VECTOR TYPES FOR CLONING DNA

VECTOR TYPES FOR CLONING DNA

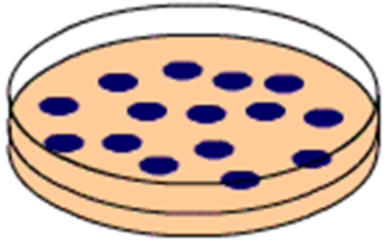
- VETTORI PLASMIDICI -> 0-10 kb

- VETTORI λ di inserzione -> 0-10 kb
- VETTORI λ di sostituzione -> 9-23 kb
- VETTORI COSMIDICI -> 30-44 kb
- VETTORI PAC (crom. artif. P1) -> 130-150 kb
- VETTORI BAC (crom. artif. batt.)-> fino a 300 kb
- VETTORI YAC (crom. artif.lievito-> 0.2-2 Mb

• Vettori per la clonazione di frammenti di DNA
VETTORI BASATI SUL BATTERIOFAGO λ

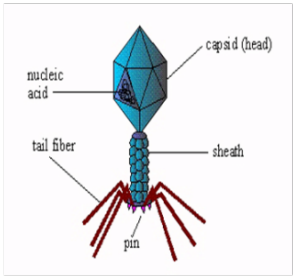
Ospiti

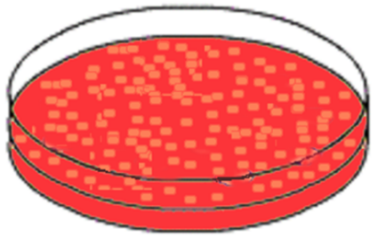
Batteri



Colonie

Virus (Batteriofagi)

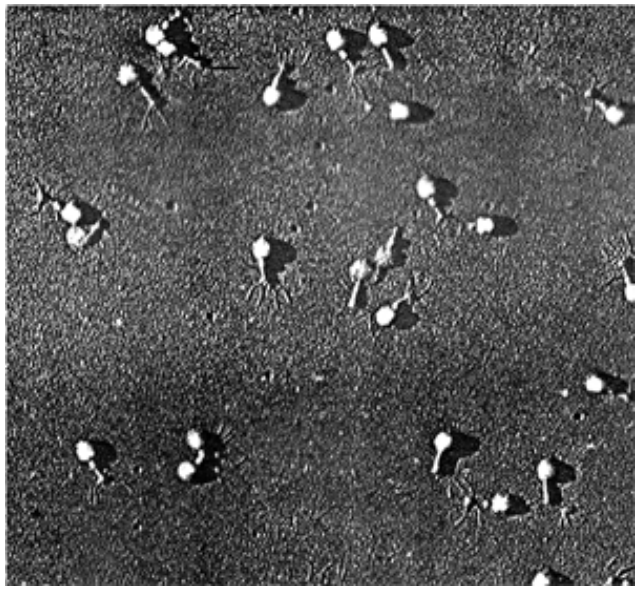


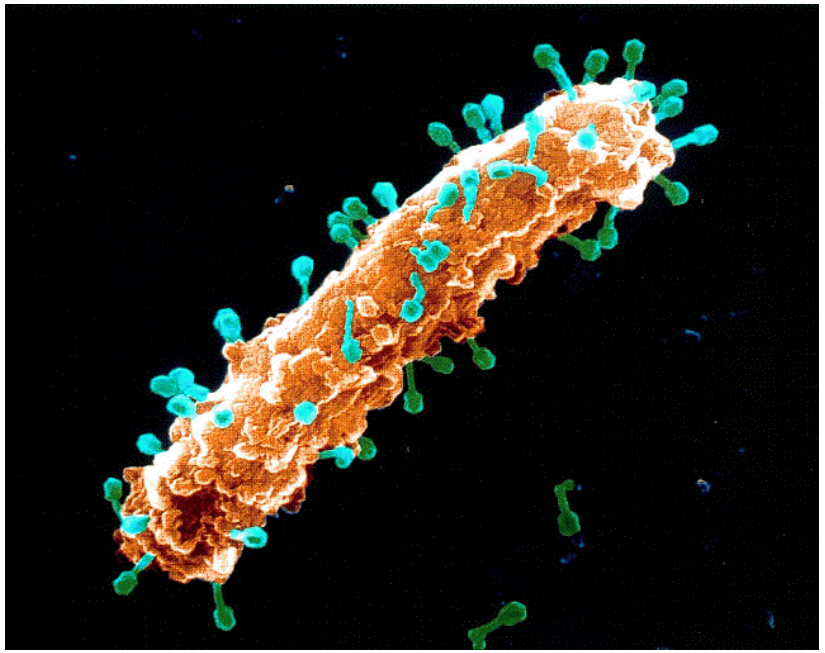


Placche di lisi

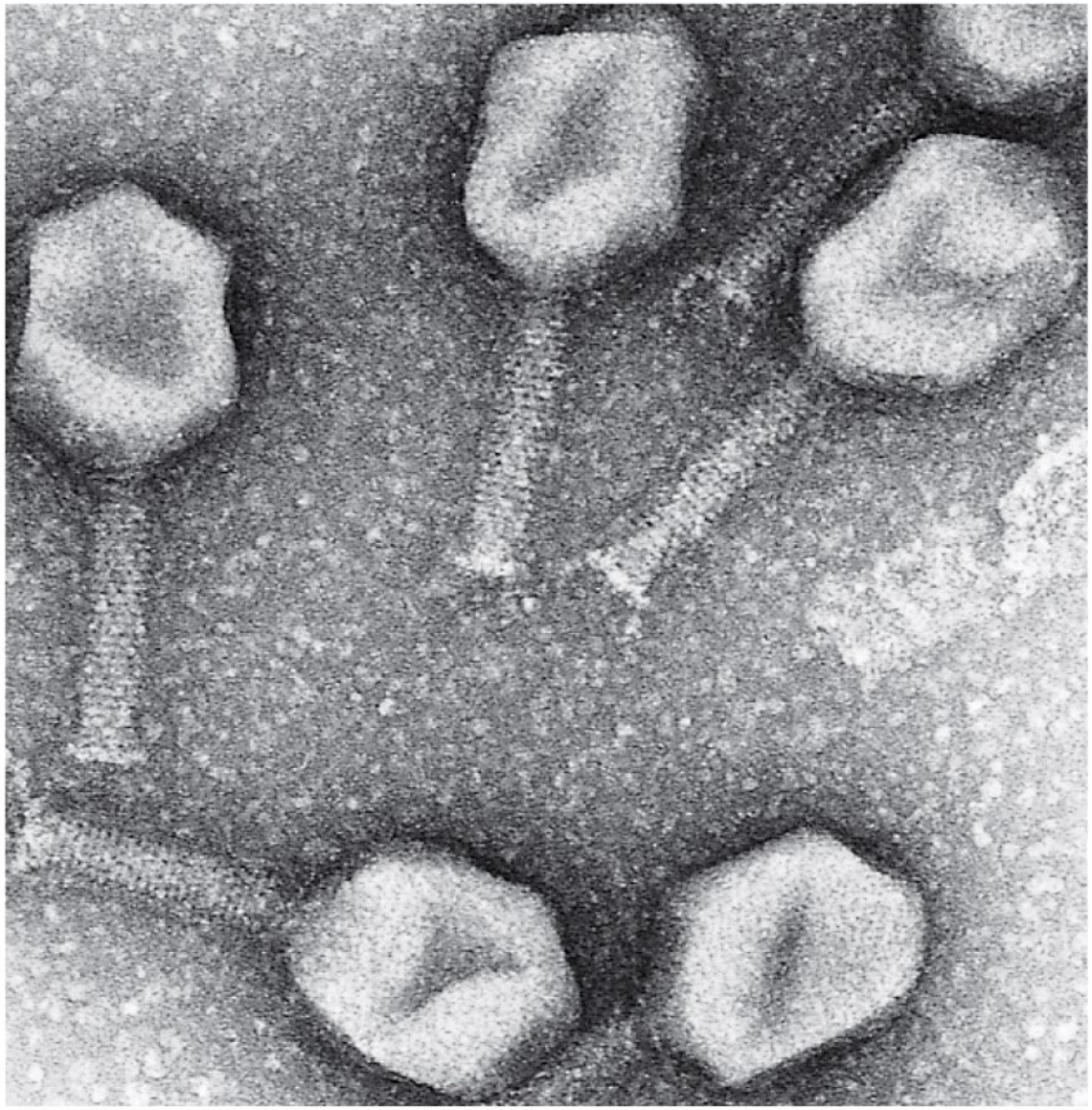
FORMAZIONE DI GENOTECHE - LIBRERIE

Vettori per la clonazione di frammenti di DNA
VETTORI BASATI SUL BATTERIOFAGO λ

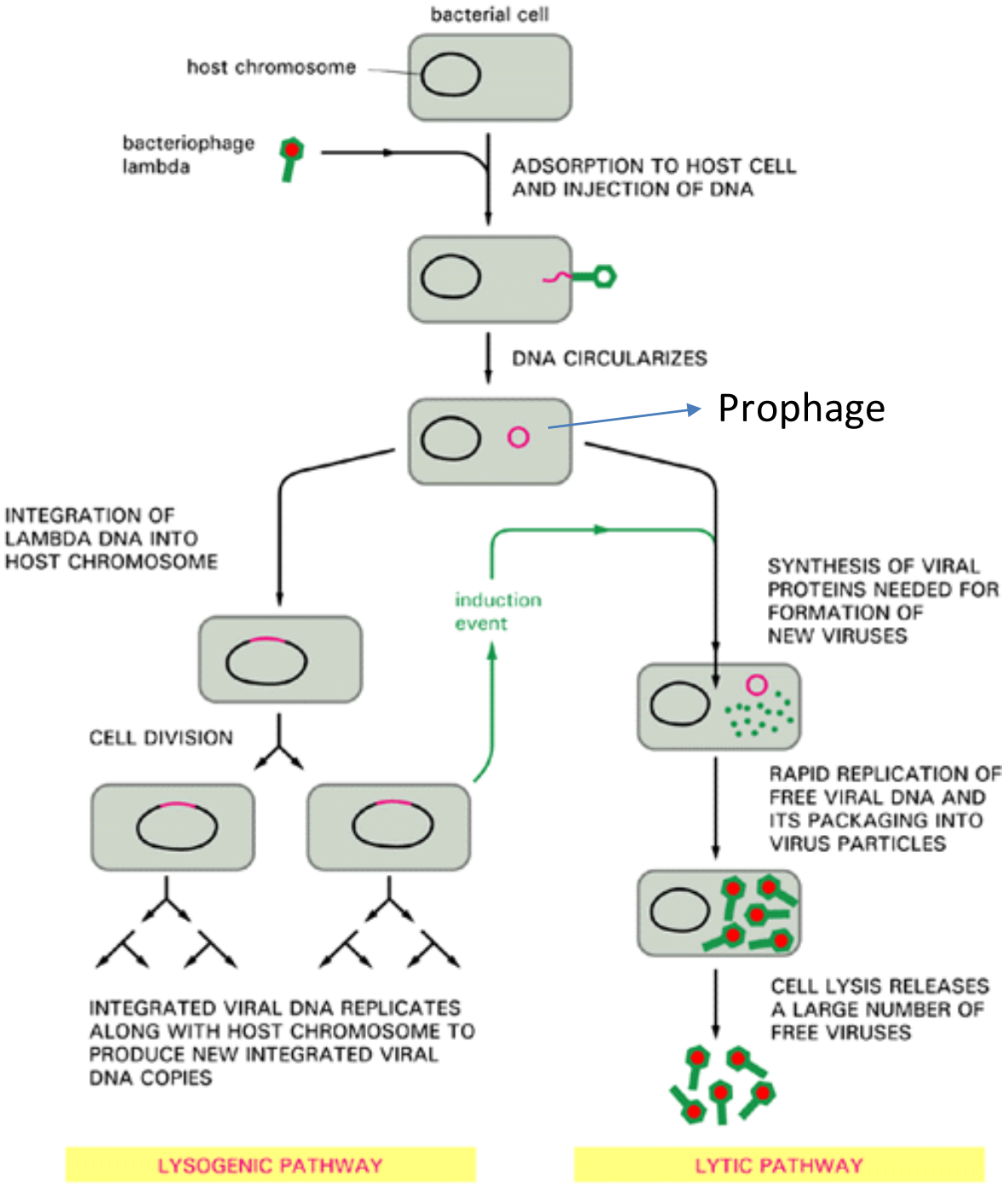




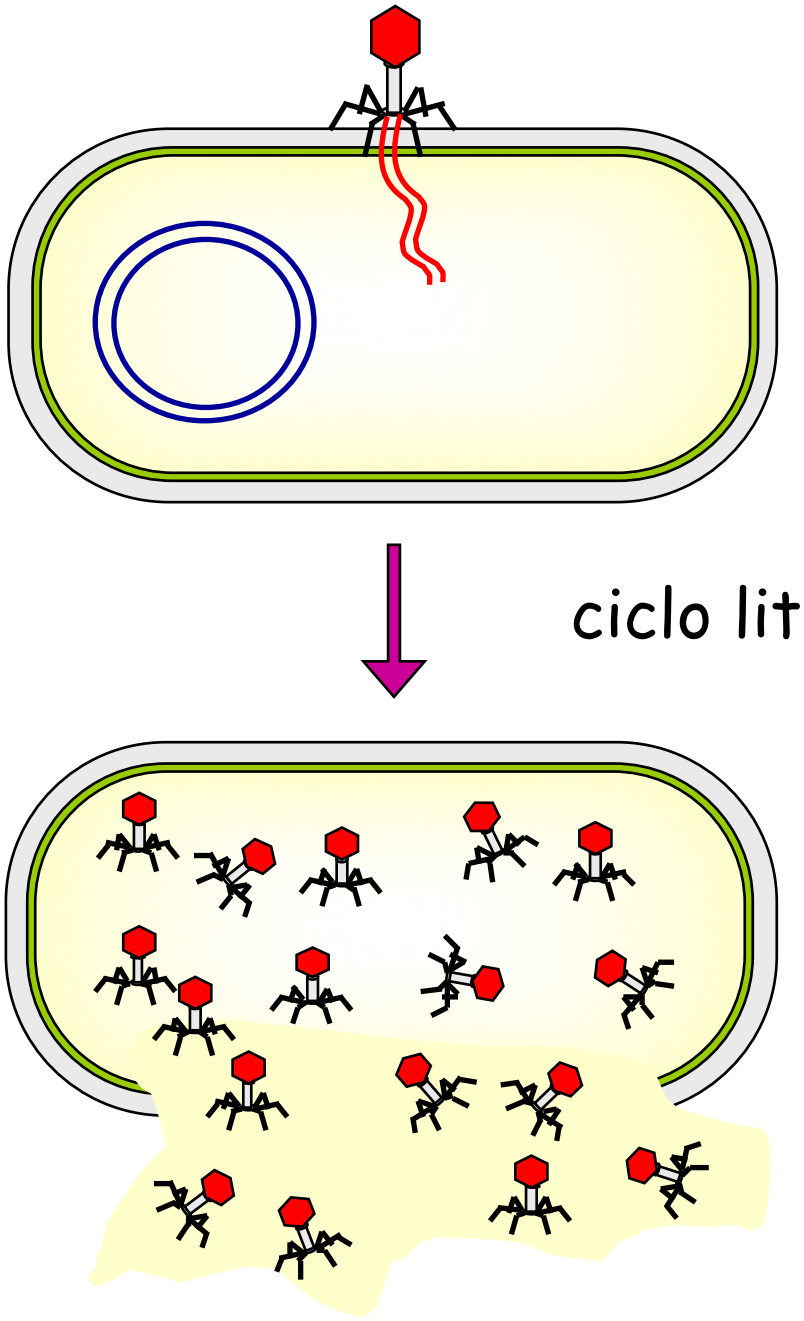
Il numero di particelle fagiche prodotte per ogni particella infettante ("burst size") è caratteristico di ciascun batteriofago. Esso può giungere a 10.000.


100 nm

Il ciclo vitale del fago lambda

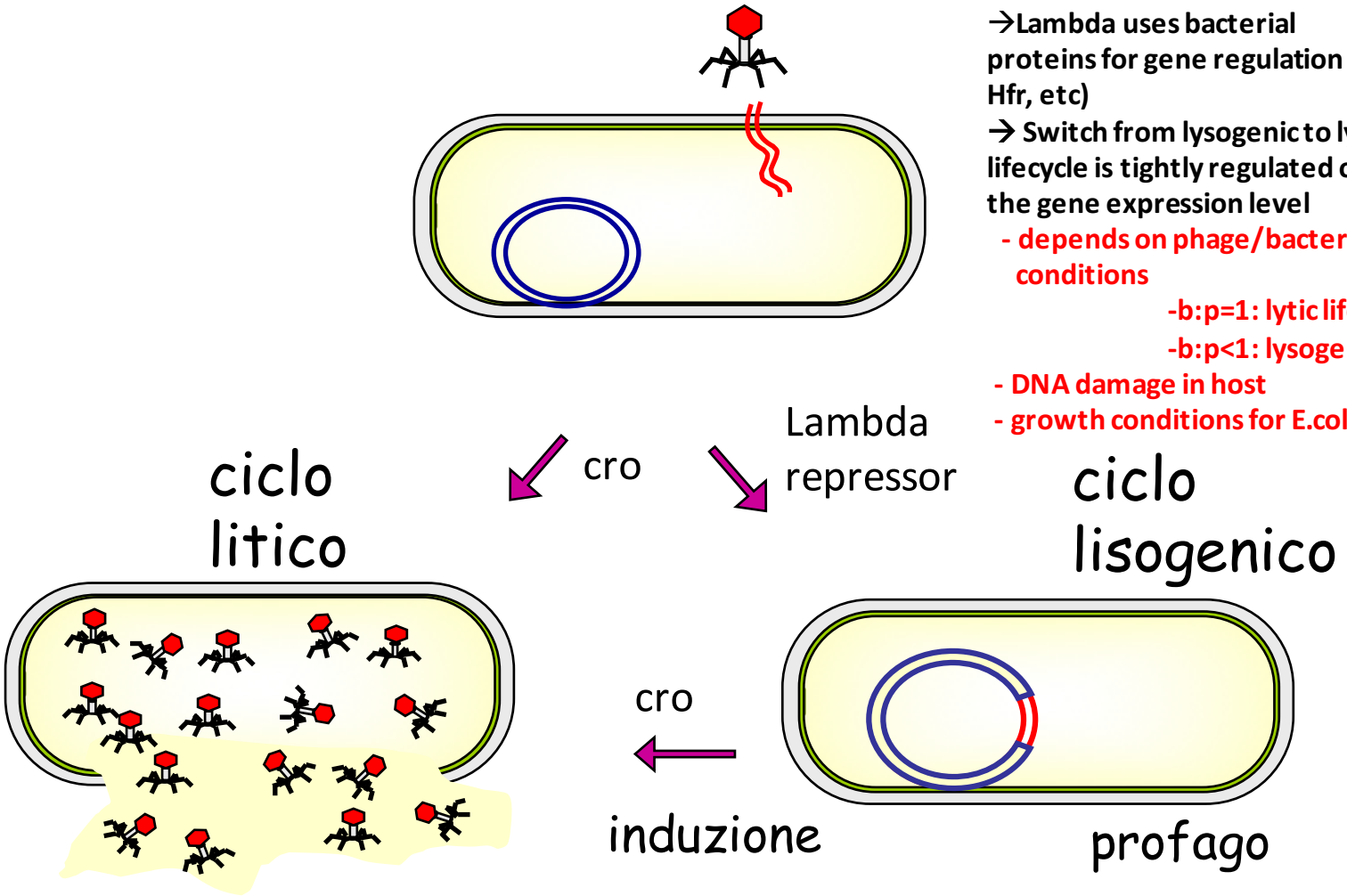


Batteriofagi virulenti



ciclo litico

Batteriofagi temperati

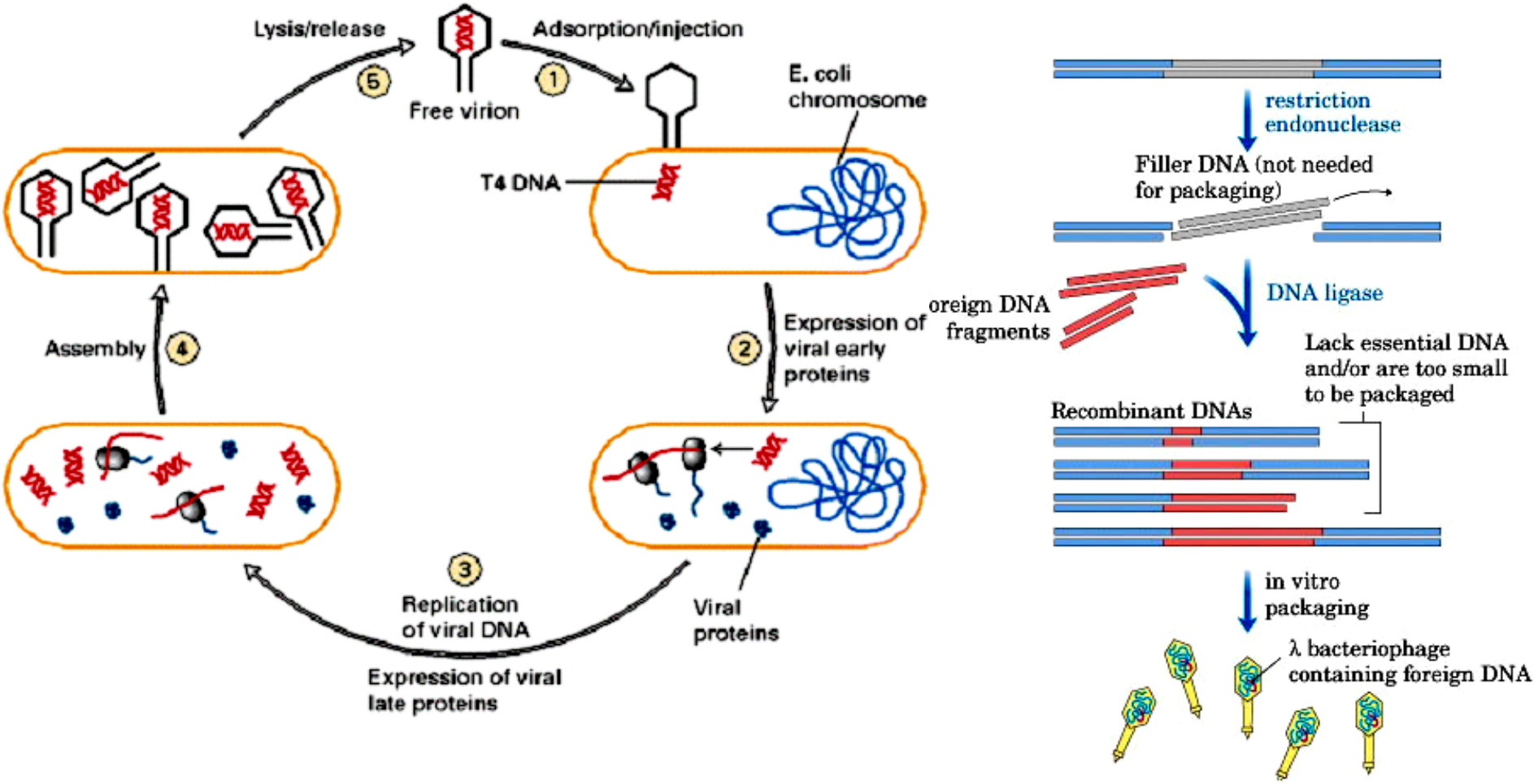


→Lambda uses bacterial proteins for gene regulation (RNA polymerase, Hfr, etc)

→ Switch from lysogenic to lytic lifecycle is tightly regulated on the gene expression level

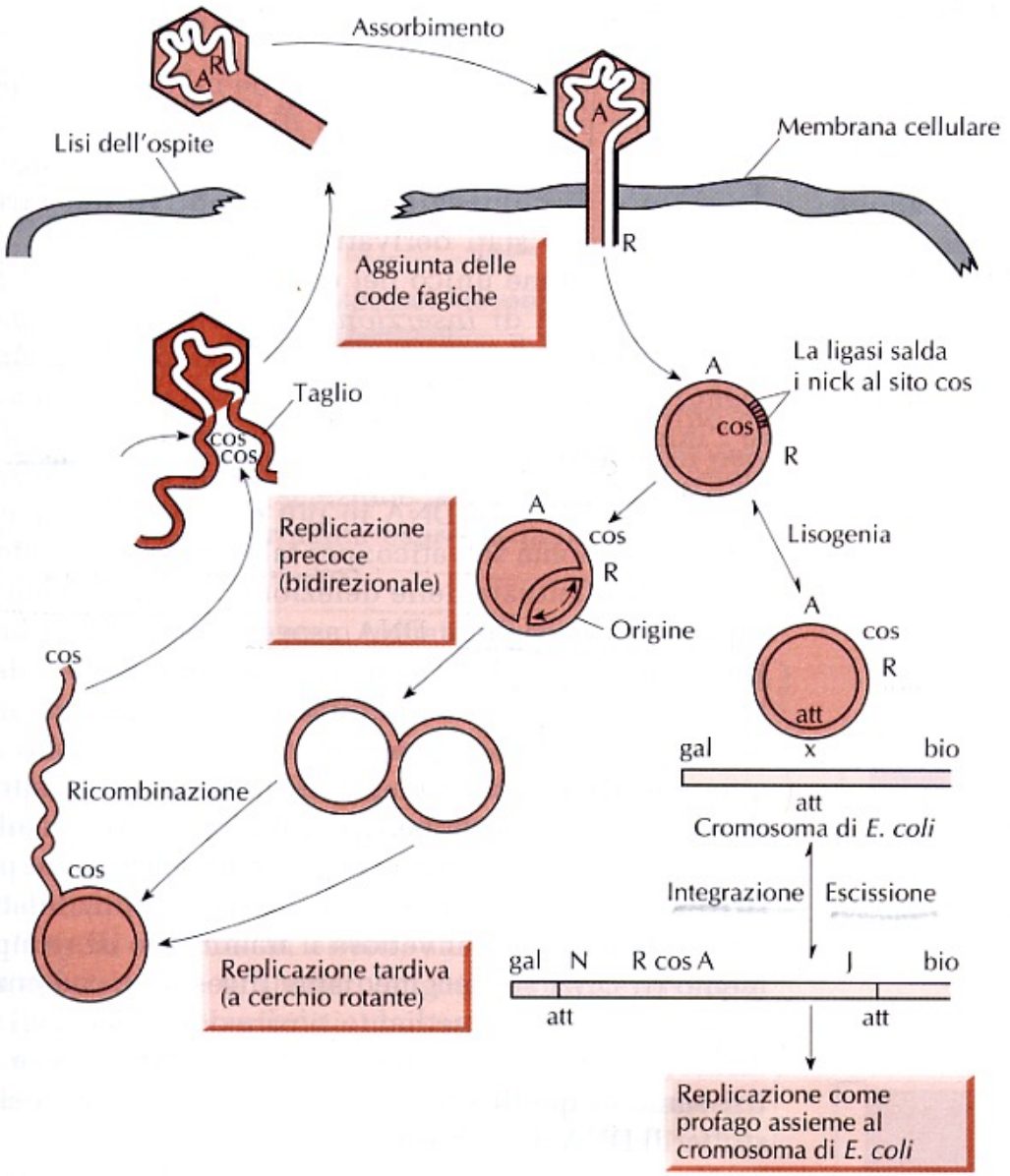
- depends on phage/bacteria ratio in culture conditions
  - -b:p=1: lytic life cycle (enough bacteria to infect)
  - -b:p<1: lysogenic life cycle
- DNA damage in host
- growth conditions for E.coli

