MOLECULAR BIOLOGY TECHNIQUES

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VECTOR ISOLATION

- In molecular biology, a vector is a DNA molecule used as a vehicle to transfer foreign genetic material into another cell.

- Intentionally designed artificial DNA construct used by molecular biologists to amplify selected pieces of DNA inserted into the construct; examples include plasmid, phage, phagemid, cosmid, fosmid, yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC)

CLONING VECTOR

Cloning vectors minimally contain

- An origin of replication
- Selectable marker gene (e.g., ampicillin resistance gene)
- Multiple cloning site containing unique restriction enzyme

Features

Modern vectors may encompass additional features besides the transgene insert and a backbone:

- **Promoter**: Necessary component for all vectors: used to drive transcription of the vector's transgene.
- **Genetic markers**: Genetic markers for viral vectors allow for confirmation that the vector has integrated with the host genomic DNA.
- **Antibiotic resistance**: Vectors with antibiotic-resistance open reading frames allow for identification of which cells have uptaken the vector through antibiotic selection.
- **Epitope**: Vector contains a sequence for a specific epitope that is incorporated into the expressed protein. Allows for antibody identification of cells expressing the vector.
- **β-galactosidase**: Vector's multiple cloning site contains sequence for β-galactosidase, an enzyme that digests galactose, to either side of the region intended for an insert. Targeting sequence: Expression vectors may include encoding for a targeting sequence in the finished protein that directs the expressed protein to a specific organelle in the cell.

VECTORS

- **Plasmid:** A small circular DNA molecule found in bacteria that replicates independently of the chromosome. As large inserts plasmids not suitable.
- **Phage:** A virus that infects bacterial hosts and may be utilized to introduce genes.
- **Lambda Phages:** DNA size required for packaging 35-50 Kb
  - Type I: Inserts less than 7 kb- Insertion phages
  - Type II: Inserts of about 20 kb- Replacement phages
- **M13:** vectors are derivatives of the single-stranded, male-specific
  - Filamentous DNA bacteriophage M13.
- **Cosmids:** hybrid plasmid vectors- insert size 40-45 kb
- **Phagemid:** Phage-plasmid vector able to replicate as single or double-stranded DNA
- **Bacterial artificial chromosome (BAC):** Insert size 100-300 kb
- **Yeast artificial chromosome (YAC):** Insert size 0.3-1.2 Mb

How are plasmids engineered?

Host DNA fragments
(i.e. coral or jellyfish
FP coding DNA)

