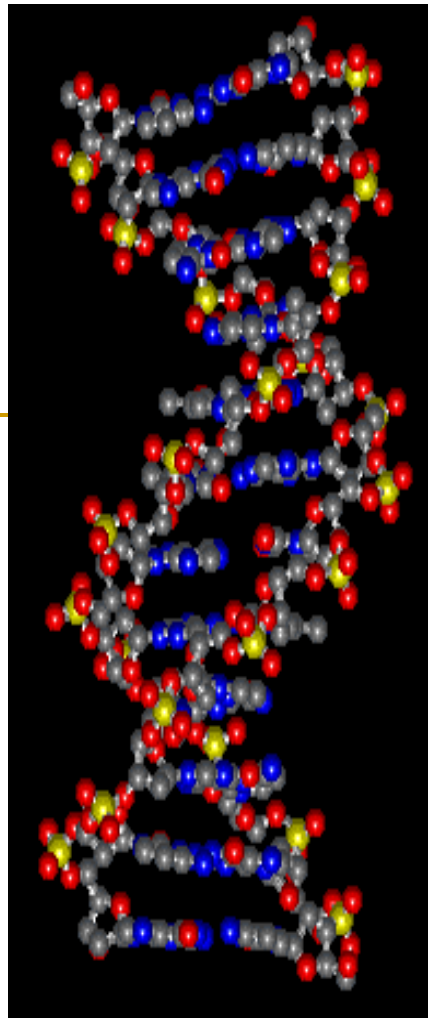
Cloning

Gene & Cell Technology



Cloning - a definition

- From the Greek - klon, a twig
- An aggregate of the asexually produced progeny of an individual; a group of replicas of all or part of a macromolecule (such as DNA or an antibody)
- An individual grown from a single somatic cell of its parent & genetically identical to it
- *Clone: a collection of molecules or cells, all identical to an original molecule or cell*

- A **clone** is an exact copy of an organism, organ, single cell, organelle or macromolecule.
  - Cell lines for medical research are derived from a single cell allowed to replicate millions of times, producing masses of identical clones.
- Gene cloning is the act of making copies of a single gene.
  - Amplified genes are useful in many areas of research and for medical applications such as gene therapy.
  - Selective amplification of genes depends on our ability to perform the essential procedures.

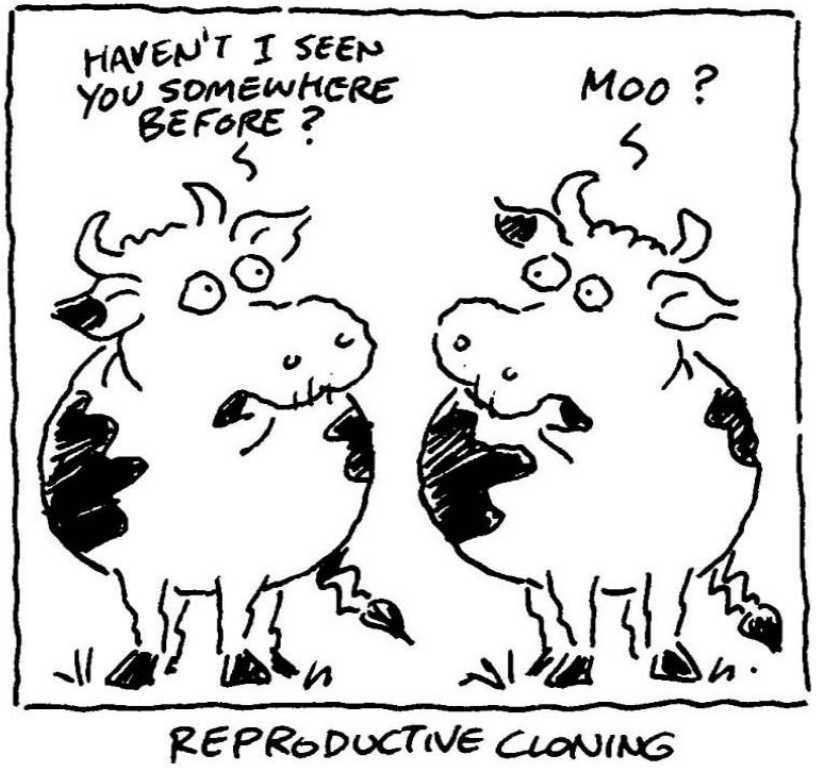
Types of Cloning

- Reproductive Cloning
  - Duplicating a person e.g. identical twins.
- Therapeutic Cloning
  - Duplicating part of a person e.g. a heart or liver, or even just a few cells.
- Gene Cloning
  - Duplicating a gene or a part of DNA.

Reproductive Cloning

- A technology used to generate an animal that has same nuclear DNA as another currently or previously existing animal
- E.G. Dolly

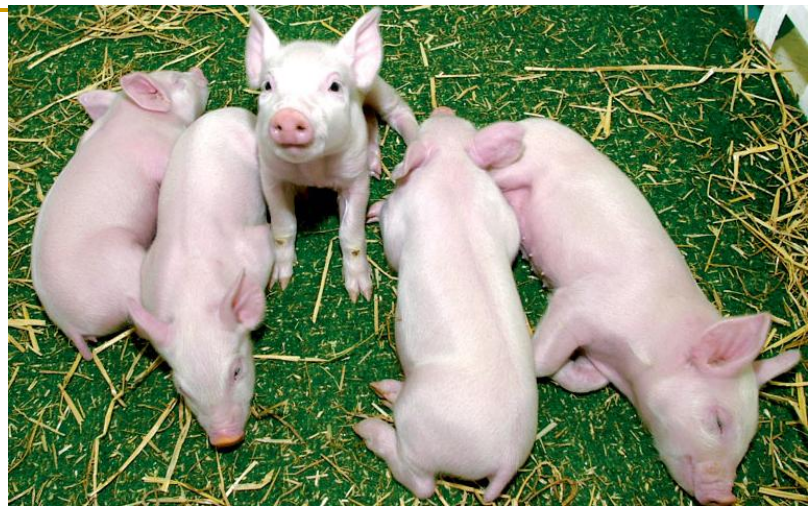
- How Is Reproductive Cloning Done?
  - Somatic cell nuclear transfer (SCNT)



Why Clone Animals?

- To answer questions of basic biology
- For pharmaceutical production.
- For herd improvement
- To satisfy our desires (e.g. pet cloning).

Carbon Copy– the First Cloned Pet

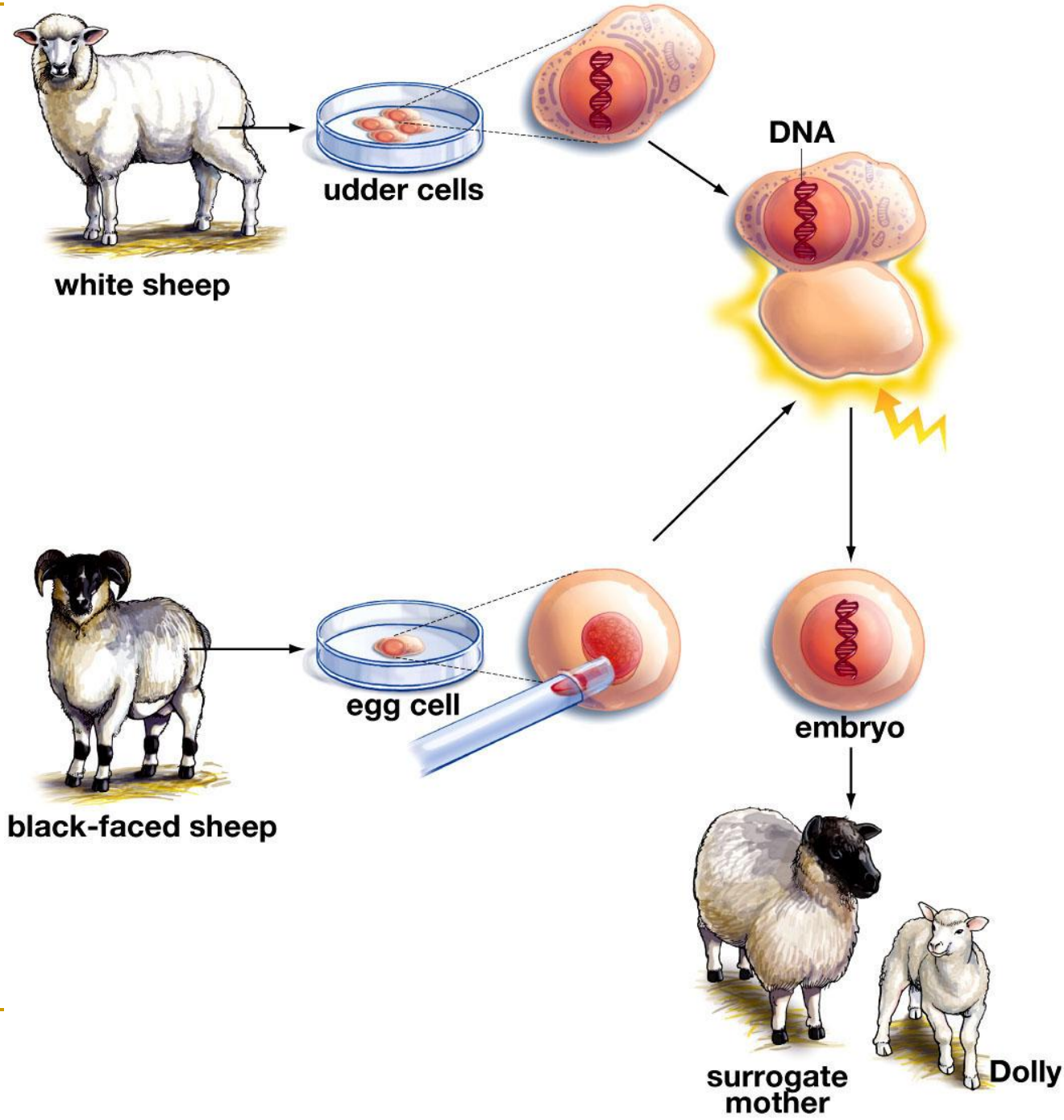


(Science 2002, 295:1443)

Dolly and her surrogate mother

Animal cloning

The Biotechnology of Reproductive Cloning

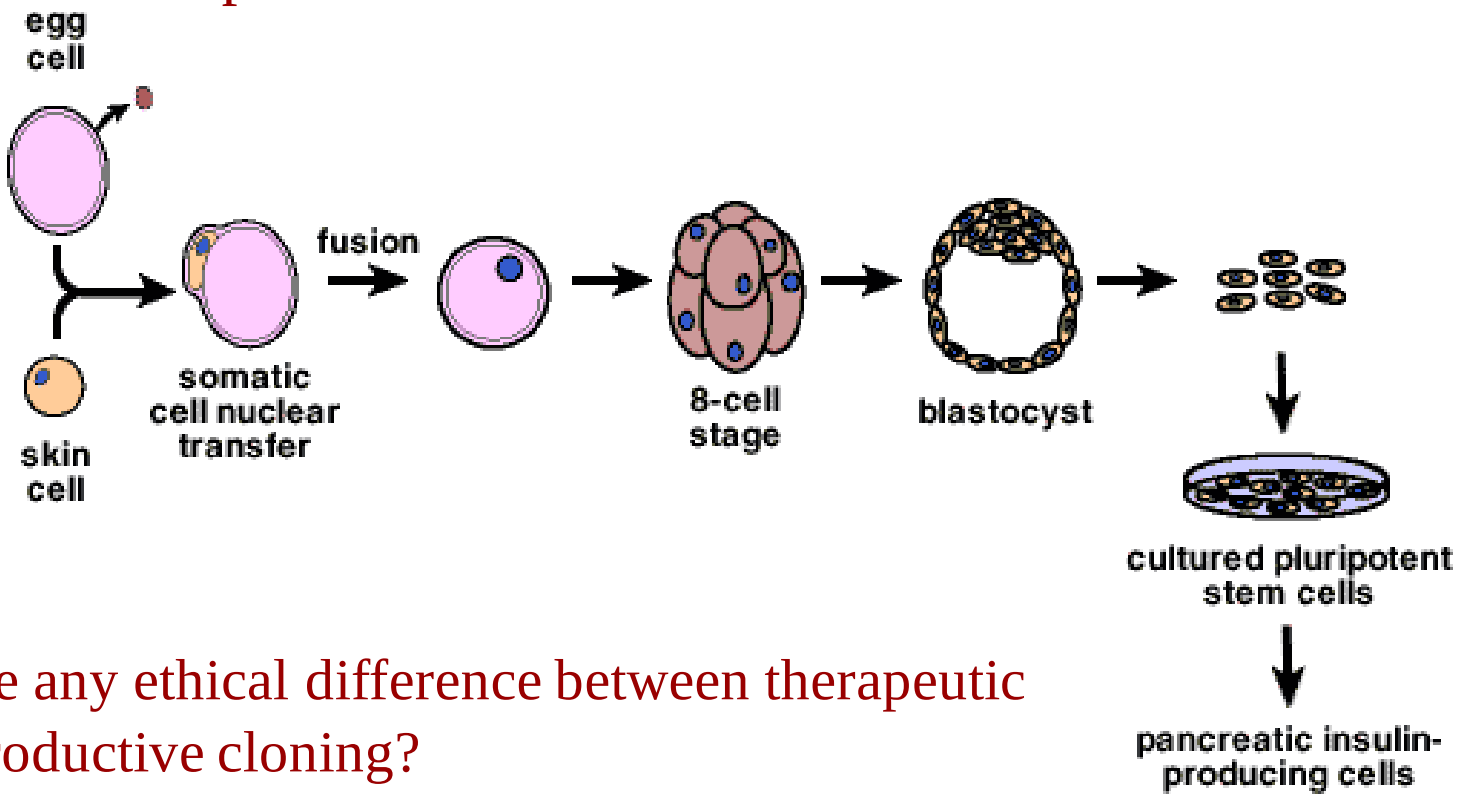


Even under the best of circumstances, the current technology of cloning is very inefficient.

Cloning provides the most direct demonstration that all cells of an individual share a common genetic blueprint.

Therapeutic Cloning

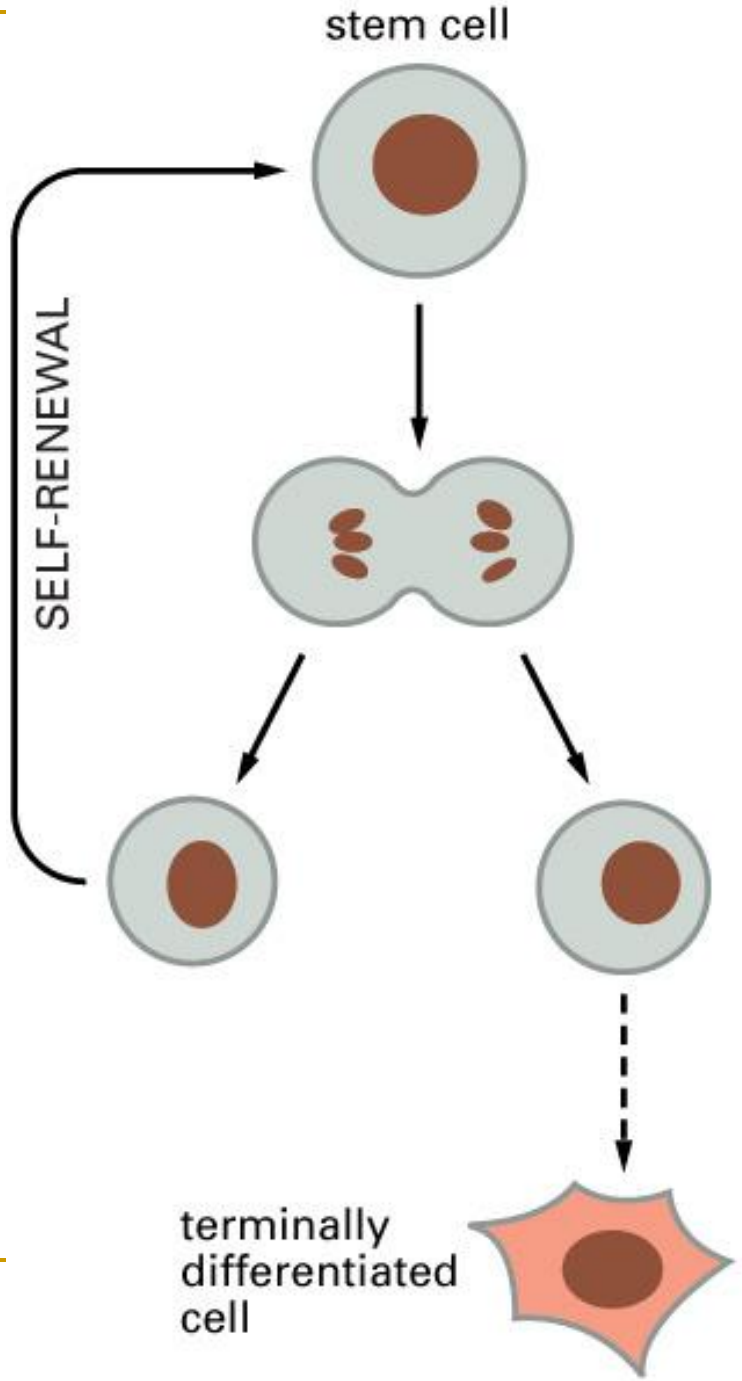
- Production of human embryos for use in research
- Goal
  - To harvest stem cells that can be used to study human development and to treat disease



Is there any ethical difference between therapeutic & reproductive cloning?

The Stem Cell Concept

A stem cell is an undifferentiated, dividing cell that gives rise to a daughter cell like itself and a daughter cell that becomes a specialized cell type.



