Lecture 4 – Vectors for Constructing Genomic and cDNA Libraries

Cloning Vectors

- **Purpose**
  - ✓**Facilitates the insertion, storage and manipulation of exogenous DNA**
- **Types**
  - ✓**Plasmids**
  - ✓**Bacteriophages**
  - ✓**Cosmids**
  - ✓**BACs, PACs and YACs**

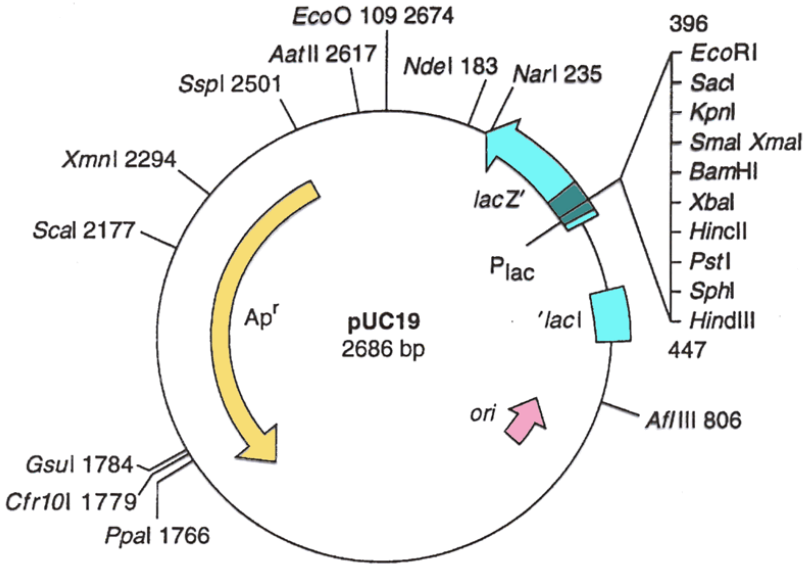
Plasmids

- **Plasmid vectors are double-stranded, circular, self-replicating, extra-chromosomal DNA molecules.**
- **Advantages:**
  - ✓ **Small, easy to handle**
  - ✓ **Straightforward selection strategies**
  - ✓ **Useful for cloning small DNA fragments (< 10kbp)**
  - ✓ **Replicate autonomously**
- **Disadvantages:**
  - ✓ **Less useful for cloning large DNA fragments (> 10kbp)**

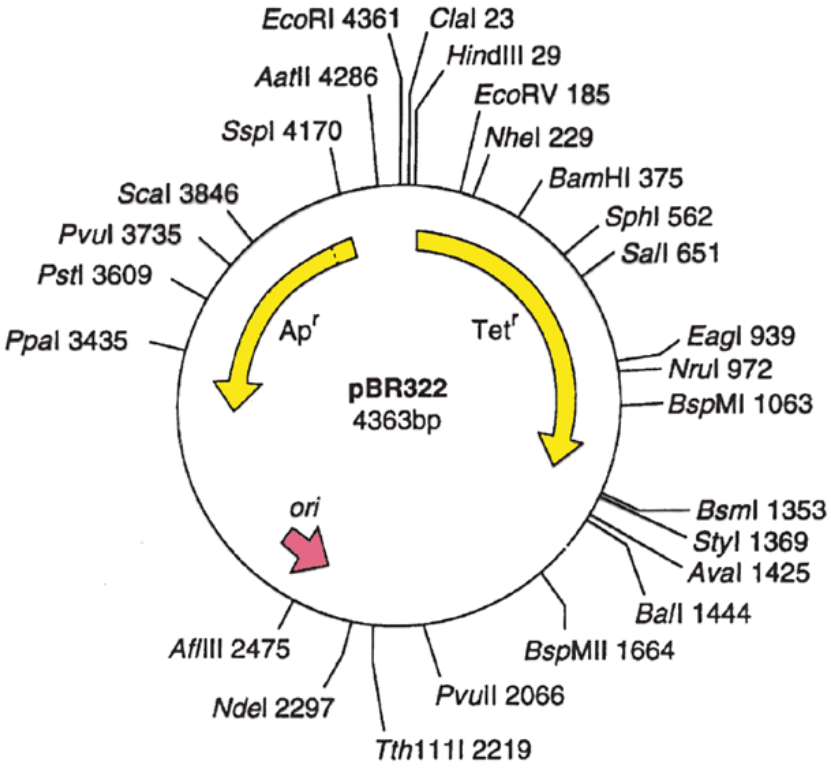
Features of Plasmid Vectors

- Contain an origin of replication, allowing for replication independent of host's genome
- Contain selective markers for antibiotic resistance and blue-white screening
- Contain a multiple cloning site (MCS)
- Easy to isolate from host cell

Examples of Plasmid Vectors



- **pUC19 is one of a series of plasmid cloning vectors created by Joachim Messing et al. at UC Davis.**
- **The designation "pUC" is derived from the classical "p" prefix (denoting “plasmid”) and the abbreviation for the University of California, where early work on the plasmid series had been conducted.**



- **pBR322 was one of the first widely used E. coli cloning vectors created in Herbert Boyer laboratory at UC San Francisco.**
- **It was named after the postdoctoral researchers who constructed it: Bolivar and Rodriguez.**

Bacteriophages

Lambda (λ) phage

- Lambda (λ) phage is a virus particle consisting of a head, containing double-stranded linear DNA (ds DNA)as its genetic material, and a tail that can have tail fibers.
- The phage particle recognizes and binds to its host, *E. coli*, causing DNA in the head of the phage to be ejected through the tail into the cytoplasm of the bacterial cell.
- Usually, a "lytic cycle" ensues, where the lambda DNA is replicated many times and the genes for head, tail and lysis proteins are expressed.
- This leads to assembly of multiple new phage particles within the cell and subsequent cell lysis, releasing the cell contents, including virions that have been assembled, into the environment.

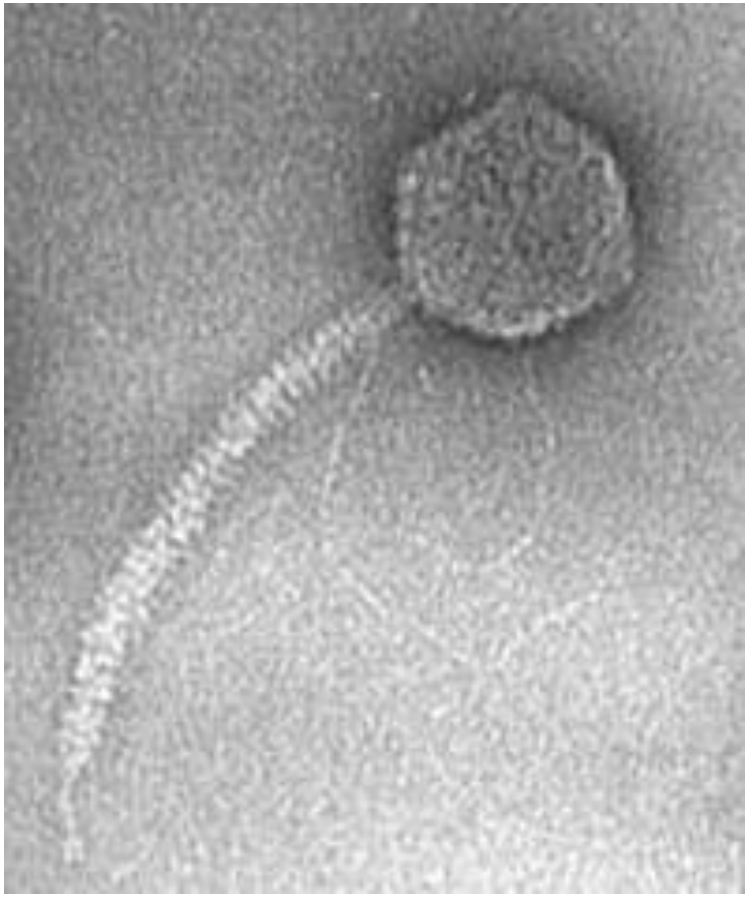
M13 phage

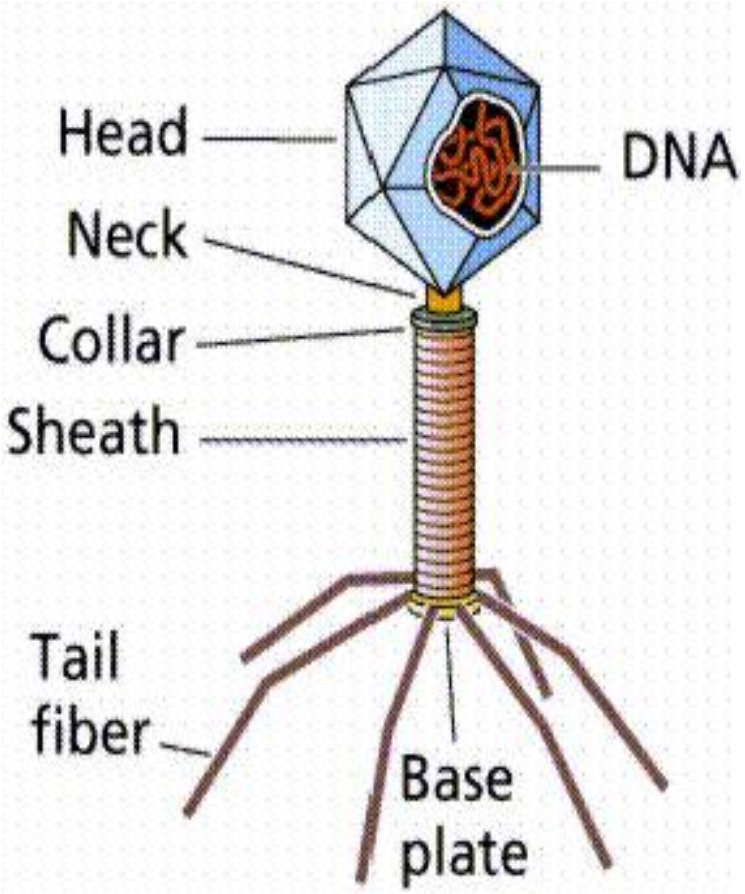
- M13 is a filamentous bacteriophage composed of circular single stranded DNA (ssDNA), which is 6407 nucleotides long encapsulated in approximately 2700 copies of the major coat protein P8, and capped with 5 copies of two different minor coat proteins (P9, P6, P3) on the ends.