DNA Plasmid Vector

Cut genomic
DNA into
fragments

Ligate (paste) fragments
into cut DNA vector

Cut plasmids
open with
restriction
enzymes

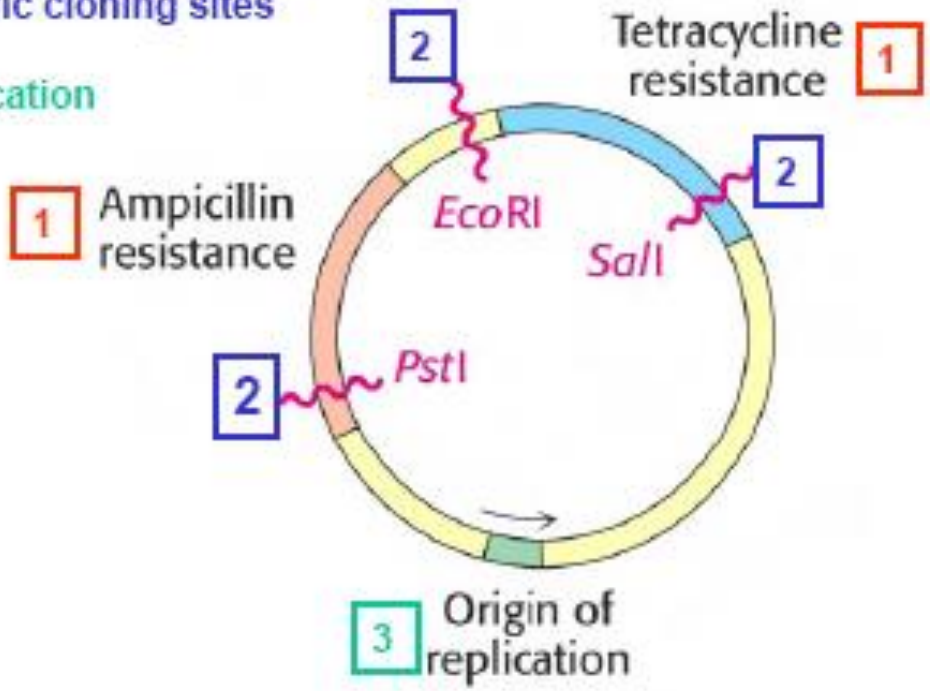
End result: Plasmid
containing FP gene

Ex: Prokaryotic (*E.coli*) Plasmid Vector: Example that satisfies requirements

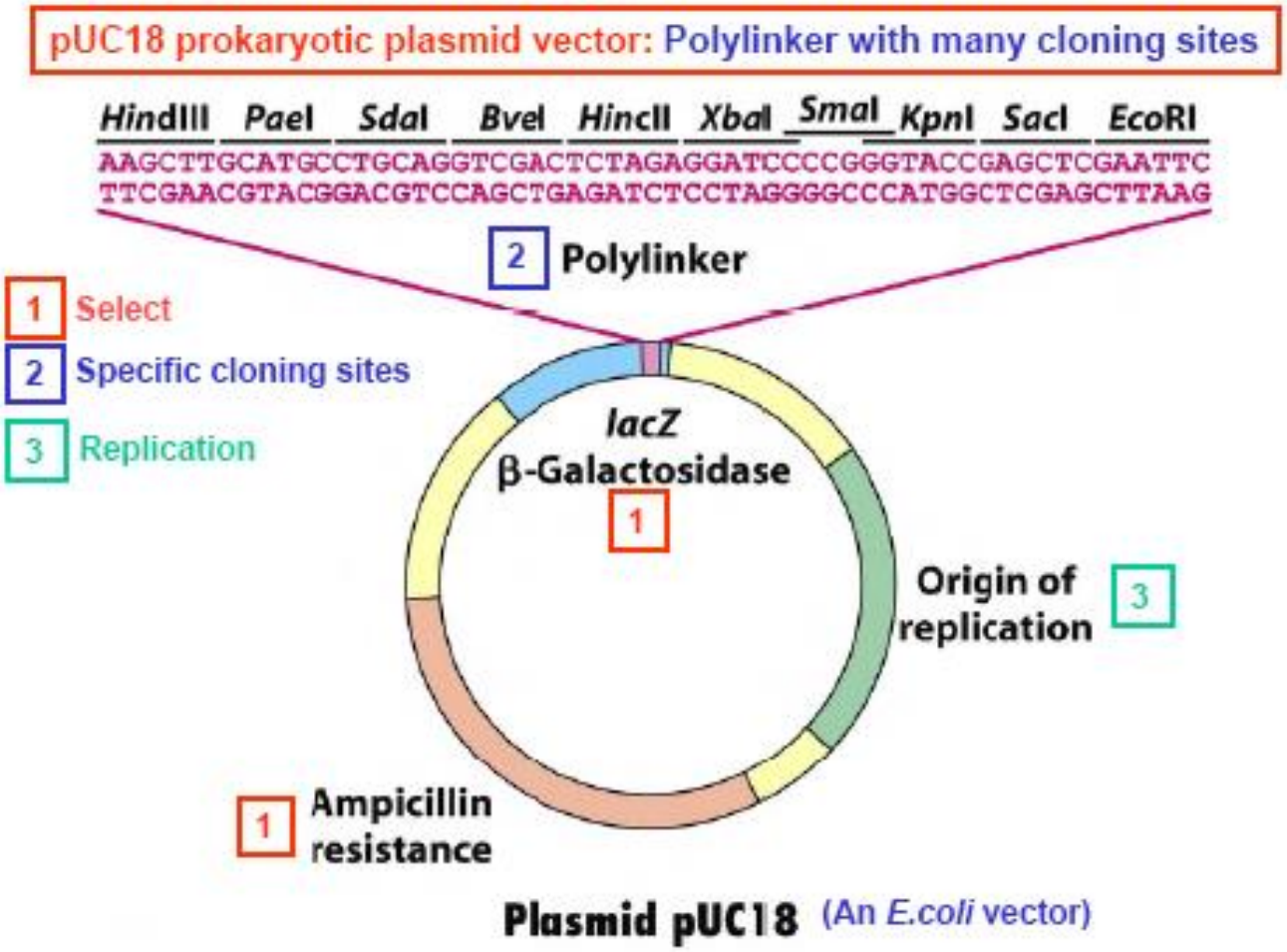
1 Select

2 Specific cloning sites

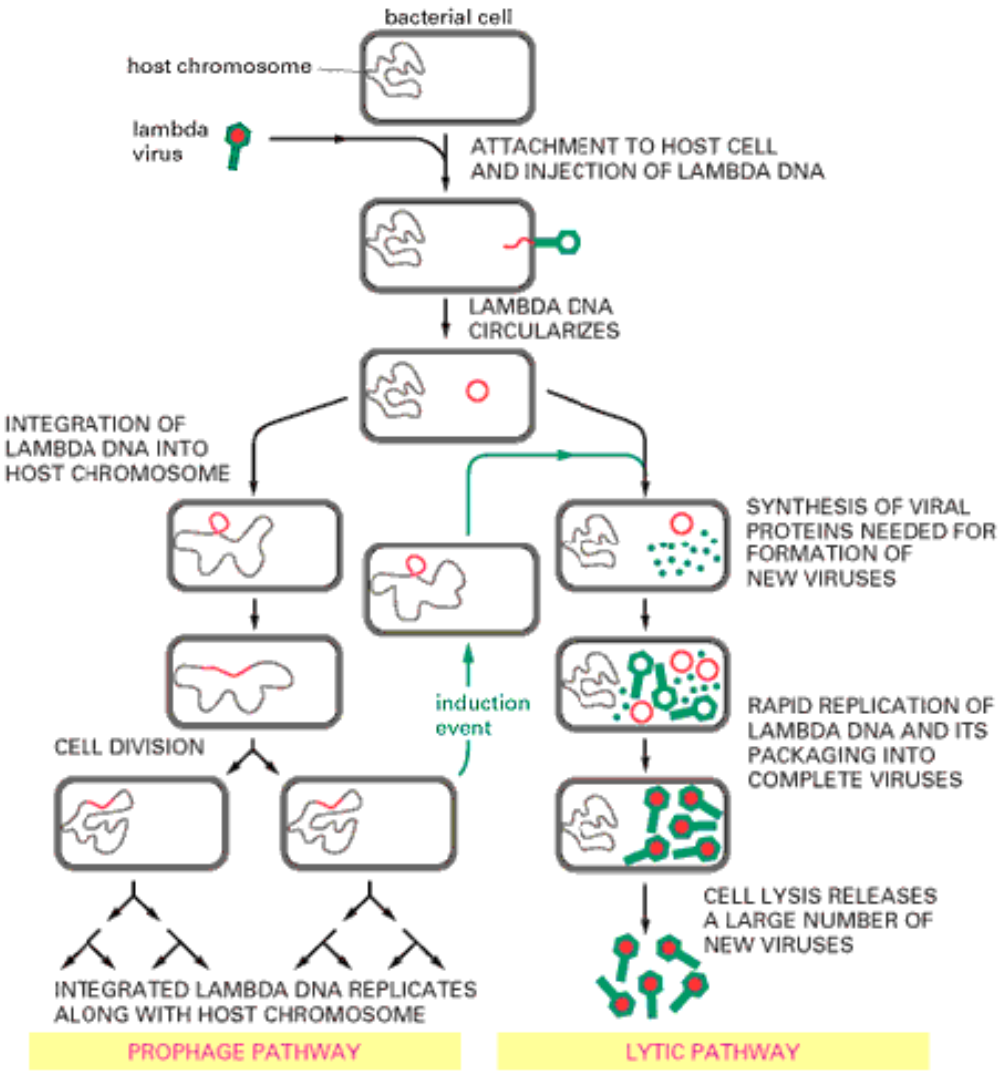
3 Replication



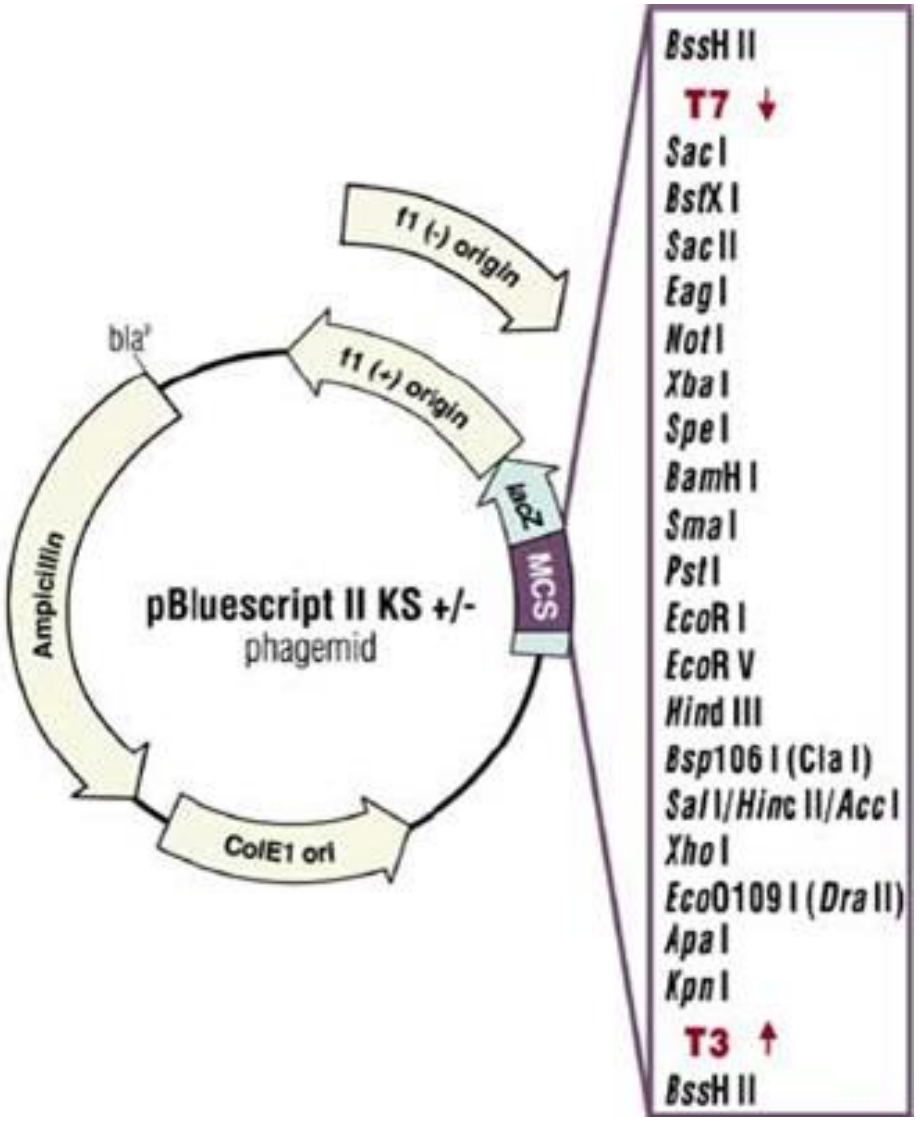
Plasmid pBR322


pUC18 prokaryotic plasmid vector: Polylinker with many cloning sites
HindIII PaeI SdaI BveI HincII XbaI SmaI KpnI SacI EcoRI
AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTC
TTCGAACGTACGGACGTCCAGCTGAGATCTCCTAGGGGCCCATGGCTCGAGCTTAAG
2 Polylinker
1 Select
2 Specific cloning sites
3 Replication
lacZ
β-Galactosidase
1
Origin of replication
3
1 Ampicillin resistance
Plasmid pUC18 (An E.coli vector)

LAMBDA LIFESTYLES



PHAGEMID



Filamentous phage origin of replication

PLASMIDS VS PHAGE

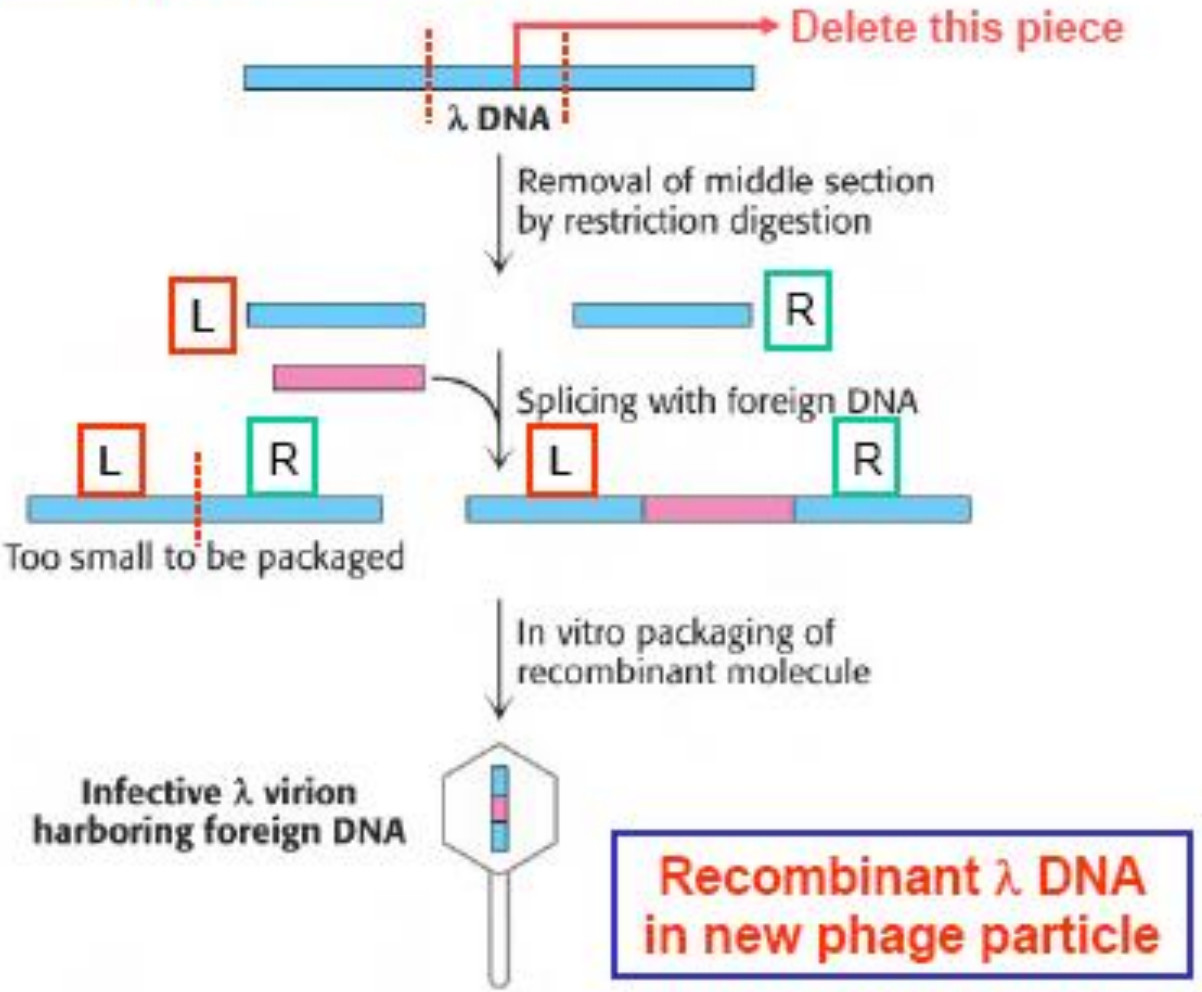
PLASMIDS

- Easy to manipulate in the lab
- Can accommodate relatively small DNA fragments
- Up to 10 kb
- Transformation inefficient

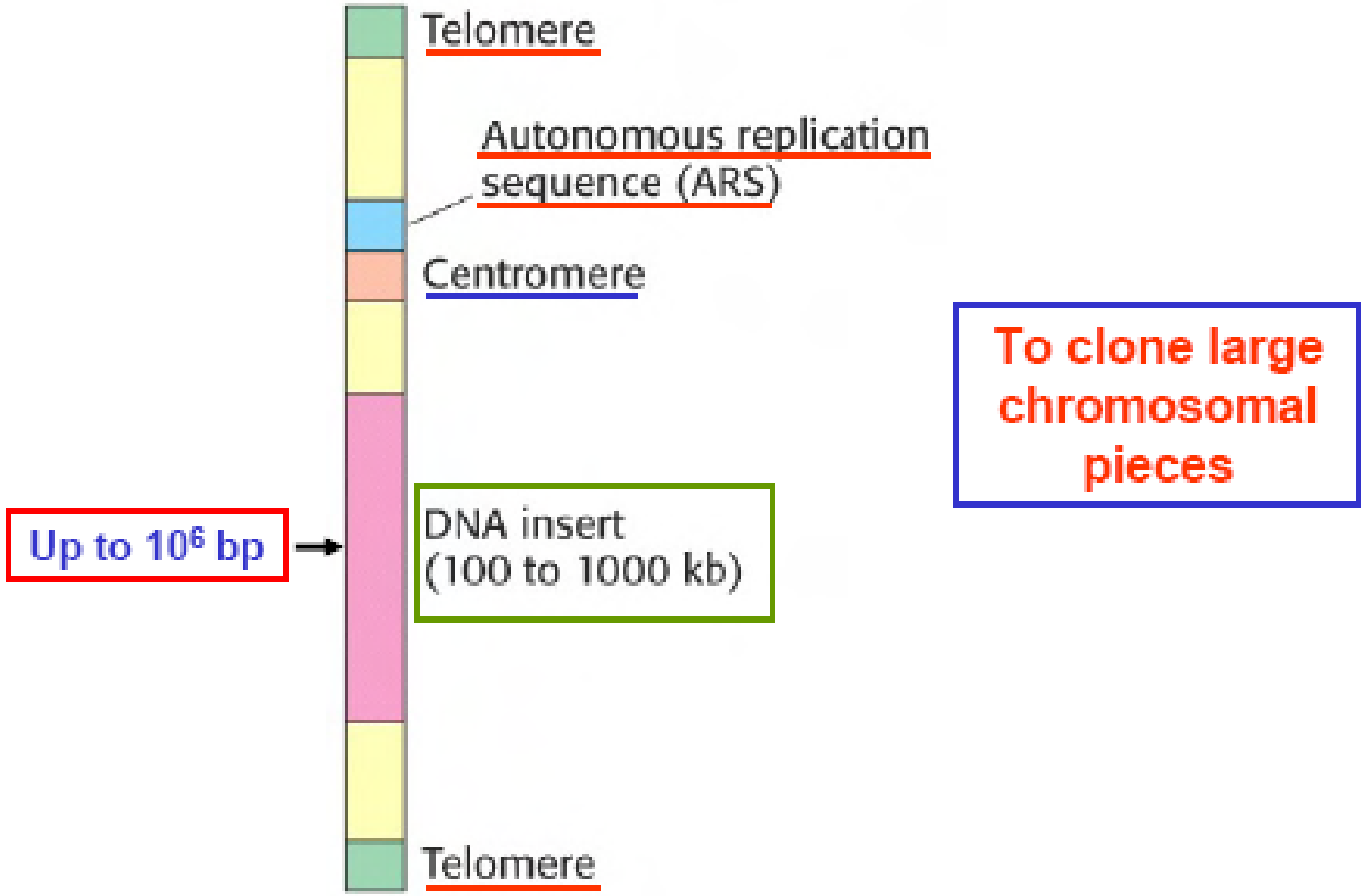
PHAGE

- Difficult to manipulate in the lab
- Can accommodate larger DNA fragments –20-40 kb
- Transfection efficient
- Have capacity to make DNA

