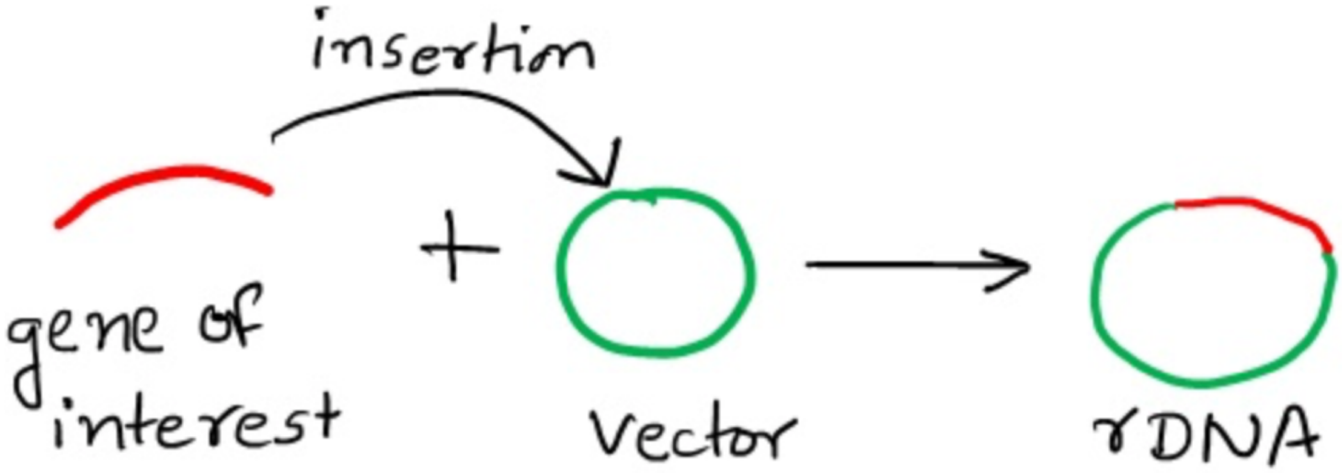
RECOMBIANANT DNA TECHNOLOGY
DNA - CLONING

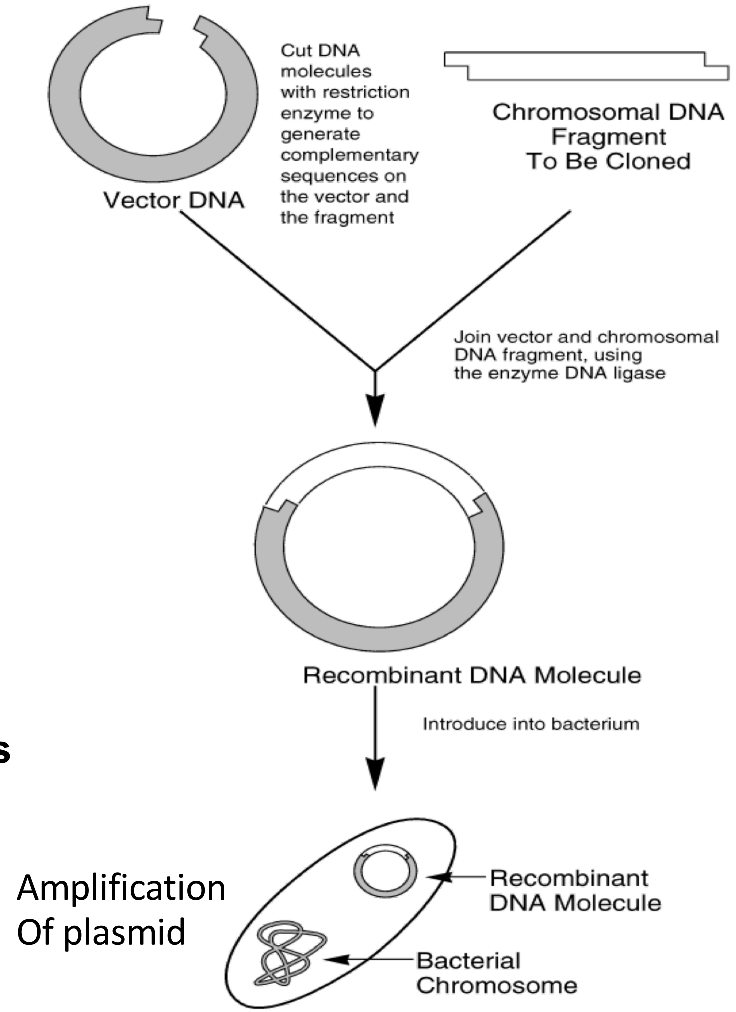


DNA RICOMBINANTE:

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

DNA cloning

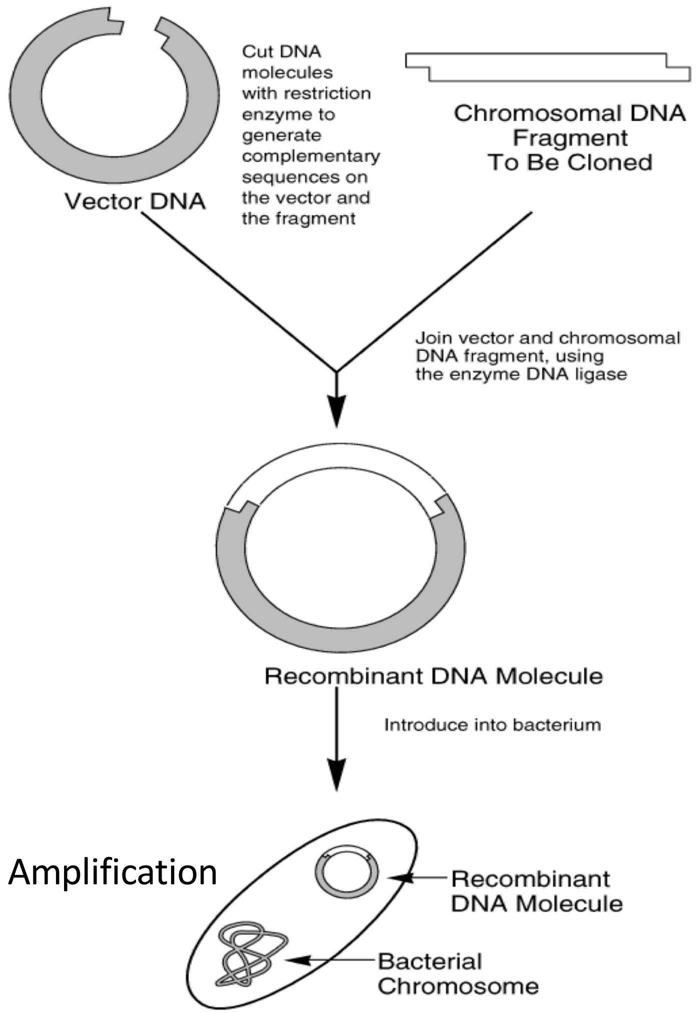
- **DNA cloning is the process of making multiple, identical copies of a particular piece of DNA**
- **It can be achieved by two different approaches:**
  - ***cell based nucleic acids (genomic DNA, plasmid DNA, cDNA after reverse transcription of RNA***
  - ***amplification of defined sections of DNA 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 → cDNA) sequence to be selected among many others and produced in an unlimited amount.**
- **This technique is the first stage of most of the molecular experiments or biotech applications:**
  - ***production of expression vectors***
  - ***cloning of PCR fragments***
  - ***cloning of DNA fragments for seqeucing***
  - ***cloning of DNA constructs for genetic engineering (gene targeting)***



DNA cloning

Avantage:

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



DNA cloning requires the use of enzymes that cut or modify DNA

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

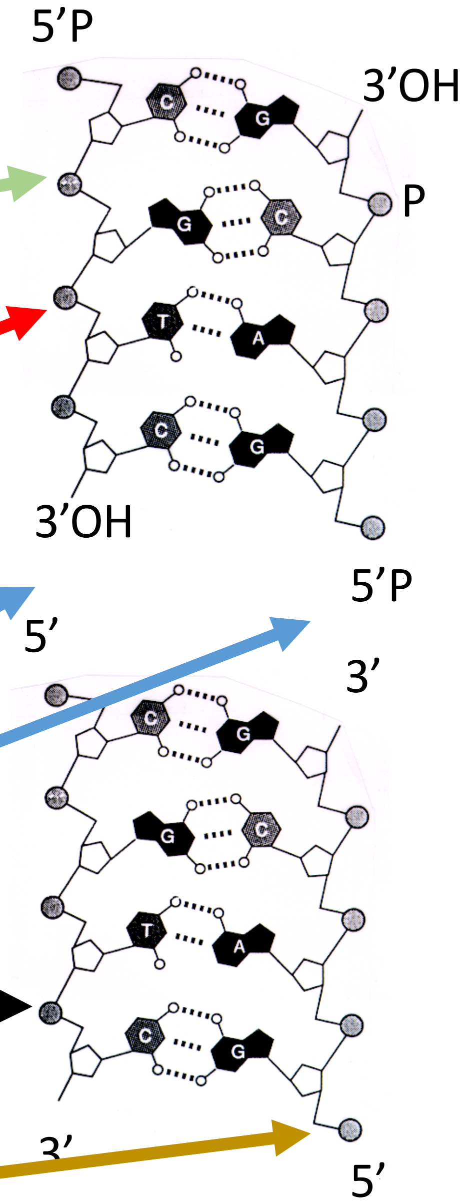
Le esonucleasi rompono
il legame al <u>termine</u> dei filamenti di DNA
(5' → 3')

