VECTOR TYPES USED IN THE LABORATORY

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1. Vectors to clone genomic fragements/cDNA (libraries

   - Bacteriophages
   - Cosmids
   - Bacterial artificial chromosomes
   - P1 derived artificial chromosomes
   - Yeast artificial chromosomes

2. Vectors to express proteins in cells and/or purify recombinant proteins

   - In bacteria
   - In yeast
   - In fly cells
   - In vertebrate cells

= Expression vectors

1. Vectors to clone genomic fragements/cDNA (libraries)
   - Bacteriophages – Phage vectors (lambda, M13)
   - Cosmids
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Engineered vector systems

2. Vectors to express proteins in cells and/or purify recombinant proteins
   - In bacteria
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Obtaining genomic information from organisms, cells, tissues…
Vectors to clone genomic fragements/cDNA (libraries)

How to generate "physical" genomic information by making **DNA libraries** :
→ get DNA piece in lambda vector → amplify using bacteria → prepare DNA → get DNA sequence

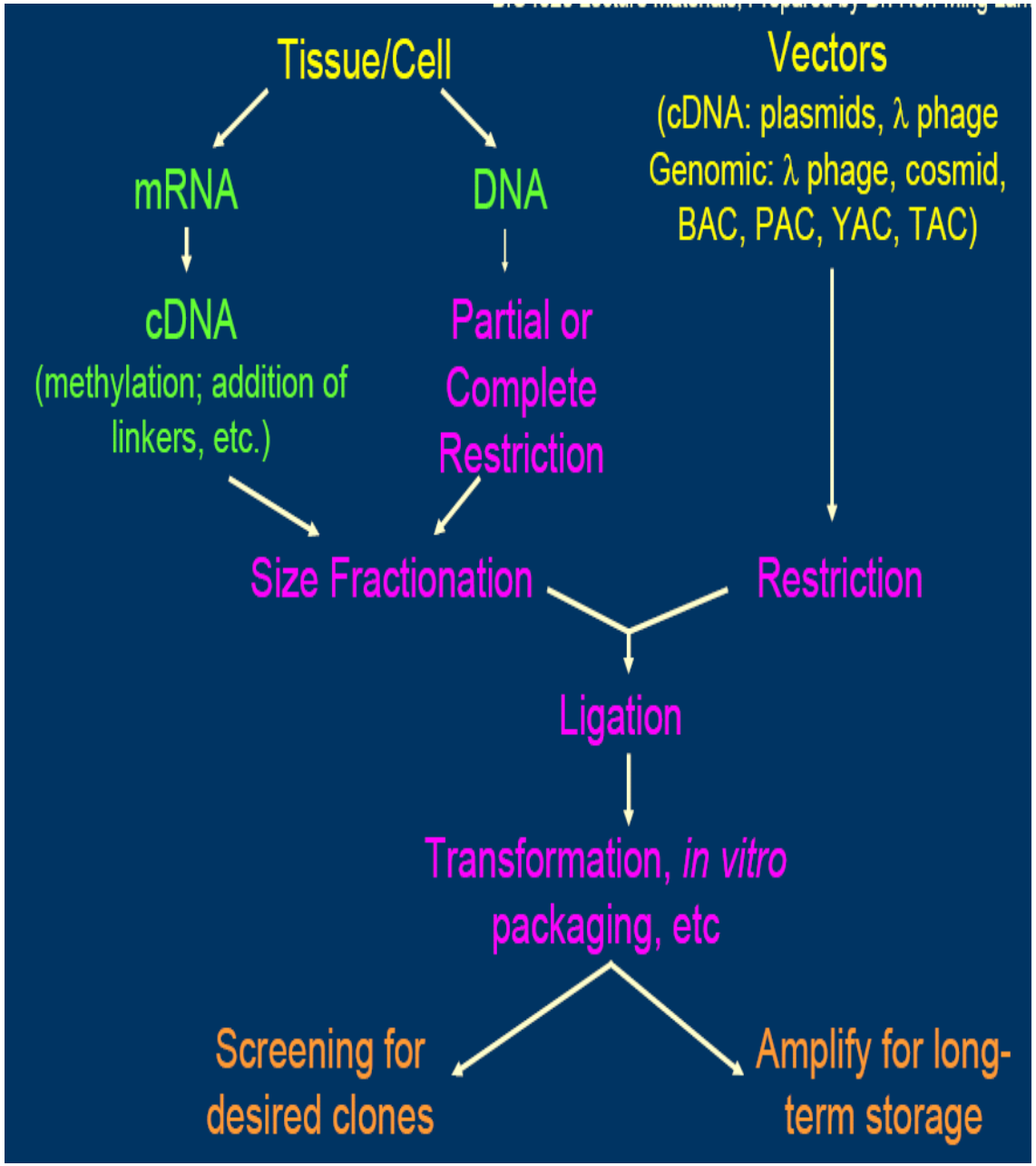
Main questions:

1. DNA sequence of the genome: get all the DNA seqeunce of the genome in hands and do DNA sequencing (→ obtain complete seqeucne of the genome) (or other library vector)

2. What genes are expressed in a given cell type?
Prepare RNAs → reverse transcription → cDNA → clone cDNA (fragments) in lambda vector (or other library vector)

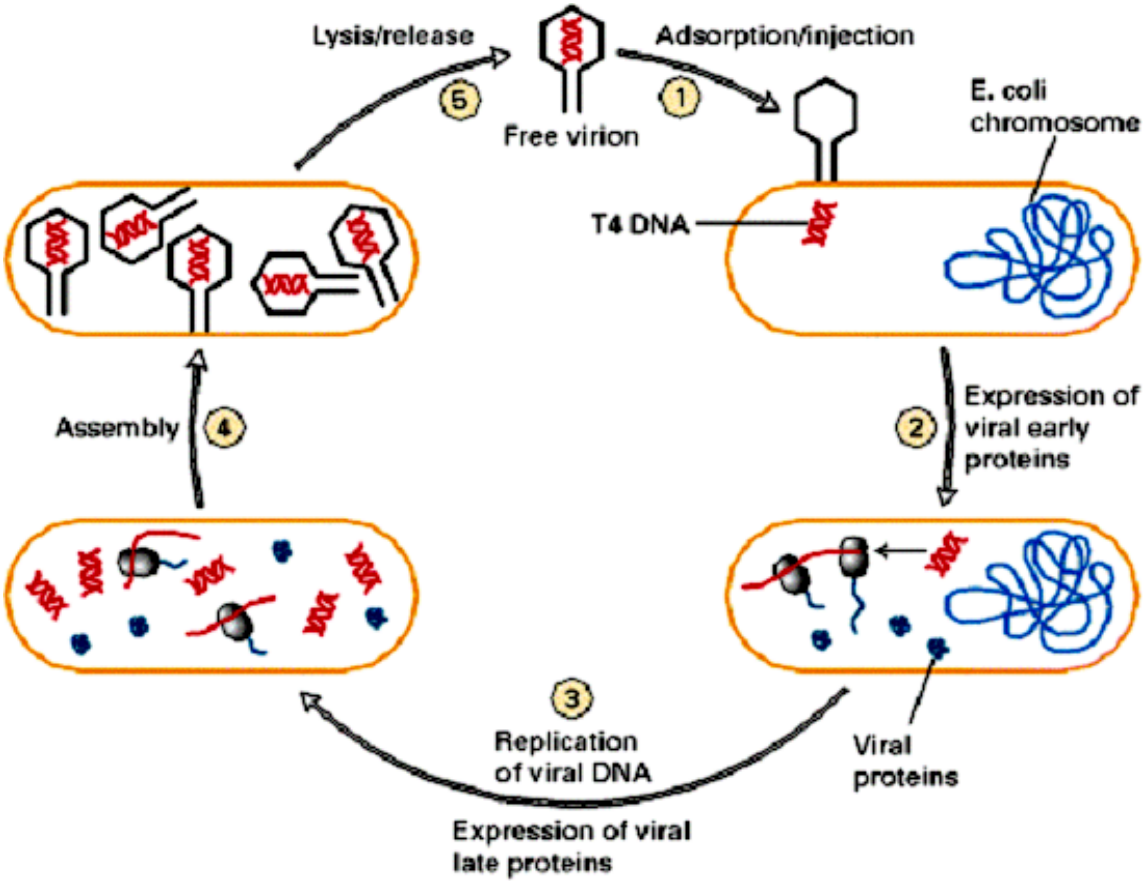
- There two main types of DNA libraries
- **Genomic library** which contains the entire human Genome (exons and introns)
- **cDNA (complementary DNA) library** that contains only expressed genomic information (only exons + UTRs)

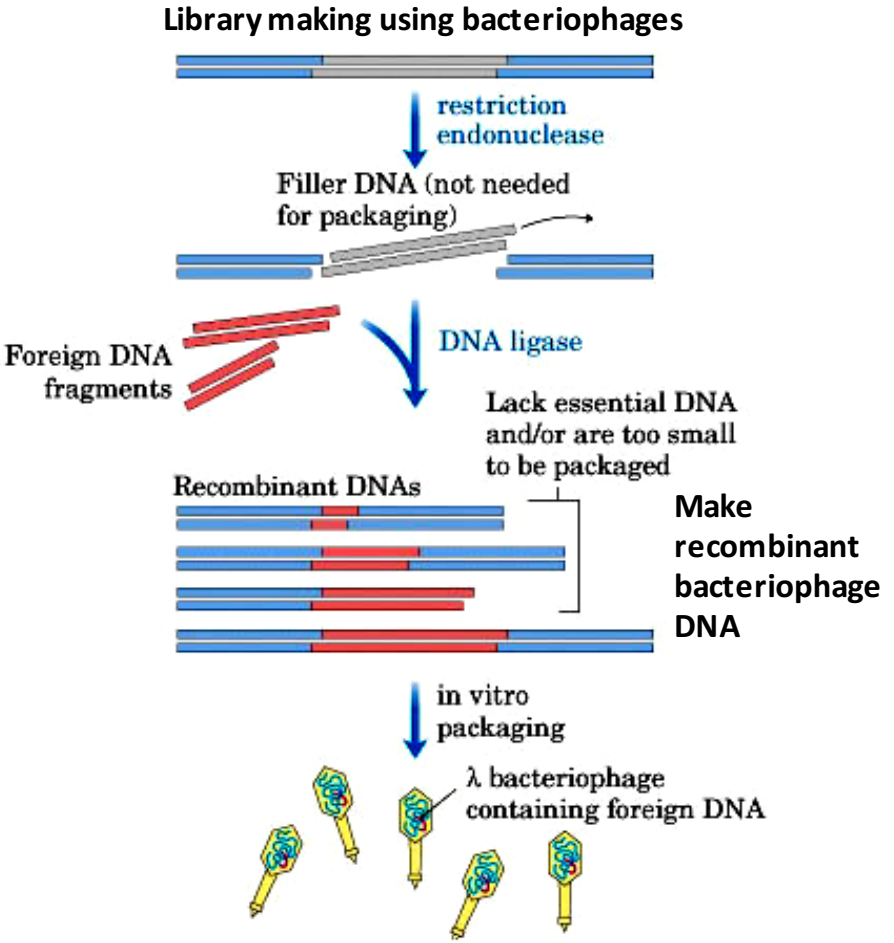
Obtaining genomic information from organisms, cells, tissues…
Vectors to clone genomic fragements/cDNA (libraries)



DNA libraries are commercially availabe

Phage vectors - Vettori fagici

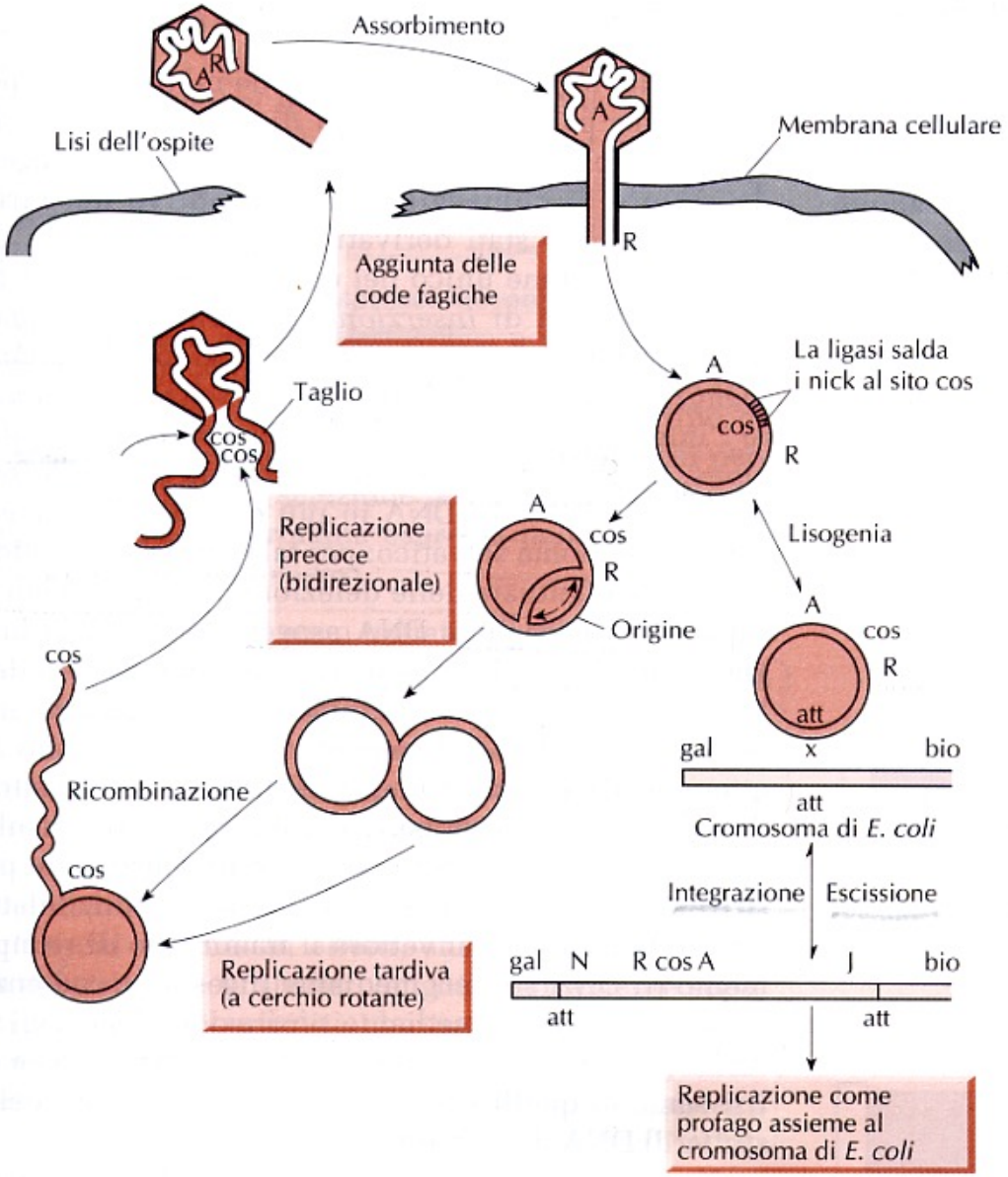




Much more on libraries
Prof. Edomi
Genetica Applicata
STB: Anno 3 Sem. 2

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

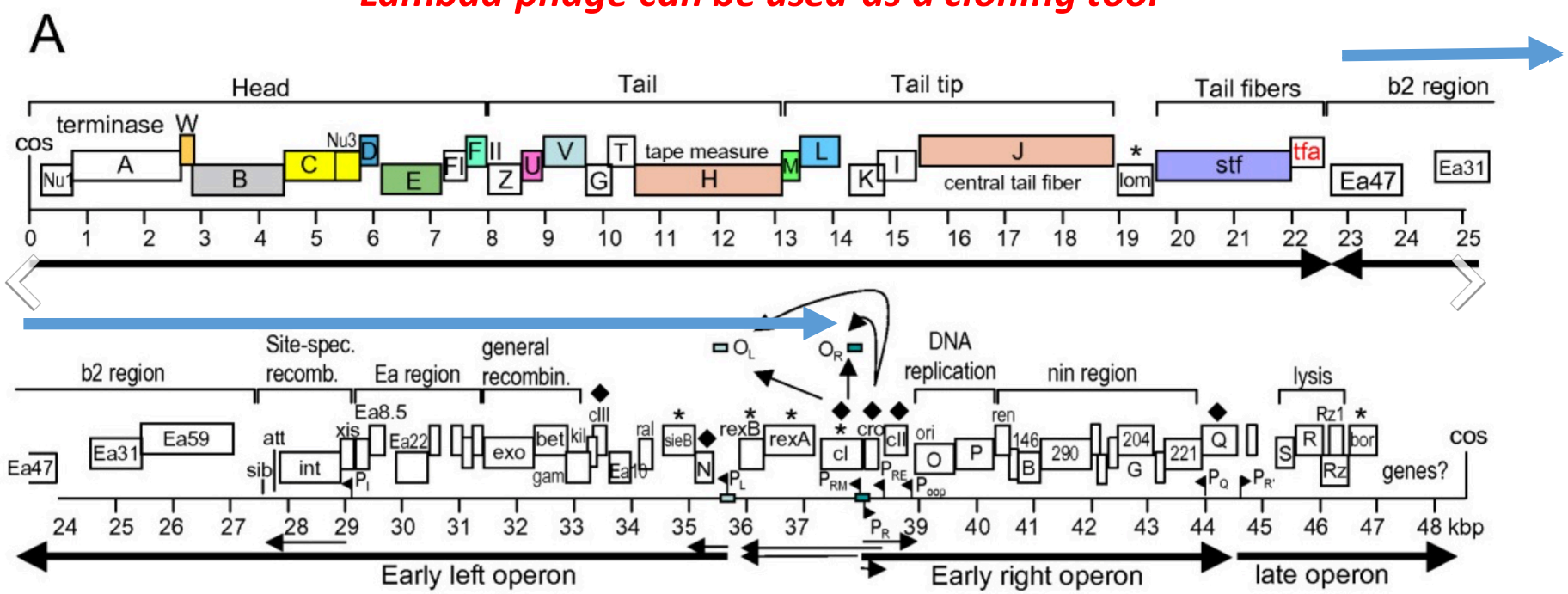
Life cycles of the Lambda phage



Using the Lambda phage 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

In the laboratory only the lytic life cycle is interesting for cloning purposes

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 (cDNA; genomic DNA frament)

→ Phage is viable

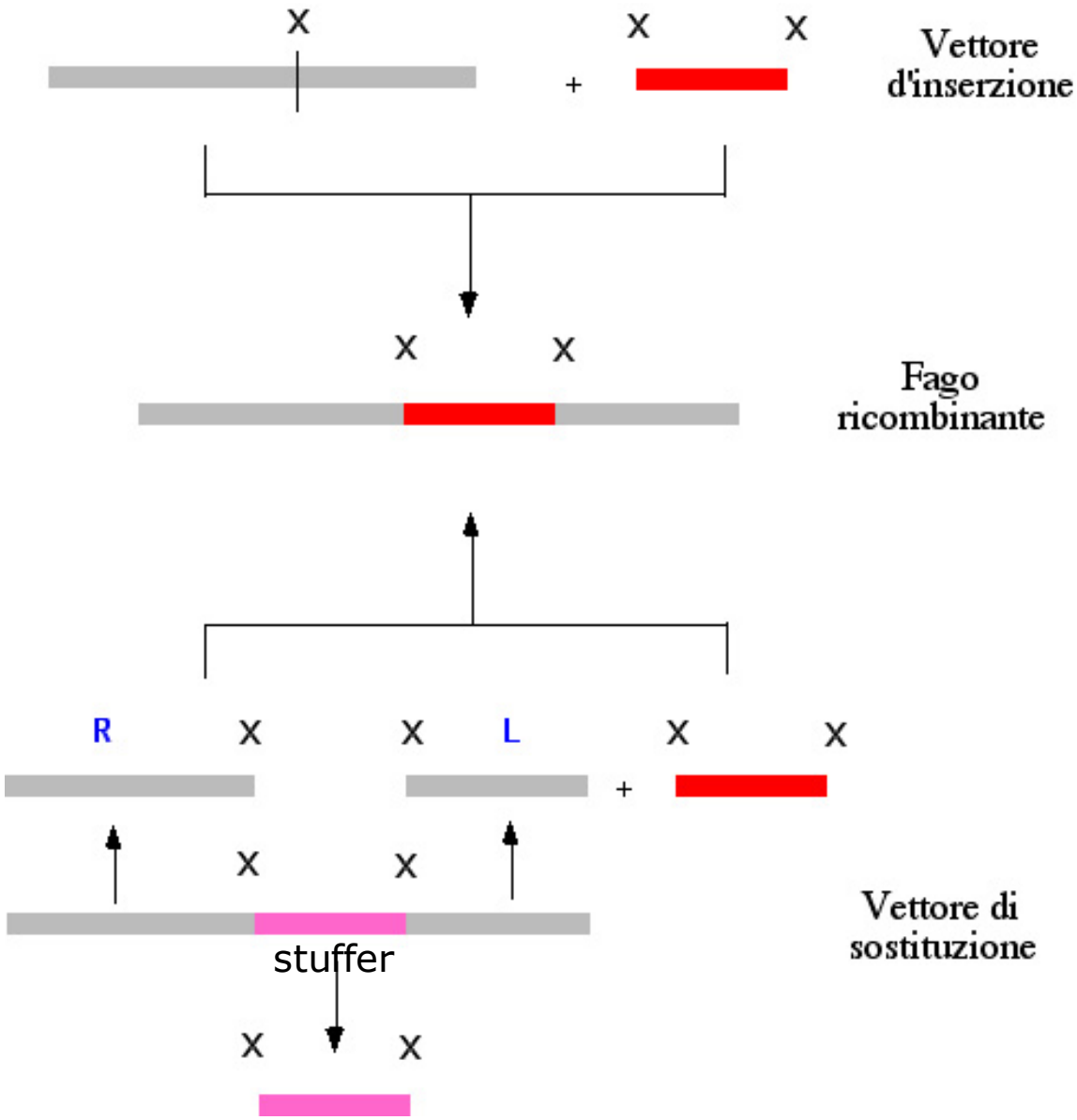
→ Phage uses lytic life cycle and amplifies DNA

Using Lambda phages as cloning vectors

Two types of λ vectors

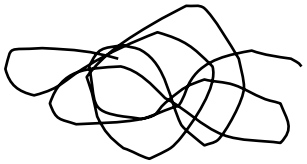
- **Insertion vector/vettori d'inserzione**, exogenouse (esogeno) DNA is inserted into unique restriction site in lamba vector

- **Subsitution vectors/vettori di sostituzione, exogenouse DNA (**esogeno) is replacing a segment of lambda DNA not required for the lytic life cycle ("stuffer").