Gene Cloning

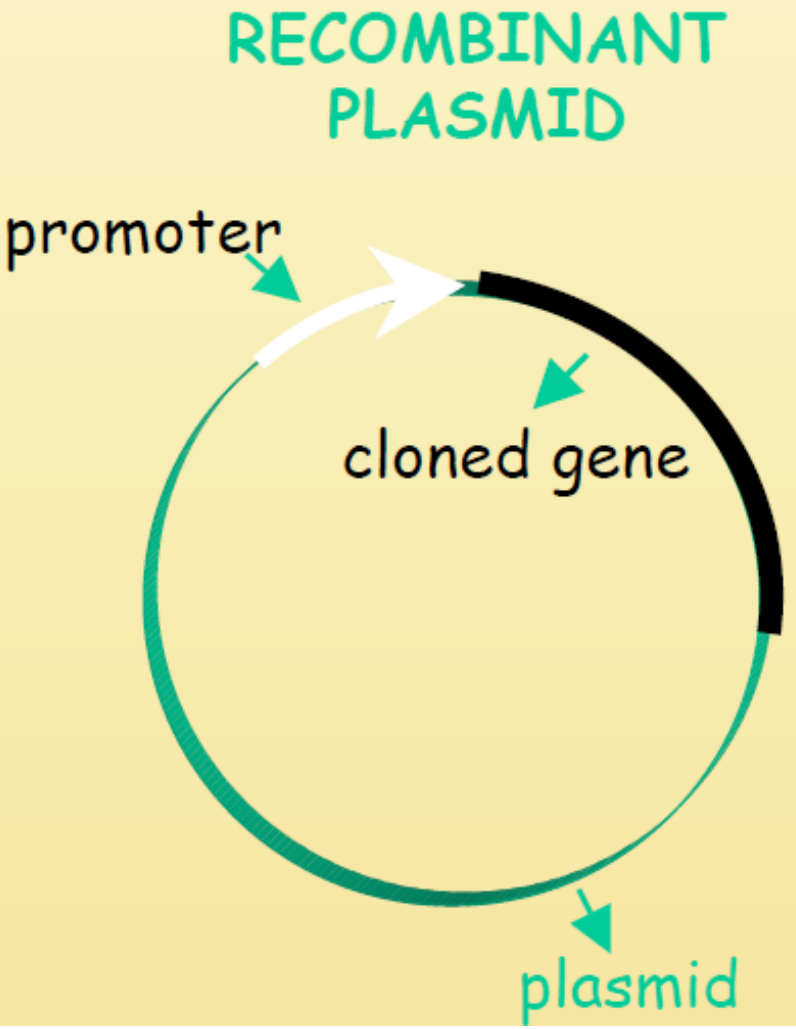
- Transfer of a DNA fragment of interest from one organism to a self-replicating genetic element such as a bacterial plasmid
- Plasmids
  - Self-replicating extra-chromosomal circular DNA molecules, distinct from normal bacterial genome

Why Clone DNA?

- A particular gene can be isolated and its nucleotide sequence determined
- Control sequences of DNA can be identified & analyzed
- Protein/enzyme/RNA function can be investigated
- Mutations can be identified, e.g. gene defects related to specific diseases
- Organisms can be 'engineered' for specific purposes, e.g. insulin production, insect resistance, etc.

What is a "cloned" gene?

- A particular stretch of DNA isolated from an entire genome
- In a bacterial plasmid
  - can produce many copies of the gene
  - can produce the protein



Cloning a gene

Genomic

- piece of DNA obtained directly from the genome
- Useful if you want regulatory information

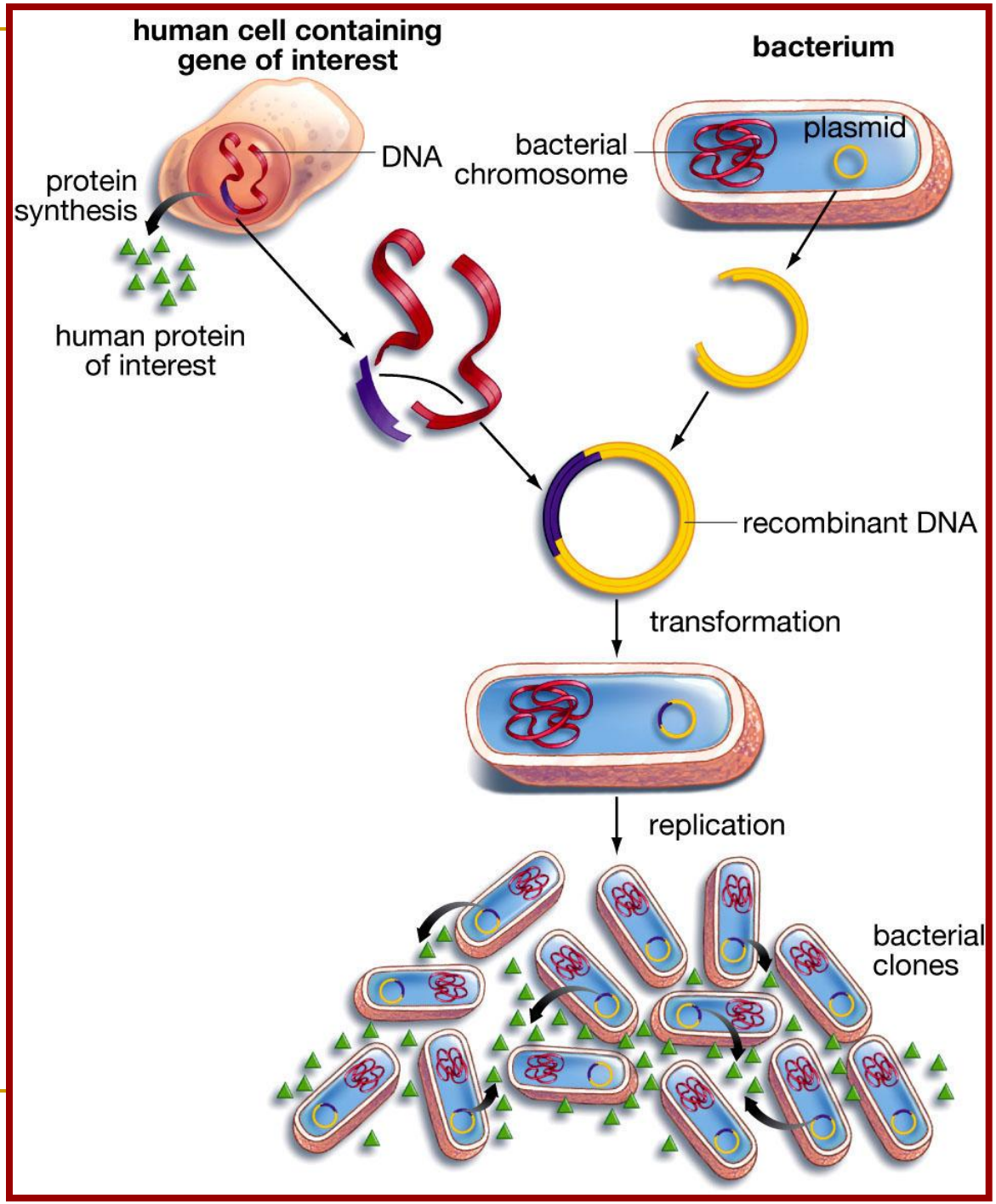
cDNA

- DNA copy of mRNA
- no introns
- no promoter or non-coding sequences
- Useful of you want protein information

Steps in Gene Cloning

1. Amplification of a specific gene
2. Cutting DNA at precise locations
3. Join two pieces of DNA
4. Selection of small self-replicating DNA
5. Method to move a vector into a host cell
6. Method to select hosts expressing recombinant DNA

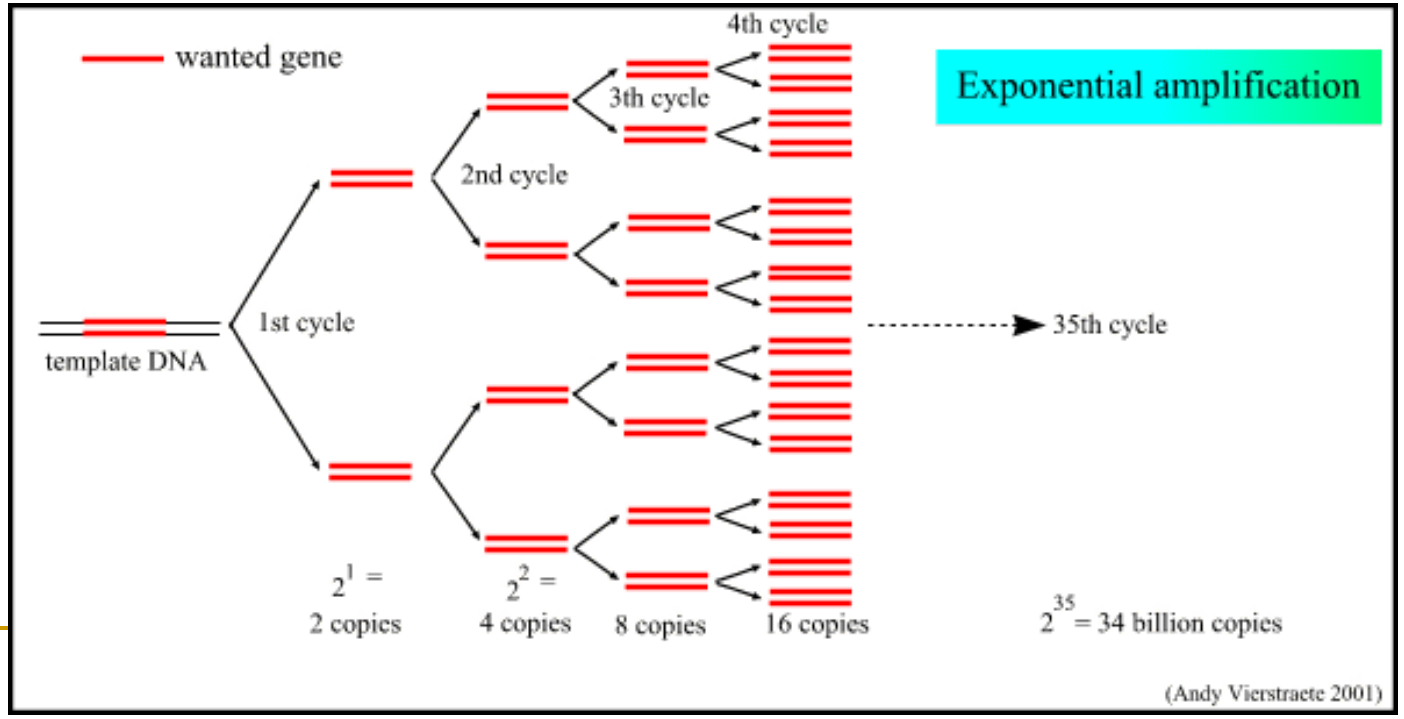
Overview of gene cloning



1. Amplification of a Specific Gene

Polymerase chain reaction

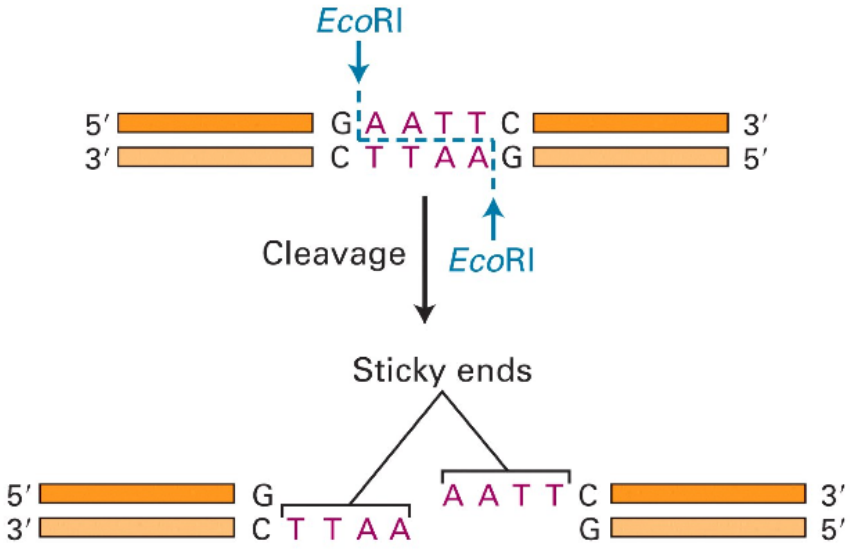
Generating millions of copies of a particular gene.



2. Cutting DNA at Precise Locations

Using Restriction endonucleases

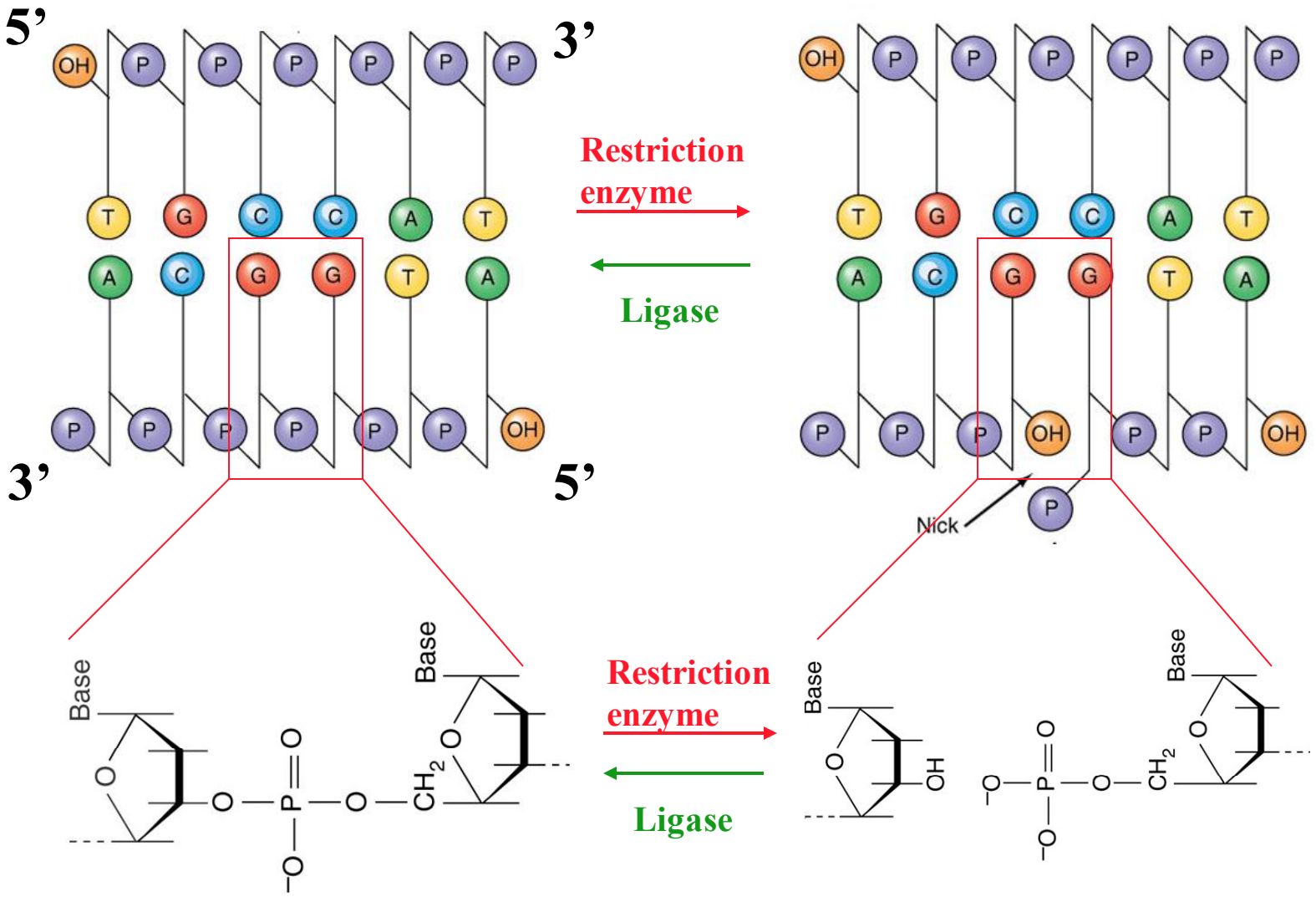
Cut DNA at specific locations based on the nucleotide sequence



3. Join Two Pieces of DNA

- In genetic research it is necessary to link two or more individual strands of DNA,
  - to create a longer strand, or
  - close a circular strand that has been cut with restriction enzymes.
- Enzymes called DNA ligases can create covalent bonds between nucleotide chains.
  - DNA polymerase I (for filling in gaps) and
  - Polynucleotide kinase (phosphorylating the 5' ends)

DNA ligase covalently links two DNA strands



DNA Ligase Function & Activity

- FUNCTION
  - DNA replication, recombination & repair
- ACTIVITY
  - Catalyze formation of phosphodiester bonds between 3'OH and 5'PO_4 of double stranded DNA
- Two classes based on co-factors
  - ATP in T4 and eukaryotes ligases
  - NADH in *E. coli* and other bacteria
- Potential problems with ligations Self-ligating vector

How will you proceed for insertion of gene into vector ?