Bacteriophages

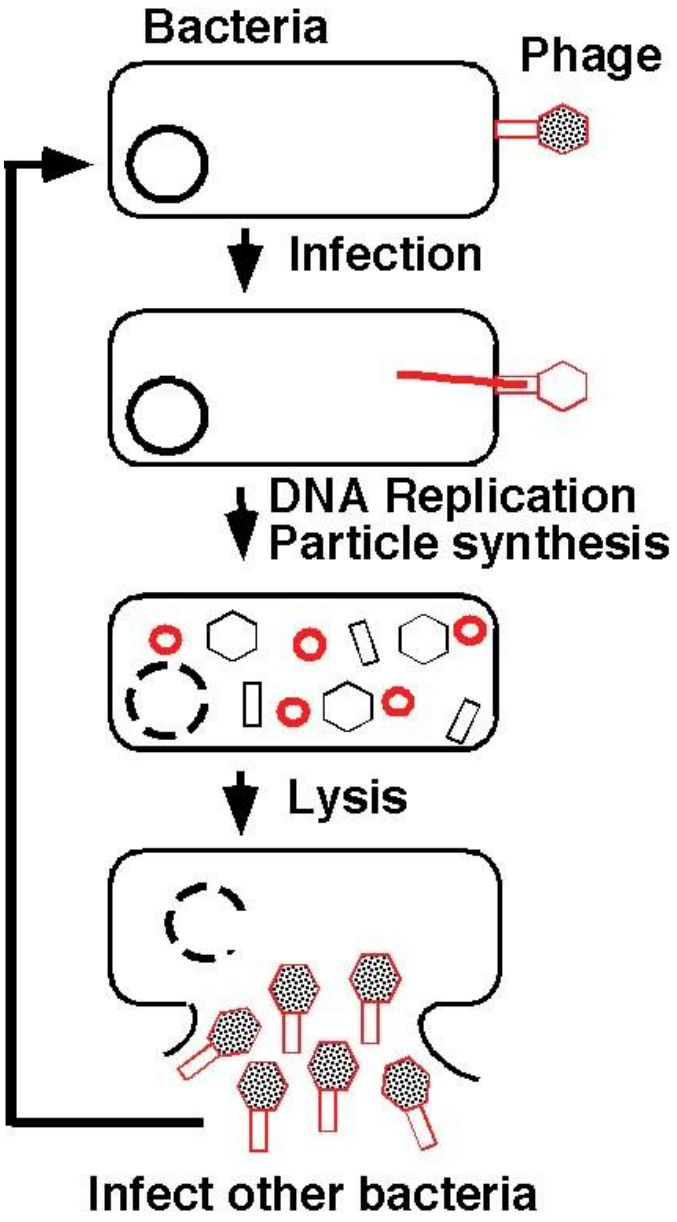
- **Advantages:**
  - ✓ **Useful for cloning large DNA fragments (10-23 kbp)**
  - ✓ **Inherent size selection for large inserts**
- **Disadvantages:**
  - ✓ **Less easy to handle than plasmid vectors**