Vettori fagici



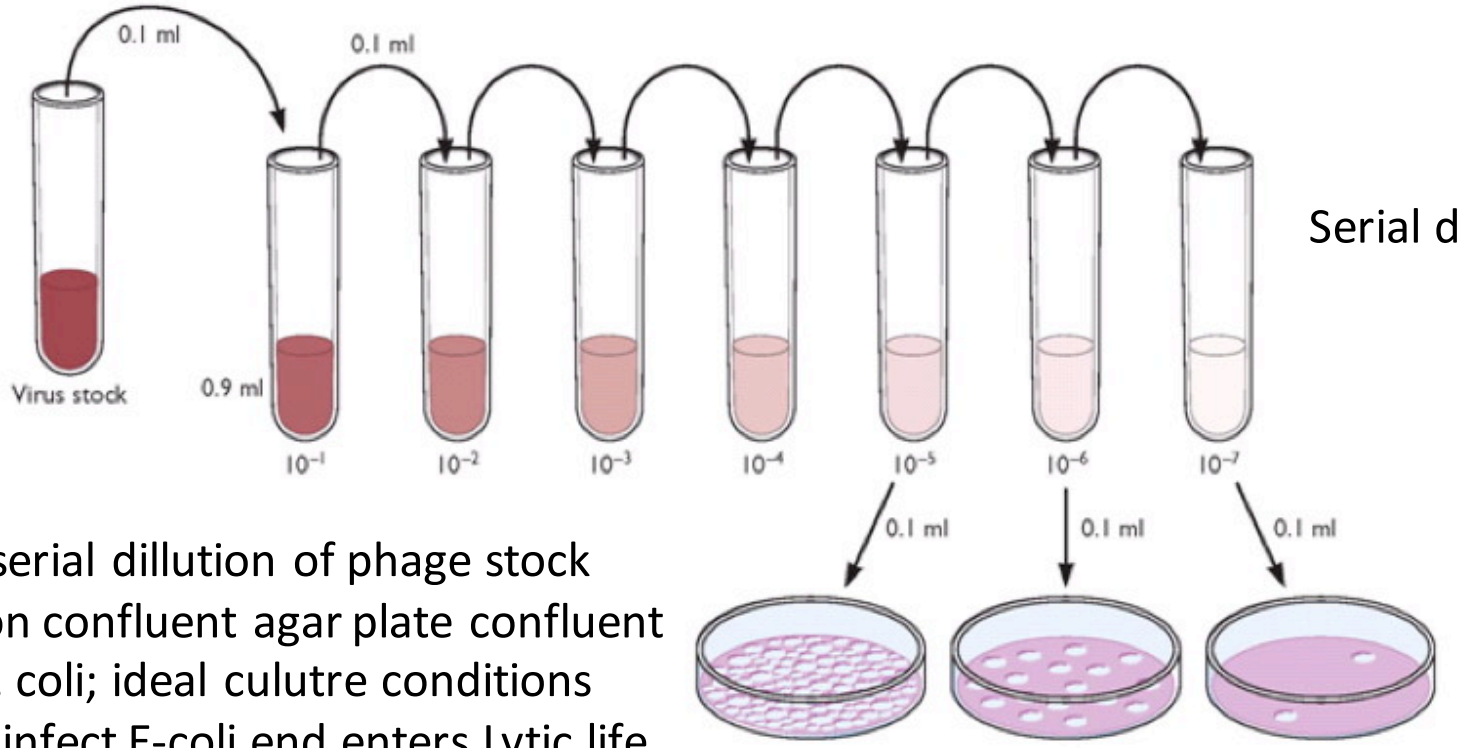
Phages can be used to amplify and trasport DNA
→ Phages are used as recombinant DNA

Life cycles of the Lambda phage



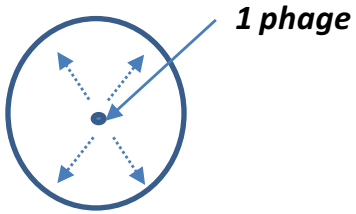
Working with phages in the lab

The plaque assay

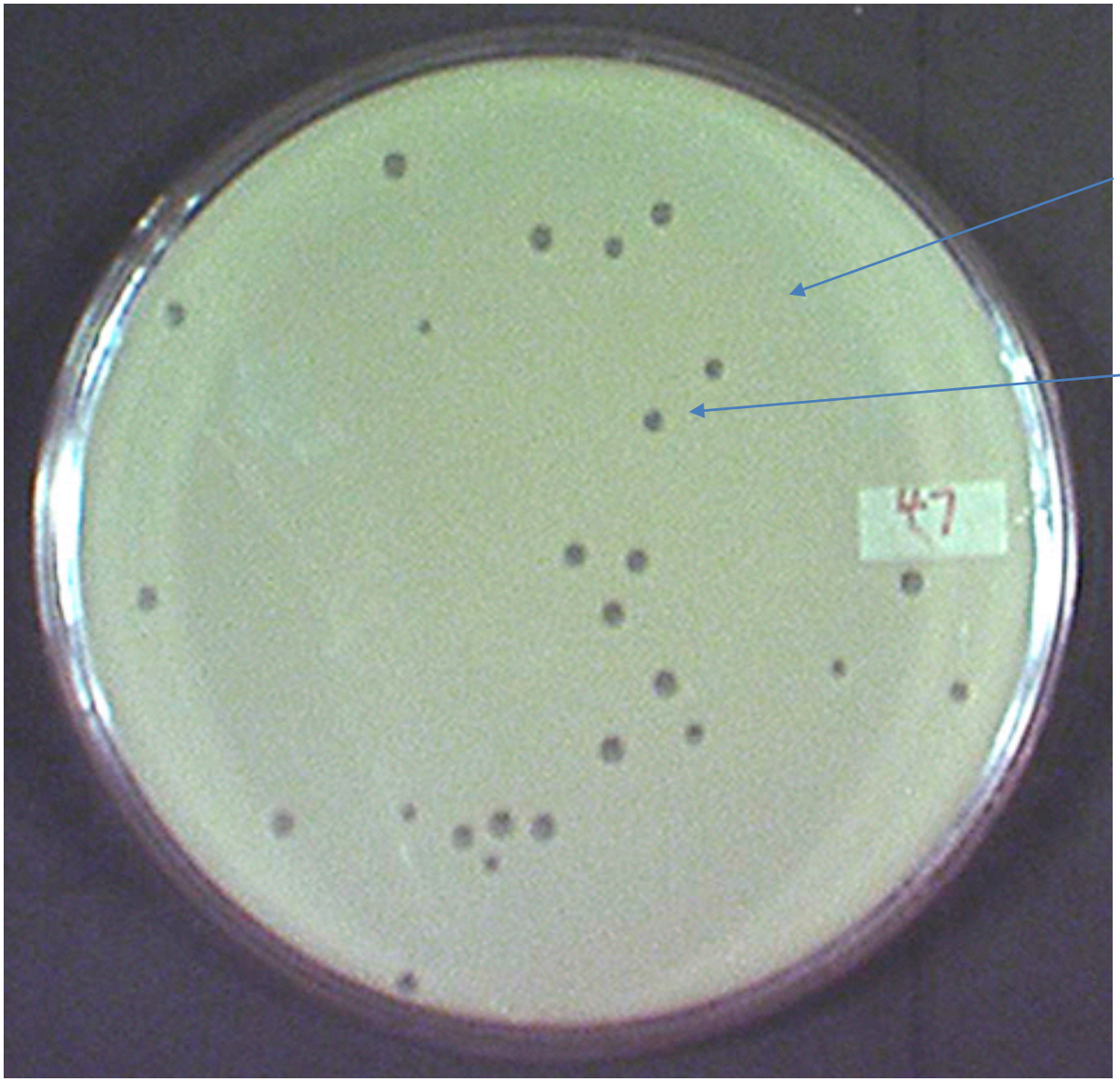


Serial dillution

- → Make serial dillution of phage stock
- → Plate on confluent agar plate confluent with E. coli; ideal culutre conditions
- → Phage infect E-coli end enters Lytic life cycle
- → Exit from cell and infection of neighbouring cells
- → Formation of plaque



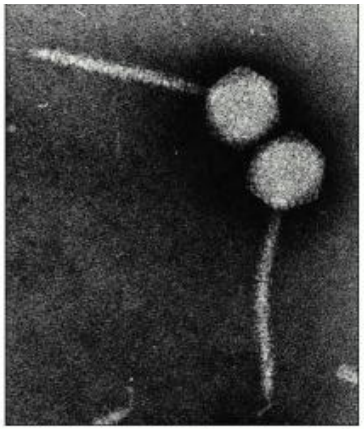
Working with phages in the lab



E-coli
(confluent)

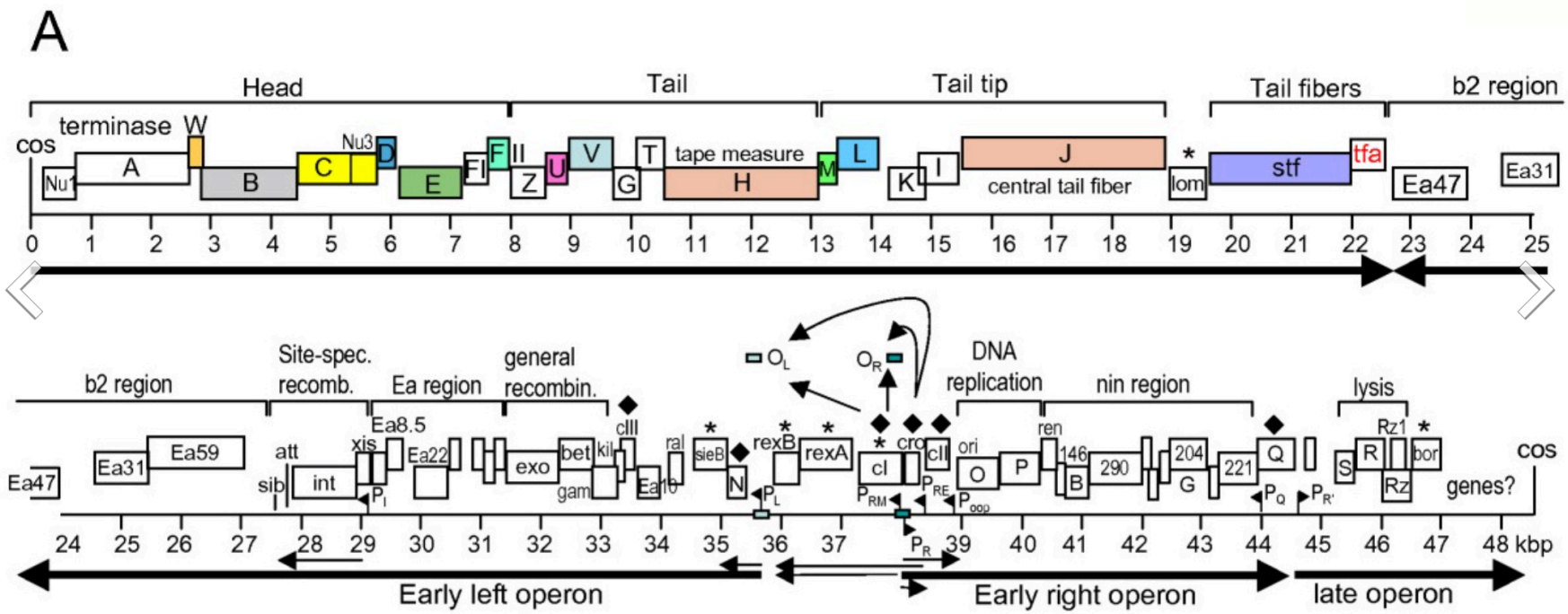
Plaque:
→ Initiated by 1 phage
→ Plaque contains millions of phages
→ Phages can be isolated and furhter amplified

Genomic structure of the Lambda phage



48 kb Length

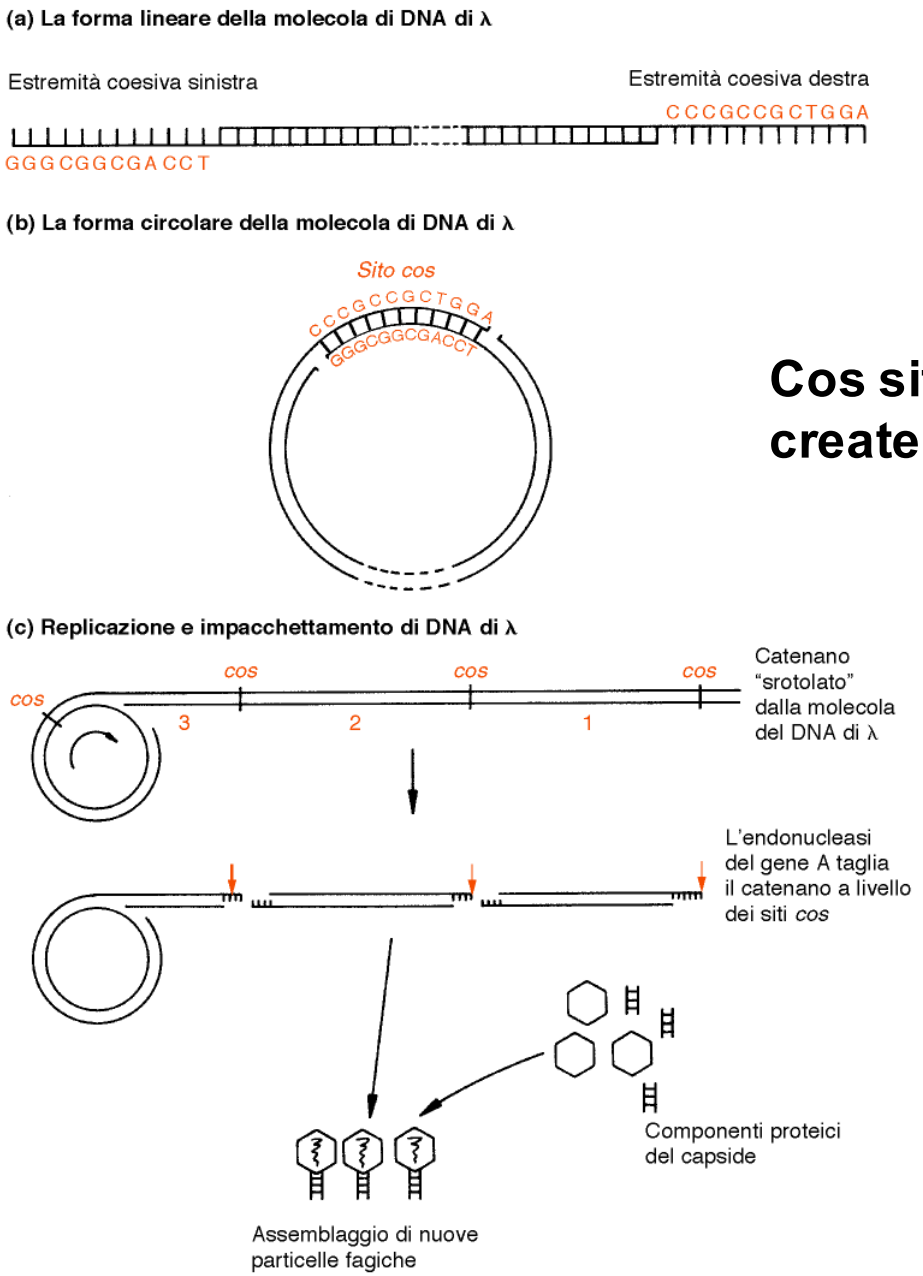
Figure 1.



→ **50 genes, predominantly encoding for proteins for capsid formation, replication, recombination and lysis**

→ **7 promoter elements that control the decision between lytic and lysogenic cycle (P_L, P_{RM}, P_R, P_{RE}, P_{AQ}, $P_{R'}$, P_I)**

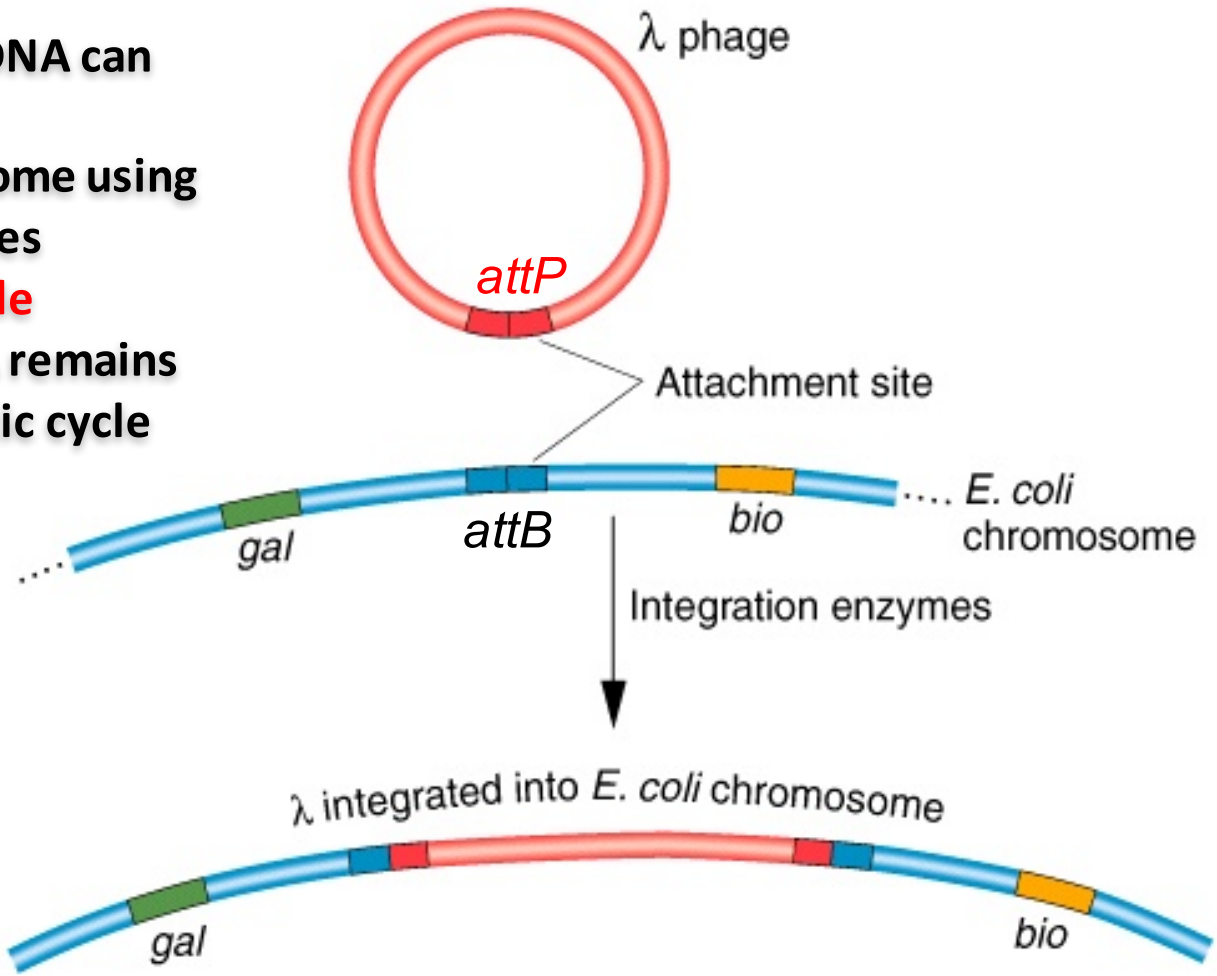
Cos sites mark the ends of phage



Cos sites → used to create circular DNA

att sites control insertion of phage DNA in genome

Circulated lambda DNA can integrate
Into the bacterial genome using attachment sites
=lysogenic cycle
If circular phage DNA remains circular = start of lytic cycle



Genomic structure of the Lambda phage

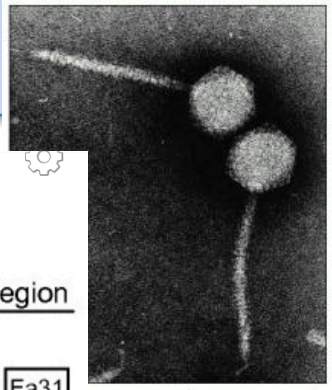
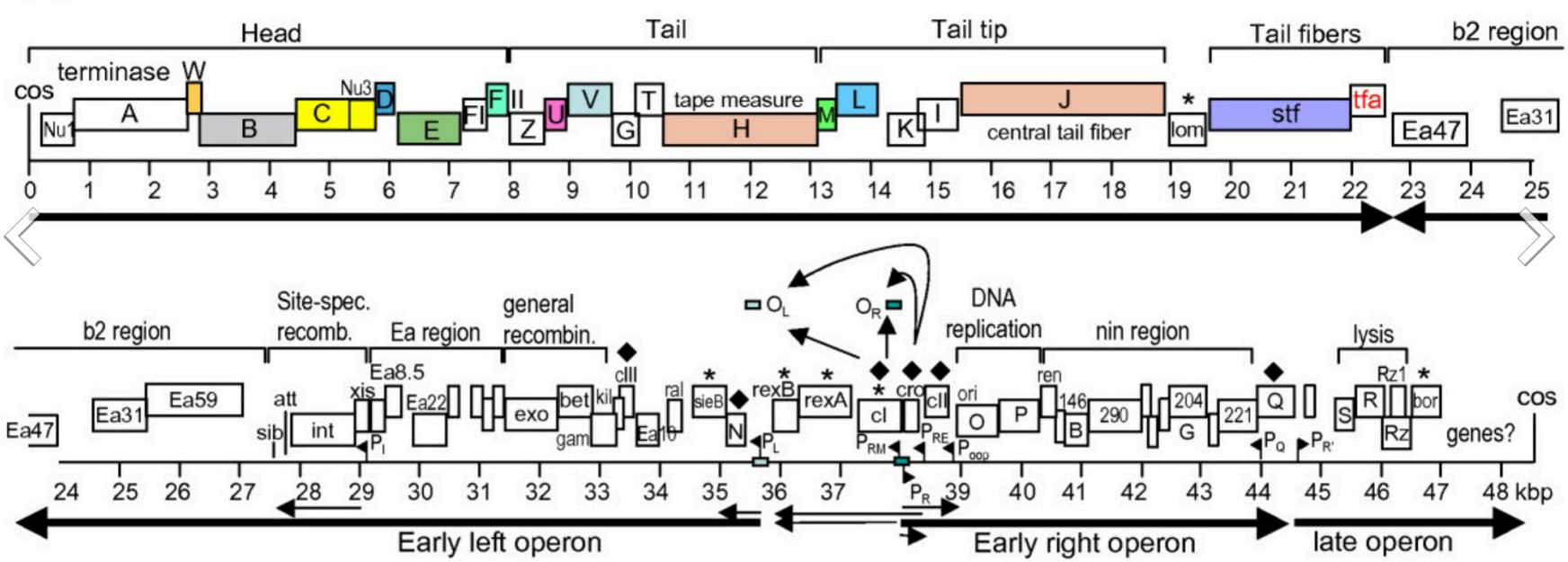


Figure 1.