Bacteriophage lambda (λ) as a cloning vector



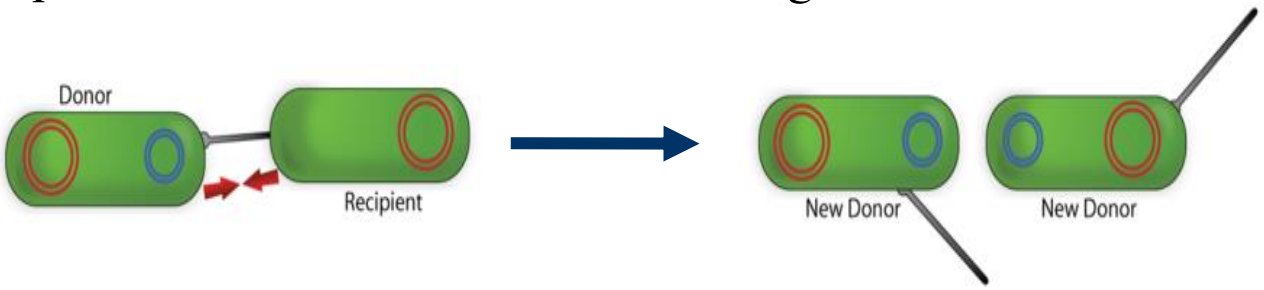
Yeast Artificial Chromosome (YAC)



To clone large chromosomal pieces

Plasmid Functional Categories

- F-plasmids: Facilitate bacterial conjugation

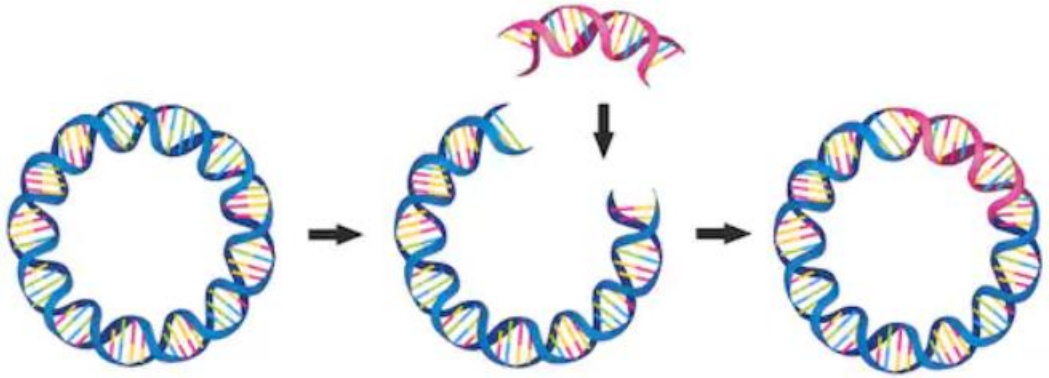


- R-plasmids: Confer resistance to antibiotics or other toxins

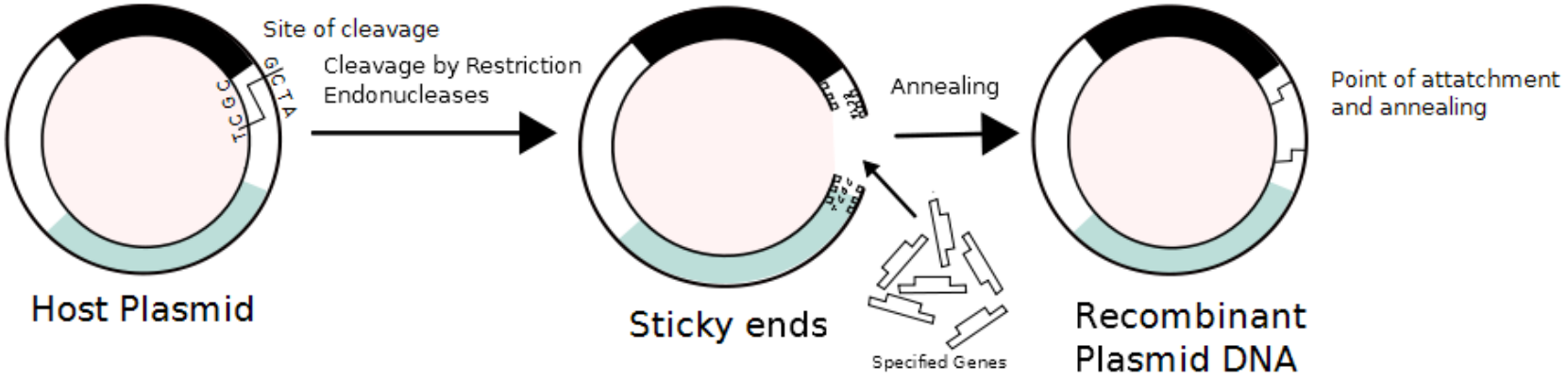
Bacteria carrying a plasmid with the gene *neomycin phosphotransferase* are capable of surviving in the presence of the antibiotic kanamycin

- **Col-plasmids:** Encode for colicines (potentially toxic to other bacteria)
- **Degradative plasmids:** Enable the breakdown of certain substances
- **Virulence plasmids:** Causes the bacteria to act as a pathogen

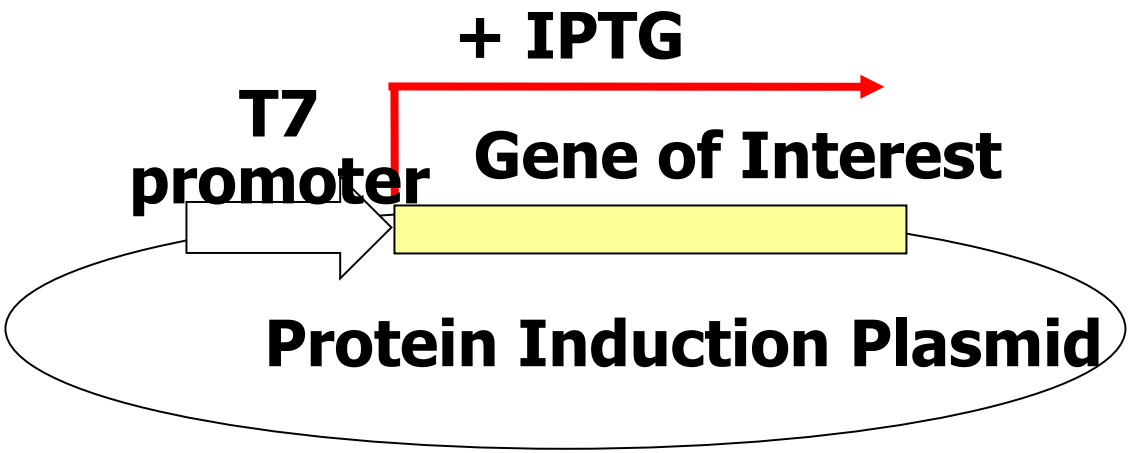
Molecular Biology Applications for Plasmids