λ Phage

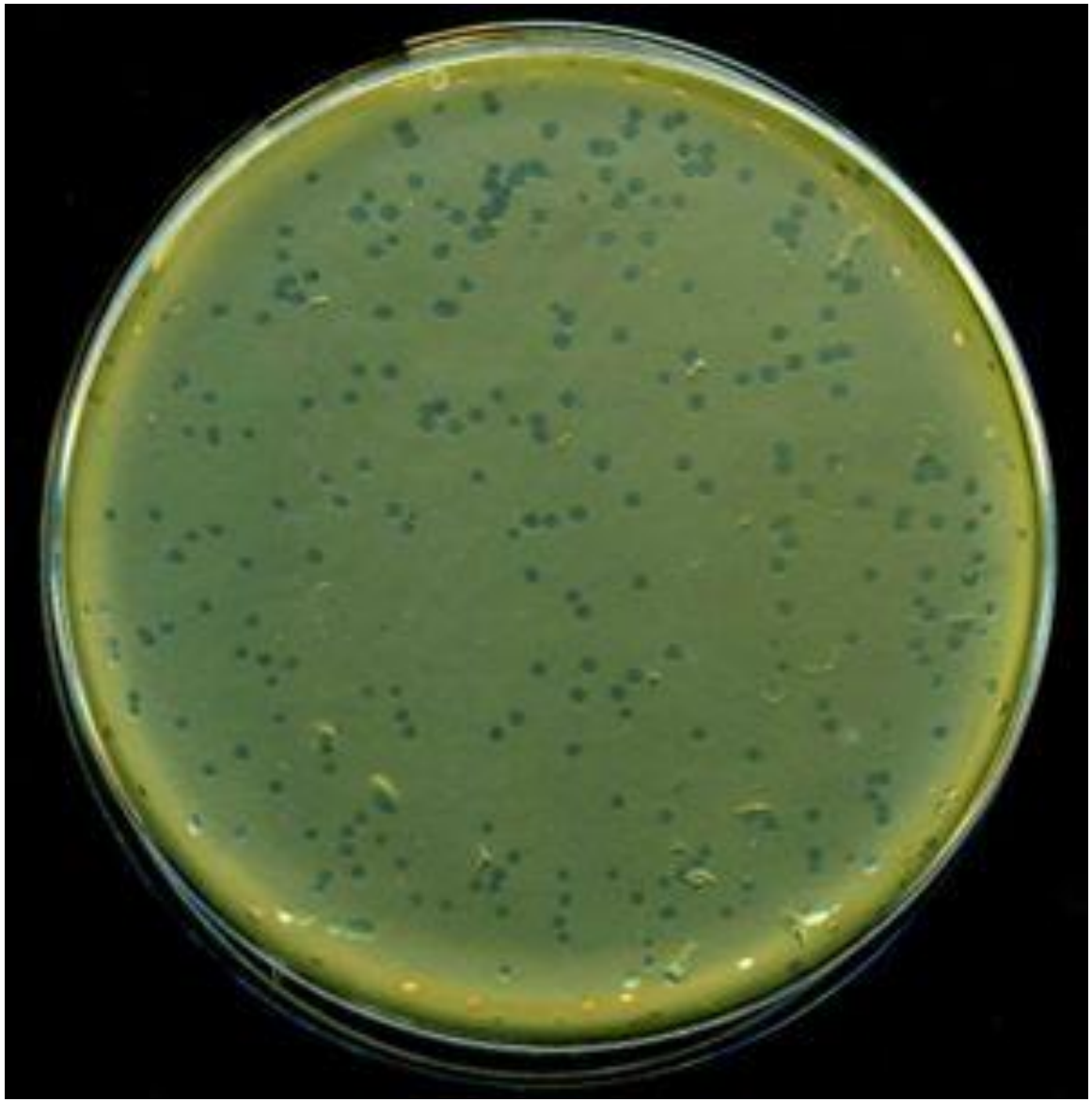




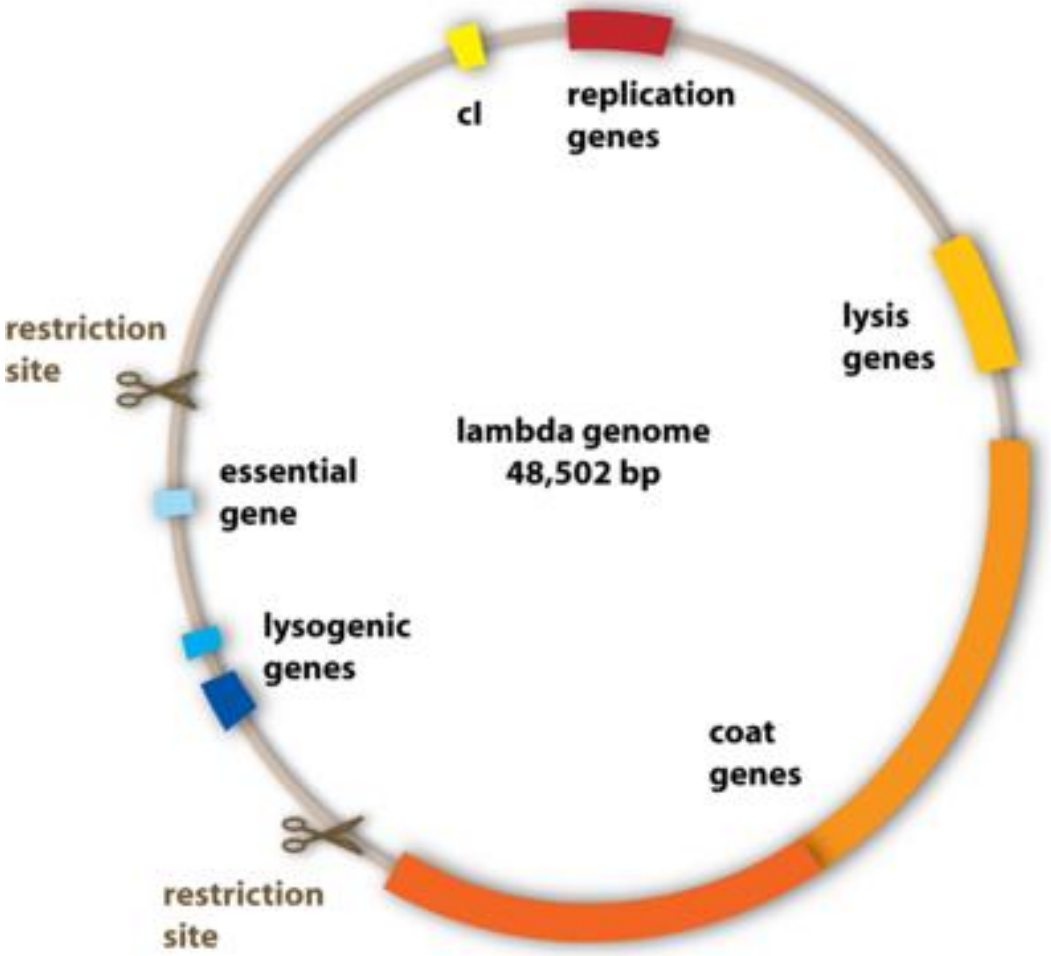
Life Cycle of λ Phage



Lysis Plaques of Lambda Phage on *E. coli*

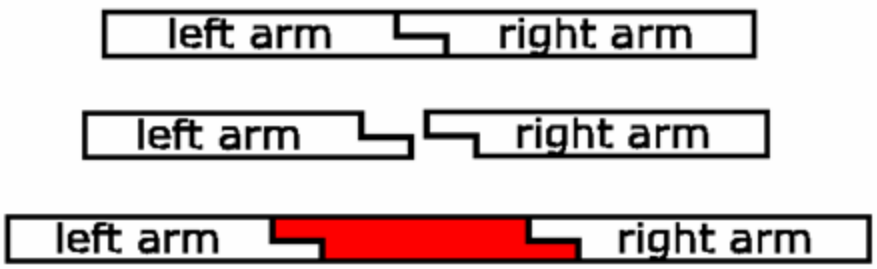


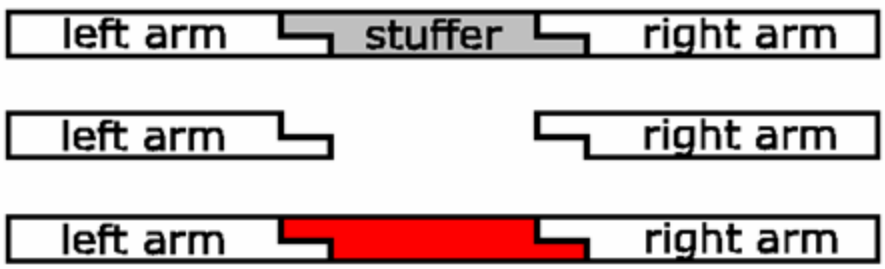
λ Phage Vector



Lambda Vector

- Infectious λ can be assembled *in vitro*.
- Foreign DNA can be incorporated into the λ genome.
- Non-essential genes are removed.
- Phage assembly can occur with 40-52 kb of DNA





- accommodates up to 7-10 kb of foreign DNA (depending on the vector)

- 13 kb “stuffer fragment” (lysogeny genes) discarded
- accommodates 11-20 kb of foreign DNA

Lambda Insertion Vectors

- Insertion vectors are the simplest form of lambda cloning vectors.
- The vector itself can be grown (must contain at least 75% of the wild-type genome length).
- Foreign DNA fragments are inserted into unique restriction sites in the vector genome.
- Packaging requirements limit insert fragment size to 0-10 kb due to the limitations on viral genome size (75% to 105% of the wild-type length = 50 kb).
- In the construction of insertion vectors, 25% of the dispensable portion of the wild-type lambda genome is eliminated.
- Because the lambda genome approximates a random 50-kb DNA sequence, most 6-mer restriction enzymes will cut several times (average frequency is 1/4000 bp).
- Removal of unwanted restriction sites involves mutagenesis of the phage DNA followed by selection for efficient growth on hosts carrying different restriction/modification systems.
- After multiple rounds of mutagenesis and selection, phage genomes lacking unwanted restriction sites are recovered.

Lambda Replacement Vectors

- **Lambda replacement vectors have a loading capacity of 10-23 kb and consists of a full-length lambda molecule with two identical restriction sites flanking a non-essential region called a stuffer fragment.**

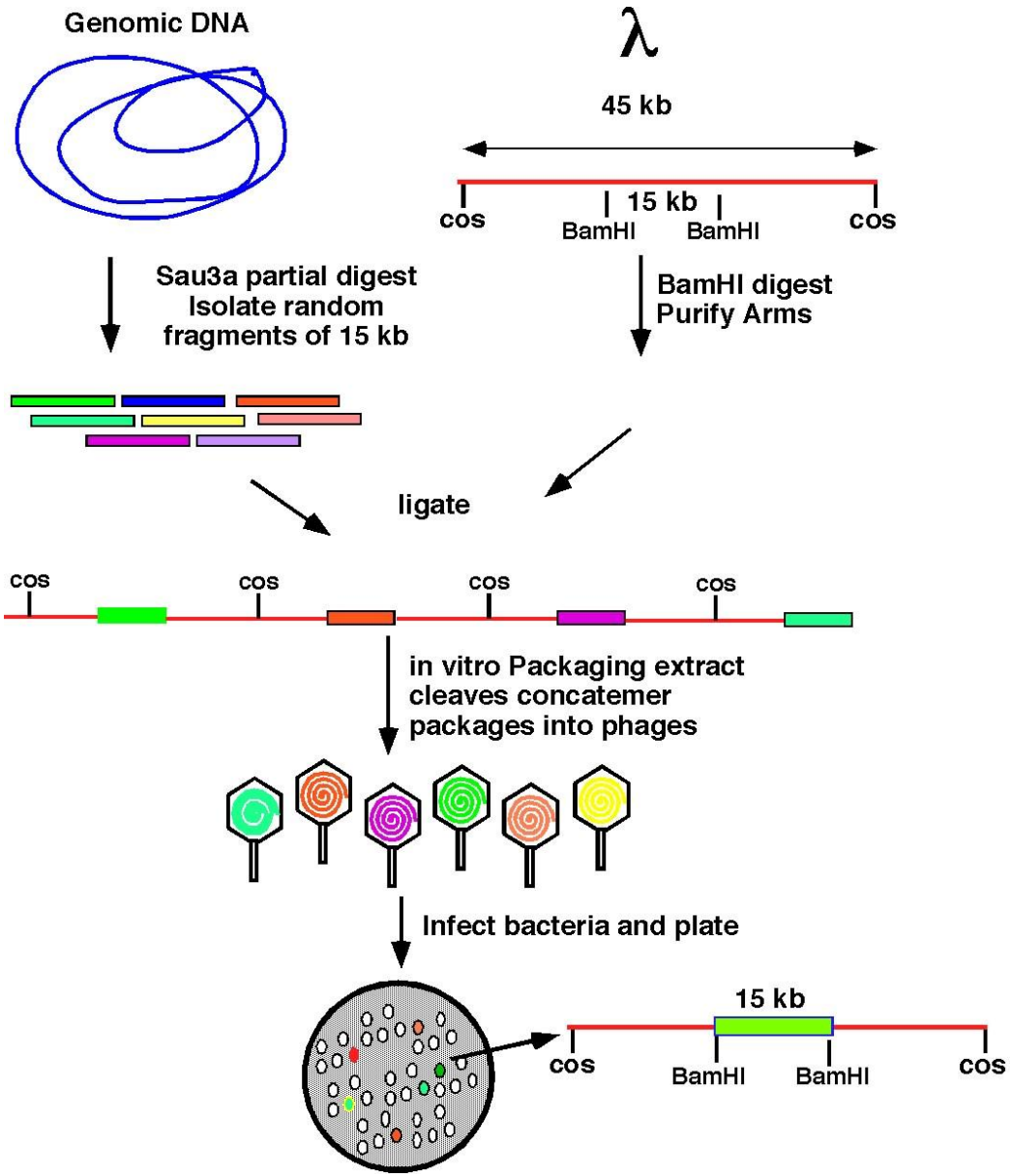
- **A stuffer fragment is deleted and replaced by foreign DNA.**