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

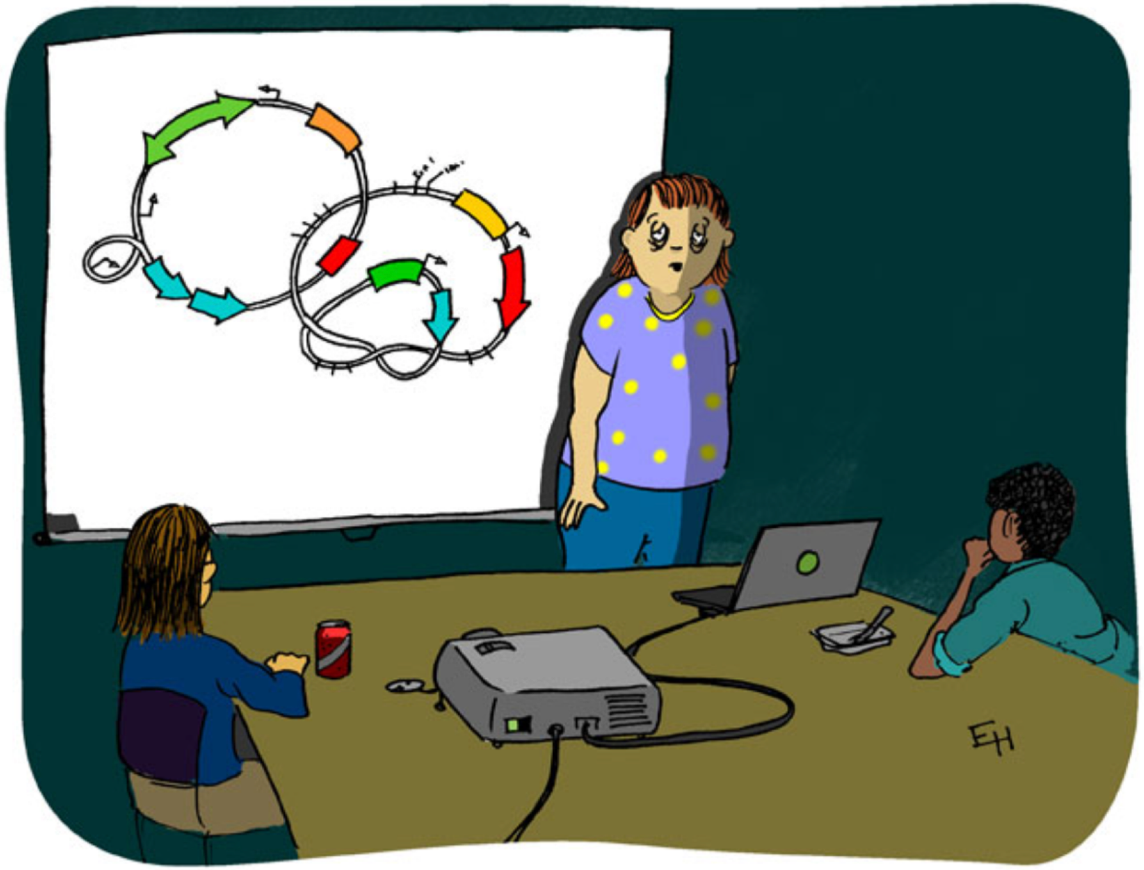
Ligasi: catalizza la formazione del legame tra due
molecole di DNA, spesso accompagnato dall'idrolisi
di una molecola come ATP

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

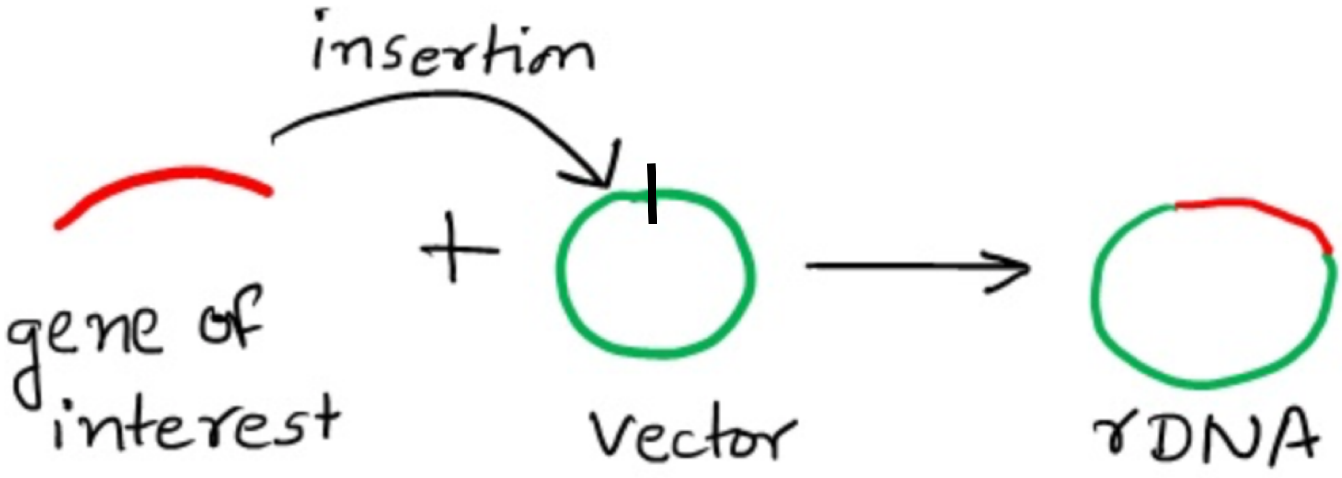
...frustrating...

