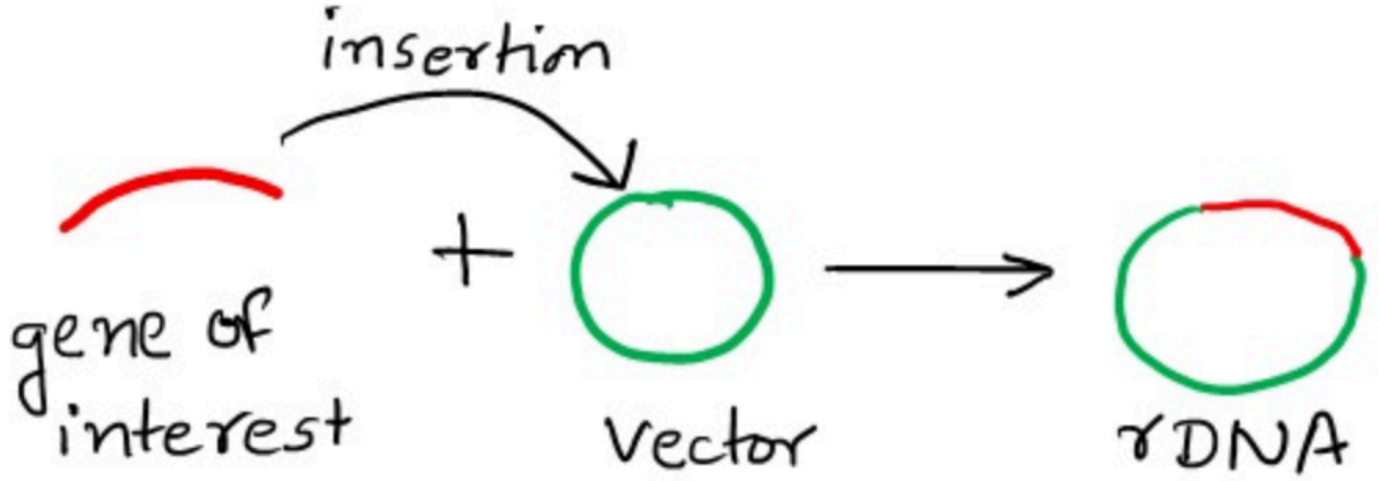
RECOMBIANANT DNA TECHNOLOGY
CLONING OF DNA FRAGMENTS INTO PLASMIDS

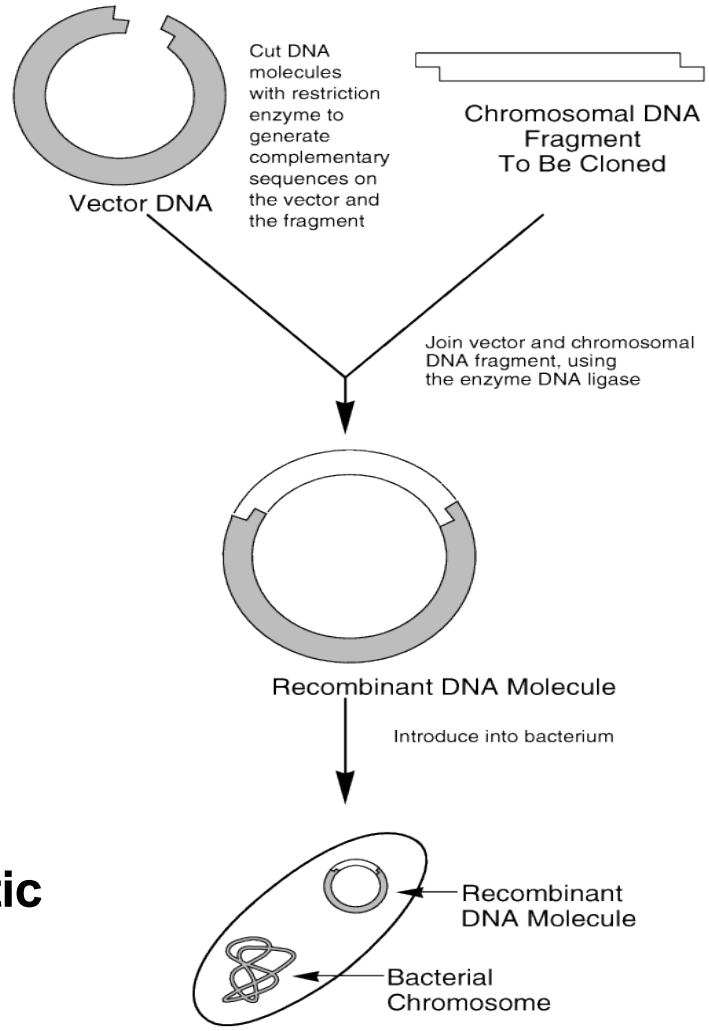


DNA RICOMBINANTE:

DUE MOLECOLE DI DNA VENGONO UNITE IN PROVETTA E FATTE RIPRODURRE IN LABORATORIO

DNA cloning

- **DNA cloning is a technique for reproducing (making copies) DNA**
- **It can be achieved by two different approaches:**
  - ***cell based nucleic acids (genomic DNA, plasmid DNA, cDNA after reverse transcription of RNA***
  - ***amplification of defined sections of DNA by using specific primers and polymerase chain reaction (PCR).***
- **a vector is required to carry the DNA fragment of interest into the host cell.**
- **DNA cloning allows a copy of any specific part of a DNA (or RNA) sequence to be selected among many others and produced in an unlimited amount.**
- **This technique is the first stage of most of the genetic engineering experiments:**
  - ***production of DNA libraries***
  - ***PCR***
  - ***DNA sequencing***



DNA cloning

- **Massive amplification of DNA sequences**
- **Stable propagation of DNA sequences using E.coli proofreading mechanisns during DNA replication (extremely low mutation rate)**
- **A single DNA molecule can be amplified allowing it to be:**
  - ***Studied - Sequenced***
  - ***Manipulated - Mutagenized or Engineered***
  - ***Expressed - Generation of Protein***

Principal enzyme types used to generate recombiant DNA

Nucleasi (per esempio: endonucleasi di restrizione)
DNA polimerasi
Fosfatasi
Ligasi
Enzimi che modificano le estremità

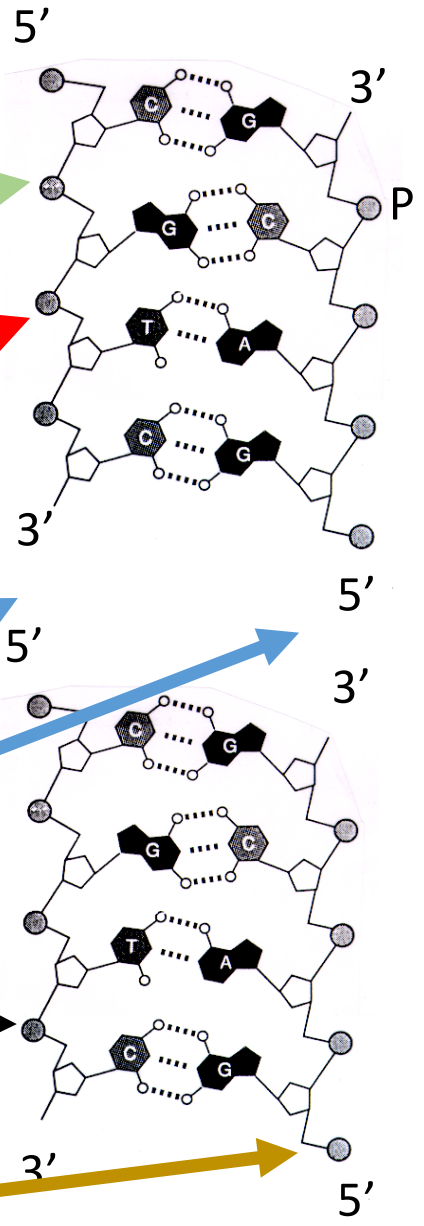
Le esonucleasi rompono
il legame al <u>termine</u> dei filamenti di DNA
(5' → 3')

Le endonucleasi rompono il legame
<u>internamente</u> nel filamento dando
prodotti sia 5' sia 3' fosfati

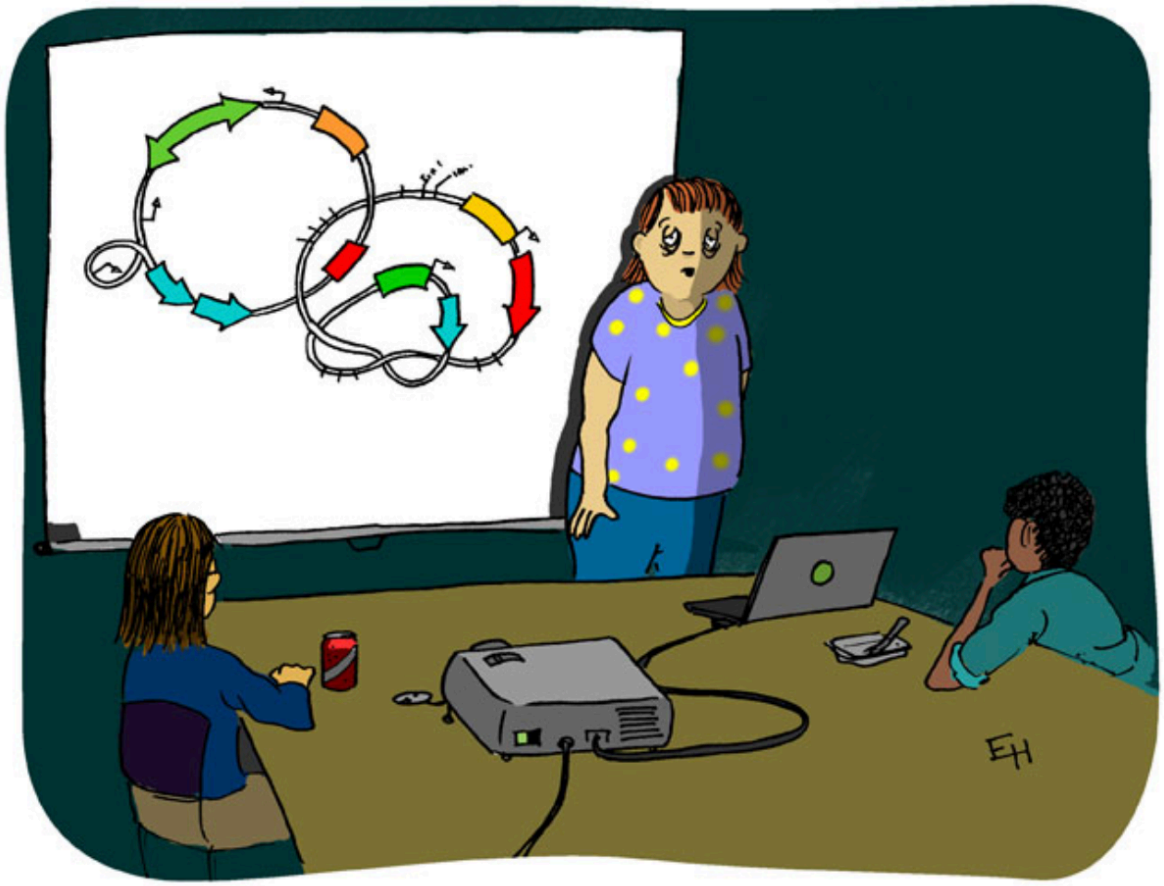
Ligasi: catalizza la formazione del legame tra due
molecole di DNA, spesso accompagnato dall'idrolisi
di una molecola come ATP

Le esonucleasi rompono il legame al
<u>termine</u> dei filament
(3'→ 5')

Phosphatasi: sono una classe di enzimi idrolasi che
catalizzano la rimozione di gruppi fosfato



DNA cloning



I wish I could report otherwise,
but the cloning is not going very well.

