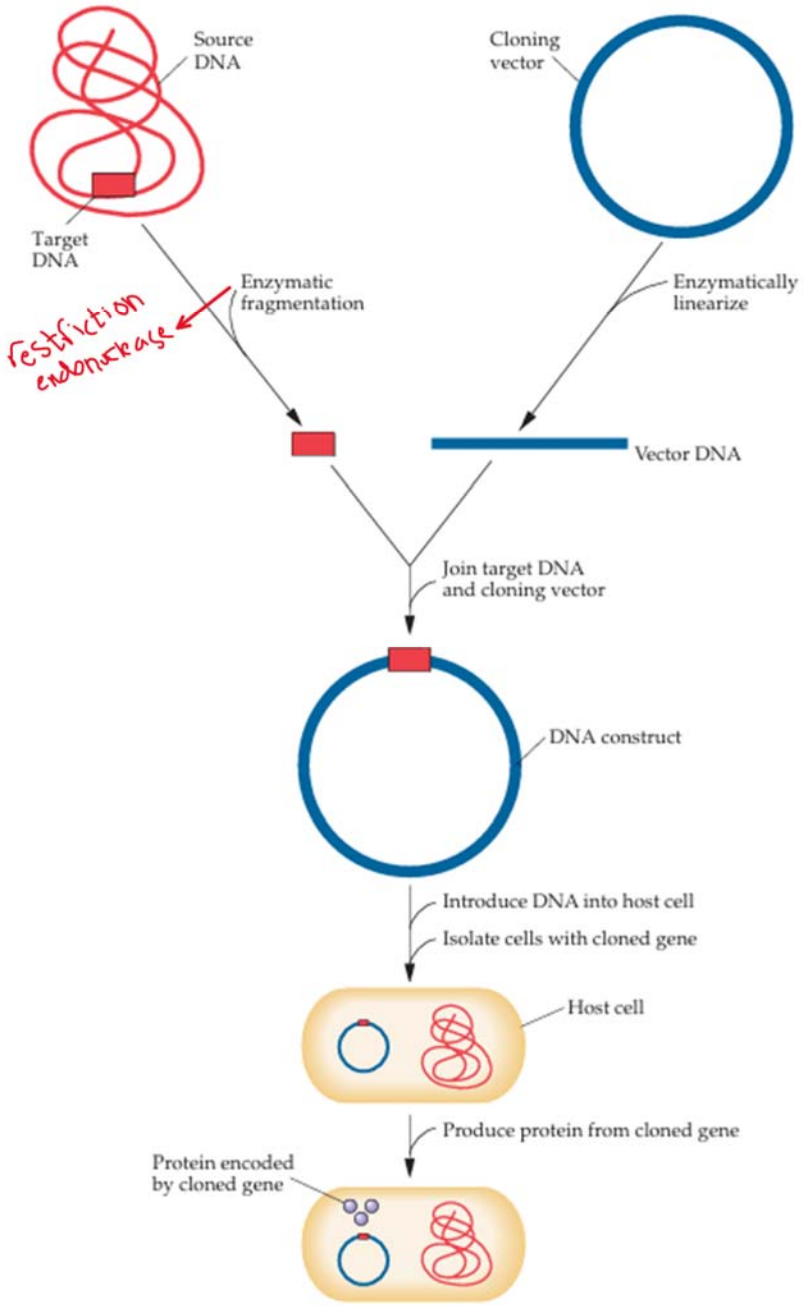
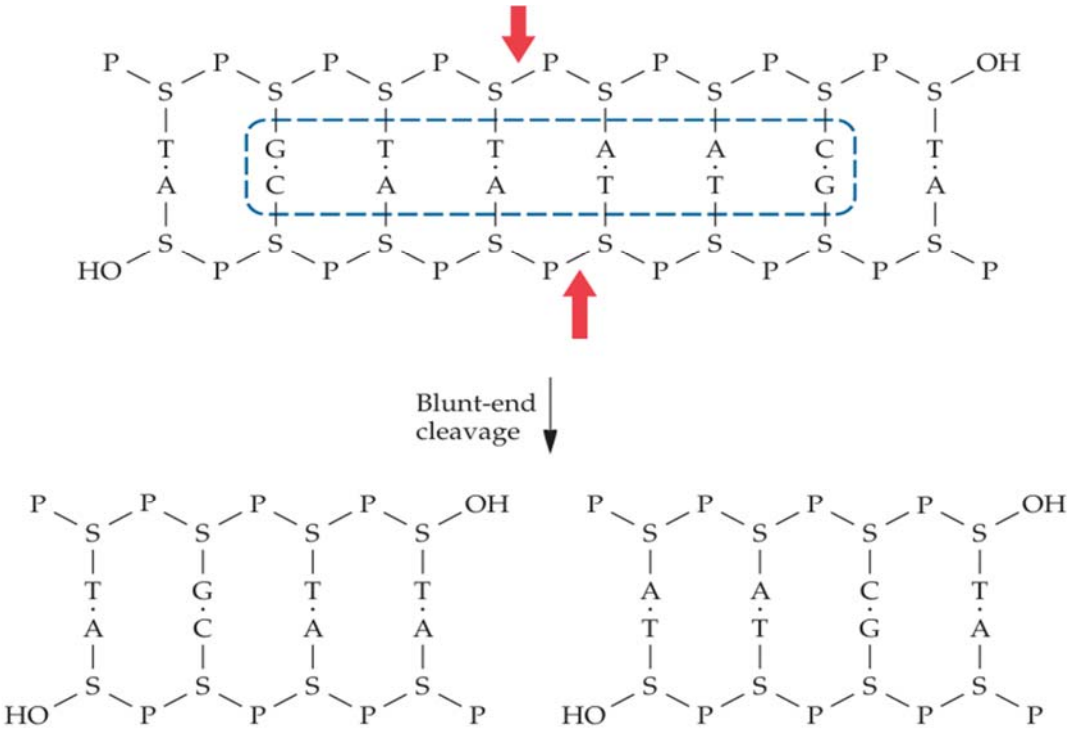
Recombinant DNA technology

- also known as molecular cloning/gene cloning.
- Includes a group of experimental protocols resulting in the transfer of the genetic information from one organism to another.