Lamda subsitution vectors

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.

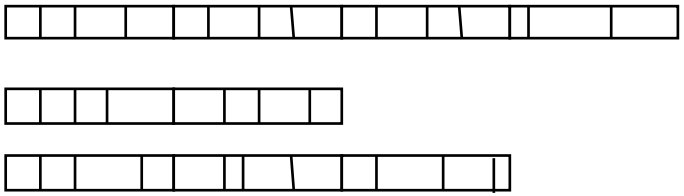


Sau3A 5′...GATC...3′ 3′...CTAG...5′

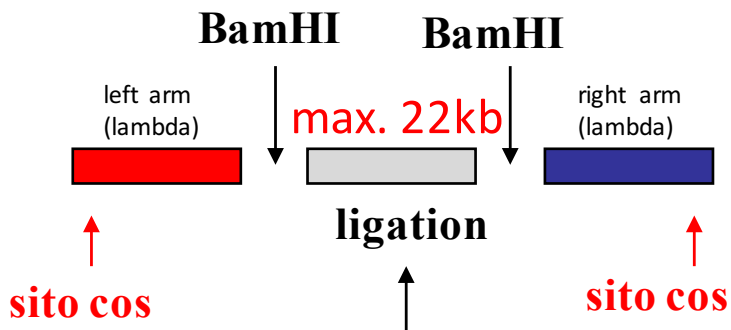
Isocaudomers

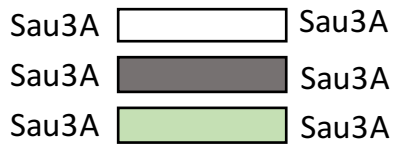
BamHI 5′...GGATCC...3′ 3′...CCTAGG...5′

digestione parziale con Sau3A



Isolate fragments With approximate size of 15 Kb (agarose gel)

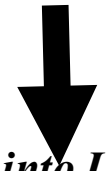




BamHI and Sau3A have cohesive ends

Genomic DNA fragments with different lengh

Also possible with cDNA obtained from reverse transcription of mRNA



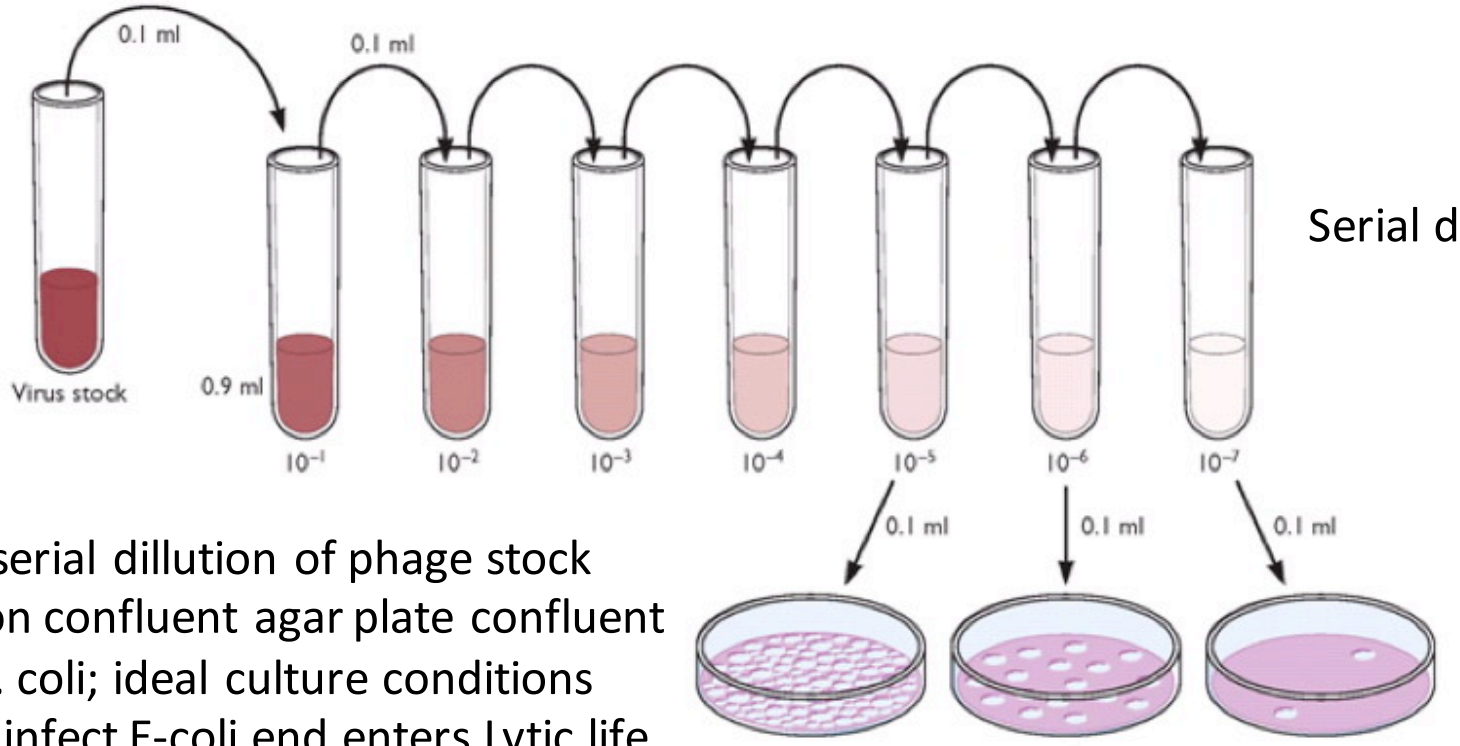
- **Packaging *in vitro into Lambda capsids***
- ***Conserve Lambda library stocks***
- ***Dilute phage library***
- ***Infect confluent E.coli culture on agar plate***

cDNA libraries

Genome libraries

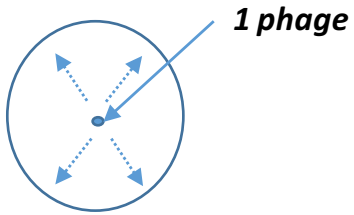
Working with phages in the lab

The plaque assay

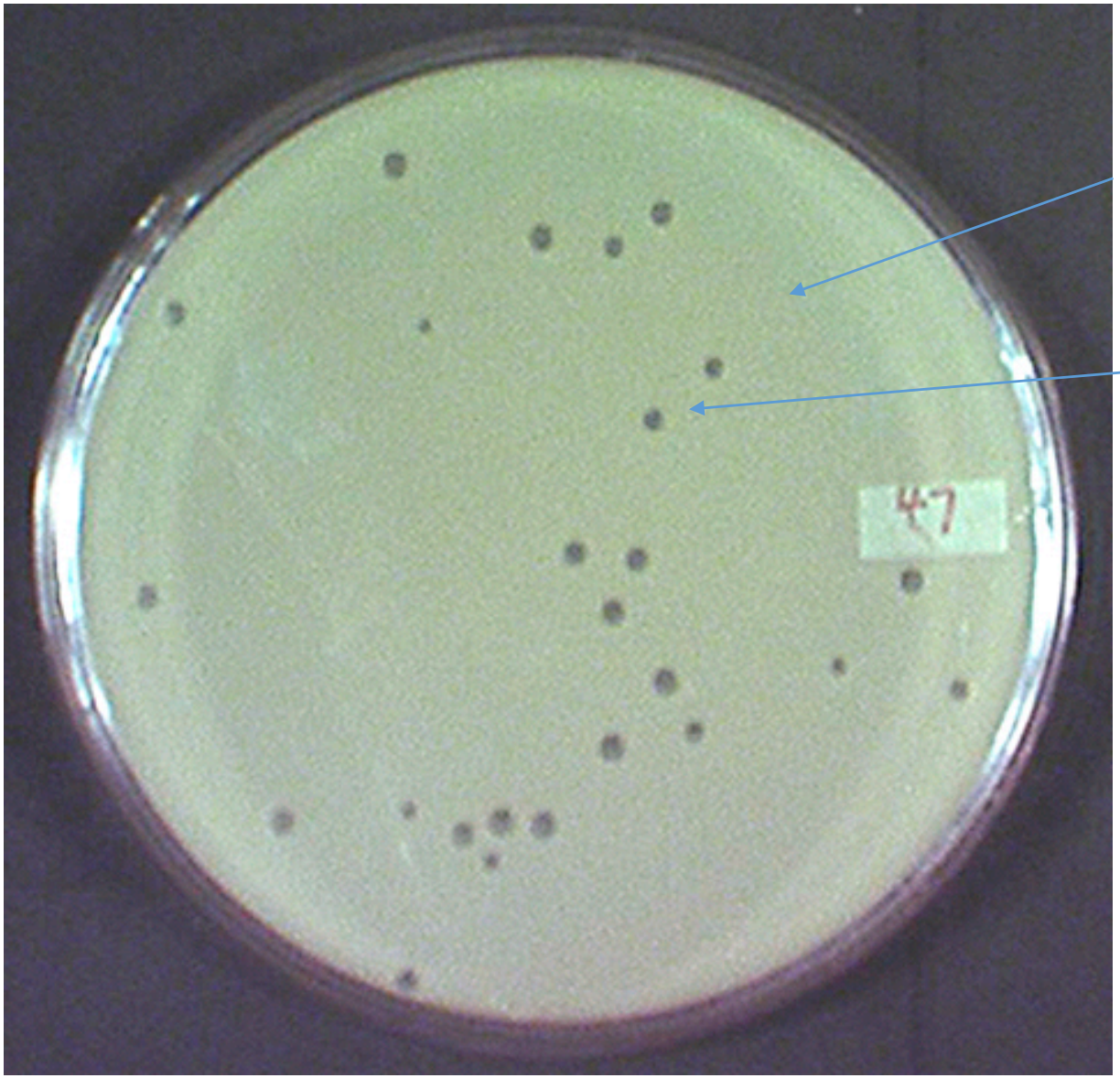


Serial dillution

- → Make serial dillution of phage stock
- → Plate on confluent agar plate confluent with E. coli; ideal culture 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 in liquid cultures
→ Prepare phage DNA
→ Sequence inserted DNA

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   Engineered vector systems

2. Vectors express and purify recombinant proteins

   - In bacteria
   - In yeast
   - In fly cells
   - In vertebrate cells

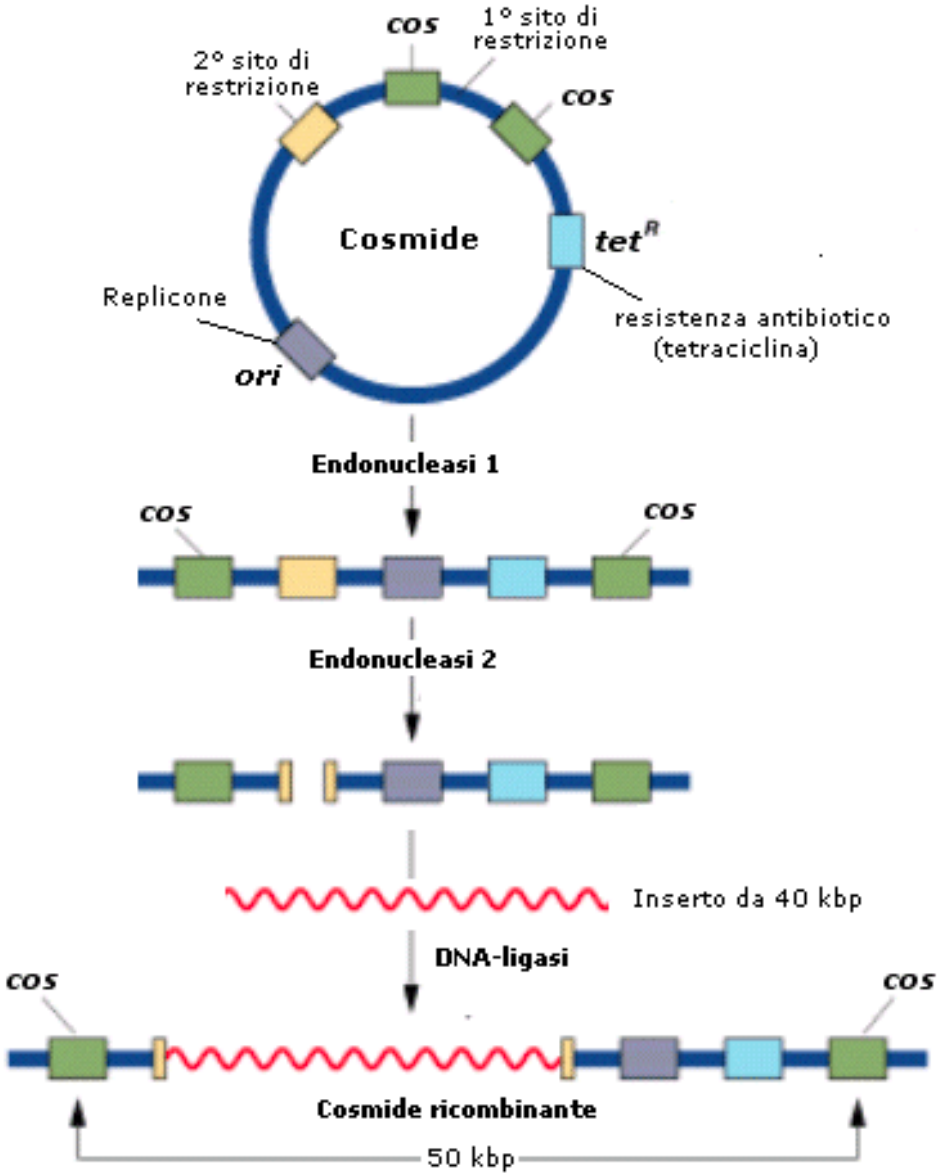
COSMIDI - COSMIDS

COSMIDS are engineered vectors that contain elements from phage and plasmid

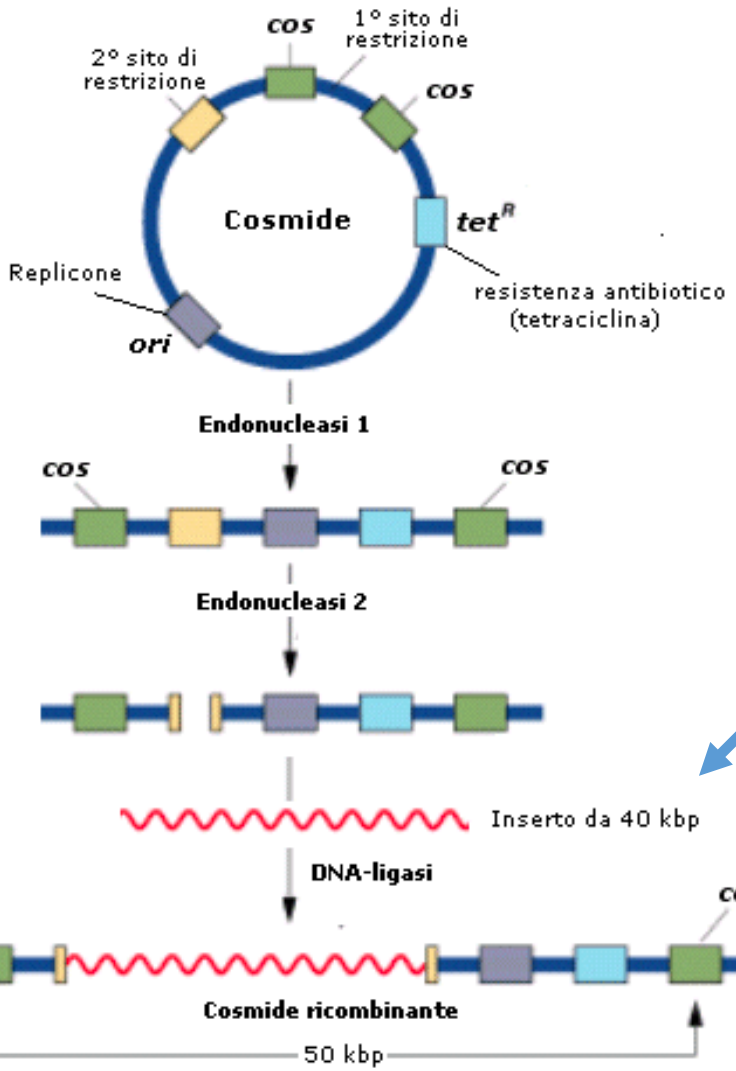
- Can carry **40kb** of insert DNA
- Can be maintained as plasmids in bacteria
- Can be shuttled as phage
- Less vectors required to cover the genome

CHARACTERISTIC FEATURES:

- Basic vector is small: 5-6 kb
- Cos sites are present; rest of lambda pahge DNA has been largely deleted
- Contains 2 cos sites for in vitro packaging → recombinant capsids are mixed with library –> cos sites are recognized and library vectors are inserted into capsid –> recombinant phage is used to infect bacteria.
- Note: 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**
- 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 re-circularize cosmid vector

Cosmid is maintained as plasmid (Tet resistance cassette)

Genome libraries

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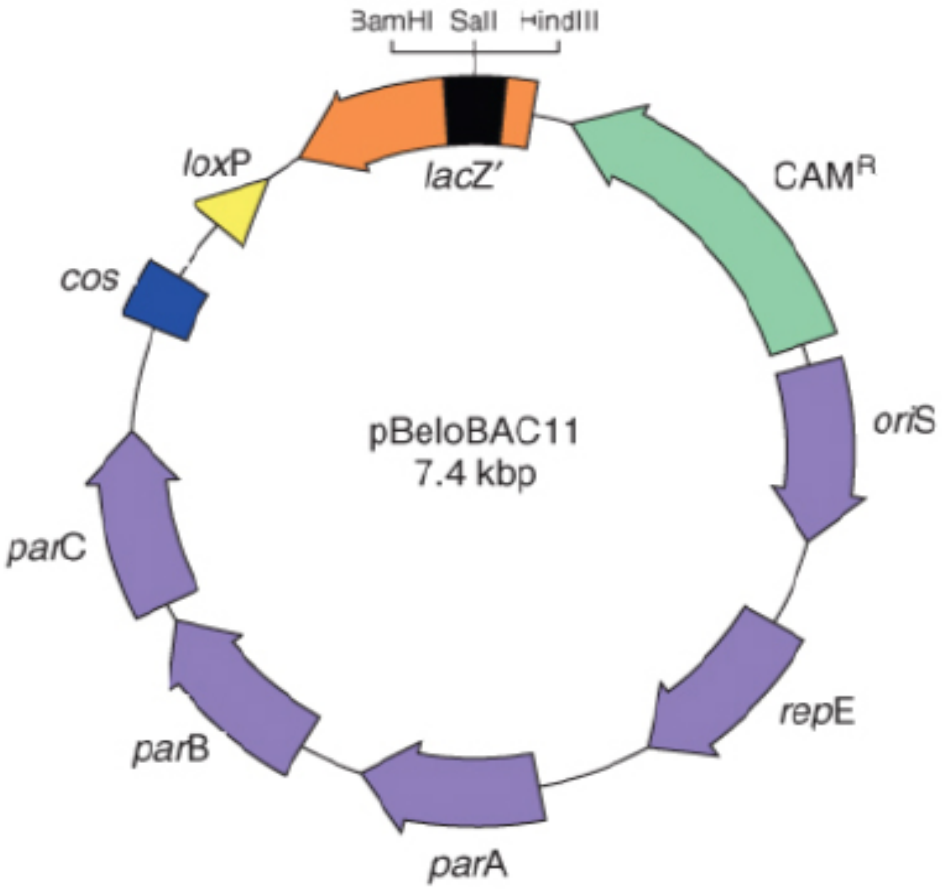
BACs – BACTERIAL ARTIFICIAL CHROMOSOMES