CLONING OF DNA FRAGMENTS

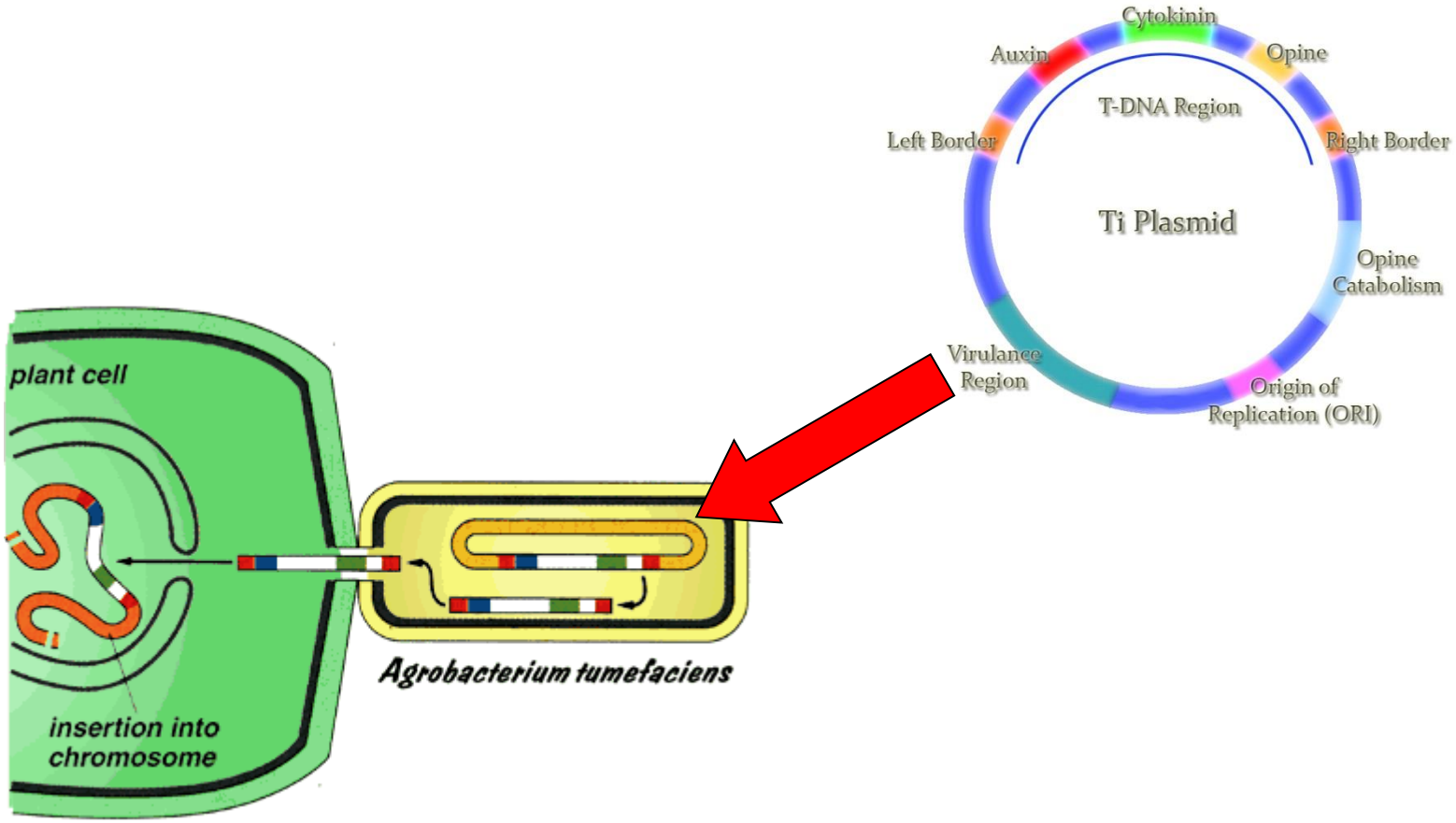


PROTEIN PRODUCTION

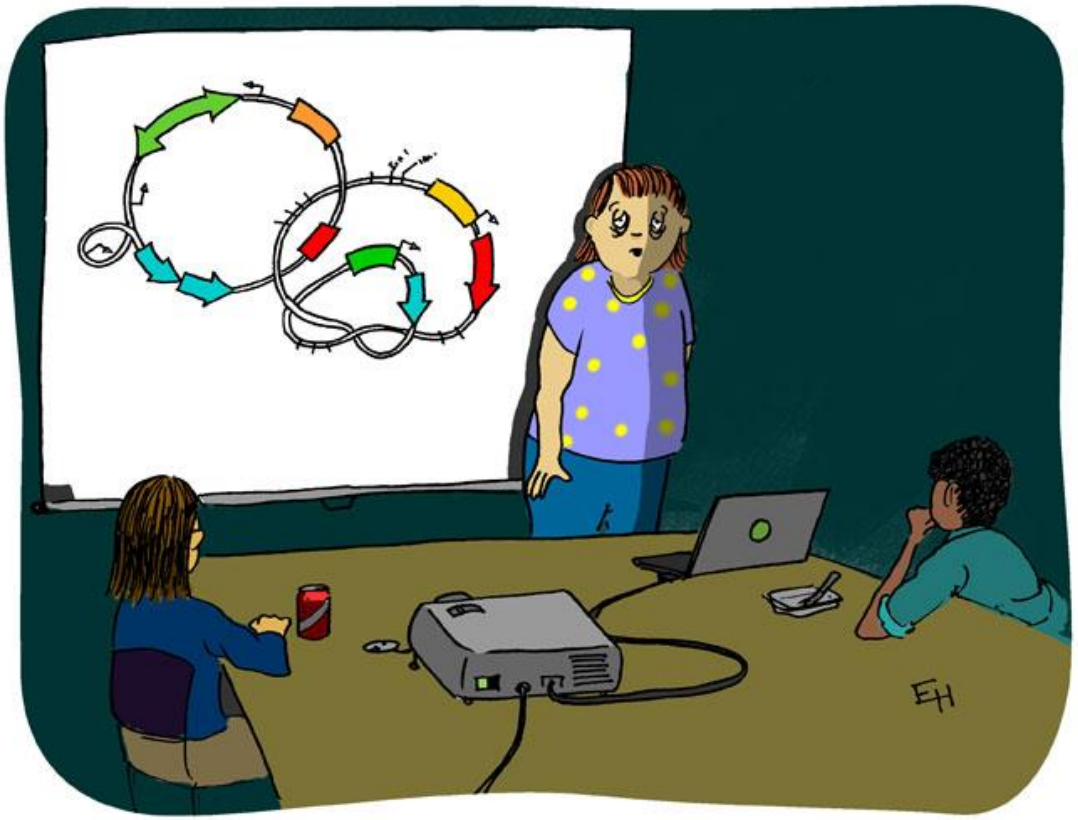


AGROBACTERIUM-MEDIATED PLANT TRANSFORMATION

A means of performing plant genetic engineering

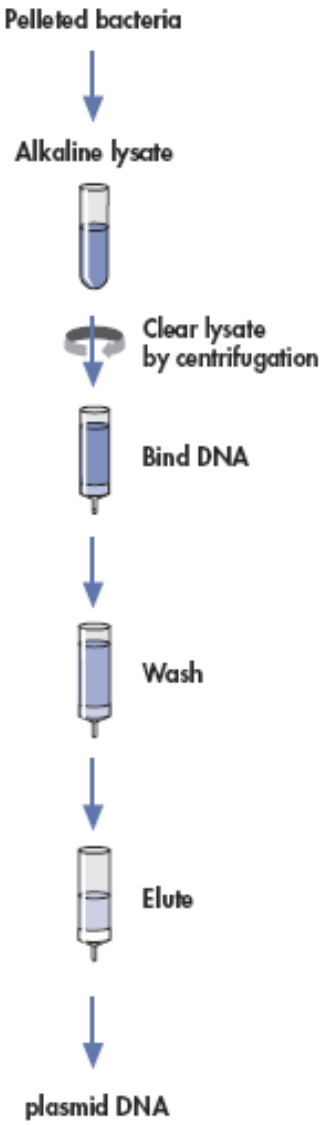


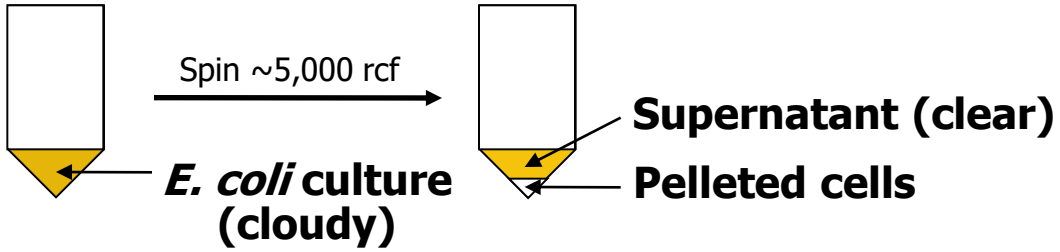
Purification of Plasmid DNA using silica-based columns



I wish I could report otherwise,
but the cloning is not going very well.

Harvest cells by centrifugation





Discard supernatant

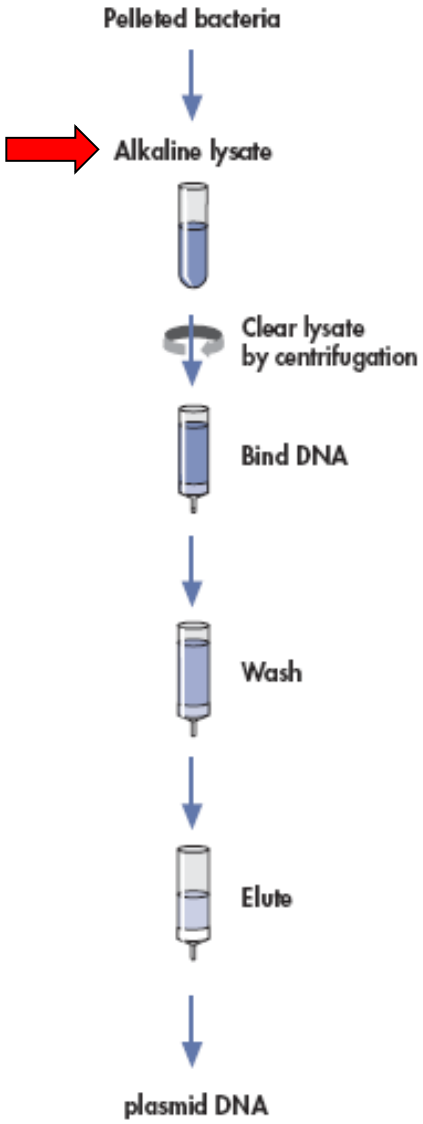
Residual media may interfere with downstream steps

Resuspend cells in buffer

Thoroughly resuspend cells, making sure that no clumps remain. P1 buffer contains:

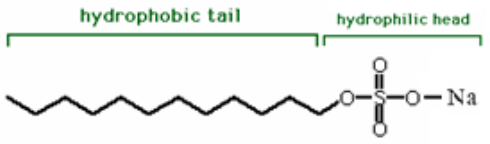
- Tris-Cl (buffering agent)
- EDTA (metal chelator)
- RNase A (degrades RNA)

Lyse cells with SDS/NaOH solution

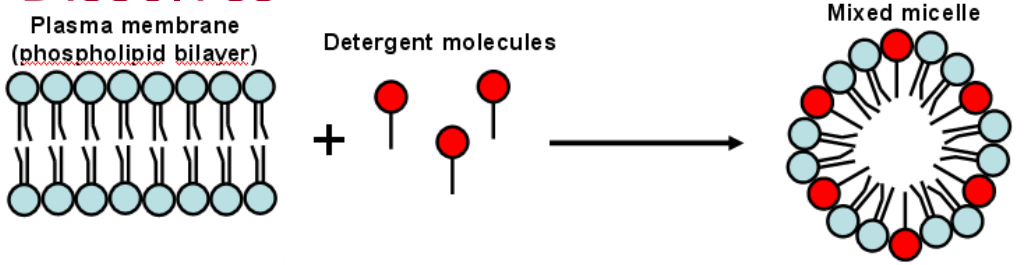


Adding buffer P2 causes solution to become viscous

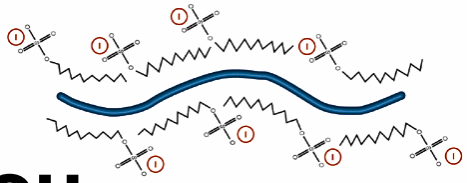
1. Sodium dodecyl sulfate



- Dissolves m

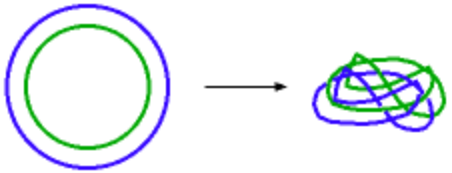


- Binds to and denatures prote

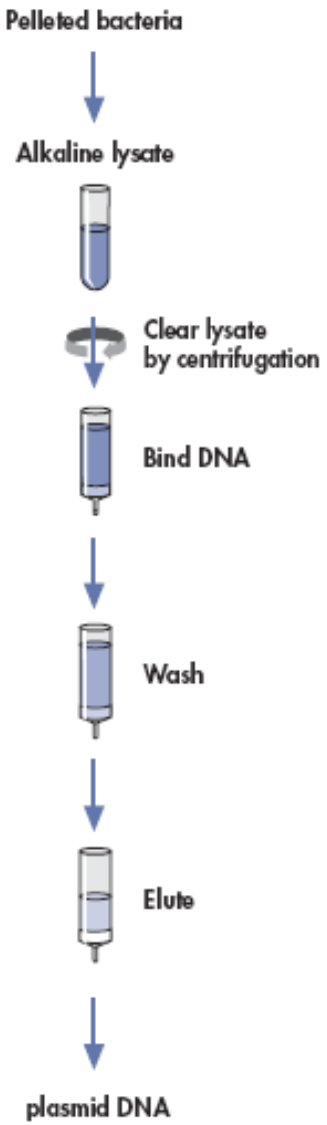


2. NaOH

- Denatures DNA



Because plasmids are supercoiled, both DNA strands remain entangled after denaturation

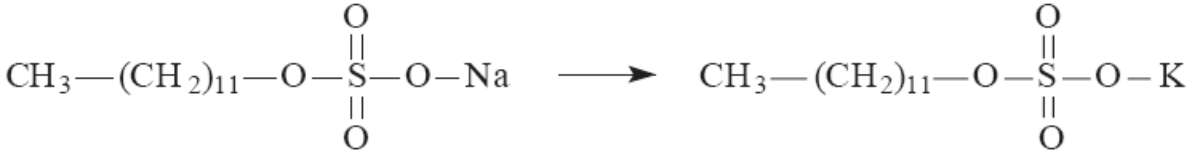


Neutralize NaOH with potassium acetate solution

Mixing with buffer N3 causes a fluffy white precipitate to form.

1. Potassium acetate / acetic acid solution

- Neutralizes NaOH (renatures plasmid DNA)
- Converts soluble SDS to insoluble PDS

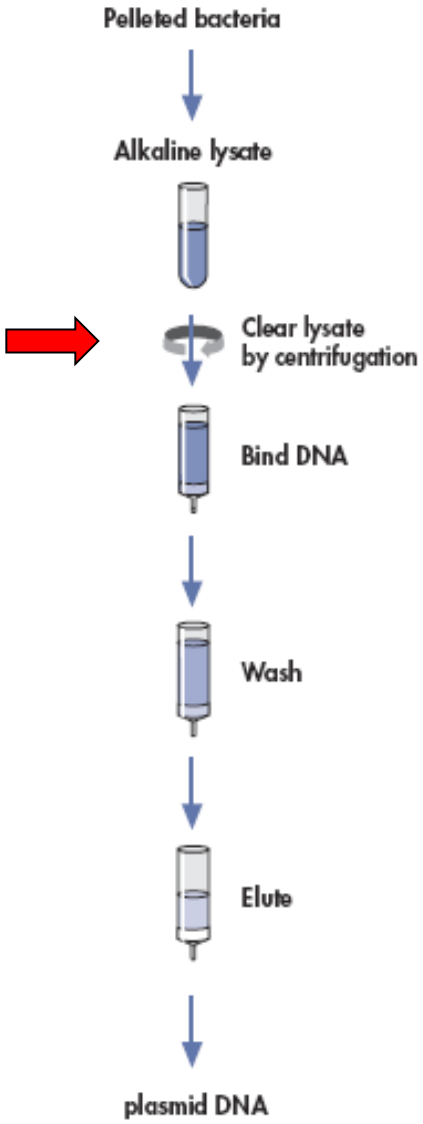


sodium dodecyl sulfate (SDS)
(H_2O sol. = 10%)

potassium dodecyl sulfate (PDS)
(H_2O sol. < 0.02%)

2. Guanidine hydrochloride (GuCl)

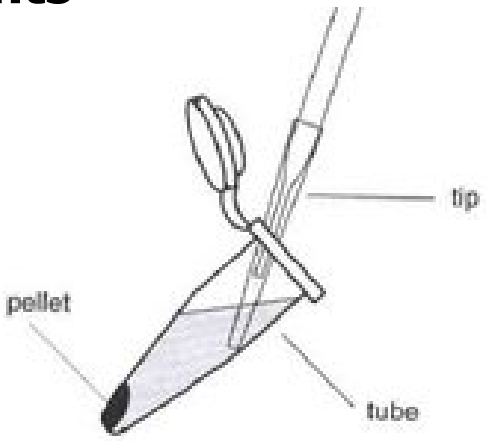
- Chaotropic salt; facilitates DNA binding to silica in later steps