- **A selection system is required to differentiate between wild-type and recombinant phage.**

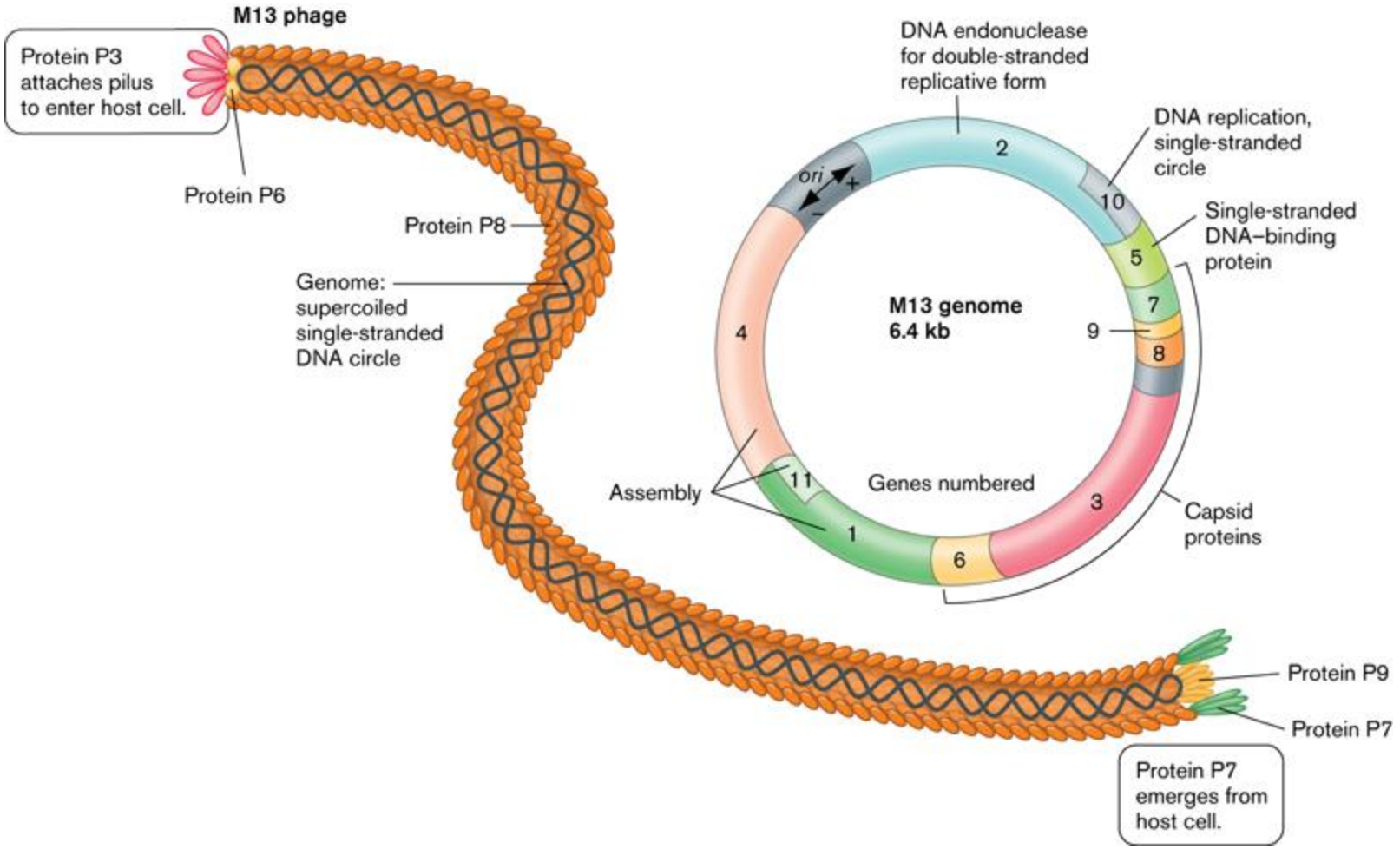
- **This is done by placing relevant genes onto the stuffer fragment, the loss of which gives rise to a detectable phenotypic signal.**

- **One example is:**
  - ✓**LacZ inactivation by inserting the lacZ gene onto the stuffer fragment**
  - ✓**Loss of the fragment and, therefore, lacZ can be monitored by the well known blue/white color discrimination.**

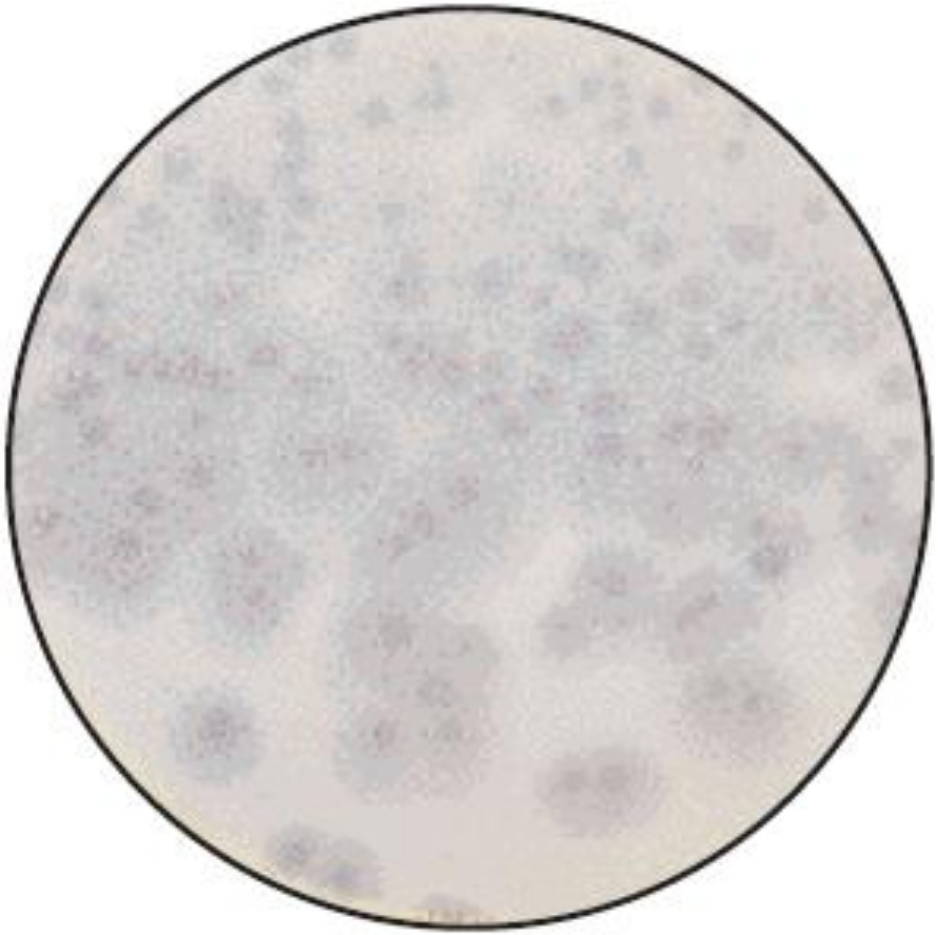
Constructing a Genomic Library in λ Phage