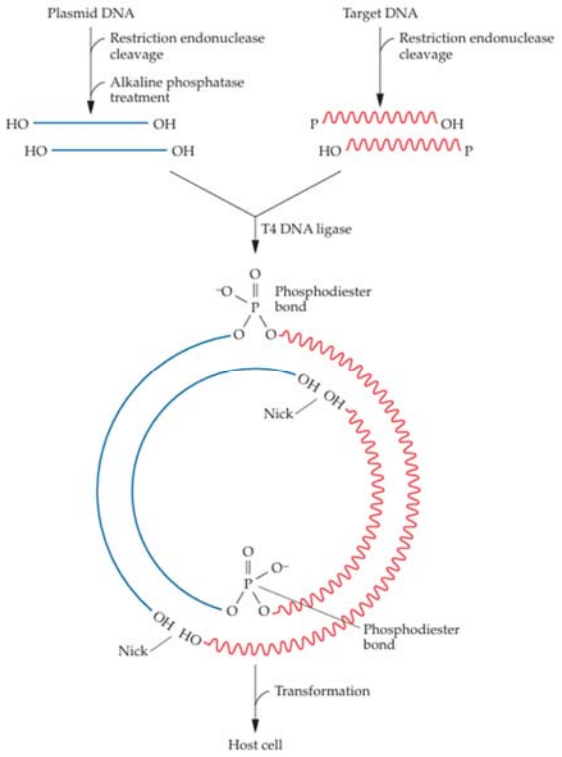
- DNA (from a source organism) is cleaved with a restriction endonuclease and inserted into a cloning vector. The DNA construct is introduced into a host cell, and those cells that carry the construct are identified and grown. If required, the cloned gene can be expressed (transcribed and translated) in the host cell, and the protein (recombinant protein) can be harvested (collected).
- Plasmids: circular, self-replicating form of DNA found in bacteria.
- Scientists use plasmids as tools to clone, transfer, and manipulate genes. Plasmids that are used experimentally for these purposes are called vectors. Researchers can insert DNA fragments or genes into a plasmid vector, creating a so-called recombinant plasmid.



- Blunt-end cleavage of a short fragment of DNA by the type II restriction endonuclease HindII. The large arrows show the sites of cleavage in the DNA back bone.
- HindII is a restriction enzyme (endonuclease) isolated from *Haemophilus influenzae*. The blue box shows the recognition site of this enzyme.

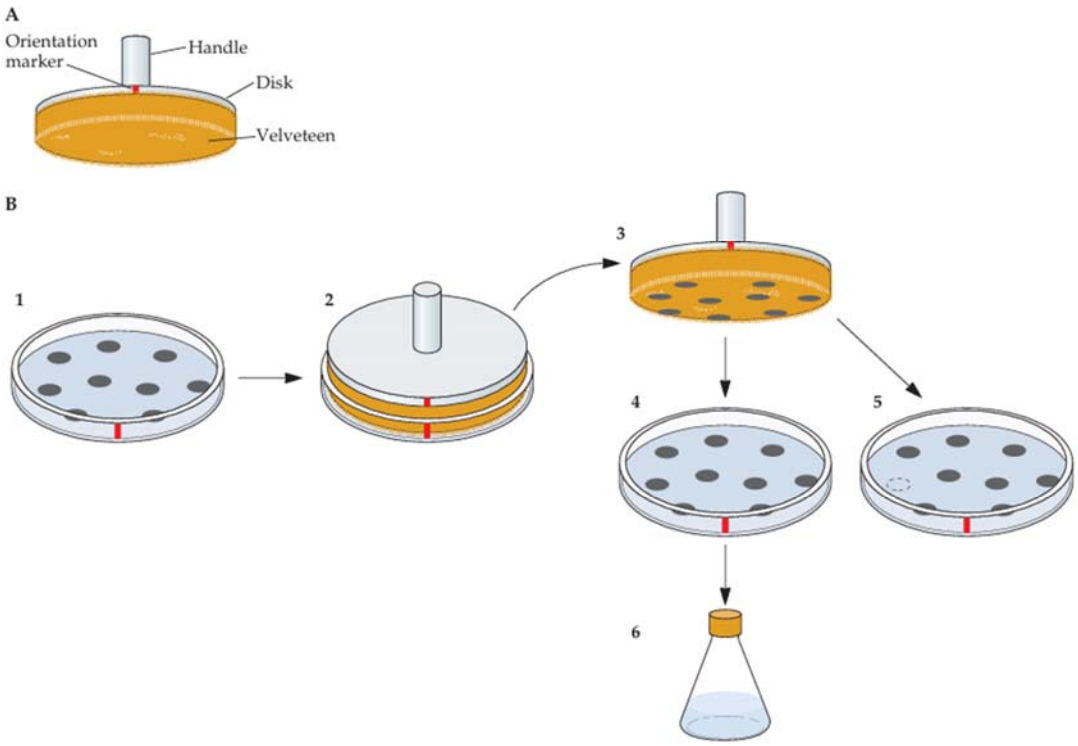
- Enzymes: used as catalysts to cut specific DNA sequence.
- DNA digestion is cutting it by a restriction enzyme.
- The only type of insulin available was the animal based one (such as pigs and cows) until Drs (Boyer, Riggs, and Itakura), made the first genetically engineered insulin in vivo (1977).
- The majority of insulin used today is produced by recombinant DNA (like Novolog).
- Restriction enzymes types of cut ends:

5' phosphate extension (sticky)	3' hydroxyl extension (sticky)	Blunt ends
NotI	PstI	PvuII
EcoRI		HpaI
BamHI		HaeII
Sau3AI		



- Cloning foreign DNA into a plasmid vector. After restriction endonuclease cleavage and alkaline phosphatase treatment, the plasmid DNA is ligated to the restriction endonuclease-digested target DNA, and two of the four nicks are sealed. This molecular configuration is stable, and the two DNA molecules are covalently joined. After introduction into a host cell, ensuing replication cycles produce new complete circular DNA molecules with no nicks.

Screening bacterial colonies for mutant strains by replica-plating:

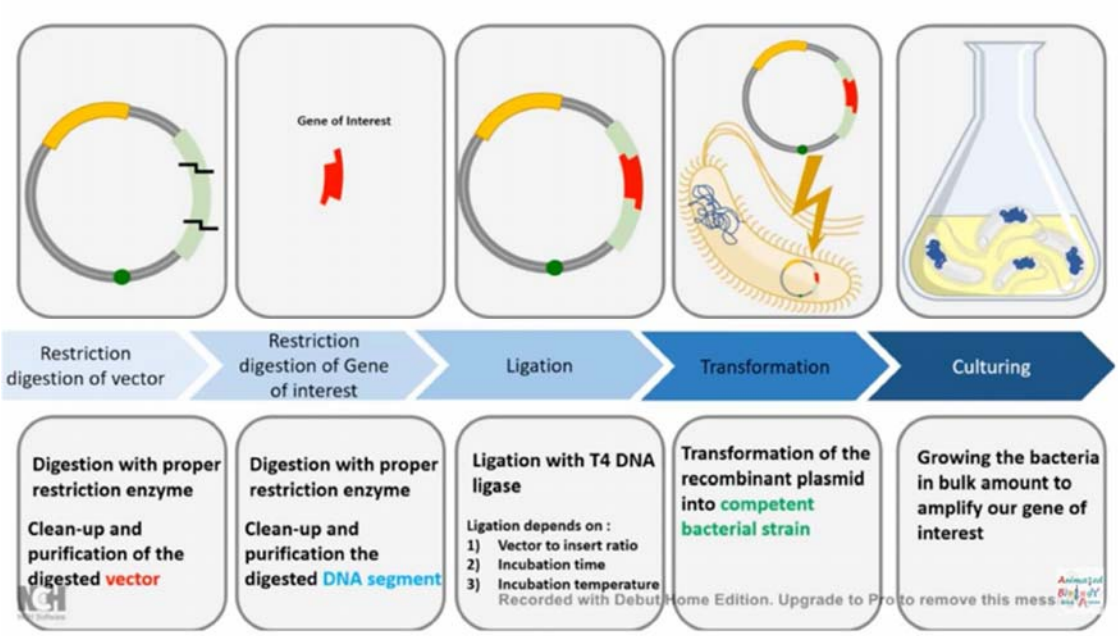


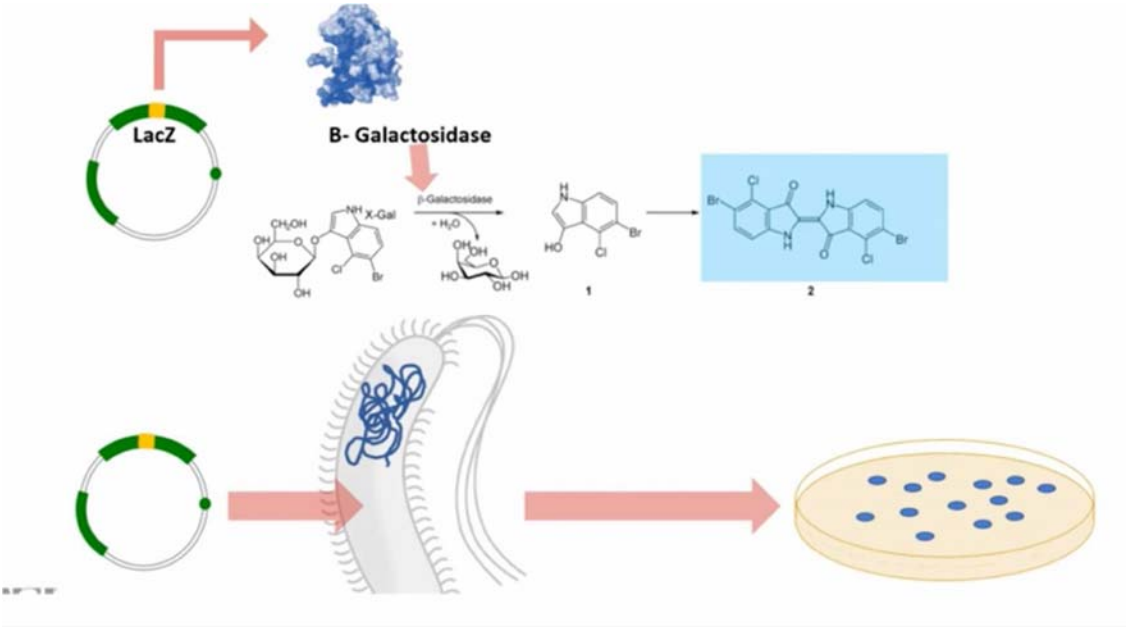
- A: Replica-plating (colony transfer) device.
- Auxotrophic mutants: are microorganisms (or cells) that have lost the ability to synthesize a specific compound, which they now require from the environment to survive. They can't produce certain essential metabolites (like amino acids, vitamins, or nucleotides), so they need to be supplied with those compounds in the growth medium.