- No restriction site on the ends of PCR amplified gene
- If there is restriction site?
  - If the sticky ends are compatible?
  - If the ends are incompatible.
    - One end is sticky and the other is blunt
    - Both ends are sticky but incompatible

Cloning DNA into a Vector

- Homopolymeric tailing with terminal transferase

GCATp GGGGGGGGGGATAC

CGTACCCCCCCCCC pTATG

- Linker ligation and restriction digestion

pCCGGAATTCCGG

GGCCTTAAGGCCp

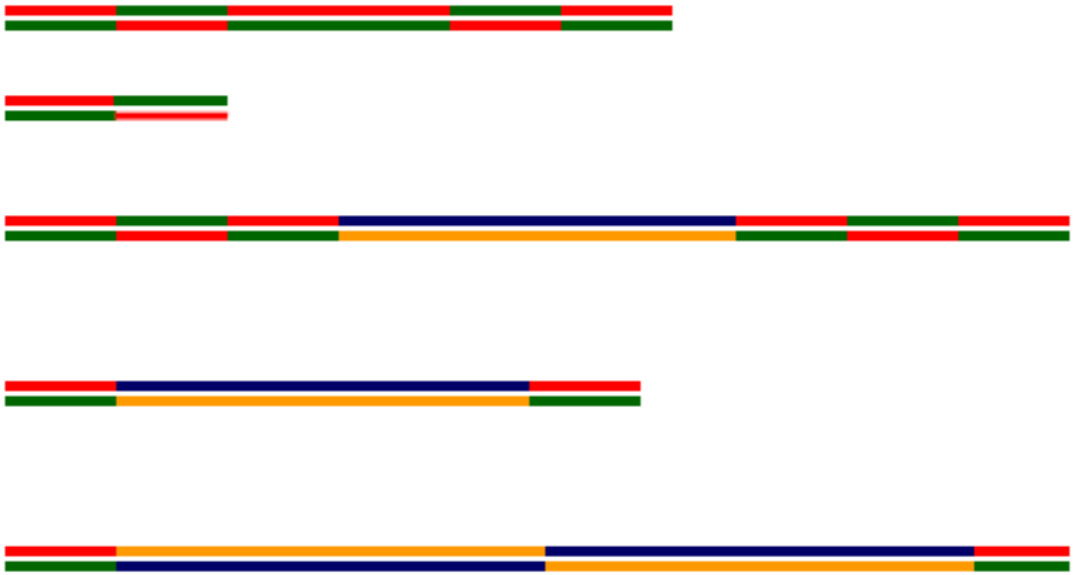
- Adapter ligation

AATTCGAACCCCTTCG

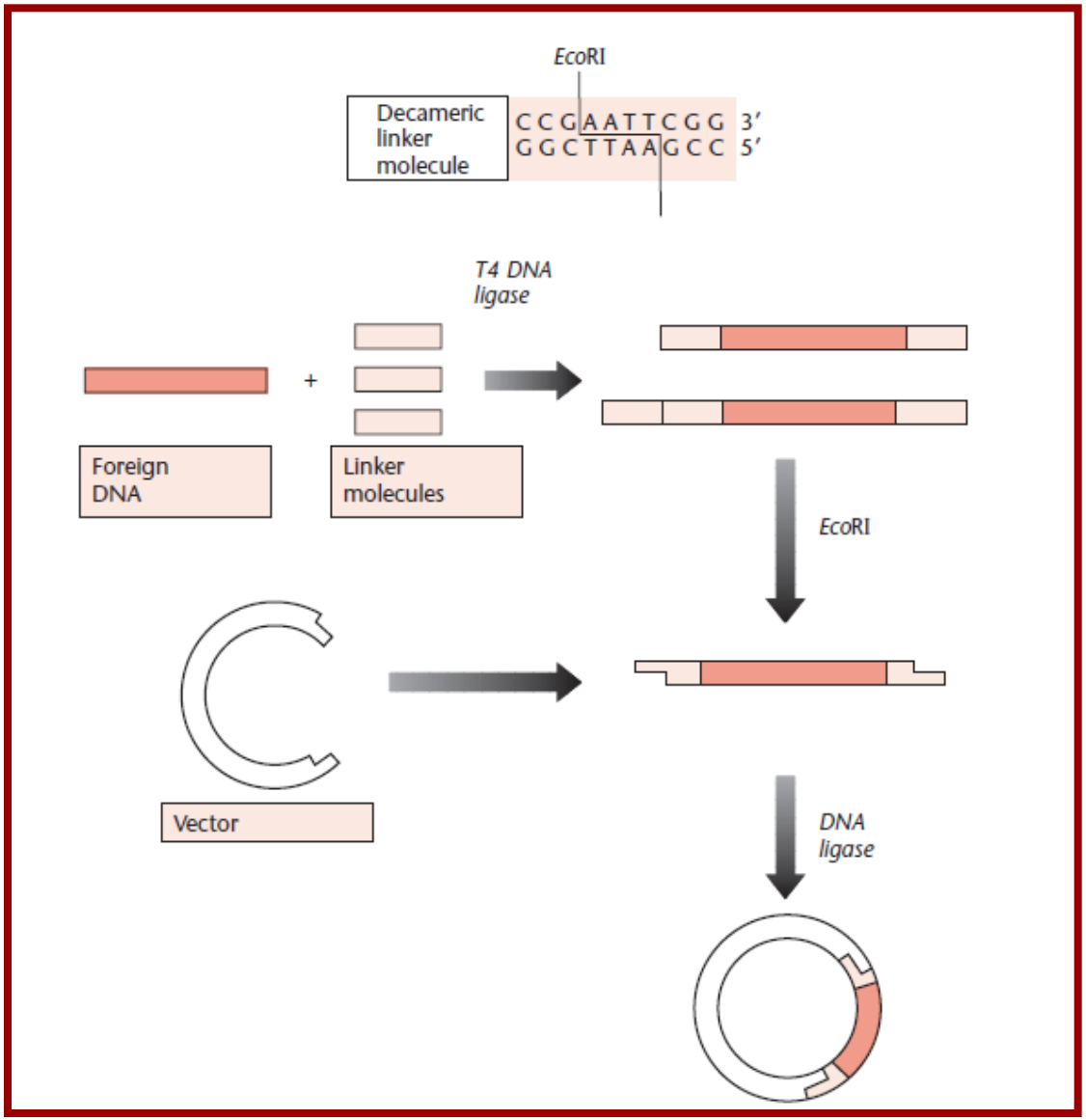
GCTTGGGGAAGCp

Products After Linker Ligation

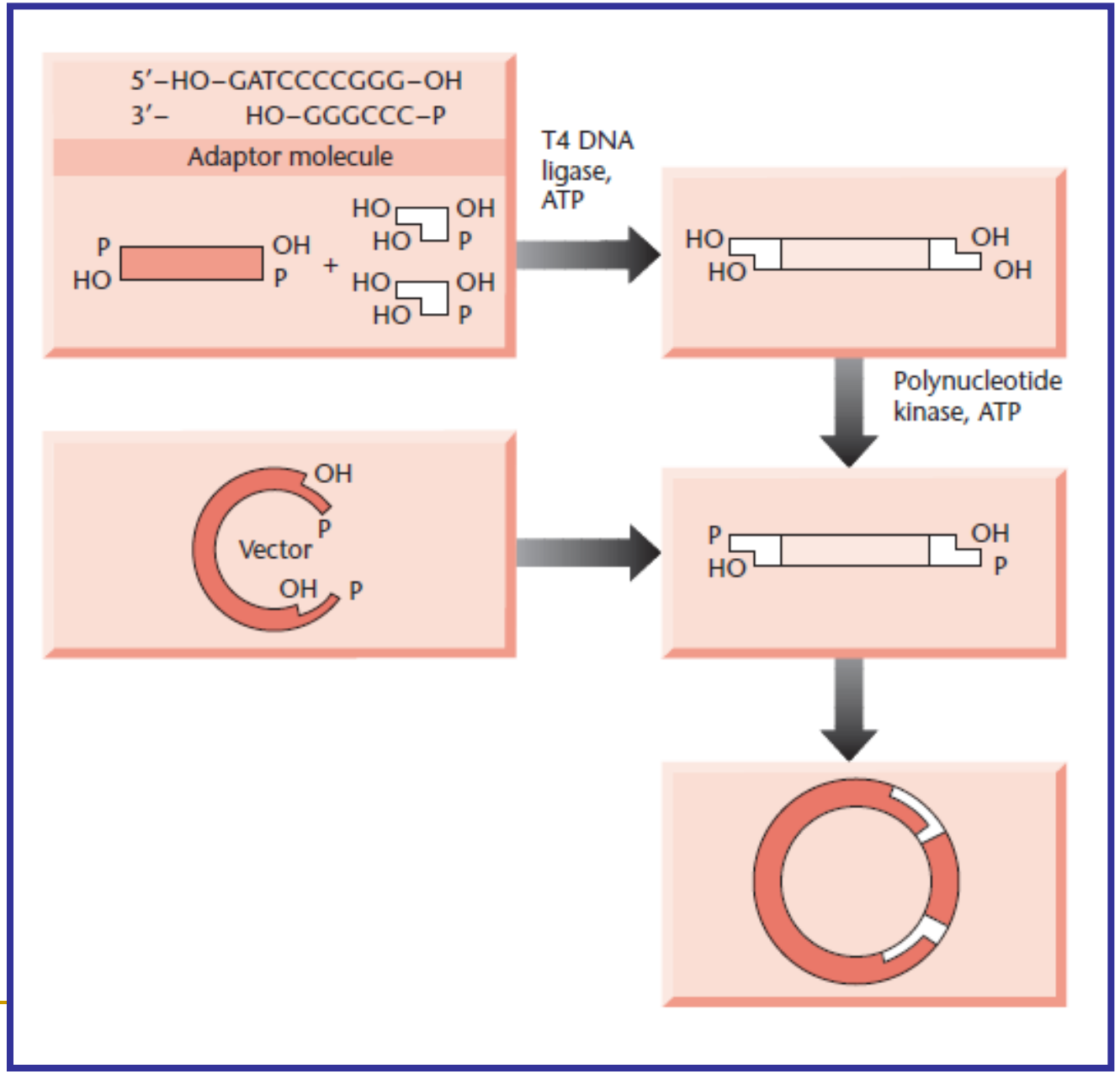
- Linker multimers
- Linker dimers
- cDNA with linker multimers
- cDNA with linkers
- Fusion cDNAs with linkers



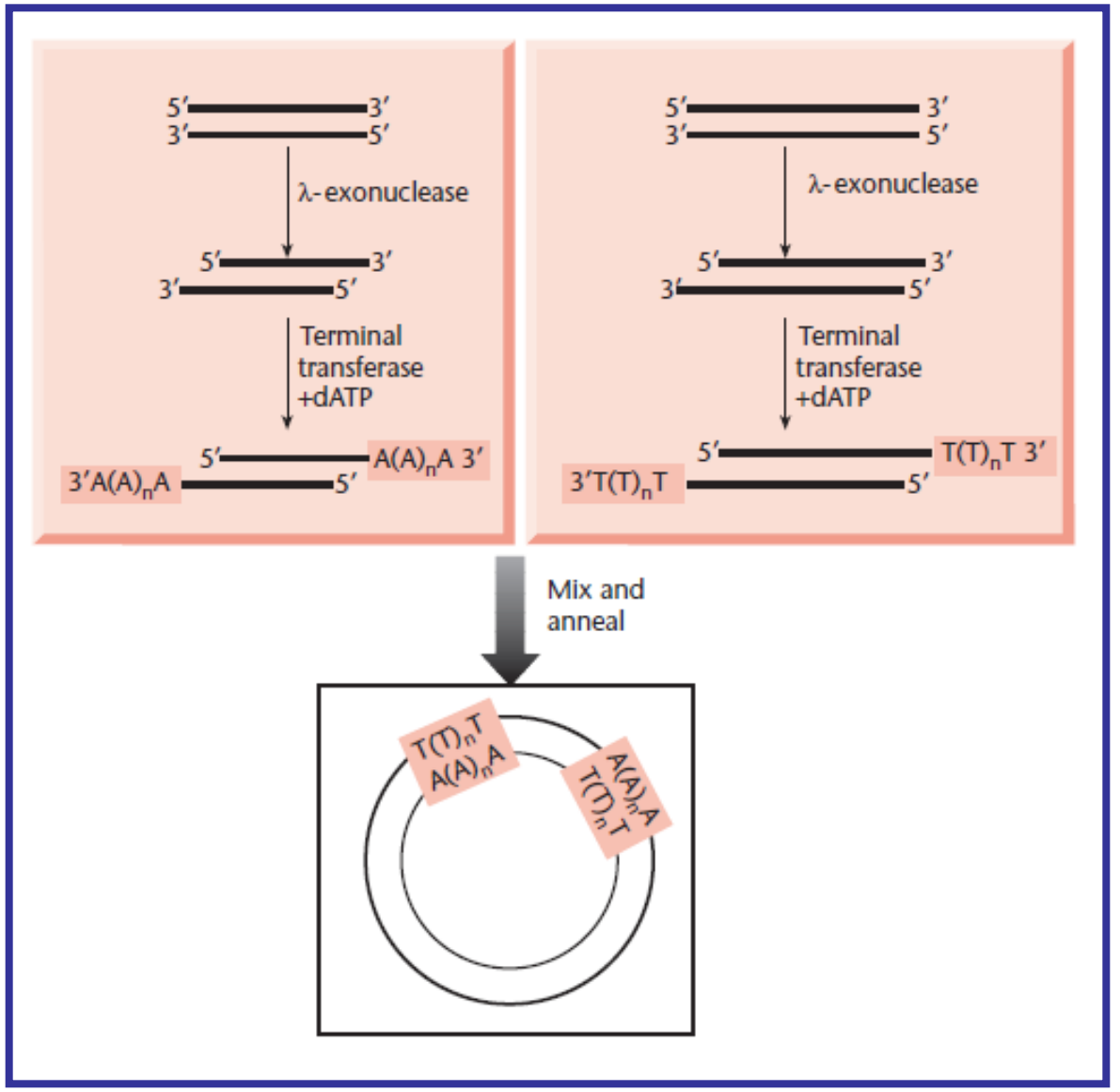
Linkers


EcoRI
Decameric linker molecule
C C G A A T T C G G 3′
G G C T T A A G C C 5′
T4 DNA ligase
+
Foreign DNA
Linker molecules
EcoRI
Vector
DNA ligase

Adapters


5′–HO–GATCCCCGGG–OH
3′– HO–GGGCCC–P
Adaptor molecule
P
HO
OH
P
+
HO
HO
OH
P
HO
HO
OH
P
T4 DNA ligase, ATP
HO
HO
OH
OH
Polynucleotide kinase, ATP
OH
P
Vector
OH
P
P
HO
OH
P

Homopolymer Tailing



Use directional cloning

- Two different restriction enzymes
- Give non-complementary sticky ends
- Prevents vector from self-ligating

Advantages & Disadvantages of Various Cloning Strategies

METHOD	ADVANTAGE	DISADVANTAGE
Tailing	Efficient ligation to vectors from long overhangs	Does not work with lambda phage vectors
Linker Ligation	Directional cloning Efficient	Loss of certain mRNAs with internal restriction sites without use of methylase, possible cloning of linkers only
Adapter Ligation	No need for restriction digestion prior to cloning unless doing directional cloning	Cloning of adapter dimers can lead to high background if not removed before ligation
RE sites introduced in cDNA synthesis	High proportion of full length, ability to use small amounts of mRNA, Directional cloning	Reduced proportion of truncated cDNAs, need to use restriction enzymes to produce sticky ends

4. Selection of Small Self-Replicating DNA

- Plasmids
  - Small circular pieces of DNA that are not part of a bacterial genome, but are capable of self-replication.
  - Plasmids are often used as “vectors” to transport genes between microorganisms.
  - In biotechnology, once the gene of interest has been amplified and both the gene and plasmid are cut by restriction enzymes, they are ligated together generating what is known as a recombinant DNA.
  - Viral (bacteriophage) DNA can also be used as a vector, as can cosmids, recombinant plasmids containing bacteriophage genes.

Properties of Good Vector -

1. It should be able to replicate autonomously.
2. It should be easy to isolate and purify.
3. It should be easily introduced into the host cells, Le., transformation of the host with the vector should be easy.
4. The vector should have suitable marker genes that allow easy detection and/or selection of the transformed host cells.
5. When the objective is gene transfer, it should have the ability to integrate either itself or the DNA insert it carries into the genome of the host cell.
6. The cells transformed with the vector containing the DNA insert (recombinant DNA) should be identifiable be selectable from those transformed by the unaltered vector.

7. A vector should contain unique target sites for as many restriction enzymes as possible into which the DNA insert can be integrated.

8. When expression of the DNA insert is desired, the vector should contain atleast suitable control elements, e.g., promoter, operator and ribosome binding sites.

The choice of vector depends largely on the host species into which the DNA insert of gene is to be cloned.

Most naturally occurring vectors do not have all the required functions; therefore, useful vectors have been created by joining together segments performing specific functions (called modules) from two or more natural entities.