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 →

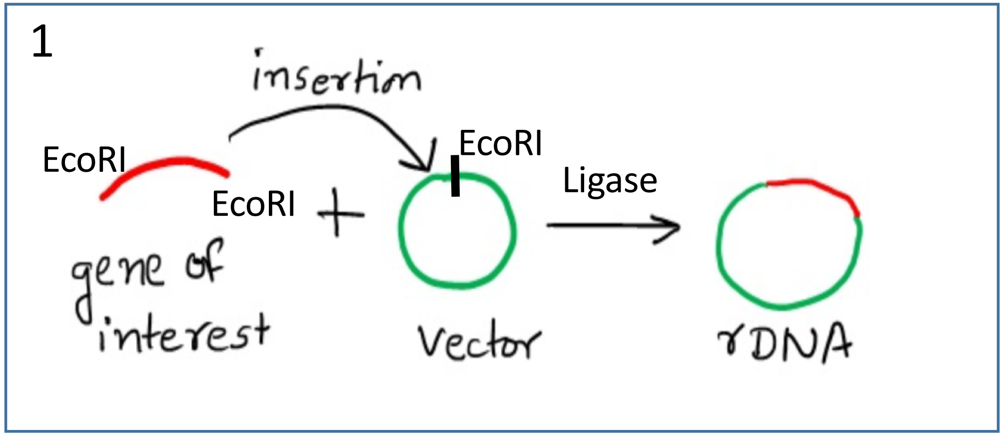
Making recombinant DNA

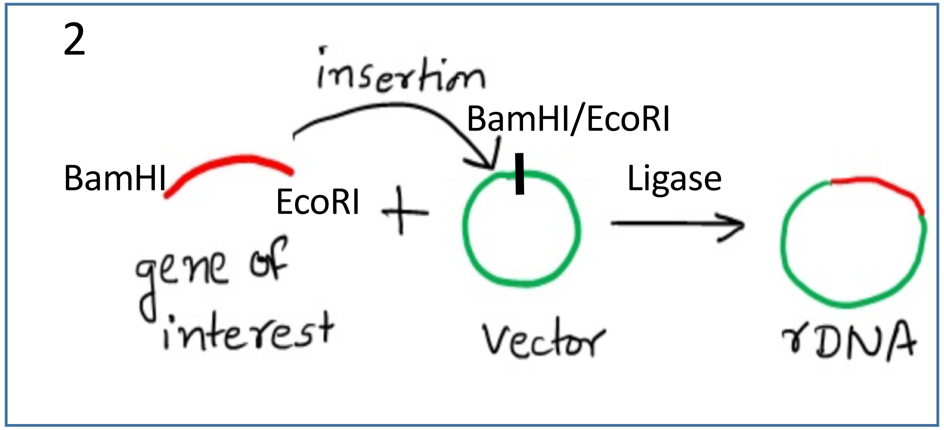
Ligase

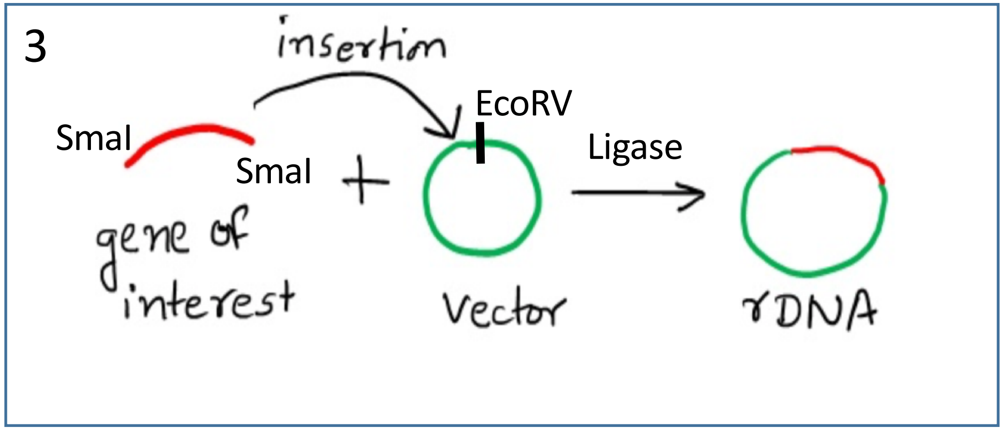


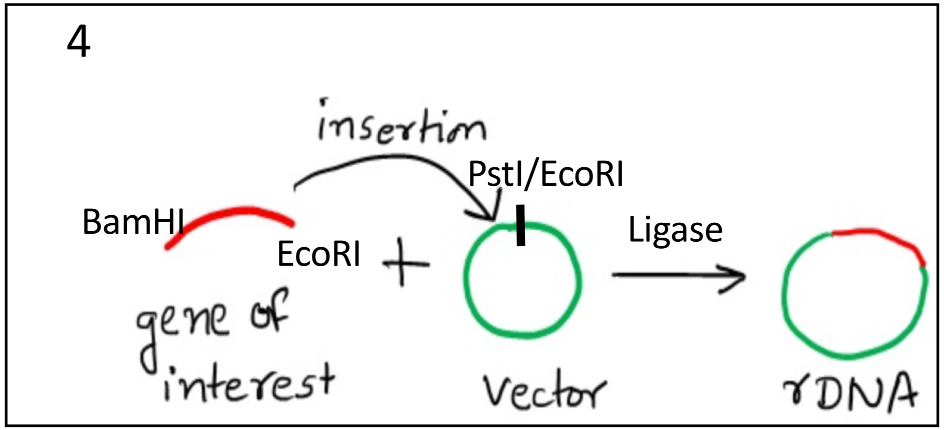
1. **Fragment and Insert are cut with 1 (the same) restriction enzymes**
2. **Fragment and Insert are cut with 2 (the same) restriction enzymes**
3. **Fragment and Insert are cut with blunting restriction enzymes**
4. **Overhangs generated after cutting are modified (filled up; or overhang digested)**

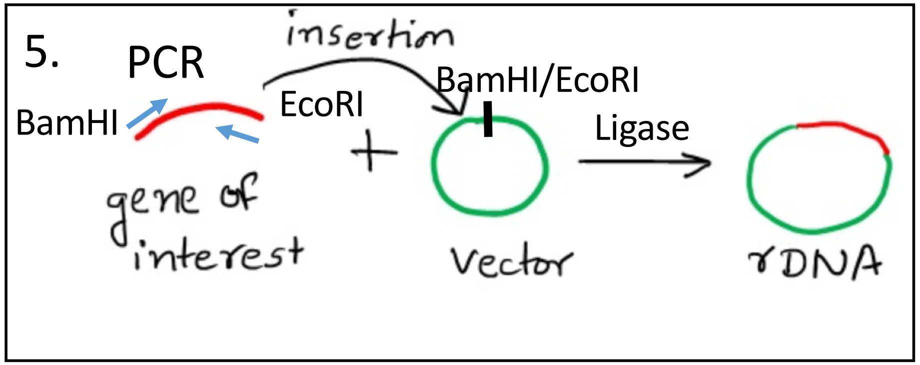
OVERVIEW OVER OTHER CLONING STRATEGIES



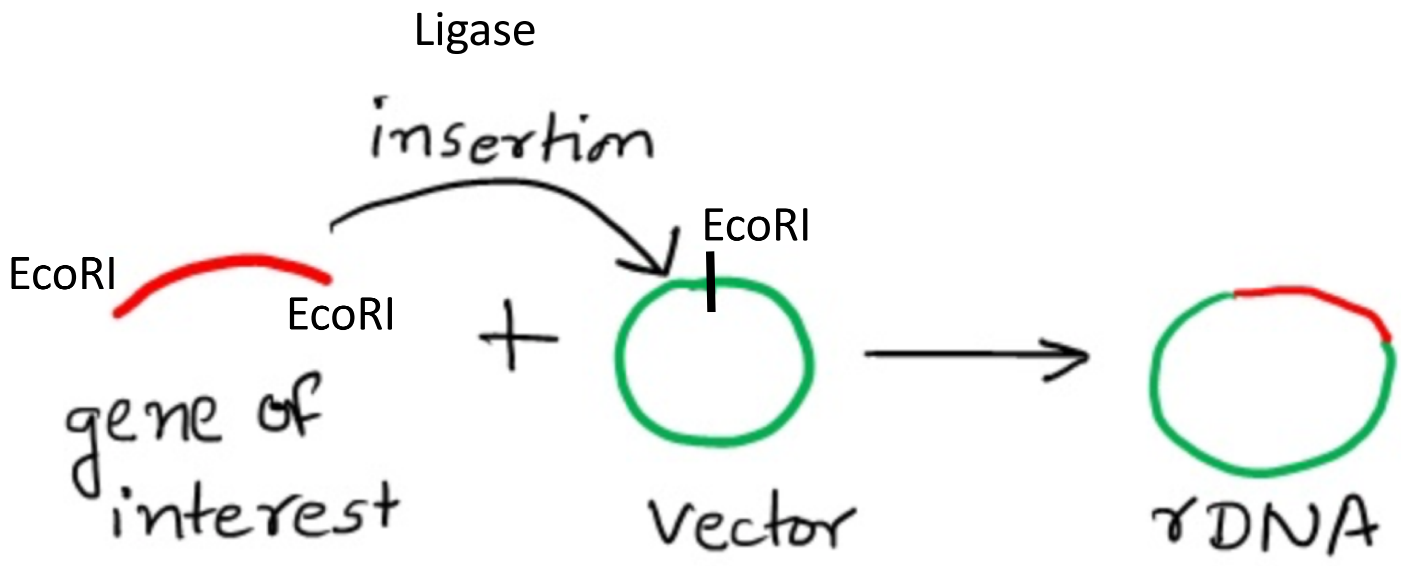






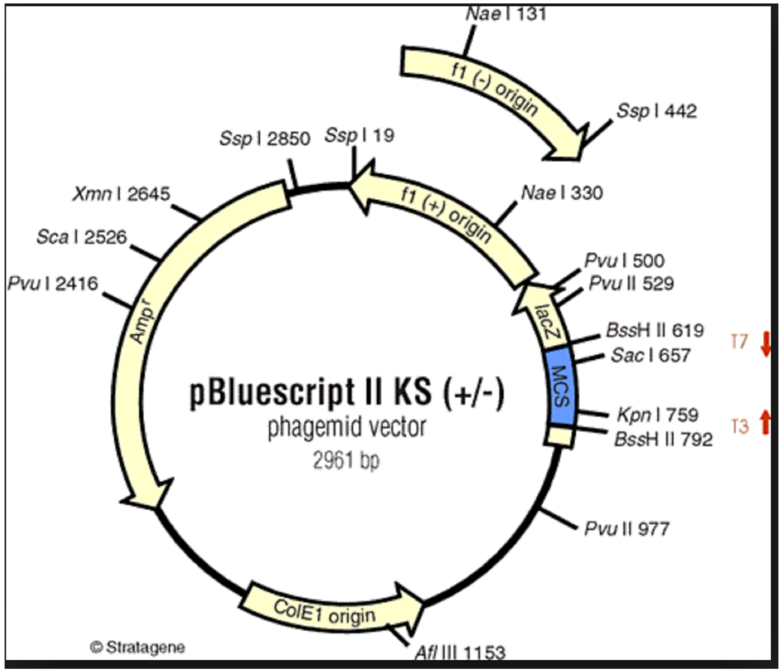


Making recombinant DNA



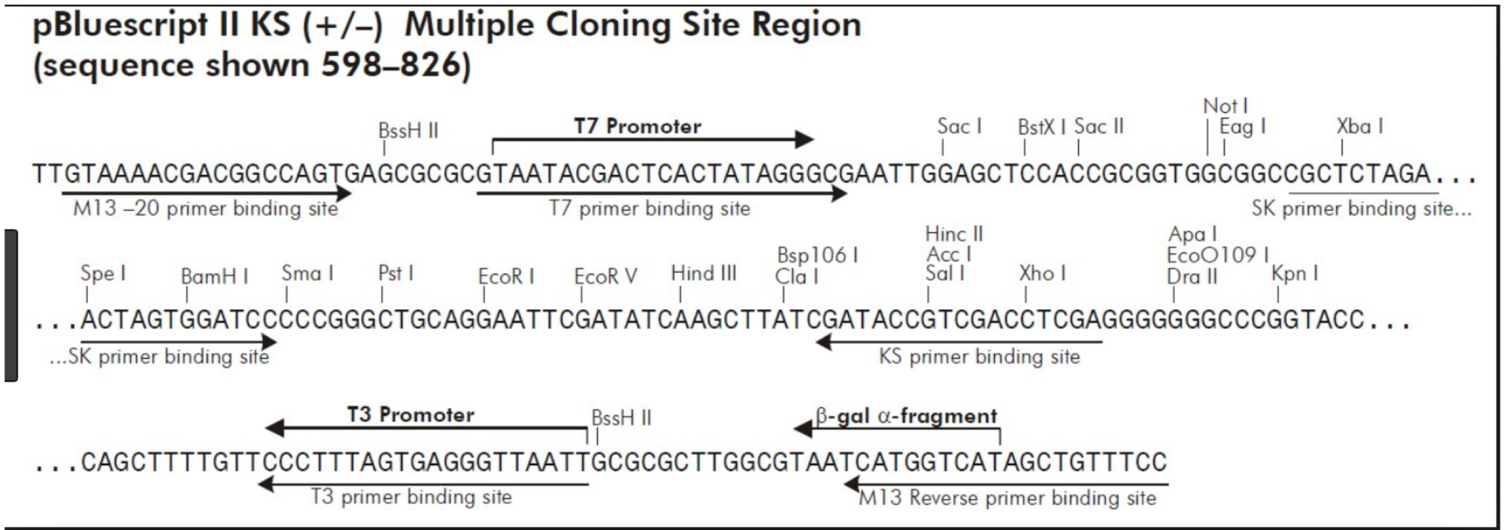
1. **Fragment and Insert are cut with 1 (the same) restriction enzymes**