M13 Phage

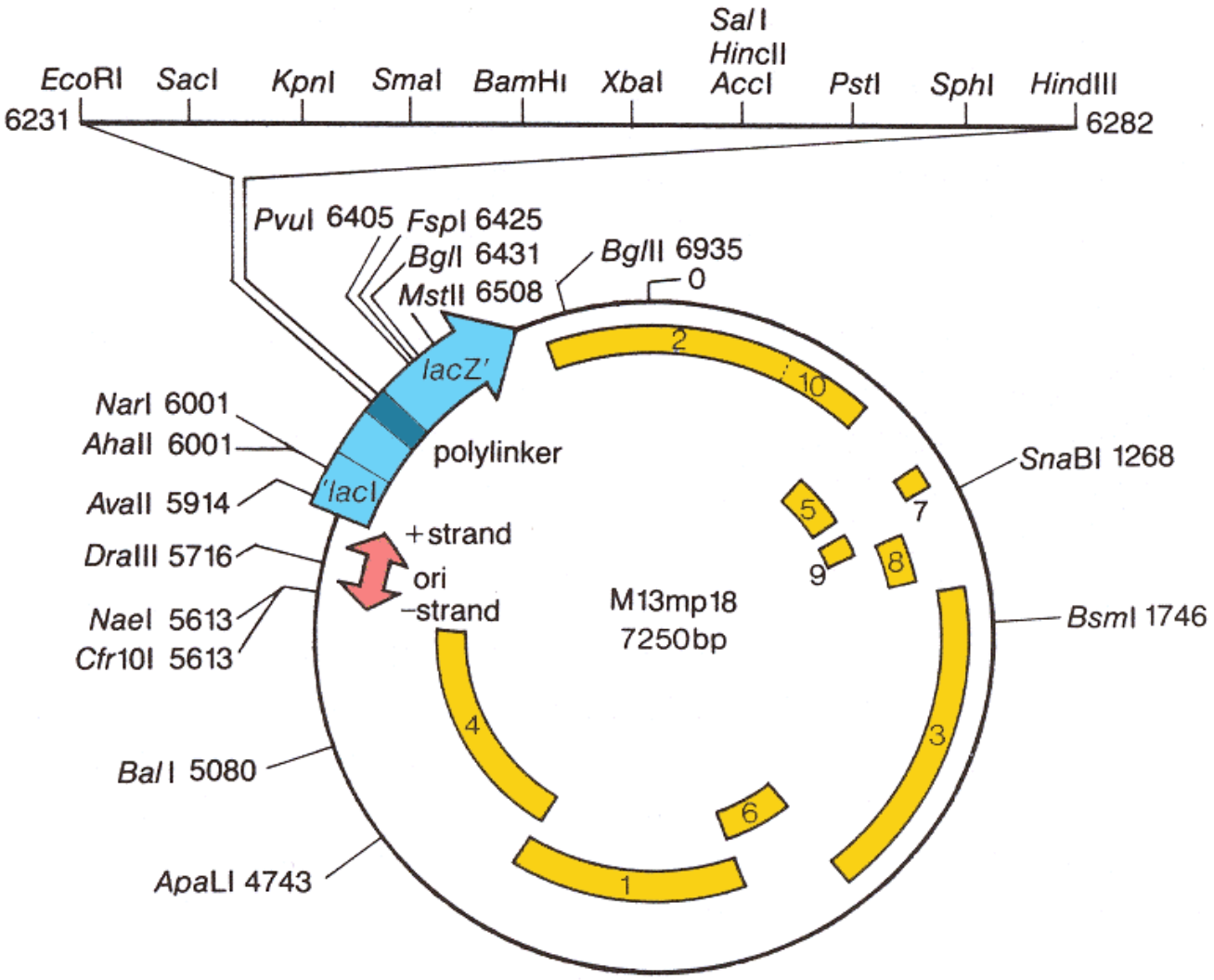

M13 phage
Protein P3 attaches pilus to enter host cell.
Protein P6
Protein P8
Genome: supercoiled single-stranded DNA circle
DNA endonuclease for double-stranded replicative form
DNA replication, single-stranded circle
Single-stranded DNA–binding protein
ori
+
−
2
10
5
7
9
8
4
M13 genome
6.4 kb
Assembly
11
Genes numbered
3
1
6
Capsid proteins
Protein P9
Protein P7
Protein P7 emerges from host cell.

Lysis Plaques of M13 Phage on *E. coli*



- **M13 and the other filamentous DNA phages are unusual in that they are released from the infected cell, without causing the death of the bacterial cell.**
- **Therefore, phage plaques of M13 are seen as areas of reduced growth in the bacterial lawn, i.e., clear zones of bacterial lysis do not occur.**

M13 Vector


SalI
HincII
EcoRI SacI KpnI SmaI BamHI XbaI AccI PstI SphI HindIII
6231
6282
PvuI 6405
FspI 6425
BglI 6431
MstII 6508
BglII 6935
0
lacZ'
'lacI
polylinker
NarI 6001
AhaII 6001
AvaII 5914
DraIII 5716
NaeI 5613
Cfr10I 5613
BalI 5080
ApaLI 4743
+strand
ori
–strand
M13mp18
7250bp
SnaBI 1268
BsmI 1746
1
2
3
4
5
6
7
8
9
10

Bacteriophage M13

- **Bacteriophage M13 has a single-stranded DNA genome.**
- **After M13 infection into a bacterial cell, a complementary DNA strand is synthesized, generating a double-stranded replicative form (RF) of the bacteriophage genome.**
- **The complementary strand then serves as a template for synthesis of new single-stranded viral DNA by a rolling circle mechanism, except that the second strand is not synthesized until a new cycle of replication is initiated.**
- **The single-stranded DNA is cut into genome-length fragments and extruded from the cell.**
- **This single-stranded DNA is useful in sequencing studies on foreign DNA cloned into M13.**
- **In addition, M13 clones can easily be subjected to site-directed mutagenesis.**

Cloning Into an M13 Vector

- **M13 vectors have been engineered to contain restriction endonuclease cut sites in the double-stranded replicative form.**

- **For cloning, the replicative form can be isolated from infected bacteria or generated artificially from the single-stranded form with a complementary synthetic oligonucleotide primer and DNA polymerase plus ligase.**

- **The double-stranded form is cut with a restriction endonuclease and the DNA is inserted much like any other cloning procedure.**

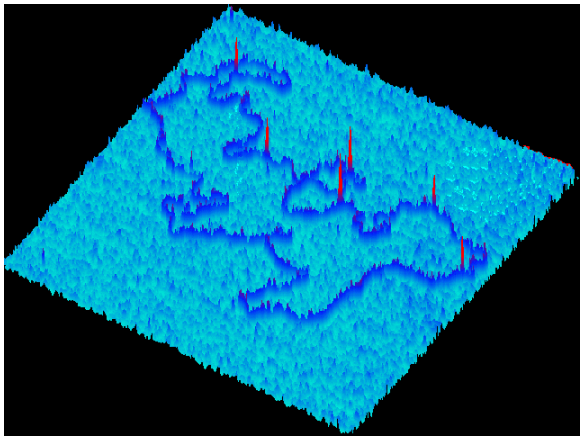
- **The double-stranded replicative form with the insert is infected into bacteria and generates single-stranded clones by the normal process of single-stranded genomic replication.**

Cosmids

- **Features**
  - ✓ **A cosmid is a type of hybrid plasmid that contains a lambda phage *cos* sequence, which is the sequence that is cut to produce the cohesive, single-stranded extensions located at the ends of the linear DNA molecules of certain phages such as lambda.**
  - ✓ **Cosmid DNA sequences originate from the lambda phage (*cos* sites + plasmid = cosmid).**
  - ✓ ***Cos* sequences are ~200 base pairs long and essential for packaging.**
  - ✓ **Cosmids are recombinant plasmids contained in phage shells.**
- **Advantages**
  - ✓ **Useful for cloning very large DNA fragments (32 - 47 kbp)**
  - ✓ **Inherent size selection for large inserts**
  - ✓ **Handle like plasmids**

Cosmid Engineering

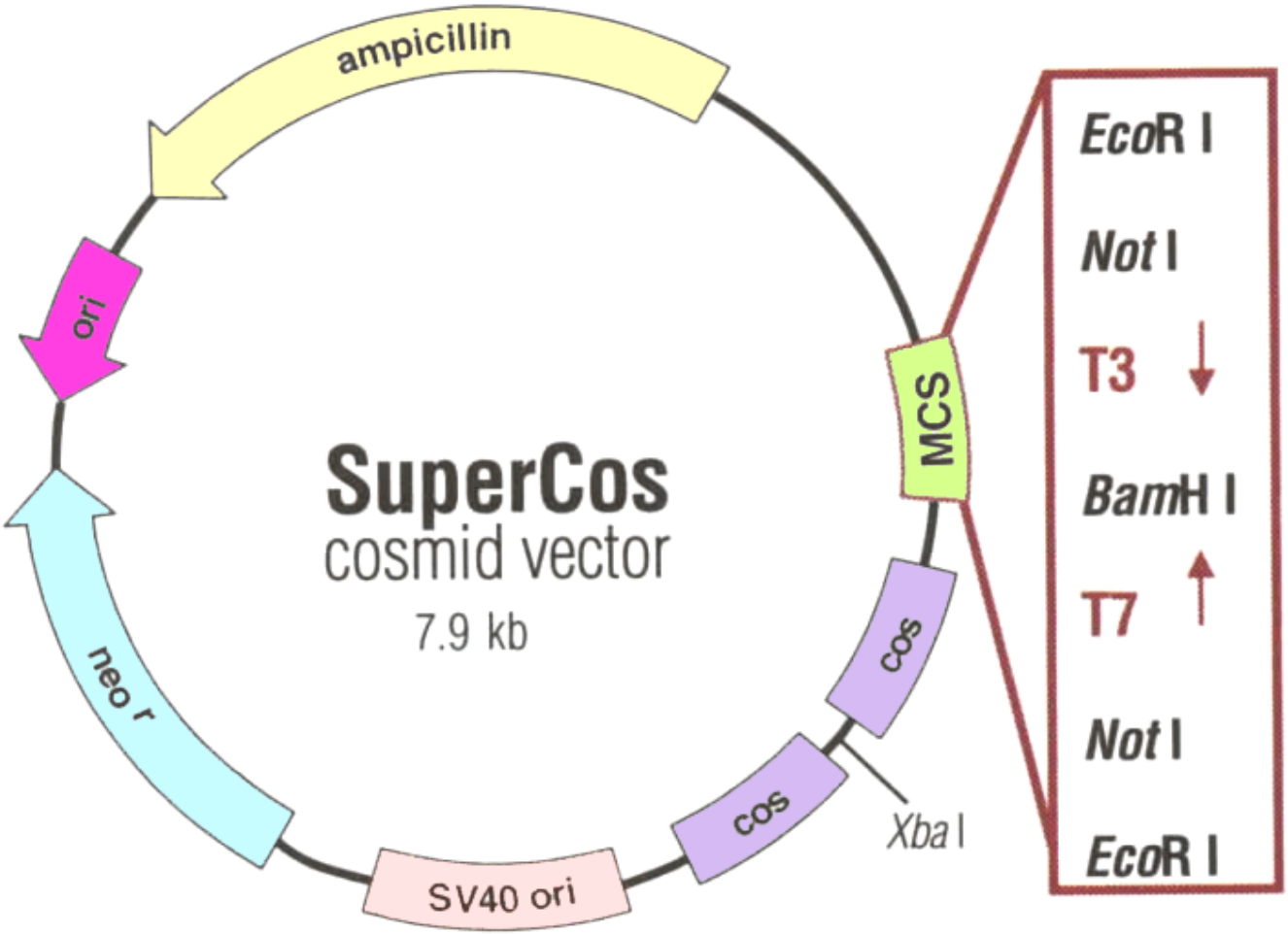
- Cosmids combine essential elements of a plasmid and lambda systems.
- Fragments from 30 - 46 kb can be accommodated by a cosmid vector.
- Cosmids are extracted from bacteria and mixed with restriction endonucleases.
- Cleaved cosmids are mixed with foreign DNA that has been cleaved with the same endonuclease.
- Recombinant cosmids are packaged into lambda caspids.
- Recombinant cosmid is injected into the bacterial cell where the R cosmid arranges into a circle and replicates as a plasmid.
- It can be maintained and recovered just as plasmids.