Left region



→ 50 genes, predominantly encoding for proteins for capsid formation, replication, recombination and lysis

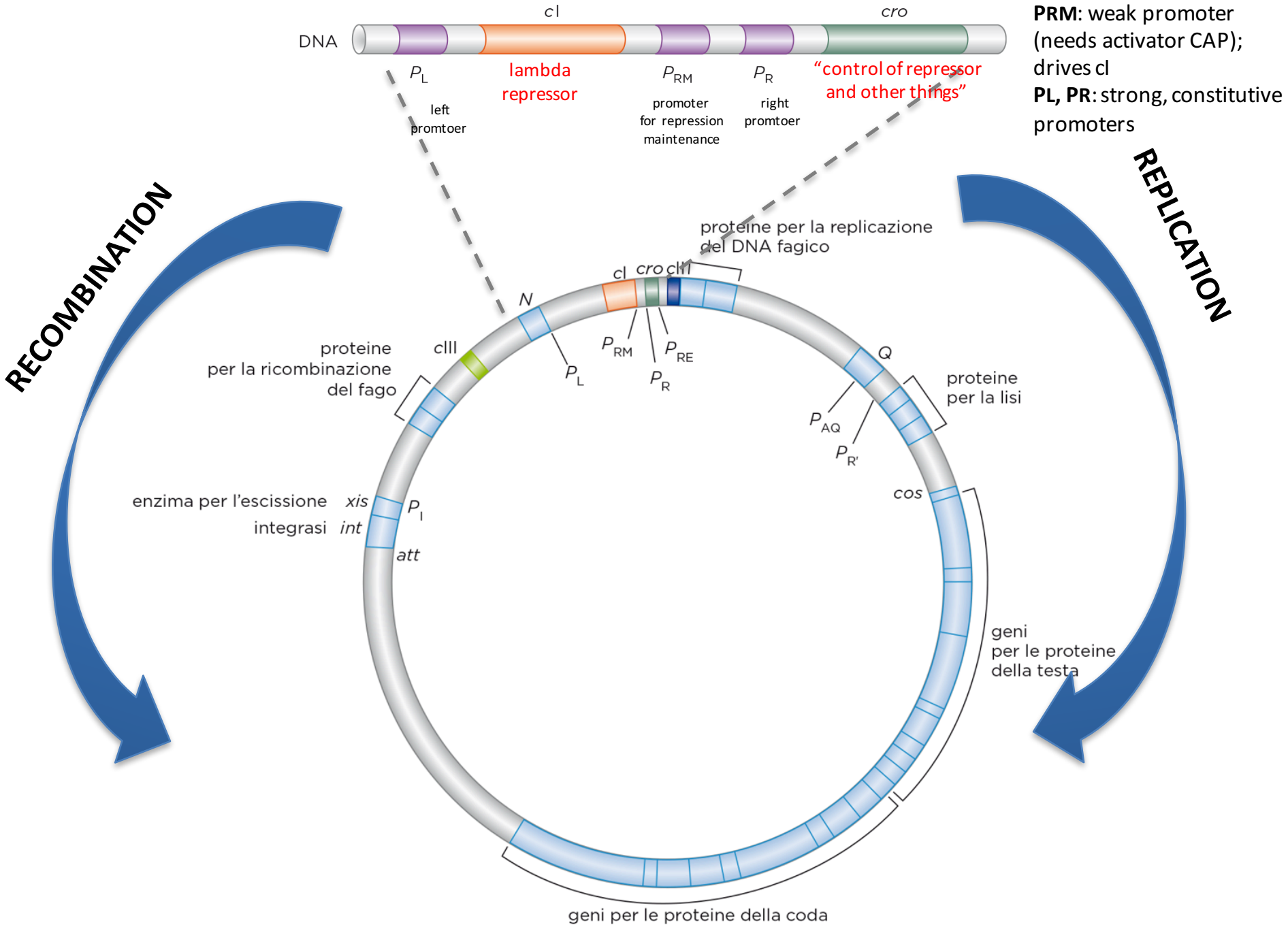
→ 7 promoter elements that control the decision between lytic and lysogenic cycle (P_L, P_{RM}, P_R, P_{RE}, P_{AQ}, $P_{R'}$, P_I)

-<u>a sinistra</u>, comprende i geni che codificano per proteine strutturali della testa e della coda;

-<u>al centro</u>, contiene geni responsabili per la lisogenia, cioè il processo che porta all'integrazione del DNA virale ed altri processi ricombinativi.

-<u>a destra</u> contiene i geni coinvolti nella replicazione del DNA e nel ciclo litico.

Genomic structure of the Lambda phage



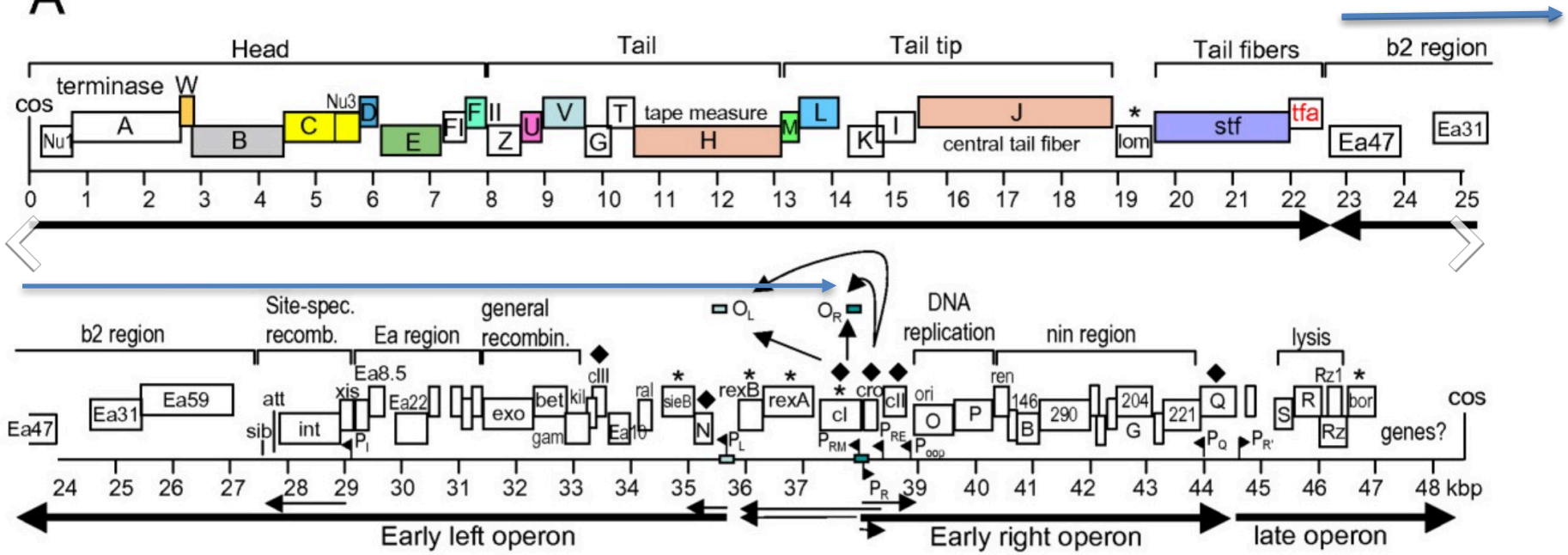
PRM: weak promoter (needs activator CAP); drives cI

PL, PR: strong, constitutive promoters

Using Phages as cloning tool to generate recombinant DNA

Figure 1.

Lambda phage can be used as a cloning tool



Sequences non required for lytic life cycle

Note: lambda phage are only viable if they contain between about 39 and 52 kbp of DNA

→ Non essential regions (=14,5 kb) can be deleted and replaced by a max of 22 kb DNA of foreign origin

→ Phage is viable

→ Phage uses lytic life cycle and amplifies DNA

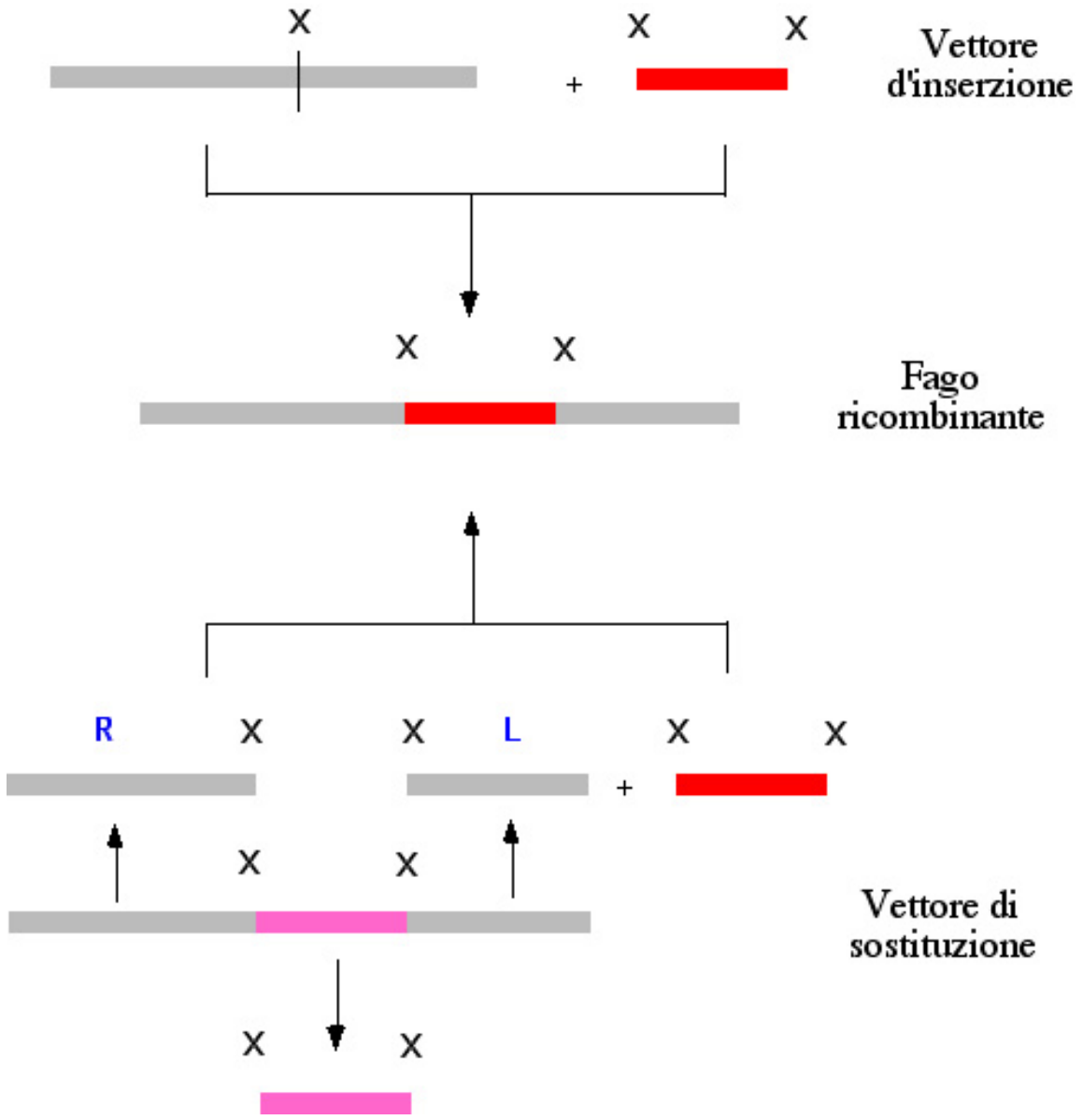
Using Lambda phages as cloning vectors

Sono stati sviluppati due tipi di vettori λ:

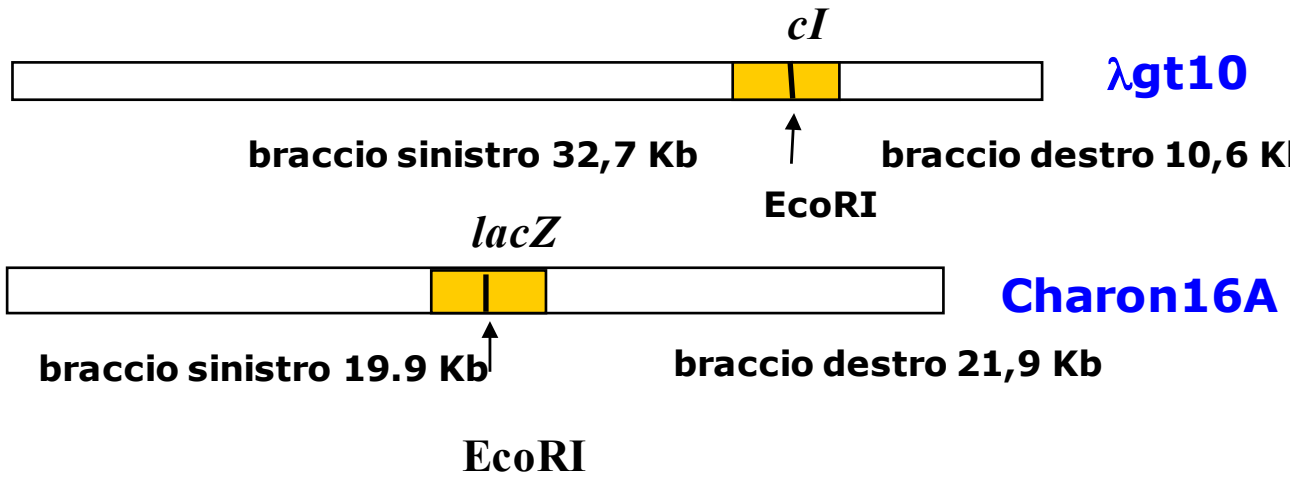
- **vettori d'inserzione**, in cui il DNA esogeno è inserito in un sito unico di restrizione;

- **vettori di sostituzione,** in cui il DNA esogeno sostituisce un pezzo di DNA del vettore (stuffer).

Using Lambda phages as cloning vectors



Vettori d'inserzione

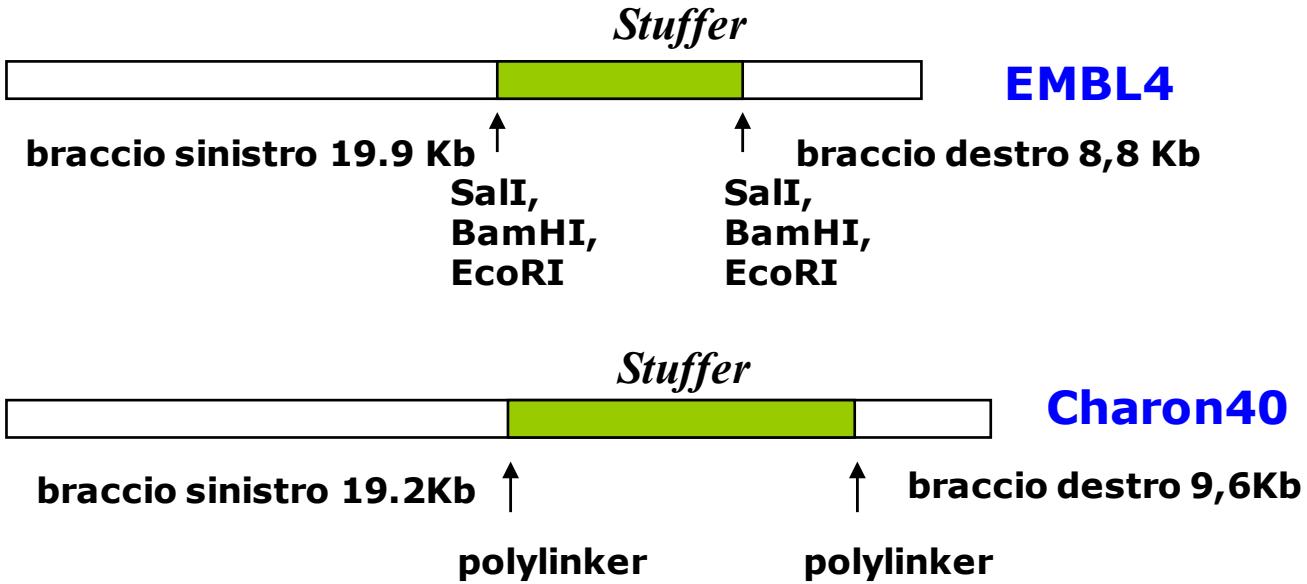


λgt10 è un buon esempio di inattivazione inserzionale. Il sito EcoRI è posizionato in mezzo al gene cI. Un inserzione al suo interno, dunque distruggerà l'integrità strutturale del repressore e il fago non sarà più in grado di entrare nel ciclo lisogeno.
Quindi i fagi con l'inserzione daranno placche chiare (placche litiche), mentre i fagi senza inserzioni avranno colonie torbide (miscela di fagi lisogeni e litici)

Charon 16A contiene l'MCS nel **gene lacZ** (α-peptide della β-galattosidasi) e i cloni ricombinanti sono identificabili attraverso lo screening bianco-blu in E.coli che esprimono il frammento ω dell β-galattosidasi.

Questi vettori sono più facili da utilizzare e possono accettare inserti di dimensioni da 8 fino a 10-12 Kb. Sono generalmente utilizzati per costruire librerie di cDNA.

Vettori di sostituzione

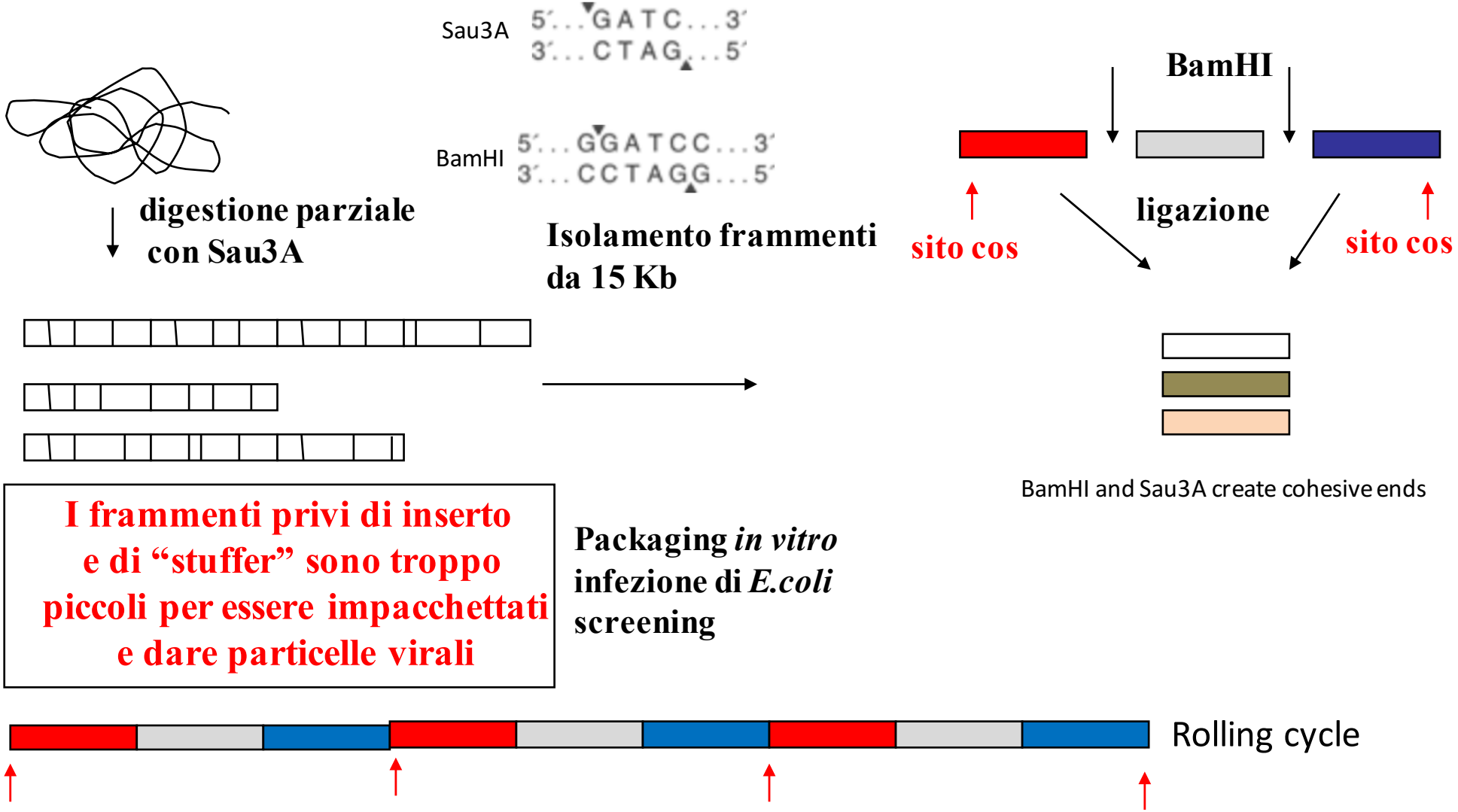


λ**EMBL4** (42Kpb) contiene uno stuffer di 14 kpb tra il braccio destro e sinistro.

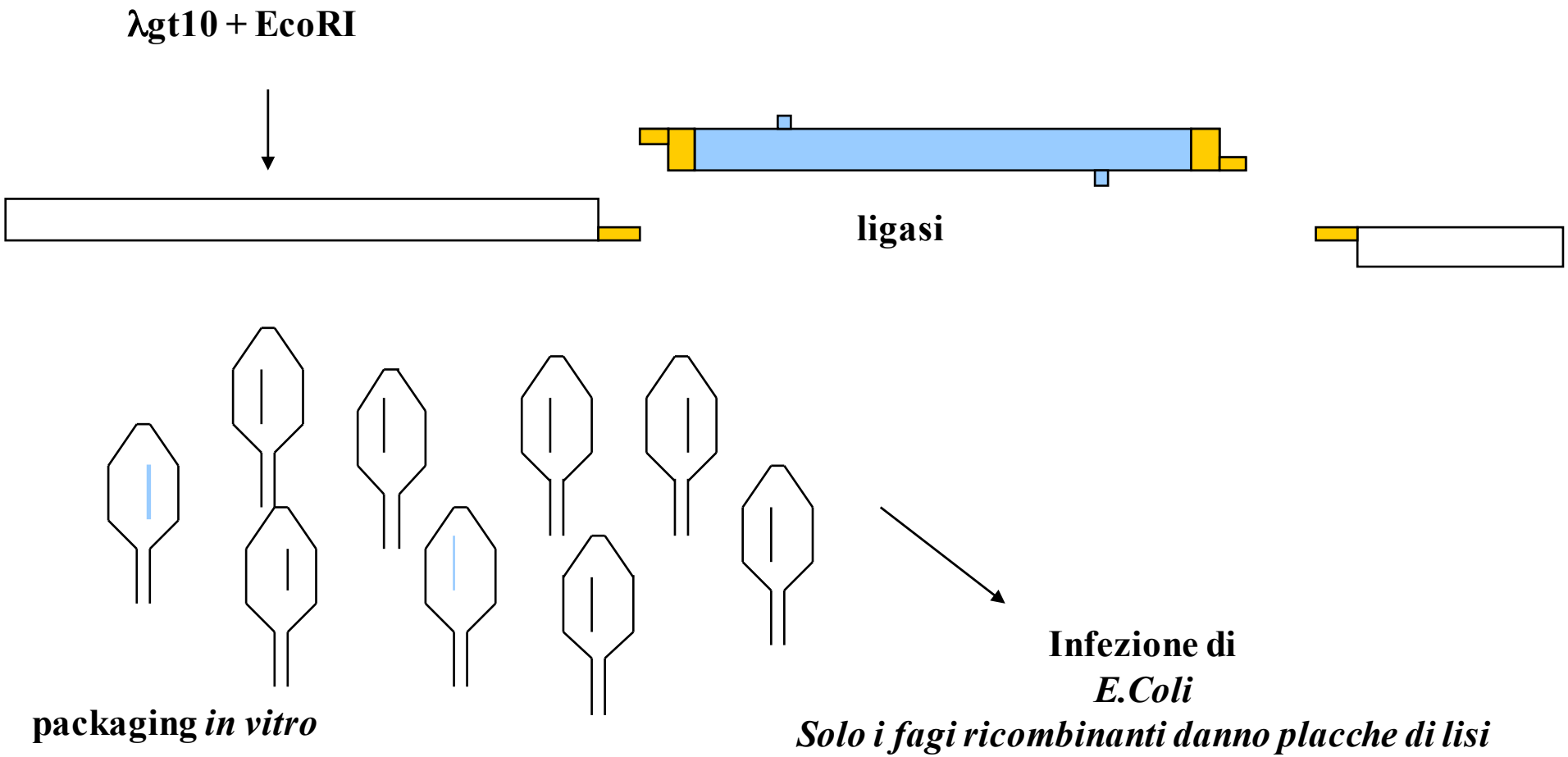
I vettori con due siti di taglio, in cui la parte centrale del DNA (frammento stuffer) può essere rimossa e sostituita con un frammento di DNA estraneo. Possono accettare inserti da 10 a 22 Kb e sono in genere utilizzati per costruire librerie genomiche.

Clonaggio in vettori di sostituzione

I vettori di sostituzione permettono di clonare frammenti di 15-20 kb. Si effettua una digestione genomica parziale con Sau3A e si purifica una popolazione intorno a i 15 kb. Si digerisce quindi un vettore di sostituzione con BamHI, complementare a Sau3A, e si ligano insieme i bracci destro, sinistro e la popolazione di digesti parziali di circa 15 Kb.

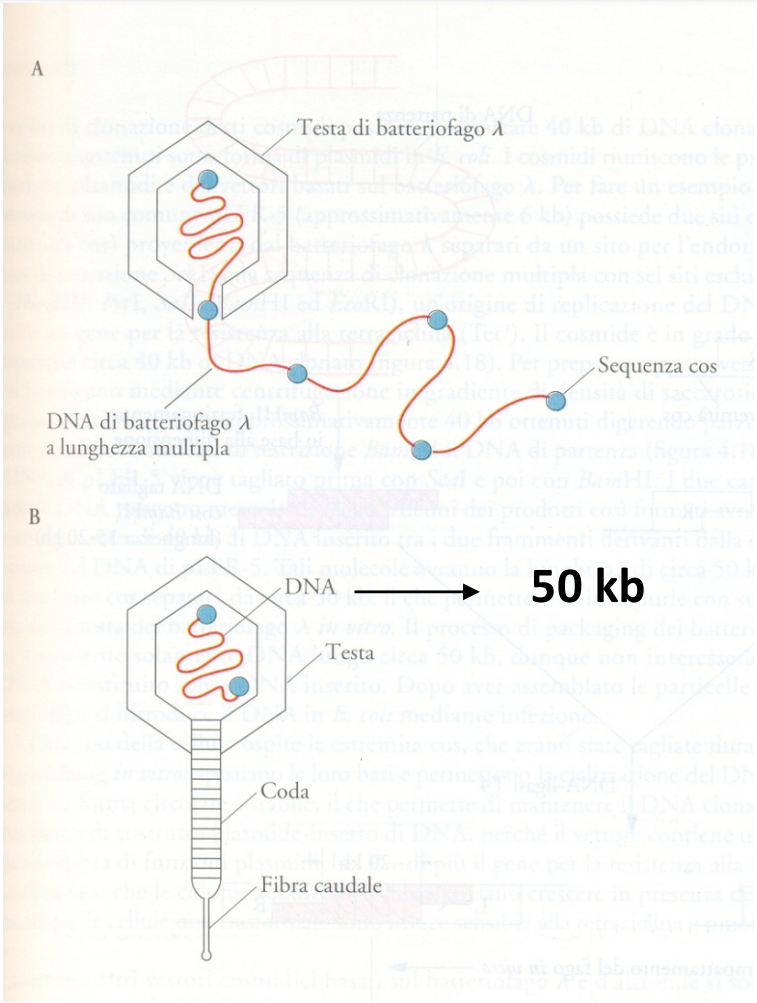


Clonaggio in vettori di inserzione



Rolling cycle

Il packaging del DNA del batteriofago l nelle teste durante il ciclo litico



Il packaging in vitro sfrutta l'esistenza di fagi mutanti, che producono teste fagiche vuote, in quanto mancanti di una proteina necessaria per il packaging, e di fagi capaci di effettuare il packaging ma incapaci di produrre le teste.