Making recombinant DNA – Plasmid features

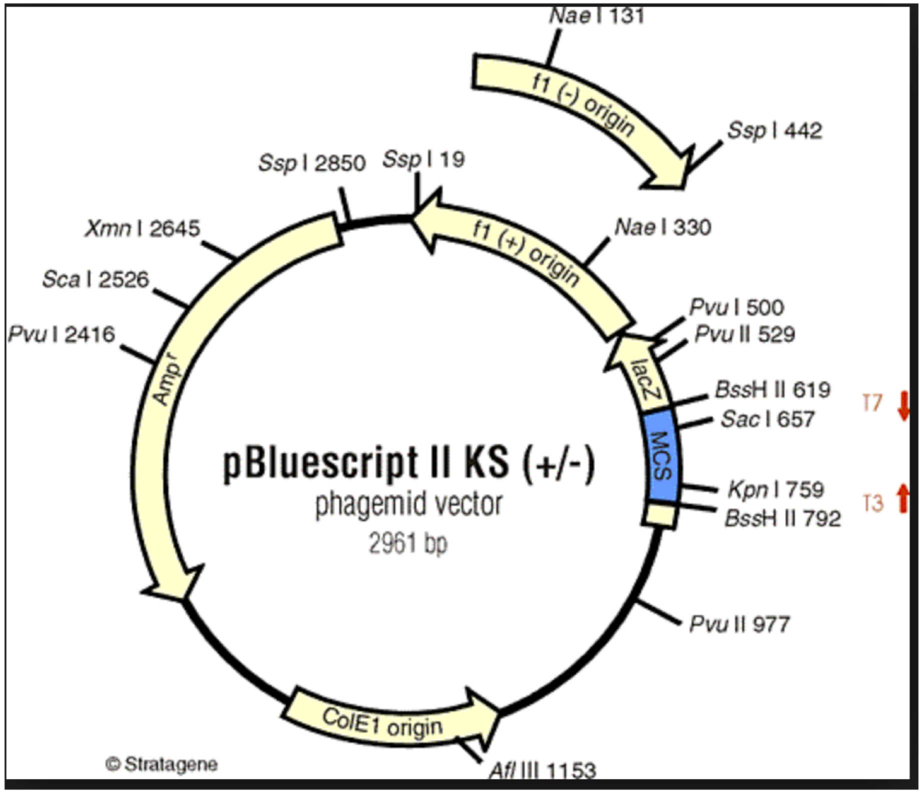


What makes a good plasmid for cloning (generating recombinant DNA)??

- **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:

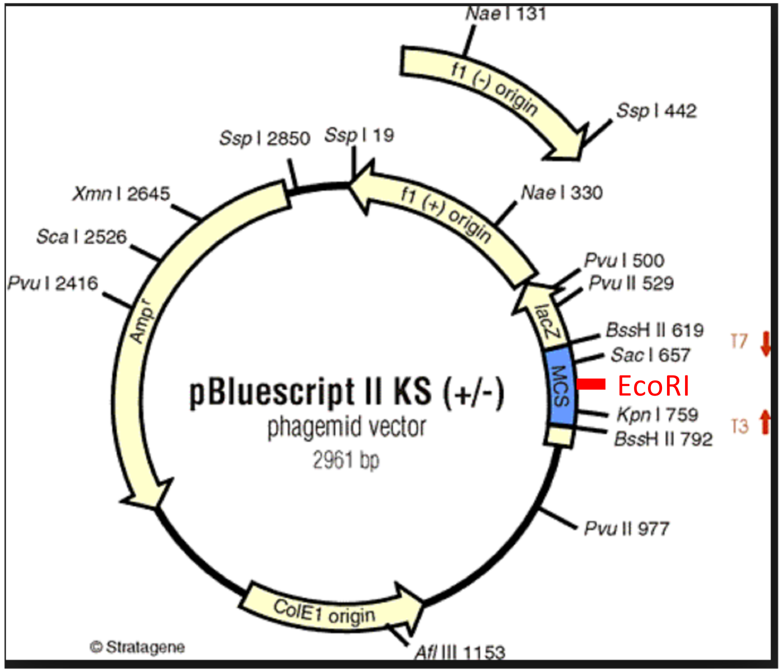
pBluescript II is a phagemid that can be secreted as single-stranded DNA in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous f1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E.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

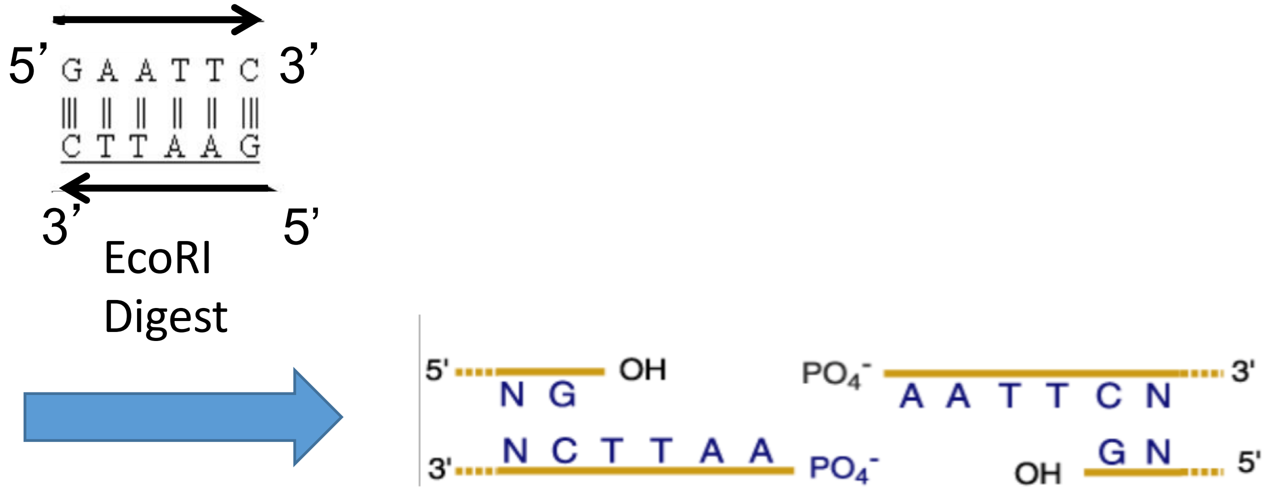
Similarly to a plasmid, a phagemid can be used to clone DNA fragments and be introduced into a bacterial host by a range of techniques, such as transformation and electroporation. However, infection of a bacterial host containing a phagemid with a 'helper' phage, for example VCSM13 or M13K07, provides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

Preparing the vector backbone





Linearized pBluescript with 5’overhangs: length: 2,9kb

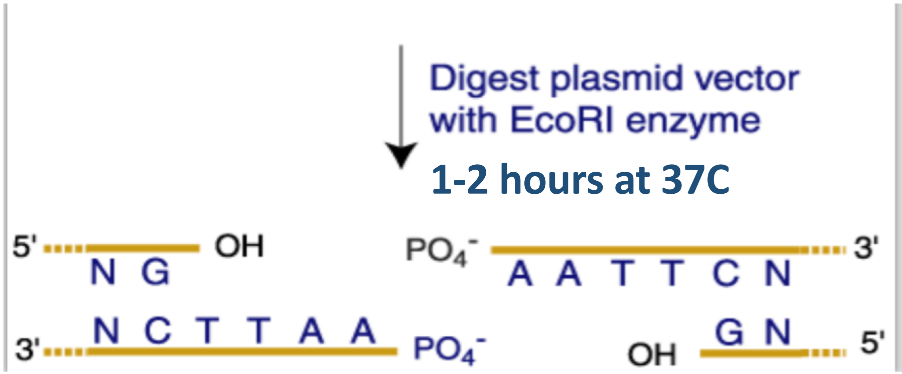
Order of solution addition	Solution	Volume(µl)
1	Nuclease free water	23.5
2	10X Buffer K	5.0
4	100 µg BSA	0.5
5	Plasmid DNA 0,1 µg/µl	20.0
3	EcoRI (20U/ µl)	1.0
Total Volume		50.0

Plasmid DNA: 2ug
EcoRI: 20Units/µl:
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: **10 fold overdigest** is recomended: = 1ug DNA + 10 units
(you are never sure about “real” activity of enzyme – storage – handling, etc)

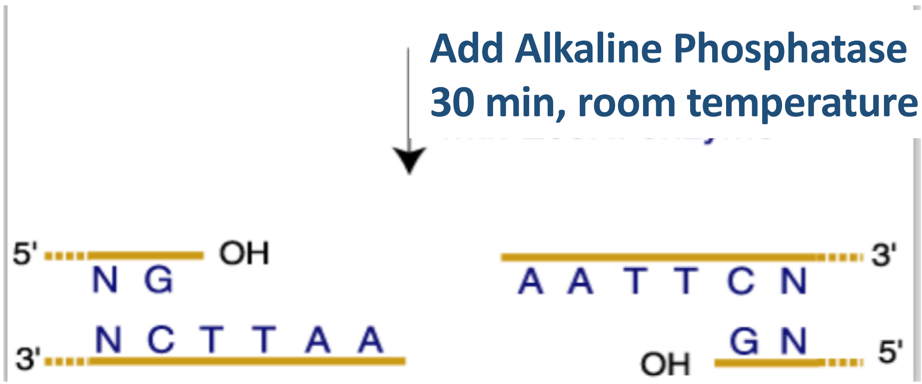
One unit of restriction endonuclease activity is defined as the amount of enzyme required to produce a complete digest of 1 µg of substrate DNA (or fragments) in a total reaction volume of 50 µl in 60 minutes under optimal assay conditions as stated for each restriction endonuclease.