Types of vectors

1. Plasmids
2. Bacteriophages
3. Cosmids
4. Phagemids
5. shuttle vectors
6. Yeast Artificial Chromosome (YACs)
7. Bacterial Artificial Chromosome (BACs)

Naturally occurring

Constructed by man

5. Method to Move a Vector into a Host Cell

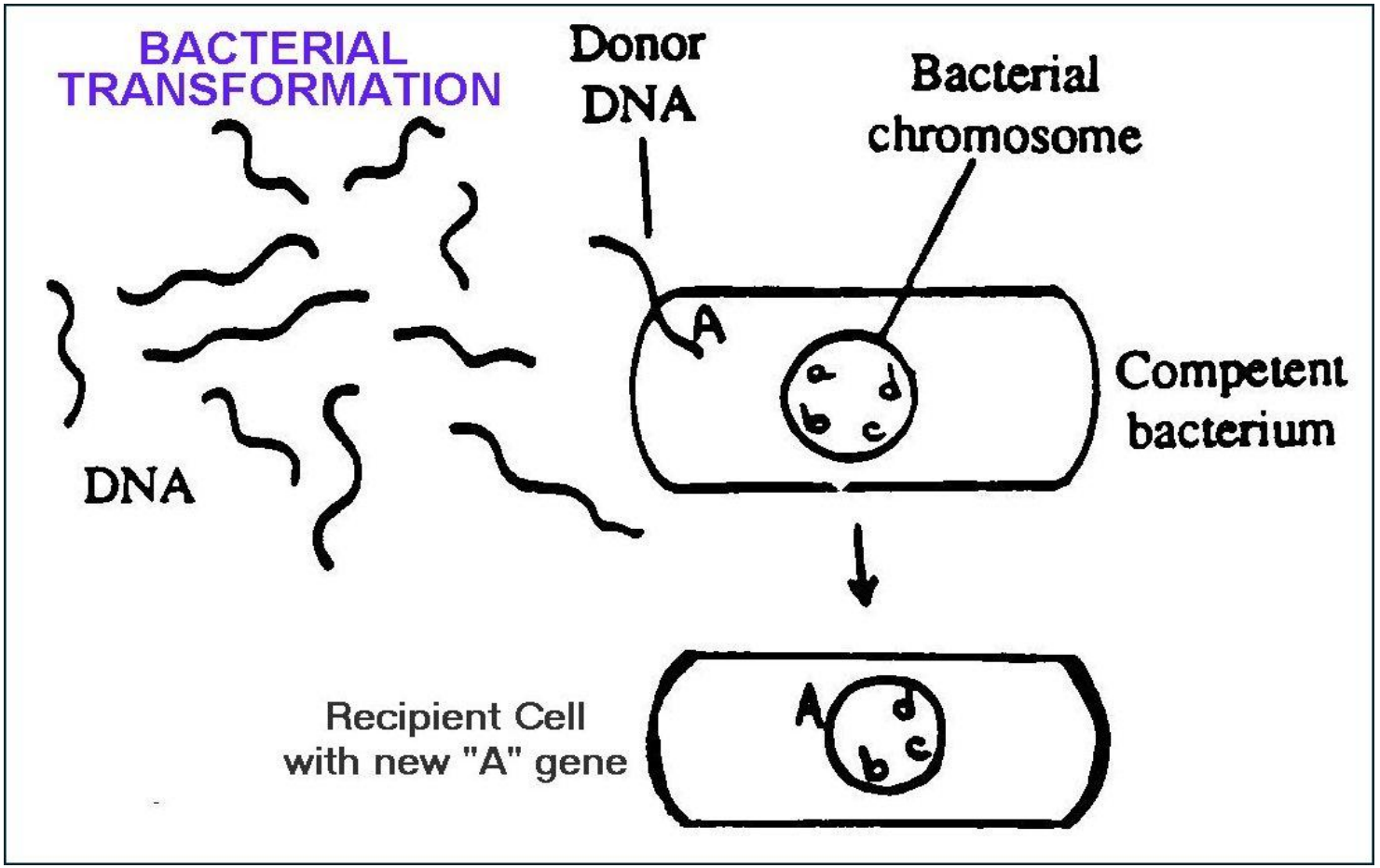
- Transformation
  - The process of transferring plasmids into new host cells
  - The host cells are exposed to a heat-shock, which makes them “competent” or permeable to the plasmid DNA.
  - The larger the plasmid, the lower the efficiency with which it is taken up by cells.
  - Larger DNA segments are more easily cloned using bacteriophage vectors or cosmids.

Properties of Good Host

A good host should have the following features:

1. Easy to transform,
2. Support the replication of recombinant DNA,
3. Free from elements that interfere with replication of recombinant DNA,
4. Lack active restriction enzymes
5. Should not have methylases since these enzymes would methylate the replicated recombinant DNA. which, as a result, would become resistant to useful restriction enzymes, and
6. Be deficient in normal recombination function so that the DNA insert is not altered by recombination events.

Competent cells and Transformation



Uptake of DNA

- Transformation
  - cell made competent to take up DNA
- Transfection
  - when the cloning vector used has aspects of a virus, the host cell can be infected (transfected) to insert the recombinant molecule
- Transduction
  - Transfer of the DNA using virus
- Microprojectiles
  - particles coated with DNA are "fired" at a cell and penetrate the membrane
- Electroporation
  - the cell is placed in an electric field such that small pores are temporarily opened in the membrane. Added DNA can enter through these pores

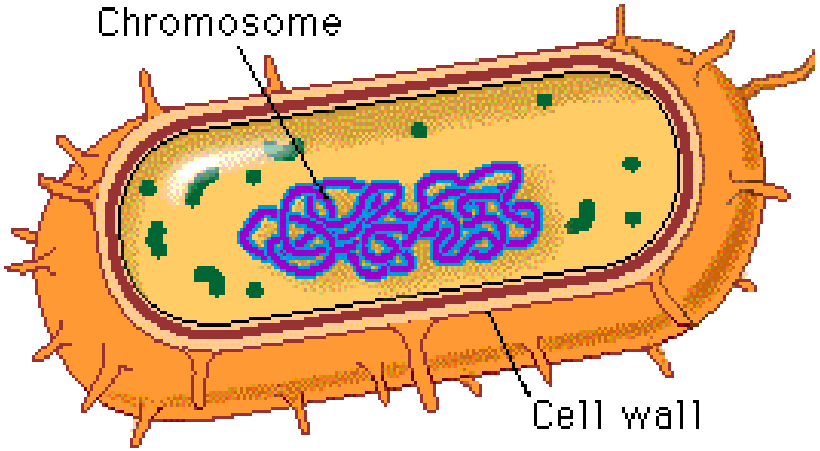
Bacterial Transformation

- Not all bacteria take up free-floating DNA in the environment.
- The genera that generally exhibit transformation include: *Bacillus*, *Streptococcus*, *Azotobacter*, *Haemophilus*, *Neisseria*, and *Thermus*.
- The recipient cells must be competent (able to transform).
- Competence is a phenotype conferred by one or more proteins.
- It has been shown that competence occurs late in the exponential phase of bacterial growth.
- The duration of competence varies from a few minutes in *Streptococcus* to hours in *Bacillus*

Competency

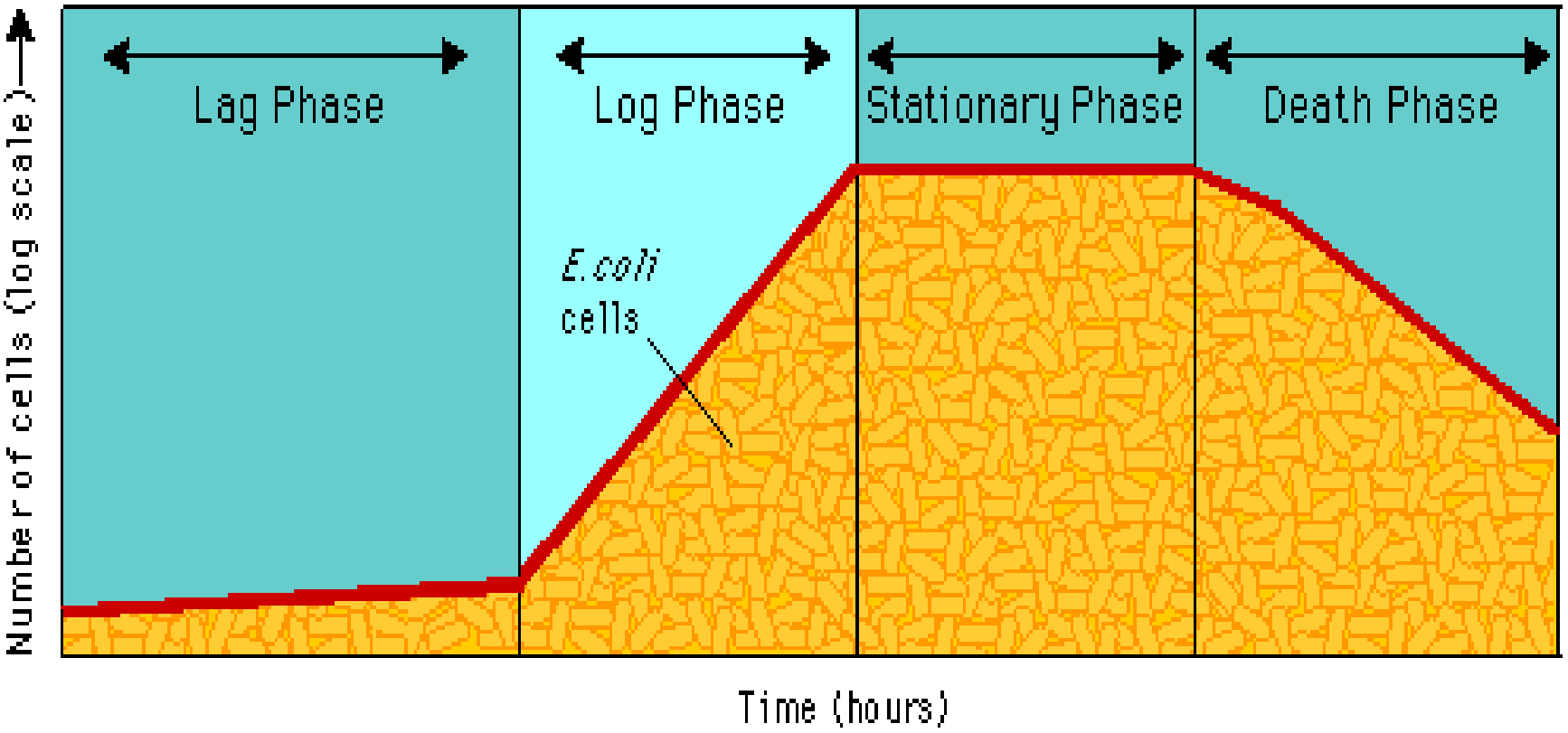
- Since DNA is a very hydrophilic molecule, it won't normally pass through a bacterial cell's membrane.
- In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA.
- This is done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium.
- DNA can then be forced into the cells by incubating the cells and the DNA together on ice, placing them briefly at 42^{o}C (heat shock), and then putting them back on ice.
- This causes the bacteria to take in the DNA. The cells are then plated out on antibiotic containing media.

E. coli bacterium

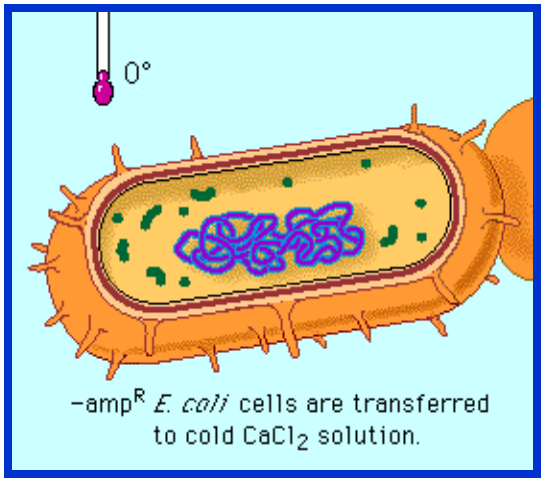


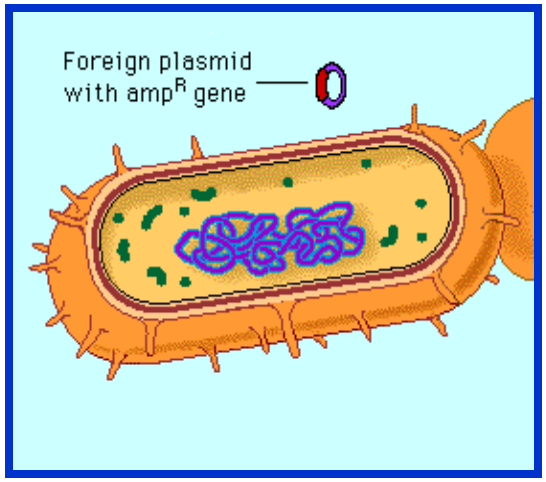
- *E.coli* is the most common bacterium in the human gut
- *E.coli* has been extensively studied
- Reproduce very rapidly
- A single cell can divide and give rise to 10^6 cells overnight (16 hrs)