….can be simple….

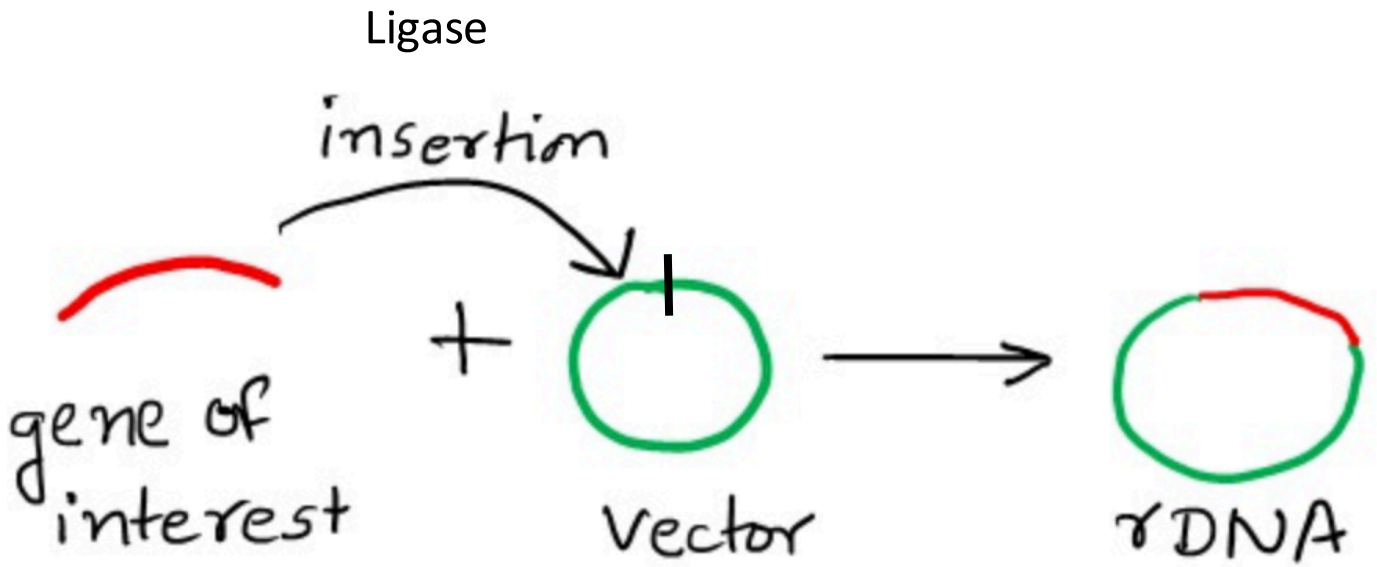
or

…frustrating…

Cloning is a fickle process that can make even the most seasoned bench scientists scream in frustration. By the time you perform a colony PCR and run the gel to check for your insert, you've invested several days in preparing these transformed cells. But then, the unthinkable happens. When you image your gel…the target band is missing.

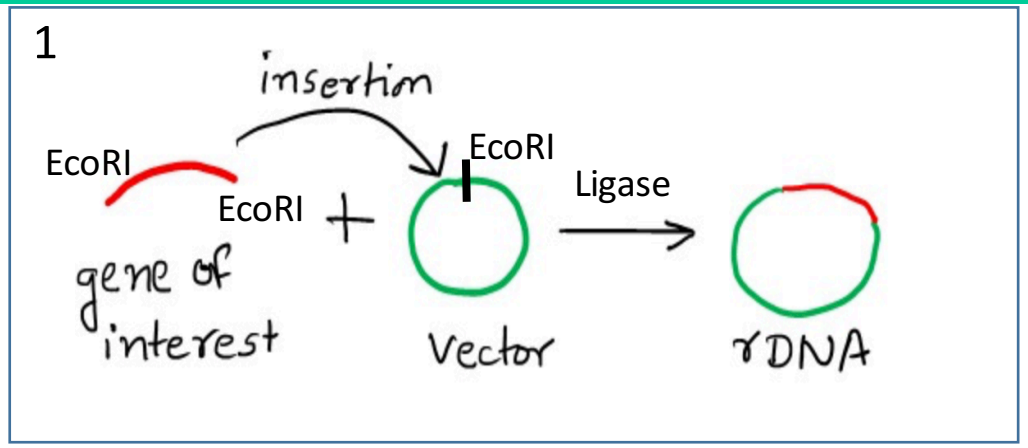
This can trigger what's known as "The 5 Stages of Failed Cloning Grief." As you work through each stage at your own pace, just know that scientists all over the world feel your pain and can empathize with you in this difficult time. Continue reading →

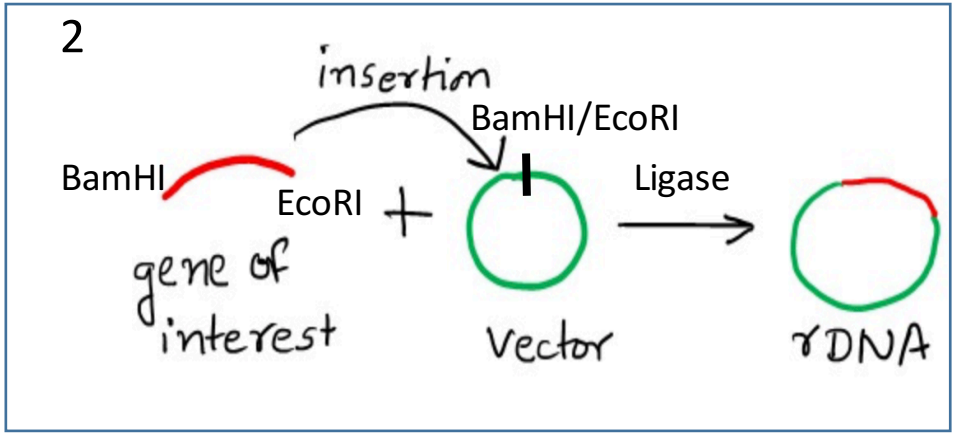
Making recombinant DNA

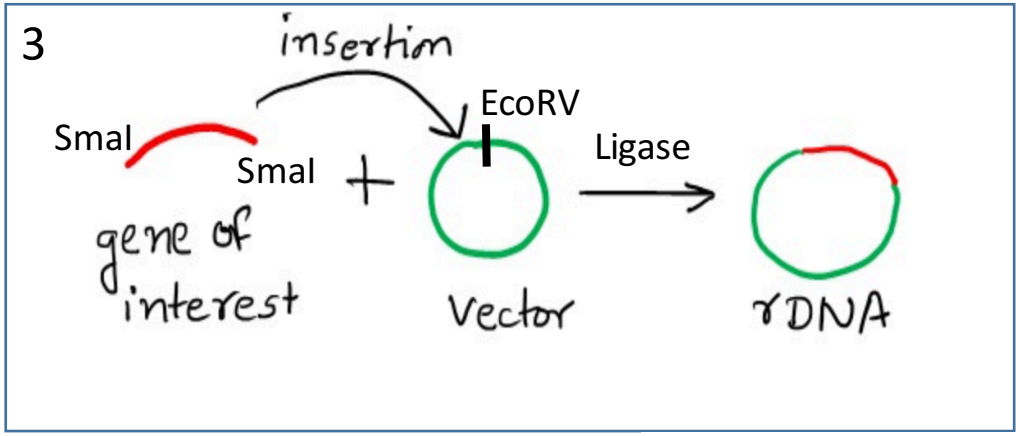


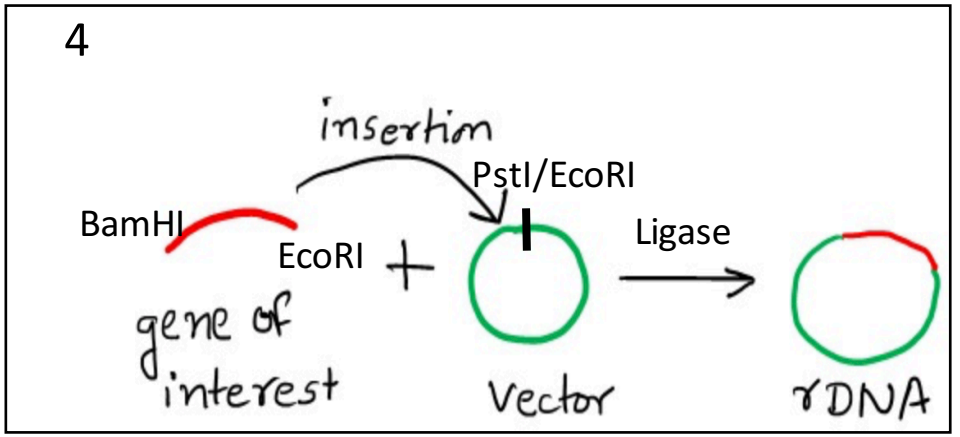
1. **Fragment and Insert are cut with 1 (the same) restriction enzymes**
2. **Fragment and Insert are cut with 2 (the same) restriction enzymes**
3. **Fragment and Insert are cut with blunting restriction enzymes**
4. **Overhangs generated after cutting are modified (filled up; or overhang digested)**