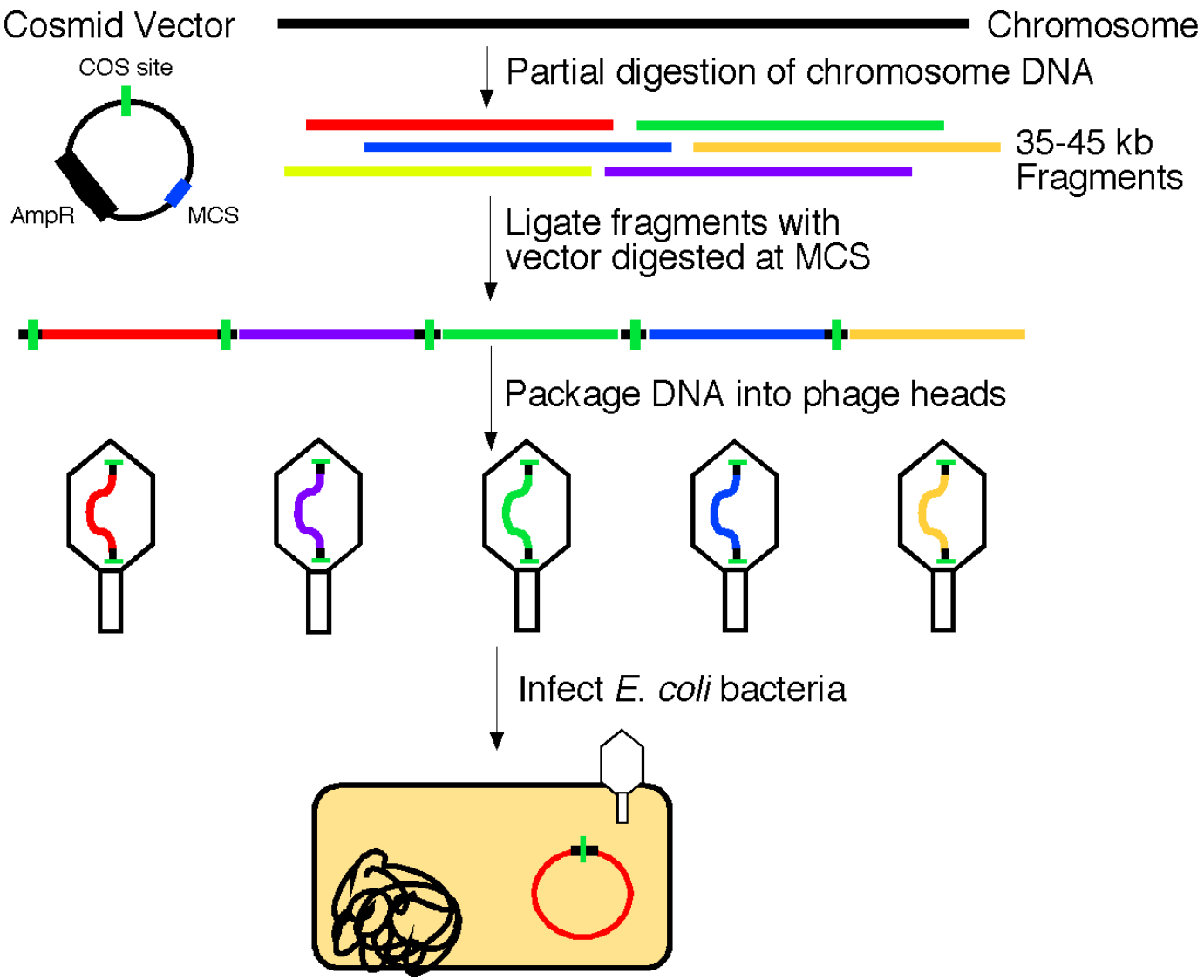
- Shown above is a 50,000 base-pair long DNA molecule bound with six EcoRI molecules, and imaged using an atomic force microscope.
- This image clearly indicates the six EcoRI "sites" and allows an accurate restriction enzyme map of the cosmid to be generated.

http://homer.ornl.gov/cbps/afmimaging.htm

Example of Cosmid Vector



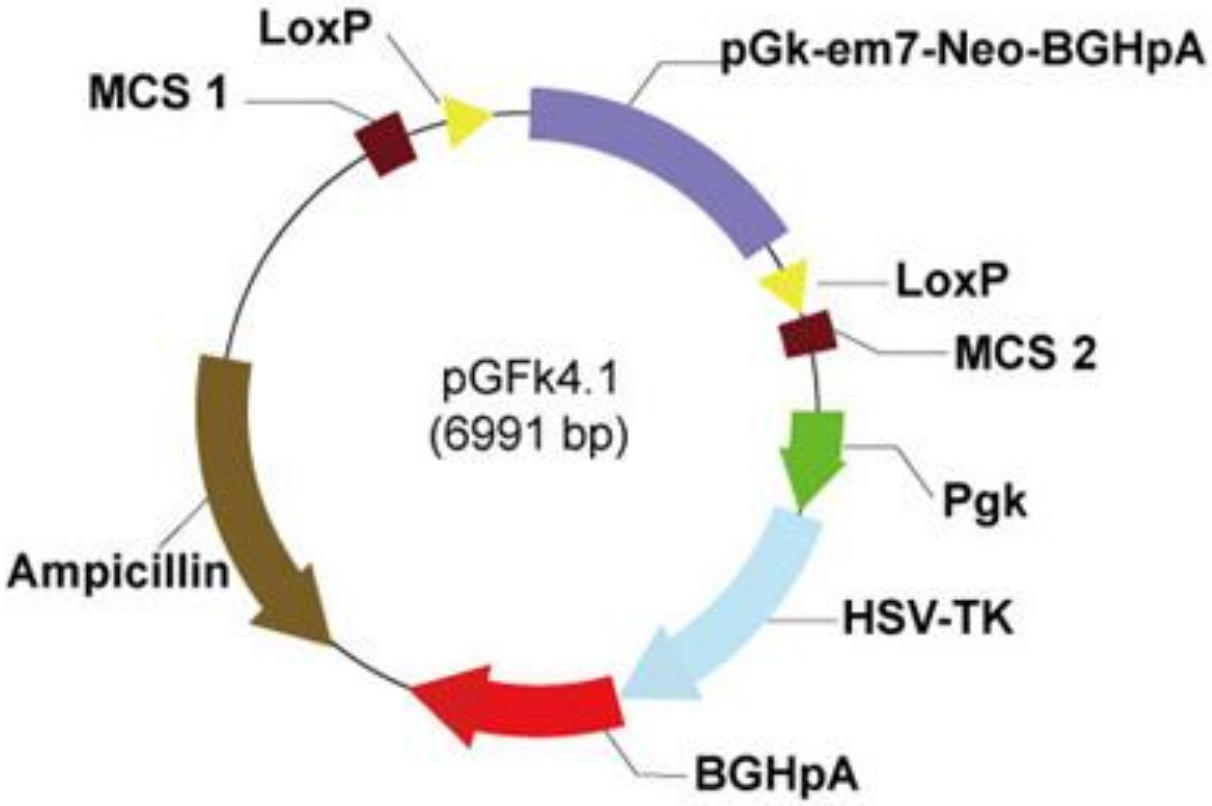
Constructing a Genomic Library in Cosmids