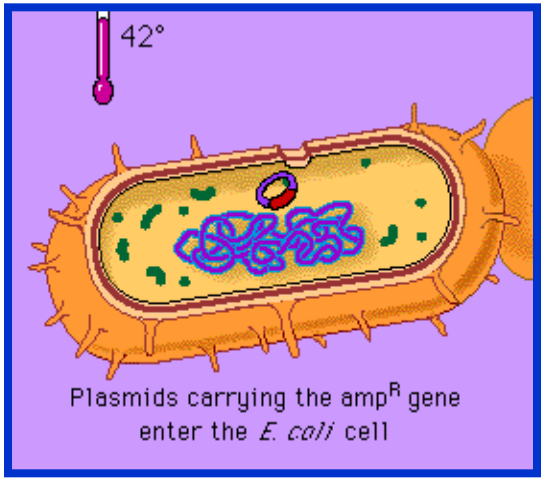
Growth Phases

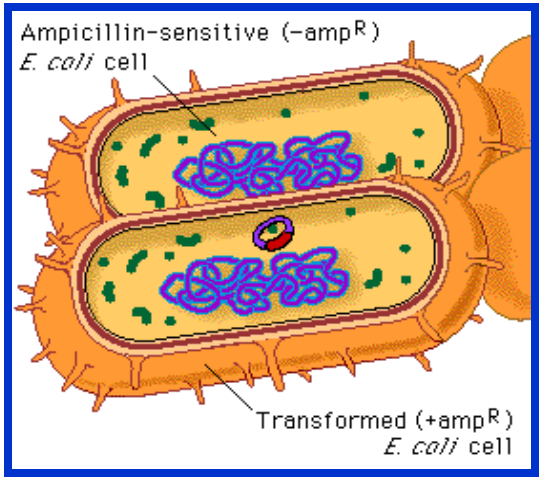


Transformation procedure

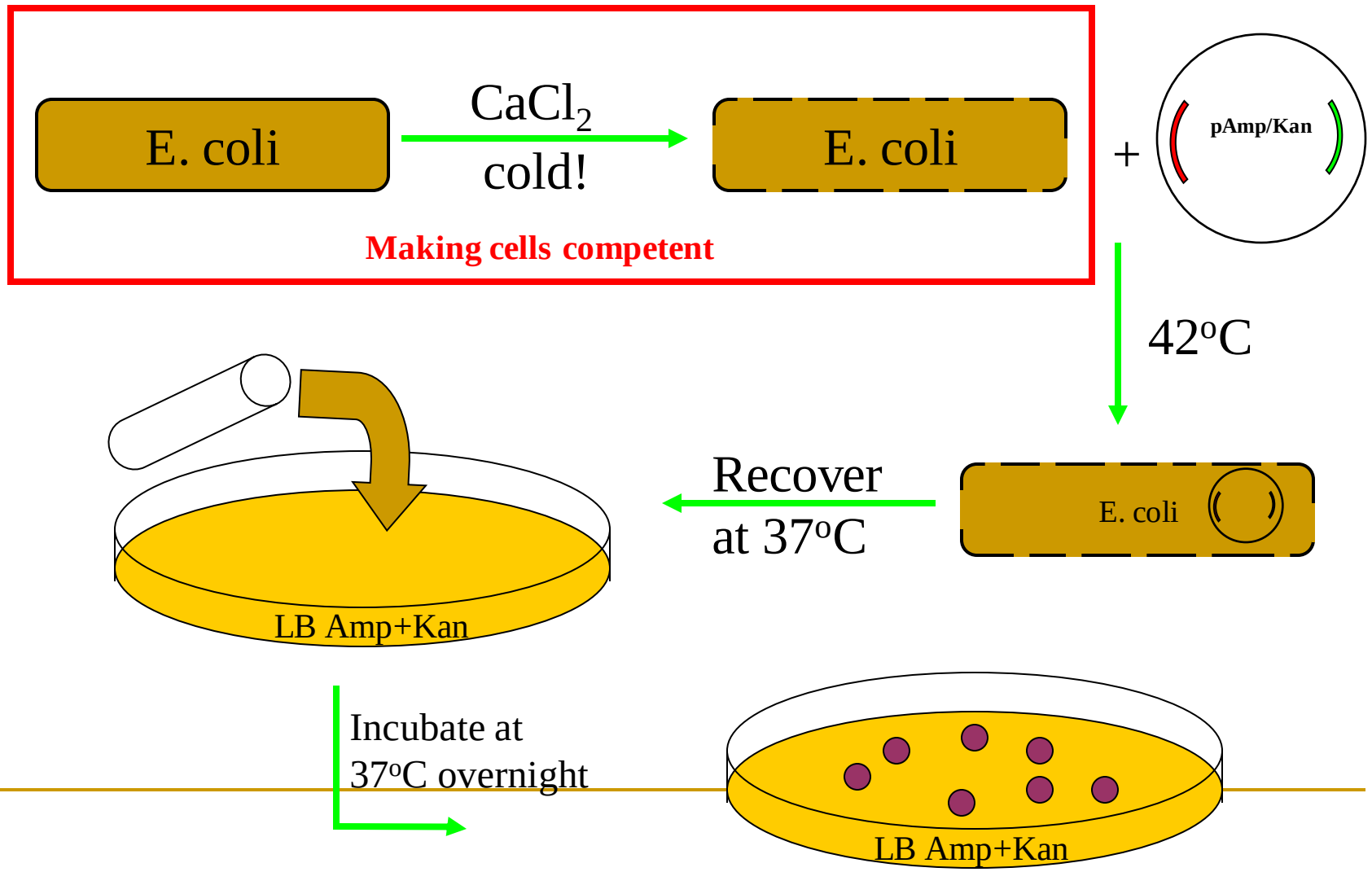








Transformation procedure

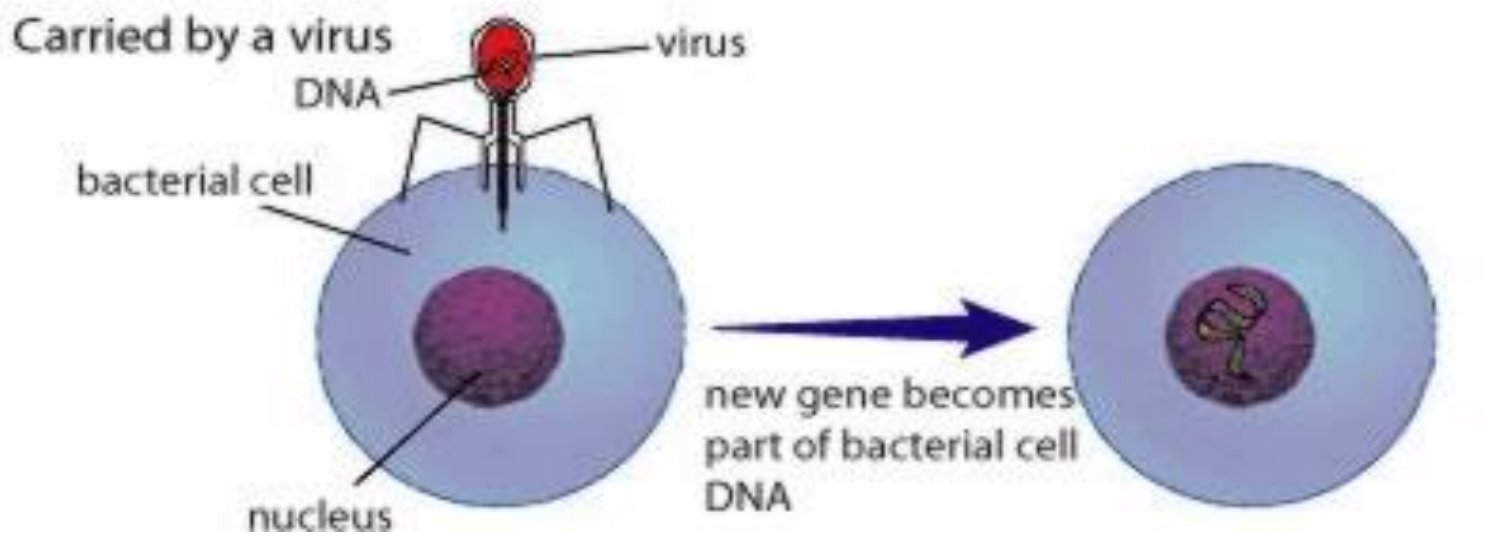


Transfection Methods

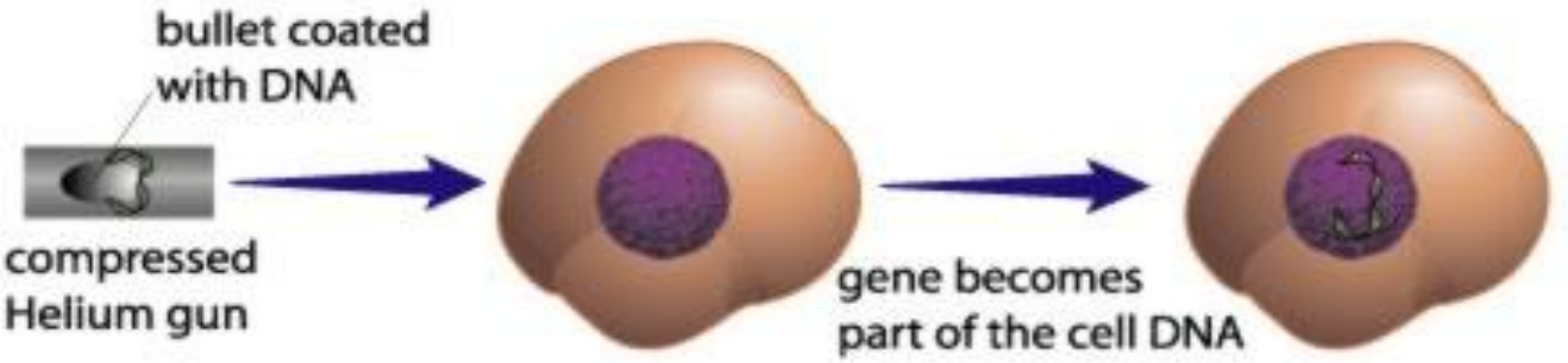
- Calcium phosphate precipitation and phagocytosis: used to transform cells of mammals.
- Lipofection: used to transform cells of animals, yeast, plants and bacteria.
  - The DNA to be transferred is placed into liposomes.
  - Since the liposomes are made up of lipids, they become part of the cell membrane of the cells and the contents - the new DNA - enters the cells.

Transduction

- Using a virus to insert DNA into a cell
- The gene is inserted into the genetic make-up of harmless viruses that then invade cells, carrying the gene into the cells.

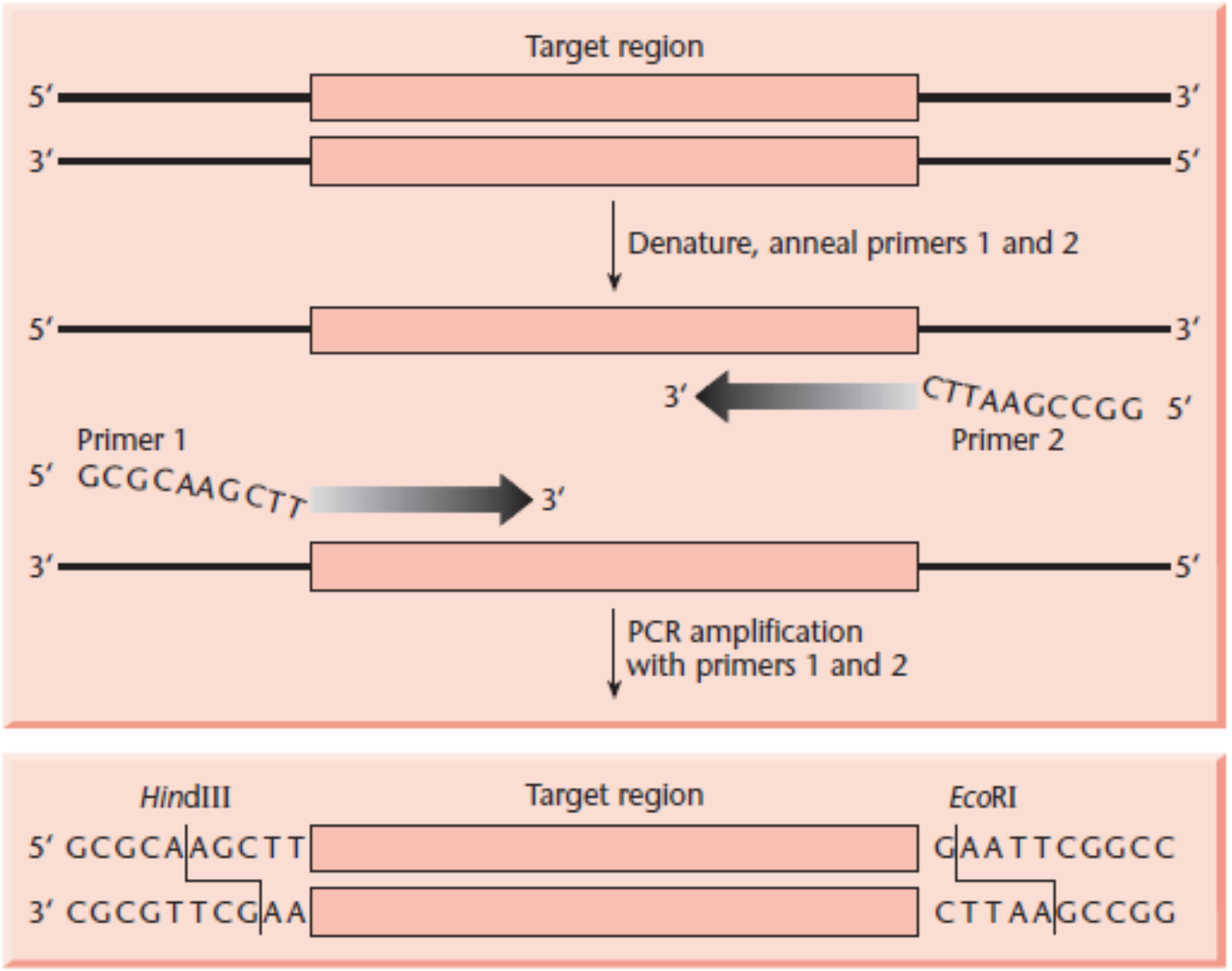


Biolistics

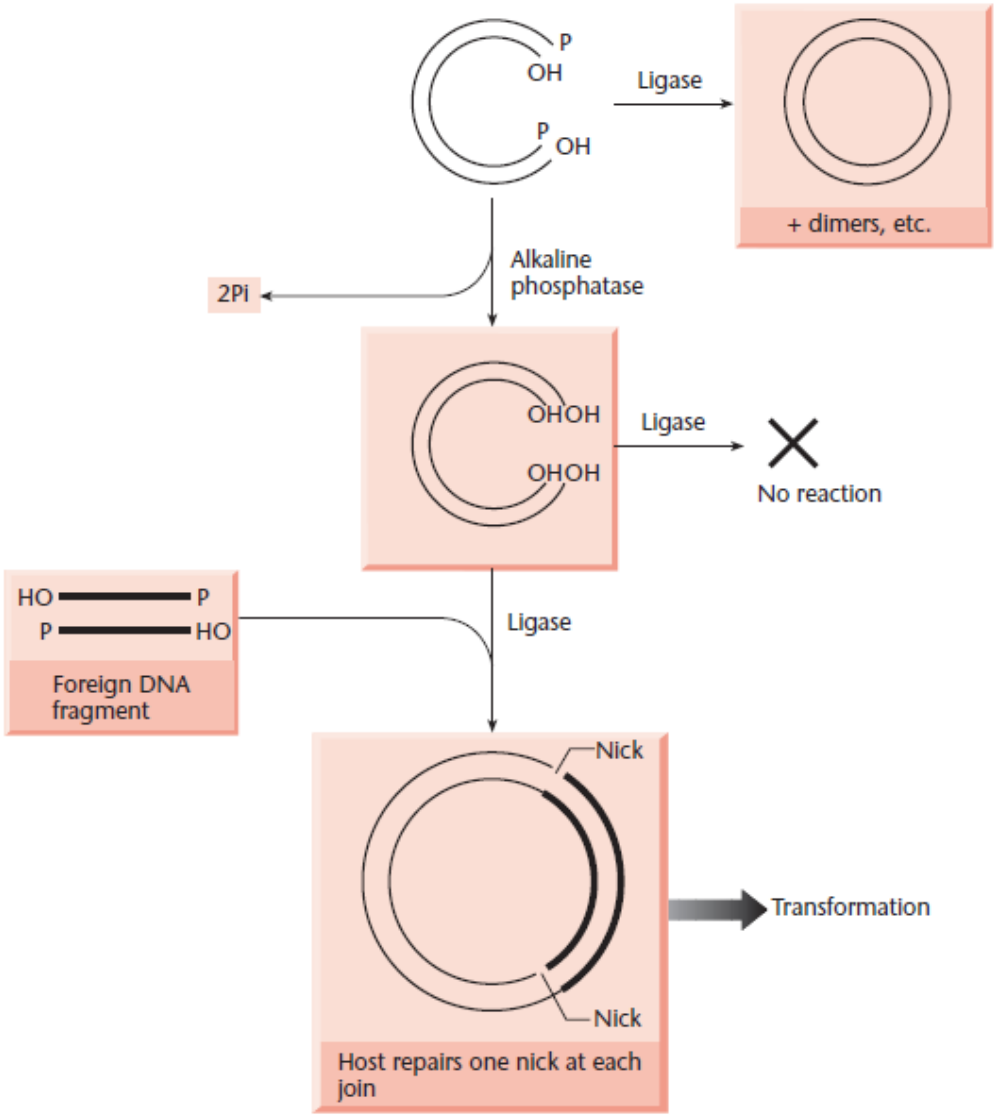


- A specially designed gene gun using compressed helium gas fires dozens of metal pieces at target cells.
- The tiny pellets, usually of tungsten or gold, are much smaller then the target cell, and coated with DNA.

RE site insertion



Alkaline phosphatase treatment



6. Method to Select Hosts Expressing Recombinant DNA

Not all cells will take up DNA during transformation.

- A **marker gene** is used to determine if a piece of DNA has been successfully inserted into the host organism.

  There are two types of marker genes:

1. **A selectable marker** (protect the organism from a **selective agent** that would normally kill it or prevent its growth)

   - Antibiotic resistance genes

     - Transformed cells can be selected based on expression of those genes and their ability to grow on media containing that antibiotic.

Antibiotic Resistance Genes Found in R-Plasmids their Proteins

Antibiotic (gene conferring resistance)	Protein produced by the gene	Mechanism of resistance
Ampicillin (amp)	Penicillinase or β-lactamase	Hydrolysis of C-N bond in β-lactam ring
Kanamycin (kan)	Kanamycin acetyltransferase*	N-acetylation of the antibiotic
Neomycin (nea)	Aminoglycoside phosphotransferase*	O-phosphorylation of the antibiotic
Streptomycin (str)	Streptomycin phosphotransferase	Phosphorylation of -OH on the antibiotic
	Streptomycin adenylate synthetase	Adenylation of the -OH on the antibiotic

* The antibiotics kanamycin and neomycin are related; hence nea product also inactivates kanamycin, and kan product inactivates neomycin as well.

Reporter proteins (Screening Makers)
(make cells containing the gene look different)

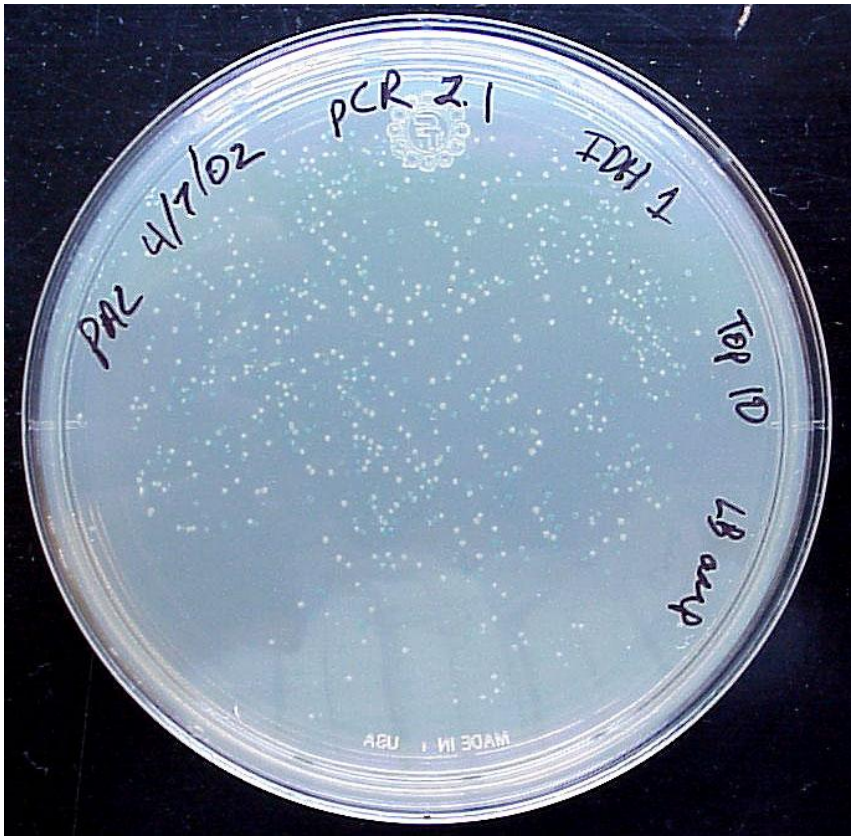
Two types commonly used:

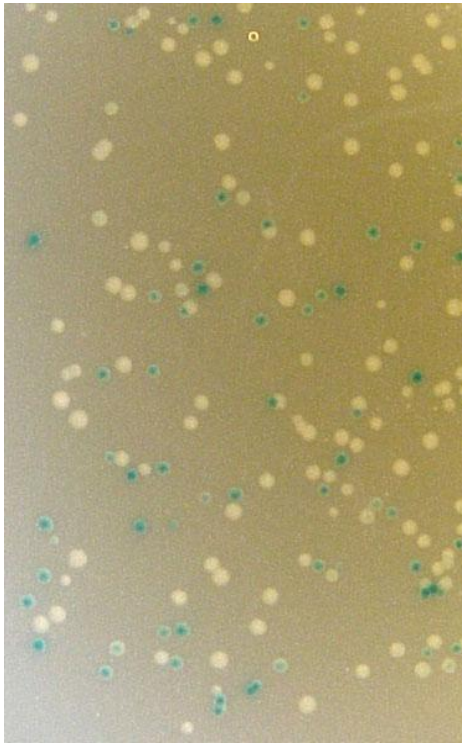
- **Green fluorescence protein (fluorescence detection)**
  - makes cells glow green under UV light. A specialized microscope is required to see individual cells. YFP and RFP can also be used to look at multiple genes at once. It is commonly used to measure **gene expression**.
- **x-gal/*lacZ* system (Color selection)**
  - The lacZ gene makes cells turn blue in special media (e.g. X-gal). A colony of cells with the gene can be seen with the naked eye.

b-galactosidase, encoded by the bacterial gene *lacZ*, cleaves the disaccharide lactose (sugar found in milk) into glucose and galactose.

b-galactosidase cleaves the colorless substrate X-gal (5-bromo-4-chloro-3-indolyl-b -galactopyranoside) into galactose and a blue insoluble product of the cleavage.