- replica-plating technique. Cells from each separated colony on a master plate adhere to the velveteen of the replica plating device after it is gently pressed against the agar surface. The adhering cells are transferred, in succession, to a petri plate with complete medium (4) and to one with selective medium (5). The pattern of the colonies is consistent among the replicated plates because the orientation markers (red squares) are aligned for each transfer. In this example, minimal medium is the selective medium used to identify colonies that require a nutritional supplement for growth (such as auxotrophic mutants). The missing colony (dashed circle) on the minimal medium (5) denotes an auxotrophic mutation. The equivalent location on the plate with complete medium (4) has the colony with the auxotrophic mutation that can be picked and grown (6). Further analysis of the isolated strain is necessary to determine the nature of the auxotrophic mutation.

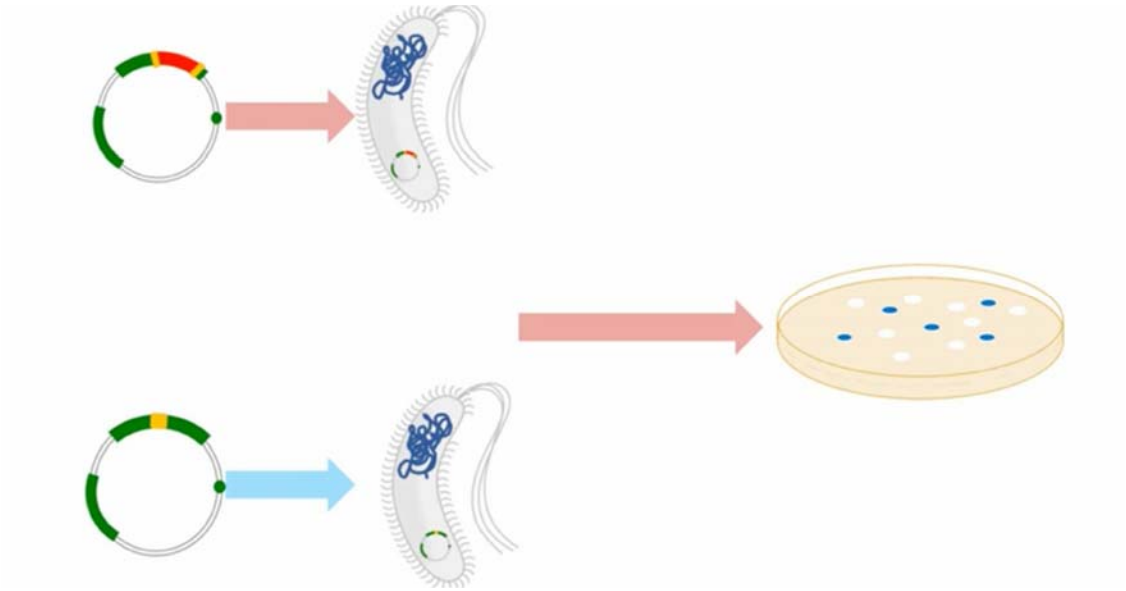
Blue-white screening:

- it is a technique commonly used in genetic engineering to identify and select bacteria that have successfully incorporated a recombinant plasmid (one that contains a piece of foreign DNA).





- The plasmid has the gene LacZ, which produces β-Galactosidase.
- β-Galactosidase breaks X-Gal, giving a blue colored product.
- If LacZ is active the colony will turn blue. (X-Gal is present on the media, and the enzyme is produced by bacteria).



- LacZ (yellow) is disrupted with an inserted gene (red), since it is disrupted, bacteria cannot make β-Galactosidase, thus, no breaking of X-Gal, no blue color. The bottom picture shows the normal (no insertion).

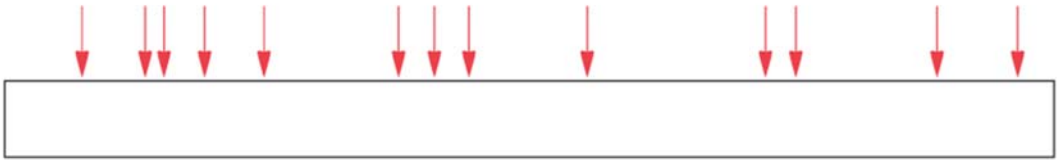
Making a Genomic Library:

- a genomic library is a collection of all DNA fragments from an organism's genome, stored inside vectors (like plasmids or phages) so they can be studied.
- Problems facing the library making: how to clone such big DNA regions? Where to clone? How to screen desired sequence from a collection of sequence?
- The human genome has 3.2×10^9 bp.
- Each lambda phage stores about 20kb of DNA.
- The genomic library is made using the λ-bacteriophage because:
  1. A large number of these bacteriophages can be screened simultaneously (at the same time), 5×10^4 phage plagues (Plaques are clear spots).
  2. Lambda phage has a higher transformation efficiency about 1000 times higher compared to a plasmid.