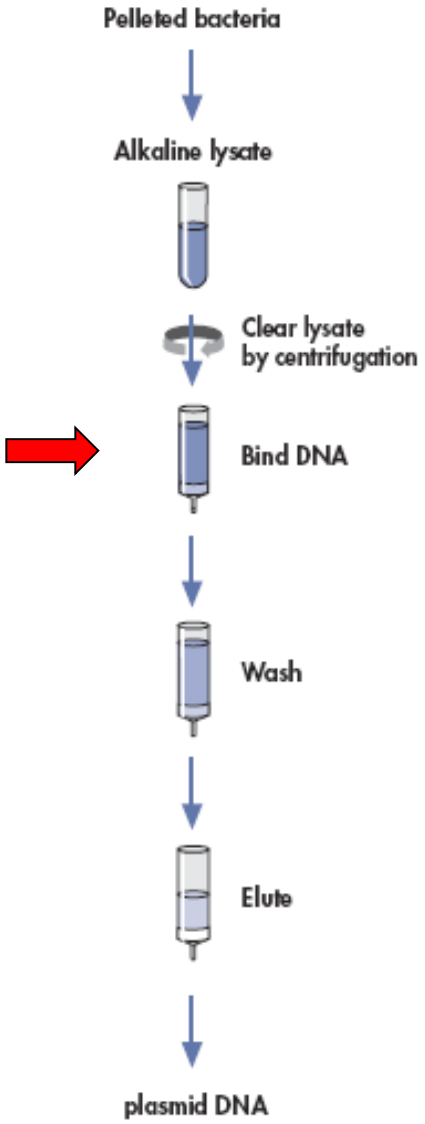
Separate plasmid DNA from contaminants by centrifugation

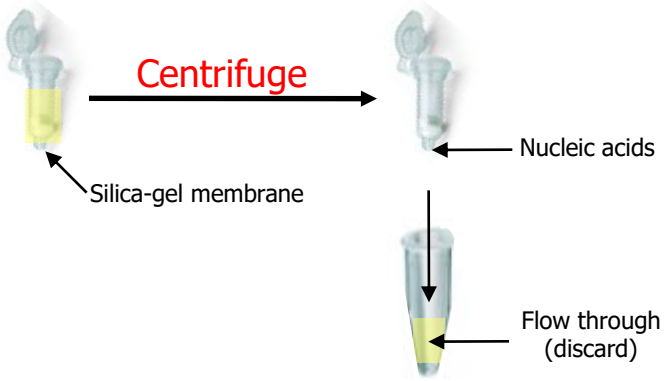
Supernatant contains:
- **Plasmid DNA**
- **Soluble cellular constituents**

Pellet contains:
- **PDS**
- **Lipids**
- **Proteins**
- **Chromosomal DNA**

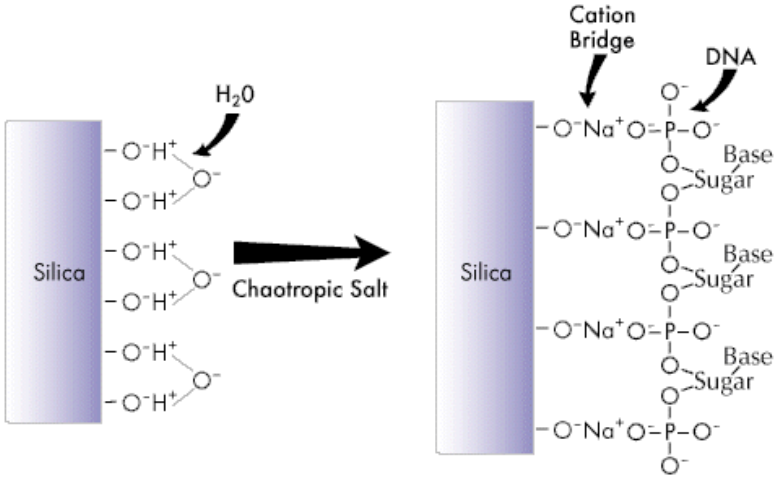


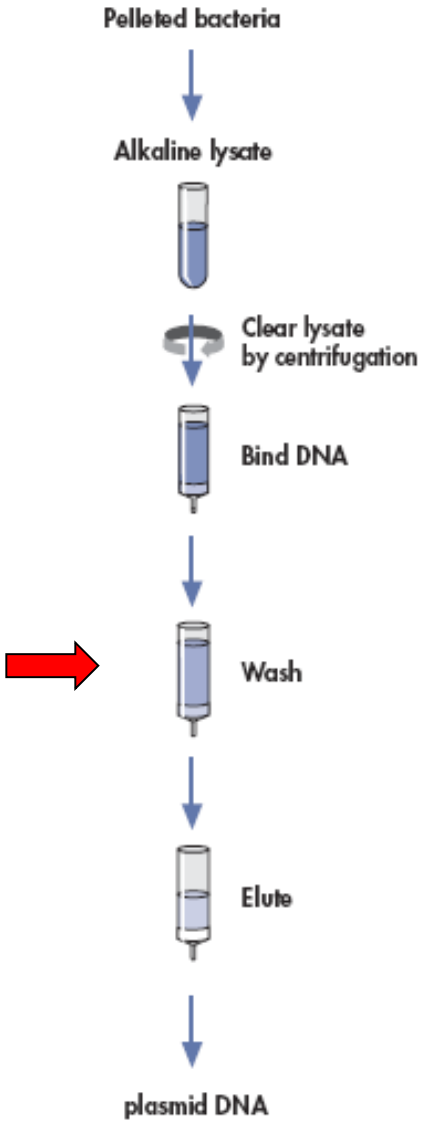
Add cleared lysate to column and centrifuge





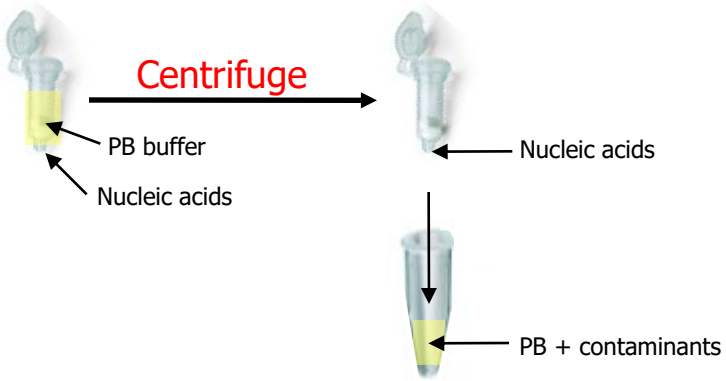
The high ionic strength and presence of chaotropic salt causes DNA to bind to the silica membrane, while other contaminants pass through the column



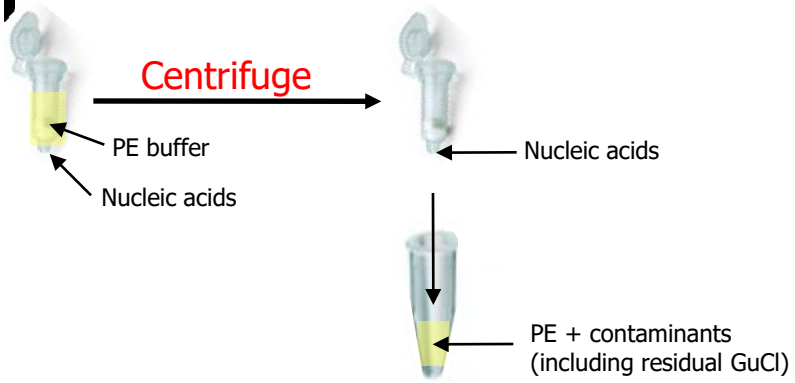


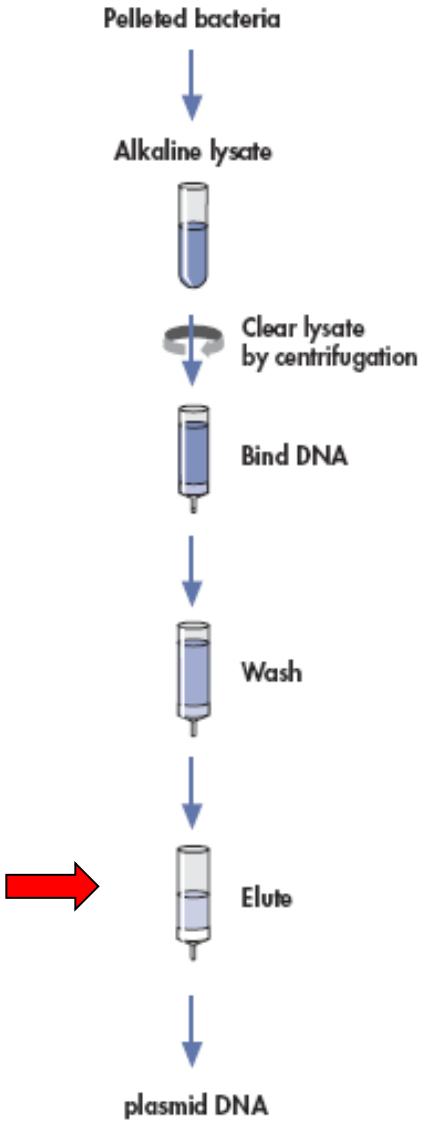
Wash the silica membrane to remove residual contaminants

Buffer PB contains isopropanol and GuCl

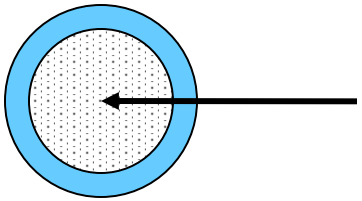


Buffer PE contains ethanol and Tris-Cl



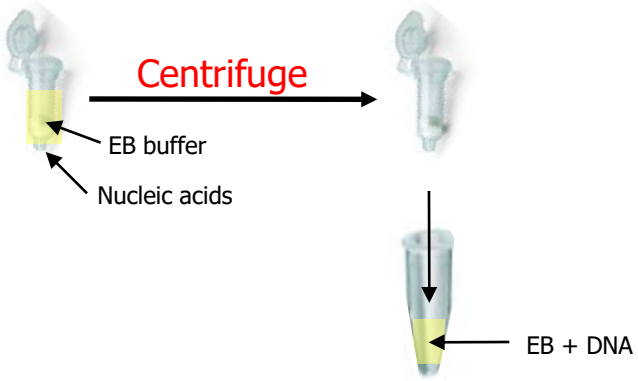


Elute purified DNA from the column

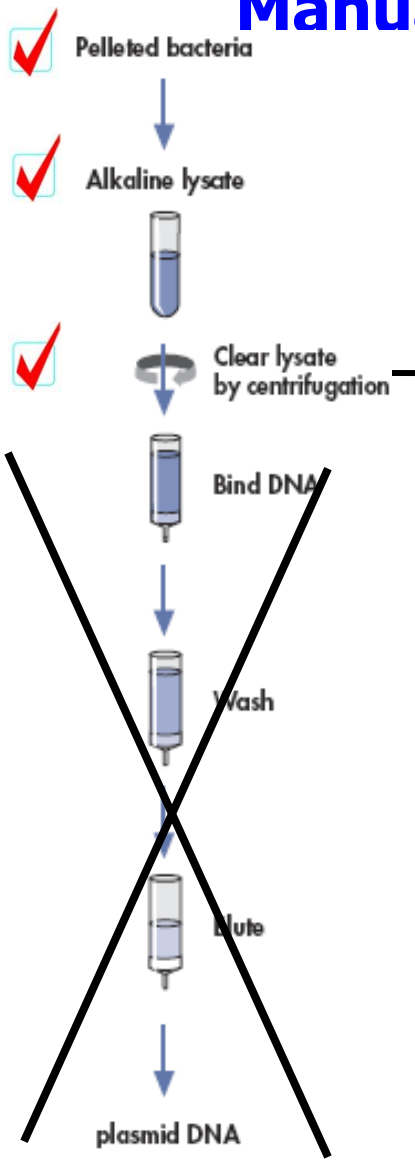


Buffer EB should be added directly to the membrane for optimal DNA recovery and to avoid possible EtOH contamination (from residual PE buffer)

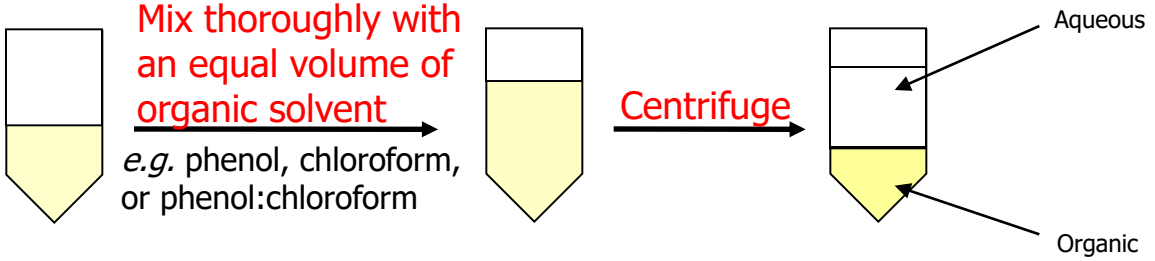
EB is 10 mM Tris-Cl (pH 8.5). TE or dH_2O may also be used.