Mescolando in provetta queste due popolazioni fagiche insieme al nostro DNA, avremo un'efficiente packaging in vitro.

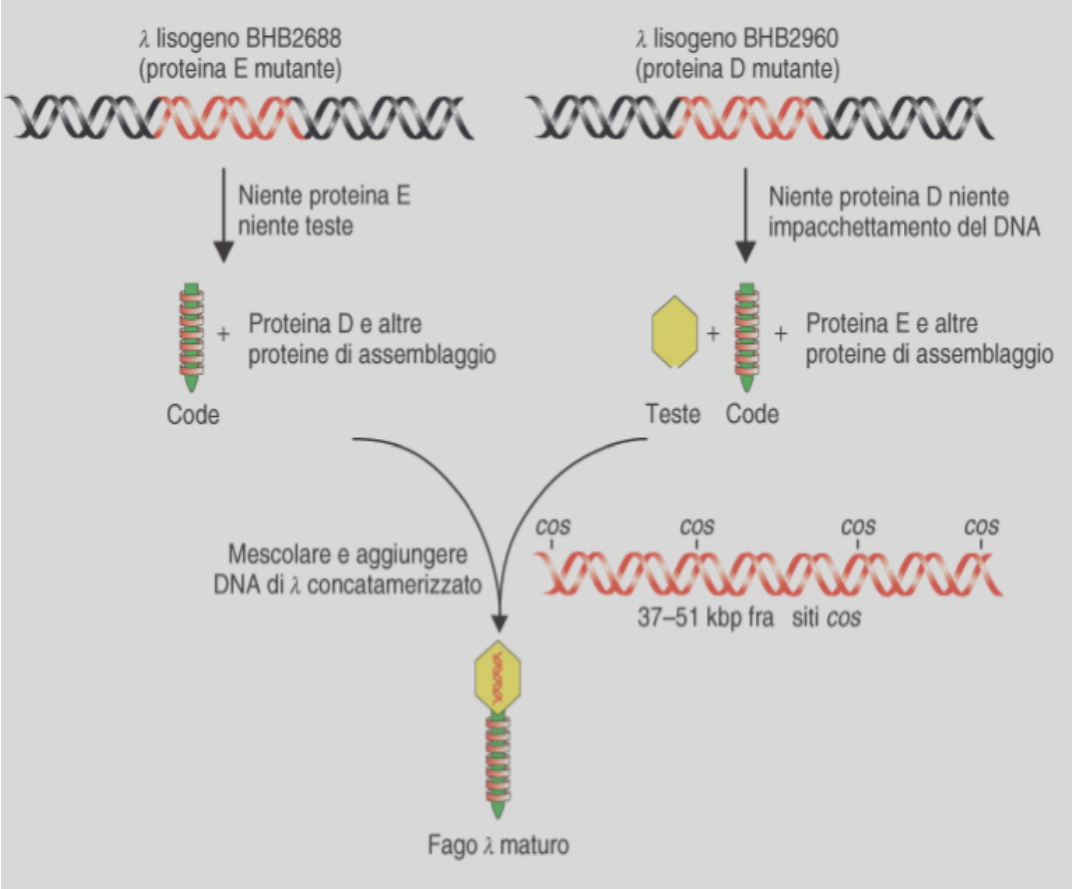
I fagi ricombinanti saranno quindi utilizzati per infettare una popolazione di batteri sensibili ai fagi in soft agar.

Il risultato sarà la produzione di placche di lisi, apparentemente simili a colonie batteriche.

Per il packaging esiste:

- un **limite inferiore**, pari al **75%** del genoma di λ (circa 37 Kbp, al di sotto il DNA non viene impaccato e il fago non è vitale)
- un **limite superiore**, pari al **105%** della lunghezza del suo DNA (circa 51Kbp)

Packaging *in vitro* di particelle fagiche



Un packaging molto efficiente *in vitro* può essere ottenuto mescolando il DNA ricombinante di λ, con due ceppi di *E.coli* che portano fagi λ lisogeni difettivi nel processo di impacchettamento:

-ceppo BHB2690, produce teste fagiche vuote, perché mancante della proteina D necessaria per il packaging del DNA;

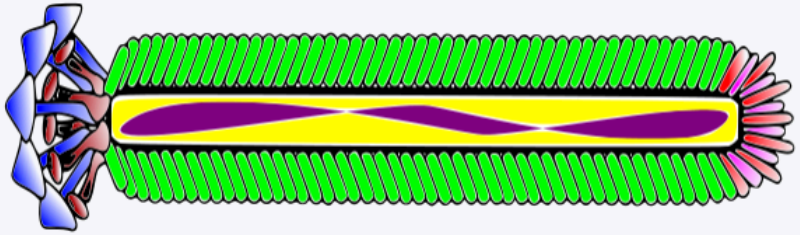
- ceppo BHB2688, non produce la proteina E, e quindi le teste, ma contiene la proteina del packaging.

Ricordiamo che:

- **1- durante un'infezione litica il DNA virale è introdotto, sotto forma di DNA lineare di circa 48.5 Kb, all'interno della cellula batterica;**
- **2- una volta all'interno della cellula, il DNA fagico ricircolarizza, sfruttando le sue estremità coesive *cos,* e si replica come molecola circolare con una replicazione di tipo Teta, simile a quella batterica. Da un certo momento in poi, lambda comincia a replicarsi con una modalità di tipo "rolling circle, cominciando a formare lunghi concatenameri di singoli genomi fagici;**
- **3- contemporaneamente si esprimono i geni strutturali che assemblano delle teste "vuote", dove vengono inseriti singole unità di lambda (un DNA di 48.5 Kb definito da due siti cos).**
- **4- infine viene assemblata la coda e i fagi lisano Il batterio e fuoriescono.**

Il fago M13

M13 bacteriophage



Blue: Coat Protein pIII; Brown: Coat Proteín pVI; Red: Coat Protein pVII; Limegreen: Coat Protein pVIII; Fuchsia: Coat Proteín pIX; Purple: Single Stranded DNA

The coat's dimensions are flexible though and the number of p8 copies adjusts to accommodate the size of the single stranded genome (6400 nt) it packages.

The phage appear to be limited at approximately twice the natural DNA content.

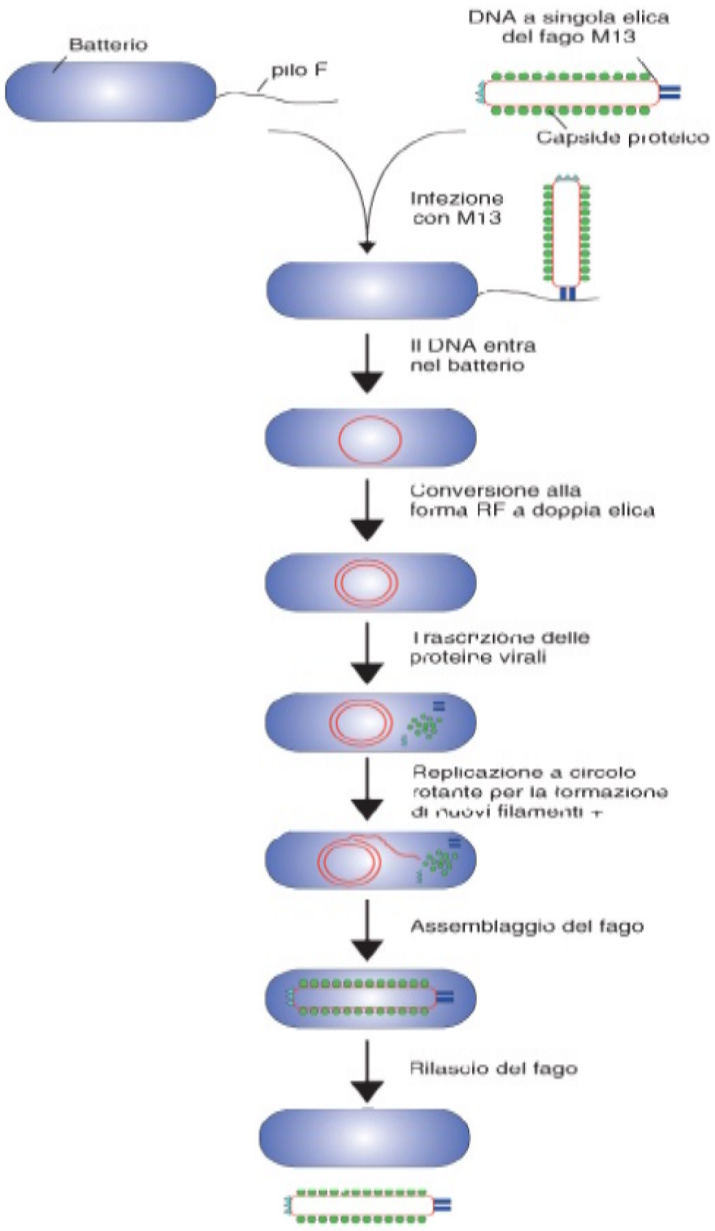
M13 is a virus that infects the bacterium Escherichia coli.

It is composed of a circular single-stranded DNA molecule encased in a thin flexible tube made up of about 2700 copies of a single protein called P8, the major coat protein.

The ends of the tube are capped with minor coat proteins.

Infection starts when the minor coat protein P3 **attaches to the receptor at the tip of the F pilus of the bacterium**.
→ Phage can only infect bacteria that have the F plasmid (encdes pilus prtoeins for conjugation)

Il fago M13



La biologia del fago M13: il ciclo vitale

•Il genoma del fagoM13 è costituito da una molecola di DNA circolare a singola elica, lunga 6407 nt.

• M13 infetta solo ceppi che portano il plasmide F. Esso infatti entra nella cellula batterica attraverso il pilo codificato dal fattore F. + strand (single stranded enters the cell)

• Il DNA viene convertito nella forma replicativa intermedia a doppio filamento (RF, double stranded replicative form). Vengono sintetizzate circa 100 copie della forma RF.

→ - strand: template for trascription of phage proteins

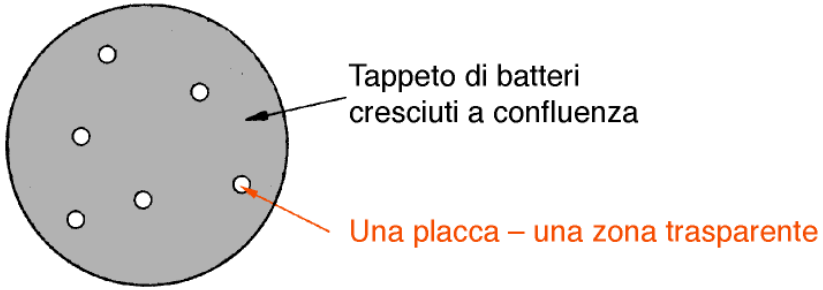
→ - strand: templete for rolling cycle replication

• Inizia la replicazione a cerchio rotante di un'unica elica del genoma virale. Vengono sintetizzate circa 1000 copie.

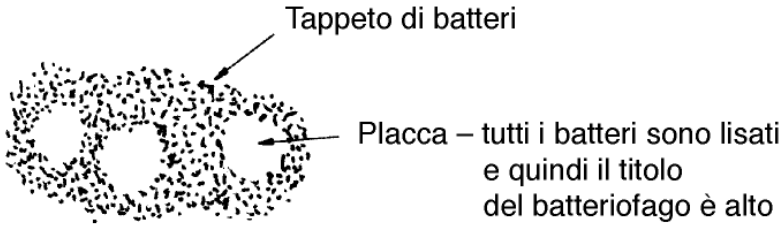
• Il genoma (+ strand) viene assemblato alle proteine per costituire le nuove particelle virali che fuoriescono dalla cellula batterica senza causarne la lisi.

Il fago M13

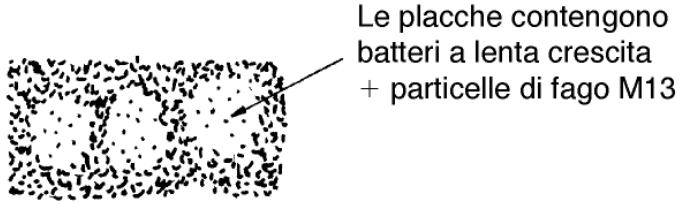
(a) Placche su un tappeto di batteri



(b) Placche litiche



(c) Placche di M13



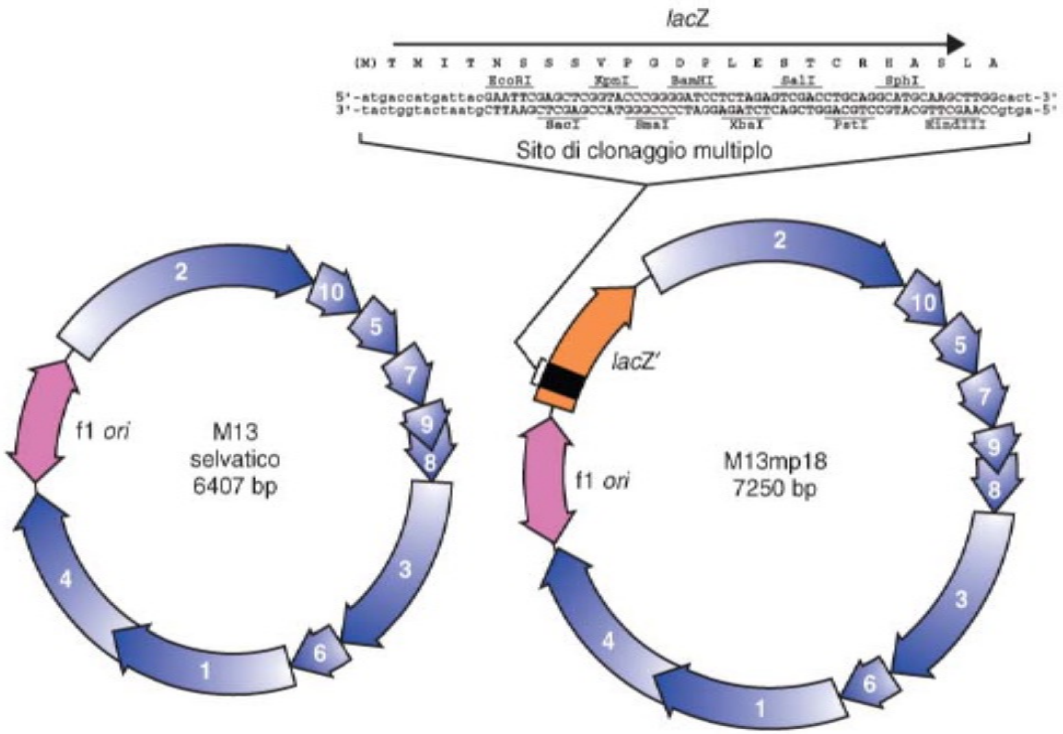
Infection with M13 is not lethal.

However, the infection causes **turbid plaques** in E. coli because infected bacteria grow more slowly than the surrounding uninfected bacteria.

It engages in a viral lifestyle known as a **chronic infection** which is neither lytic nor temperate.

However a decrease in the rate of cell growth is seen in the infected cells.

Il fago M13



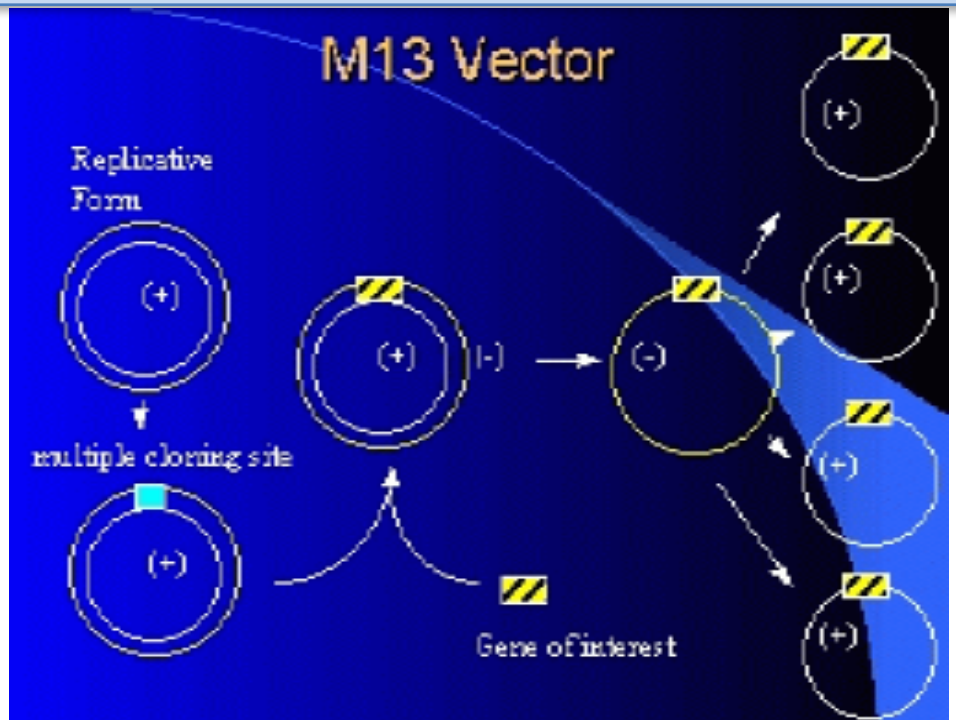
Il genoma del fago M13, nella sua forma replicativa intermedia RF, viene utilizzato come vettore di clonaggio.

holds f1 ori: amplifaction of DNA as Single stranded DNA.

Modificazioni del genoma selvatico per l'ottimizzazione del vettore:

- Aggiunta del gene *lacZ'* come marcatore genetico per la selezione bianco/blu delle placche positive contenenti il genoma ricombinante
- Aggiunta di un polylinker all'interno del gene lacZ'
- Eliminazione dei siti di restrizione naturali

Tappe del clonaggio di DNA a singolo filamento nei vettori derivati dal fago M13



Si linearizza il vettore M13 a doppio filamento con un enzima di restrizione, come se fosse un plasmide.

• Si mescola il vettore linearizzato con l'inserto avente estremità coesive compatibili con quelle del vettore. Si aggiunge la ligasi.

•Il prodotto della reazione di ligasi viene inserito nelle cellule *E. coli* mediante trasformazione.

• Si selezionano le placche di colore bianco contenenti il vettore ricombinante e si scartano le blu contenenti il vettore virale senza inserto.

- Viral (+) strand DNA enters cytoplasm
- Complementary (-) strand is synthesized by bacterial enzymes
- DNA Gyrase, a type II topoisomerase, acts on double-stranded DNA and catalyzes formation of negative supercoils in double-stranded DNA
- Final product is parental replicative form (RF) DNA
- A phage protein, pII, nicks the (+) strand in the RF
- 3'-hydroxyl acts as a primer in the creation of new viral strand
- pII circulizes displaced viral (+) strand DNA
- Pool of progeny double-stranded RF molecules produced
- Negative strand of RF is template of transcription
- mRNAs are translated into the phage proteins

Clonando l'inserto nell'orientamento opposto si ottengono copie multiple dell'elica complementare

Tappe del clonaggio di DNA a singolo filamento nei vettori derivati dal fago M13

Perché si clona DNA a singolo filamento?

►Per sequenziare l'inserto clonato con il metodo di inserto Sanger
- Per mutare l'inserto con le tecniche di mutagenesi sito-specifica
- Per ottenere sonde di ibridazione a singola elica

Vantaggi del vettore M13
- La forma replicativa RF a doppio filamento può essere manipolata come un normale plasmide

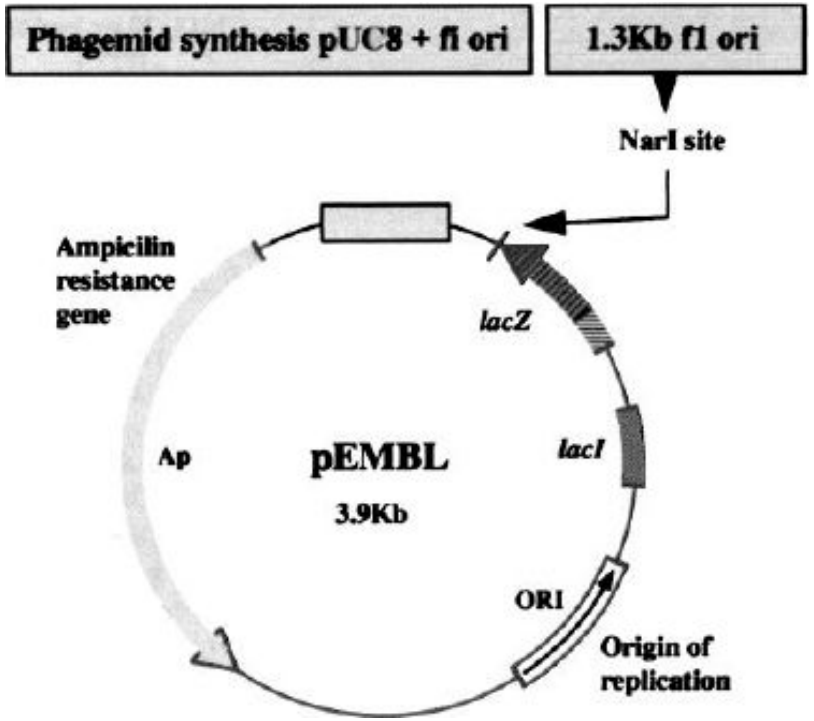
Svantaggi del vettore M13
- limit if insert size is low(2 kb)
- relative low copy number
- phage protein toxic at high concentration

Modified vectors pased on M13 phage : PHAGEMIDE VECTORS

phagemid vectors
= plasmid that holds an M13 origin of replication
e.g. pEMBL18 & pEMBL19

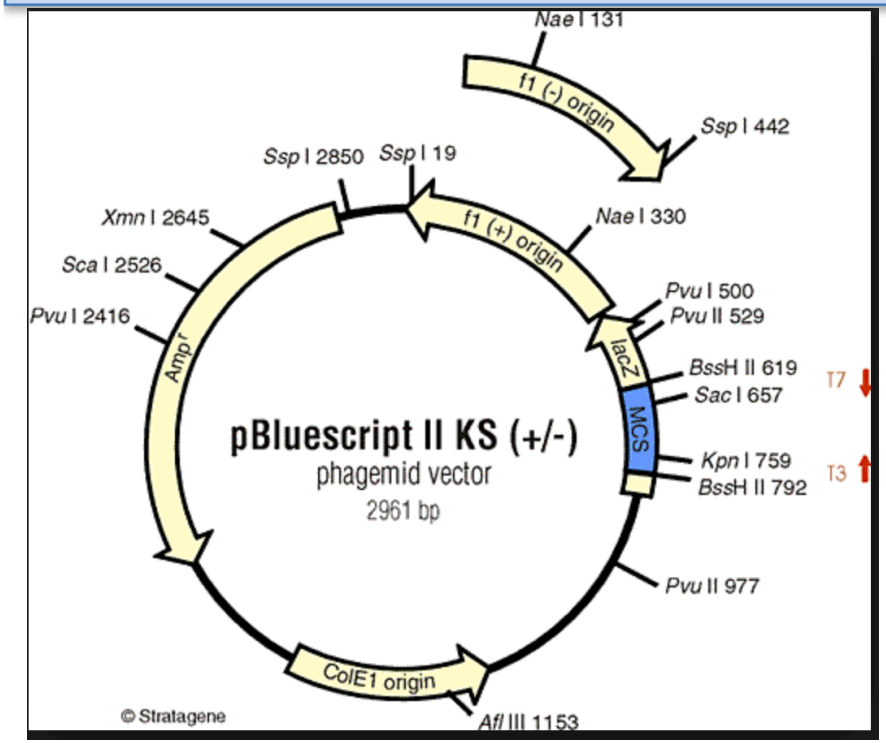
– pUC18 & pUC19 + M13 origin of replication
maintained in host cell like regular plasmid
– high copy number, lots of copies of cloned DNA

– if M13 **helper phage** infects cell containing pEMBL
– phage proteins package single-stranded plasmid
– in phage particles – collected like regular phage



Note – helper phage genomes packaged too
– but not as many present as plasmid
– get 100 fold excess of plasmid packaged
helper phage contamination not a problem for sequencing
– use "universal" primers
– homologous to plasmid sequences
– no cross-reaction with phage sequences

Modified vectors based on M13 phage : IMPROVED PHAGEMIDE VECTORS



Amp^R : Ampicilin resistance
ColE1 origin: origin of replication in bacteria
MCS: multiple cloning site
Lac Z: beta galactosidase: cleves H-Gal → blue color (colonies)
T7; T3: Promoter for transcriptio of RNA polymerase of T3 and T7 phage RNA Polymerase (short sequences often used for seuencing using primers; same for M13)

F1 (-) (+) origin:

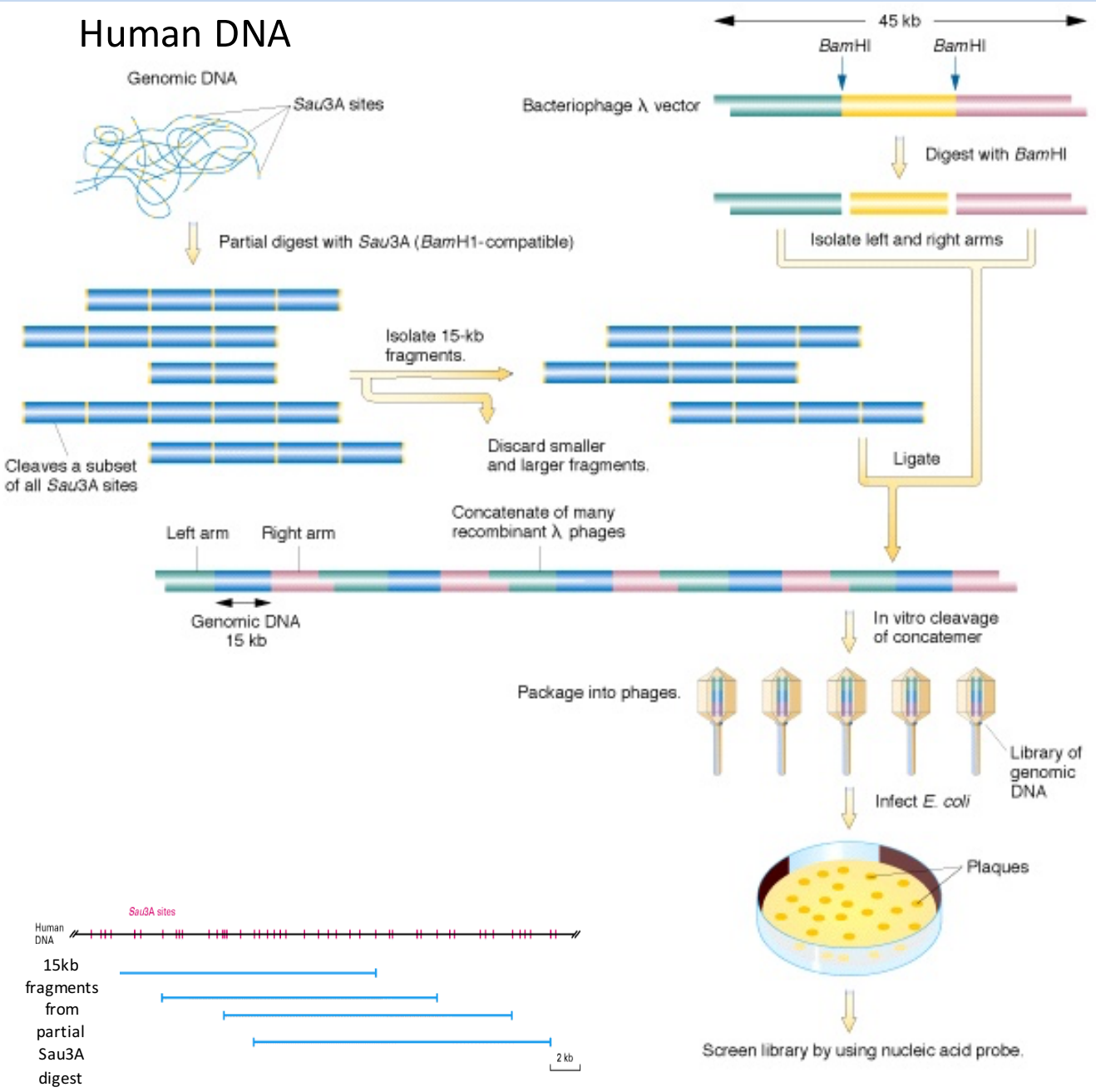
pBluescript II is a phagemid that can be secreted as single-stranded DNA in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous f1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E. coli* with the F+ phenotype (containing an F´ episome), pBluescript II phagemids will be secreted as single-stranded f1 "packaged" phage when the bacteria has been infected by a helper phage. Since these filamentous helper phages (M13, fl) will not infect *E. coli* without an F´ episome coding for pili, **it is essential to use XL1-Blue MRF´ or a similar strain containing the F´ episome.**[7,8]

Similarly to a plasmid, a phagemid can be used to clone DNA fragments and be introduced into a bacterial host by a range of techniques, such as transformation and electroporation. However, infection of a bacterial host containing a phagemid with a 'helper' phage, for example VCSM13 or M13K07, provides the necessary viral components to enable single stranded DNA replication and packaging of the phagemid DNA into phage particles. The 'helper' phage infects the bacterial host by first attaching to the host cell's pilus and then, after attachment, transporting the phage genome into the cytoplasm of the host cell. Inside the cell, the phage genome triggers production of single stranded phagemid DNA in the cytoplasm. This phagemid DNA is then packaged into phage particles. The phage particles containing ssDNA are released from the bacterial host cell into the extracellular environment.

Ampicillin

- Ampicillin binds to and inhibits a number of enzymes in the bacterial membrane that are involved in the synthesis of the ***gram negative*** cell wall.
  - Therefore, proper cell replication cannot occur in the presence of ampicillin.
- The ampicillin resistance gene (***amp^r***) codes for an enzyme (***b-lactamase***) that is secreted into the periplasmic space of the bacterium where it catalyzes hydrolysis of the b-lactam ring of the ampicillin.
  - Thus, the gene product of the amp^r gene **destroys the antibiotic**.
- Over time the ampicillin in a culture medium or petri plate may be substantially destroyed by b-lactamase.
  - When this occurs, cell populations can arise which have "lost" the plasmid.

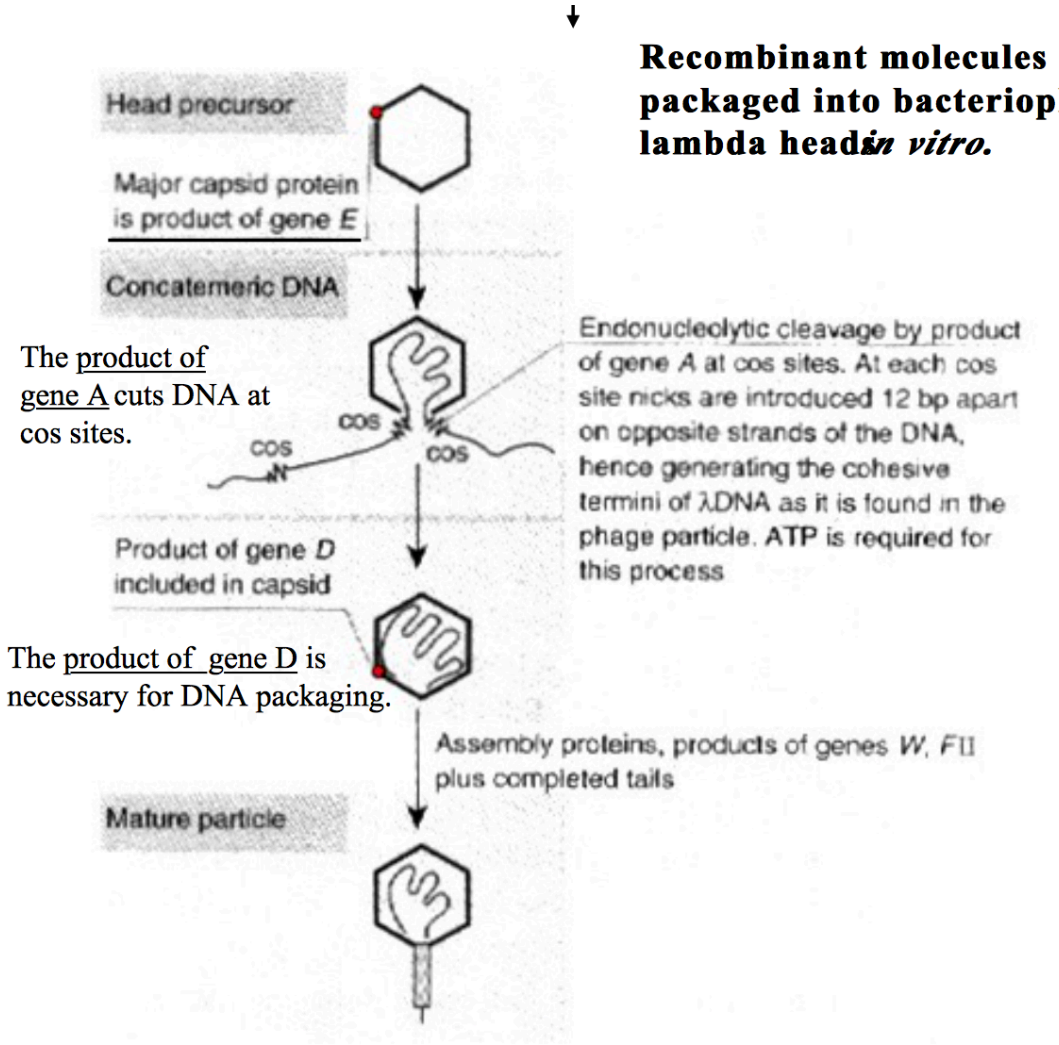
Generation of a genome library using the phage lambda



Key issues:

1. Partial digest with Sau3A generates a vast amount of DNA fragments covering the entire genome many times
2. Fragments with 15kb size are isolated to clone fragments with max. size into the lambda vector
3. Lambda phage cut with BamHI to liberate segments that are dispensable for the lysogenic life cycle
4. Lamda arm are isolated. Central region discarded
5. Ligation: Lamda arms + Genomic DNA; Sau3A is compatible with BamHI
6. Ligation generates a random assembly of arms with genomic DNA insert.
7. IN VITRO PACKAGING ENSURES THAT ONLY FUNCTIONAL FRAGMENTS ARE INSERTED INTO CAPSID
8. Cos—left arm –GENOMIC DNA—right arm-Cos
9. A pool of phages will be generated = PHAGE LIBRARY
10. Bacteria on plate are infected with phage-library
11. Individual plaques contain monoclonal phage+genomic DNA insert

In vitro packaging of phage library

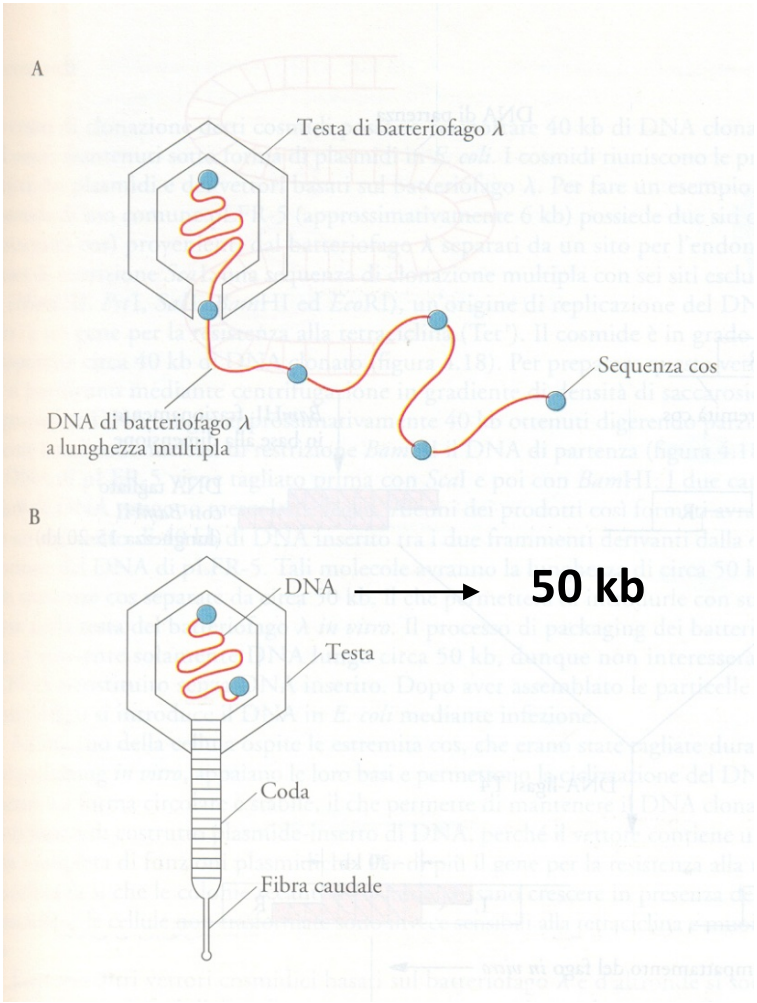


Recombinant molecules are packaged into bacteriophage lambda heads *in vitro*.

→ Efficienza di trasformazione del DNA di λ con un inserto clonato: 10^4-10^3 placche/µg di DNA

→ Impaccamento in vitro 10^6 placche/µg di DNA

In vitro packaging of phage library



Il packaging in vitro sfrutta l'esistenza di fagi mutanti, che producono teste fagiche vuote, in quanto mancanti di una proteina necessaria per il packaging, e di fagi capaci di effettuare il packaging ma incapaci di produrre le teste. Mescolando in provetta queste due popolazioni fagiche insieme al nostro DNA, avremo un'efficiente packaging in vitro. I fagi ricombinanti saranno quindi utilizzati per infettare una popolazione di batteri sensibili ai fagi in soft agar.
Il risultato sarà la produzione di placche di lisi, apparentemente simili a colonie batteriche.

Per il packaging esiste:

- un **limite inferiore**, pari al **75%** del genoma di λ (circa 37 Kbp, al di sotto il DNA non viene impaccato e il fago non è vitale)
- un **limite superiore**, pari al **105%** della lunghezza del suo DNA (circa 51Kbp)

How many genomic clones must be screened to find your gene?

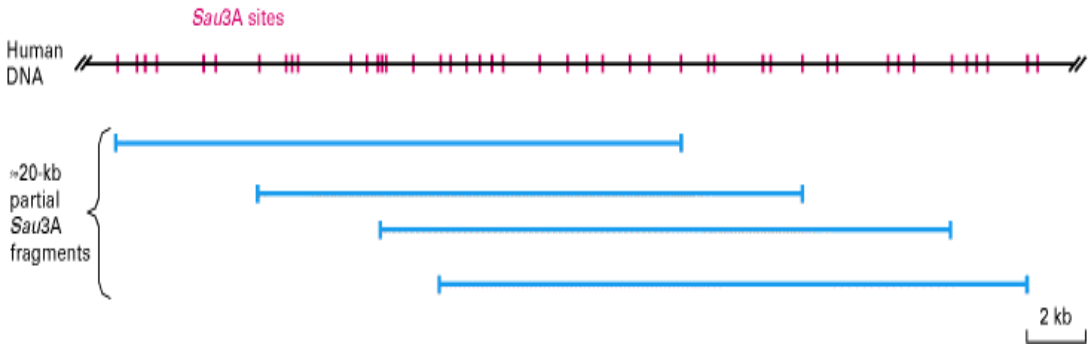
Theoretically, you will need to screen N clones where N=ln(1-P)/ln(1-f) where

P=the probability of finding your gene and

f=the average size of the cloned genomic sequence in your vector divided by the total genome size.

How many clones must you screen to find your gene in a human gene library packaged into a lambda library with 99% certainty?

N=ln(1-0.99)/ln(1-20kb/2.8 x 10^6kb)= 6.4 x 10^5 clones (lambda phages + insert)



- In order to sequence the entire genome it is necessary to produce overlapping sequences.
- Using a technique called chromosome walking, it is possible to order to genomic clones.
- The human genome contains 3 × 10^9 base pairs. Each vector contains 20kb that means that it is necessary to generate about 6,4 x 10^5 phages.
- You can screen 5 × 10^4 plaques on each petri dish meaning that you can contain all the human genome on 20-30 petri dishes.
- If plasmids were used instead of λ phage it would take 5000 petri dishes.

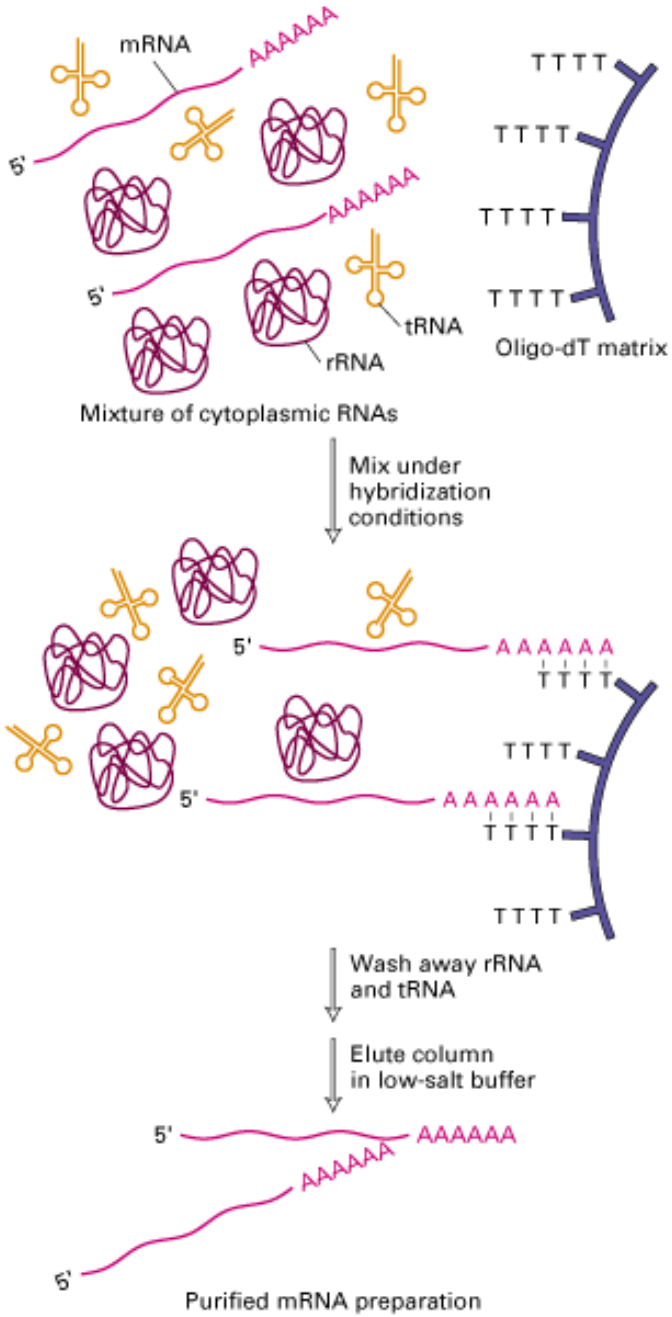
Generation of an expression library using the phage lambda

First steps in making a cDNA library:

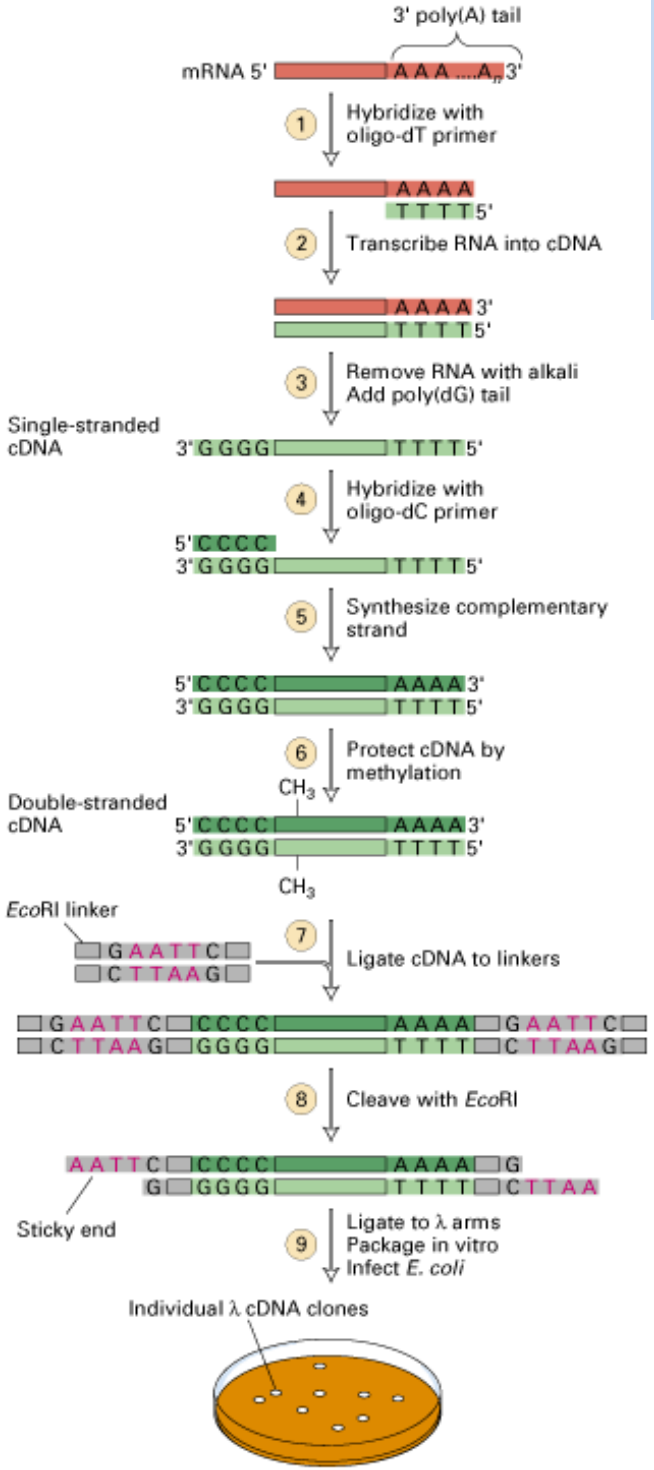
1. Preparation of RNA from different human tissue
2. Purification of polyadenylated mRNA using oligo(dT)-cellulose

Note: selection of the proper source (organ, tissue) of the RNA is critical here!

→ There exist lung cDNA libraries, brain tissue libraries, etc….



Construction of an expression library using the phage lambda system



1. Isolated mRNA libraries need to be converted into dsDNA to be cloned into a vector
2. Generate cDNA
3. Treat with EcoRI methylase to protect from digest by EcoRI (later)
4. Ligate adapters to termini
5. Adapters contain restriction sites that can be used to clone cDNAs into phage vector (here: EcoRI; depeded on the phage type used for library construction
6. Adaptor cut and ligated – random – to lampda phage arms (here cut with EcoRI); note: inserts are protected by DNA methyalation
7. Concatamer formation
8. In vito packaging ensures that functional lambda vectors+insert are packaged
9. Infection of E.coli
10. Observation of plaques

How to screen a Lambda library

TO SCREEN THE LIBRARY YOU NEED A "TOOL" THAT CAN IDENTIFY A SEQUENCE/PROTEIN OF INTEREST

A. RADIOACTIVELY LABELLED PROBE

- Using a **DNA probe (min. ca. 100 bp double stranded)** with a homologous sequence (e.g., a homologous cDNA or gene clone from a related species)

- Using an **oligonucleotide probe (20 nucleotides single stranded)** based on a known amino acid sequence (requires purification of the protein and some peptide sequencing)

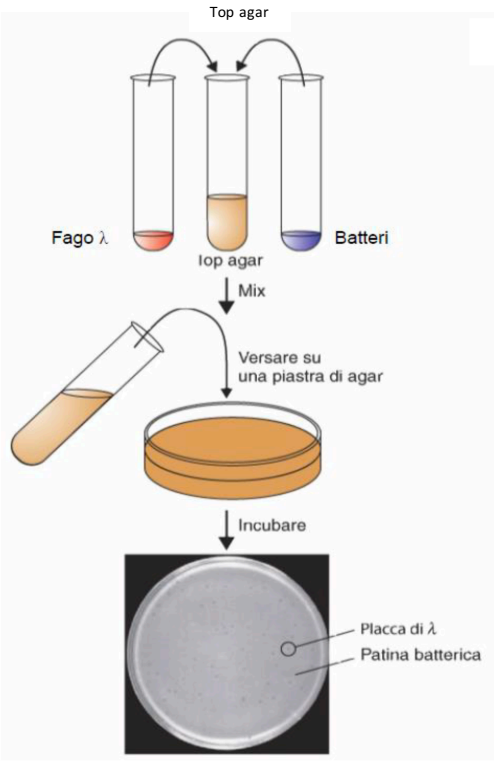
- Differential screening (labelled cDNA probes)

B. ANTIBODY AGAINST A PROTEIN

- Special case: Using an antibody against the protein of interest (note: this requires use of an expression vector)

How to screen a Lambda library

MAKING MASTER PLATES AND PLAQUE LIFT



Step 1:
- get in vitro packaged lambda library (correct titer)
- get bacteria suspension (correct density)
-Prepare »top agar» = soft agar that is plated in top of normal agar (solid)

Step 2: Mix top agar with bacteria and Phage library

Step 3: Plate on top of agar plate and incubate for 12-16 hours at 37°C

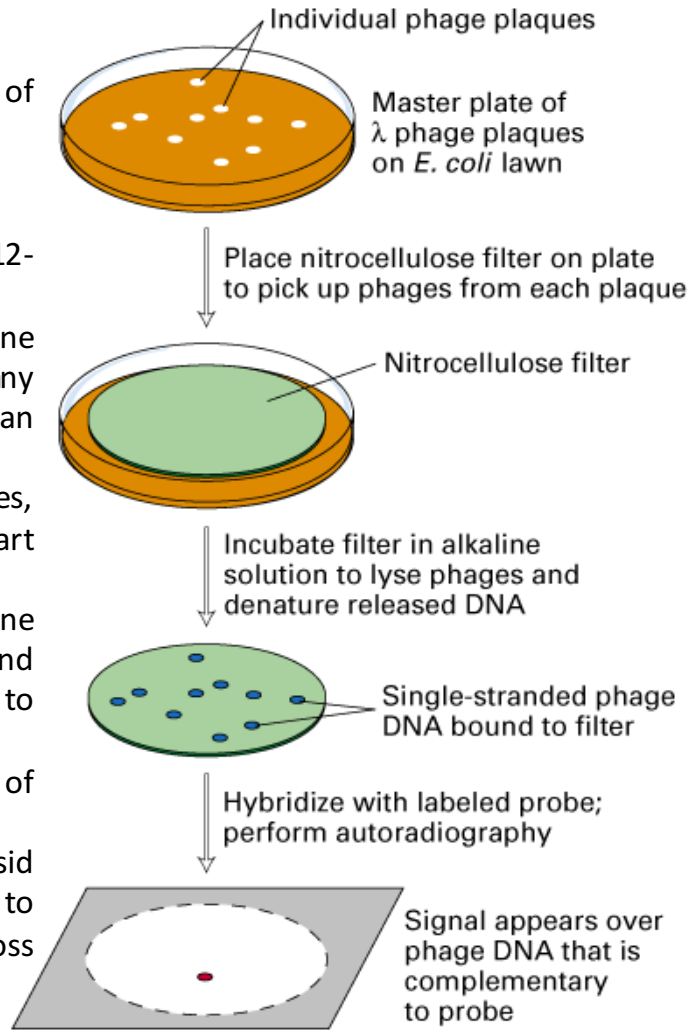
Step 4: Take out plates from incubator and determine number and size of plaques (remember how many plaques are necessary to cover the entire human genome/transcriptome)
= **MASTER PLATE** (you'll have dozens of master plates, each having a collection of inserts covering the a part of the respective genome/transcriptome)