Preparing the vector backbone

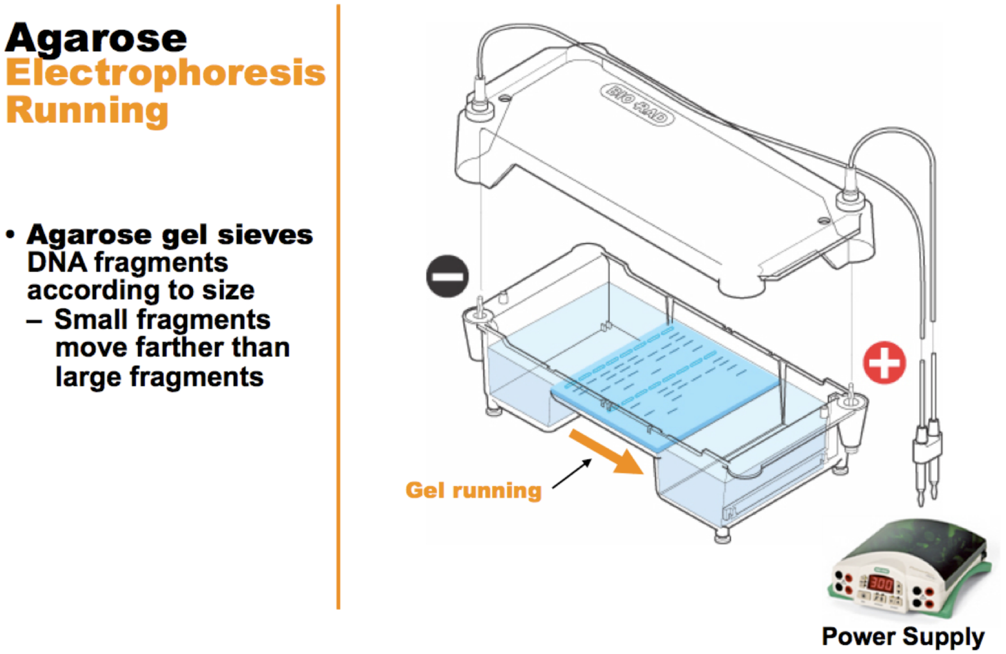
- **Alkaline phosphatase removes 5' phosphate groups from <u>DNA</u> and RNA. It will also remove phosphates from nucleotides and proteins. These enzymes are most active at alkaline pH.**



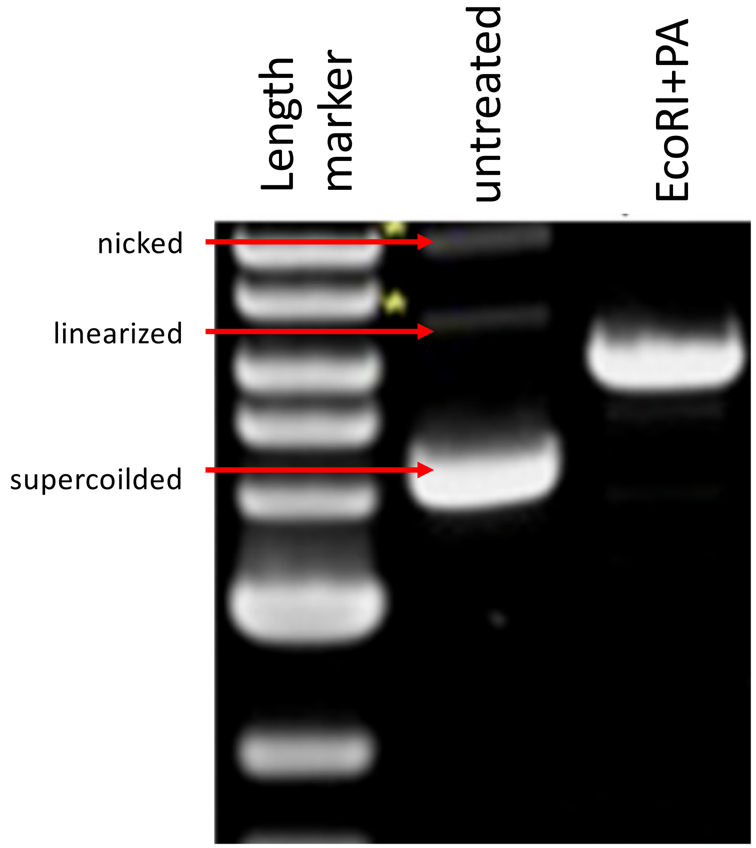
- **In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby greatly facilitates ligation of other DNA fragments into the vector**



Preparing the vector backbone



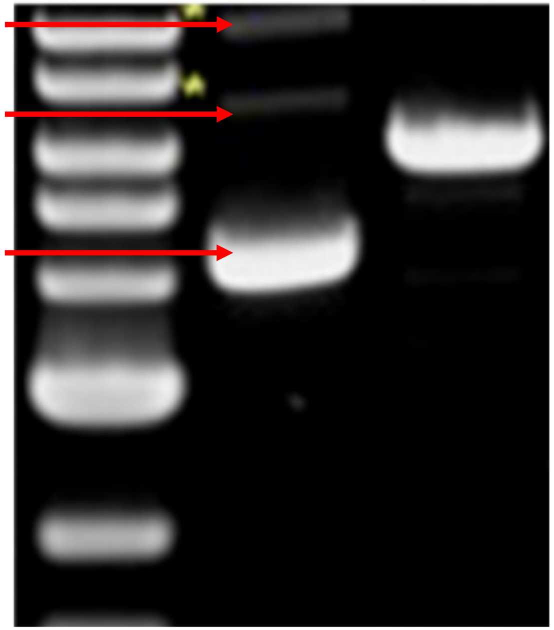
Digest run on agarose gel (gel contains Ethidiumbromide; or samples contains DNA colouring agent)