- BAC (bacterial artificial chromosomes): is a type of plasmid used to clone very large pieces of DNA, 100kb-300kb (way bigger than the lambda phage that can only contain 20kb).
- BAC has multiple sites:
  1. MCS (multiple cloning site): restriction sites.
  2. Antibiotic resistance gene: to select the bacteria that successfully took up the BAC.
- Genomic DNA contain the sequences for both introns and exons.
- Genomic DNA library contain representation for all genes.
- The cDNA clone will only contain sequences found in the mRNA, not the entire gene, while, the genomic clone could have the sequences of the entire gene.
- cDNA library would contain representation for all the genes that are expressed in that cell in a given point of time.

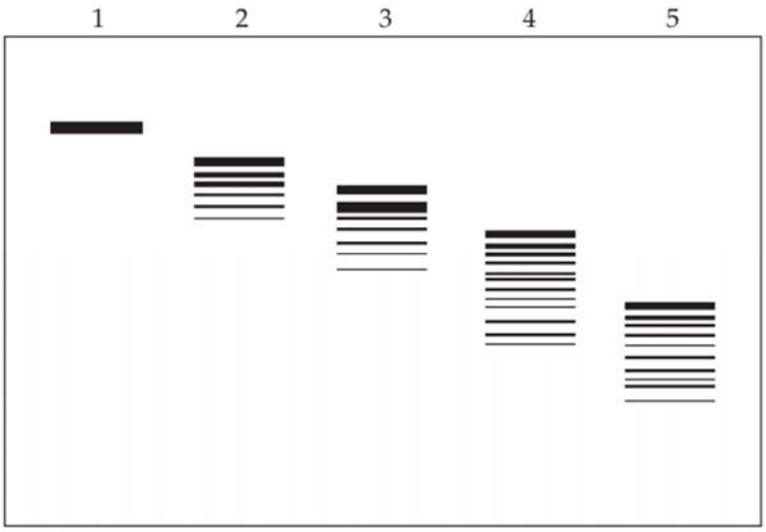
Effect of increasing the time of restriction endonuclease digestion:

A



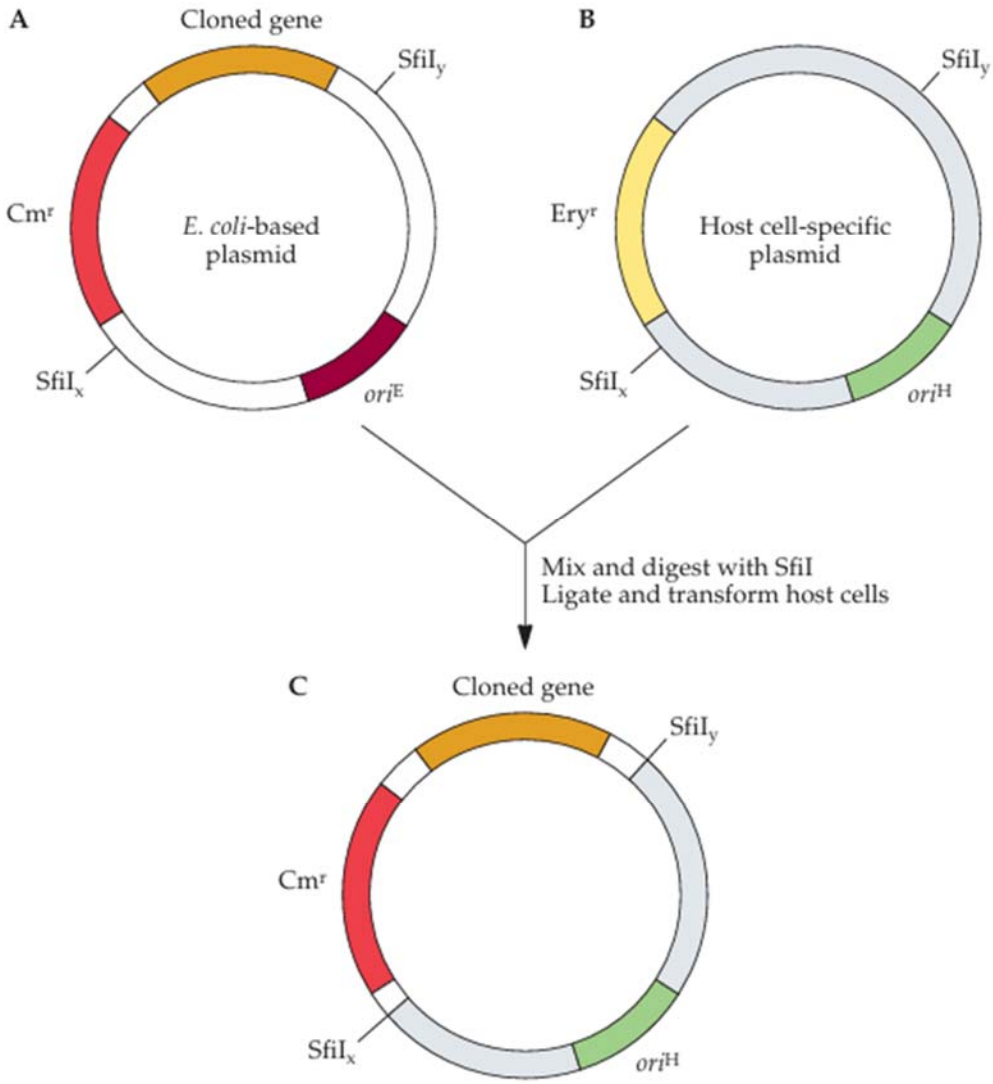
- The restriction endonuclease sites (arrows) of a DNA molecule are shown.