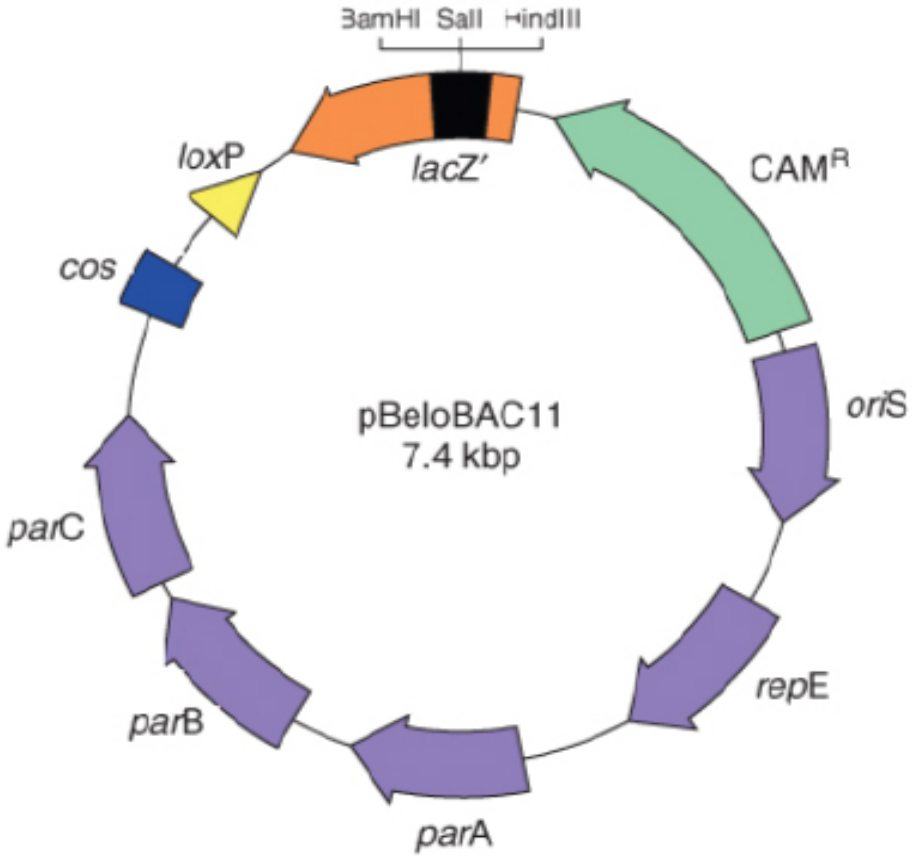


BACs are engineered vectors that contain elements from the F plasmid

- Can carry **300kb** of insert DNA
- Transformed into bacteria
- Can be maintained as **circular vector** in bacteria (oriS)
- Chloramphelicol resitance (CAM^R)
- Copy number control: 1-2 copies per bacteria
- Segregation system of F-plasmid → very stable vector (par region)
- Cos site optional for phage packaging (remember max. size for packaging)
- Ideal for genomic libraries

Genome 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:

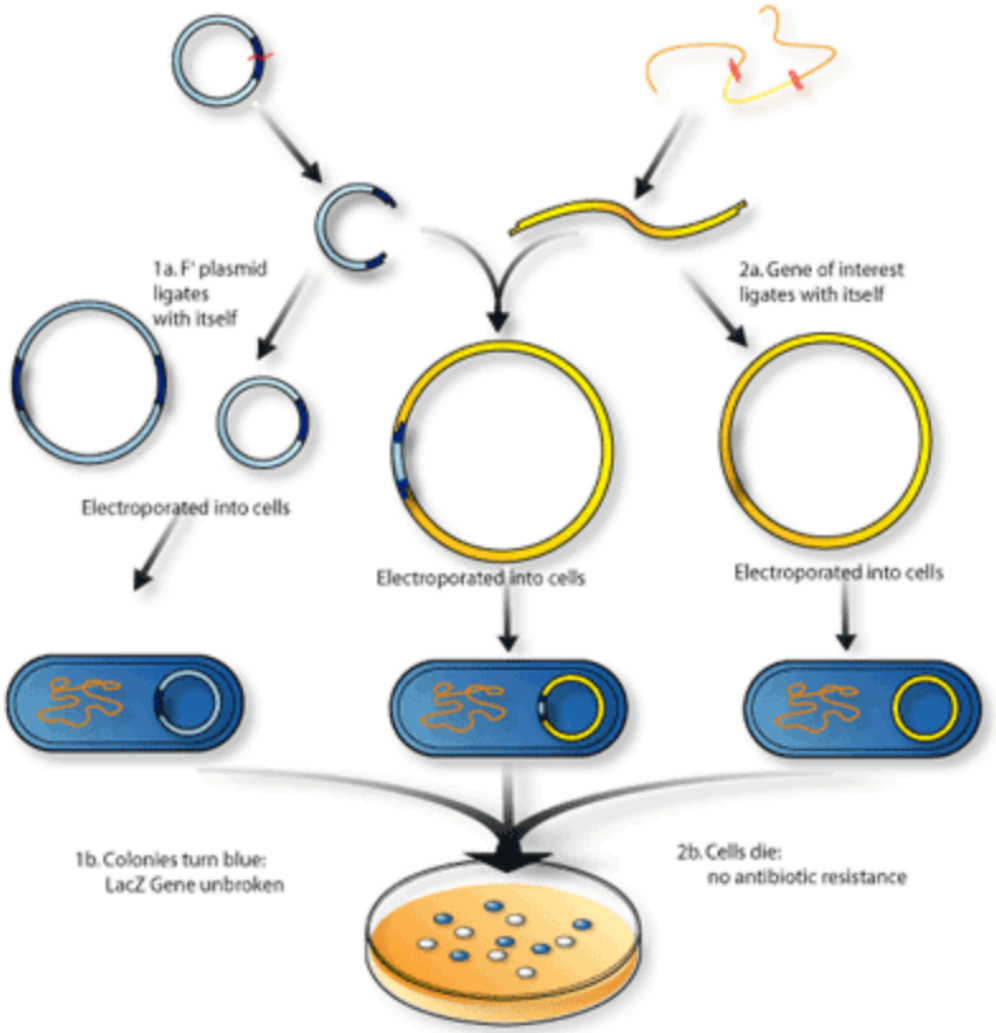
➢**Cos**: **for cutting by lambda terminase; optinal packaging**

➢**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



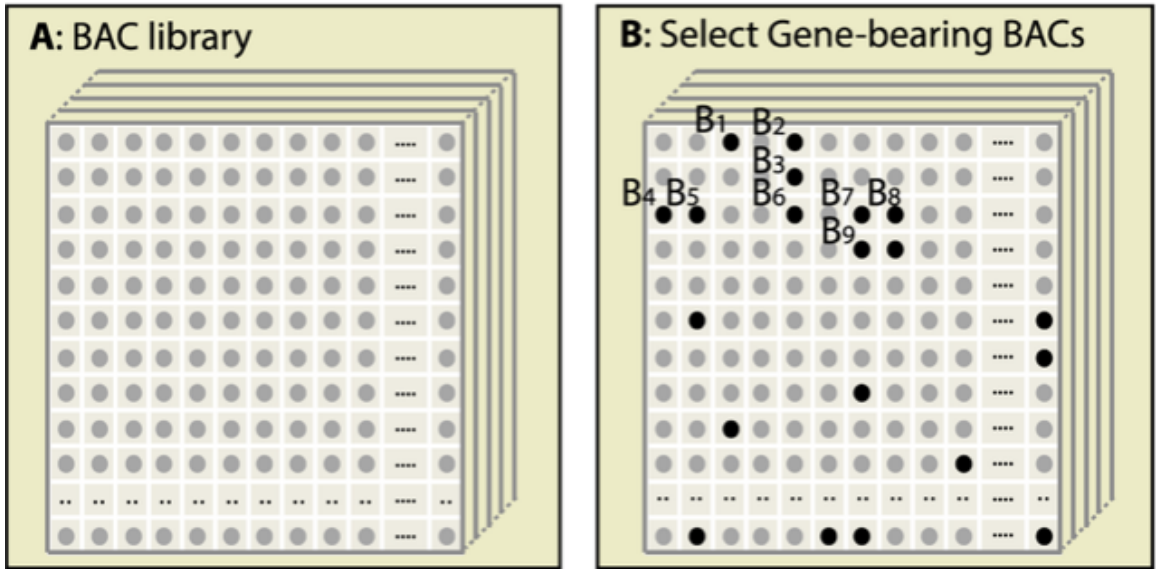
Screening on bacterial colonies

- 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**

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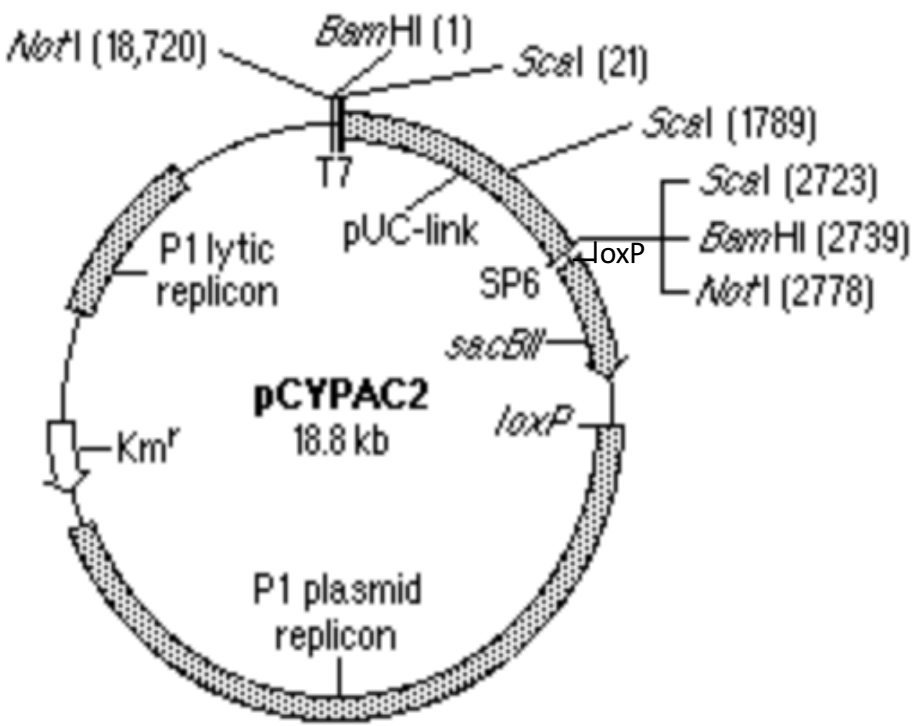
PAC (P1 derived Artificial Chromosome)

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

A



Fragments to be cloned into PACs:

150 kb (max. 300 kb)

Natural P1 phage: The genome of the P1 phage is moderately large, around 93Kbp in length. In the viral particle it is in the form of a linear double stranded DNA molecule. Once inserted into the host it circularizes and replicates as a plasmid.

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, ideal to maintain vector in cell**

- P1 lytic replicon: **IPTG inducible promoter**. IPTG to bacteria, lytic life cycle of P1 is triggered → generation of high copy number.

YAC - Yeast artificial chromosomes

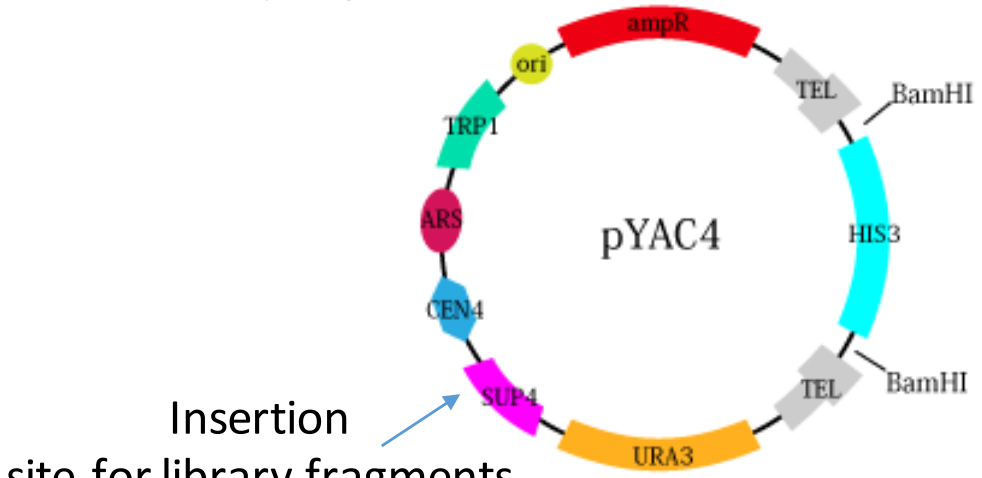
1. **CEN4**: 125 bp centromeric region, allowing segregation in yeast
2. **TEL**: telomere repeat; yeast 13bp repeats; stabilize linear YAC
3. **ARS**: autonomously replication region sequence; ensures replication in yeast.
4. **Ori, Amp^R**: replication and selection in E.coli
5. Selectable marker for yeast ; URA3,… many posibilities;
6. Yeast strains used have mutations that are compensated by **auxotrophic markers** found on the YACe vector:

   A. Trp marker. Yeast strain is Trp$^-$ and cannot grow in the absence of Trypotphane. YAC contains Trp marker for **complementation** → yeast with YAC library vector can grow

   B. Ura3 marker (Orotidine 5'-phosphate decarboxylase): Yeast is ura3- and cannot produce uracil → do not grow in media lacking uracil. Yeast with YAC can grow in media lacking uracil Identifying yeast colonies that have YAC with insert from colonies with YAC non carrying and insert:
7. SUP4: This gene compensates for a sup$^-$mutation in the yeast host cell. Sup$^-$: causing the accumulation of red pigment. The host cells are normally red. Cells 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

Cloning of fragments: <1Mb

Very long: instable, risk of recombination, breaks



Gli YAC ricombinanti vengono trasformati in un ceppo di lievito ura3$^-$, trp1$^-$ o ade2 -, sup4$^-$

I ricombinanti vengono identificati come colonie rosse **che crescono in terreno privo di uracile e triptofano**

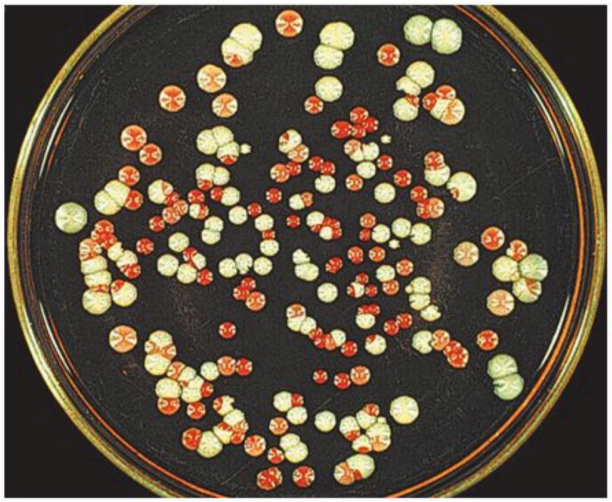
Ade2-fosforibosilamino-imidazolo-carbossilasi mutata

SUP4 → **Colonie bianche Non Ricombinanti**

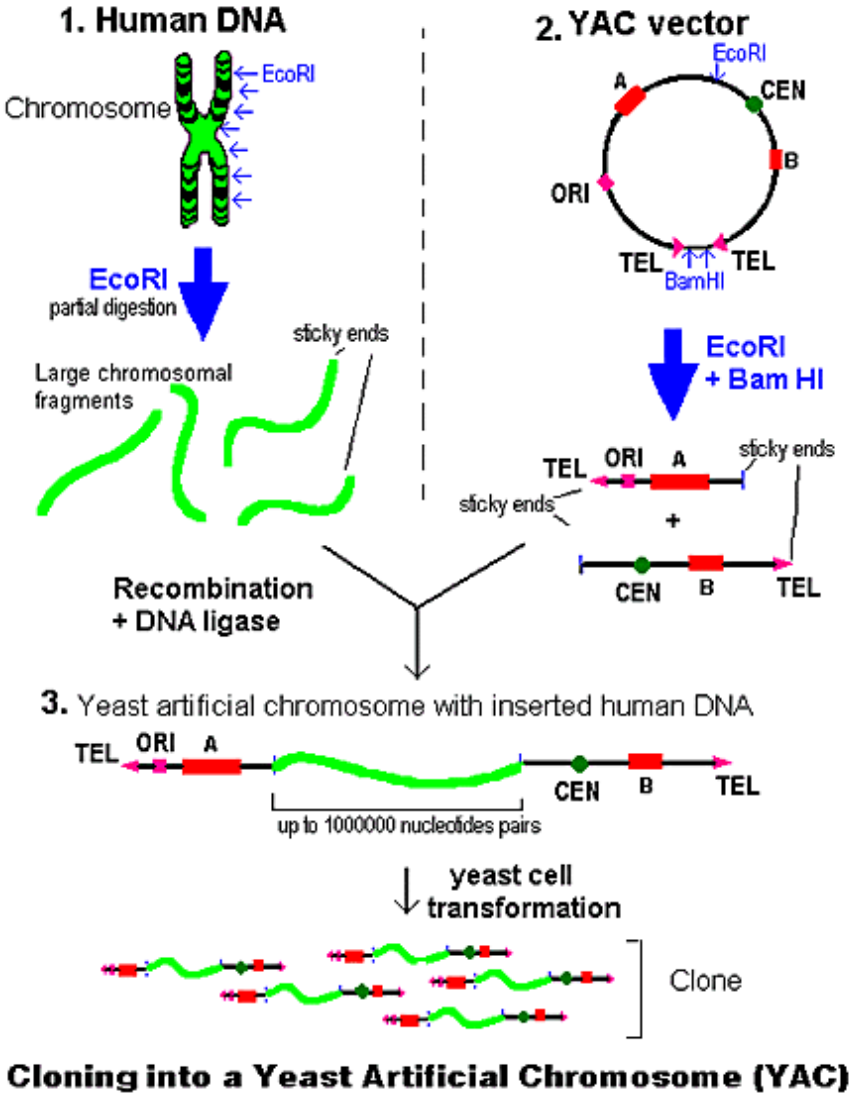
~~SUP4~~ → **Colonie rosse Ricombinanti**

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)

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Vettori di espressione per la produzione di proteine

A cosa possono servire le proteine ricombinanti?

-PROTEINE DI INTERESSE TERAPEUTICO.

-PROTEINE DI INTERESSE COMMERCIALE (ENZIMI).

-PROTEINE DA UTILIZZARE COME ANTIGENI PER LA PRODUZIONE DI ANTICORPI POLICLONALI E MONOCLONALI.

-REAGENTI PER LA RICERCA BI BASE E APPLICATA.

Produzione di proteine

Brand	Generic	Company	Therapeutic category	Indications
Humulin	Insulin	Eli Lilly	Diabetes	Diabetes
Humatrope	Recombinant Somatropin	Eli Lilly	Hormones	Growth failure
Genotropin	Somatropin	Pfizer	Hormones	Growth failure
Saizen	Somatropin	Serono	Hormones	Growth failure
Nutropin/Protropin	Somatropin/Somatrem	Genentech	Hormones	Growth failure
Intron A &	Interferon alpha-2b/	Schering-Plough	Anti-infective	Viral infections
Avonex	Interferon beta-1a	Biogen Idec	Multiple sclerosis	Chronic inflammatory demyelinating polyneuropathy
Betaseron/Betaferon	Interferon beta-1b	Schering AG	Multiple sclerosis	Multiple sclerosis
Procrit/Eprex	Epoetin alpha	J&J	Blood modifier	Anaemia
Epogen	Epoetin alpha	Amgen	Blood modifier	Anaemia
NeoRecormon	Epoetin beta	Roche	Blood modifier	Anaemia
Kogenate	Factor VIII	Bayer	Blood modifier	Haemophilia
NovoSeven	Factor VIIa	Novo Nordisk	Blood modifier	Haemophilia
Benefix	Factor IX	Wyeth	Blood modifier	Haemophilia
Fabrazyme	Agalsidase beta	Genzyme	Enzymes	Fabry disease
Replagal	Agalsidase alfa	TKT Europe	Enzymes	Fabry disease
Pulmozyme	Dornase alpha	Genentech	Enzymes	Cystic fibrosis
Activase/Actilyse	Alteplase	Genentech	Blood factor	Myocardial infarction

Produzione di proteine

Monoclonal Antibodies

Brand	Generic	Company	Therapeutic category	Indications
ReoPro	Abciximab	Eli Lilly	Blood modifier	Acute coronary syndrome
Rituxan	rituxumab	Genentech	Cancer	Non-Hodgkin's lymphoma
Herceptin	Trastuzumab	Genentech	Cancer	Breast cancer
Synagis	Palivizumab	MedImmune	Respiratory	Respiratory syncytial virus
Campath	Alemtuzumab	Schering AG	Cancer	Non-Hodgkin's lymphoma
Humira	Adalimumab	Abbott Labs	Anti-arthritic	Rheumatoid arthritis
Xolair Omalizumab	Omalizumab	Genentech	Respiratory diseases	Paediatric asthma, peanut allergies
Erbitux	Cetuximab	Imclone Systems	Cancer	Colon cancer
Avastin	Bevacizumab	Genentech	Cancer	Colon cancer

Vettori di espressione per la produzione di proteine

QUALI SISTEMI ETEROLOGHI UTILIZZARE PER L'ESPRESSIONE DEI GENI ?

E' virtualmente possibile esprimere geni in sistemi di ogni tipo utilizzando vettori d'espressione appropriati,in funzione di esigenze specifiche.