BACs, PACs and YACs

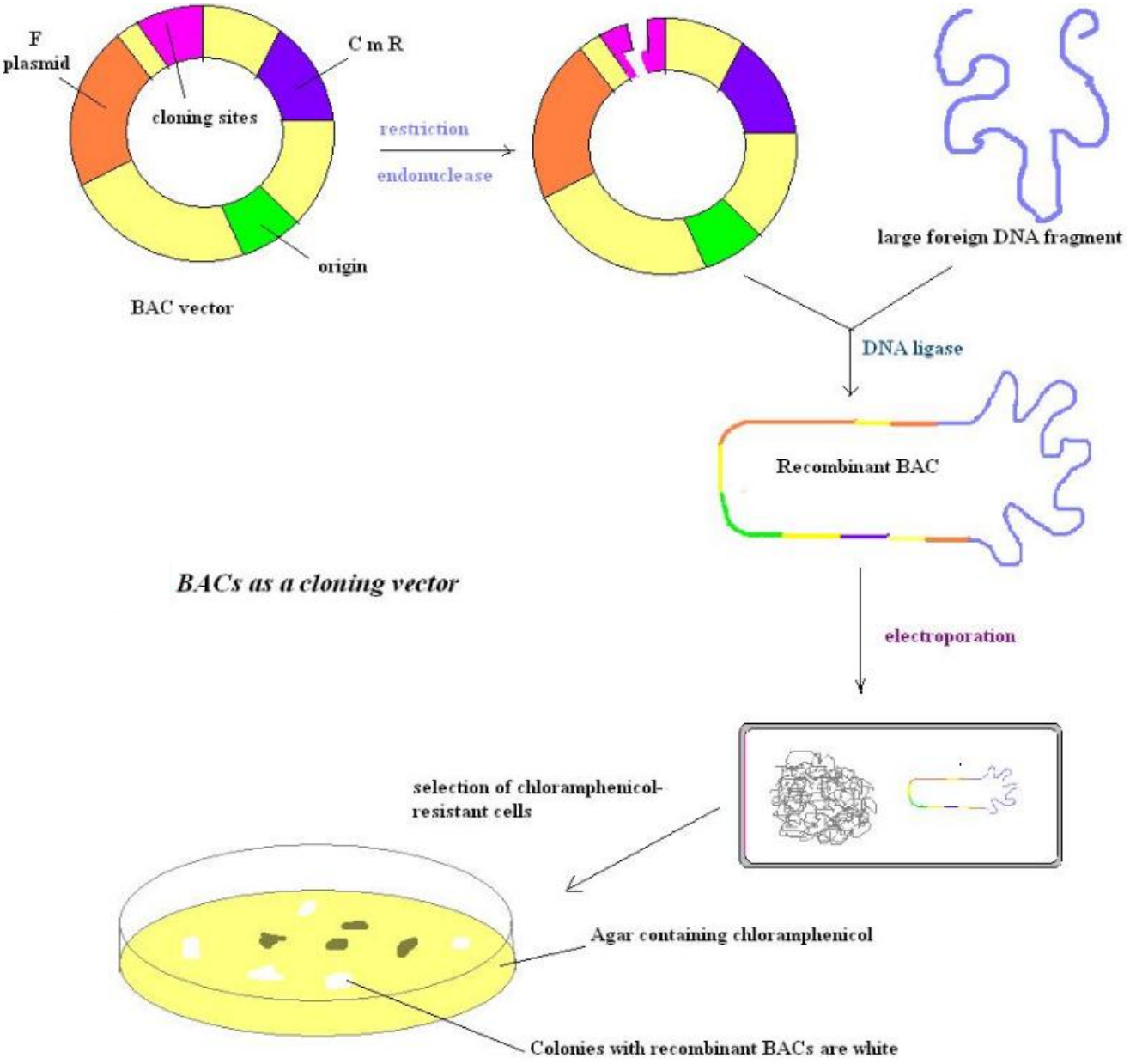
- **Features**
  - ✓ **BAC: Bacterial Artificial Chromosome – DNA construct based on a functional fertility plasmid (F-plasmid)**
  - ✓ **PAC: P1-derived Artificial Chromosome – DNA construct derived from DNA of P1 bacteriophage**
  - ✓ **YAC: Yeast Artificial Chromosome – originated from a bacterial plasmid; contains additionally a yeast centromeric region (CEN); autonomously replicating sequence (ARS) which is a yeast origin of DNA replication; a cluster of unique restriction sites; and a selectable marker and a telomere region at the end of each arm**
- **Advantages**
  - ✓ **Useful for cloning extremely large DNA fragments (100 - 2,000 kbp), depending on the artificial chromosome vector used**
- **Disadvantages**
  - ✓ **Not easy to handle extremely large DNA molecules**

Example of a BAC

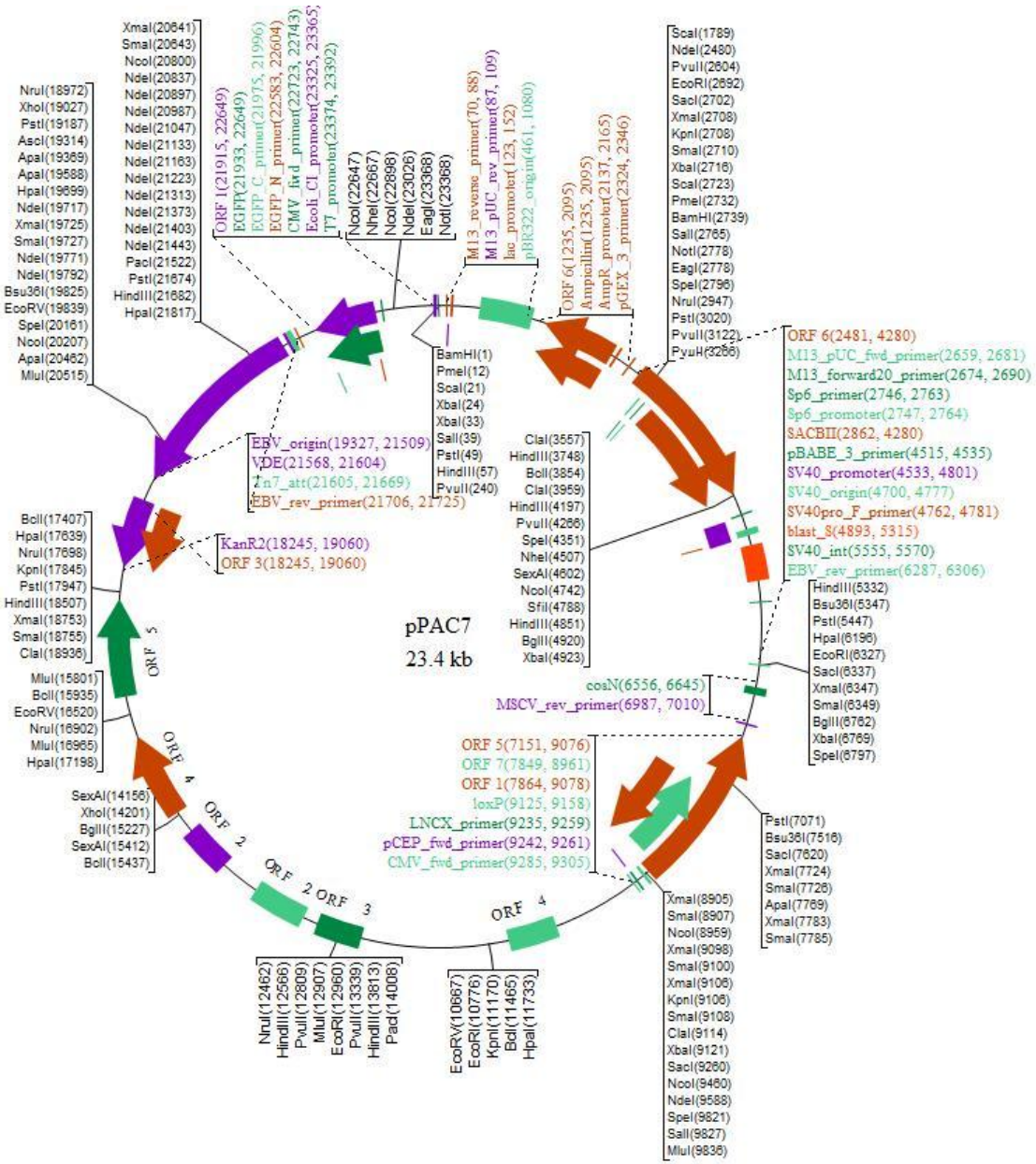


- **Cre-Lox recombination consists of a single enzyme, Cre recombinase, which recombines a pair of short target sequences called the *Lox* sequences.**
- **This system can be implemented without inserting any extra supporting proteins or sequences.**
- **The Cre enzyme and the original *Lox* site called the *LoxP* sequence are derived from bacteriophage P1.**