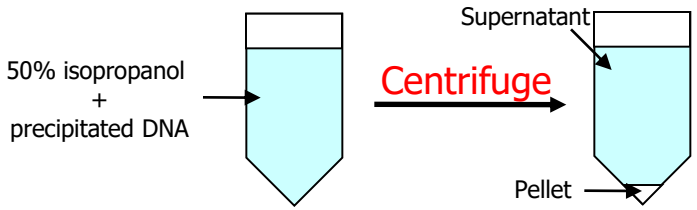
Manual alkaline lysis preparation of plasmid DNA



- **Organic extraction (optional)**



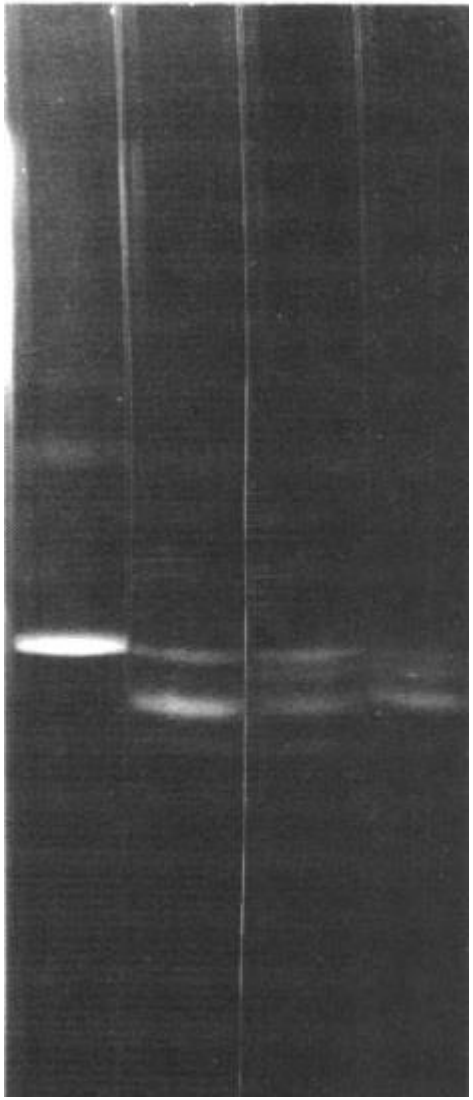
- **Precipitate DNA with isopropanol (1:1 volume)**



- **Wash pellet with 70% EtOH (to remove salts)**

- **Dissolve pellet with TE (or other aqueous solution**

ASSESSING PLASMID PREPARATION

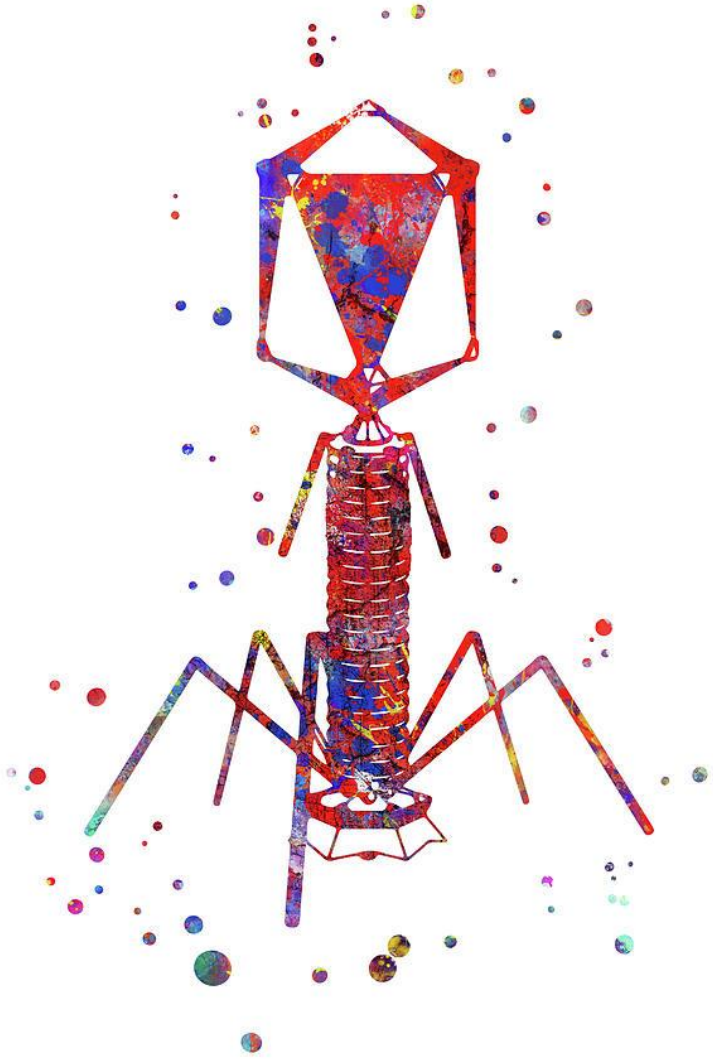


- Quantify abundance (A_{260}) and purity (A_{260}/A_{280})
- Verify by restriction digestion
- Run undigested plasmid to see if it is mostly supercoiled

GENE INSERTION

- Gene insertion, the incorporation of exogenous genetic material into a genome, can occur naturally or can be artificially induced.
- In nature, mobile elements called insertion sequences.
- Encode only the information necessary for their insertion into DNA.
- Depending upon the insertion sequence, they can insert at specific regions or at random.
- Genes can also be inserted via the use of insertion vectors.

VIRUSES ARE GENE VECTORS



- Viruses can thus be engineered to deliver DNA, which has been introduced into the viral genome prior to infection. Gene insertion can also be achieved mechanically by microinjection of the gene into a cell, or ballistically by shooting gold beads coated with the gene of interest into the cell.
- The insertion of the genetic material is a random event.with no control yet possible for the routine insertion of DNA at precise locations.
- large numbers of cells must be screened in order to identify an insertion event at a desired location.
- For example, the technique of insertional mutagenesis relies upon such screening to detect the functional disruptions in the host cell caused by insertional disruption of the gene encoding the function.

INTRONS

- Deliver genes to the host DNA more precisely.
- Introns, are capable of inserting themselves into DNA
- Do so by recognizing specific sequences.
- Modifying both the intron and the target sequence can also change the site of insertion.
- This raises the possibility that introns may be useful in gene therapy, with the delivery of DNA to correct the defect in a gene.

RECOMBINANT DNA PRODUCTION

- In order to characterize a gene or study the biological function of its protein, it is important to isolate and amplify the gene. Recombinant DNA Technology is a method for Gene Isolation & Amplification

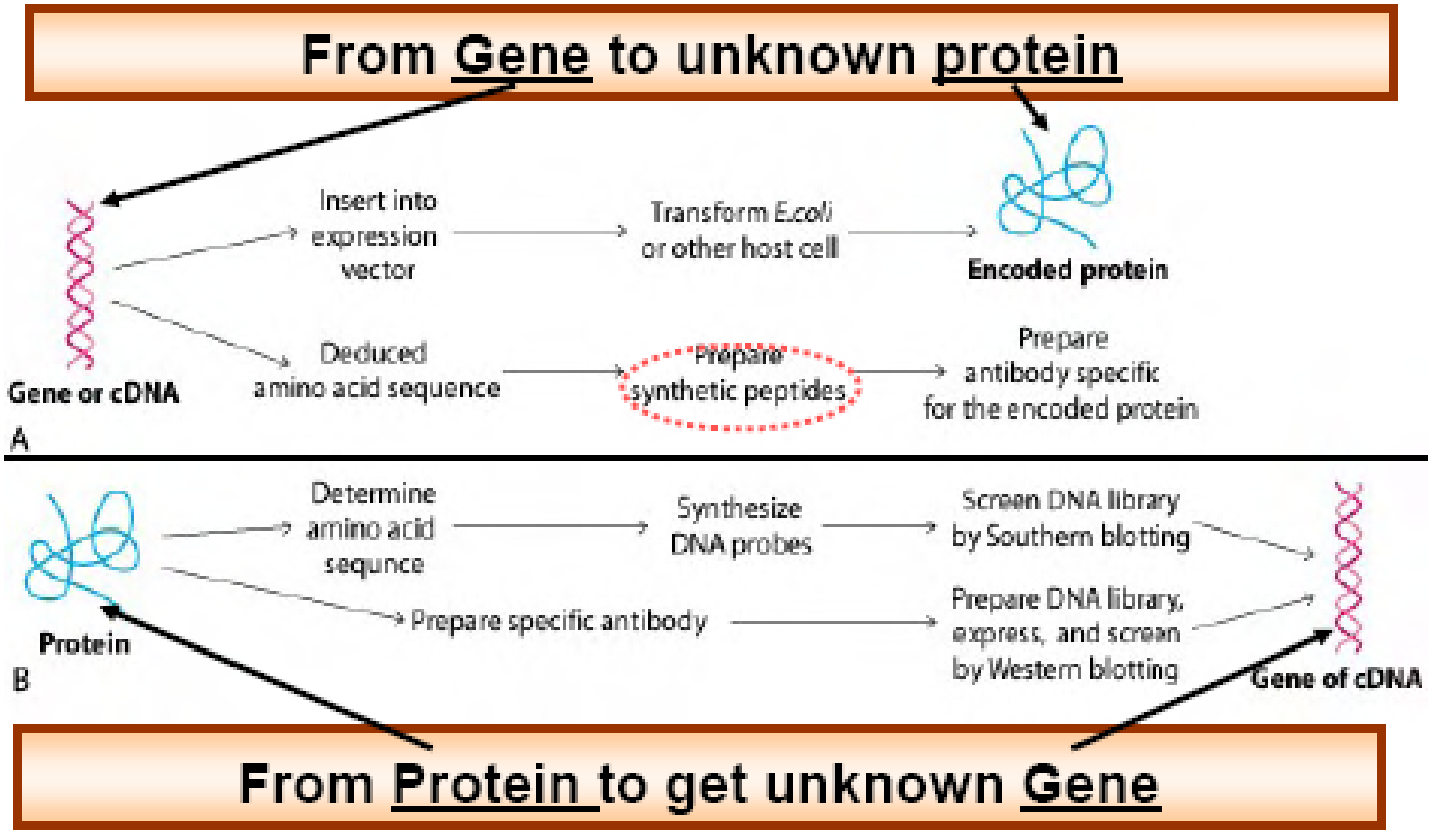
- The main purpose of rDNA technology is to make large amounts of DNA (gene) &/or its Proteins (Ex: Insulin)

Recombinant DNA technology

(started in mid-late 1970's)

- An incredibly powerful set of tools for gene manipulation.
- Methods associated with this "technology" make genetic engineering a reality.
- DNA (genes), RNA, and protein structure and function can be altered by design for beneficial (or detrimental – biological warfare/terrorism?) results.