I più diffusi:

- ***Escherichia coli,***
- ***Bacillus subtilis,***
- ***Lieviti (yeast)***
- ***cellule d'insetto/sistemi virali (Insect cells)***
- ***cellule vegetali***
- ***cellule di mammifero in coltura (mammalian cells)***

L'espressione in E.coli è di gran lunga la più semplice e, forse, per questo la più utilizzata come prototipo di espressione genica in sistemi eterologhi.

Codon usage in different organisms

Table 3.2 The genetic code and codon usage in *E. coli* and humans

Codon	Amino acid	Frequency of use in:	
		E. coli	Humans
GAG	Glutamic acid	0.30	0.59
GAA	Glutamic acid	0.70	0.41
CGG	Arginine	0.08	0.19
CGA	Arginine	0.05	0.10
CGU	Arginine	0.42	0.09
CGC	Arginine	0.37	0.19
AGG	Arginine	0.03	0.22
AGA	Arginine	0.04	0.21
CCG	Proline	0.55	0.11
CCA	Proline	0.20	0.27
CCU	Proline	0.16	0.29
CCC	Proline	0.10	0.33
UGA	Stop	0.30	0.61
UAG	Stop	0.09	0.17
UAA	Stop	0.62	0.22

Problem:

The expression of human cDNAs is often inefficient

Codoni sononimi:

Tre dei 64 codoni sono segnali di terminazione della sintesi proteica (codoni di stop o di terminazione), mentre gli altri 61 codificano per i 20 amminoacidi. Nei casi in cui uno stesso amminoacido è codificato da più codoni (da 2 a 6), questi si chiamano codoni sinonimi.

Se alcuni tRNA sono rari in quell'ospite, allora alcuni codoni non verranno riconosciuti e funzioneranno come codoni di stop, causando sintesi premature; oppure potrebbero non corrispondere all'inserzione dell'aminoacido desiderato (codon bias).

Bisognerà modificare il cDNA ricombinante da clonare in modo che contenga tra i sinonimi quei codoni per i tRNA più frequenti nell'ospite; oppure bisognerà ingegnerizzare l'ospite affichè esprima i tRNA rari (ceppi Rosetta di E. coli).

1. Vectors to clone genomic fragements/cDNA (libraries
    - Bacteriophages
    - Cosmids
    - Bacterial artificial chromosomes
    - P1 derived artificial chromosomes (PAC)
    - Yeast artificial chromosomes

2. Vectors express and purify recombinant proteins
    - **In bacteria**
    - In yeast
    - In fly cells
    - In vertebrate cells

Pro and Cons of expressing human proteins in bacteria

Vantaggi:

- Vasta scelta di vettori di clonaggio
- Vasta scelta d ceppi
- Controllo relativamente semplice dell' espressione a livello genetico
- Buona resa della proteina ricombinante (25% del totale delle proteine)
- La proteina ricombinante può essere espressa come proteina di fusione
- La proteina ricombinante può essere disegnata per essere secreta nel terreno di crescita
- Economico

Svantaggi:

- La proteina ricombinante mancherà di modificazioni post-trasduzionali
- L' attività biologica del ricombinante può essere diverso dalla proteina naturale
- Carico metabolico molto pesante con overespressione di proteine che a volte porta alla formazione di inclusion bodies, aggregati che rendono difficoltosa la purificazione del prodotto e possono ridurre la sua attività biologica

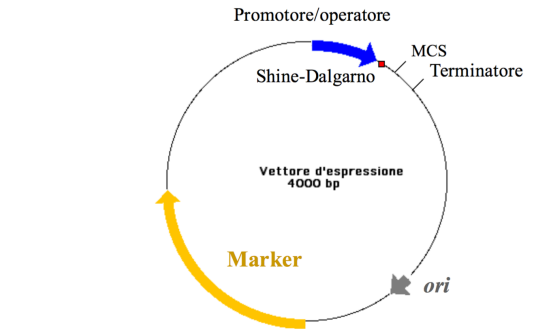
Fattori influenzanti l'espressione dei geni clonati per ottimizzare l'espressione di proteine di mammifero nei batteri

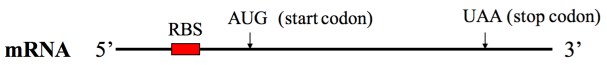
- **Il promotore** (batterico/eucariotico): la regione legante l'RNA polimerasi e segnali regolanti l'espressione genica (iniziare la sintesi del trascritto di mRNA)

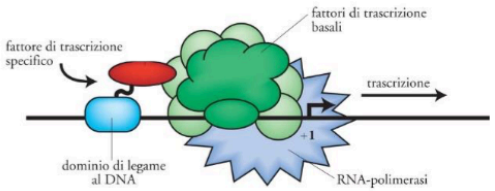
- **cDNA**: più usato, rispetto al clonaggio di DNA genomico contenente introni

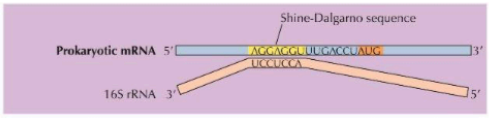
- **L'inizio della traduzione**: **sequenza di Shine-Dalgarno legante il 3' dell'rRNA 16S (nei batteri) e la sua distanza dal codone d'inizio della traduzione,** per inserire un appropriato sito di legame al ribosoma e un codone d'inizio correttamente posizionato. Inoltre, alcuni organismi non usano l'AUG, ma codoni d'inizio alternativi (GUG, UUG, CUG), anche il **sito d'inizio dovrà essere opportunamente modification base all'ospite.**

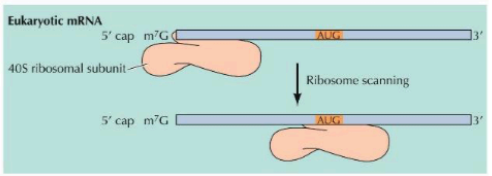
- **Codoni sononimi** (diversi ma codificanti lo **stesso aminoacido**): la frequenza d'inserimento dell'aminoacido dipenderà dalla **disponibilità nella cellula del tRNA** specifico per ciascun codone.

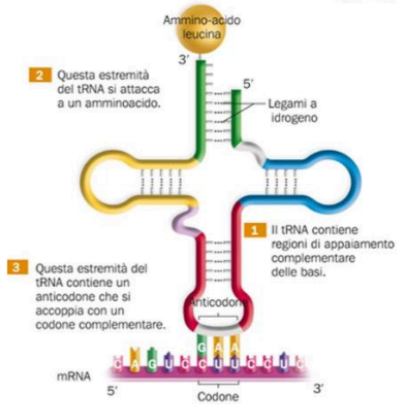


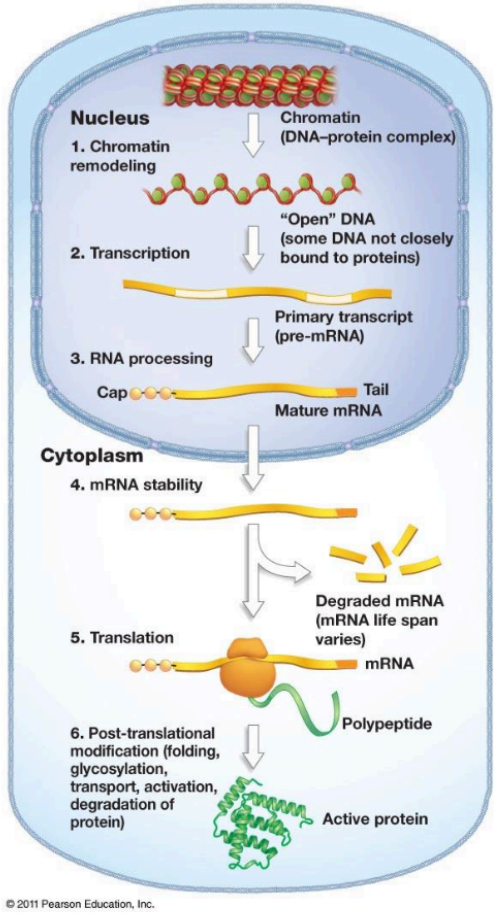












VETTORI D'ESPRESSIONE (Batterie)
caratteristiche generali

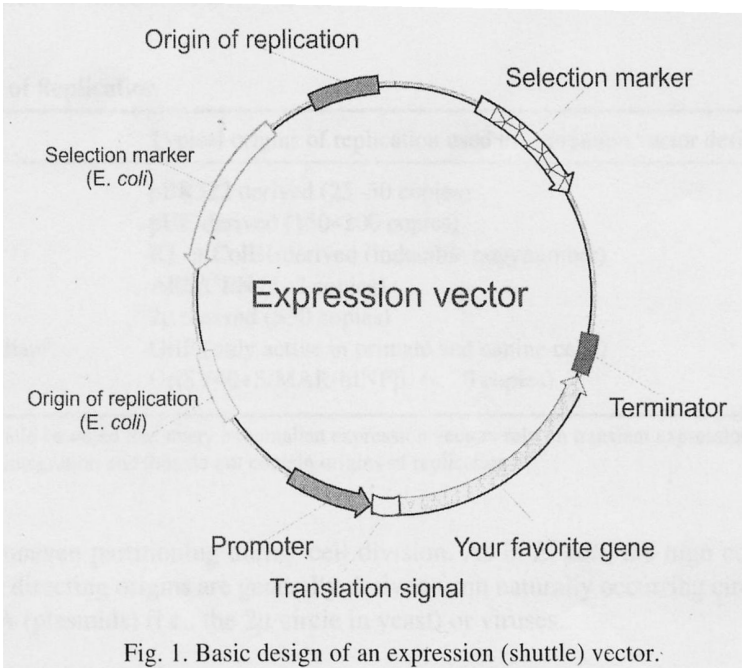
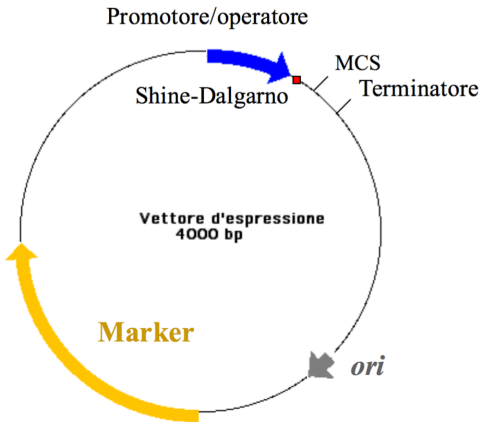
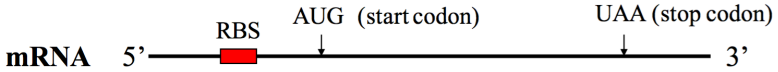


Fig. 1. Basic design of an expression (shuttle) vector.





Elementi standard
Origine di replicazione
Marker di selezione
Promotore (T7, T3, SP6; normalmente inducibile)
Terminatori di trascrizione
Segnali per ottimizzare mRNA
Codoni di terminazione della traduzione

Elementi genetici specifici per diverse applicazioni:
Sequenze segnale per secrezione
Molecole di fusione
Peptidi per purificazione (tags)
Ecc.

Subito dopo la sequenza di Shine-Dalgarno deve essere presente un codone di inizio, quasi **sempre AUG (in una piccola percentuale di casi può essere presente il codone GUG).**

La spaziatura ottimale tra **SD e AUG è di 8 bp**

E' importante che la sequenza nucleotidica tra la SD e il codone d'inizio non sia disturbata da strutture secondarie (es. hairpin loops) che possono interferire drasticamente con il legame al ribosoma e la conseguente traduzione.

Caratteristiche del prodotto proteico ricombinante

Geni in procarioti possono avere:
-Espressione Costitutiva
-ESPRESSIONE REGOLATA (= inducibile!!! Es. Lac Operon)

Una produzione continua può provocare:
-Inibizione Funzioni Cellula
-Perdita Energia
-Perdita Plasmide

Nella produzione di proteine eterologhe in batteri vengono utilizzati spesso promotori forti e regolabili

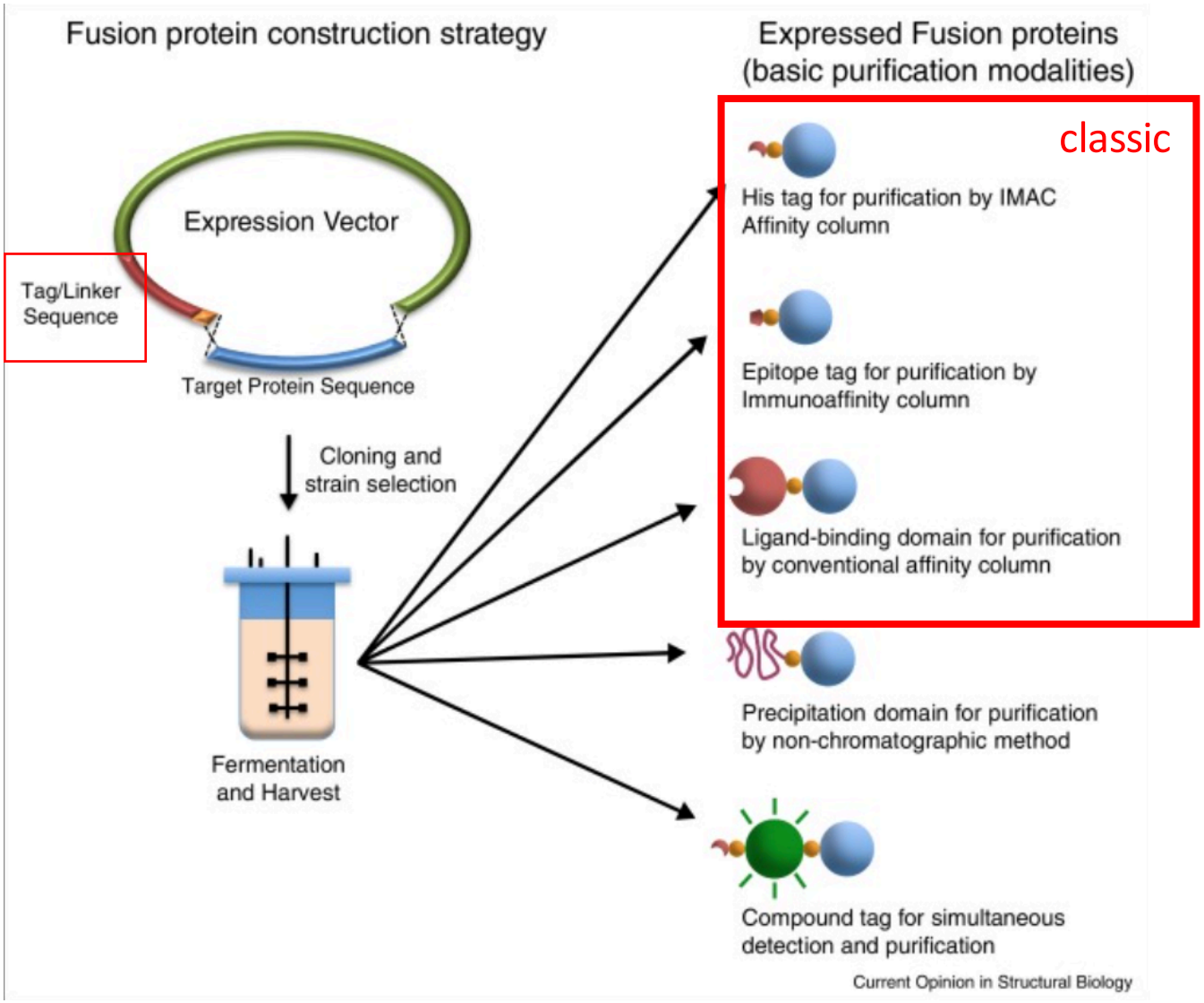
Promotori per la espressione di proteine ricombinanti in E. coli

Table 4 Some *E. coli* promoter systems that are in use for heterologous protein production and their characteristics

Expression system based on	Induction (range of inductor)	Level of expression	Key features	Original reference
lac promoter	Addition of IPTG 0.2 mM (0.05–2.0 mM)	Low level up to middle	Weak, regulated suitable for gene products at very low intracellular level Comparatively expensive induction	Gronenborn (1976)
trc and *tac* promoter	Addition of IPTG 0.2 mM (0.05–2.0 mM)	Moderately high	High-level, but lower than T7 system Regulated expression still possible Comparatively expensive induction High basal level	Brosius et al. (1985)
T7 RNA polymerase	Addition of IPTG 0.2 mM (0.05–2.0 mM)	Very high	Utilizes T7 RNA polymerase High-level inducible over expression T7*lac* system for tight control of induction needed for more toxic clones Relative expensive induction Basal level depends on used strain (pLys)	Studier and Moffatt (1986)
Phage promoter p_L	Shifting the temperature from 30 to 42 °C (45 °C)	Moderately high	Temperature-sensitive host required Less likelihood of "leaky" uninduced expression Basal level, high basal level by temperatures below 30 °C No inducer	Elvin et al. (1990)
tetA promoter/operator	Anhydrotetracycline 200µg/l	Variable from middle to high level	Tight regulation Independent on metabolic state Independent on *E. coli* strain Relative inexpensive inducer Low basal level	Skerra (1994)
araBAD promoter (P_{BAD})	Addition of L-arabinose 0.2 % (0.001–1.0 %)	Variable from low to high level	Can fine-tune expression levels in a dose-dependent manner Tight regulation possible Low basal level Inexpensive inducer	Guzman et al. (1995)
$rhaP_{BAD}$ promoter	L-rhamnose 0.2 %	Variable from low to high level	Tight regulation Low basal activity Relative expensive inducer	Haldimann et al. (1998)

Sistemi per la purificazione di proteine ricombinanti in E. coli

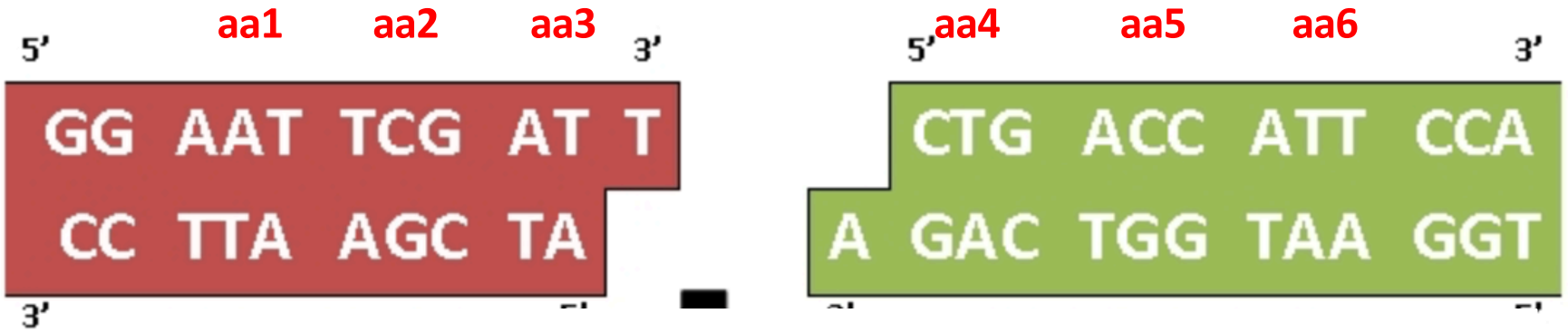
Si preferisce esprimere proteine di fusione (Tag + proteina di interesse), per la loro maggiore stabilità, gli alti livelli di espressione e la relativa facilità con cui si purificano.