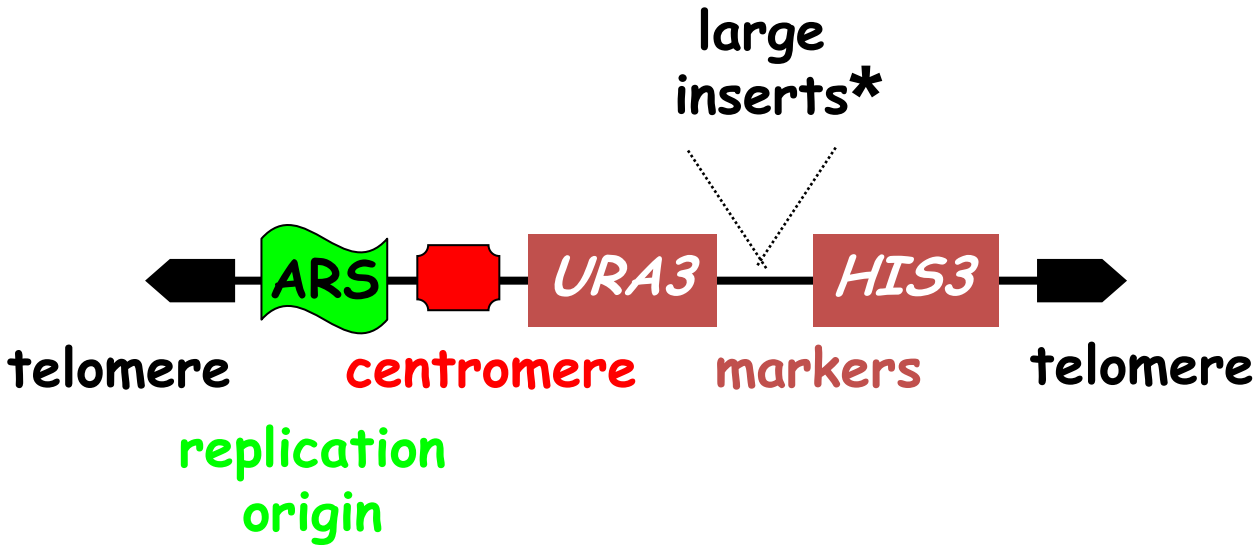
Cloning in a BAC



Example of a PAC

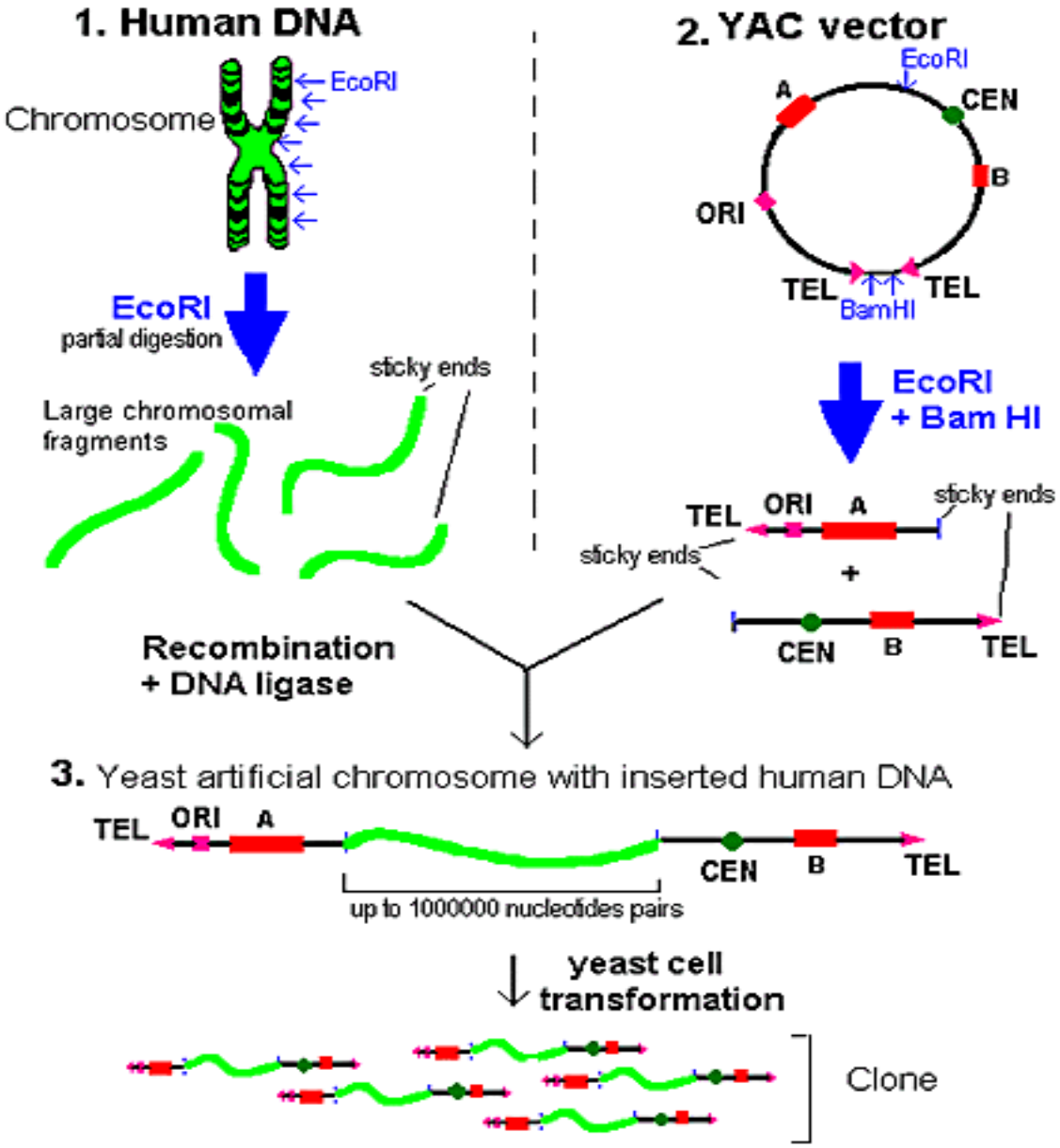


Example of a YAC

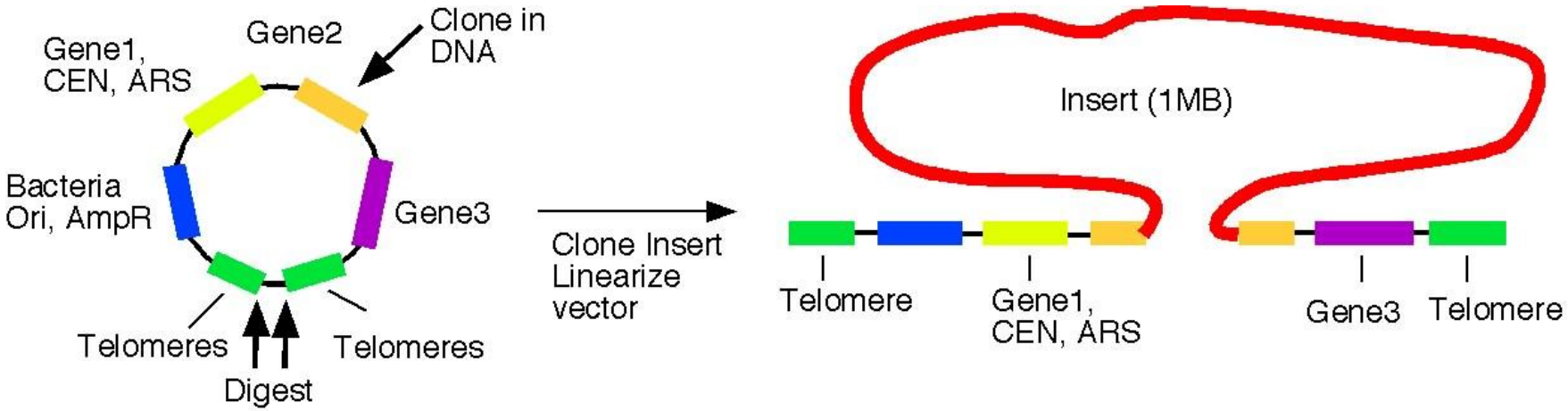


*Capable of carrying inserts of 200-2000 kbp in yeast

Cloning in a YAC



Constructing a Genomic Library in a YAC



ARS – autonomously replicating sequence

CEN – yeast centromeric region

Expression Vectors

- Expression vectors enable a particular gene to be not only amplified but also expressed in a host cell.
- The vector is constructed to contain appropriate regulatory sequences, such as a promoter and operator, so that the host-cell machinery can transcribe the gene and translate the resultant messenger RNA to synthesize the corresponding protein.
- Such vectors are thus essential for the manufacture of, for example, mammalian proteins by bacterial host cells.
- Expression vectors used in prokaryotes are typically based on plasmids or phages, or plasmid–phage hybrids (phagemids).
- Some eukaryote proteins are extensively modified during or following their synthesis, for example by the addition of carbohydrate groups.
- Prokaryote host cells are unable to accomplish these modifications, and therefore expression systems based on eukaryotic cells must be used instead.
- For example, vectors based on baculovirus, a DNA virus of insects, are used successfully in cultures of insect cells.
- Secretion vectors allow both expression and secretion of the novel protein by the host cell, by ensuring that the expressed protein carries a signal peptide that allows it to be transported across the plasma membrane.