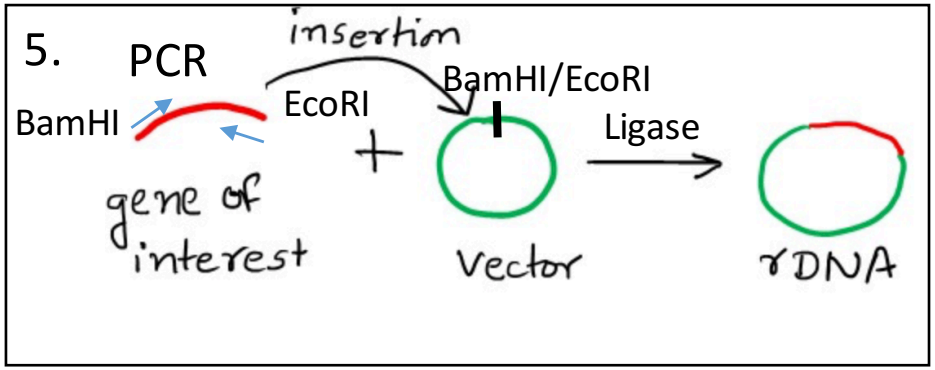
OVERVIEW OVER OTHER CLONING STRATEGIES



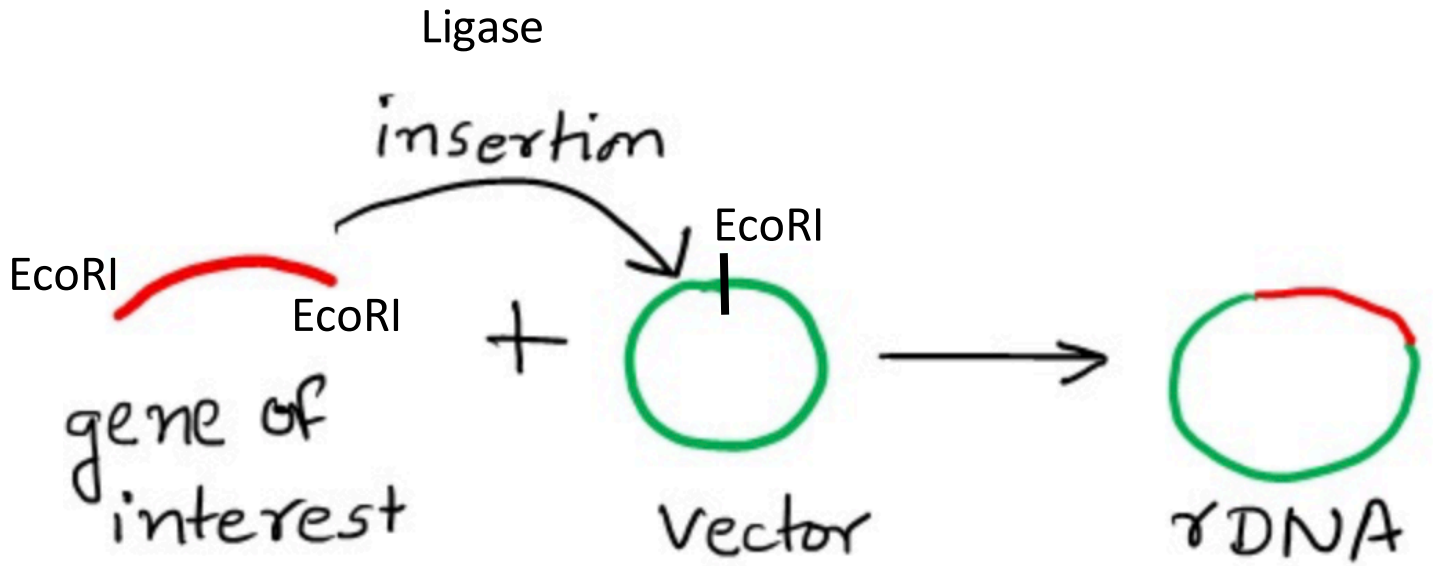




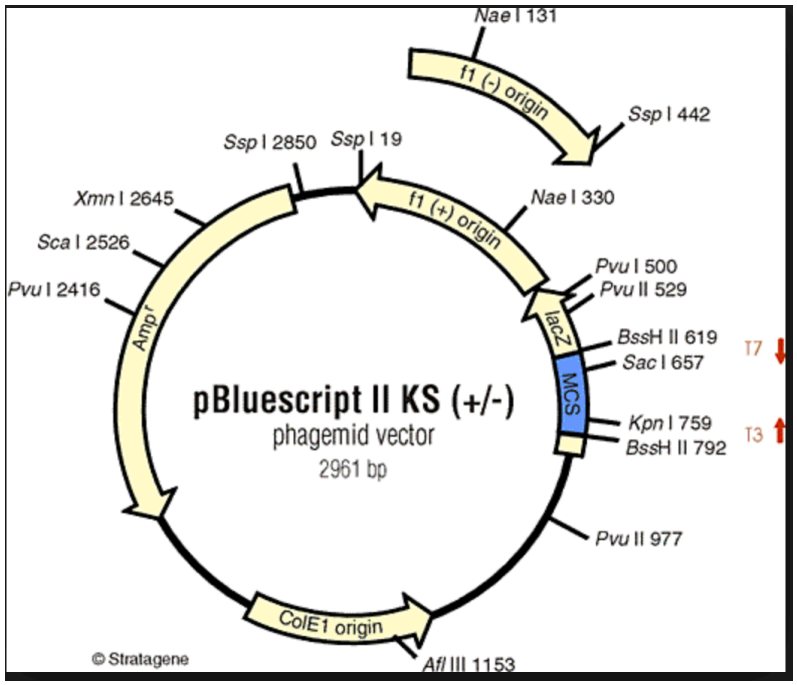




Making recombinant DNA

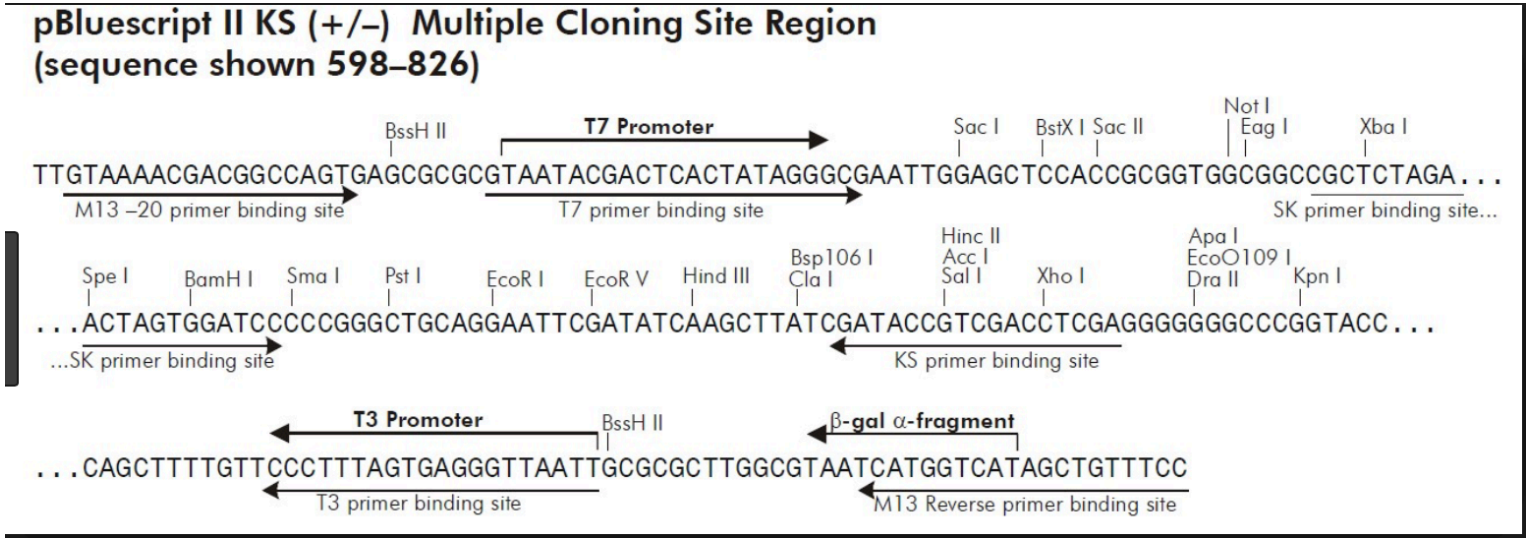


Making recombinant DNA – Plasmid features

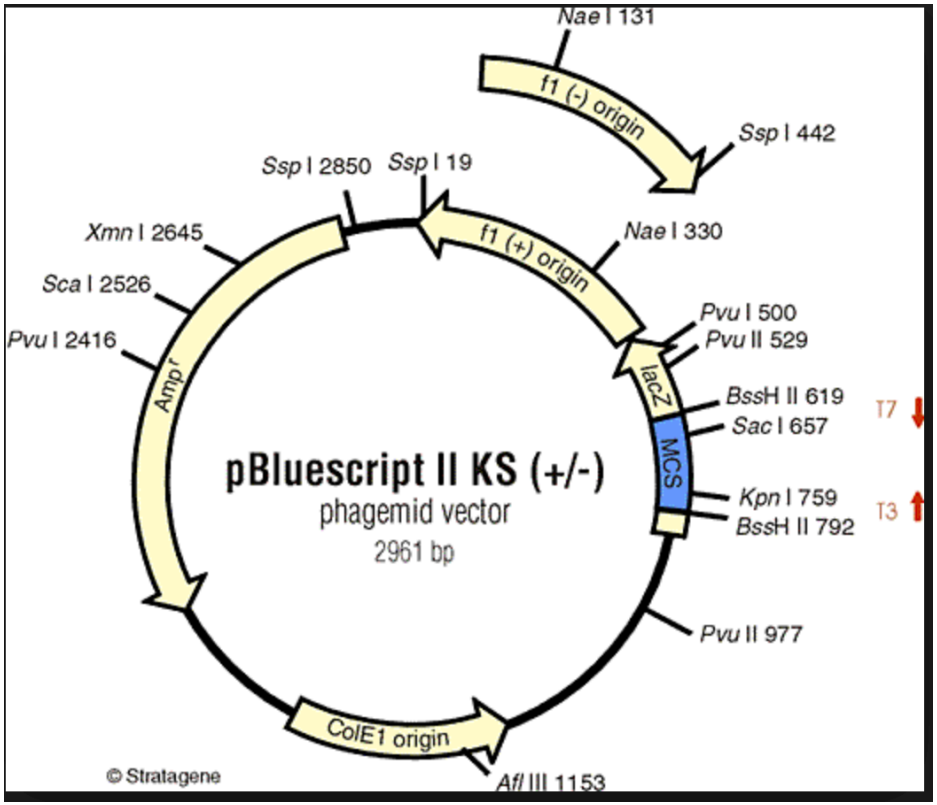


What makes a good plasmid for cloning (generating recombinant DNA)??

- **oriC**, an origin of replication. Gotta start making new plasmid somewhere.
- a **selectable marker**: This is usually an antibiotic resistance of some sort, to give the bacteria with plasmids a selective advantage in specific media.
- a **multiple cloning site (MCS) inside a scorable marker**. The MCS allows us to cut the plasmid, insert new DNA, and re-ligate; the scorable marker allows us to see if the plasmid does indeed have an insert, because the insert will disrupt expression of the marker. This is seen in the use of the lac-Z-alpha fragment in blue/white screening.
- and it should be **small**, with a high **copy number.**



Making recombinant DNA – Plasmid features



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 transcription of RNA polymerase of T3 and T7 phage RNA Polymerase (short sequences often used for seuencing using primers; same for M13)

F1 (-) (+) origin:

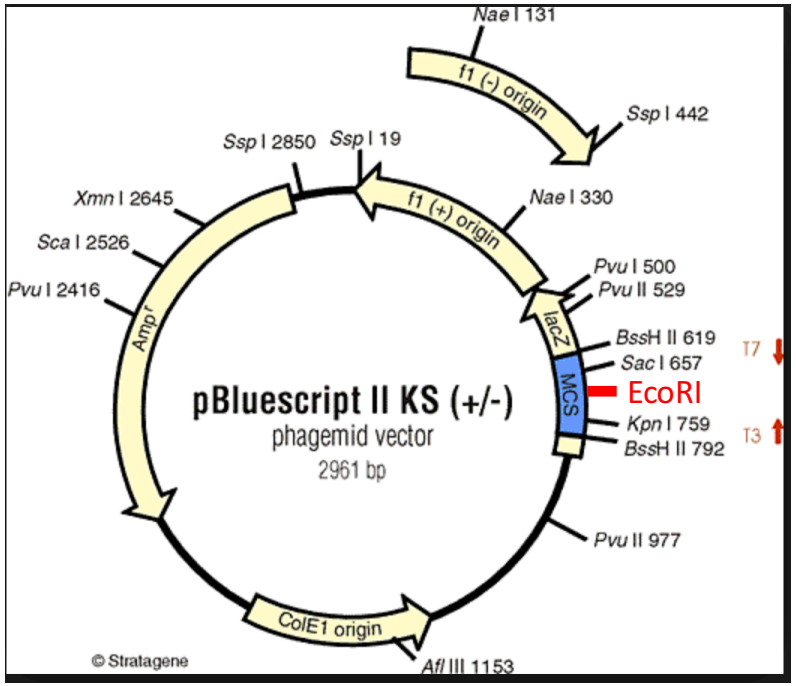
pBluescript II is a phagemid that can be secreted as single-stranded DNA in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous f1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E. 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, fI) 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]

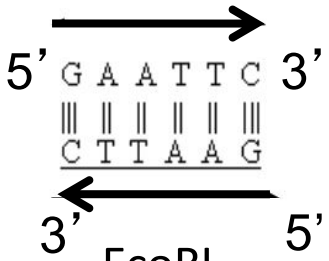
Similarly to a plasmid, a phagemid can be used to clone DNA fragments and be introduced into a bacterial host by a range of techniques, such as transformation and electroporation. However, infection of a bacterial host containing a phagemid with a 'helper' phage, for example VCSM13 or M13K07, provides 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.