Step 5: **PLAQUE LIFT:** place nitrocellulose membrane on top of soft agar. Punch wholes into membrane and agar plate to allow the alignment of the membrane to the pattern of the plaques for each plate

Step 6: remove nitrocellulose membrane; phages of plaques are attached to membrane

Step 7: let membrane float on alkaline solution; capsid proteins and DNA get denatured; DNA attaches to membranr. After that renatualization and UV cross linking = covalent attachment of DNA to membrane

Step 8: Hybridization with probes



How to screen a Lambda library

LABELLING OF A PROBE

1. Labelling by incorporation of [alpha-^{32}P]-dCTP by klenow fragement (exo$^-$)

Step 1: Get dsDNA for making the probe
→ Depends on scientific question; for example:
- You have the yeast gene and want the human homolog → take yeast gene/cDNA cloned into a vector
- You have a human cDNA (from mRNA) and want the entire gene → take human cDNA cloned in a vector
- You have a piece of cDNA of a human gene and want the entire cDNA → take piece of human cDNA cloned in a vector

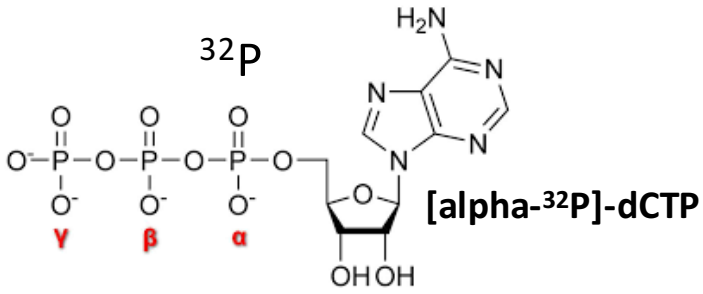
Step 2: Use restriction enzymes to cut out and gel-purify your fragment of interest

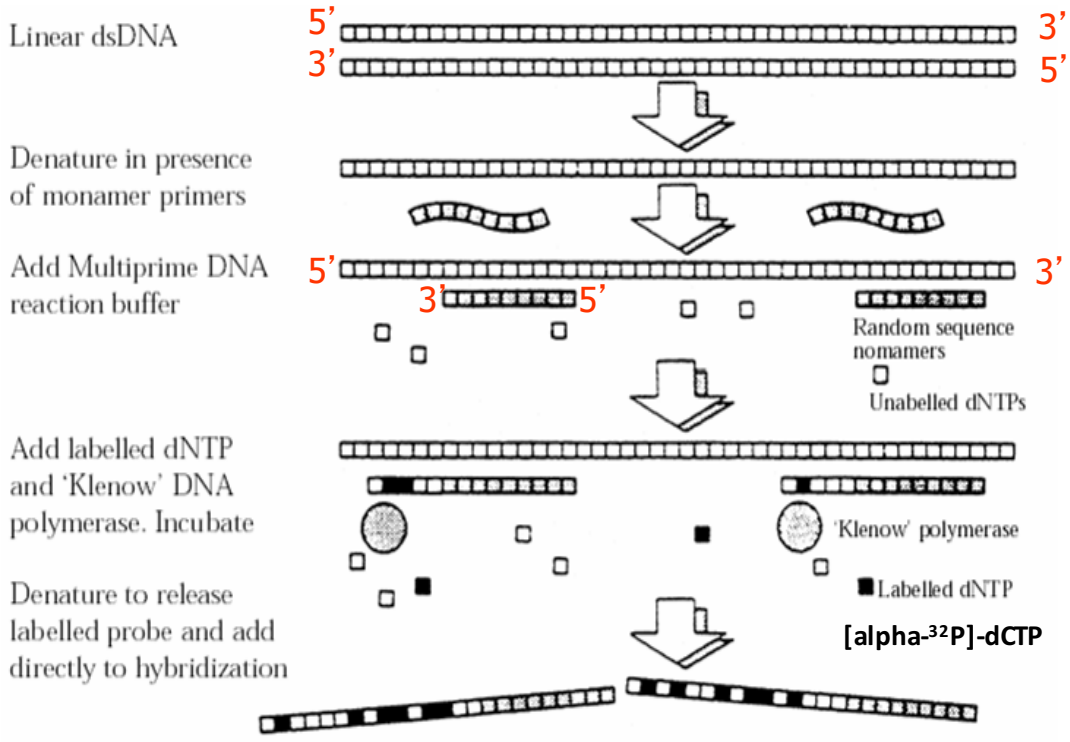
Step 3: denature dsDNA; add non-labelled mix if dNTPs and random 9-mer primers, buffer

Step 4: reanneal random primers to your DNA of interest and add [alpha-32P]-dCTP and the Klenow fragment. A fraction of primers that anneal perfectly will prime DNA synthesis by Klenow → radioactive [alpha-32P]-dCTP will be incorporated

Step 4: purify labelled fragments and remove non-incorporated [alpha-32P]-dCTP.

Step 5: hybridization





^{32}P is a high energy beta emitter and and decays into sulfur-32 by beta decay
Half-life: 14,3 days
Protection required
A beta particle, also called beta ray or beta radiation, is a high-energy, high-speed electron (or positron) emitted by the radioactive decay of an atomic nucleus during the process of beta decay.

Screening a genomic library using DNA hybridization to a (radio-)labeled DNA probe

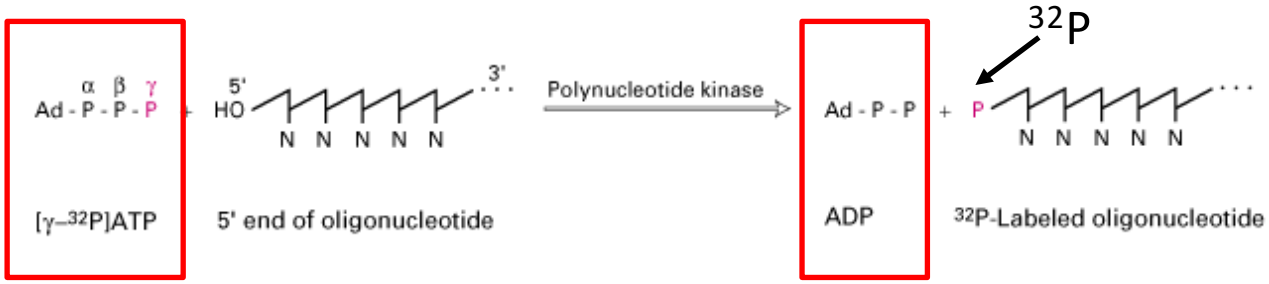
Note: The probe can be:

- A labelled DNA fragment

How to screen a Lambda library

LABELLING OF A PROBE

2. Labelling of terminus of oligonucleotide using Polynucleotide Kinase (PNK) and [gamma-^{32}P]-ATP



Step 1: Get our oligonucleotide of interest (single stranded DNA; ca. 20mer

→ Depends on scientific question; for example:

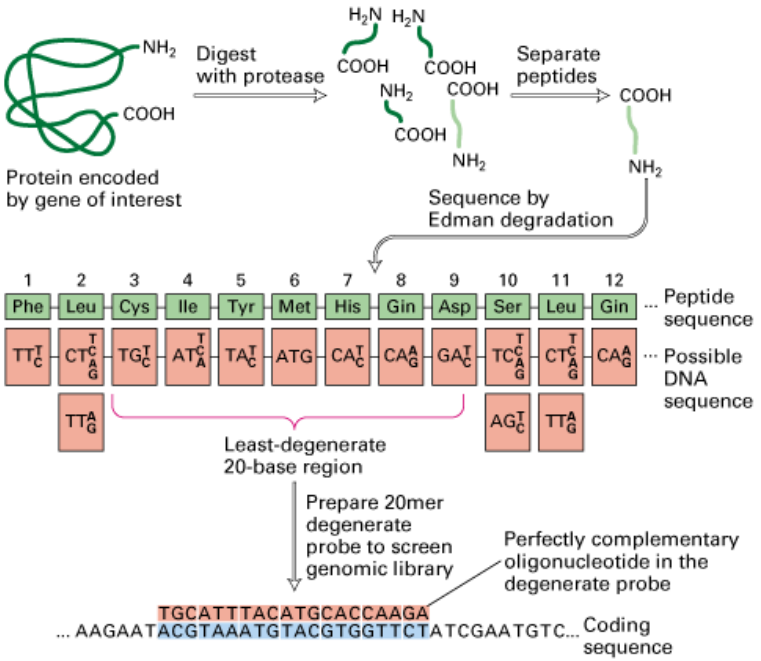
- You know the peptide sequence of a yeast protein and want to get the human homolog gene → convert peptide sequence into DNA sequence (genetic code) → design mix of oligonucelotides that "encode" peptide sequence
- You want the entire cDNA of a human gene → if you know the sequence design oligonucleotide

Step 2: Order oligonucleotide

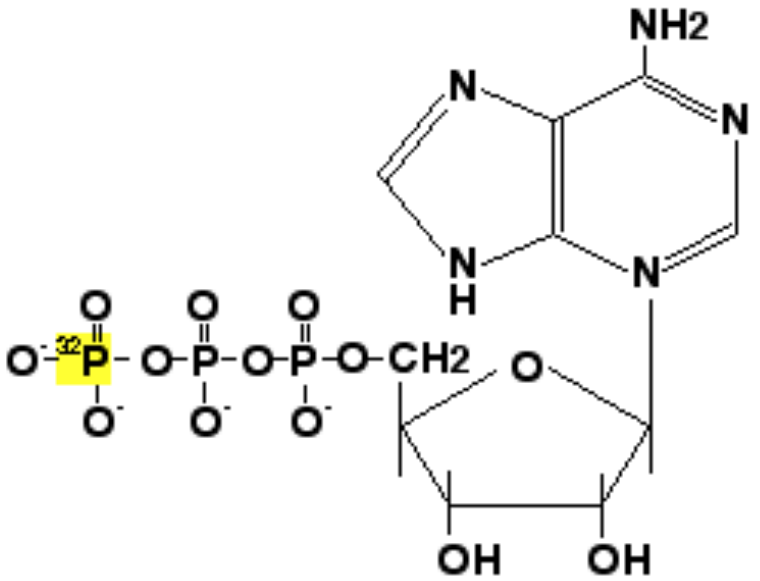
Step 3: Mix oligonucleotide with PNK and [gamma-32P]-ATP, buffer = PNK reaction

Step 4: purify labelled oligonucleotides and remove non-incorporated [gamma-32P]-ATP

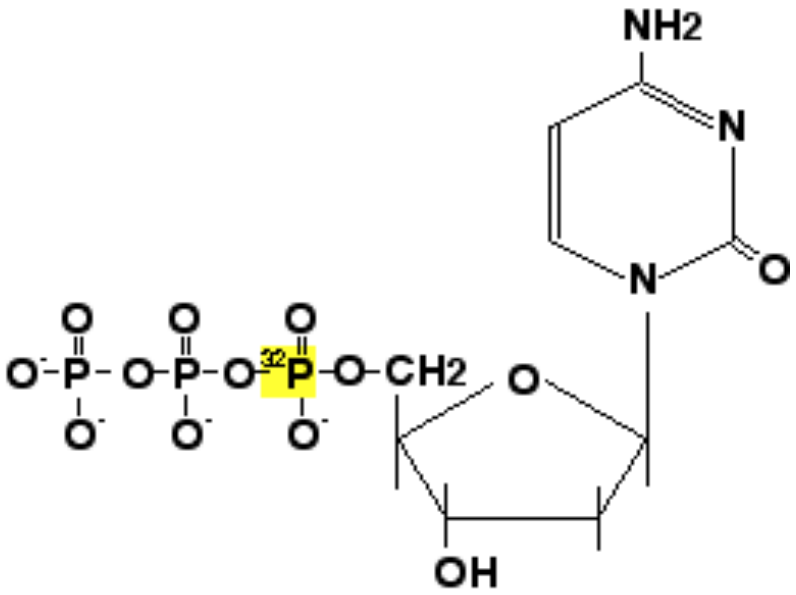
Step 5: hybridization



REMEMBER THE DIFFERENCE



[γ-^{32}P]ATP



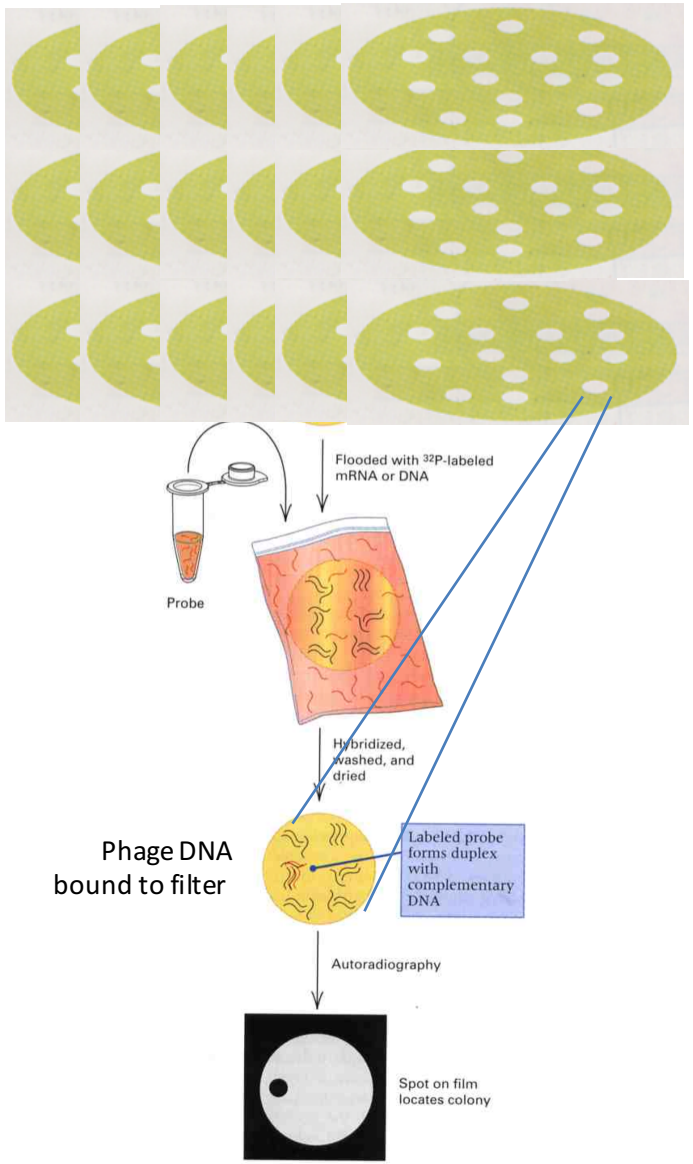
[α-^{32}P]dCTP

PNK

KLENOW

How to screen a Lambda library

Hybidization of probes to nitrocelluloase mambranes



Step 1: Dozens of nitrocellulose membranes Obtained by plaque lift – plaque number must be high enough to cover the entire genome/transcriptome

Step 2: pre-hybridize membranes with hybrization buffer at 65°C

Step 3: denature radioactive probe (oligonucleotide or dsDNA probe)

Step 4: Hybridize with radioactive probe over night at 65°C; probes will anneal specifically to DNA but als non-specifically to other DNA (imperfect base pairing)

Step 5: remove hybridization solution and wash membranes with washing buffer. Ensures that probe remains hybridized with perfectly matched sequences (perfect base pairing)

Step 6: wrap membranes in plastic and expose to X-ray film

Step 7: go back to Master plate, re-align the X-Ray film to the master plate (remember you have punched holes into the membrane-agar)
find plaque related to signal on X-ray (position); pick plaque and amplify lambda virus in E-coli, purify virus; isolate DNA and sequence insert = GET THE FULL SEQUENCE/GENE IDENTITY

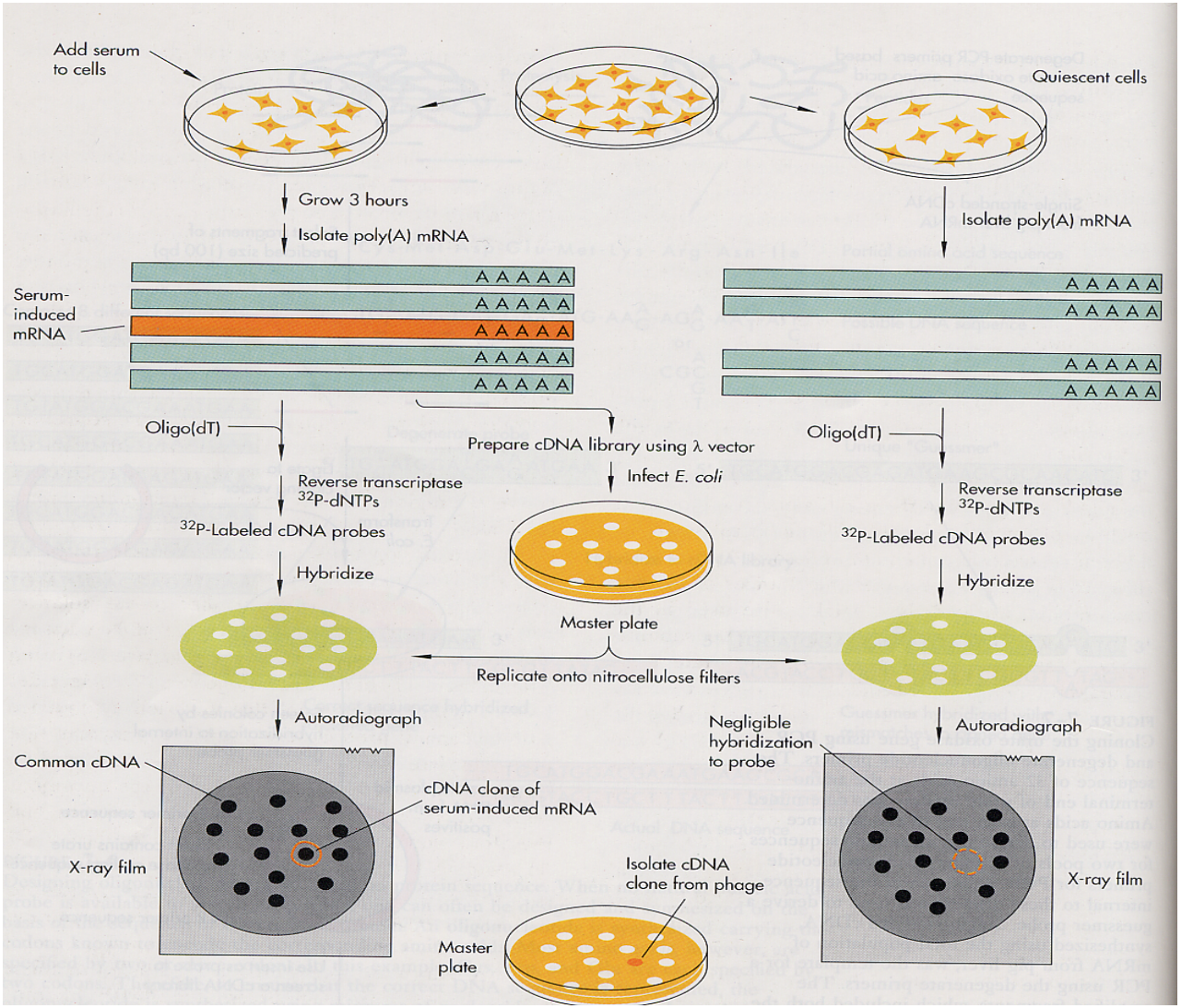
Differential screening: identifying gene that show different expression in 2 biological situations

Simple scientific question: what are genes linked with cell proliferation?

Hela cells:
Sample 1: grown in medium without serum → do not proliferate
Sample 2: grown in medium with serum → cell proliferation
Sample 1 has different gene expression than Sample 2

Step 1: make cDNA expression library from Sample 2 = Master plate (number of plaques cover entire transcriptome multiple times)
Step 2: Make probes:
Probe 1: radioactively labelled cDNA from Sample 1
Probe 2: radioactively labelled cDNA from Sample 2
Step 3: make 2 plaque lifts from each master plate of the cDNA lambda library (they are dublcates where each plaque as the same position on the membrane)
Step 4: hybridize each probe on a duplicate master plate membrane
Step 5: compare autoradiographs
Result:

- most plaques will give a signal on both dublicated membrane = gene expressed in Sample 1 and Sample 2 (not differentially expressed)
- Some signals will be only observed in plaque lift hybidized with radioactive cDNA from Sample 2 = genes expressed in serum grown Hela cells –> potential gene linked with cell proliferation

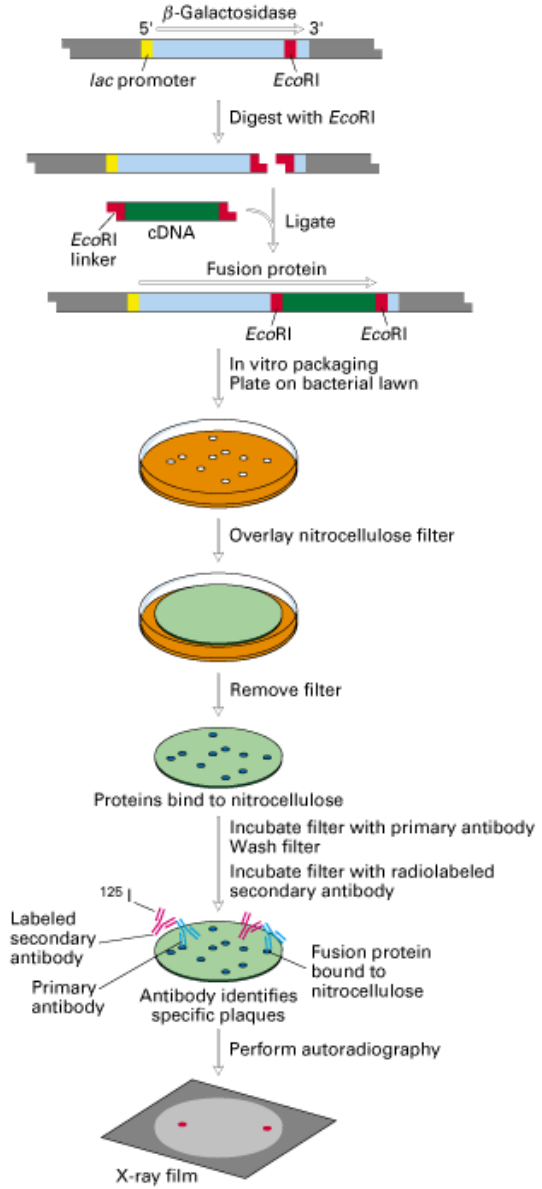


Step 6: go back to Master plate, find plaque related to signal on X-ray (position); pick plaque and amplify lambda virus in E-coli, purify virus; isolate DNA and sequene cDNA insert = GET THE GENE IDENTITY

How to screen a Lambda library

USING ANTIBODIES AS A PROBE

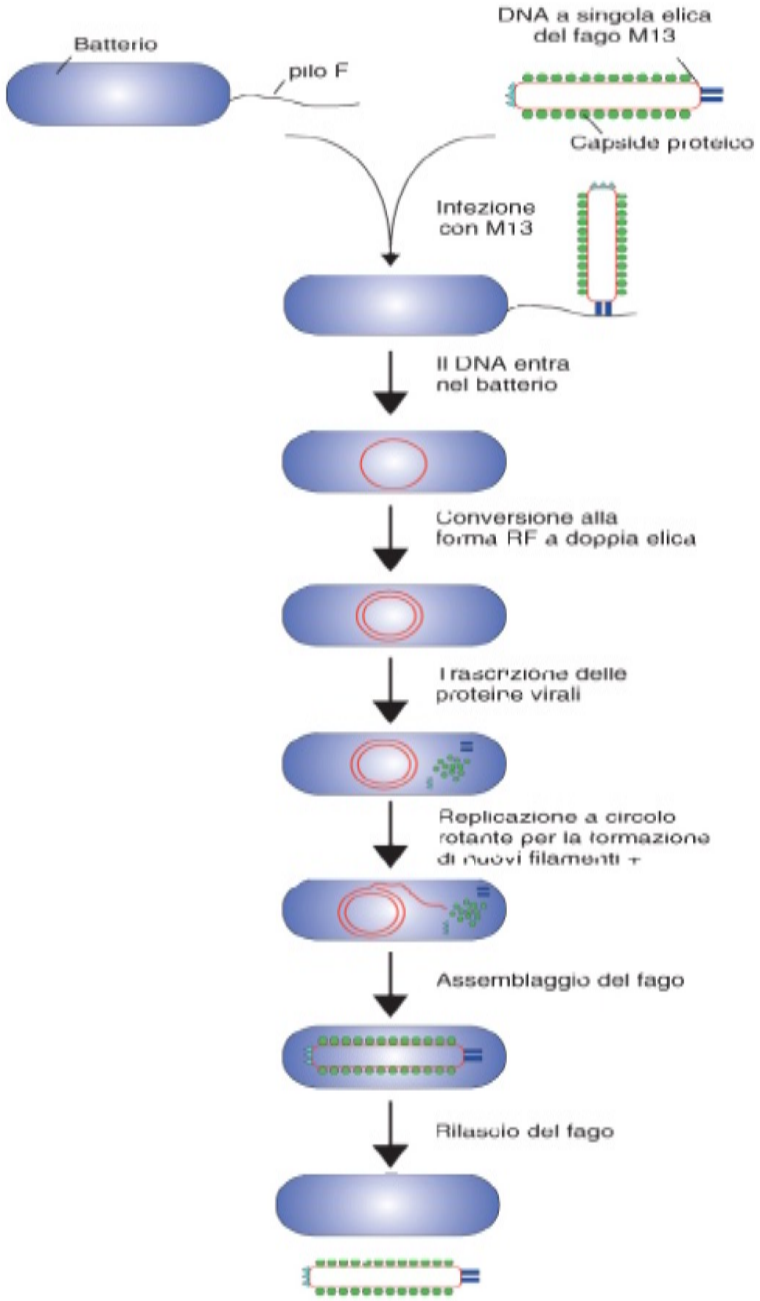
3. Immunological screening of an expression cDNA library with a primary antibody



Special lambda phage design:

- Beta-Gal gene under the control of the lac promoter inserted into lambda phage; lacZ contains a EcoRI site
- cDNA inserts are generated as usual; treated with EcoRI methylase
- Ligation of EcoRI adapters
- Digest withe EcoRI
- Ligation with lambda phage arms (previously digested by EcoRI)
- cDNA inserts are flanked by left arm, containing phage DNA and lacZ and right arm containing rest of lambda phage
- In vitro packaging
- Infection of E.coli and generation of Master plate
- Lac promoter drives expression of beta-Gal-cDNA fusion; → production of fusion protein; NOTE: beta Gal is not functional → blue plaque when added X-Gal
- Make plaque lift
- Incubate membranes with primary antibody; primary antibody will bind to proteins encoded by a beta-Gal – fusion protein, expressed by infected bacteria in a particular plaque.
- Detection – option 1: primary antibody is labelled by ^{125}I (chemical reaction)
- Detection – option 2: use secondary antibody fused to horseradish peroxidase → chemical reaction creating light
- Expose to X-ray film
- Go back to master plate and identify correct plaque.