- Tag is a short peptide sequence fused to the protein of interest (NH2 o COOH terminus of protein) that can be used the purify the recombiannt protein
- Tag can be captured by a special surface of a solid resin (in suspension)
- Rest of cell lysate is discarded
- Recombinant protein bound to resin
- Washing
- elution

Sistemi per la purificazione di proteine ricombinanti in E. coli

TAG and Protein must be "in frame)

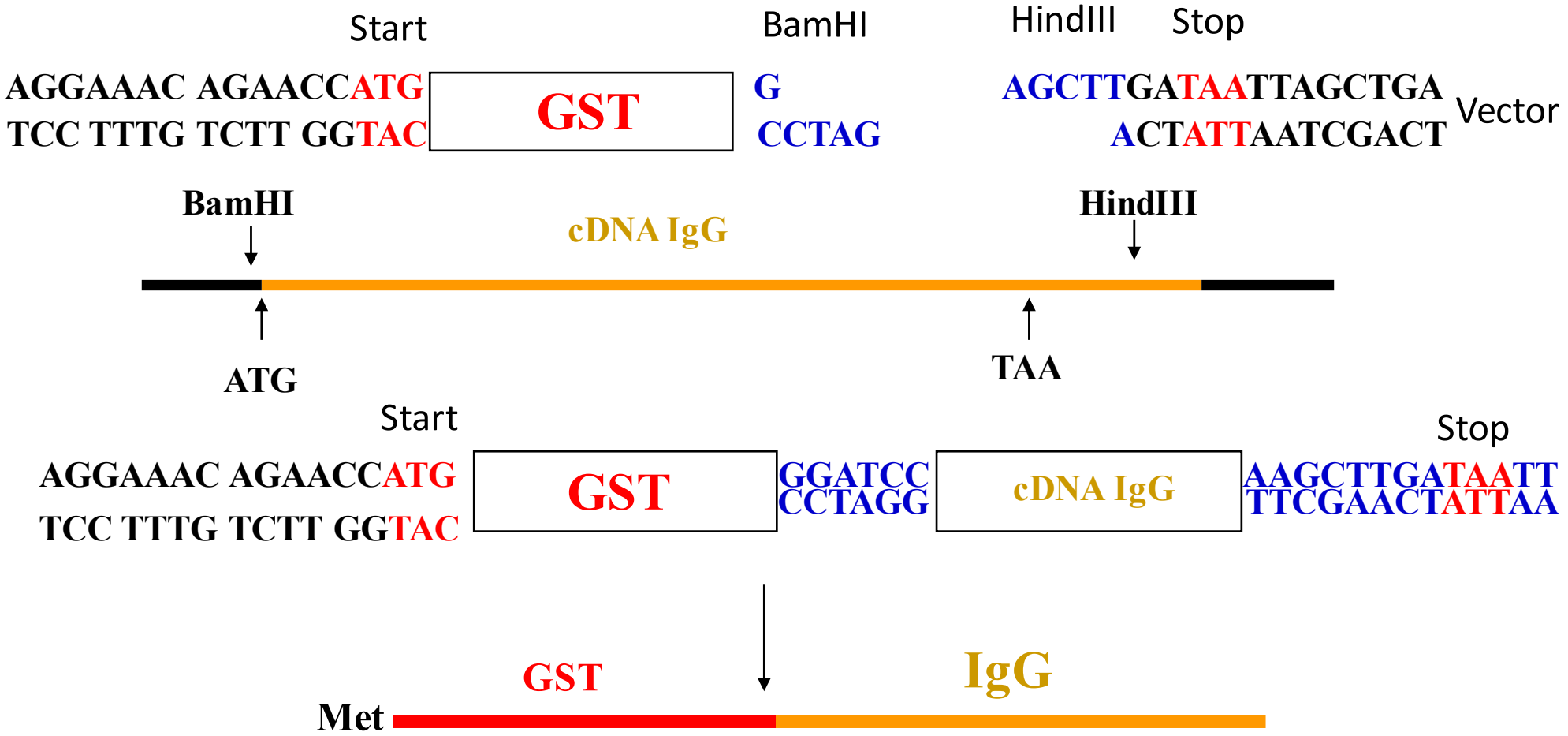


Tag (last 3 AA) **Protein of interest**

Sistemi per la purificazione di proteine ricombinanti in E. coli

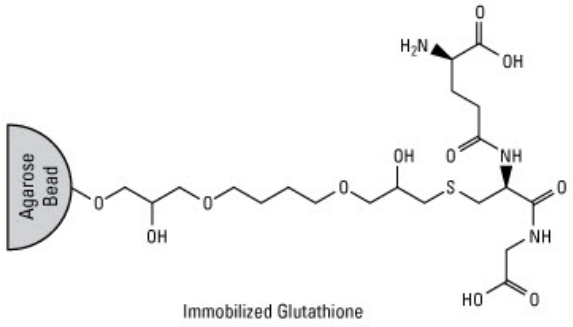
GST-tagged proteins:

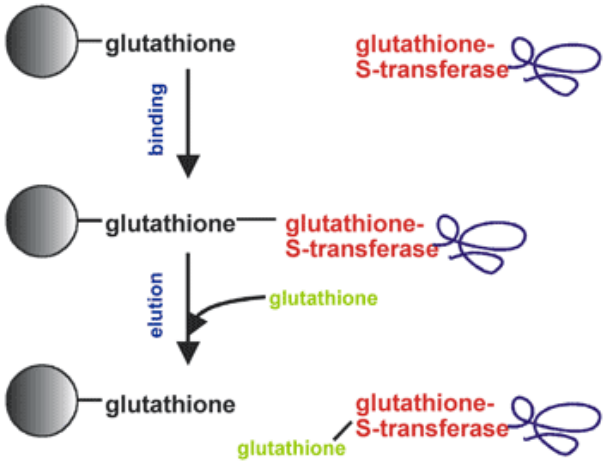
A convenient method of protein expression and subsequent purification is to fuse a protein with a glutathione-S-transferase (GST) domain. The DNA encoding for this 25 kDa protein domain is ligated in-frame with the gene for the desired protein so that, upon expression, your desired protein is fused to the GST domain. This is an incredible help in protein purification, since **GST binds glutathione extremely strongly.** The general purification strategy is thus to bind the GST fusion protein on a column of immobilized glutathione, wash away all the other stuff, and then elute the protein.

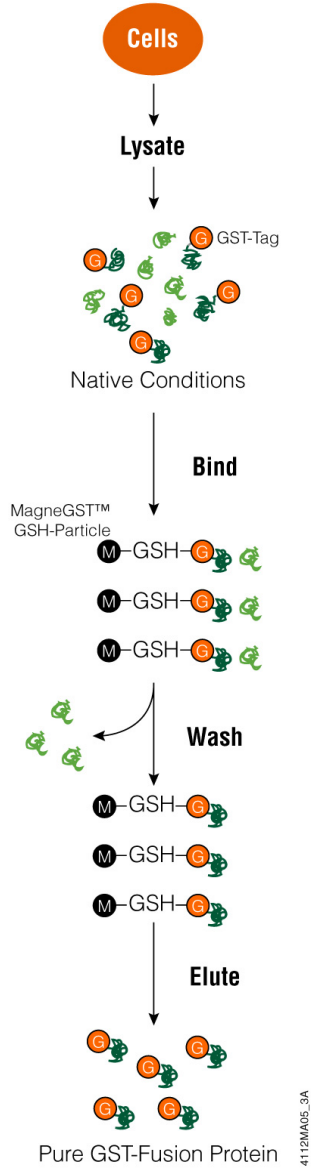


Sistemi per la purificazione di proteine ricombinanti in E. coli

1. Construction of GST-tagged cDNA in frame. Cloned into bacterial expression vector.
2. Transformation of bacteria
3. Growth of bacteria
4. Induction of protein expression via the use of an inducible prmoter
5. Cell harvest and lysis
6. Binding of recombiant protein to resin with immbilized gluthation
7. Bond protein can be eluted with excess of gluthation (in elution buffer – competition).







Sistemi per la purificazione di proteine ricombinanti in E. coli

AGGAAAC AGAACCATG
TCC TTTG TCTT GGTAC

HisHisHisHisHisHis

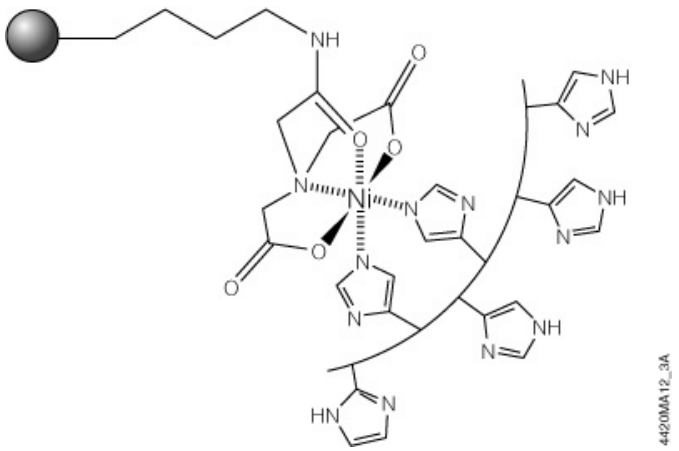
GGATCC
CCTAGG

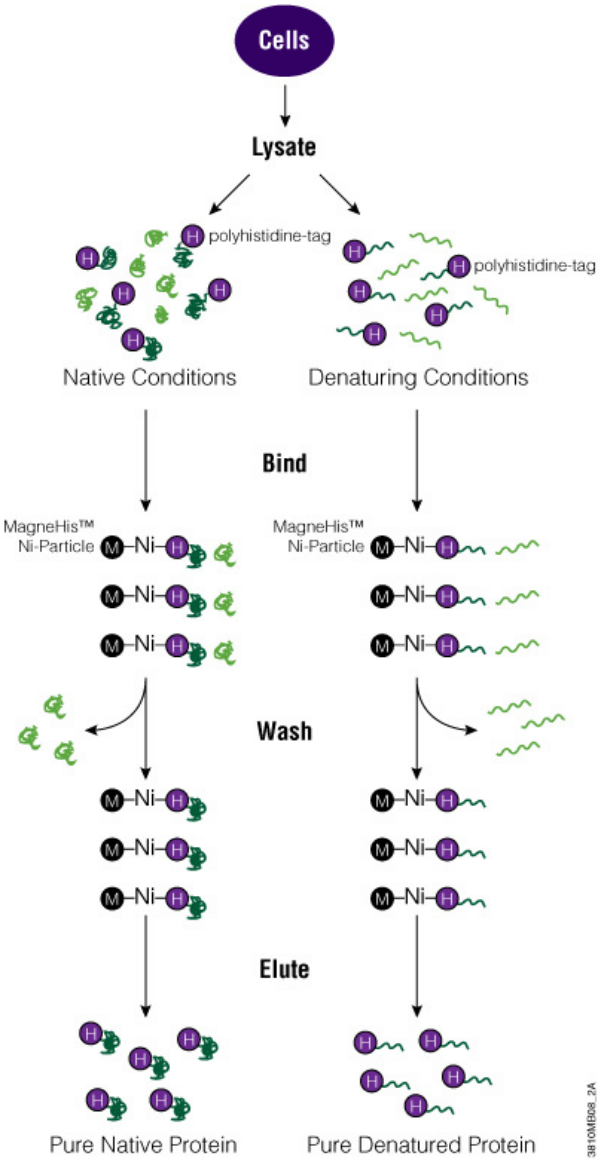
cDNA IgG

AAGCTT
TTCGAA

HISTIDINE (His)-tagged proteins:

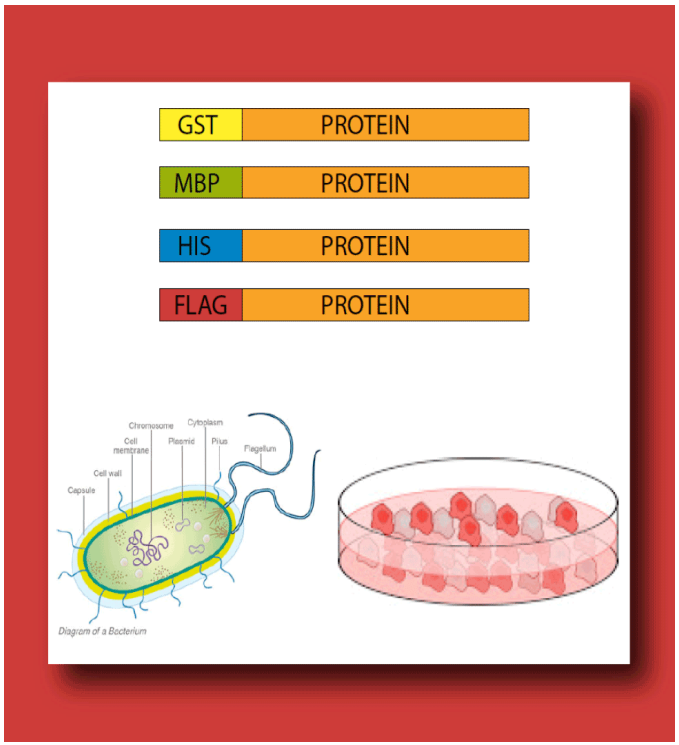
In general, proteins possess more or less the ability to coordinate metal ions on their surface, Histidine is strongly involved in the coordinate bond with metal ions. Therefore, if a number of histidines are added to the end of the protein by genetic engineering, the affinity of the protein for the metal ion is remarkably increased and the basic idea is that purification can be easily carried out. When a protein having a His tag is brought into contact with a carrier on which a metal ion such as nickel is immobilized under the condition of pH 8 or higher, the histidine residue chelates the metal ion and binds to the carrier.

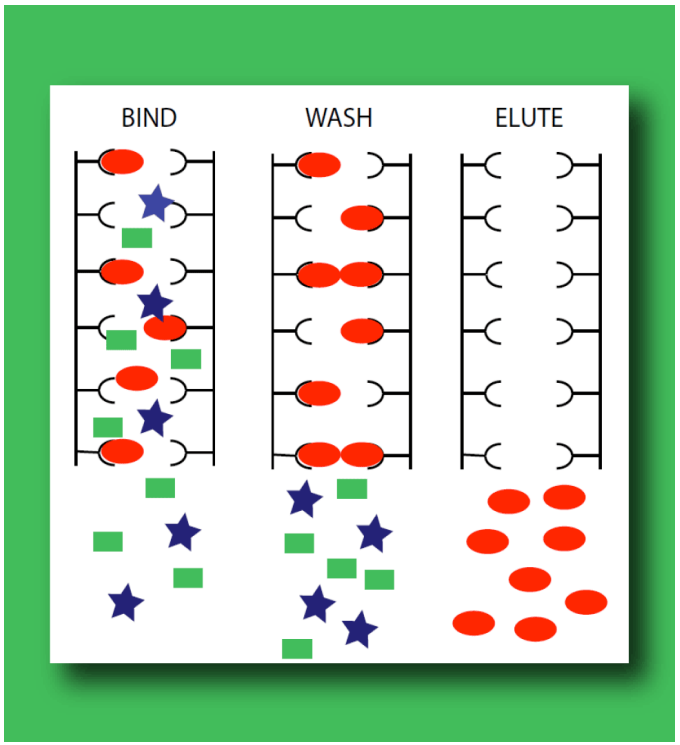


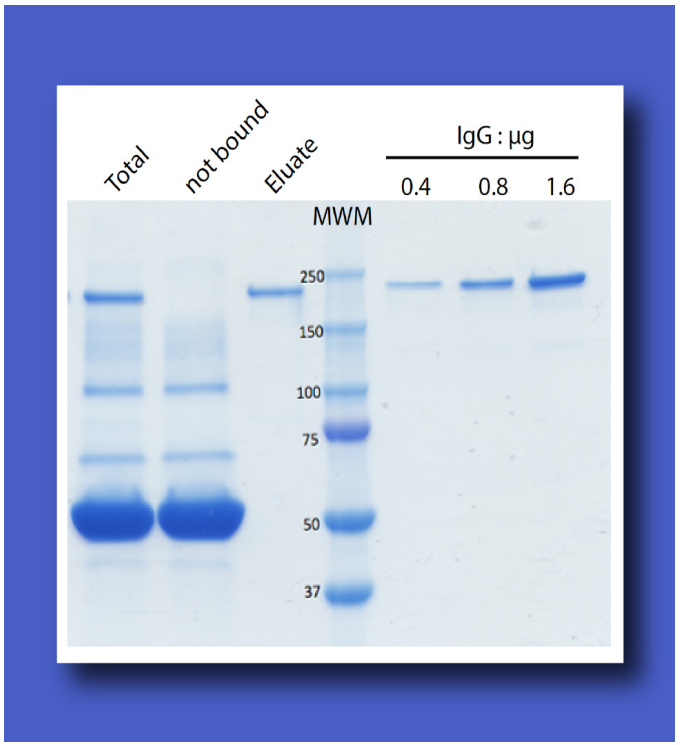


COMMON TAGs FOR EXPRESSION OF RECOMBINAT PROTEINS IN E. COLI

- Calmodulin binding peptide (CBP)
- 6xHis
- Proteina A (IgG binding domain)
- Chitin binding domain (CBD)
- Glutatione S-transferasi (GST)
- MBP (Maltose binding protein)
- Strep tag (Streptavidin binding tag)







1. Vectors to clone genomic fragements/cDNA (libraries
    - Bacteriophages
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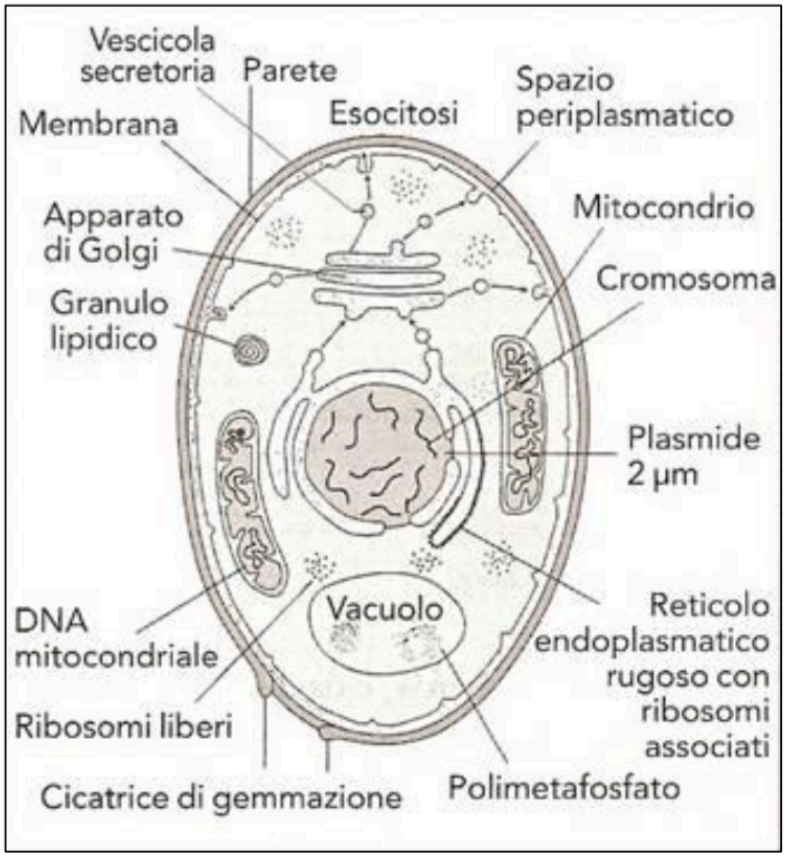
    - Bacterial artificial chromosomes
    - P1 derived artificial chromosomes (PAC)
    - Yeast artificial chromosomes

2. Vectors express and purify recombinant proteins
    - In bacteria
    - **In yeast**
    - In fly cells
    - In vertebrate cells

Trasformazione di cellule eucariotiche di lievito (e funghi filamentosi)

❑ **Inserimento di DNA ricombinante in cellule di *Saccaromices cerevisiae*:**

- Biochimica cellulare e i meccanismi di regolazione del lievito sono **simili agli eucarioti superiori**
- Trasduzione del segnale e regolazione della trascrizione da parte di **ormoni steroidei simile ai mammiferi**
- Molti geni sono omologhi di geni umani, tra questi molti coinvolti nella divisione cellulare
- **Sistemi modello di studio**
- Possono **esprimere proteine** eterologhe (farmaci proteici)
- Rappresentano **modelli di screening di farmaci**



► **Produzione di sferoplasti (treatment of call wall with enzmes such as zymolyase, glusulase or lyticase) e trattamento con polietilenglicole**

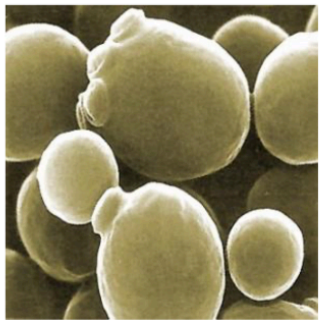
► **Elettroporazione**

Vettori di espressione per la produzione di proteine in lievito

Sono funghi, organismi eucariotici unicellulari di forma tonda o ellittica

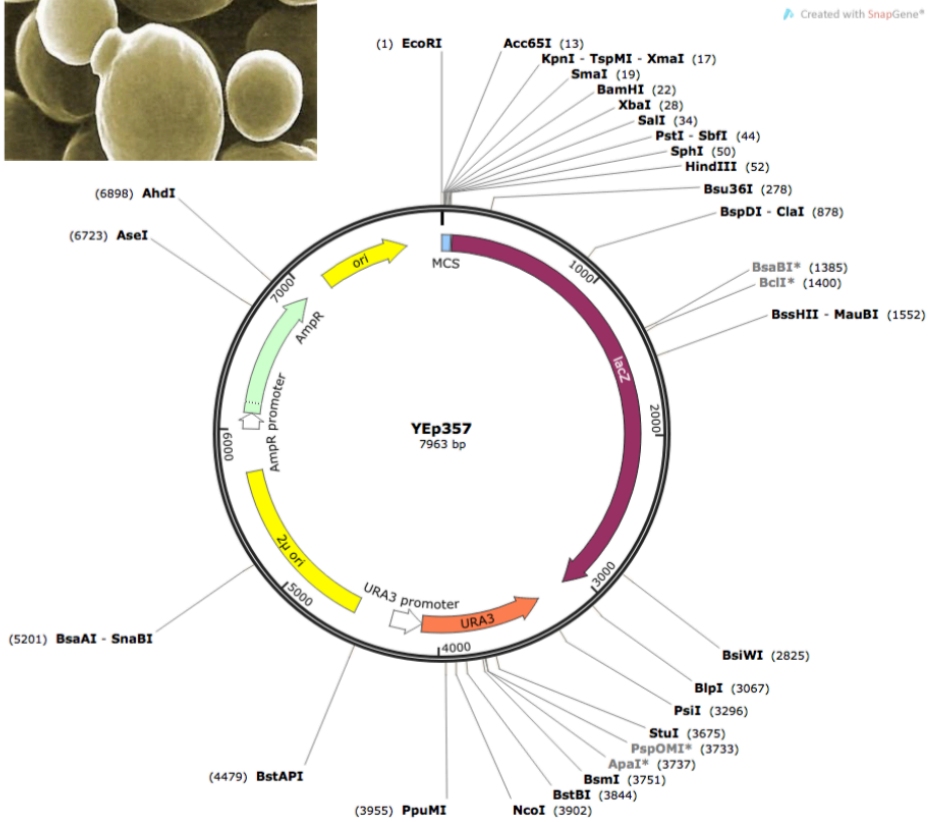
Dimensioni: 3-50 micron

Più di 1000 specie, ma i più usati (per lievitare il pane e fermentare bevande alcoliche) appartengono agli Ascomiceti: *Saccharomyces cerevisiae*



Yest Episomal Plasmid (**Yep; repliconi episomali**):

1. Si replicano autonomamente ad alto numero, **20-100 copie per cellula**, grazie all'aggiunta la **sequenza di replicazione autonoma *ars***. Essendo instabili, tendono ad essere persi durante la duplicazione delle cellule di lievito.
2. Hanno un ***ori* batterica**, che consente la loro crescita e manipolazione anche nei batteri (*vettori shuttle*)
3. Hanno anche un **marcatore per la resistenza agli antibiotici**, per la selezione in *E. coli*
4. Ma è necessario anche un secondo marcatore di selezione rispetto ai batteri, poiché i lieviti sono sensibili a un numero ridotto di antibiotici, sono quindi usati altri marcatori di selezione, come la **«complementazione di mutazioni auxotropiche», associate alla produzione di enzimi coinvolti nella biosintesi di aminoacidi o altre molecole: *trp1*** (triptofano), ***leu2*** (leucina), his3 (istidina) o ***ura3*** (uracile).