B



- As the duration of restriction endonuclease treatment is extended, cleavage occurs at an increased number of sites (lanes 1 to 5). Lane 1 represents the size of the DNA molecule at the time of addition of restriction endonuclease. Lanes 2 to 5 depict the extents of DNA cleavage after increasing exposures to restriction endonuclease.

Vector backbone exchange:

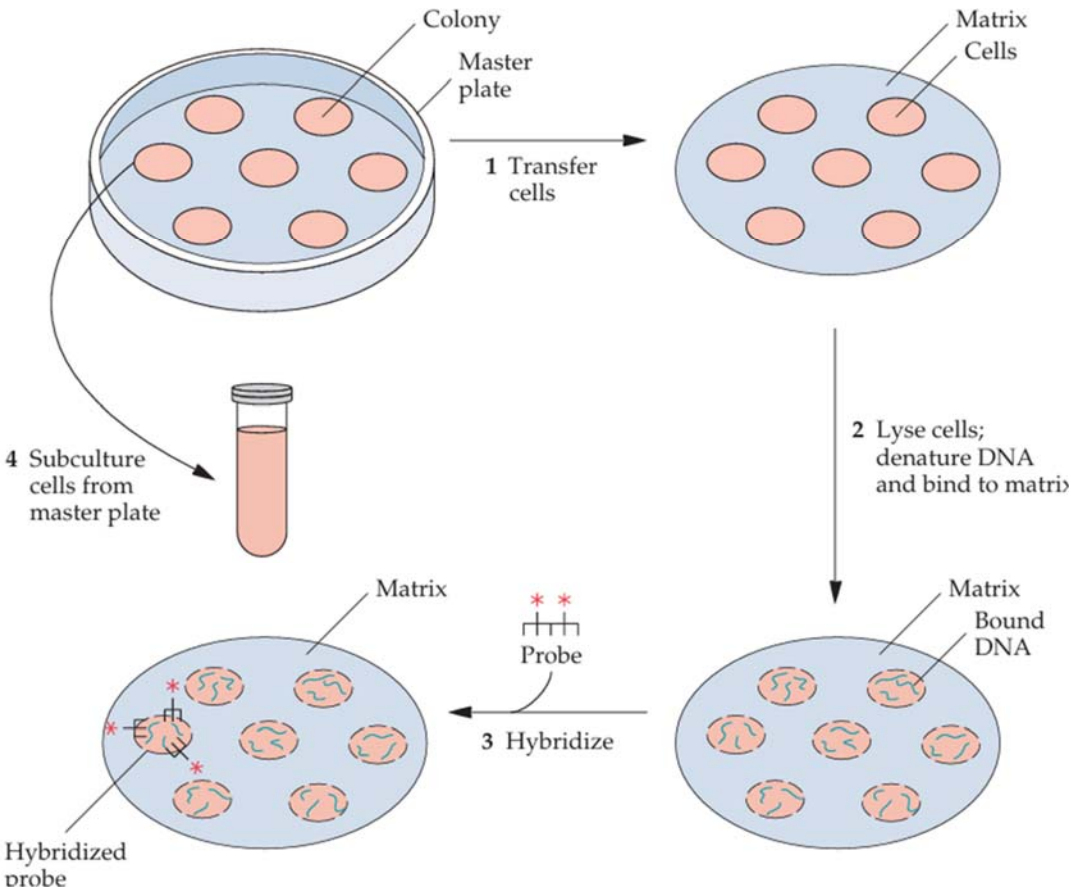


- Shown are an E. coli-based plasmid with a cloned DNA sequence, a chloramphenicol resistance gene (Cmr), and an E. coli-specific origin of replication (oriE) (A) and a host cell-specific plasmid with a host-specific origin of replication (oriH) and an erythromycin resistance gene (Eryr). that have each been engineered with SfiIx and SfiIy recognition sites.
- Treatment of the plasmids shown in panels A and B with SfiI generates two fragments from each plasmid with SfiIx and SfiIy extensions. Several

different circular chimeric DNA molecules are formed after base pairing between complementary extensions and ligation (not shown).