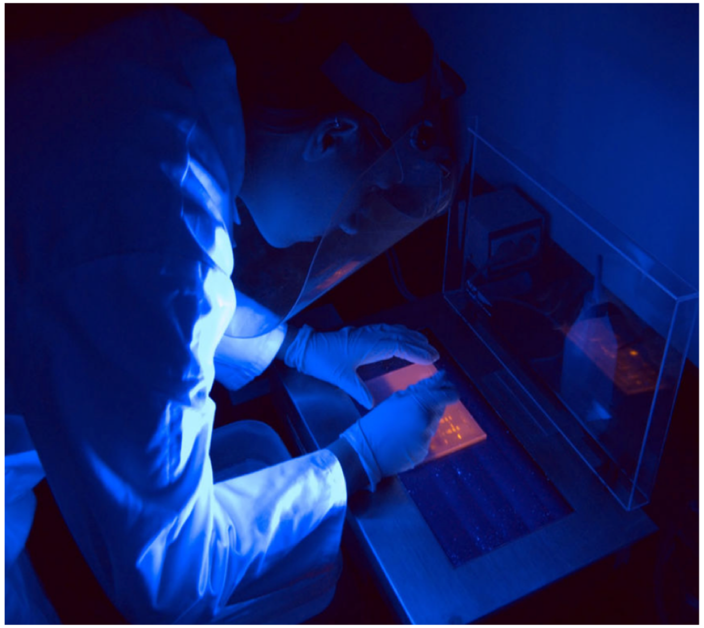


Length marker: mix of DNA fragments with defined length

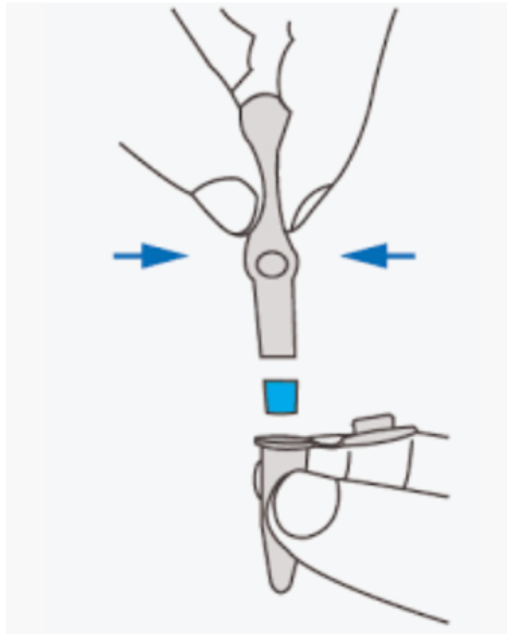
Linearized pBS: 2900 nt

Making recombinant DNA – Plasmid features



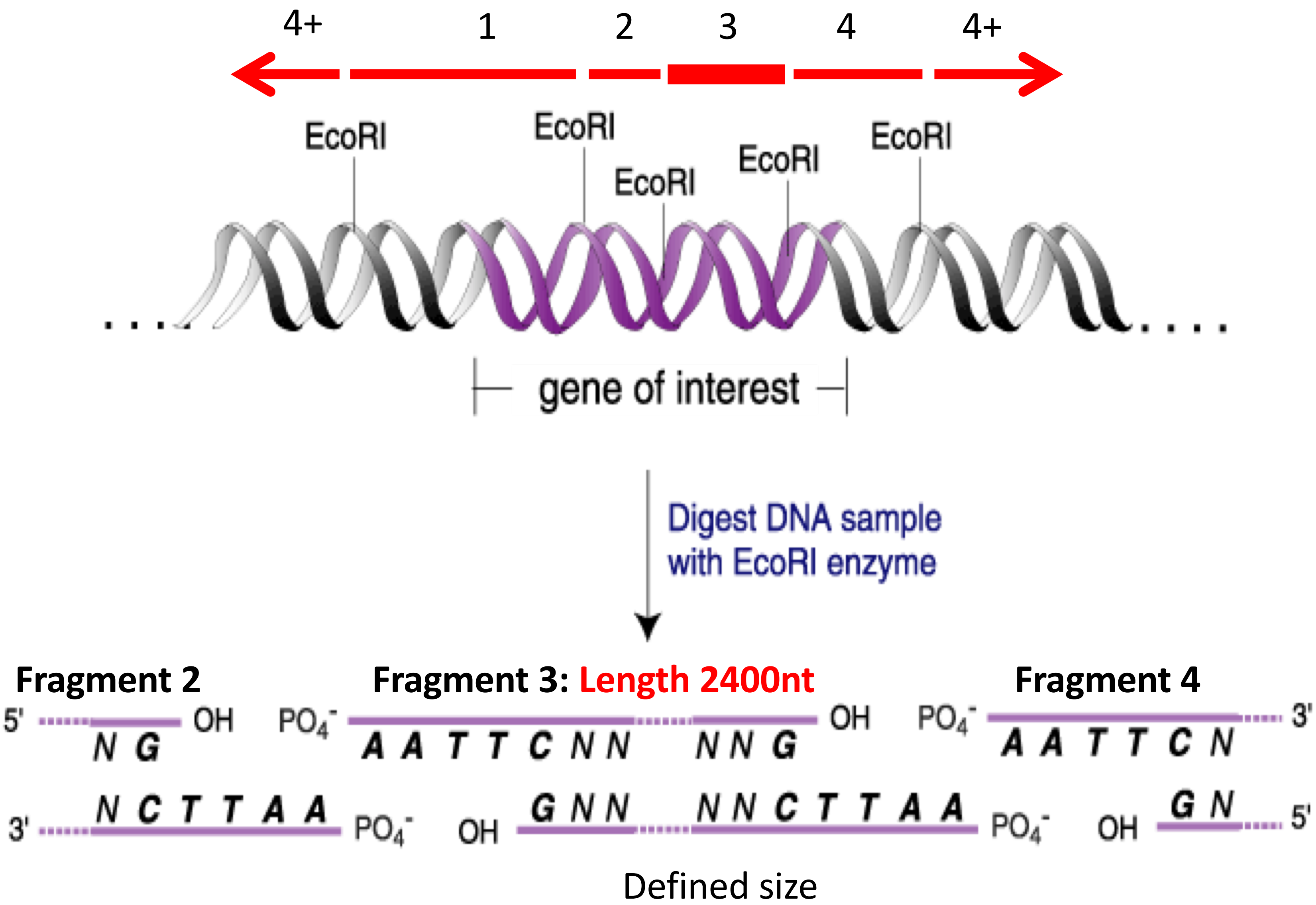


Cut out band from gel using a scalpel blade

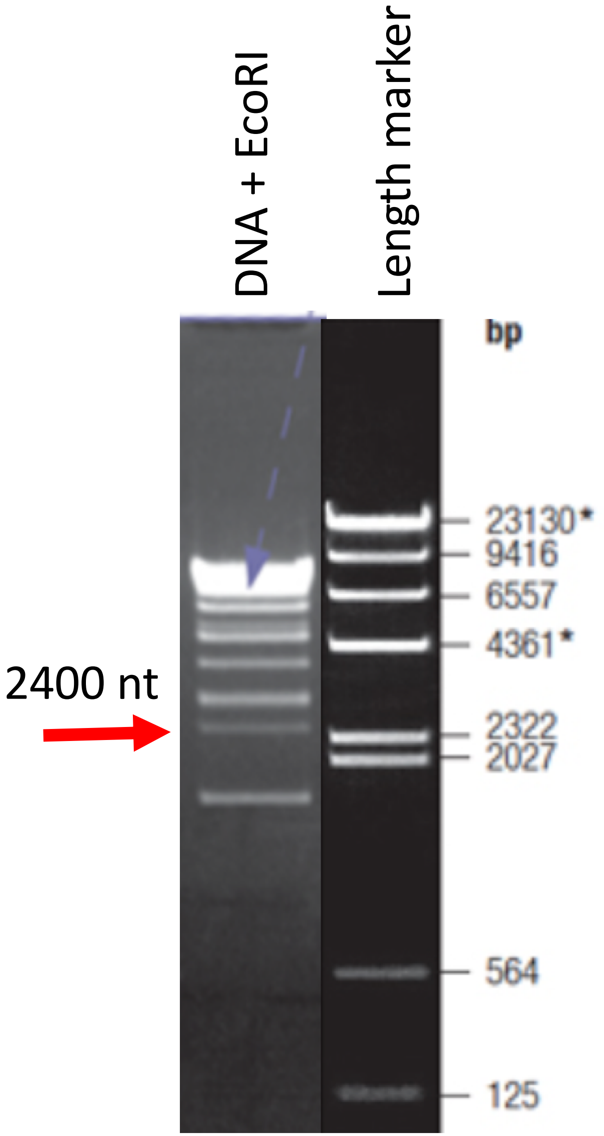


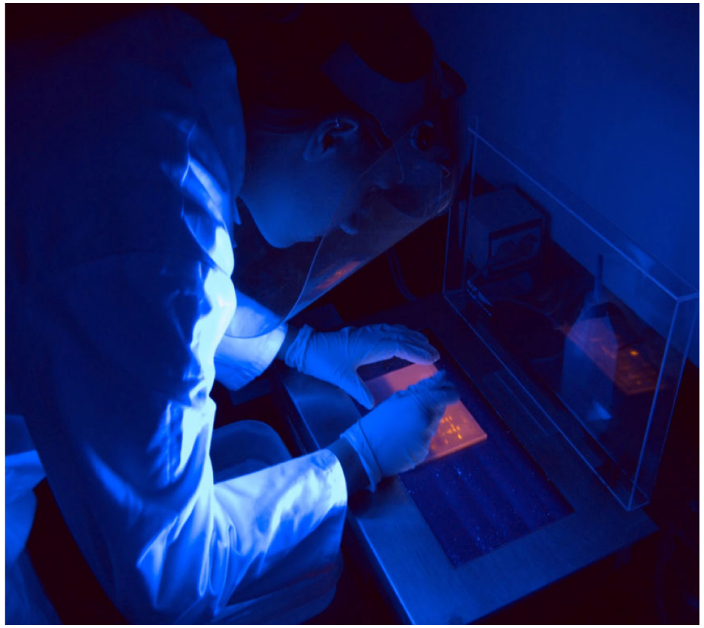
Purify DNA by eliminating agarose (commercial kit)
Determine concentration and itnegrity of purified plasmid DNA
(ca. 50% loss of starting material)

Preparing the insert



Preparing the insert





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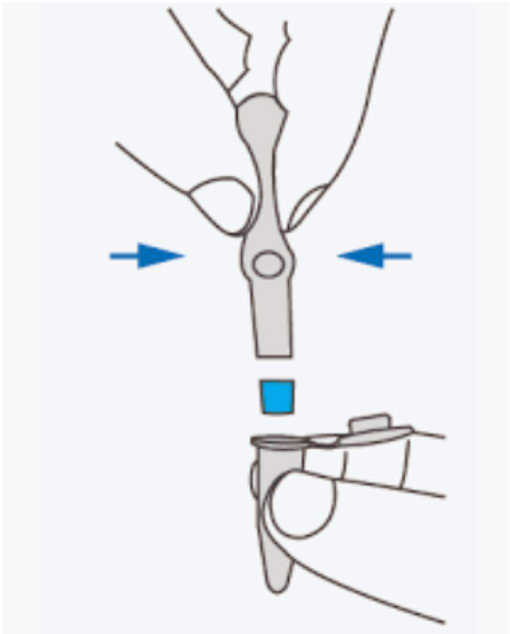