Vettori di espressione per la produzione di proteine in lievito

Nuovi costrutti hanno anche un centromero
Yest Centromero Plasmid (**YCp**):
Le sequenze centromeriche si associano a proteine specifiche che formano un complesso multiproteico capace di legare i microtubuli del fuso responsabili della segregazione, consentendo una corretta distribuzione delle copie episomali durante la divisione cellulare.
Basso numero di copie **1-2 copie per cellula**.
Vantaggiosi quando il gene clonato è dannoso per l'ospite, o se ne vuole studiare la regolazione (studi d'induzione dell'espressione)

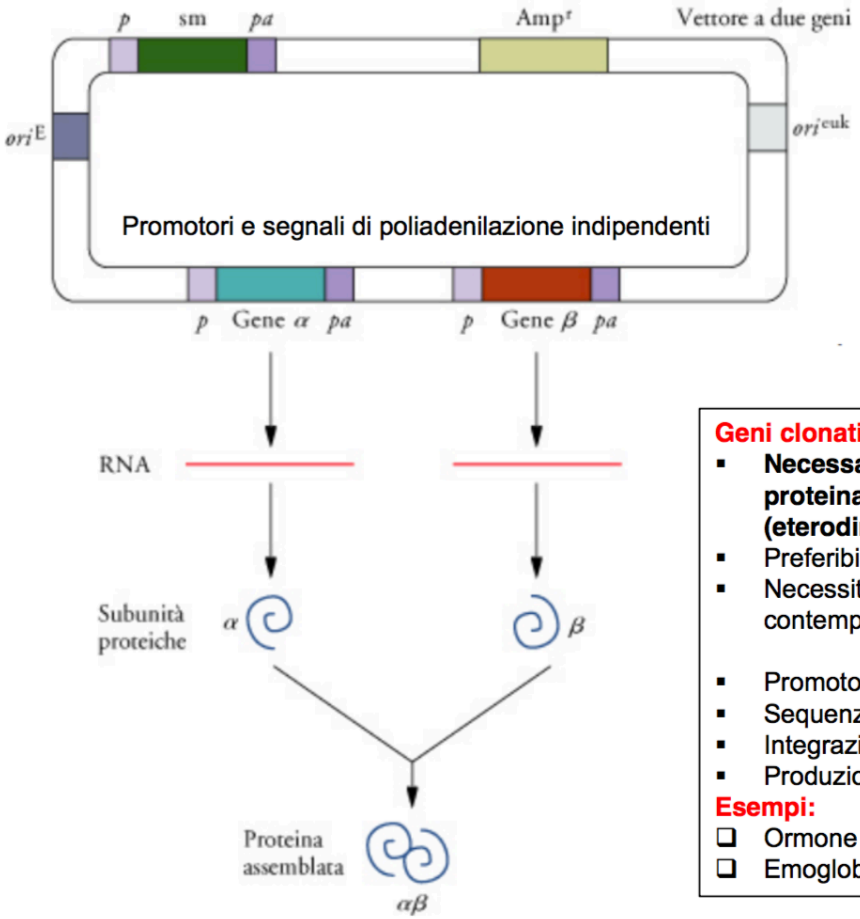
Yest Integrating Plasmid (**YIp**):
Si replicano autonomamente, ma si integrano anche nel cromosoma di lievito per ricombinazione omologa, hanno bassa frequenza di trasformazione, ma sono più stabili

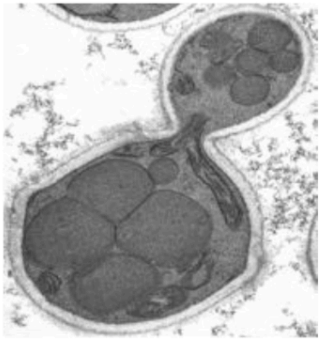
Yest Artificial chromosomes (**YAC**):
Usati per clonare frammenti molto grandi

Vettori di espressione per la produzione di proteine in lievito

espressione di 2 geni in una stessa cellula

vettori a doppia cassetta





Hansenula polymorpha

Geni clonati in tandem:

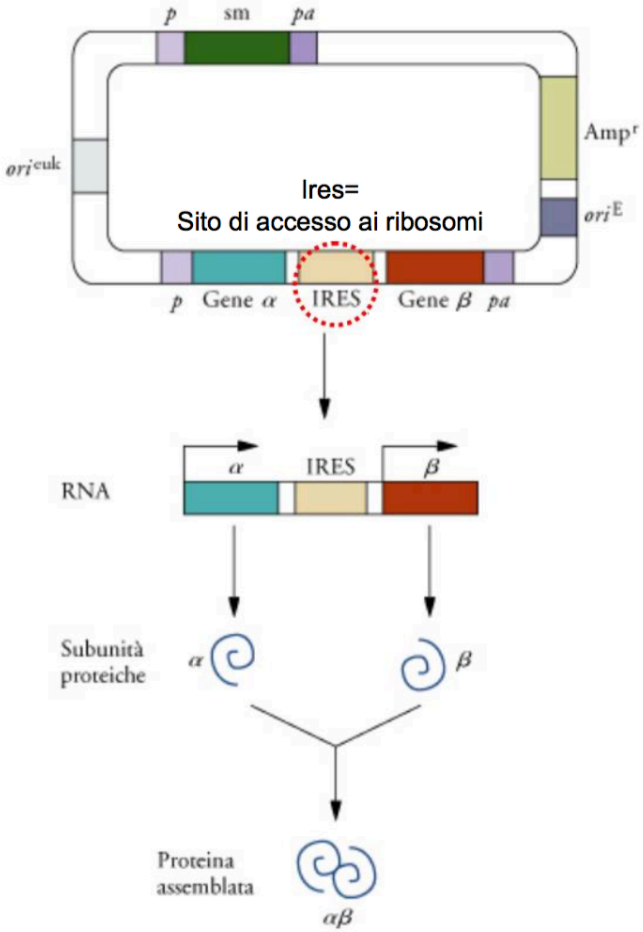
- **Necessaria quando la forma attiva di una proteina è costituita da 2 proteine diverse (eterodimero)**
- Preferibile all'assemblaggio *in vitro*
- Necessità di esprimere le subunità contemporaneamente e stechiometricamente

- Promotore MOXp (metanolo ossidasi)
- Sequenza di arresto della trascrizione MOXt
- Integrazione fortuita dopo varie generazioni
- Produzione del tetramero α2β2 funzionale

Esempi:

- ❑ Ormone stimolatore della tiroide (αβ)
- ❑ Emoglobina ($\alpha_2\beta_2$)

vettori dicistronici

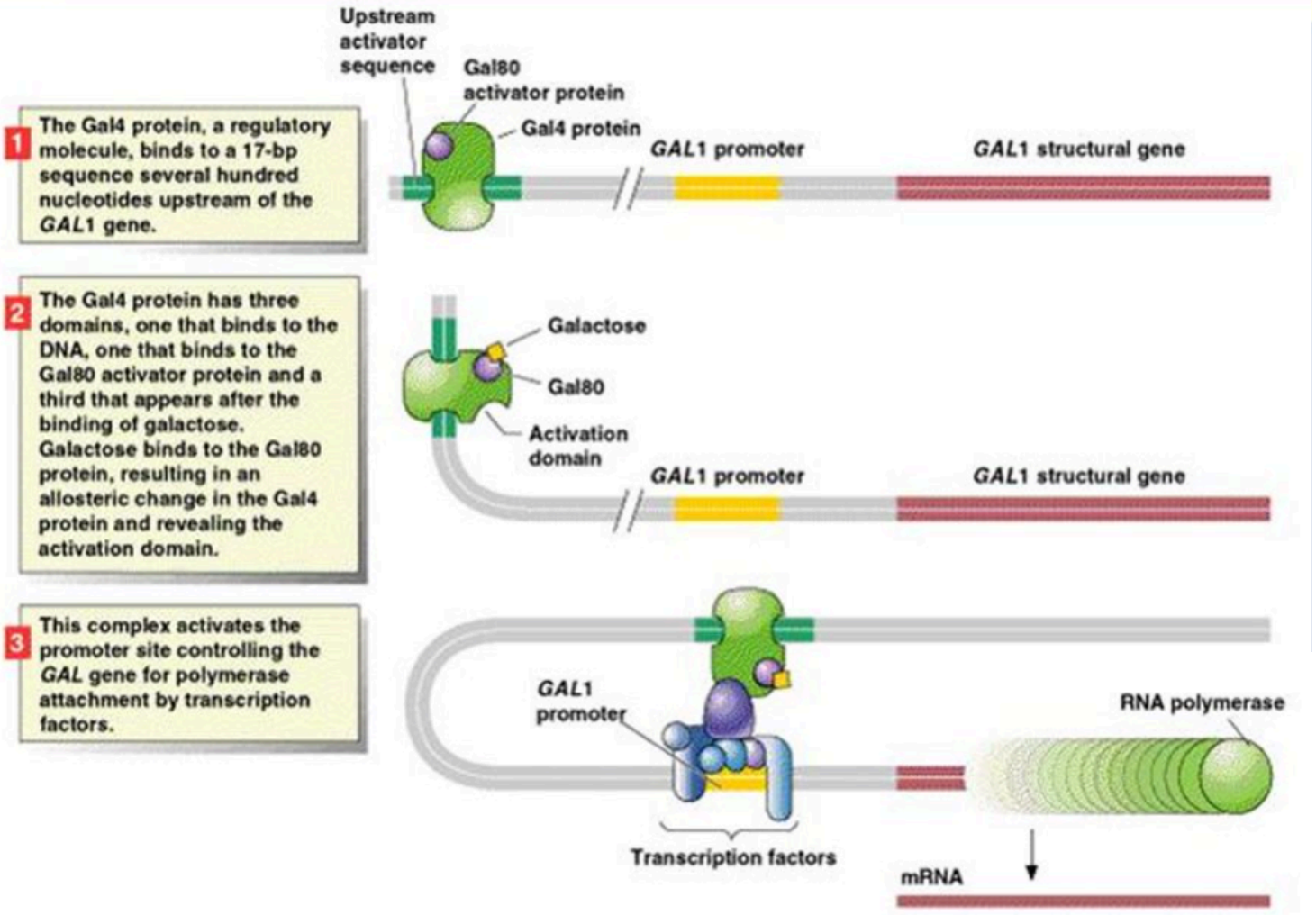


Promoters to express recombinant proteins in yeast cells

Table 7.1 Promoters for *S. cerevisiae* expression vectors

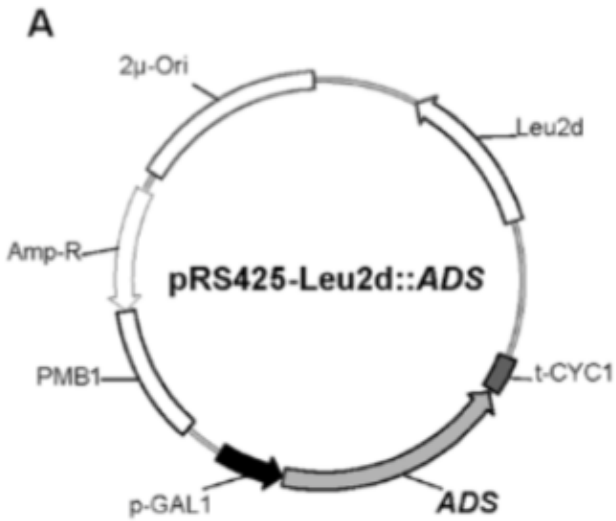
Promoter	Expression conditions	Status
Acid phosphatase (*PH05*)	Phosphate-deficient medium	Inducible
Alcohol dehydrogenase I (*ADHI*)	2–5% Glucose	Constitutive
Alcohol dehydrogenase II (*ADHII*)	0.1–0.2% Glucose	Inducible
Cytochrome c_1 (*CYC1*)	Glucose	Repressible
Gal-1-P Glc-1-P uridyltransferase	Galactose	Inducible
Galactokinase (*GAL1*)	Galactose	Inducible
Glyceraldehyde-3-phosphate dehydrogenase (*GAPD, GAPDH*)	2–5% Glucose	Constitutive
Metallothionein (*CUP1*)	0.03–0.1 mM copper	Inducible
Phosphoglycerate kinase (*PGK*)	2–5% Glucose	Constitutive
Triose phosphate isomerase (*TPI*)	2–5% Glucose	Constitutive
UDP galactose epimerase (*GAL10*)	Galactose	Inducible

The GAL1 promoter: an inducible gene expression system



Grow S.cerevisiae in liquid medium without galactose but glucose (glucose blocks activation of GAL1 promoter)

Induce recombinant protein expression by switching energy resouce to galactose



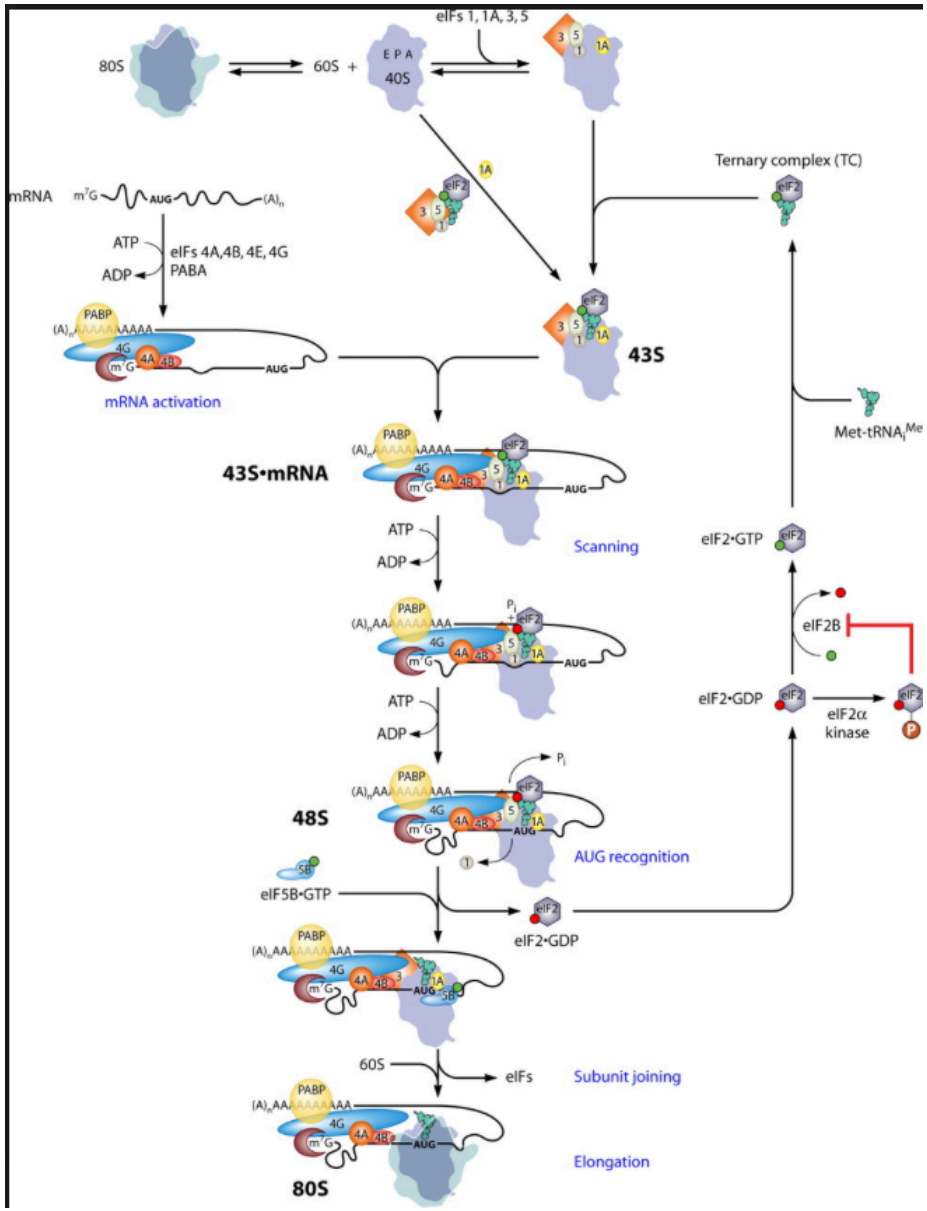
Pre-requesites for recombinant protein expression in S- cerevisiae

1. Cap-dependent translation:

- **Cap structure** and the cap binding proteins are responsible for proper ribosome binding to mRNA and recognition of the correct **initiation codon.**
- The first AUG codon in the 5'end of mRNA functions as the initiation codon.
- **Kozak sequence** may be present around the initiation codon. (ACC**AUG**G)

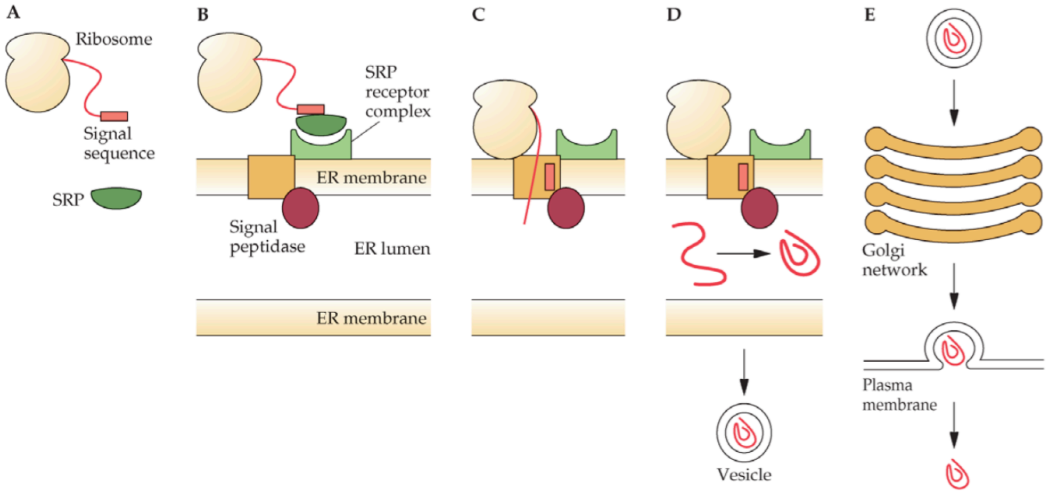
2. Cap-independent translation:

Ribosome binding to mRNA occurs through 'internal ribosome entry site' (IRES) on mRNA.



Secretion of recombinant proteins from yeast cells

Gene expression in the new host	Posttranslational modifications	Protein secretion and intracellular transport	Downstream processing
– Regulation of gene expression – Molecular biology methods	– Translocation to the secretory pathway – Folding and modifications – Glycosylation	– The secretory pathway: ER → Golgi → secret. vesicles → PM – Secretion to the growth medium	– Chemical character of the protein – Protein engineering – Recovery & purification



Signal sequence of protein is recognized by signal recognition particle

- SRP directs secretory protein to the translocation machinery on the ER-membrane
- Translation and translocation process are coupled
- After translocation proteins undergo: – Folding
– Post-translational modifications
– Is transported through secretory pathway
– Released into the medium

Secretion

The yeast secretory pathway is very similar to that in higher eukaryotes.

N-terminal signal sequences for co-translational translocation of screted proteins into the ER are removed by a signal peptidase. Examples of popular signal sequences used for secretion of heterologous proteins -these of Pho5, Suc2 and the a -factor.

