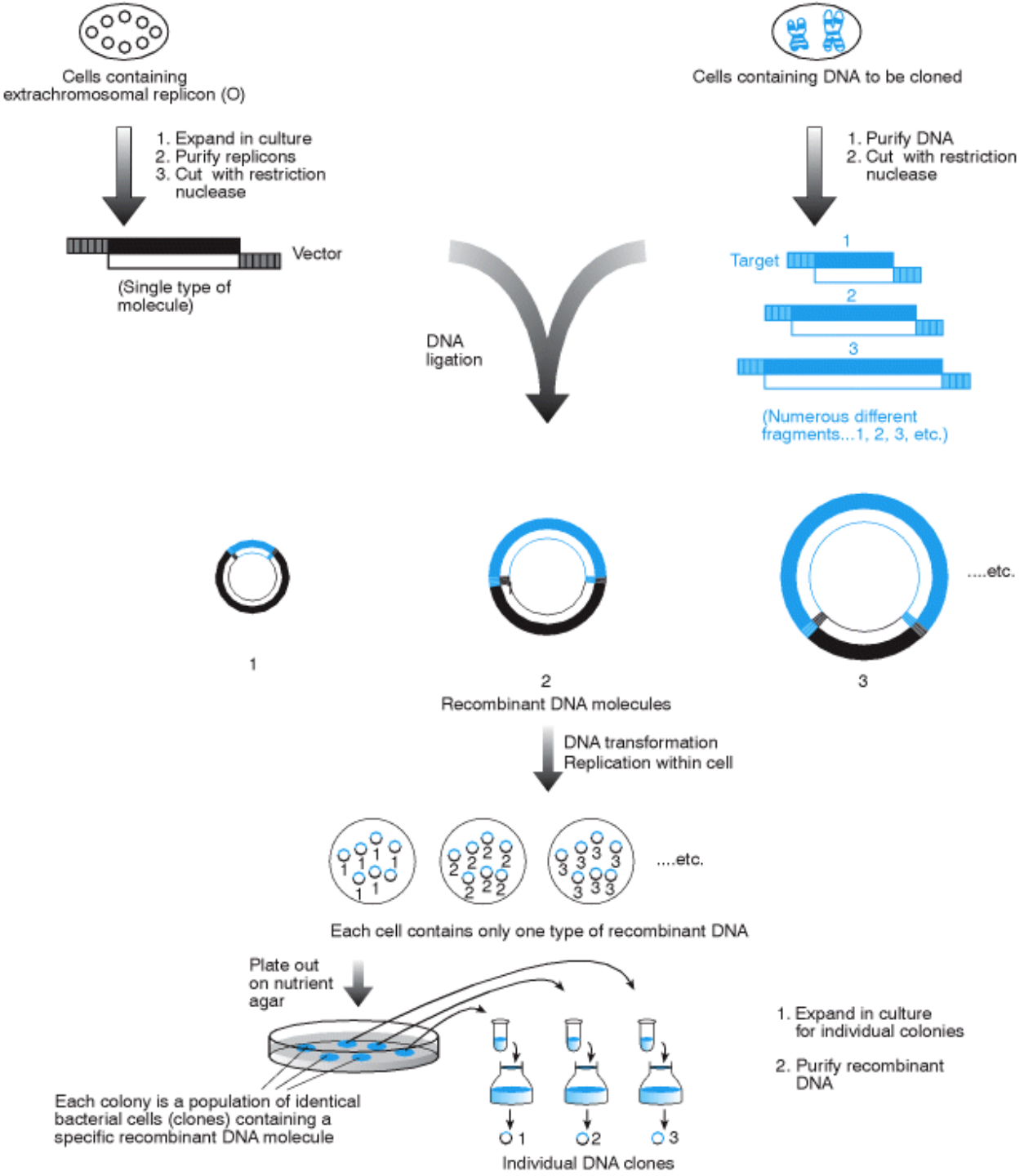
- Electroporation
  - Cell/DNA mix given high voltage electric shock
  - 2.5kvolts, ~5msec
  - useful for high efficiency transformation
  - 10^9 transformants / µg of DNA