Il batteriofago M13 come vettore per il clonaggio di DNA a singolo filamento



La biologia del fago M13: il ciclo vitale

•Il genoma del fagoM13 è costituito da una molecola di DNA circolare a singola elica, lunga 6407 nt.

• M13 infetta solo ceppi che portano il plasmide F. Esso infatti entra nella cellula batterica attraverso il pilo codificato dal fattore F.

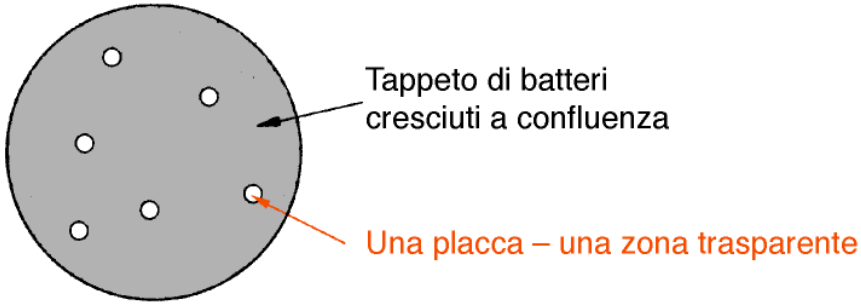
• Il DNA viene convertito nella forma replicativa intermedia a doppio filamento (RF). Vengono sintetizzate circa 100 copie della forma RF.

• Inizia la replicazione a cerchio rotante di un'unica elica del genoma virale. Vengono sintetizzate circa 1000 copie.

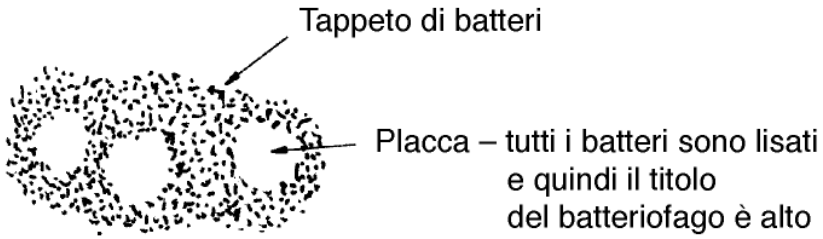
• Il genoma viene assemblato alle proteine per costituire le nuove particelle virali che fuoriescono dalla cellula batterica senza causarne la lisi.

Plaque types : M13 and Lambda type phages

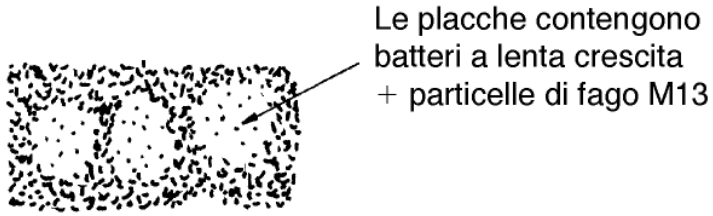
(a) Placche su un tappeto di batteri



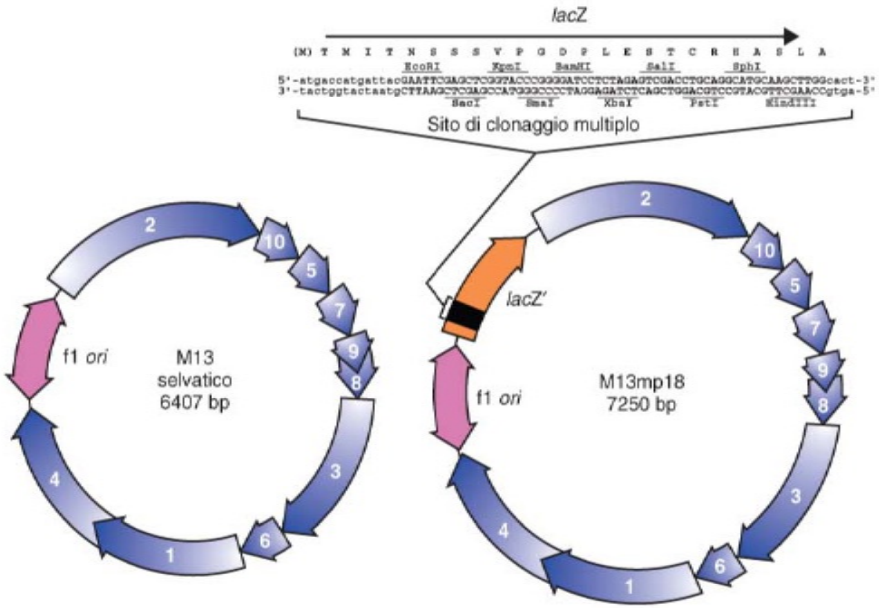
(b) Placche litiche



(c) Placche di M13



Il batteriofago M13 come vettore per il clonaggio di DNA a singolo filamento

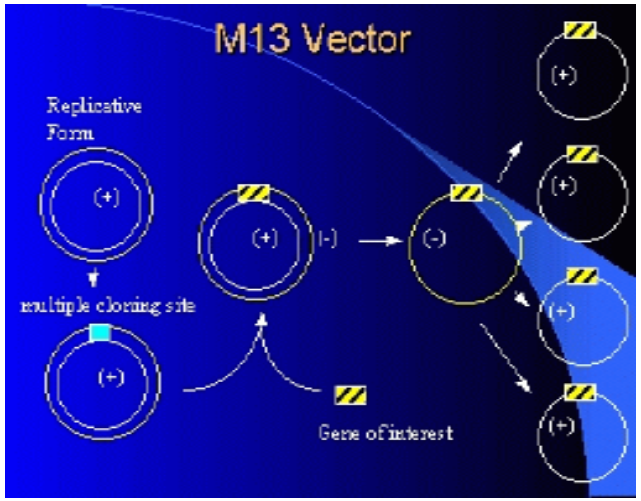


Il genoma del fago M13, nella sua forma replicativa intermedia RF, viene utilizzato come vettore di clonaggio.

Modificazioni del genoma selvatico per l'ottimizzazione del vettore:

- Aggiunta del gene *lacZ'* come marcatore genetico per la selezione bianco/blu delle placche positive contenenti il genoma ricombinante
- Aggiunta di un polylinker all'interno del gene lacZ'
- Eliminazione dei siti di restrizione naturali

Tappe del clonaggio di DNA a singolo filamento nei vettori derivati dal fago M13



Clonando l'inserto nell'orientamento opposto si ottengono copie multiple dell'elica complementare

Si linearizza il vettore M13 a doppio filamento con un enzima di restrizione, come se fosse un plasmide.

- **Si mescola il vettore linearizzato con l'inserto avente estremità coesive compatibili con quelle del vettore. Si aggiunge la ligasi.**

- **Il prodotto della reazione di ligasi viene inserito nelle cellule *E. coli* mediante trasformazione.**

- **Nell'ospite batterico il vettore verrà replicato prima in maniera bidirezionale producendo copie a doppia elica e poi a cerchio rotante producendo copie a singola.**

- **Si selezionano le placche di colore bianco contenenti il vettore ricombinante e si scartano le blu contenenti il vettore virale senza inserto.**

Vantaggi del fago M13 e vettore a singolo filamento

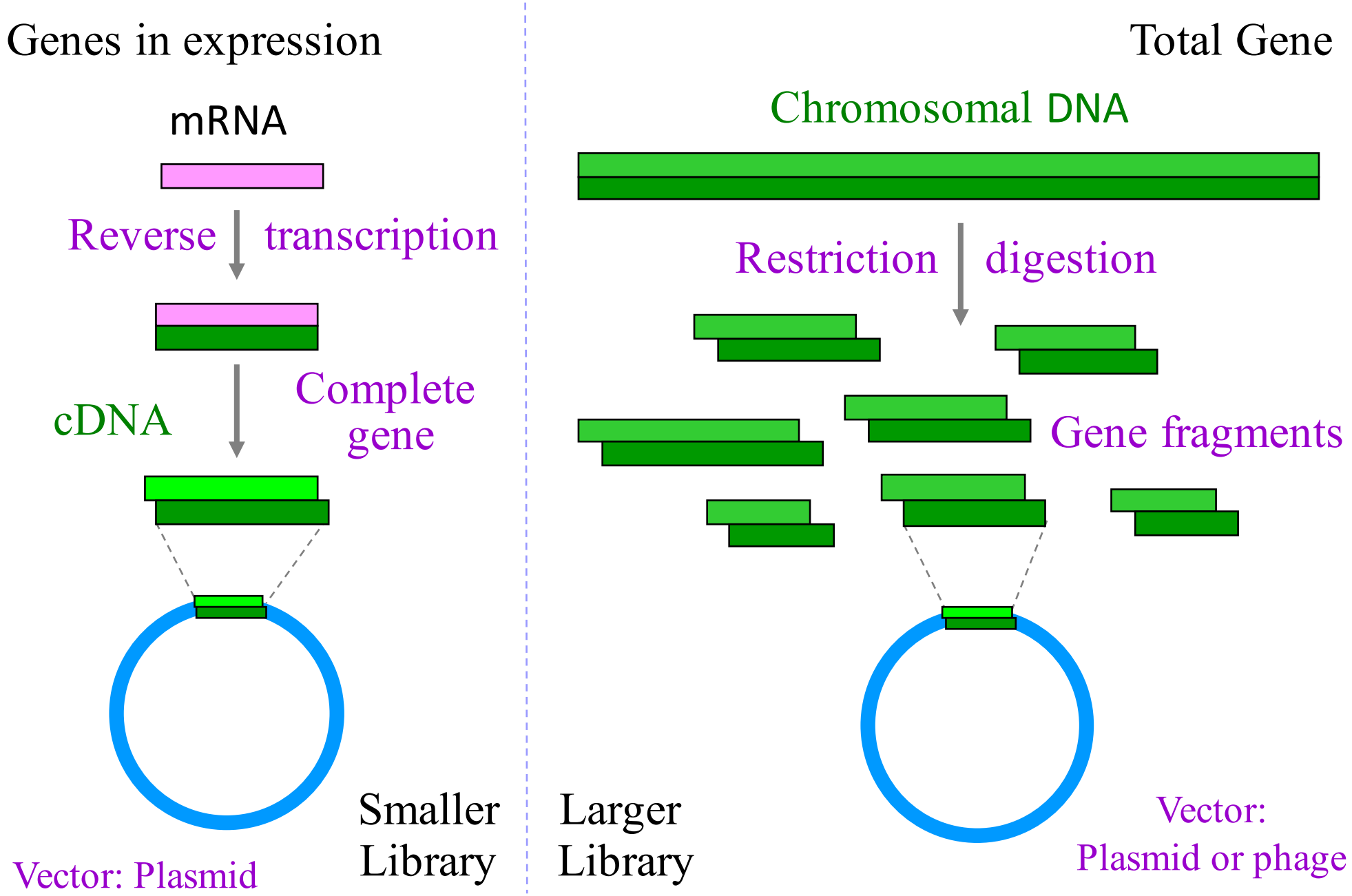
- Clonaggio non limitato dall'impaccamento, aumento delle dimensioni dell'inserto (teoricamente, fino a 6 volte quelle dell'M13 wild type)
- Nella forma replicativa a doppio filamento può essere manipolato come un plasmide
- Il vettore nella sua forma a singolo filamento può essere usato nel clonaggio per:

❑ Sequenziamento diretto sul singolo filamento

❑ Produzione di DNA mutato: mutagenesi sito-specifica mediante oligonucleotidi

❑ Produzione di sonde d'ibridazione a singola elica

Two Libraries： cDNA Library vs Genomic Library



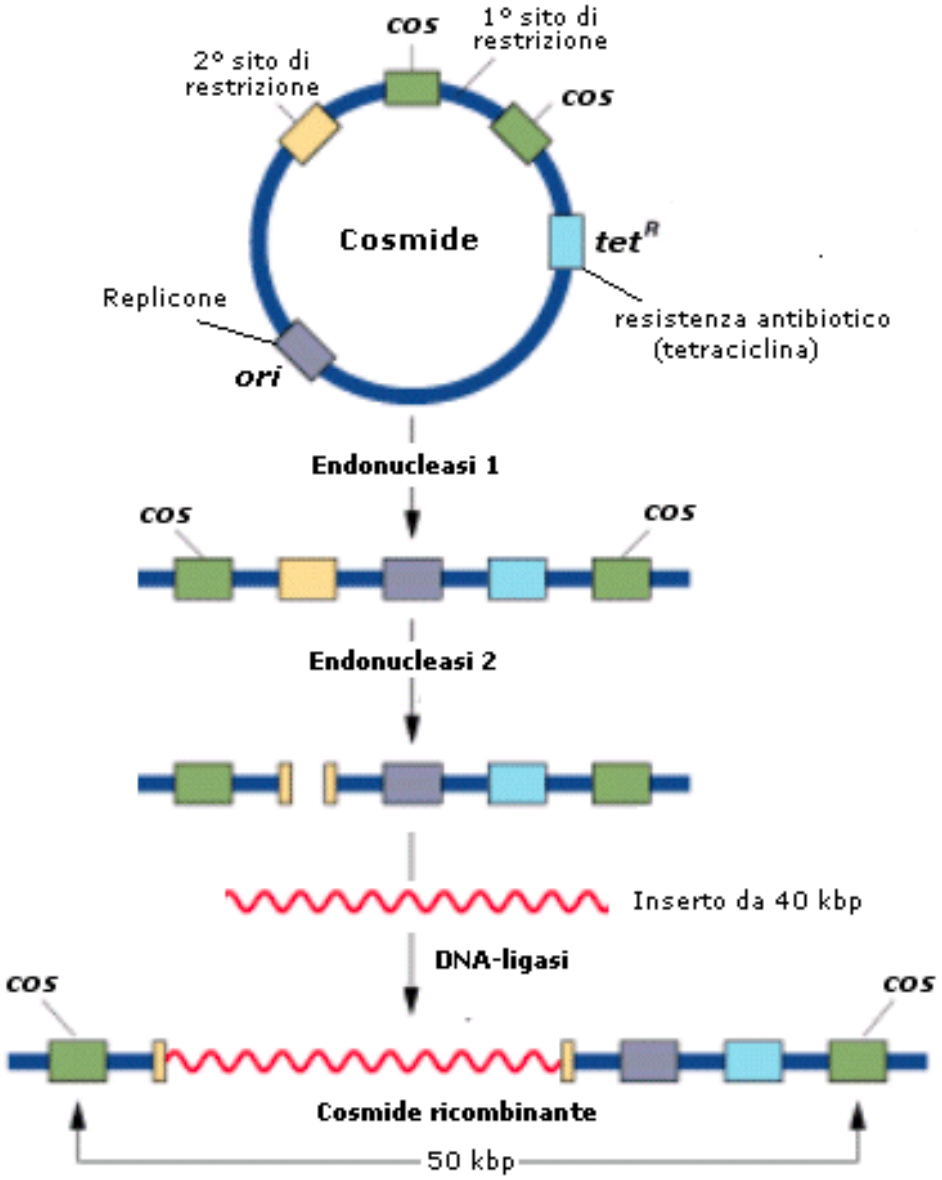
COSMIDI - COSMIDS

COSMIDS are engineered vectors that contain elements from phage and plasmid

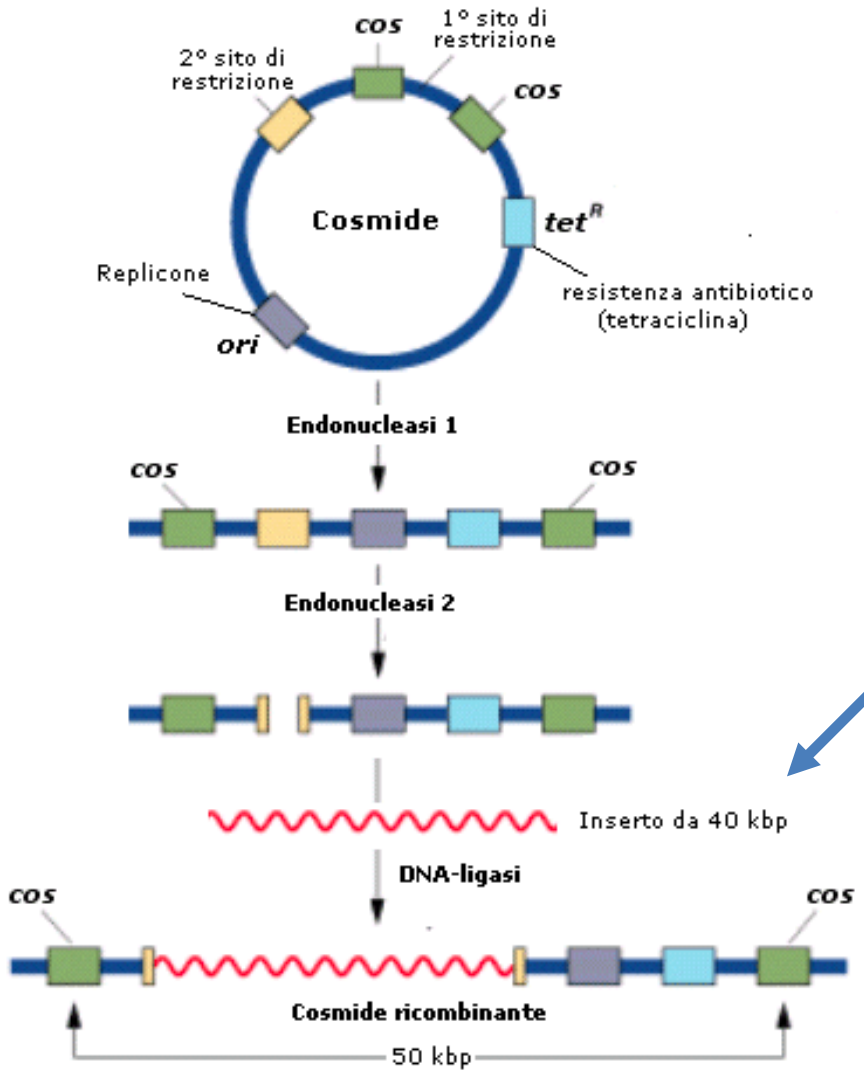
- Can carry 40kb of insert DNA
- Can be maintained as plasmids in bacteria
- Can be shuttled as phage
- Less vectors required to cover the genome

CHARACTERISTIC FEATURES:

- Basic vector is small: 5-6 kb
- Contains 2 cos sites for in vitro packaging
- Insertion of cosmid using a phage is more efficient that via transformation (cosmids are long)
- No replication system of phage → no plaques
- Uses bacterial origin of replication
- Contains cloning sites
- Carries markers for selection in bacteria



GENERATION OF COSMID LIBRARIES

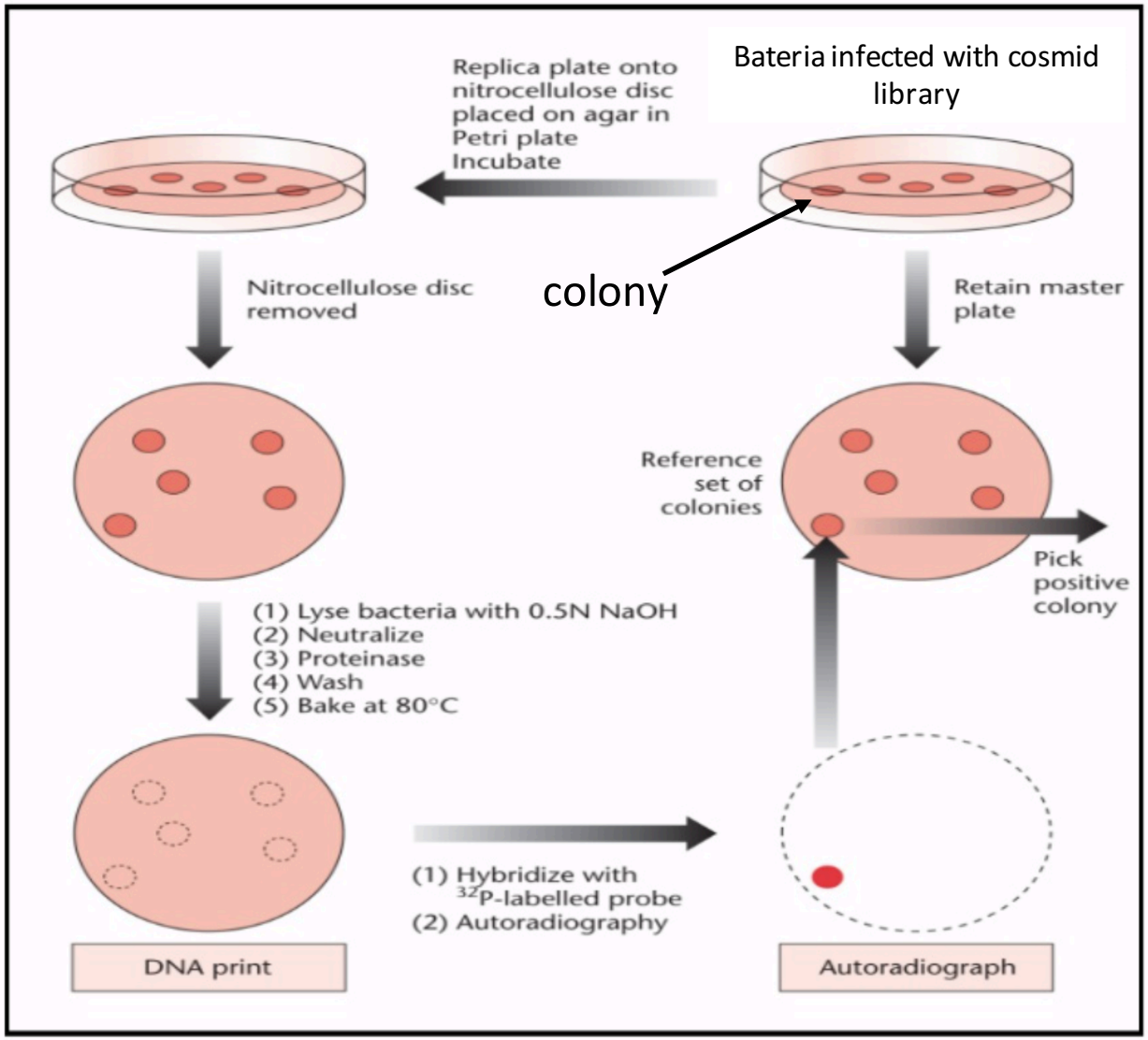


Partial digest with Endonuclase #2;
Dephosphorylation of overhangs

- Selection of 40kb fragments;
- Ligation with cosmid vector arms;
- In vitro packaging
- Infection of E-coli

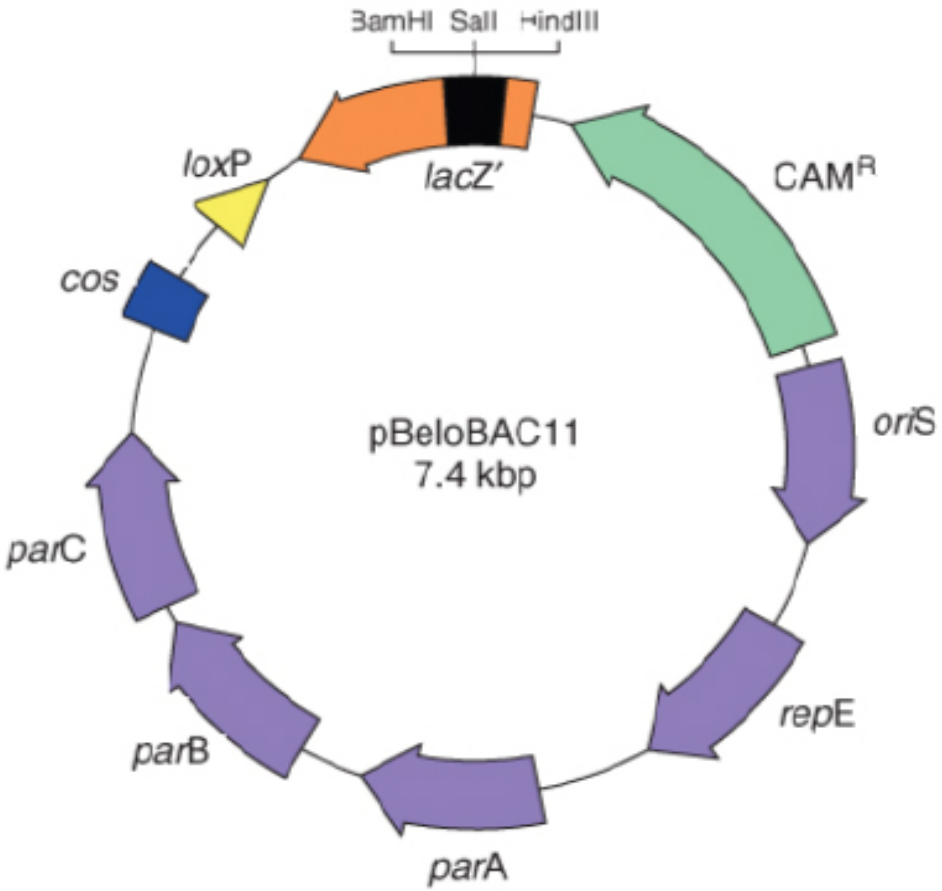
ATTENTION: when entered in bacteria; cos sites recircularize cosmid vector
Cosmid is maintained as plasmid (Tet resistance cassette)

Screening a cosmid library



NOTE:
For doing a screenting a **COLONY LIFT** has to be done.
→ Cosmids amplify in bacteria as plasmids
Works the same like a plaque lift.