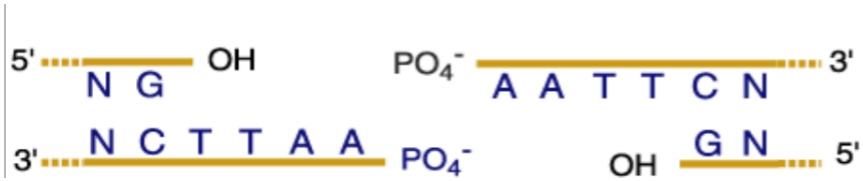
Ampicillin

- 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.

Making recombinant DNA – Plasmid features







Linearized pBS with 5'overhangs

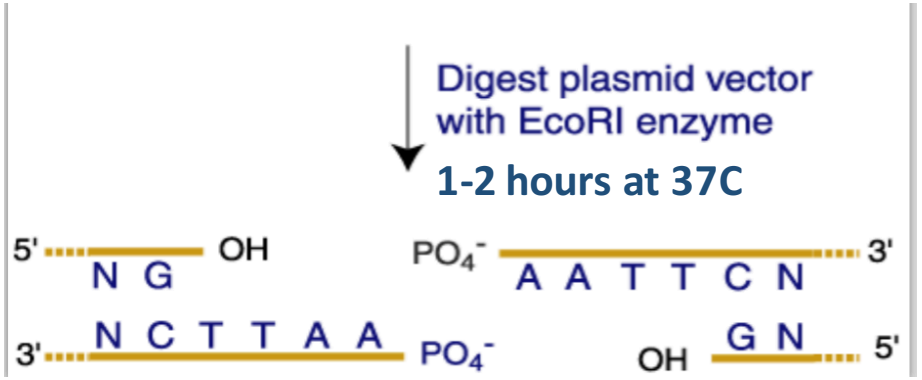
Order of solution addition	Solution	Volume(µl)
1	Nuclease free water	23.5
2	10X Buffer K	5.0
4	100 µg BSA	0.5
5	Plasmid DNA	20.0
3	EcoRI (20U/ µl)	1.0
Total Volume		50.0

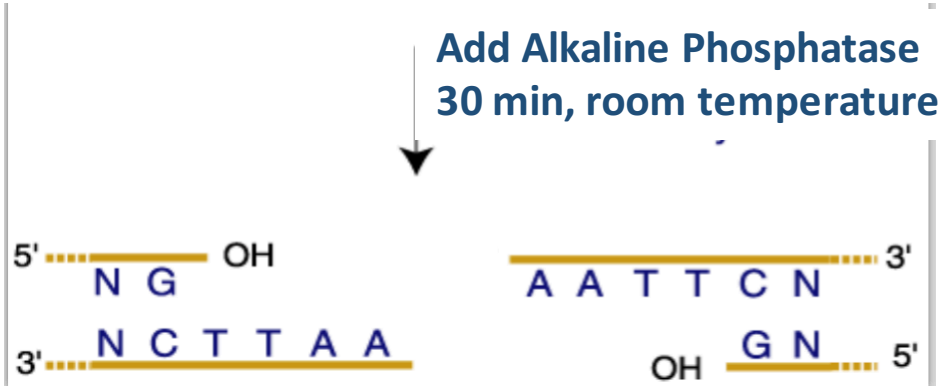
Plasmid DNA: ca. 2-5ug
EcoRI: 20Units/ul:
DEFINITION: 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes
For practical reasons: **5-10 fold overdigest** is recomended: = 1ug DNA + 5-10 units
(you are never sure about "real" activity of enzyme – storage – handling, etc)

One unit of restriction endonuclease activity is defined as the amount of enzyme required to produce a complete digest of 1 µg of substrate DNA (or fragments) in a total reaction volume of 50 µl in 60 minutes under optimal assay conditions as stated for each restriction endonuclease.

Making recombinant DNA – Plasmid features

- Alkaline phosphatase removes 5' phosphate groups from DNA and RNA. It will also remove phosphates from nucleotides and proteins. These enzymes are most active at alkaline pH.
- In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby greatly facilitates ligation of other DNA fragments into the vector

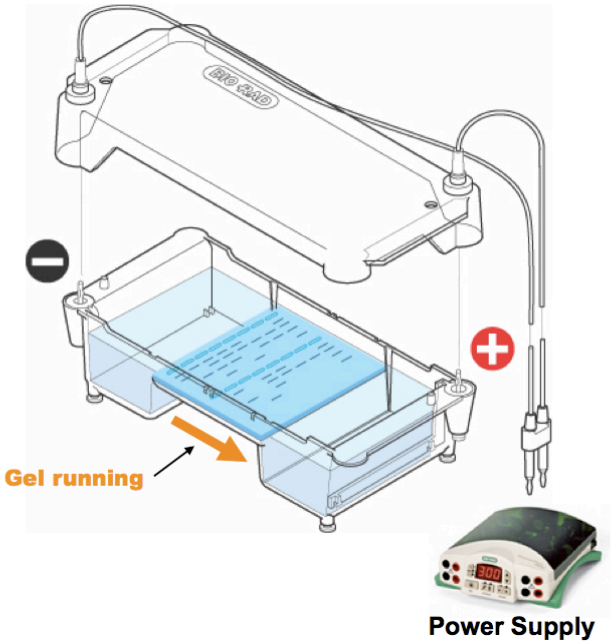




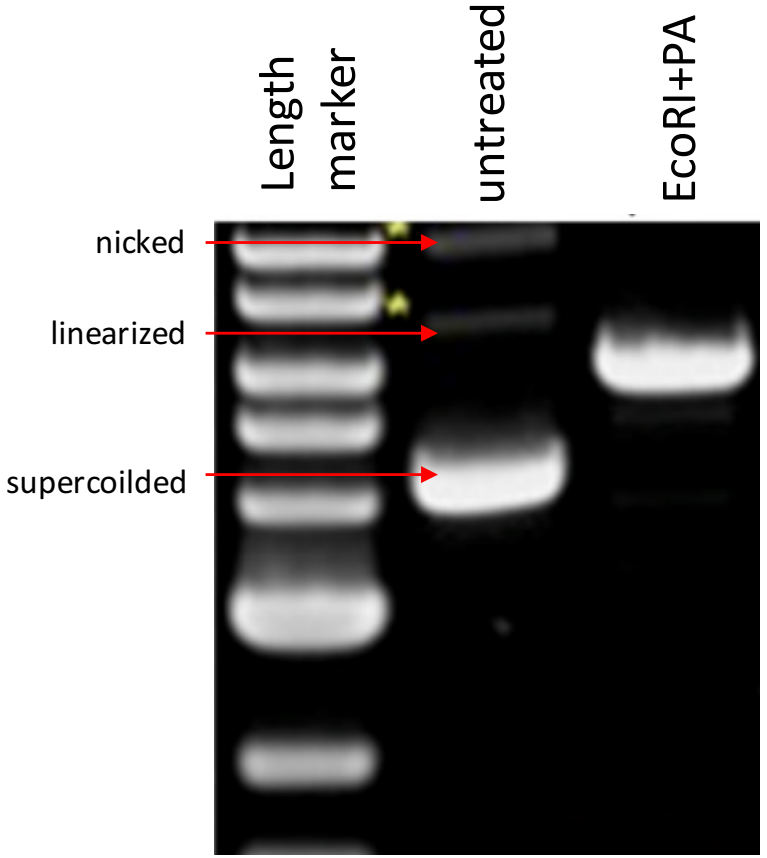
Making recombinant DNA – Plasmid features

Agarose Electrophoresis Running

- **Agarose gel sieves DNA fragments according to size**
  - **Small fragments move farther than large fragments**



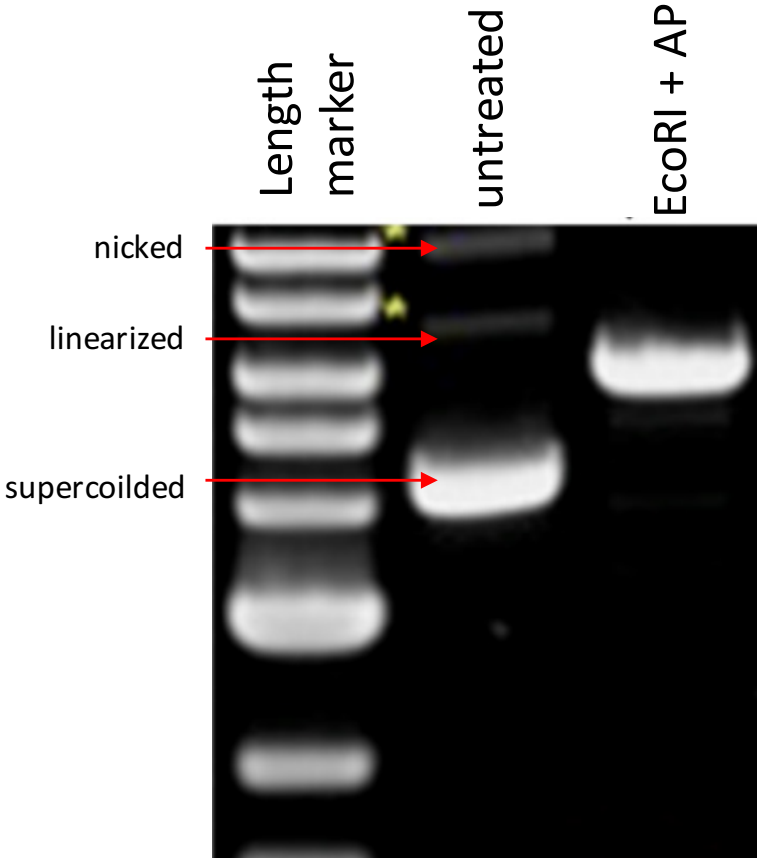
Digest run on agarose gel (gel contains Ethidiumbromide; or samples contains DNA colouring agent)