Blue/white selection after ligation. X-gal and IPTG are added to LB ampicillian plates prior to spreading the transformed cells.

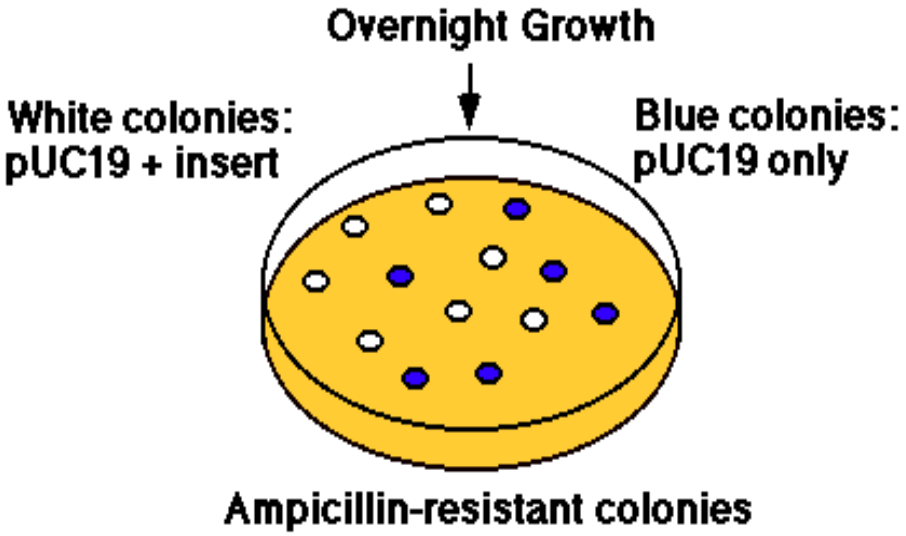




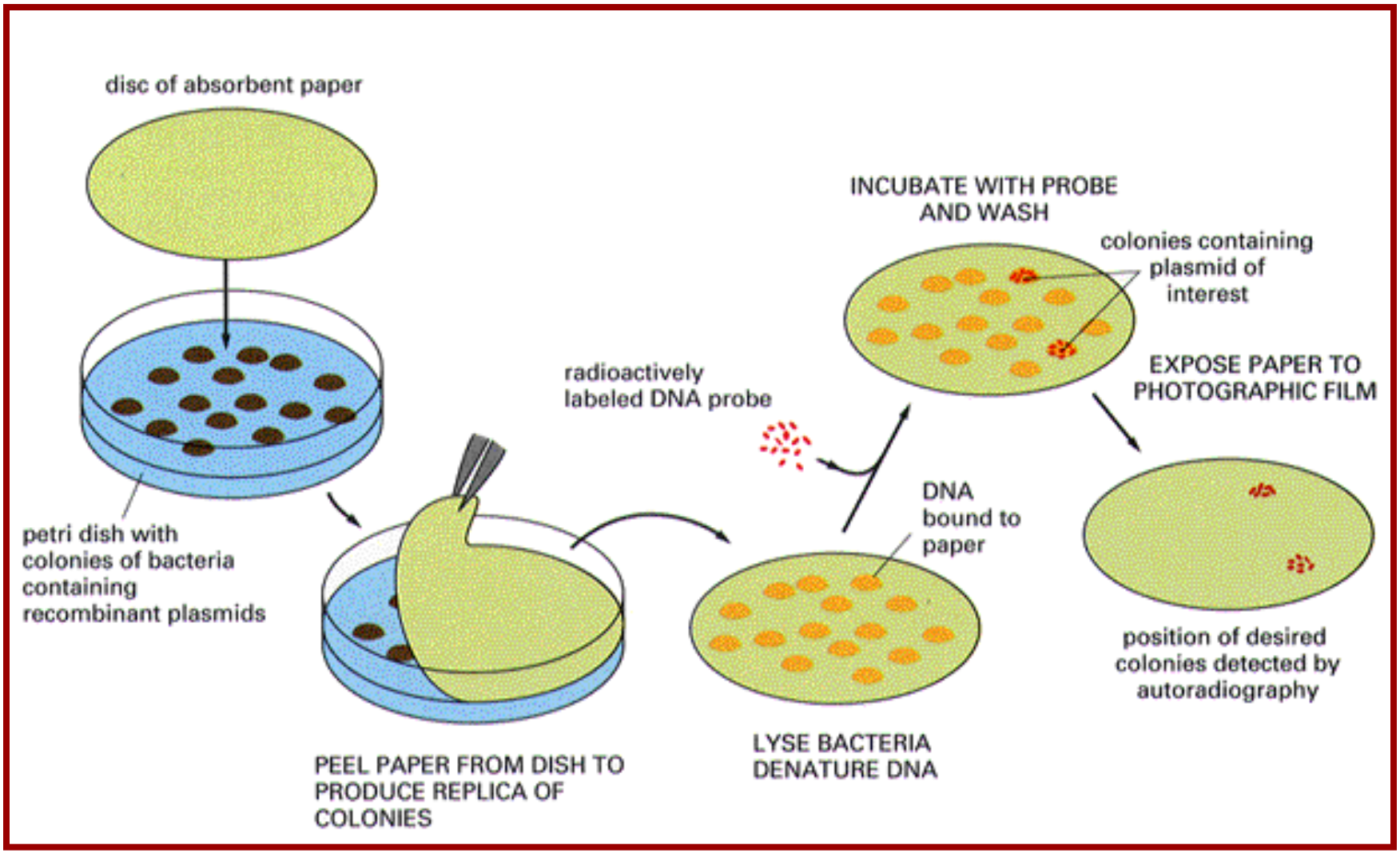
Screening

Screening can involve:

1. Phenotypic screening- the protein encoded by the gene changes the colour of the colony
2. Using antibodies that recognize the protein produced by a particular gene

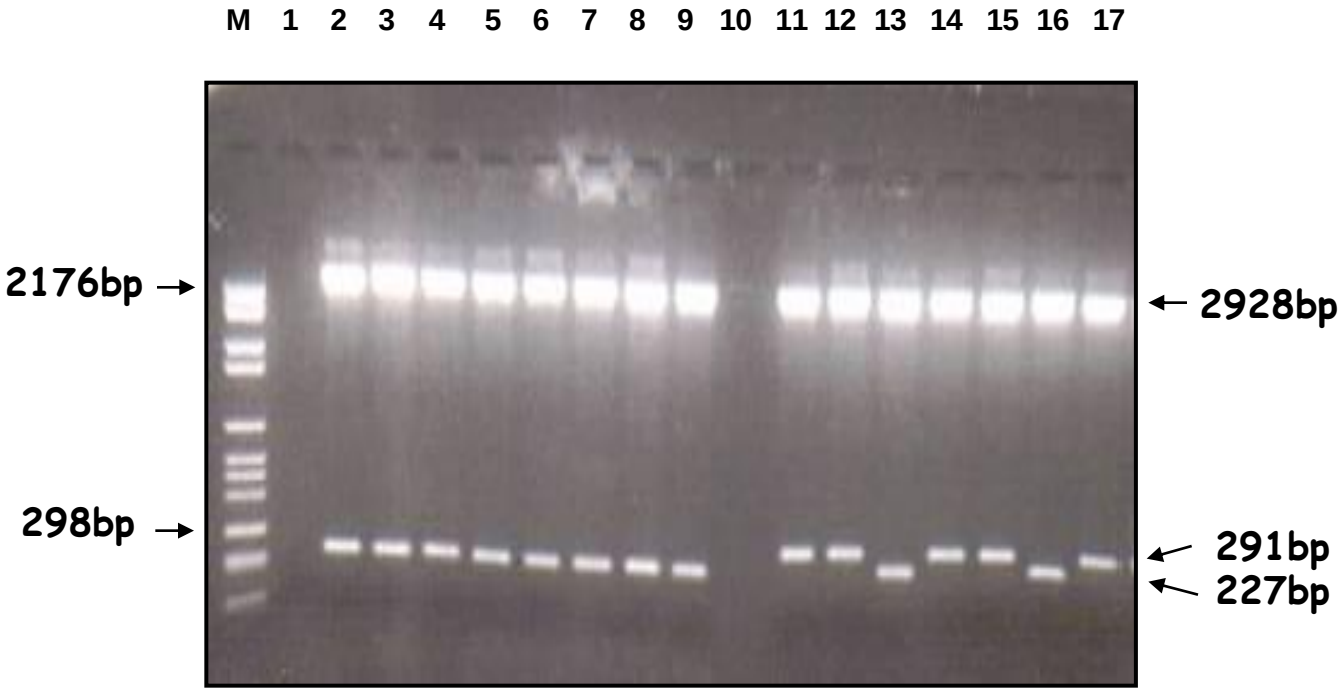


Detecting the DNA sequence of a cloned gene with a probe (DNA hybridization)



Restriction analysis

- Isolate the plasmid DNA
- Digest with restriction endonucleases
- Analysis through Gel electrophoresis



Transformation efficacy

- Supercoiled plasmids are most easily taken up, yielding transformation efficiencies in the range 10^6 - 10^{10} transformants/µg of DNA.
- Relaxed circular DNA (nicked or covalently closed) gives efficiencies about 10- to 100-fold less than supercoiled.
- Linear DNA is down by another factor of 10- to 100-fold from relaxed circular DNA.

Host systems for cloning

- Bacteria (*E. coli*)
- Yeast
- Mammalian cells
- Plant Cells

Reasons for using *E. coli* in Gene Cloning

1. Genetic Simplicity

Relatively small genome. *E. coli* cells only have about 4,400 genes whereas humans contain approximately 30,000.

Also, live their entire lifetime in a haploid state, with no second allele to mask the effects of mutations during protein engineering experiments.

2. Growth Rate

Grow much faster than more complex organisms. *E. coli* grows normally at a rate of one generation per 20 min under typical growth conditions.

Allows for preparation of log-phase cultures overnight and genetic experimental results in mere hours instead of several days, months or years.

3. **Safety**

   Present in intestine of humans and animals as normal flora where it helps provide nutrients (vitamins K and B12) to its host.

   E. coli are generally relatively innocuous if handled with reasonable hygiene.

4. **Conjugation and the Genome Sequence**

   E. coli is the most highly studied microorganism and an advanced knowledge of its protein expression mechanisms make it simpler to utilize for experiments where expression of foreign proteins and selection of recombinants is essential.

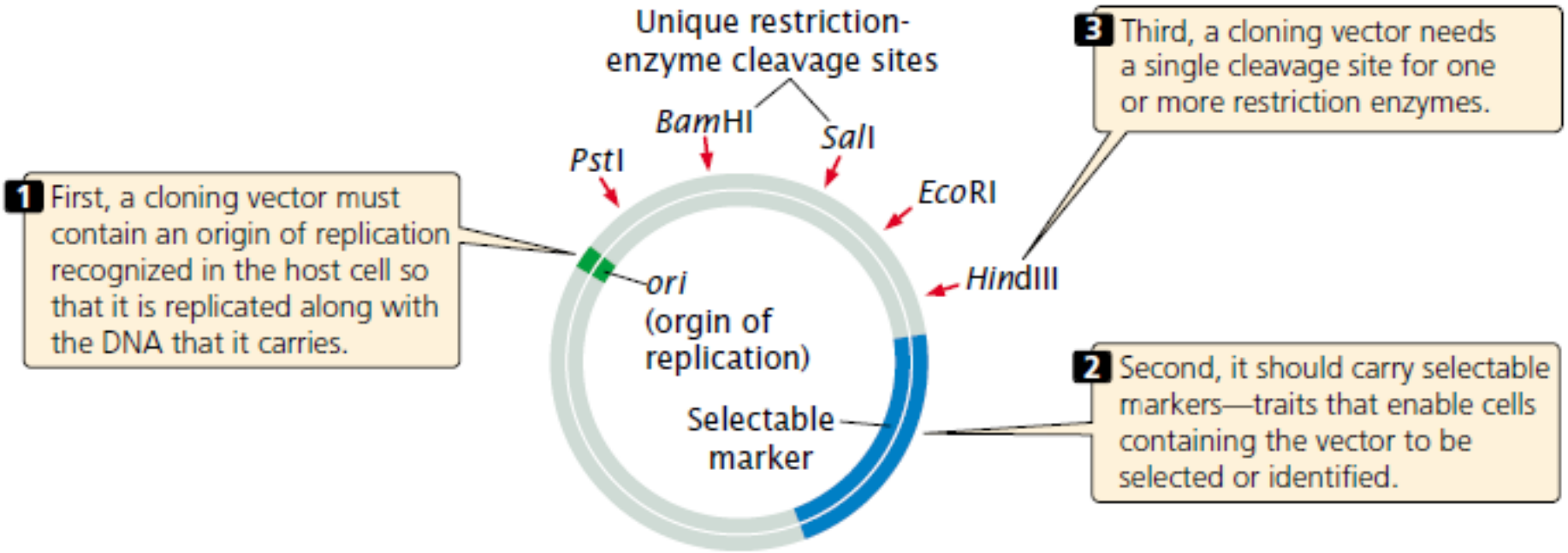
5. **Ability to Host Foreign DNA**

   Most gene cloning techniques were developed using this bacterium and are still more successful or effective in *E. coli* than in other microorganisms.

   E. coli is readily transformed with plasmids and other vectors, and preparation of competent cells is not complicated.

   Transformations with other microorganisms are often less successful.

Choosing a cloning vector



Cloning vectors : Common Features

1. **Suitable size:** Cloning vectors are **small, circular, double-stranded DNA** molecules. The vector DNA contributes as little as possible to the overall size of recombinant molecules. This assures that a cloned fragment constitutes a large percentage of amplified and isolated plasmid DNA, making it easier to prepare large quantities of insert DNA.

2. **An origin of replication:** Cloning vectors contain a **replicon**, that is a stretch of DNA that permits DNA replication of the plasmid independent of replication of the host chromosome.

   This element contains the site at which DNA replication begins or the origin of replication and genes encoding RNAs and/or proteins that are necessary for plasmid replication.

   The replicon largely determines the copy number of the plasmid,

   (the number of plasmid molecules maintained per bacterial cell)

3. **A selectable Marker:** Cloning vectors contain selectable markers for distinguishing cells transformed with the vector from non-transformed cells.

4. **Cloning site:** Cloning vectors contain unique cloning sites for the introduction of DNA fragments. The cloning sites in most general-purpose vectors used today consist of a **multiple cloning site** or a **polylinker**

(cloning region where a number of restriction enzyme cleavage sites are immediately adjacent to each other)

5. **Markers for DNA insertion:** Cloning vectors contain an element for screening for the recombinant clones. (reporter gene) [*lacZ* (b-galactosidase) gene]

6. **High copy number**

   Desirable but not essential

   To maximize the yield, the copy number in each cell should be as high as possible.

   1. **Relaxed:** High copy number plasmids 20 or more copies per bacteria
   2. **Stringent**: Low copy number plasmids less than 20 copies per **cell**

7. **Disablement**

   Plasmid is disables so that it cannot spread to other bacteria by conjugation

   Removal of mob gene (responsible for plasmid mobilization)

Vectors for Transformation of genes (Self-Replicating DNA)

- Nature did not deliver plasmid vectors ready-made for genetic engineering.
- The most useful plasmid vectors were themselves constructed by genetic engineering using **R plasmids**

(plasmids or autonomously replicating DNA elements that carry one or more drug-resistance genes)

The R plasmids are the cause of serious medical problems, for various bacteria can acquire R plasmids and thereby become resistant to the normal drugs used for treatment of infections.

Conversion of R plasmid to useful vector ?

- Elimination of extraneous DNA
- Removal of multiple restriction enzyme cleavage sites

For cloning, the plasmid should possess only one cleavage site for at least one restriction enzyme, and this should be in a non-essential region.

Digestion (various restriction enzymes)

Hybridization of self-complementary ends

Ligation to produce combinations of scrambled fragments

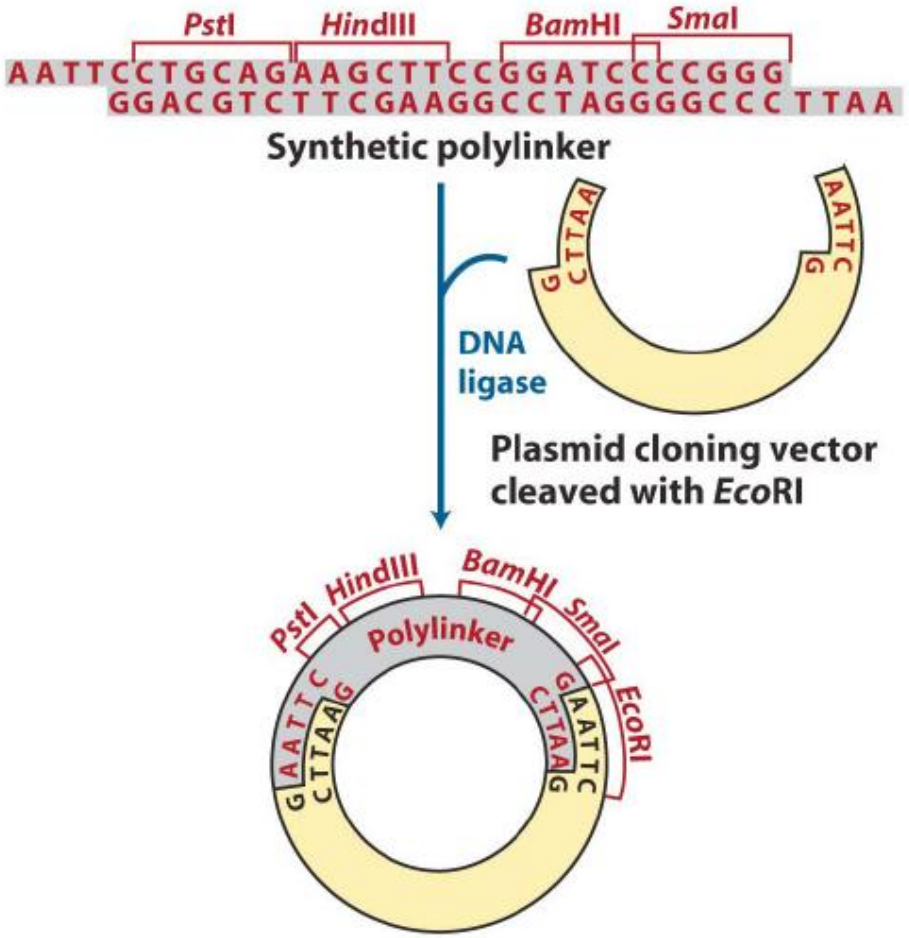
Transformation into cells.