Recombinant proteins produced in Yeast

VACCINES
- Hepatitis B virus surface antigen
- Malaria circumsporozoite protein
- HIV-1 envelope protein

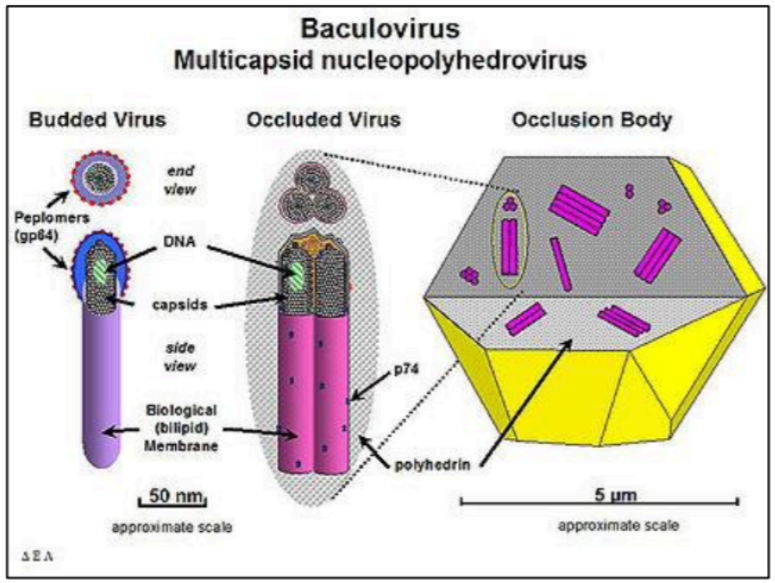
DIAGNOSTICS
- Hepatitis C virus protein
- HIV-1 antigens

HUMAN THERAPEUTIC AGENTS
- Epidermal growth factor
- Insulin
- Insulin-like growth factor
- Platelet-derived growth factor
- Proinsulin
- Fibroblast growth factor
- Granulocyte-macrophage colony-stimulating factor
- α_1 antitrypsin
- Blood coagulation factor XIIIa

1. Vectors to clone genomic fragements/cDNA (libraries
   - Bacteriophages
   - Cosmids
   - Bacterial artificial chromosomes
   - P1 derived artificial chromosomes (PAC)
   - Yeast artificial chromosomes

2. Vectors express and purify recombinant proteins
   - In bacteria
   - In yeast
   - **In fly cells – insect cells**
   - In vertebrate cells

I baculovirus - Autographa californica nucleopolyhedrovirus (AcMNPV)



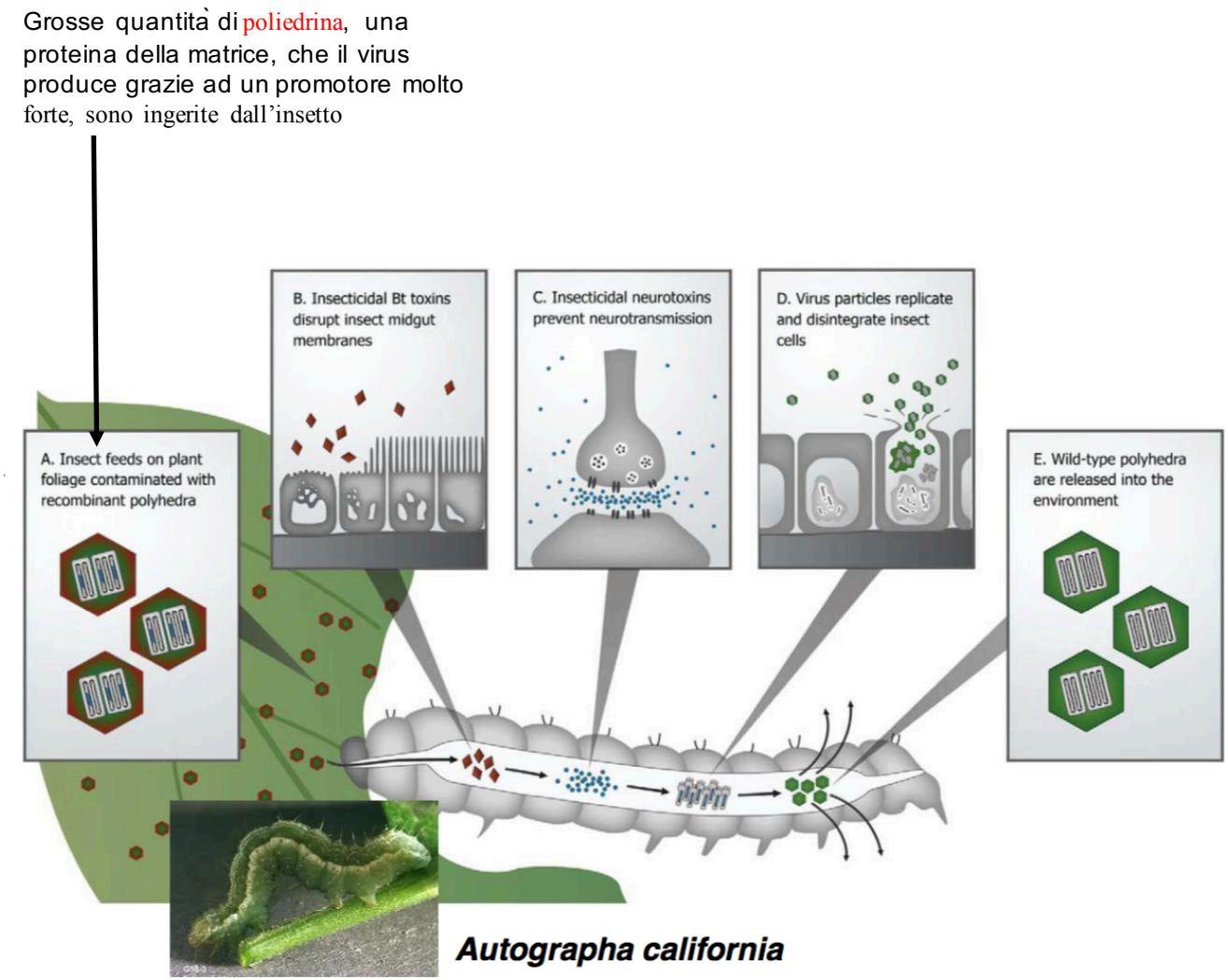
Autographa californica

The baculovirus life cycle involves two distinct forms of virus. Occlusion derived virus (ODV) is present in a protein matrix (polyhedrin or granulin) and is responsible for the primary infection of the host while the **budded virus (BV)** is released from the infected host cells later during the secondary infection

Typically, the initial infection occurs when a susceptible host insect feeds on plants that are contaminated with the occluded form of the virus. The protein matrix dissolves in the alkaline environment of the host midgut (stomach), releasing ODV that then fuse to the columnar epithelial cell membrane of the host intestine and are taken into the cell in endosomes. Nucleocapsids escape from the endosomes and are transported to nucleus. This step is possibly mediated by actin filaments. Viral transcription and replication occur in the cell nucleus and new BV particles are budded out from the basolateral side to spread the infection systemically. During budding, BV acquires a loosely fitting host cell membrane with expressed and displayed viral glycoproteins.
Baculovirus infection can be divided to three distinct phases:
Early (0–6 h post-infection),
Late (6–24 h p.i.)
Very late phase (18–24 to 72 h p.i.)
While BV is produced in the late phase, the ODV form is produced in the very late phase acquiring the envelope from host cell nucleus and embedded in the matrix of occlusion body protein. These occlusion bodies are released when cells lyse to further spread baculovirus infection to next host. The extensive lysis of cells frequently causes the host insect to literally disintegrate, thus the reason for the historic name "wilting disease." The complete ODV-polyhedrin particles are resistant to heat and light inactivation, whereas the naked BV virion is more sensitive to environment

I baculovirus - Autographa californica nucleopolyhedrovirus (AcMNPV)

Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) has a double-stranded circular DNA genome of approximately 130 kb that contains more than 150 open reading frames. The ability of AcMNPV to infect insect cells has led to its use in multiple protein expression systems and as plant insecticides. AcMNPV also infects a variety of mammalian cell types, with the exception of certain hematopoietic cell lines, although its genome does not replicate or integrate into mammalian chromosomes.



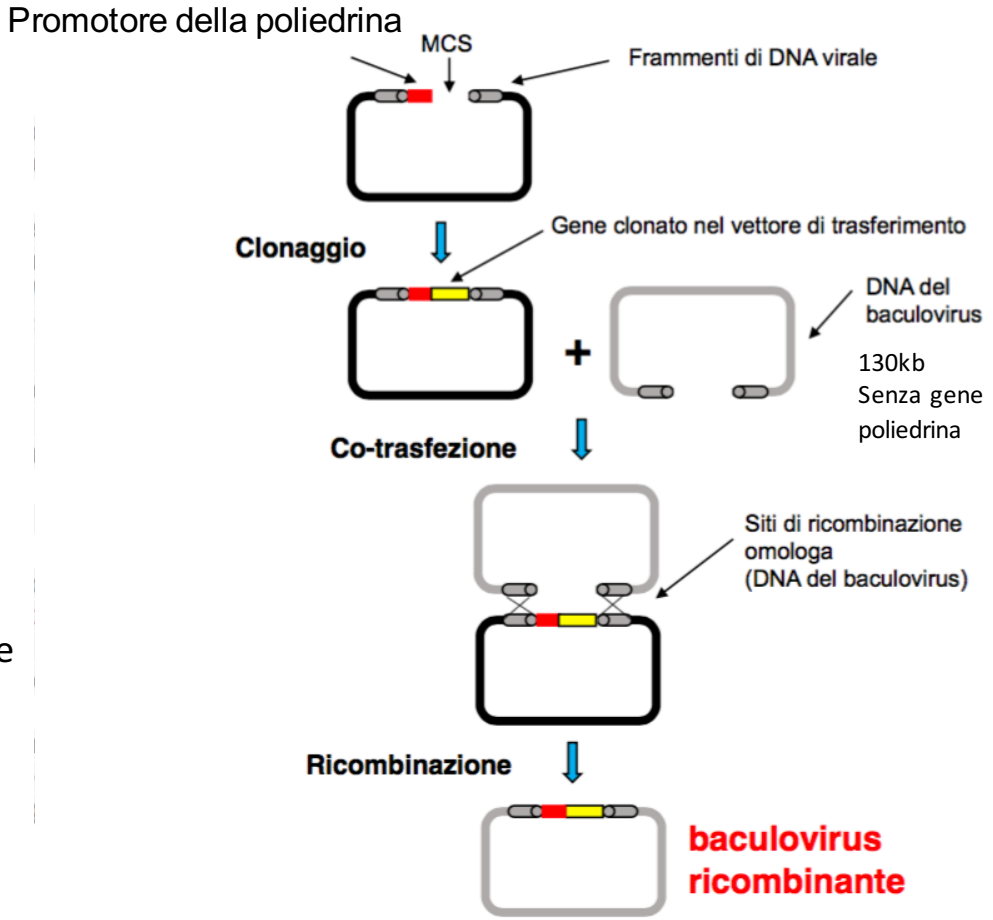
Use of baculovirus for expression of recombinant protein

1. I baculovirus (AcMNPV) usati per infettare le cellule d'insetto: Il promotore della poliedrina è usato per l'espressione del gene esogeno

3. DNA virale è troppo grande (130kb): il gene da esprimere deve essere inserito nel gene della poliedrina usando un vettore di trasferimento più piccolo (contenente anche un ori per la sua manipolazione in E. coli)

4. Ricombinazione del vettore di trasferimento col DNA virale.

5. Presenza di un gene marcatore, come la β-galattosidasi nel vettore di trasferimento per selezionare i ricombinanti (placche blu), sostituito spesso con la GFP (Green Fluorescent Protein), che consente di identificare più facilmente i ricombinanti illuminandoli con una lunghezza d'onda appropriata (emissione 509 nm)



Commonly used cell lines are sf9 & sf21 derived from the pupal ovarian tissue of the fall army worm spodoptera frugiperda and high five derived from the ovarian cells of the cabbage looper

Use of baculovirus for expression of recombinant protein

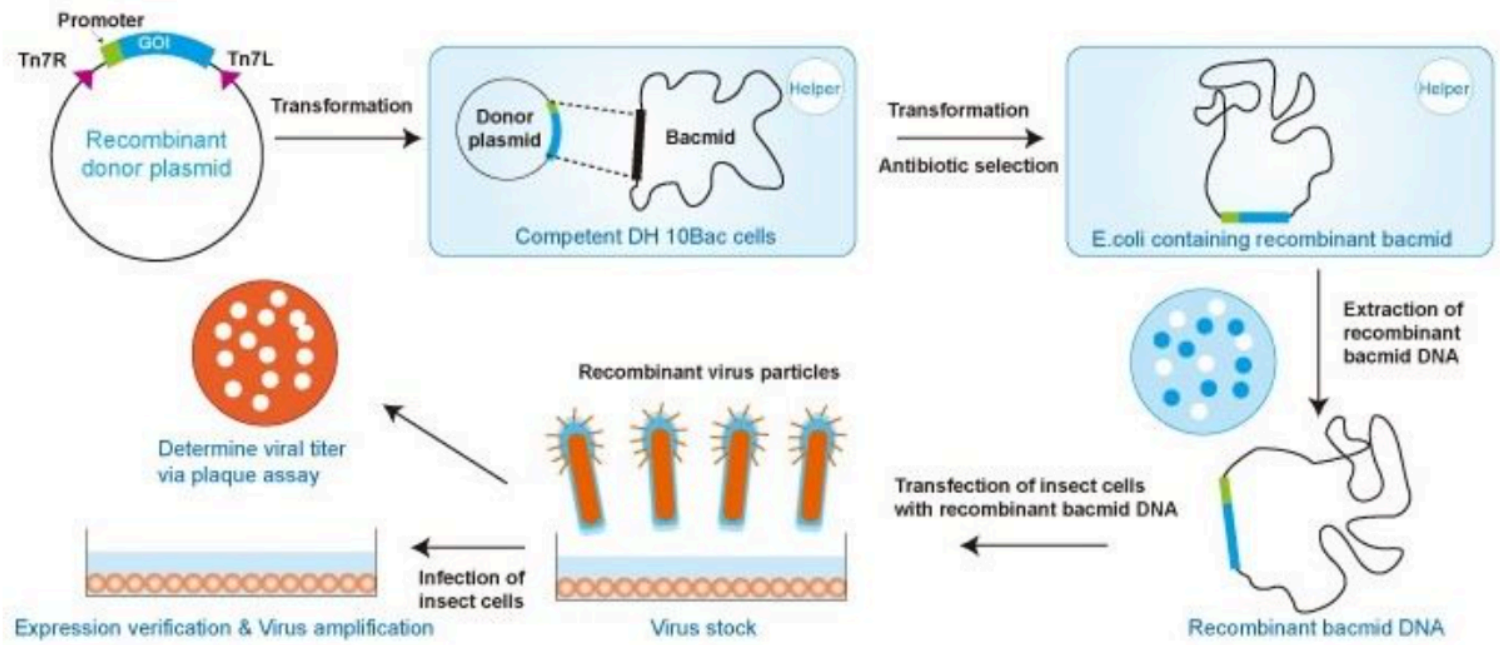


Figure 1. Baculovirus-insect cell expression system

6. I virus ricombinanti non produrranno poliedrina, pero producono il capside normale
7. Col virus ricombinante viene effettuata l'infezione su larga scala utillizzando cellule sf9
8. Alti livelli di proteina prima della lisi delle cellule dell'ospite: la proteina eterologa (prodotte all'interno della cellula, oppure secrete nel mezzo di coltura: proteine di fusione, **His6, GST**, Proteina legante il maltosio, epitopi) viene raccolta dopo 4-5 giorni dall'infezione
9. Coltura dell'ospite più costosa rispetto al lievito, ma possibile l'inserimento delle modificazioni post-traduzionali e la co-espressione (nel baculovirus) di geni che codificano per enzimi di glicosilazione necessari per la corretta modificazione della proteina

Use of baculovirus for expression of recombinant protein

PROs:

- The polyhedrin gene is **not required** for the continuous production of infectious virus in insect cell culture. Its sequence is replaced with that of the heterologous gene.
- The polyhedrin gene promoter is very **strong.** This determines a very high level of production of recombinant protein.
- Very late expression allows for the production of very toxic proteins.
- **This system is capable of post-translational modifications**

CONs:

- Expensive.
- Glycosylation in insect cells is different (insect cells unable to produce complex N-linked side chains with penultimate galactose and terminal sialic acid) from that in vertebrate cells, therefore, a problem for therapeutic proteins.
- A large fraction of the RP can be poorly processed and accumulates as aggregates.
- Discontinuous expression: baculovirus infection of insect cells kills the host and hence the need to reinfect fresh cultures for each round of protein synthesis.
- Inefficient for production on a commercial scale

Adenosina deaminasi	Emagglutinina del virus influenzale	Proteina dell'involucro di HIV-1
Anticorpi monoclonali del topo	Eritropoietina	Proteina precursore dell'amiloide β
Antigene del capside del rotavirus della scimmia	Fosfatasi alcalina umana	Proteina trasportatrice multifarmaco
Antigene del carbonchio	Glicoproteina 50 del virus della pseudorabbia	Proteina del capside di HSV
Antigene del virus sinciziale respiratorio	Glicoproteina del virus della rabbia	Proteine del poliovirus
Antigene tipo 1 del virus dengue	Interferon α	Proteine della malaria
Attivatore tissutale del plasminogeno	Interferon β	Recettori accoppiati con la proteina G
Antigene di neutralizzazione del virus della malattia della lingua blu	Lipasi pancreatica umana	Regolatore della conduttanza Transmembrana della fibrosi cistica
	Proteina del Lassavirus	Rodopsina bovina

1. Vectors to clone genomic fragements/cDNA (libraries
    - Bacteriophages
    - Cosmids
    - Bacterial artificial chromosomes
    - P1 derived artificial chromosomes (PAC)
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2. Vectors express and purify recombinant proteins
    - In bacteria
    - In yeast
    - In fly cells – insect cells
    - **In vertebrate cells**

Introduction of vectors into mammalian cells

Expression systems

Transient

High expression over limited time (2-3 days)
Plasmid is not segregated by mechanism and will get lost in a few days

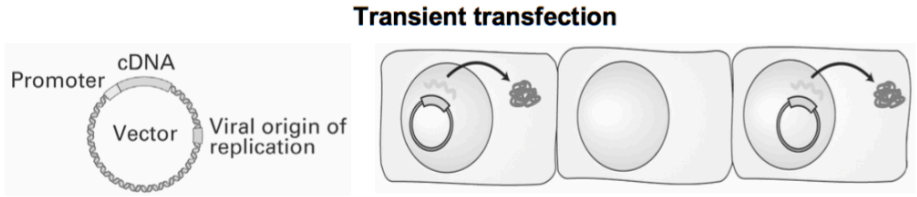
- → **Electroporation**
- → **Lipofection**
- → **Calciumphosphate**

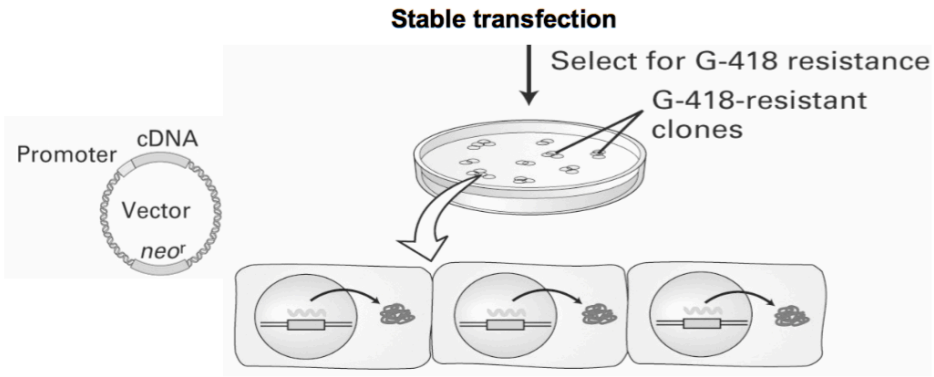
Stable

Continuous expression; requires stable integration of vector into genomic DNA; vector must contain a selectable marker

- → **Electroporation**
- → **Lipofection**
- → **Calciumphosphate**
- → **Viral transduction (stable and episomal)**

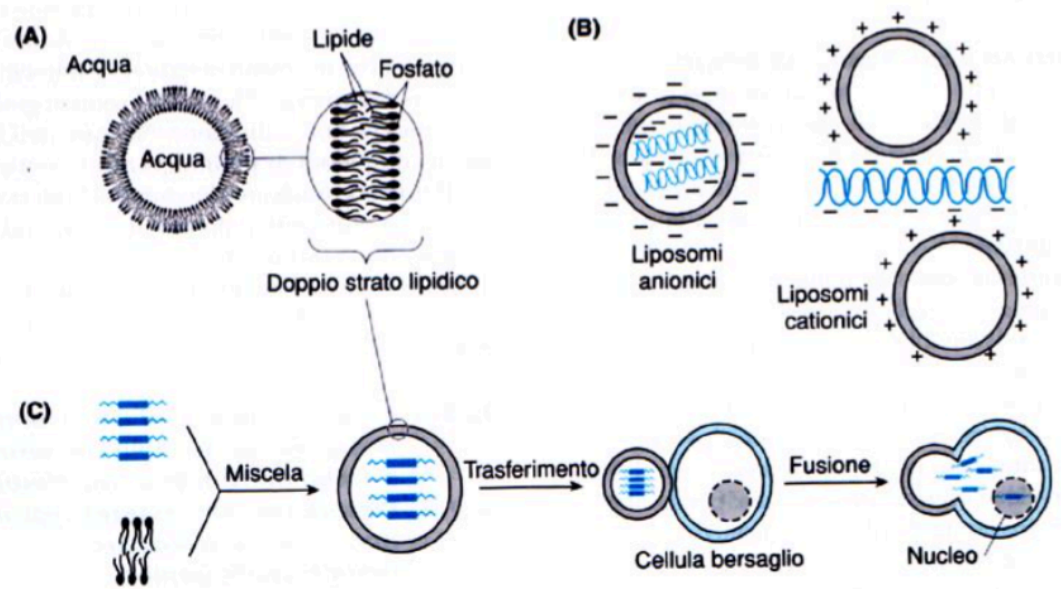
After introduction of vector→apply selection for genome integrants
Alternative SV40 system allows episomal maintenance of plasmid