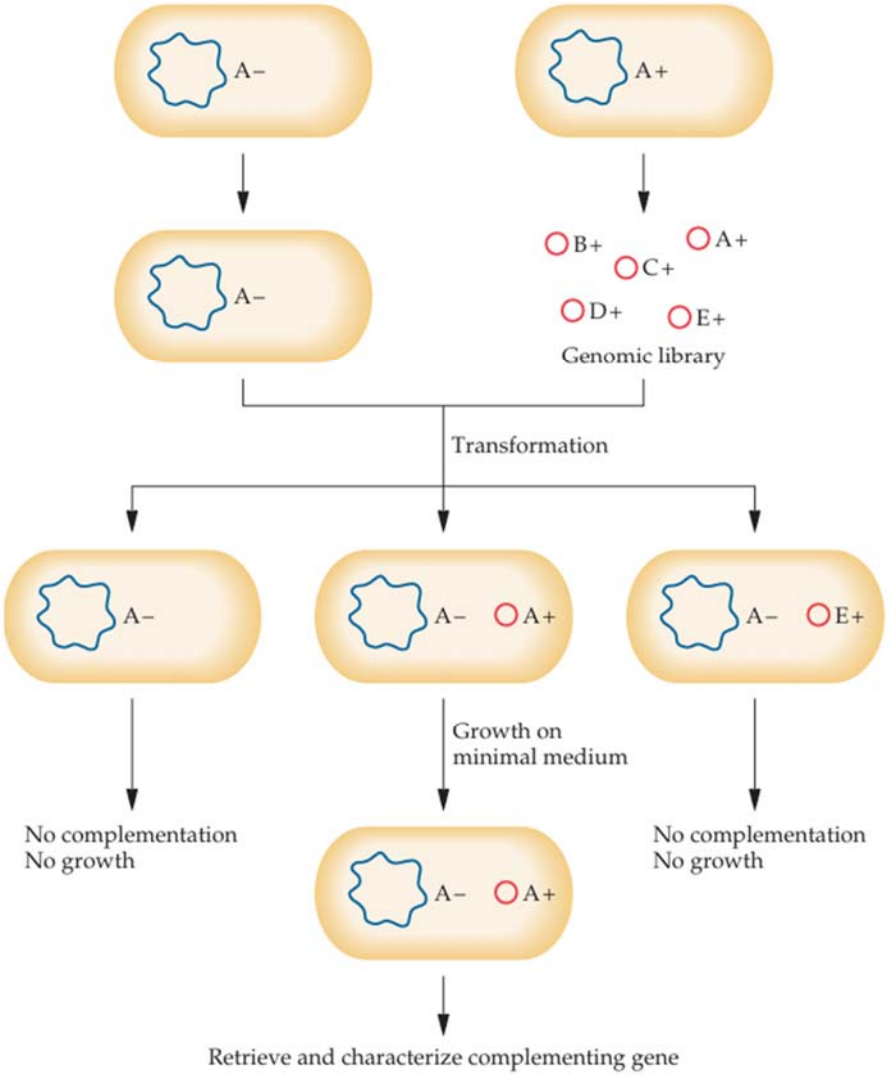
- After transformation of the mixture of chimeric DNA molecules into the host cell, only cells carrying a chimeric plasmid that has an origin of replication that functions in the host cell, as well as the cloned DNA sequence and the chloramphenicol resistance gene from the E. coli-based plasmid, will be selected on medium containing chloramphenicol.

Screening a library with a labeled DNA probe (colony hybridization):



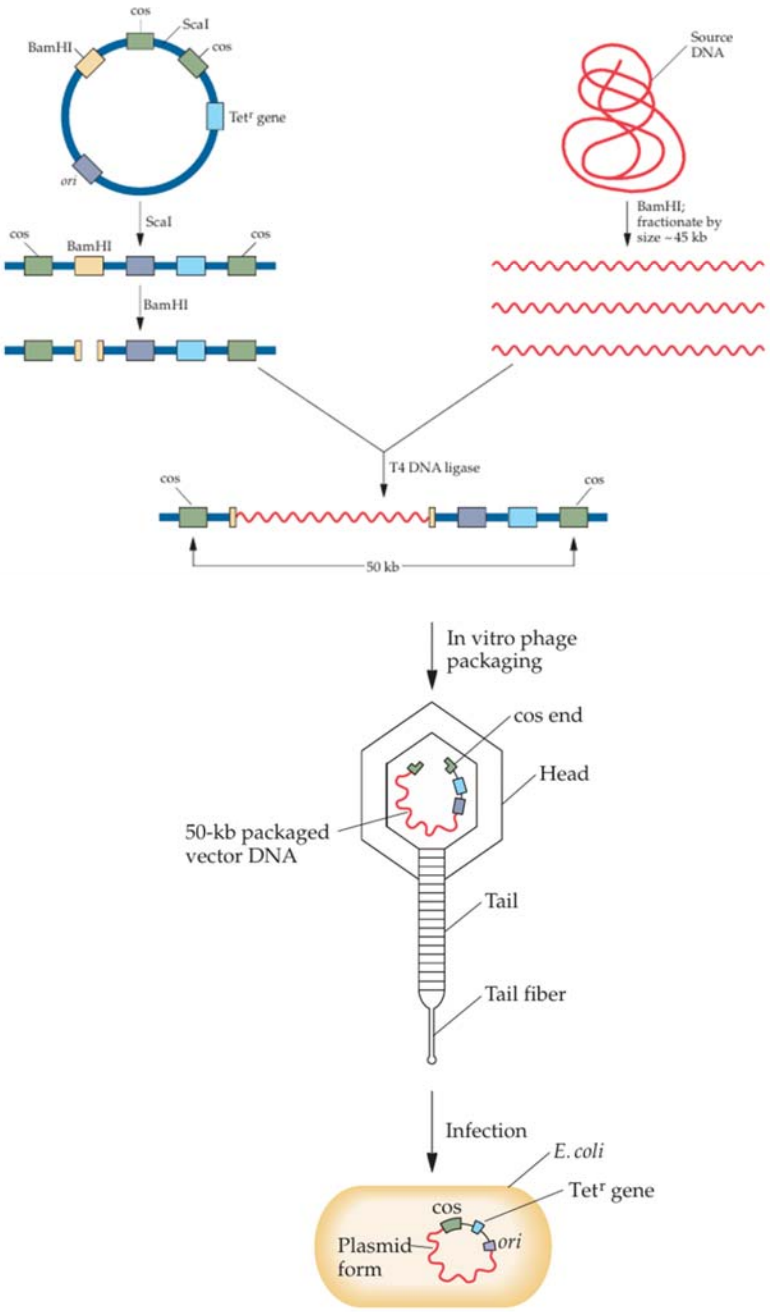
- Cells from the transformation reaction are plated onto solid agar medium under conditions that permit transformed, but not non-transformed, cells to grow.
- From each discrete colony formed on the master plate, a sample is transferred to a solid matrix, such as a nitrocellulose or nylon membrane. The pattern of the colonies on the master plate is retained on the matrix.
- The cells on the matrix are lysed, and the released DNA is denatured, deproteinized, and irreversibly bound to the matrix.
- A labeled DNA probe is added to the matrix under hybridization conditions. After the nonhybridized probe molecules are washed away, the matrix is processed by autoradiography to determine which cells have bound labeled DNA.
- A colony on the master plate that corresponds to the region of positive response on the X-ray film is identified. Cells from the positive colony on the master plate are sub-cultured because they may carry the desired plasmid–cloned DNA construct.