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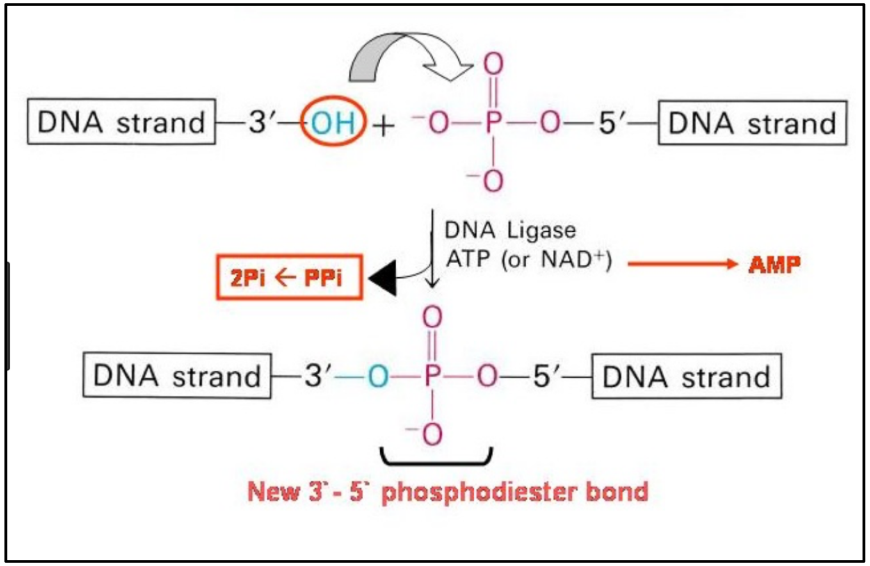
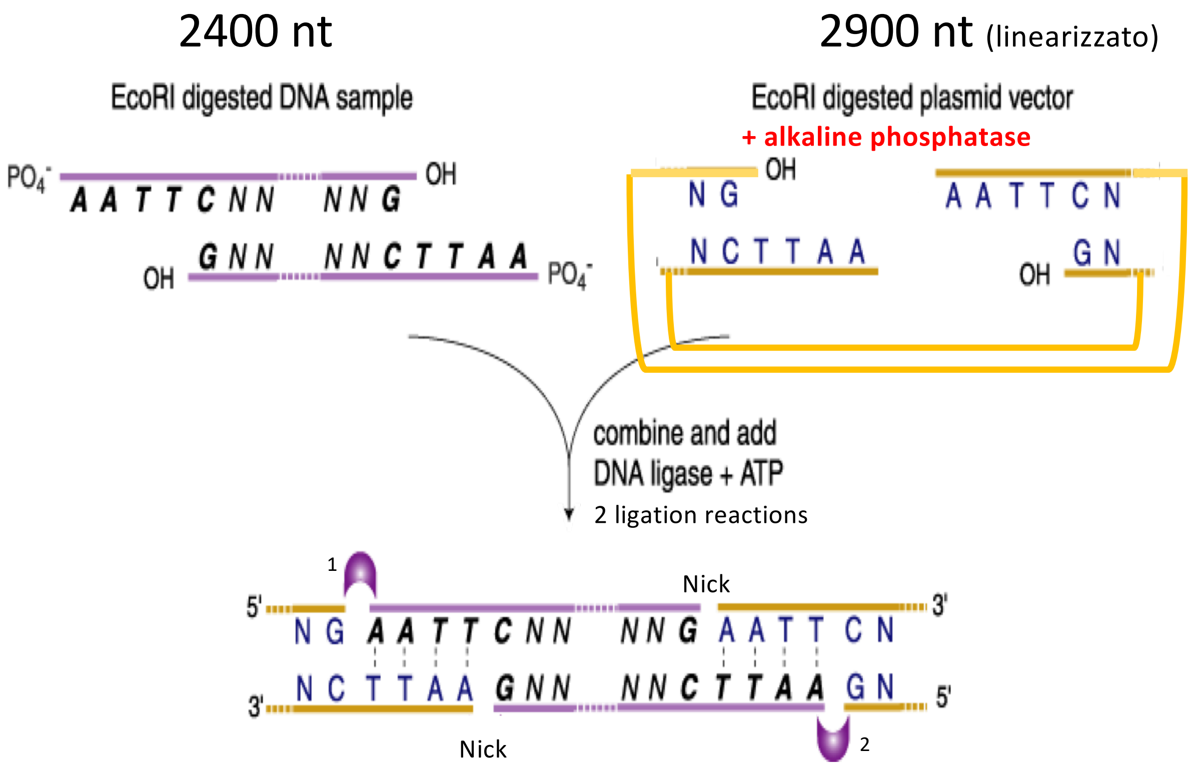
Ligating 2 vector backbone (de-P) and fragment 3 with DNA Ligase





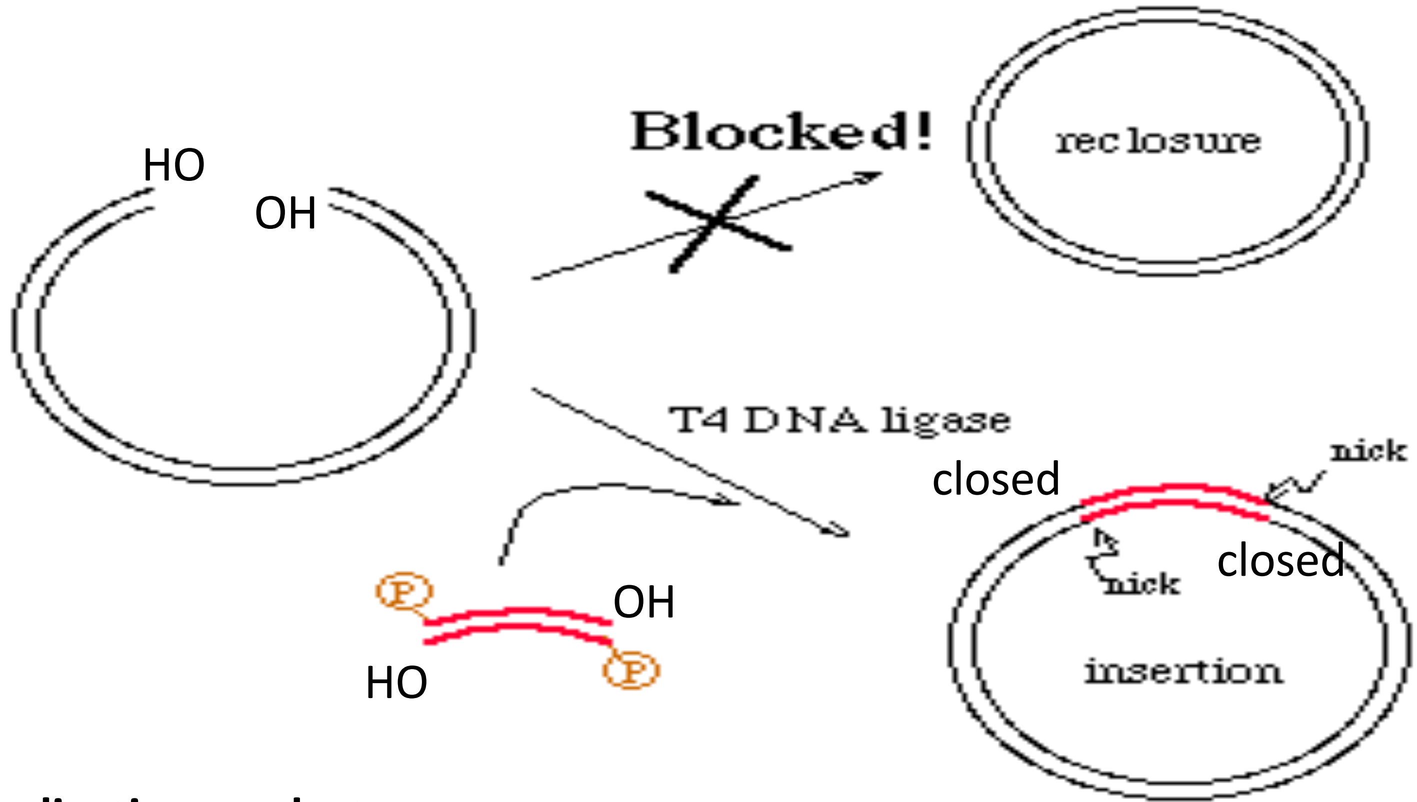
Sticky-end Ligations: 50 ng linearized plasmid + 3 fold molar excess of insert (=124 ng)
→ Increases probability of insert – plasmid ligation

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 vector backbone (de-P) and fragment 3 with DNA Ligase

Effect of de-phosphorylation on ligation



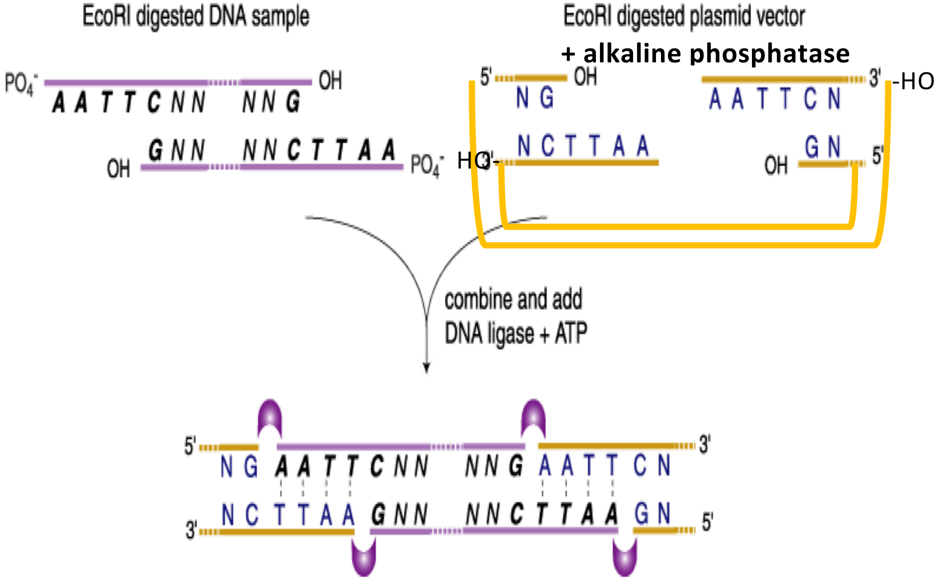
- **2 ssDNA nicks present in ligation product**
- **Ligation product stable due to 2 ligation reactions («closed»)**
- **ssDNA nicks are recognized by the DNA damage signalling**
- **Bacterial DNA damage repair closes nicks**

Ligating 2 fragments with DNA Ligase

GOLDEN RULE for sticky-end ligations:
50 ng linearized plasmid + <u>3 fold molar excess of insert</u>

2400 nt

2900 nt (linearizzato)



HOW TO CALCULATE

Fragments can be considered to contain on average an equal distribution between dATP, dGTP, dCTP and dTTP (Avg. MW = 327.0)

No need to calculate molecular weight of fragments to calculare molar excess

→ Work with proportions based on legnth of fragements (equivalent to MW)

STEP 1

$$\left(\frac{50\ ng}{2900}\right) = \left(\frac{Y\ ng}{2400}\right)$$

$$\left(\frac{50\ ng}{2900}\right) \times 2400 = Y\ (41{,}4\ ng)$$

41,4 ng insert = 50 ng vector: equimolar

STEP 2

$$\left(\left(\frac{50\ ng}{2900}\right) \times 2400\right) x3 = 124{,}13\ ng$$

124,13 ng insert = 50ng vector: 3 fold molar excess

REAL SETTING: USE CONTROLS!!