Selection on the basis of drug resistance and replication

Test digestion and electrophoresis

The plasmid pBR322 possesses single restriction enzyme cleavage sites for more than twenty enzymes including *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Pvu*II, and *Sal*I.

Creation of a polylinker or multiple cloning site



Types of vectors

1. Plasmids
2. Bacteriophages
3. Cosmids
4. Phagemids
5. shuttle vectors
6. Yeast Artificial Chromosome (YACs)
7. Bacterial Artificial Chromosome (BACs)
8. Human Artificial Chromosomes (HACs)

Naturally occurring

Constructed by man

Bacteriophage M13

- The size of inserts is about 1,500 bps, i.e. the fragments grown in M13 are ready to be sequenced.
- M13 contains single--stranded inserts, i.e. there is not going to be a denaturing step in preparation of M13 insert for sequencing.
- The disadvantage of this particular vector is a large cloning bias. M13 is prone to loosing (refusing to amplify with) certain types of sequences.

Plasmid vectors

Double-stranded circular DNA sequences that are capable of automatically replicating in a host cell.

- Plasmid vectors minimalistically consist of an
  - origin of replication
  - a multiple cloning site
  - Selectable Marker
  - Polyadenylation and translation termination sequence (in case of Expression vectors)

Plasmids are able to carry only 1-20 kbp of transgene

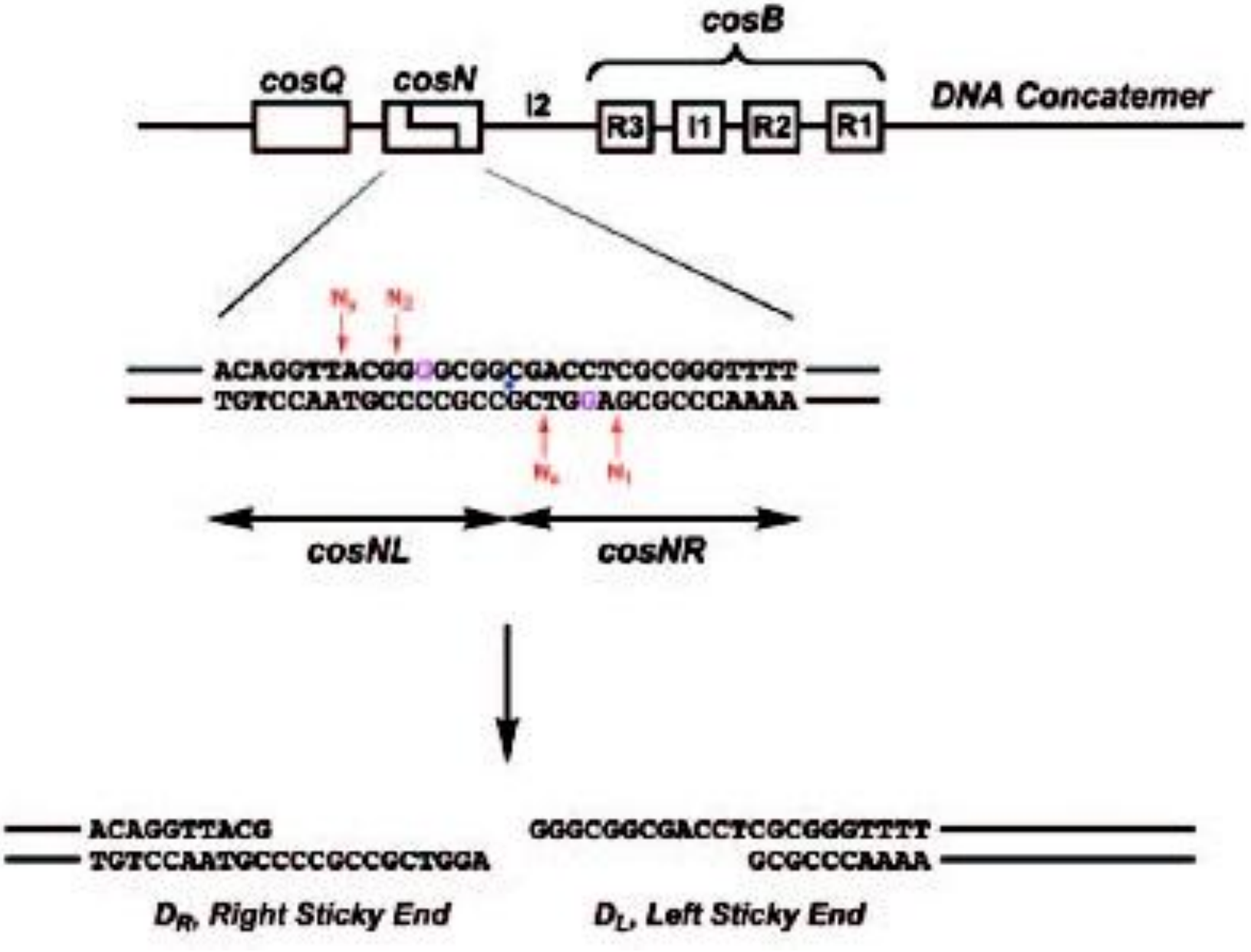
Cosmids

- A hybrid plasmid that contains *cos sequences*, DNA sequences originally from phage Lambda.
- Used to build genomic libraries.
- Able to contain 37 to 52 kbp of DNA.
- Can replicate as plasmids if they have a suitable origin of replication.
- They contain a gene for selection (Marker).
- Can also be packaged in phage capsids, which allows the foreign genes to be transferred into or between cells by transduction.

Cosmids (cont…)

- *Cos* sequences are ~200 base pairs long and essential for packaging.
- They contain a *cosN* site where DNA is nicked at each strand, 12bp apart, by.
- This causes linearization of the circular cosmid with two cohesive ends of 12bp.
- The DNA must be linear to fit into a phage head.
- The *cosB* site holds the terminase while it is nicking and separating the strands.
- The *cosQ* site of next cosmid (as rolling circle replication often results in linear concatemers) is held by the terminase after the previous cosmid has been packaged, to prevent degradation by cellular DNases.

Cosmid


cosB
cosQ
cosN
I2
R3
I1
R2
R1
DNA Concatemer
ACAGGTTACGGCGCGGCGACCTCGCGGGTTTT
TGTCCAATGCCCCGCCGCTGGAGCGCCCAAAA
cosNL
cosNR
ACAGGTTACG
TGTCCAATGCCCCGCCGCTGGA
GGGCGGCGACCTCGCGGGTTTT
GCGCCCAAAA
D_R, Right Sticky End
D_L, Left Sticky End

Figure

The *cos* region of a lambda concatemer. Upper panel: The *cosQ, cosN* and *cosB* subsites within a *cos* site in concatemeric DNA. The *cosB* subsite is composed of the I1 and R-elements, as indicated. The I2 region lies between *cosN* and the R3 element. Middle panel: The nucleotide sequence of *cosN*, with the *cosNL* and *cosNR* half-sites indicated. The center of symmetry of *cosN* is indicated with a dot. Terminase normally nicks the duplex at N1 and N2 sites indicated with arrows. In the absence of ATP, terminase incorrectly nicks the duplex at Nx and/or Ny sites. Lower panel: Strand separation by terminase yields the matured DR and DL ends of the lambda genome, as shown.

Artificial Chromosomes

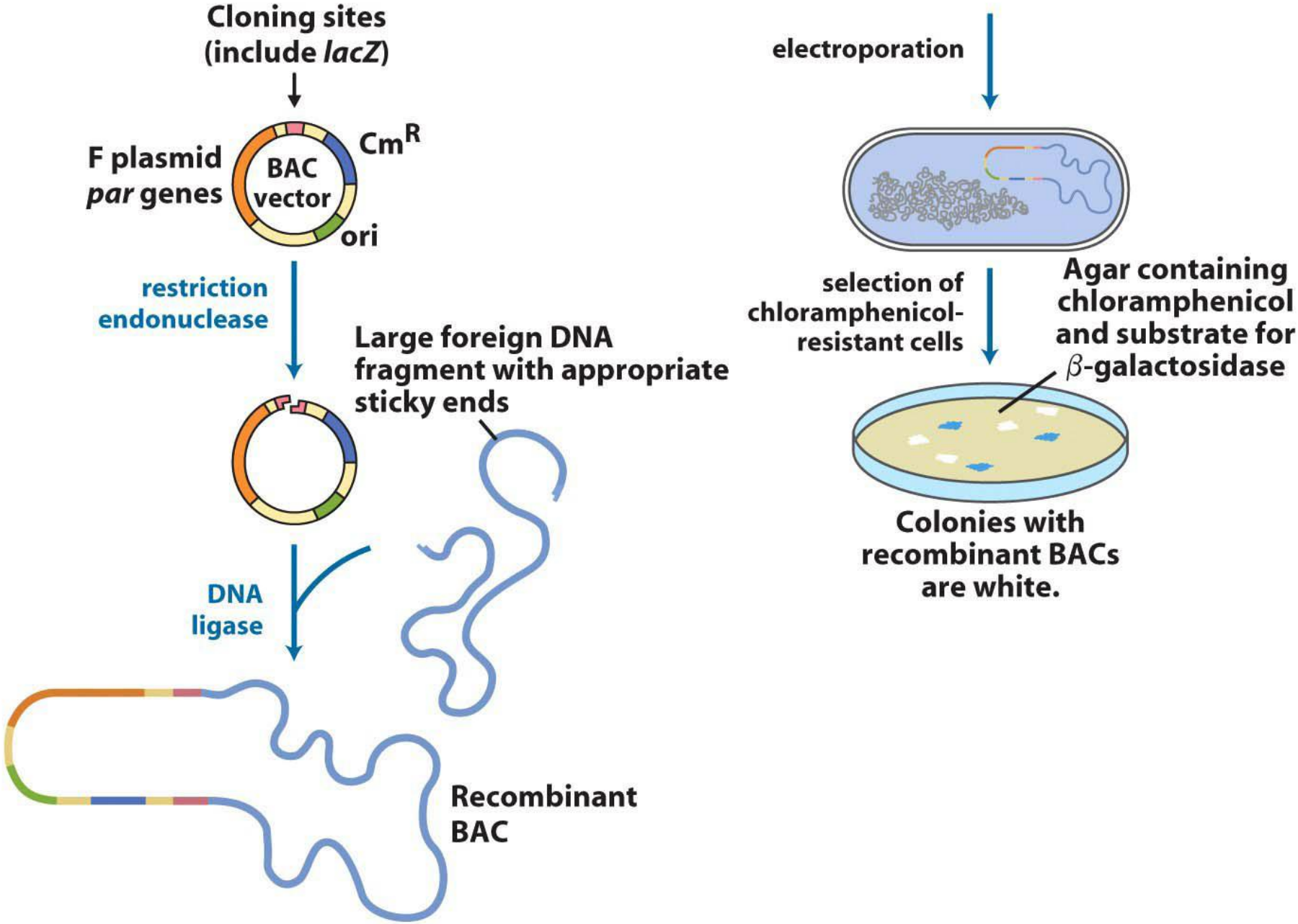
Bacterial artificial chromosome (BAC)

DNA construct, based on a functional fertility plasmid (or F-plasmid), used for transforming and cloning in bacteria, usually *E. coli.*

F-plasmids contain partition genes that promote the even distribution of plasmids after bacterial cell division.

The bacterial artificial chromosome's usual insert size is 150-350 kbp, but can be greater than 700 kbp.

BACs are often used to sequence the genome of organisms in genome projects, for example the Human Genome Project.


Cloning sites
(include lacZ)
F plasmid
par genes
BAC
vector
CmR
ori
restriction
endonuclease
Large foreign DNA
fragment with appropriate
sticky ends
DNA
ligase
Recombinant
BAC
electroporation
selection of
chloramphenicol-
resistant cells
Agar containing
chloramphenicol
and substrate for
β-galactosidase
Colonies with
recombinant BACs
are white.

Artificial Chromosomes

Yeast artificial chromosome (YAC)

- Used to clone large DNA fragments (larger than 100 kb and up to 3000 kb)
- Artificially constructed chromosome containing
  - Telomeric,
  - Centromeric, and
  - Replication origin sequences
- Useful for eukaryotic protein products with posttranslational modifications as yeasts are themselves eukaryotic cells.
- YACs have been found to be more unstable than BACs, producing chimeric effects.

Artificial Chromosomes

Human artificial chromosome (HAC)

First appeared in 1997.

Can act as a new chromosome in a population of human cells.

Instead of 46 chromosomes, the cell could have 47 with the 47th being very small, roughly 6-10 megabases in size.

Useful in expression studies as gene transfer vectors and are a tool for elucidating human chromosome function.

Grown in HT1080 cells, they are mitotically and cytogenetically stable for up to six months.

Viral Vectors

- Viral vectors are genetically-engineered viruses carrying modified viral DNA or RNA that has been rendered non-infectious, but still contain
  - Viral promoters and
  - The transgene,
- Allows translation of the transgene through a viral promoter.
- Viral vectors frequently are lacking infectious sequences, they require helper viruses or packaging lines for large-scale transfection.

- Viral vectors are often designed for permanent incorporation of the insert into the host genome, and thus leave distinct genetic markers in the host genome after incorporating the transgene.

  For example,

  Retroviruses leave a characteristic retroviral integration pattern after insertion, that is detectable and indicates that the viral vector has incorporated into the host genome.

Purposes of cloning

- Expression of gene
- Library construction

Cloning vs. Expression Vectors

1. Expression vectors (expression constructs)

   Specifically express the transgene in the target cell, and generally have a promoter sequence that drives expression of the transgene.

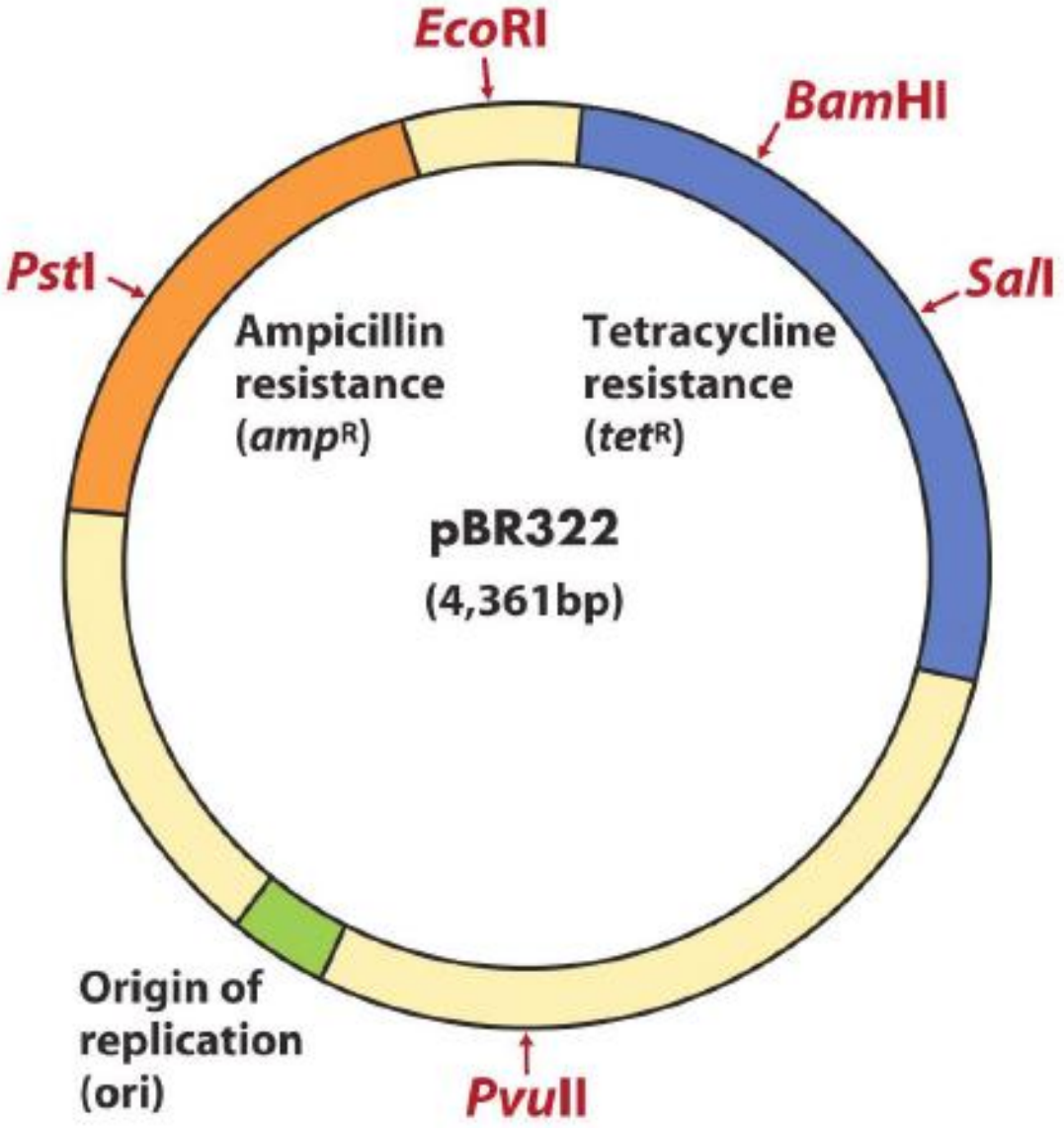
2. Transcription vectors (Cloning vectors)

   Transcription vectors are used to amplify their insert.