Gene cloning by functional complementation:



- Host cells that are defective in a certain function, e.g., A−, are transformed with plasmids from a genomic library derived from cells that are normal with respect to function A, i.e., A+. Only transformed cells that carry a cloned gene that confers the A+ function will grow on minimal medium. The cells that show complementation are isolated, and the insert of the vector is studied to characterize the gene that corrects the defect in the mutant host cells.

A cosmid cloning system:



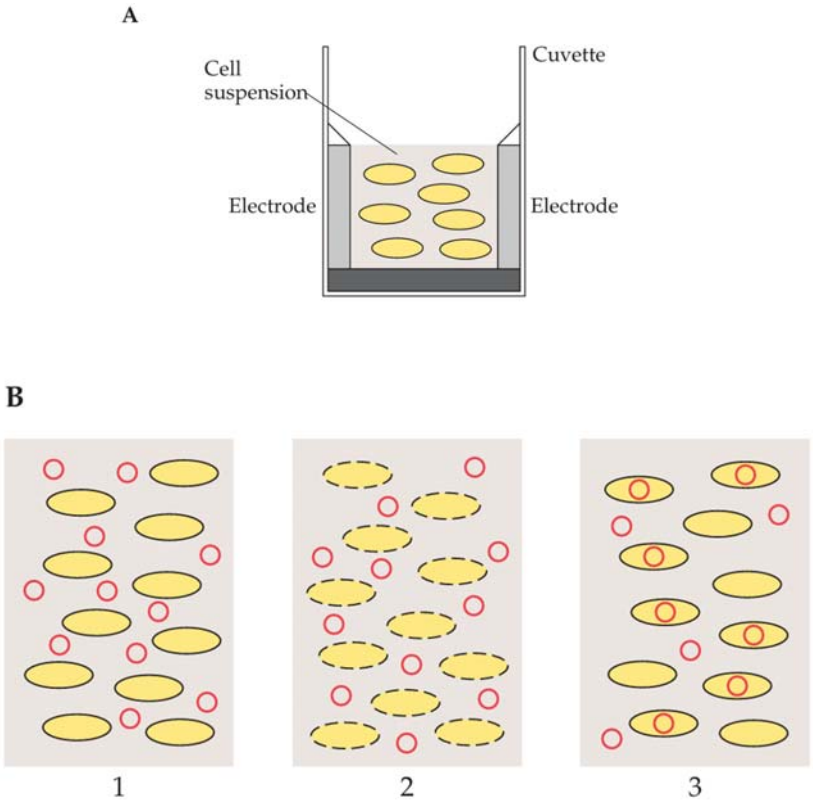
- The cosmid contains an E. coli origin of replication (ori) that allows the cosmid to be maintained as a plasmid in E. coli; two intact cos sites closely flanking a unique ScaI site; a unique BamHI site near, but outside, one of the cos sites; and a Tetr gene.

- The source DNA is cut with BamHI and fractionated by size to isolate molecules that are about 45 kb long. The plasmid DNA is cut with ScaI and BamHI. The two DNA samples are mixed and treated with T4 DNA ligase.
- After ligation, some of the joined DNA molecules will have a 45-kb piece of DNA inserted into the BamHI site of the plasmid; when this happens, the two cos sequences are about 50 kb apart. These molecules are packaged into bacteriophage λ heads in vitro, and infective particles are formed after the addition of tail assemblies.
- Infective bacteriophage λ delivers a linearized DNA molecule with cos extensions into E. coli. After entry into the host cell, the cos ends base pair and the DNA ligase of the host cell seals the nicks.
- The circular DNA molecule that is created in this way persists as a plasmid in the host cell. In this case, transformed cells can be identified because they are resistant to the antibiotic tetracycline.

Genetic Transformation of Prokaryotes:

- Heat shock: by treating mid-log-phase cells with ice-cold calcium chloride (CaCl2) and then exposing them for 2 minutes to a high temperature (42°C). This treatment creates transient openings in the cell wall that enable DNA molecules to enter the cytoplasm.
- transformation frequency of about 1 transformed cell per 1,000 cells (10^{-3}).
- The transformation efficiency is approximately 10^7 to 10^8 transformed colonies per microgram of intact plasmid DNA.
- Electroporation: By subjecting bacteria to a high voltage
- For E. coli, the cells (~50 microliters) and DNA a single pulse of 25 microfarads, 2.5 kilovolts, and 200 ohms is administered for about 4.6 milliseconds.

- Transformation efficiencies of 109 transformants per microgram of DNA for small plasmids (~3 kb) and 106 for large plasmids (~136 kb).
- Similar conditions are used to introduce BAC vector DNA
- Plasmids containing inserts that are longer than 100 kb. Very little is known about the mechanism



- (A) Electroporation cuvette with a cell suspension between two electrodes.
- (B) (1) Cells (yellow) and DNA (red) in suspension in an electroporation cuvette prior to the administration of high-voltage electric field (HVEF) pulses.
- (2) HVEF pulses induce openings in the cells (dashed lines) that allow entry of DNA into the cells.
- (3) After HVEF pulsing, some cells acquire exogenous DNA, and the HVEF-induced openings are resealed.