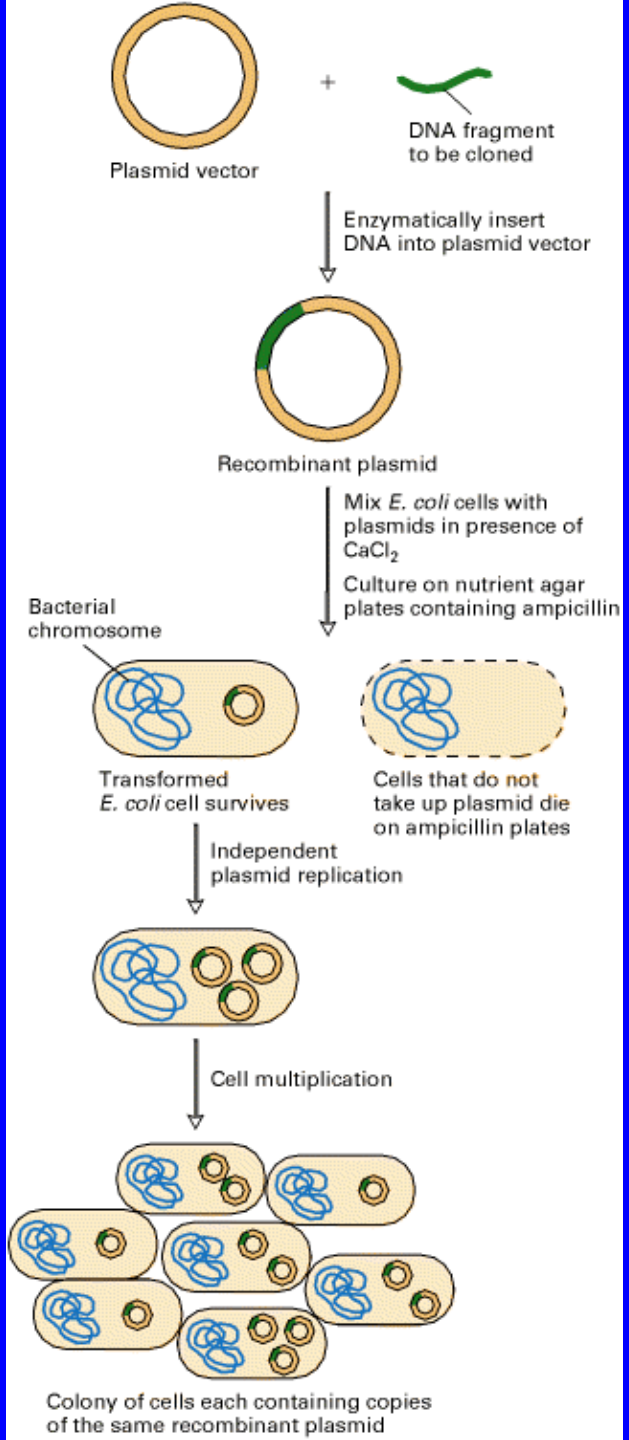
Transformation of Bacteria

- 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




Cells containing extrachromosomal replicon (O)
Cells containing DNA to be cloned
1. Expand in culture
2. Purify replicons
3. Cut with restriction nuclease
1. Purify DNA
2. Cut with restriction nuclease
Vector
(Single type of molecule)
DNA ligation
1
Target
2
3
(Numerous different fragments...1, 2, 3, etc.)
....etc.
1
2
Recombinant DNA molecules
3
DNA transformation
Replication within cell
....etc.
Each cell contains only one type of recombinant DNA
Plate out on nutrient agar
Each colony is a population of identical bacterial cells (clones) containing a specific recombinant DNA molecule
1
2
3
Individual DNA clones
1. Expand in culture for individual colonies
2. Purify recombinant DNA


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