   Only capable of being transcribed but not translated: they can be replicated in a target cell but not expressed.

   Lack crucial sequences that code for polyadenylation sequences and translation termination sequences in translated mRNAs, making protein expression from transcription vectors impossible.

Cloning Vector

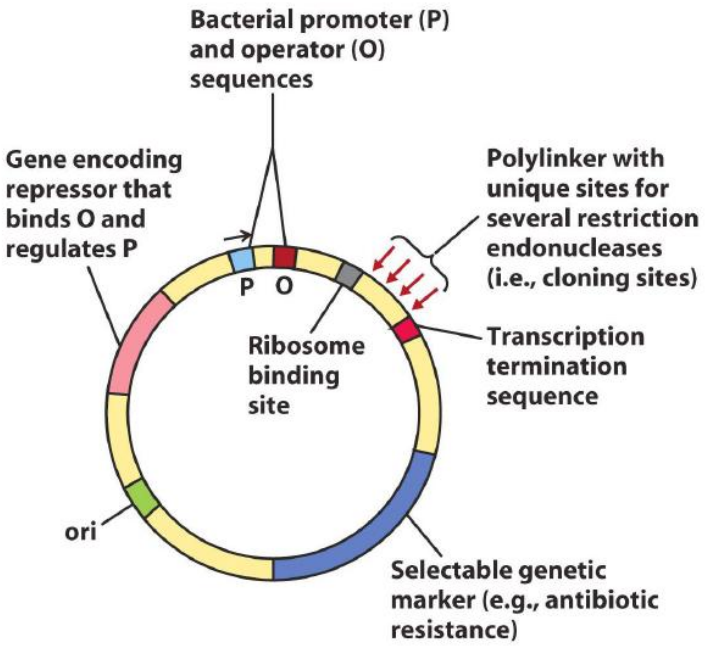


Expression

Why?

You want the cloned gene to make its product, normally a protein.

- Identifying gene from library requires expression.
- To overproduce the protein and purify it.
- For *in vivo* studies of the protein.



Expression vectors

Vectors that can yield the protein products of the cloned genes

Two elements that are required for active gene expression:

1. a strong promoter and
2. a ribosome binding site near an initiating ATG codon.

The main function of an expression vector is to yield the product of a gene, therefore a strong promoter is necessary.

The more mRNA is produced, the more protein product is made.

Expression vectors: Basic Construction

- Plasmid vectors
  - Contain prokaryotic (facilitate bacterial propagation), eukaryotic and viral sequences (transcriptional elements and selectable markers)
- Viral vectors
  - Essentially inactivated viruses into which genes are cloned

Mammalian Expression vector

Have

1. The ability to constitutively and inducible express the proteins
2. The ability to produce a large quantity of protein that is post-translationally modified and appropriately folded
3. The ability to characterize the impact of specific mutations on cell metabolism
4. The ability to stably alter cellular phenotype as a function of transgene expression

- The primary factors concerning the expression vector are

1. The type of promoter/enhancer sequence
2. The type of expression (Transient or Stable)
3. The Degree of expression
4. Efficiency of transfection

1. The type of promoter/enhancer sequence

- Promoters for Constitutive expression
  - Expression of a gene that is transcribed at a constant level. E.g. **?**
- Promoters for Inducible expression
  - Expression of a gene that is transcribed under controlled level.

Constitutive Expression

- A gene that is transcribed continually compared to a facultative gene which is only transcribed as needed.
- Typically a constitutive gene is transcribed at a relatively constant level across many or all known conditions.
- The expression is unaffected by experimental conditions.
- The housekeeping gene's products are typically needed for maintenance of the cell.

  Example:

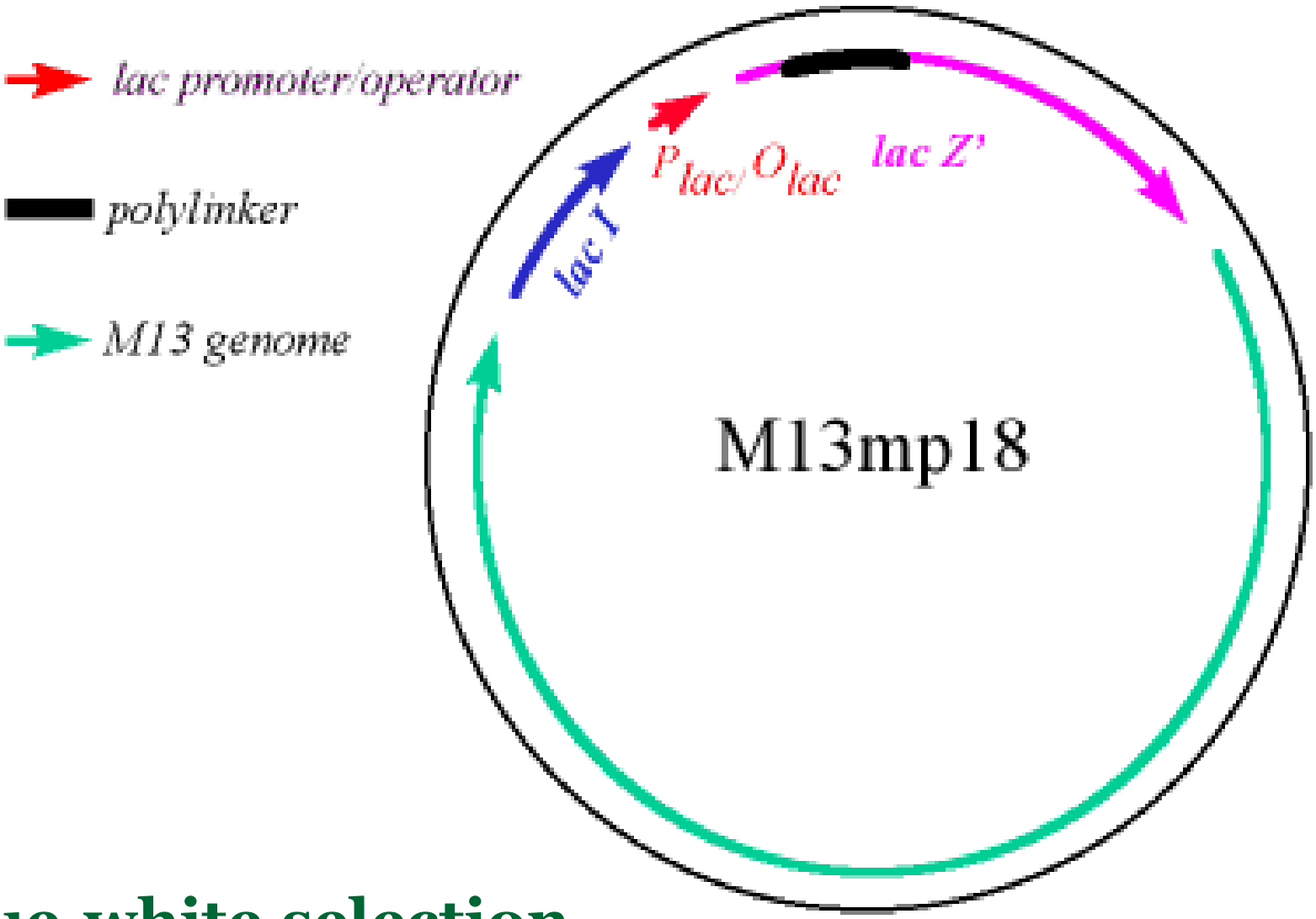
  Examples include actin and ubiquitin.

Inducible Expression

- Regulated gene expression
- Expression is regulated at transcription level and is transcribed as needed.
- Examples
  - The proto-oncogene (ABL)

Inducible system

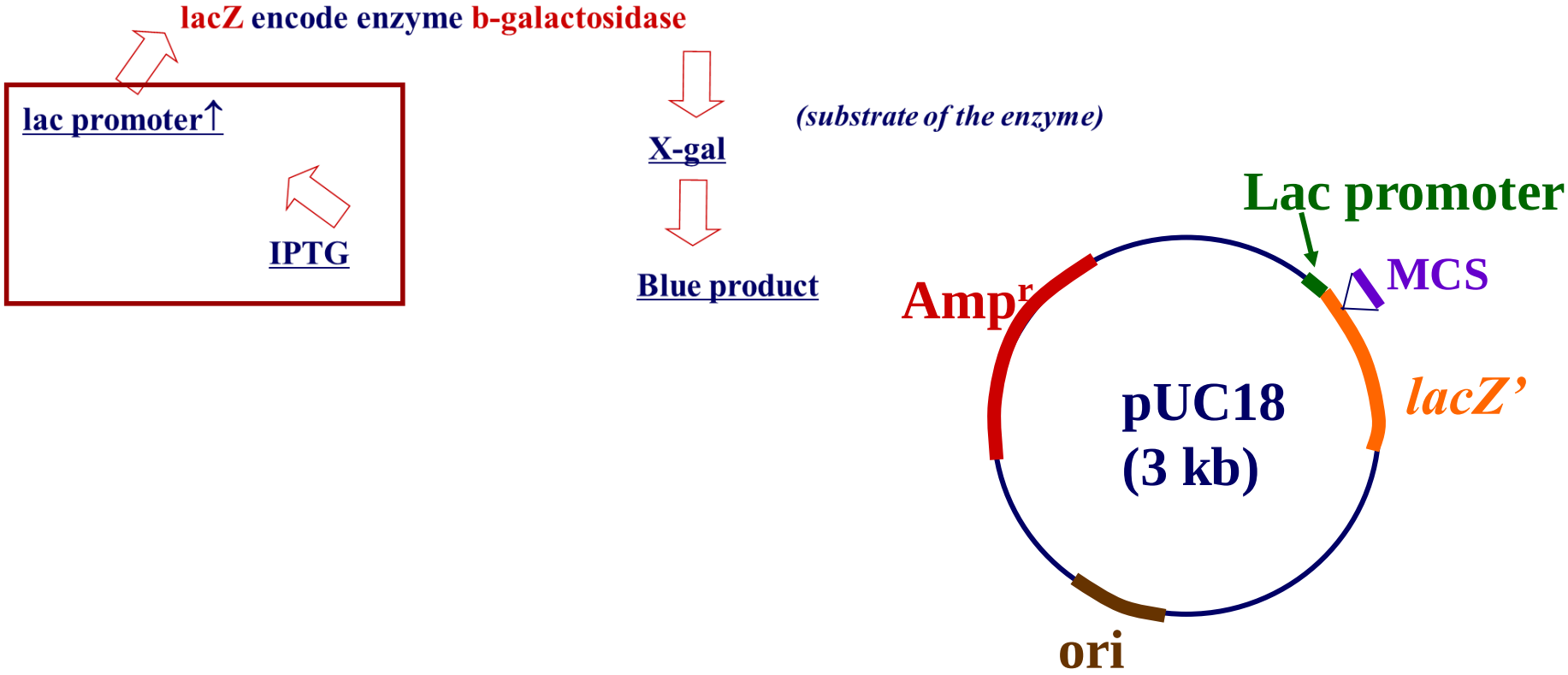
- Protein produced in a large quantity in bacteria can be toxic, so it is advantageous to keep a cloned gene repressed before expressing it.
- Solution is to keep the cloned gene turned off by placing it downstream of an inducible promoter that can be turned off.
- IPTG strongly induce *lac* promoter



Blue-white selection

Blue white screening

Screening by insertional inactivation of the lacZ gene



The insertion of a DNA fragment interrupts the ORF of lacZ' gene, resulting in non-functional gene product that can not digest its substrate x-gal.

Commonly used Promoter/Enhancer elements

1. ß-actin
   - Moderate to strong constitutive cellular transcriptional enhancer
2. Cytomegalovirus (CMV)
   - Strong viral transcriptional enhancer
3. Adenovirus inverted terminal repeats (ITR)
   - Weak viral transcriptional enhancer
4. ß-interferon
   - Virus inducible: enhancer under negative control
5. Metallothionein II (MT II)
   - Inducible by heavy metals, phorbol esters (TPA) and glucocorticoids: tends to be " leaky".

- Negative control
  - Prevention of gene expression by the binding of specific repressor molecules to operator sited
- Positive control
  - Enhancement of gene expression through binding of specific expressor molecule to promoter sites.

2. The type of expression

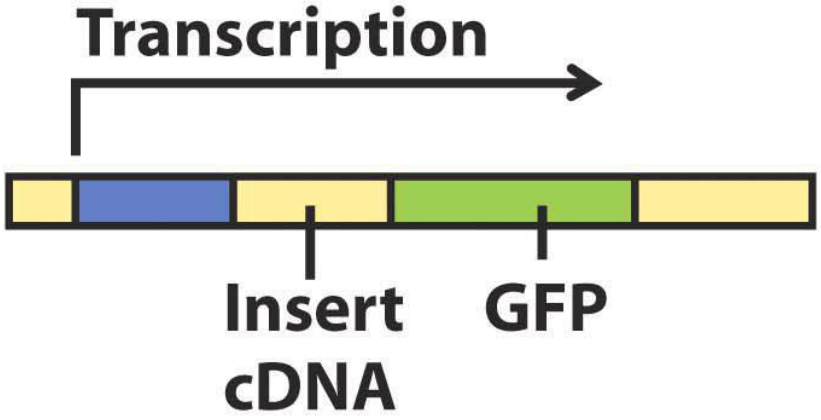
Transient expression

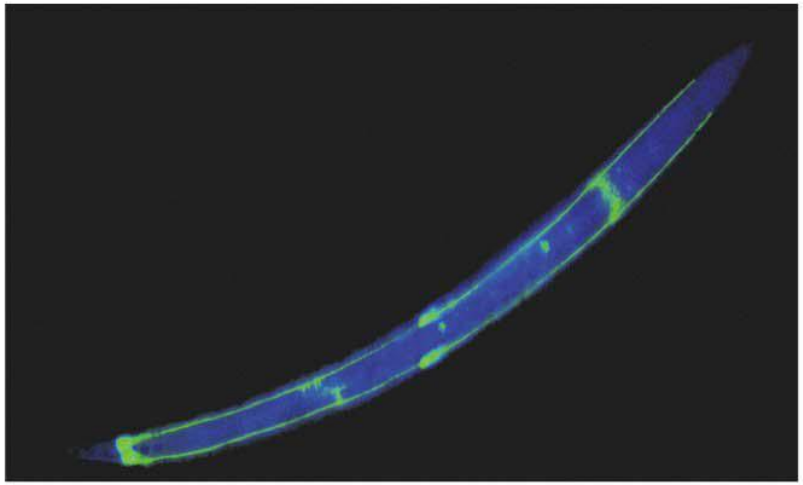
- Useful for
  - Studying elements that regulate gene expression. or
  - When it is important to have experimental results within short time frame.
- Burst of gene expression between 12 and 72 hrs after transfection followed by deterioration in expression of transgene because of cell death or loss of the expression plasmid.

- The optimal time to assay transient expression depends on
  - The cell type
  - The cell doubling time and
  - The characteristics of the vector regulatory elements.
- Transient expression system is evaluated in terms of protein product synthesized in the transfected cells
  - Activity of reporter gene that is not expressed in the cell type used.
  - Evaluated through reporter gene expression. e.g.
    - ß-galactosidase (ß-gal),
    - green fluorescent protein (GFP)
    - firefly luciferase (Luc)

Type of Tags

- Fusion protein
  - Fluorescent proteins
  - One example is the green fluorescent protein or GFP





Stable expression

- Moderate to high level of expression when coupled with an enrichment or selection scheme
- Useful when large quantities of protein expression is required
- For stable expression,
  - The expression vector can either integrate into the host cell genome or
  - Be maintained as an extachromosomal (episomal) element, under condition of chronic selection through selectable marker
- The selectable marker on the expression vector facilitates enrichment of cells that contain the transgene of interest.

- If the promoter/enhancer complex can modulate transcription of the transgene, then expression can be modified (e.g., enhanced or decreased) by compounds (e.g., hormones, metals, antibiotics) that are added to the growth medium.
- A typical vector used to enrich for successfully transfected cells will carry a gene essential for the survival of a given cell line that is either defective in the gene or void of the gene altogether.
- Classic selectable markers include
  - herpes simplex virus thymidine kinase (tk),
  - dihydrofolate reductase (dhfr),
- These genes can only be used in cells deficient in TK or DHFR, respectively.

3. Degree of Gene Expression

The level of transgene expression is influenced by

1. The number of gene copies within the cell
2. The rate of transcription of the gene
3. The stability of the mRNA transcript, and
4. The position of integration with regards to the genomic environment and the flanking DNA

- The number of gene copies within a cell depends, in part, on the number of copies that enter the cell during transfection.
- If the vector contains or is co-transfected with a gene for a drug resistance marker, the rate of expression of the gene of interest can be amplified under increasing concentrations of the selective drug.
- This process can occur if the vector is integrated into the DNA or if it is contained as an extrachromosomal particle within the cell.

4. Method of DNA Delivery/

Efficiency of transfection

- Transformation
- Transfection
- Transduction

The Next Step?

Thanks