Ways to use recombinant DNA Technology



Current and Future Applications of RECOMBINANT DNA TECHNOLOGY

- Complete chromosome gene maps
- Whole genome sequencing by shotgun approach
- Discovery of molecular bases of development, evolutionary relationships
- New proteins with new functions (or old proteins with new functions!)
- Human hormone synthesis in bacteria
- Antiviral agents
- AIDS vaccine development
- New pharmacological agents (proteins, RNA, DNA)
- Antisense RNA therapy (RNAi, gene silencing)

Current and Future Applications of RECOMBINANT DNA TECHNOLOGY

- Medical diagnostic reagents (gene probes) for detection of genetic diseases, infections and cancers
- Gene therapy: delivery with disarmed viruses to alleviate diseases caused by known gene defects.
- Agricultural revolution with animals having altered traits, more nutritious plants, heat/drought resistant crops, etc.
- Forensics - molecular detectives

TABLE 12.6 SOME PROTEIN PRODUCTS OF RECOMBINANT DNA TECHNOLOGY

Product	Made in	Use
Human insulin	*E. coli*	Treatment for diabetes
Human growth hormone (HGH)	*E. coli*	Treatment for growth defects
Epidermal growth factor (EGF)	*E. coli*	Treatment for burns, ulcers
Interleukin-2 (IL-2)	*E. coli*	Possible treatment for cancer
Bovine growth hormone (BGH)	*E. coli*	Improving weight gain in cattle
Cellulase	*E. coli*	Breaking down cellulose for animal feeds
Taxol	*E. coli*	Treatment for ovarian cancer
Interferons (alpha and gamma)	*S. cerevisiae*; *E. coli*	Possible treatment for cancer and viral infections
Hepatitis B vaccine	*S. cerevisiae*	Prevention of viral hepatitis
Erythropoietin (EPO)	Mammalian cells	Treatment for anemia
Factor VIII	Mammalian cells	Treatment for hemophilia
Tissue plasminogen activator (TPA)	Mammalian cells	Treatment for heart attacks

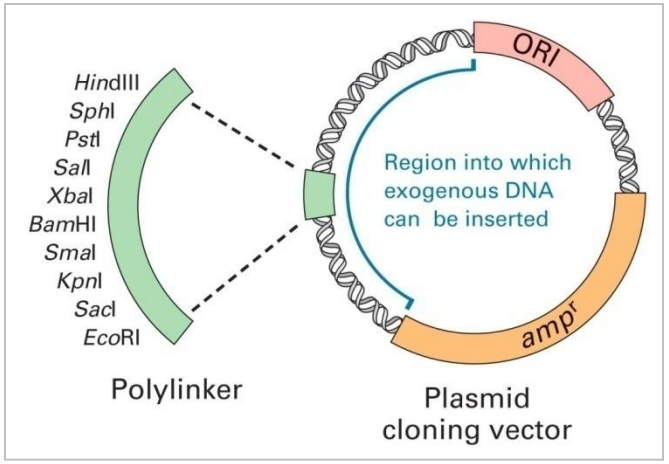
Table 12.6

OUTLINE

- Components
- Steps in Recombinant DNA Technology
- Selectable markers
- Analysis of Results
- Trouble-shooting of Rec DNA Technology
- Comparing PCR &) – Rec DNA Technology

COMPONENTS

- Vector – Plasmid or virus. Should have:
  - ✓ Antibiotic Resistance gene &
  - ✓ Lac Z gene for color selection
- DNA or Gene to Clone
- Restriction Enzyme
- DNA Ligase
- Antibiotics
- Colorless X-galactose dye
- A host, usually bacteria



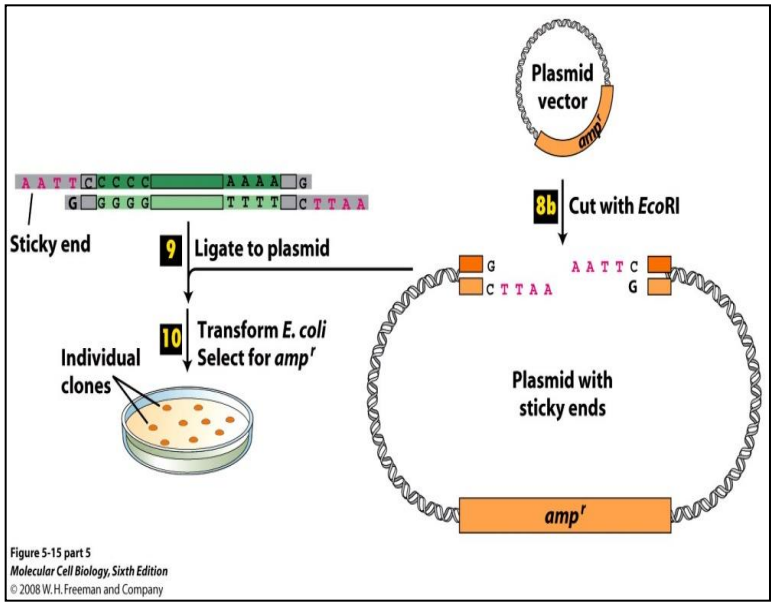
Plasmid Vector with **AmpR** - (Ampicillin Resistance gene)

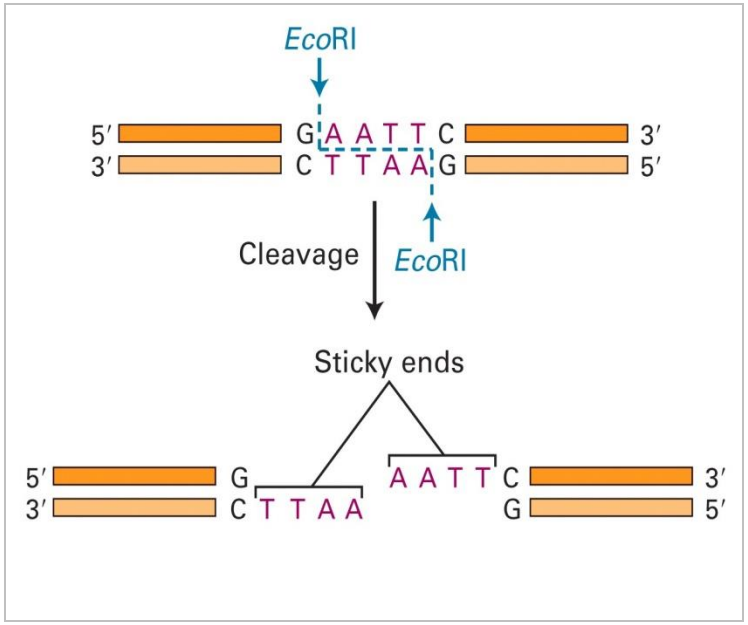
INVOLVES 5 STEPS

- Restriction Enzyme Digestion
- Ligation
- Transformation of Host
- Growth of Transformant
- Selection for Recombinant

RESTRICTION ENZYME DIGESTION

Digest the DNA to Clone & the Vector with SAME Restriction Enzyme. Ex: EcoRI, to generate complementary sticky ends that can base pair





LIST OF POSSIBLE RESTRICTION ENZYMES

TABLE 5-1 Selected Restriction Enzymes and Their Recognition Sequences

ENZYME	SOURCE MICROORGANISM	RECOGNITION SITE*	ENDS PRODUCED
*Bam*HI	*Bacillus amyloliquefaciens*	↓ -G-G-A-T-C-C- -C-C-T-A-G-G- ↑	Sticky
*Sau*3A	*Staphylococcus aureus*	↓ -G-A-T-C- -C-T-A-G- ↑	Sticky
*Eco*RI	*Escherichia coli*	↓ -G-A-A-T-T-C- -C-T-T-A-A-G ↑	Sticky
*Hin*dIII	*Haemophilus influenzae*	↓ -A-A-G-C-T-T- -T-T-C-G-A-A- ↑	Sticky
*Sma*I	*Serratia marcescens*	↓ -C-C-C-G-G-G- -G-G-G-C-C-C- ↑	Blunt
*Not*I	*Nocardia otitidis-caviarum*	↓ -G-C-G-G-C-C-G-C- -C-G-C-C-G-G-C-G- ↑	Sticky

*Many of these recognition sequences are included in a common polylinker sequence (see Figure 5-13).

Table 5-1
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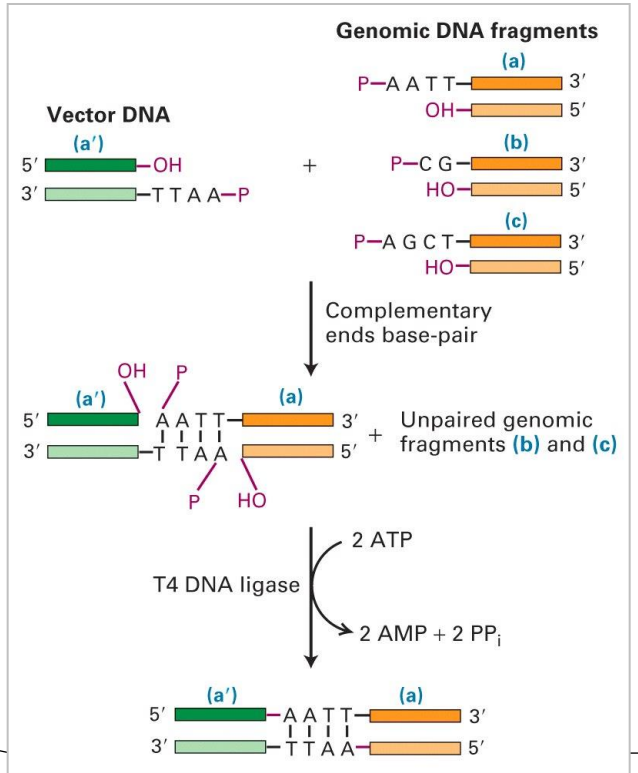
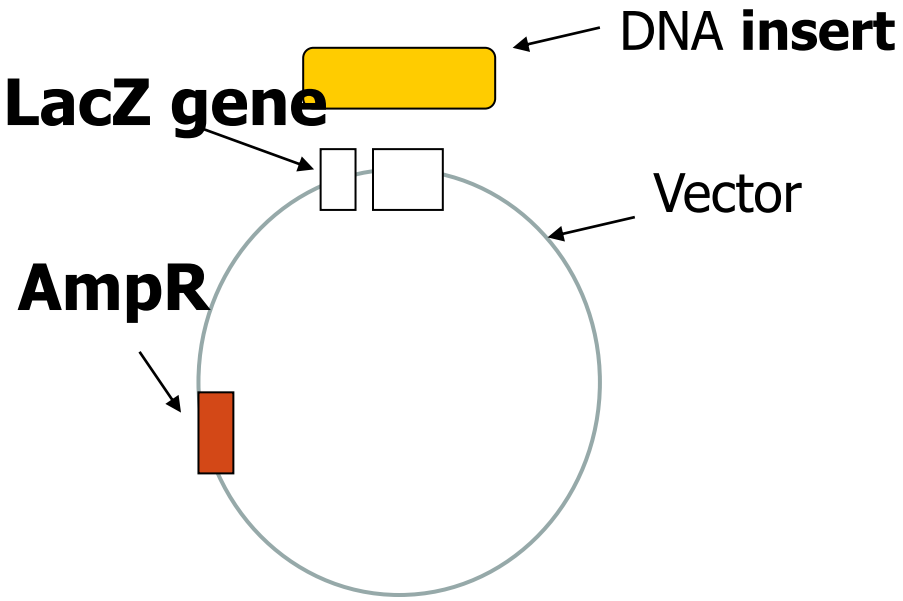
STEPS IN RECOMBINANT DNA TECHNOLOGY

LIGATION

- DNA Ligase seals the ends of DNA to clone & the vector DNA
- The DNA now inserted into Vector DNA => **Recombinant DNA**