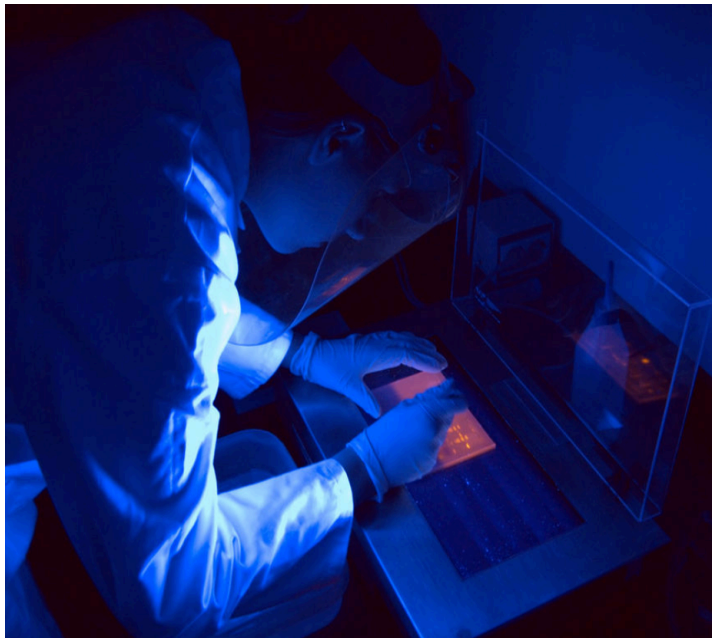


Length marker: mix of DNA fragments with defined length

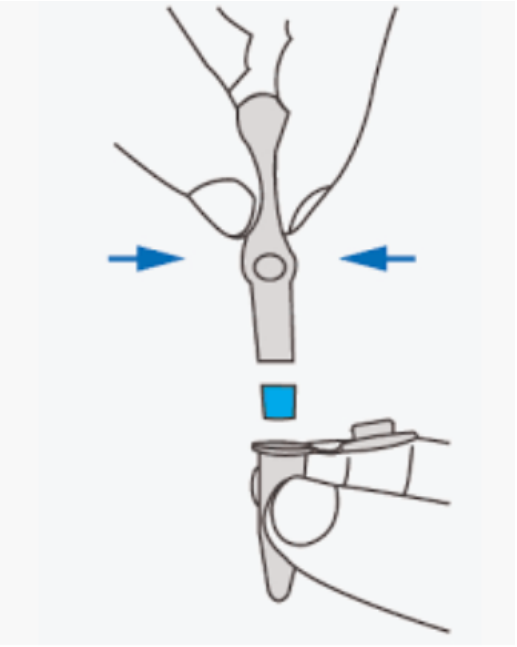
Linearized pBS: 2900 nt

Making recombinant DNA – Plasmid features



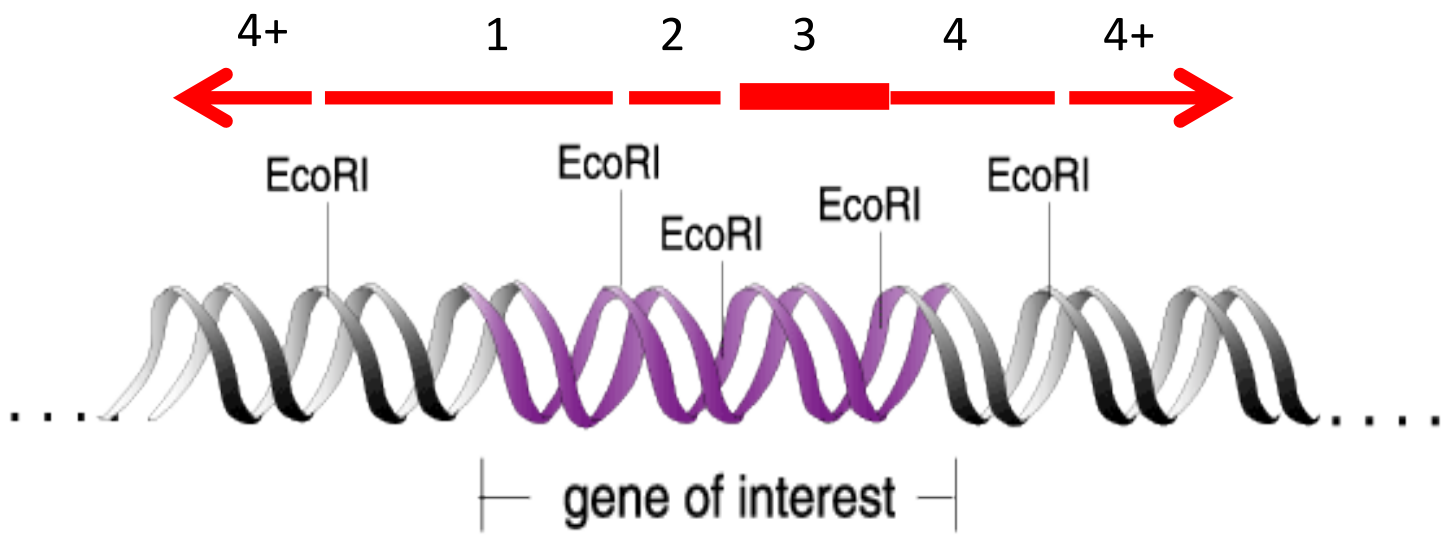


Cut out band from gel using a scalpel blade



Purify DNA and eliminate agarose
Determine concentration of purified plasmid DNA (ca. 50% loss of starting material)

Preparing the insert



Digest DNA sample with EcoRI enzyme

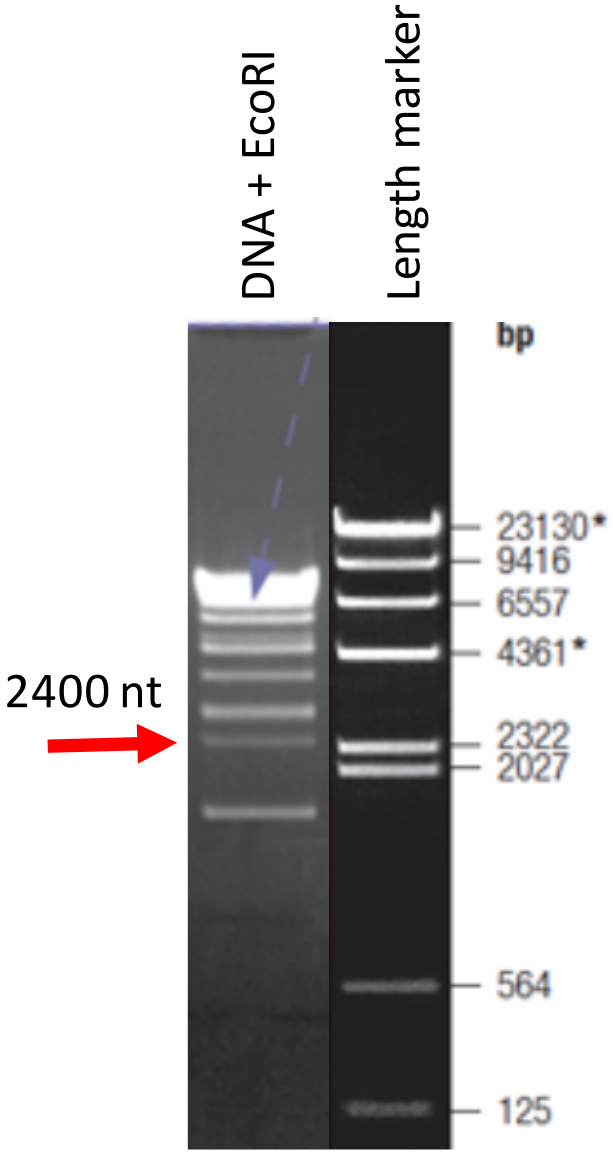
Fragment 3: 2400nt

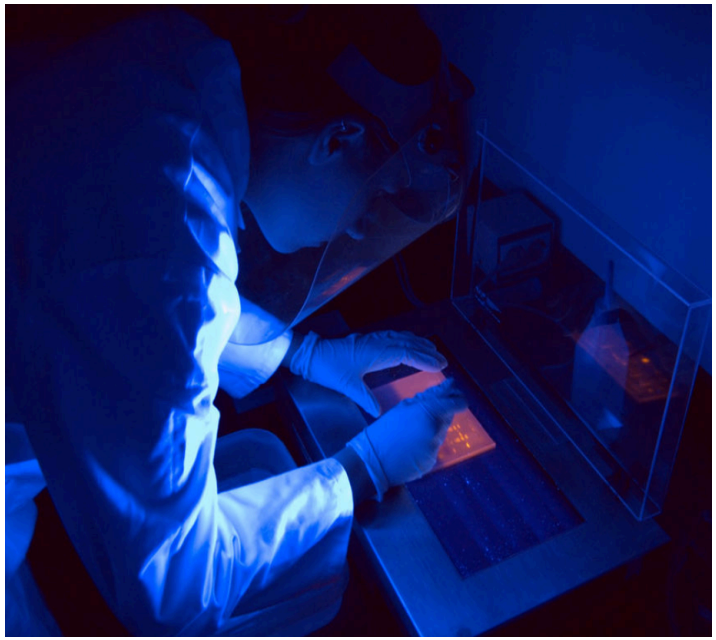
5' ..... N G OH PO_4^- A A T T C N N ..... N N G OH PO_4^- A A T T C N ..... 3'

3' ..... N C T T A A PO_4^- OH G N N ..... N N C T T A A PO_4^- OH G N ..... 5'