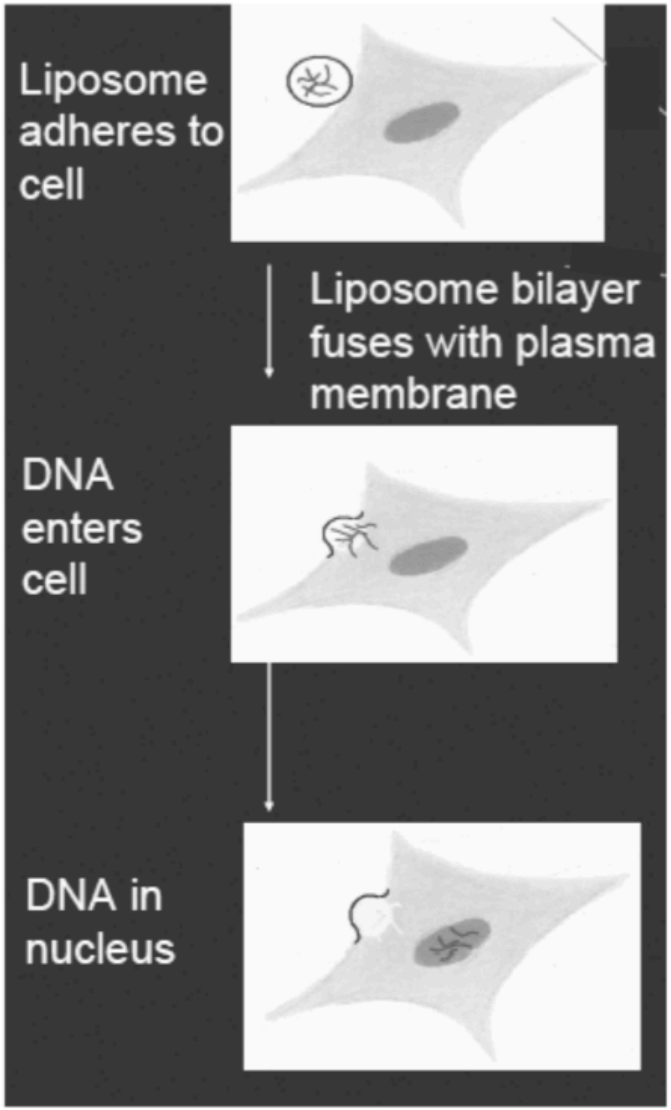




- **INCUBAZIONE CON DNA CO-PRECIPITATO CON FOSFATO DI CALCIO O DIETILAMINOETIL-DESTRANO (DEA)**
- **ELETTROPORAZIONE**
- **LIPOSOMI**
- **VEICOLAZIONE DI DNA DA PARTE DI UN VIRUS (Retrovirus, Lentivirus)**

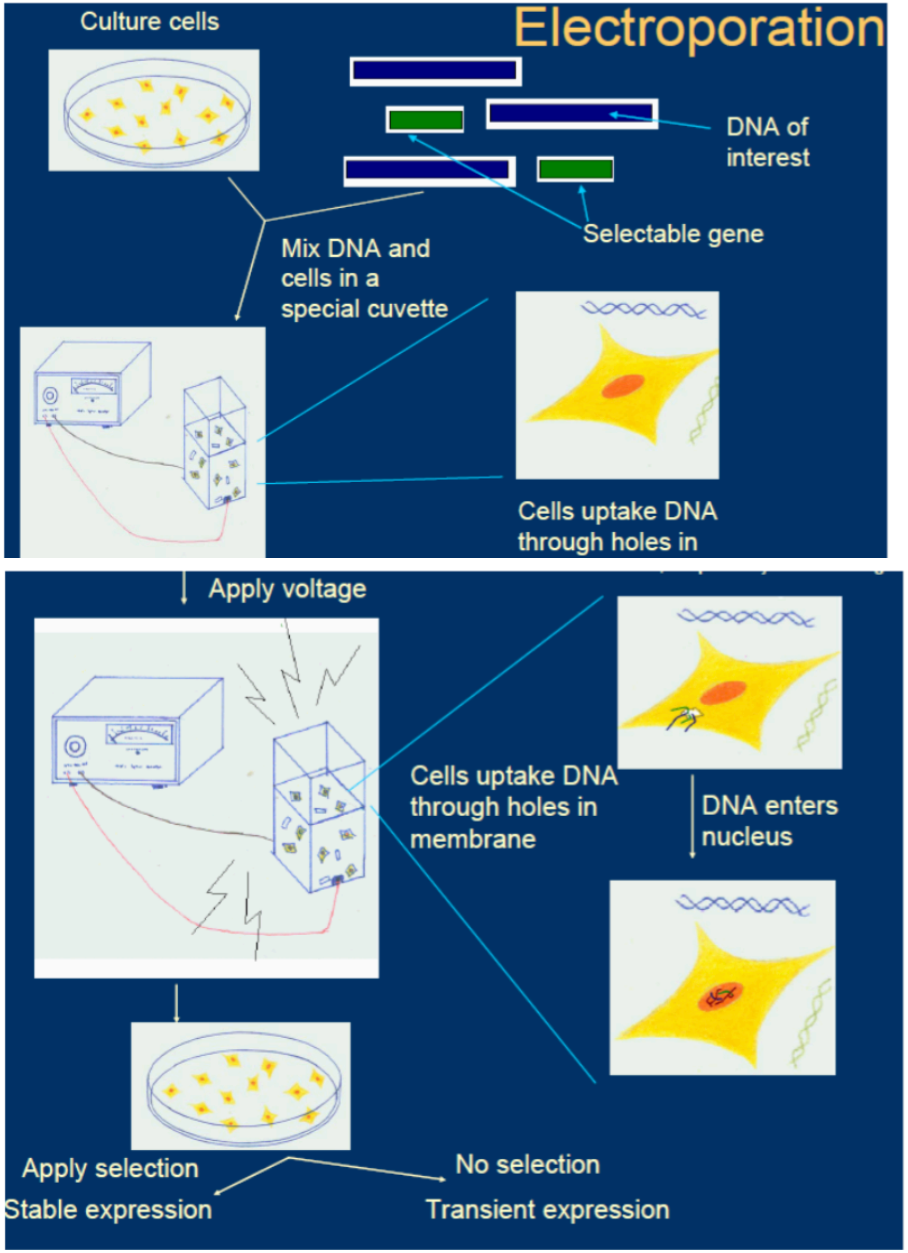
LIPOFECTION OF MAMMALIAN CELLS





Lipids form sphers and integrate DNA
Lipids enter cell and fuse with nuclear membrane
DNA (vector) ready for trasncription

ELECTORPORATION OF MAMMALIAN CELLS



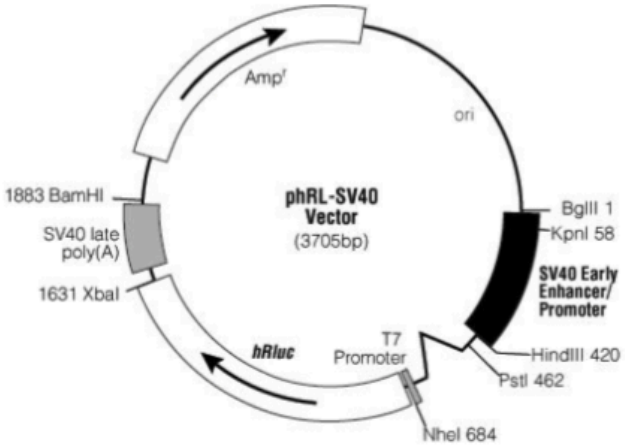
Vettori d'espressione di proteine in cellule di mammifero

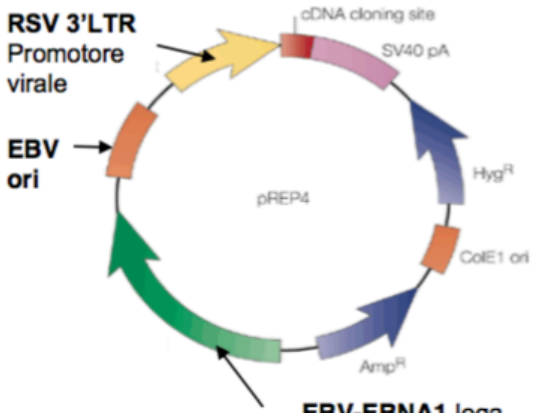
La produzione di proteine animali in ospiti artificiali, come batteri, lieviti, insetti presenta sempre il problema potenziale del non corretto processamento dell'mRNA e la produzione di una proteina inattiva o parzialmente attiva

La resa della proteina ricombinante può essere migliorata inserendo un introne del gene clonato, perché il cDNA può contenere siti di splicing criptici, mentre la presenza dell'introne **rende meno probabile lo splicing alternativo sui siti criptici**

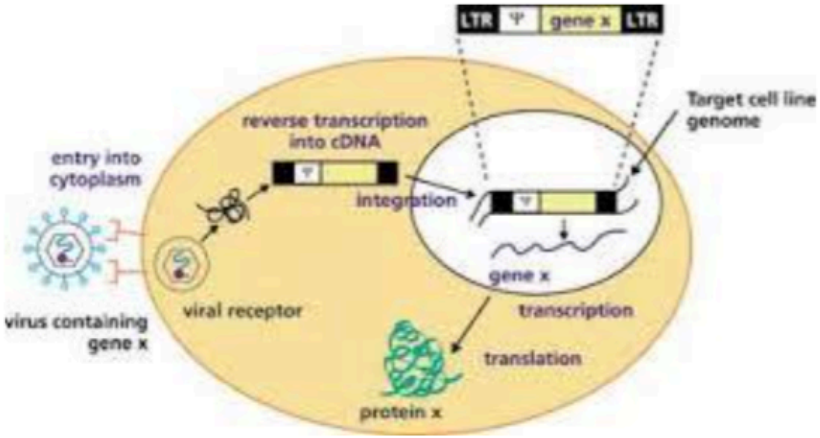
1. **Nelle cellule di mammifero un plasmide non si replica autonomamente:** è possibile con **vettori aventi l'origine di replicazione del virus SV40** (simian virus 40, causante negli animali immunodeficienza cronica, lesioni a livello renale e cerebrale) che si possono replicare in maniera episomale nelle cellule **COS** (linea cellulare di fibroblasti renali di scimmia). Avrà vita limitata, transiente, si diluirà man mano che le cellule si dividono, ma è tecnologicamente più facile (più rapida la produzione del clone)
2. **Cloni stabili in forma extracromosomica:** contengono **l'origine di replicazione del virus Eptein-Barr (EBV)**
3. Inserimento del DNA esogeno in un cromosoma delle cellule di mammifero mediante **Retrovirus. Integrazione nel DNA nucleare, si duplica quando la cellula si divide, integrazione stabile**

Per esprimere il gene clonato sono necessari **promotori forti:** derivati da virus animali o da geni di mammifero altamente espressi **SV40**, citomegalovirus (**CMV**), herpes simplex virus (**HSV**); oppure **actina β, timidina chinasi, ormone della crescita bovino**





EBV-EBNA1 lega il DNA virare e lo mantiene come DNA episomico circolare



COS cells are fibroblast-like cell lines derived from monkey kidney tissue. COS cells are obtained by immortalizing CV-1 cells with a version of the SV40 virus that can produce large T antigen but has a defect in virus genome replication.
When an expression construct with an SV40 promoter is introduced into COS cells, the vector can be replicated substantially by the large T antigen.

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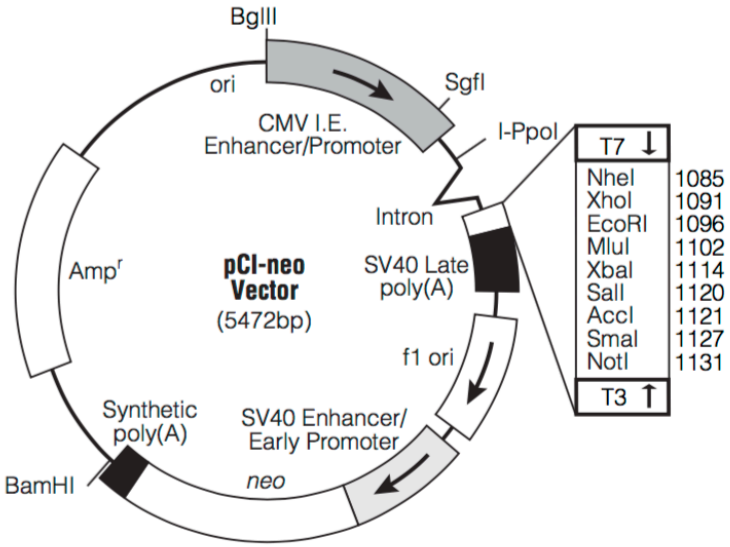
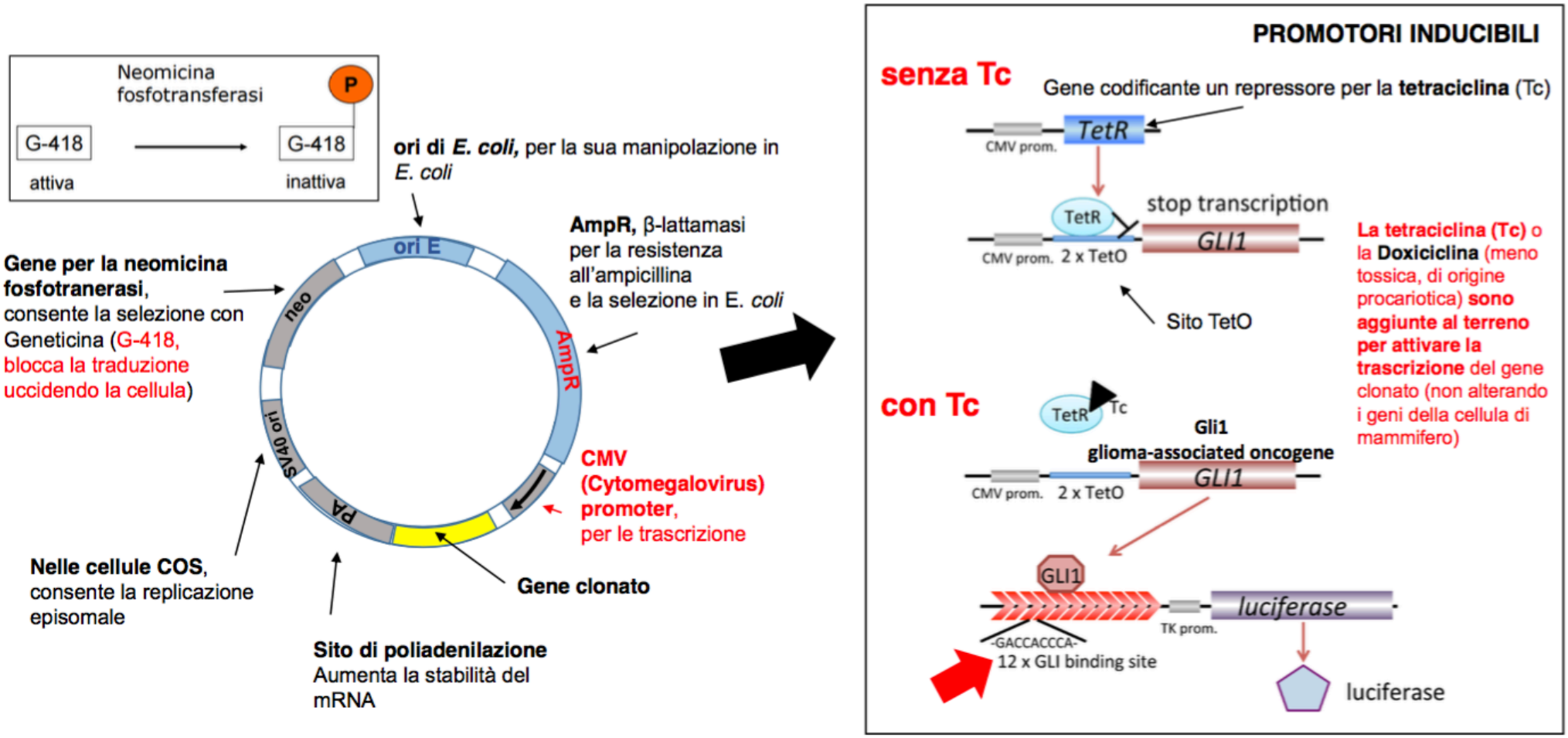


COS cells are fibroblast-like cell lines derived from monkey kidney tissue. COS cells are obtained by immortalizing CV-1 cells with a version of the SV40 virus that can produce large T antigen but has a defect in genomic replication.
When an expression construct with an SV40 promoter is introduced into COS cells, the vector can be replicated substantially by the large T antigen. These COS cells are genetically modified to produce the T antigen from their own genome.

pCI-neo Sequence Reference Points:

CMV immediate-early enhancer/promoter region	1–750
Chimeric intron	890–1022
T7-EEV sequencing primer binding region	1053–1074
T7 RNA polymerase promoter (–17 to +2)	1067–1085
Multiple cloning region	1085–1137
T3 RNA polymerase promoter (–17 to +3)	1140–1158
SV40 late polyadenylation signal	1167–1388
Phage f1 region	1483–1938
SV40 enhancer and early promoter	2000–2418
SV40 minimum origin of replication	2316–2381
Coding region of neomycin phosphotransferase	2463–3257
Synthetic polyadenylation signal	3321–3369
β-lactamase (Amp^r) coding region	3780–4640

Vettori d'espressione in cellule eucariotiche: *ori* derivante da SV40



-G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells

-Puromycin is an aminonucleoside antibiotic, derived from the Streptomyces alboniger bacterium, that causes premature chain termination during translation taking place in the ribosome.

-Hygromycin is active against both prokaryotic and eukaryotic cells. It acts by inhibiting polypeptide synthesis. It stabilizes the tRNA-ribosomal acceptor site, thereby inhibiting translation.

Vettori d'espressione in cellule eucariotiche: aggiunta di marcatori e segnali

1. **Addizione di sequenze per la localizzazione** (nucleo, citoplasma, mitocondri, reticolo endoplasmatico)

2. **Aggiunta di segnali per la secrezione, come un peptide segnale**

   - riconosciuto dal meccanismo di secrezione, trasportato attraverso la membrana. Utile se la proteina clonata è anch'essa secreta, poco efficace se è citoplasmatica
   - Stabilizza il prodotto del gene clonato proteggendolo dalle proteasi
   - Evita l'accumulo della proteina nel citoplasma e la formazione dei corpi di inclusione insolubili.
   - Facilitano la purificazione
   - Possono essere utilizzate come antigene per produrre anticorpi
   - Consentono lo *screening* di genoteche di cDNA

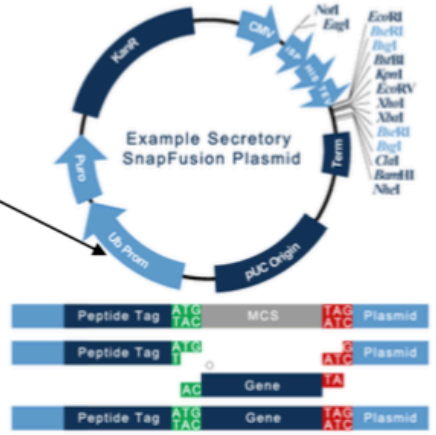
3. **Aggiunta di aminoacidi al N-terminale o C-terminale, come marcatore** (***tag***, utile per la purificazione):

   - **Epitopo**, peptide amminoacidico riconosciuto da un anticorpo monoclonale (purificazione per affinità)
   - **Coda amminoacidica**, es. con residui di istidina

4. **Per eliminare il peptide tag dalla proteina clonata: si inseriscono tra le due sequenze** (peptide e proteina) **siti di taglio per proteasi non batteriche specifiche**

   - Endopeptidasi
   - Trombina
   - Fattore Xa

IPS (*In-plane switching*) is a screen technology for liquid crystal displays
HIS, *TEV*-cleavable His6 fusion tag on its N-terminus
TEV, *Tobacco Etch Virus nuclear-inclusion*-a endopeptidase (protease)



Comparison of expression systems

Characteristics	*E. coli*	Yeast	Insect cells	Mammalian cells
Cell growth	rapid (30 min)	rapid (90 min)	slow (18-24 h)	slow (24 h)
Complexity of growth medium	minimum	minimum	complex	complex
Cost of growth medium	low	low	high	high
Expression level	high	low-high	low-high	low-moderate
Extracellular expression	secretion to periplasm	secretion to medium	secretion to medium	secretion to medium
Posttranslational modifications				
Protein folding	refolding usually required	refolding may be required	proper folding	proper folding
N-linked glycosylation	none	high mannose	simple, no sialic acid	complex
O-linked glycosylation	no	yes	yes	yes
Phosphorylation	no	yes	yes	yes
Acetylation	no	yes	yes	yes
Acylation	no	yes	yes	yes
gamma-Carboxylation	no	no	no	yes