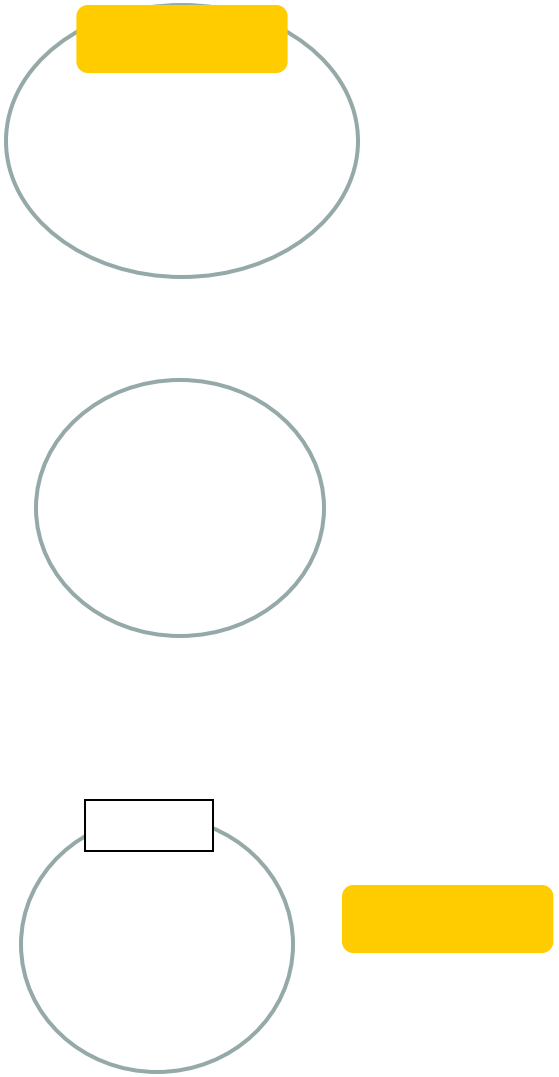




Ligation of sticky ends of vector and DNA insert

LIGATION RESULTS

- Some vectors will ligate to the insert.
- Some vectors will re-ligated without insert
- Other vectors & inserts will not be ligated



TRANSFORMATION OF HOST

- Recombinant DNA plus all other ligation products are mixed with host solution
- Chemicals are used to activate uptake by host
- Host with vector or recombinant DNA is called Transformant (transformed host)
- Host will now become antibiotic resistant
- Transformants can replicate DNA insert

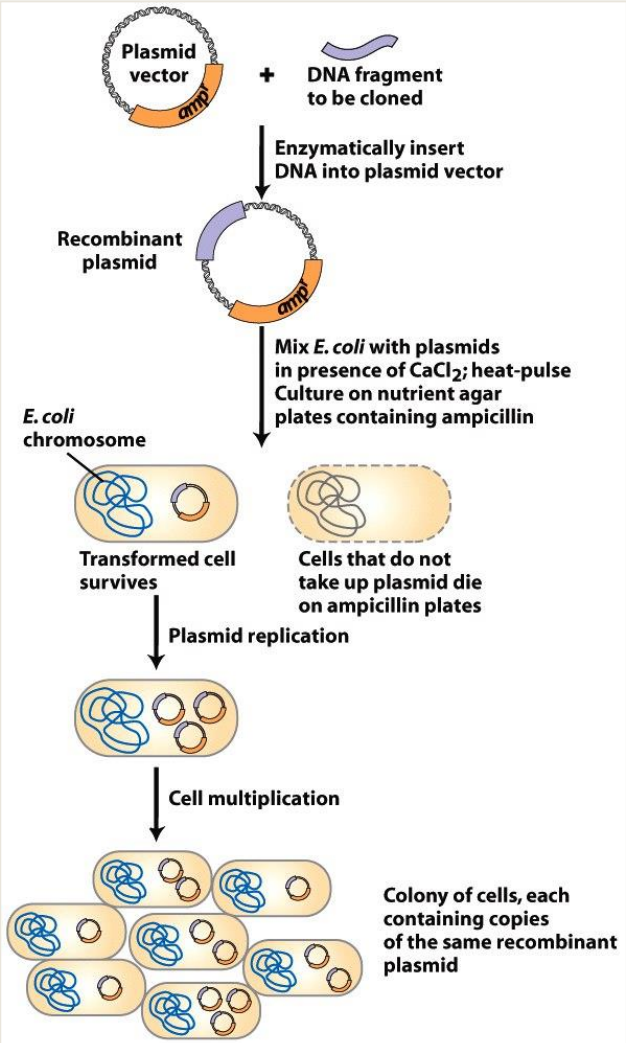


Figure 5-14
Molecular Cell Biology, Sixth Edition

TRANSFORMATION RESULT

- Some hosts will not uptake anything
- Some will uptake vector alone
- Some will take Recombinant DNA

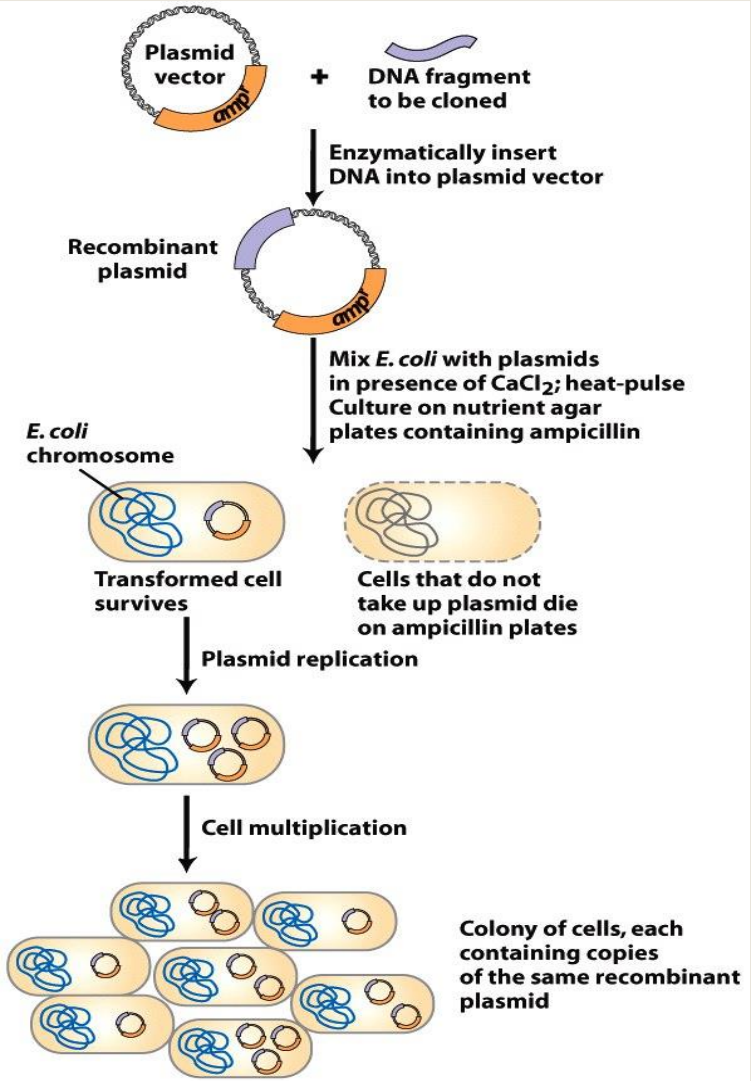
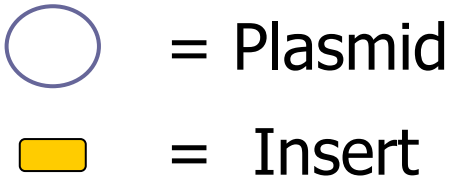
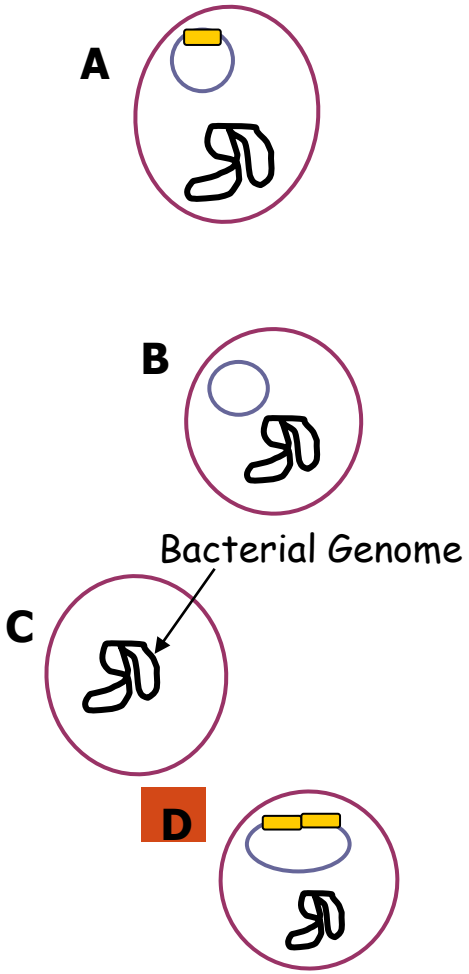


Figure 5-14
Molecular Cell Biology, Sixth Edition

TRANSFORMATION RESULT

- A.= Host transformed by “a
- B. = Host transformed by “b”
- C. = Host not transformed
- D. = Host transformed by “a” with 2 inserts

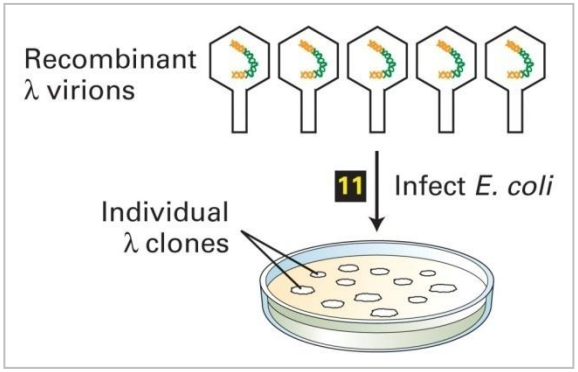




- Vectors ligated to the insert
- Vectors ligated without insert
- Vectors ligated to 2 copies of insert

GROWTH OF TRANSFORMANT

- Transformants and other products are cultured in a dish containing:
  - ✓ nutrients
  - ✓ Antibiotic
  - ✓ a colorless dye called X-galactose
- LacZ breaks down this dye forming blue color.



Recombinant l virions form plaques

- Transformants containing plasmid or recombinant plasmid DNA will from COLONIES (bumps) on the dish
- Transformants containing virus vector or recombinant viral DNA will from PLAQUES (dents)

SELECTION FOR RECOMBINANT

X-galactose in nutrient dish allows selection of successful ligation of DNA insert that:

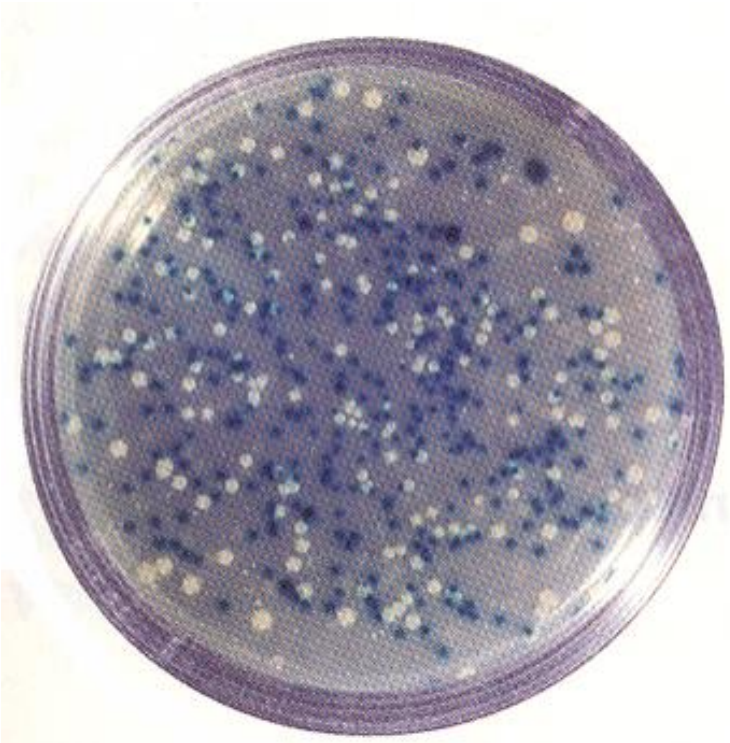
- Inactivation of LacZ gene
- White color (no blue color)

Ampicillin in nutrient allows:

- Selection of successful transformation

ANALYSIS OF RECOMBINANT DNA TECHNOLOGY RESULTS

Selection for Recombinant



Agar dish after rec DNA Technology

Shows **both blue and white** (clear) colonies.

WHY **both blue and white** (clear) colonies?

Which of the colonies is the color we want?

If you forgot to add antibiotics in the plate,
What results would you expect?