Defined size

Preparing the insert





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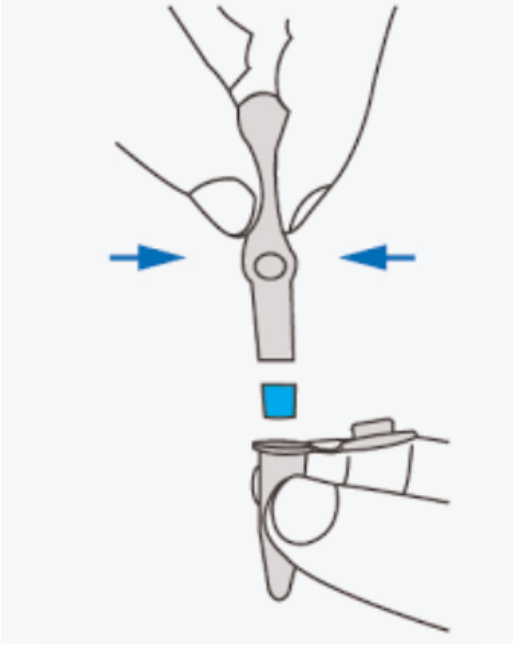
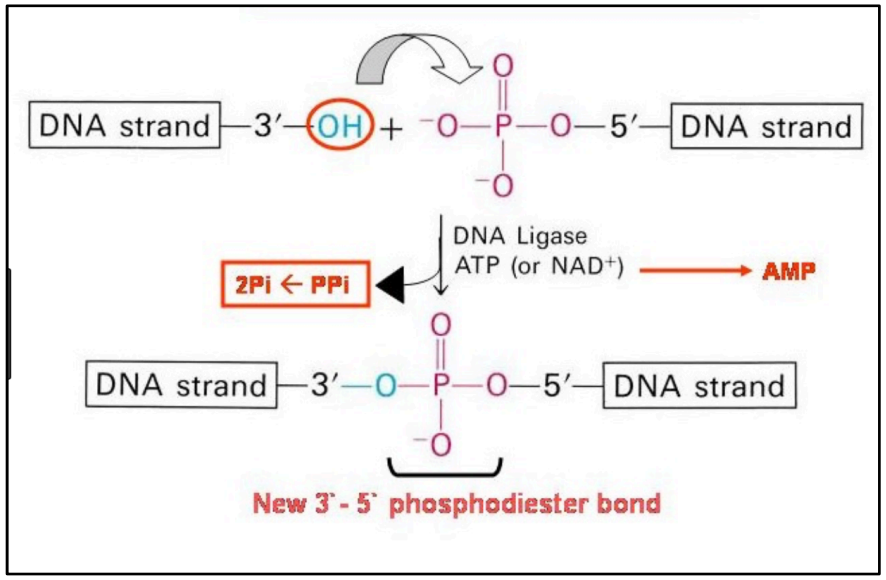
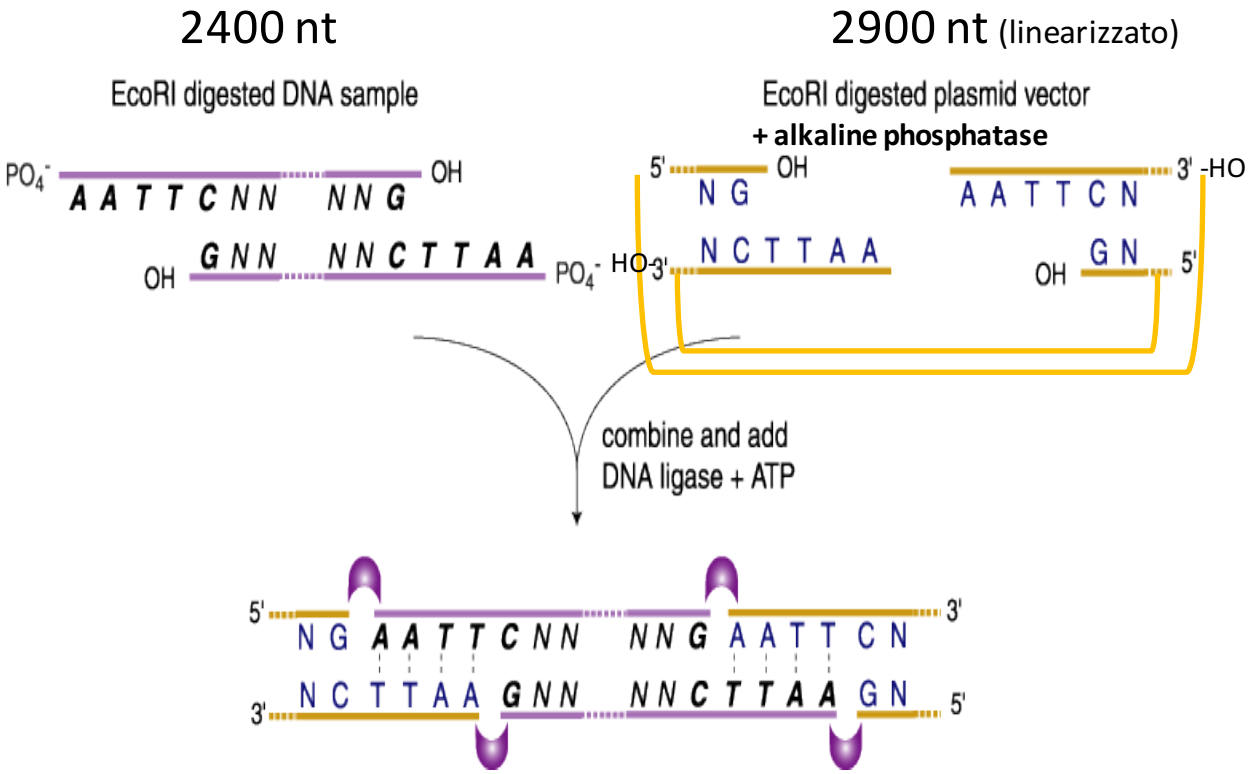


Purify DNA and eliminate agarose
Determine concentration of purified plasmid DNA
(ca. 50% loss of starting material)

Ligating 2 fragements with DNA Ligase



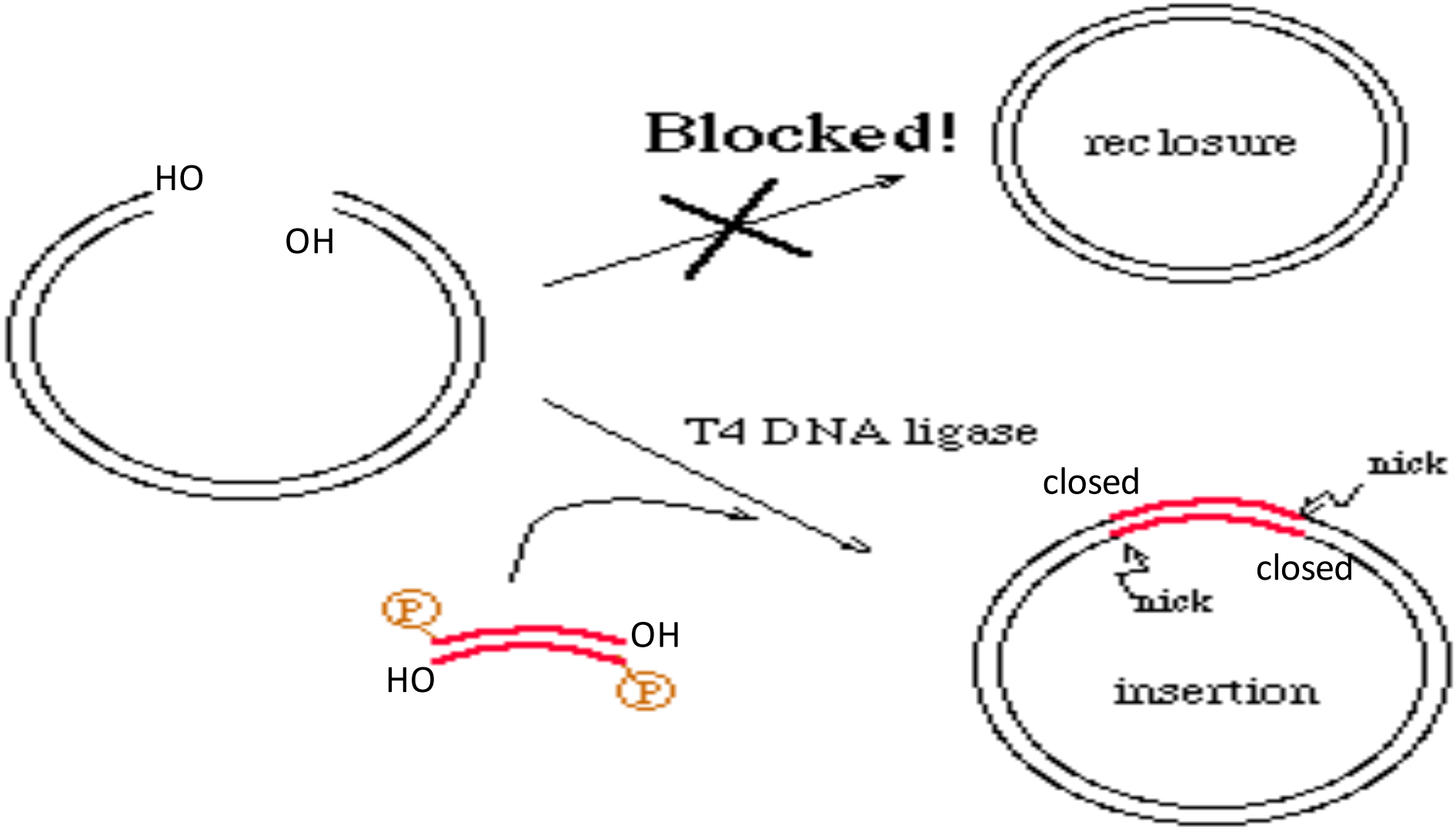


Sticky-end Ligations: 50 ng linearized plasmid + 3 fold molar excess of insert (=124 ng)

Dephosphorylation by alkaline phosphatase prevents re-ligation of EcoRI site of plasmid !!!

2400 nt fragment was NOT dephosphorylated → Ligation between 5'Phosphate + 3'OH of linearized plasmid is possible!!! (*however will not be amplified as plasmid in bacteria!!!*)

Ligating 2 fragements with DNA Ligase



NICKS (ssDNA lesion/break) ARE TOLERATED – **REPAIRED BY BACTERIA**

REAL SETTING: USE CONTROLS!!

1. Control: LIGATION 1: 50ng plasmid + LIGASE
2. Control: LIGATION 2: 124ng INSERT + LIGASE
3. LIGATION TO MAKE RECOMBINANT DNA
   50ng plasmid; EcoRI, de-phosph + 124ng INSERT + LIGASE

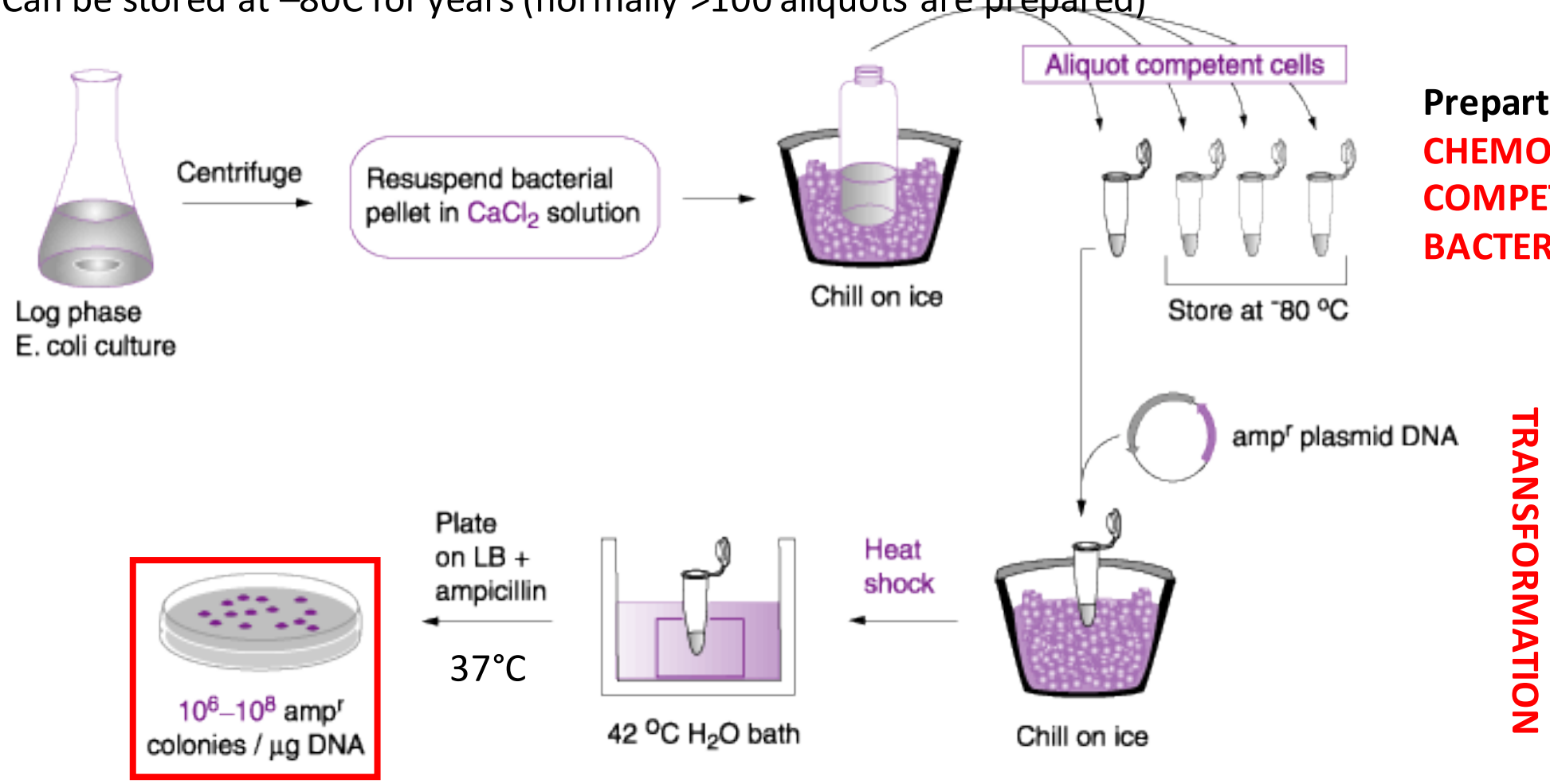
HOW TO TRANSFER LIGATION PRODUCTS INTO BACTERIA?