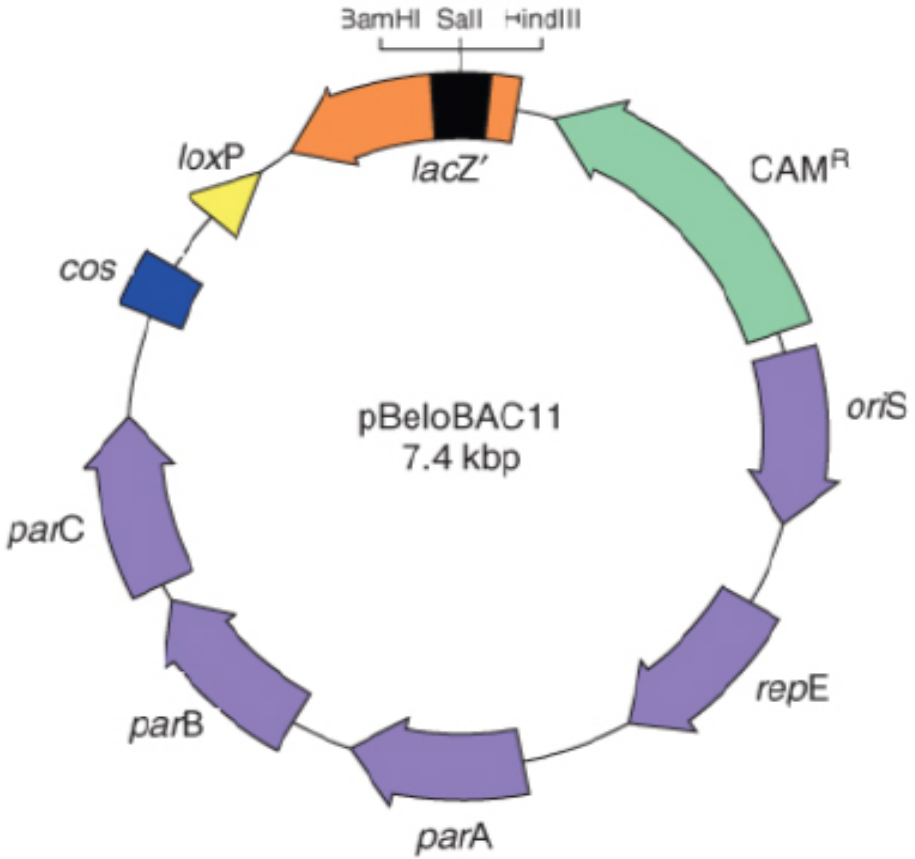
BACs – BACTERIAL ARTIFICIAL CHROMOSOMES



BACs are engineered vectors that contain elements from the F plasmid

- Can carry **300kb** of insert DNA
- Can be maintained as **circular vector** in bacteria
- Copy number control: 1-2 copies per bacteria
- Segregation system of F-plasmid → very stable vector
- Ideal for genomic libraries

BACs – BACTERIAL ARTIFICIAL CHROMOSOMES



1.**Components of the F plasmid**
- **oriS**: origin of replication
- **repE**:plasmid replication and copy number control
- **parA,parB**: correct partitioning of BAC to daughter cells; stability of BAC

2.Selectable marker in E.coli: Chloramphenicol resistance (CAM^R)

3.Segmento di clonaggio:

➢**BamHI e HindIII**: cloning sites for insertion of DNA fragments (<300kb)

➢**T7 + SP6**: promoters to eventually do in votro transcription using T7 or SP6 RNA polymerases (for example probe generation)

4.OPTIONAL:

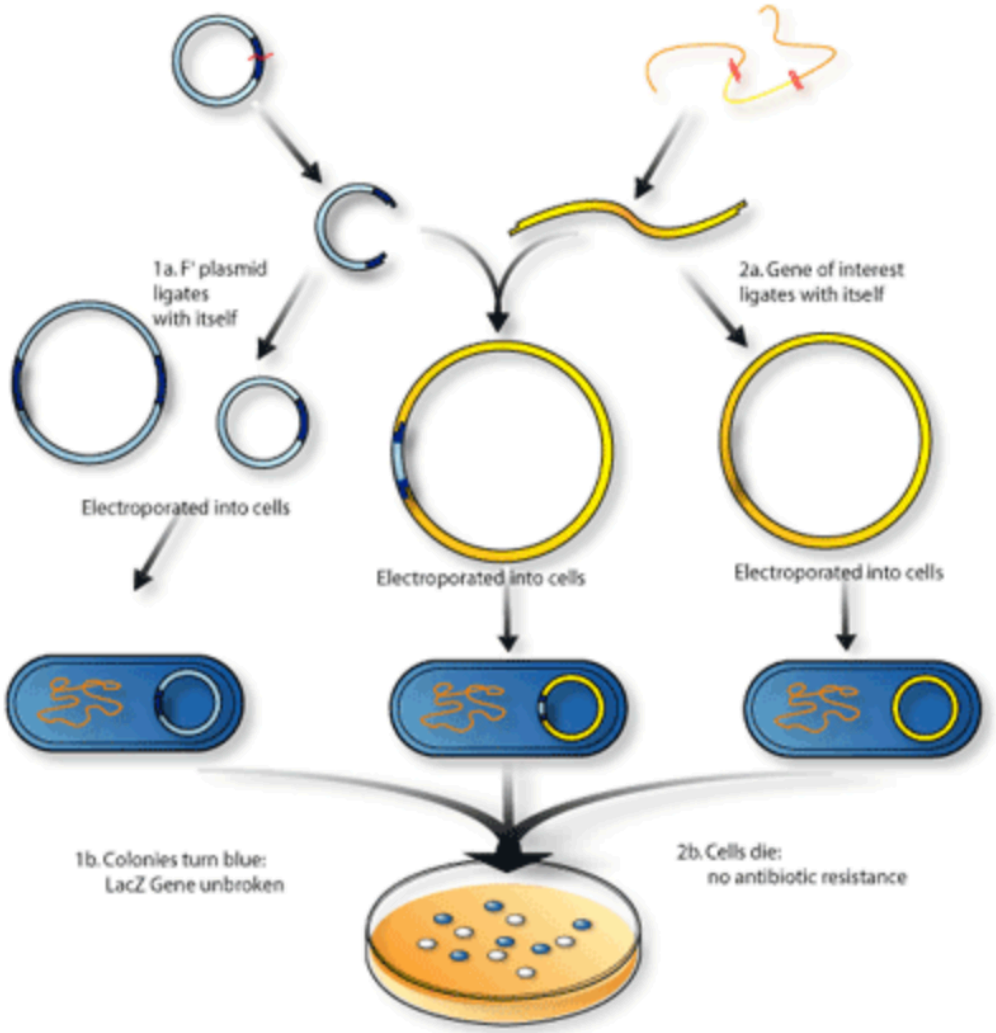
➢**Cos**: **for cutitng by lambda terminase**

➢**LoxP**: **fir cutting by the cre endonucease of the phasge P1**

➢**LacZ** (beta-galacosidase):blue-white selection to selct for colonies hilding BACs with insert

Chloramphenicol acetyltransferase (or CAT) is a bacterial enzyme that detoxifies the antibiotic chloramphenicol and is responsible for chloramphenicol resistance in bacteria.This enzyme covalently attaches an acetyl group from acetyl-CoA to chloramphenicol, which prevents chloramphenicol from binding to ribosomes. A histidine residue, located in the C-terminal section of the enzyme, plays a central role in its catalytic mechanism.

GENERATING A BAC LIBRARY



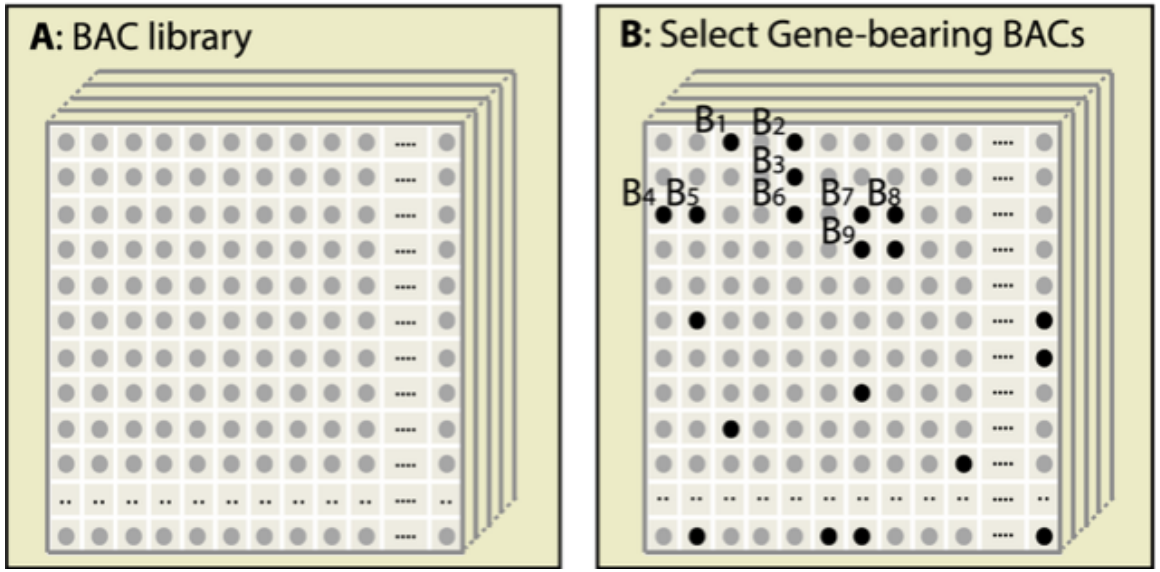
Screening on bacterial colonies

- Cut genomic DNA with 2 different restriction enzymes
- Size select
- Cut BAC vector with same restriction sites
- Ligate genomic DNA into BAC vector
- Electroporate bacteria
- Plate on agar plate
- Make blue-white selection

→White colonies: with insert

→Blue colonies: without insert

BAC libraries are commercially available:



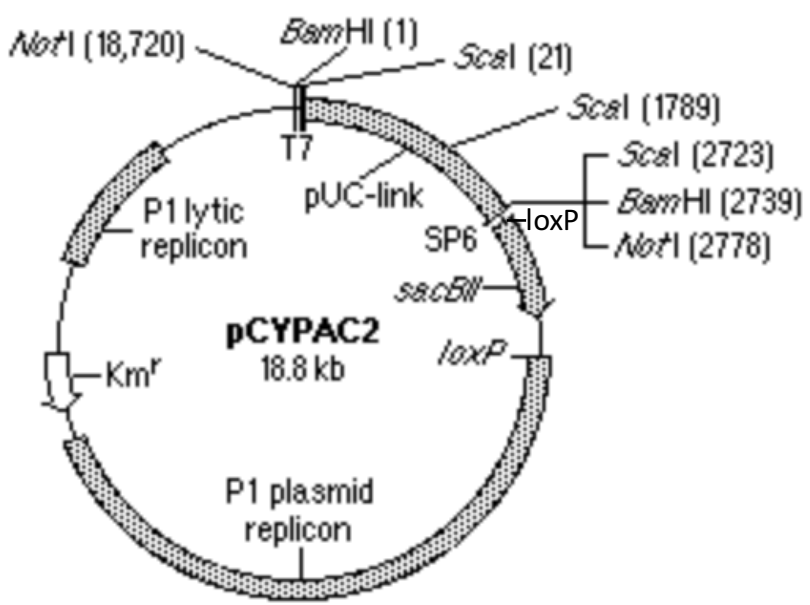
→ BAC library vectors covering multiple times the human/mouse/other genome have been spotted one by one on a nitrocellulose membrane
→ Commercially available
→ A. Hybridize membrane collection with radioactive probe (on gene of interest)
→ B. Identify BAC clones that contain insert of interest
→ Order the respective BAC vectors from BAC library vendor

→ **APPLICATION: for example for building gene targeting vector to make a knock-out for a gene in mouse (later!!!)**

PAC (P1 derived Artificial Chromosome)

pCYPAC2: used for the human genome project: sequencing of the entire genome

A



Fragments to be cloned into PACs: 150 kb (max. 300 kb)

PACs combine the advantages of BACs and the P1 phage

Structural Elements:

- puC19-link: contains cloning site for insertion of genomic fragments (BamHI, BamHI-ScaI)
- SP6; T7: in vitro transcription
- Km^r: Kamnamycin resistance gene for selection in bacteria
- SacBII: Selection for PACs with insert. In the case of empty vector SacBII is transcribed and produces levansucrase that converts succhrose to lavan = toxic for Bacteria
- loxP site: cleavage by P1 endonuclease Cre
- P1 plasmid replicon: replication of vector; copy number control (1-2 vectors per cell), packaging, genomic stability
- P1 lytic replicon: **IPTG inducible promoter**. IPTG to bacteria, lytic life cycle of P1 is triggered → generation of high copy number.

PAC cloning

1. Partial digest of genomic DNA (ScaI, BamHI)

2. Digest vector (ScaI, BamHI) + dephosphorylation; creation of left and right arm

3. Ligation

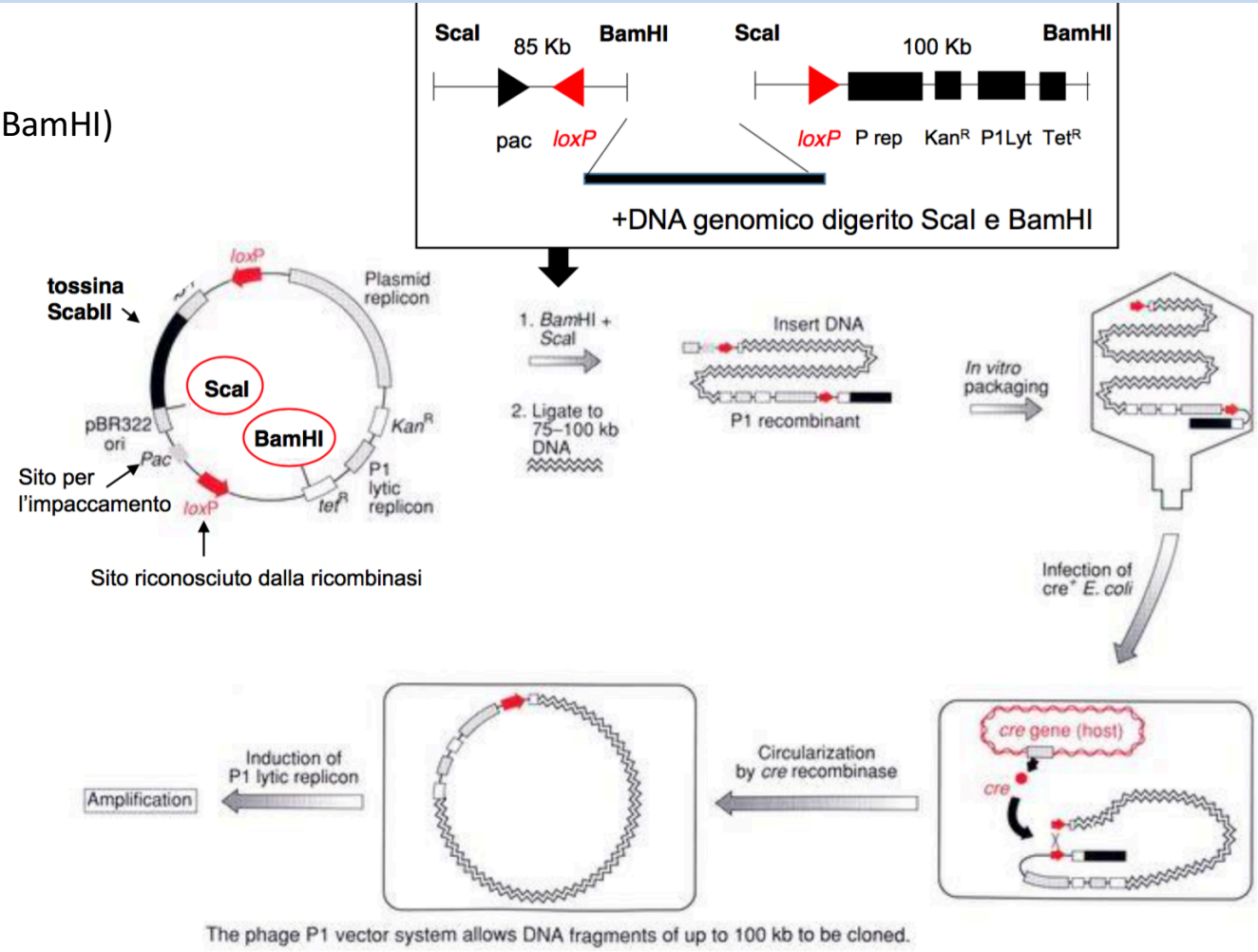
4. In vitro packaging

5. Infection of bacteria expressing Cre recombinase, grown on succhrose containing plates

6. Cre cuts loxP sites, excission of ScabII, religation of loxP sites → circulatization.

7. Origin of P1 replicon ensures copynumber of 1-2 per bacteria

7. Induction of lytic life cycle possible via the application of IPTG (P1Lyt) → high copy number

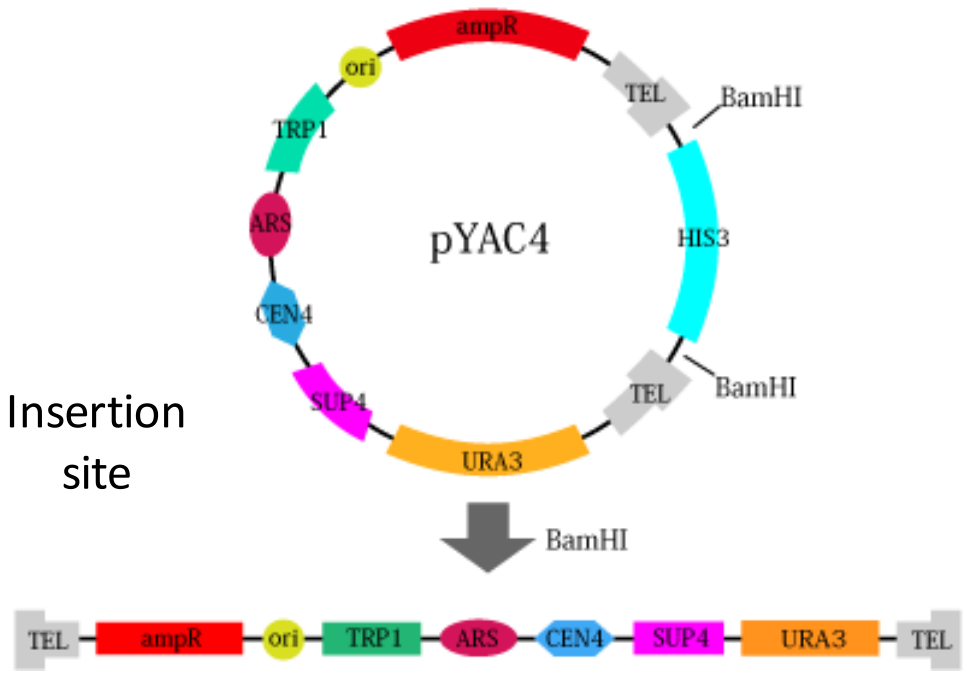


The phage P1 vector system allows DNA fragments of up to 100 kb to be cloned.

YAC - Yeast artificial chromosomes

Cloning of fragments: <1Mb
Very long: instable, risk of recombination, breaks

1. **CEN4**: 125 bp centromeric region, allowing segregation in yeast
2. **TEL**: telomere repeat; yeast 13bp repeats; stabilize linear YAC
3. **ARS**: autonomously replication region sequence; ensures replication in yeast.
4. **Ori, Amp^R**: replication and selection in E.coli
5. Selectable marker for yeast His3; URA3,… many posibilities; ceppi che hanno internalizzato il vettore vengono identificati per la loro capacità di complementare il difetto nutrizionale, pertanto vengono fatti crescere su un terreno privo dello specifico nutriente.
6. SUP4: This gene compensates for a mutation in the yeast host cell that causes the accumulation of red pigment. The host cells are normally red, and those transformed with YAC only, will form colorless colonies. Cloning of a foreign DNA fragment into the YAC causes insertional inactivation of the gene, restoring the red color. Therefore, the colonies that contain the foreign DNA fragment are red



Gli YAC ricombinanti vengono trasformati in un ceppo di lievito Ura3-, trp1- e ade2 -
I ricombinanti vengono identificati come colonie rosse **che crescono in terreno privo di uracile e triptofano**

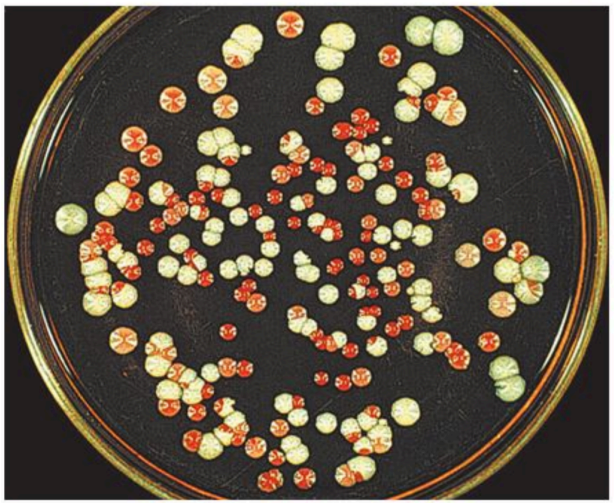
Ade2-fosforibosilamino-imidazolo-carbossilasi mutata

SUP4 → **Colonie bianche Non Ricombinanti**

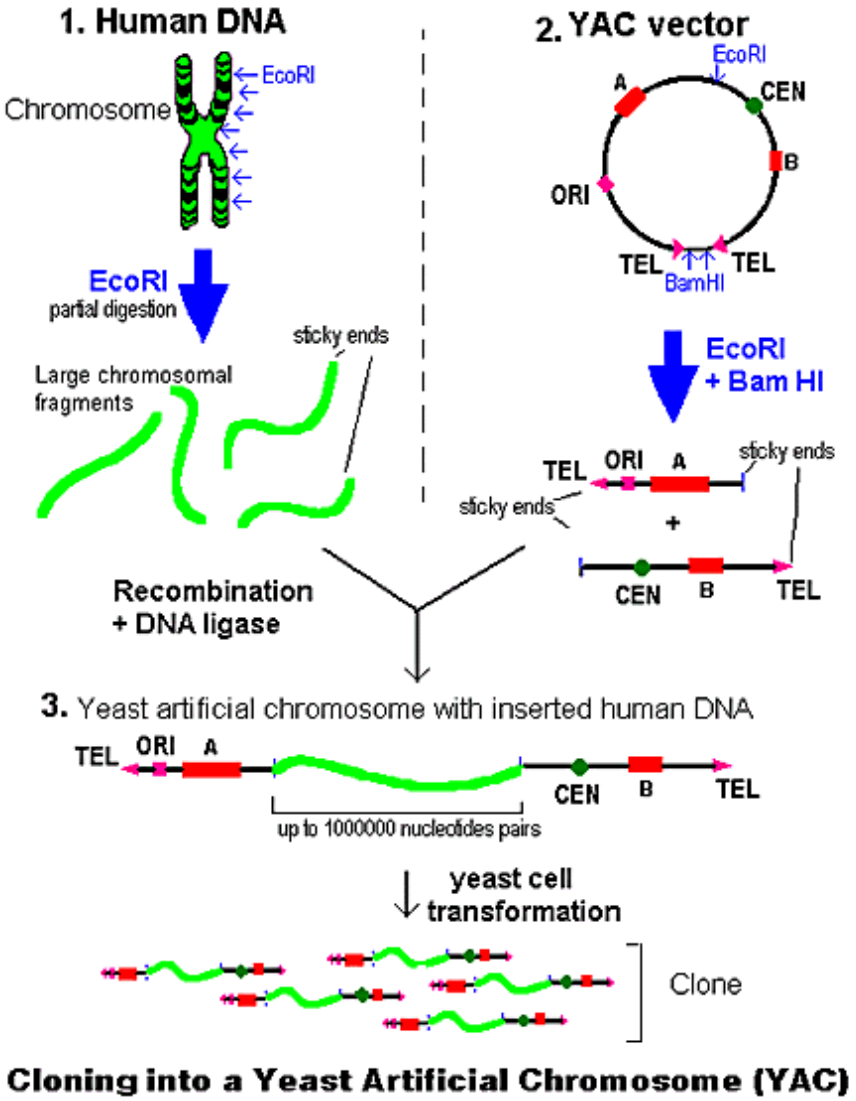
~~SUP4~~ → **Colonie rosse Ricombinanti**

YAC cloning of genomic libraries

1. Parial digest of genomic DNA with EcoRI
2. Digest empty YAC with EcoRI and BamHI, dephosphorylation
3. Select for genomic fragments with app. 100 kb (or more) and ligate with YAC arms
4. Introduce into yeast
5. Linear YAC construct stabilized by telomere and ARS, CEN



Colonie rosse ricombinanti



Cloning into a Yeast Artificial Chromosome (YAC)