TRANSFORMATION: Insertion of ligated productis into bacteria

$CaCl_2$ and cold environment makes membrane permeable without killing the cells

= **CHEMOCOMPETENT BACTERIA - metodo del $CaCl_2$ – (calcio cloruro)**

(Can be stored at –80C for years (normally >100 aliquots are prepared)

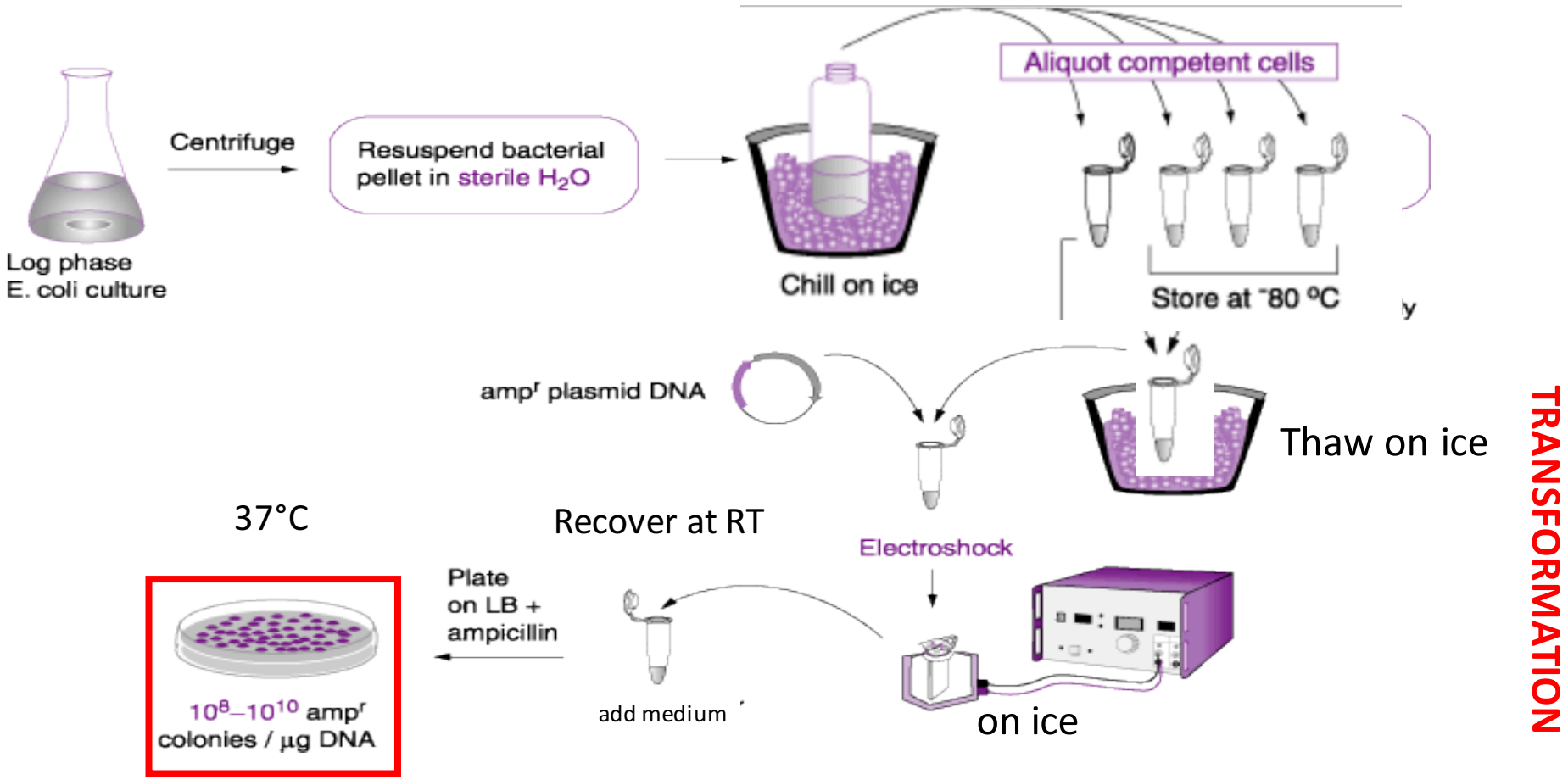


Compent bacteria are put on ice until bacteria are thawn; add ligation product; induce heat shock (42°C); DNA can enter the bacteria;

add liquid media to allow bacteria to recover; plate on media plate containing amplicilin (37°C)

TRANSFORMATION: Insertion of ligated productis into bacteria

H_2O and cold environment makes membrane permeable without killing the cells
= **ELECTROCOMPETENT BACTERIA**
(Can be stored at –80°C for years (normally >100 aliquots are prepared))



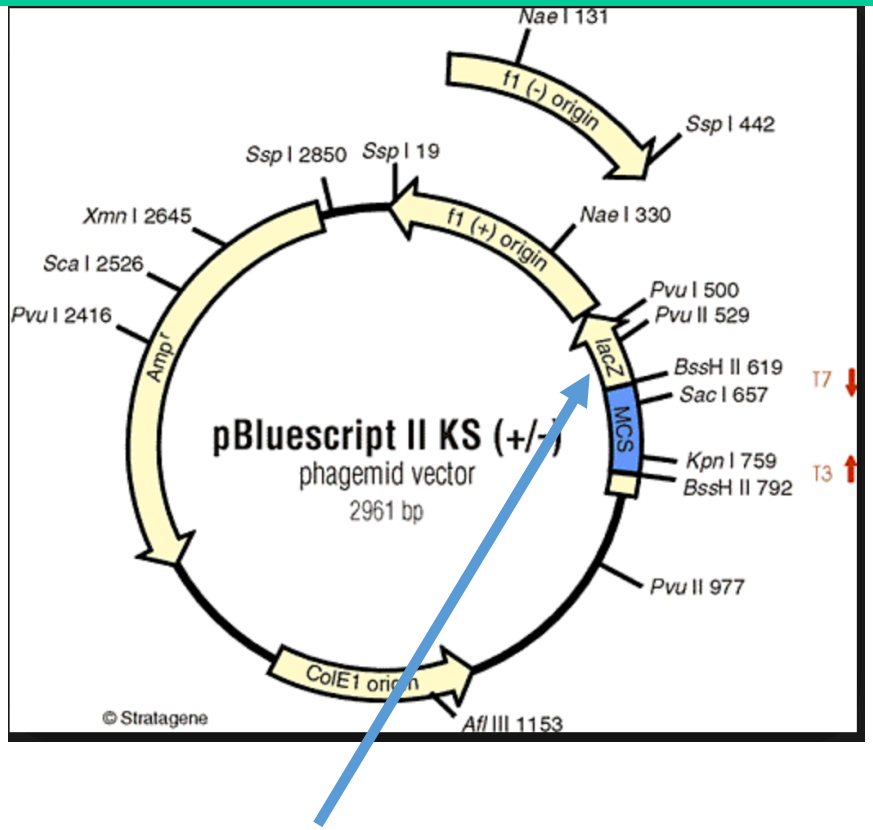
Compent bacteria are put on ice until bacteria are thawn; add ligation product; induce electroshock; DNA can enter the bacteria;
add liquid media to allow bacteria to recover; plate immediately on media plate containing amplicilin

EVALUATING THE SUCCESS OF DNA CLONING EVENTS

1. Control: LIGATION 1: 50ng plasmid; EcoRI, de-phosph + LIGASE
2. Control: LIGATION 2: 124ng INSERT + LIGASE
3. LIGATION TO MAKE RECOMBINANT DNA
   50ng plasmid; EcoRI, de-phosph + 124ng INSERT + LIGASE

1. EASY IDENTIFICATION OF SUCCESSFULL DNA CLONING EVENTS

BLUE-WHITE SELETION



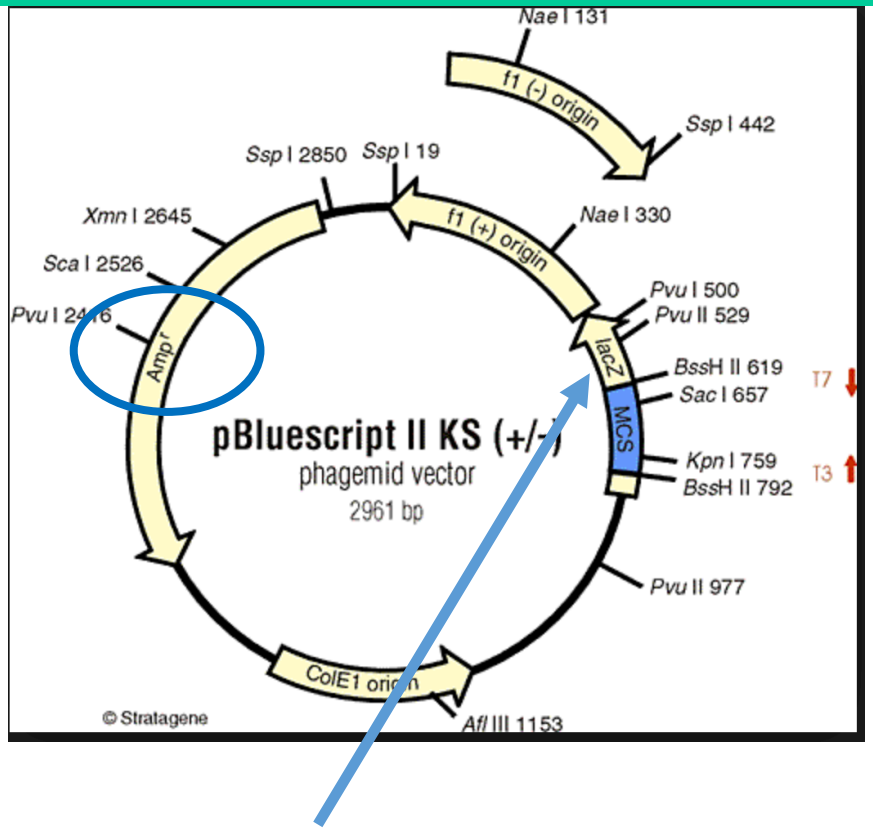
LacZ open reading frame:
MCS does not impair alpha LacZ
beta-galactosidase forms and converts X-Gal to blue colorant → blue colonies

α -complementation

- **The portion of the *lacZ* gene encoding the first 146 amino acids (the α -fragment) are on the plasmid**
- **The remainder of the *lacZ* gene is found on the chromosome of the host.**
- **If the α -fragment of the *lacZ* gene on the plasmid is intact (that is, you have a non-recombinant plasmid), these two fragments of the *lacZ* gene (one on the plasmid and the other on the chromosome) complement each other and will produce a functional β -galactosidase enzyme.**

1. EASY IDENTIFICATION SUCCESSFULL DNA CLONING EVENTS

BLUE-WHITE SELETION SCREEN



- *lacZ* gene not expressed constitutively
- must use IPTG as inducer to induce *lacZ* expression (isopropyl-β-D-thio-galactoside)
- IPTG and X-Gal are added to solid media in petri dish and also Ampilicin

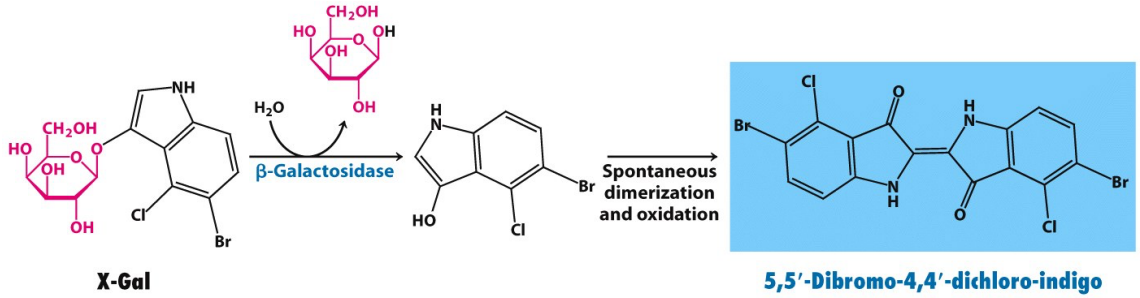


Figure 31.5
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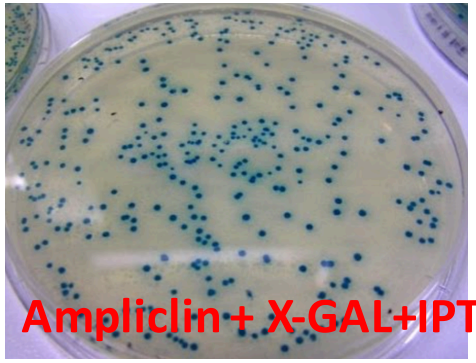
LacZ open reading frame:
MCS does not impair alpha LacZ
beta-galactosidase forms and converts X-Gal to blue colorant → blue colonies

- Note: small inframe insertions may not inactivate α peptide → you may still get blue colonies (often lighter – less activity

1. EASY IDENTIFICATION SUCCESSFULL DNA CLONING EVENTS
BLUE-WHITE SELETION SCREEN

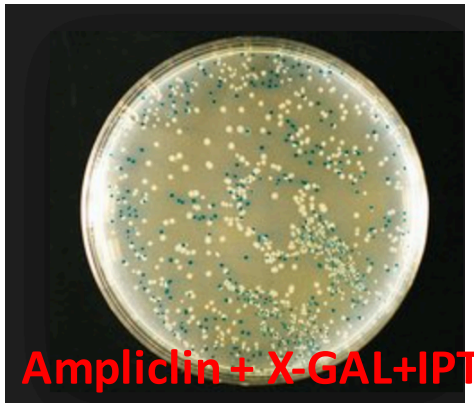
CONTROL LIGATION 1: 50ng plasmid; EcoRI, de-phosph + LIGASE



46 blue colonies

1. EcoRI cut; some vector molecules not dephosphorylated → re-ligation (blue) or:
2. Not all vector cut by EcoRI (blue)

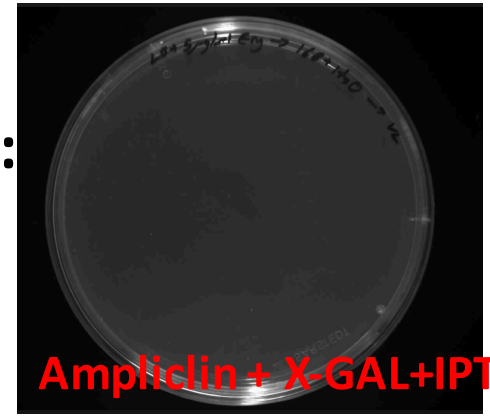
LIGATION : 50ng plasmid; EcoRI, de-phosph + 124ng INSERT + LIGASE



200 white colonie
40 blue colonies

1. EcoRI cut; some vector molecules not dephosphorylated → re-ligation (blue)
2. Not all vector cut by EcoRI (blue)
3. **SUCCESSFULL DNA CLONING EVENTS (WHITE)**

CONTOL LIGATION 2: 124ng INSERT + LIGASE



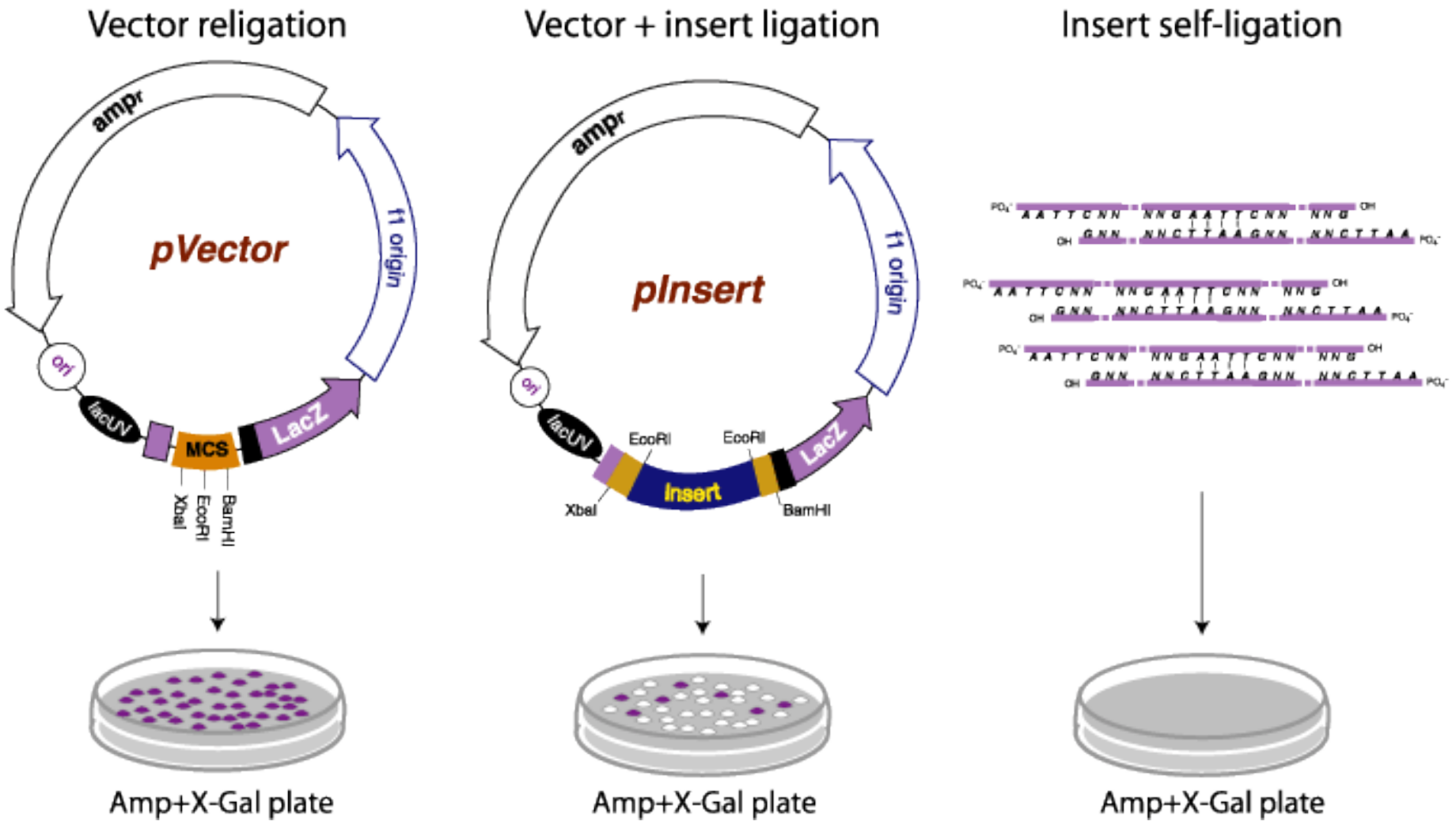
No colonies

1. **NO COLONIES: no vector backbone present**

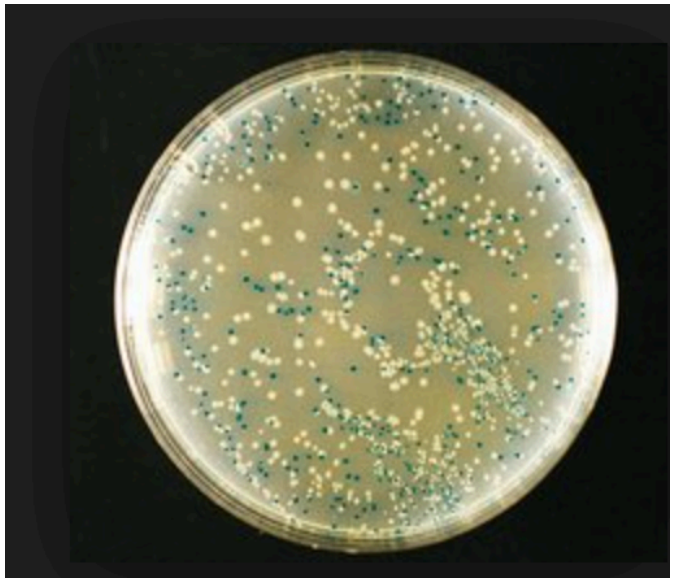
For example

1. EASY IDENTIFICATION SUCCESSFULL DNA CLONING EVENTS

BLUE-WHITE SELETION SCREEN



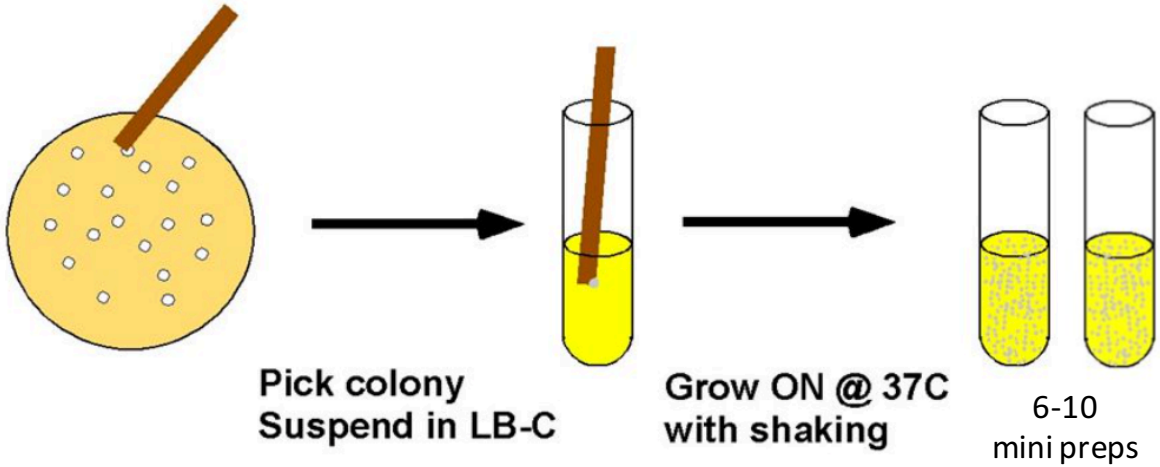
2. DNA PREPARATION AND CONTROL DIGEST



In general: pick 6-10 white colonies with sterile pipette tip

Preparation. Grow the bacteria

Grow an overnight (ON) culture of the desired bacteria in 2-5 ml of LB medium containing the appropriate antibiotic for plasmid selection. Incubate the cultures at 37°C with vigorous shaking.



Next day: harvest bacteria by centrifugation and prepare plasmid DNA