1.Negative control 1: LIGATION 1: 50ng plasmid + LIGASE

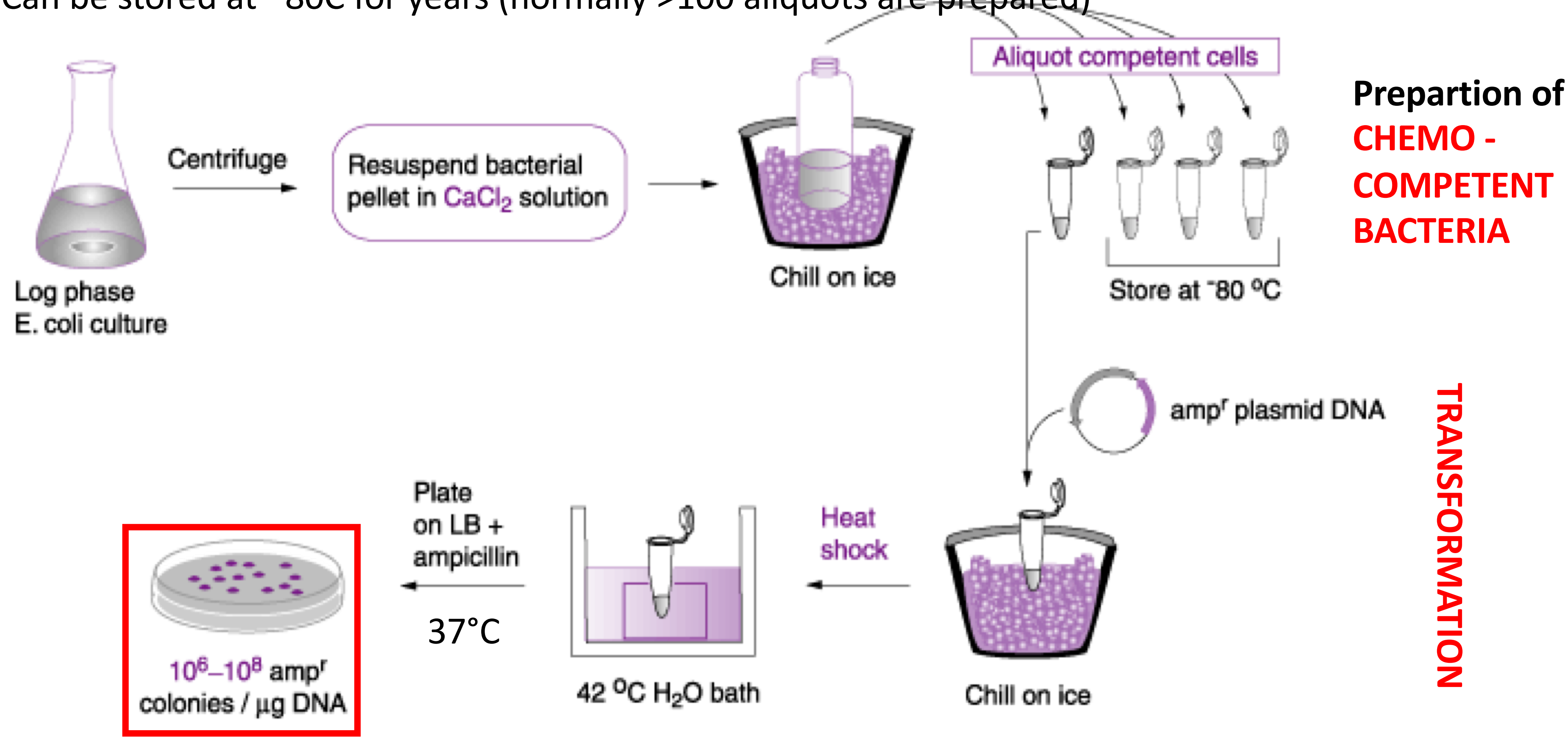
2. Negtive control 2: LIGATION 2: 124ng INSERT + LIGASE

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

HOW TO TRANSFER LIGATION PRODUCTS INTO BACTERIA?

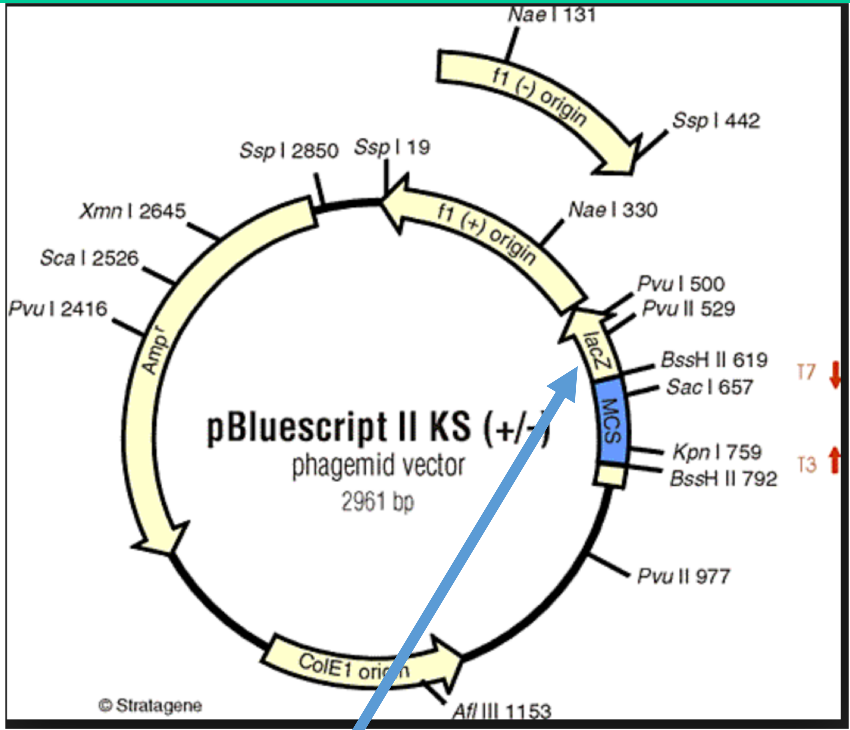
TRANSFORMATION: Insertion of ligated productis into bacteria

$CaCl_2$ 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)

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



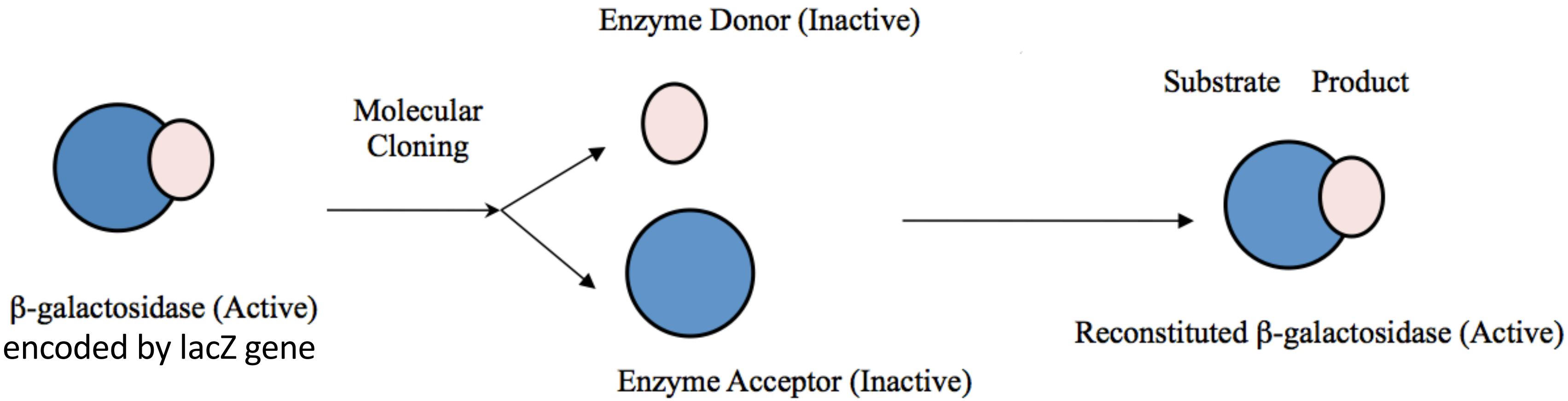
LacZ: open reading frame for the alpha subunit of beta galactosidase gene : MCS does not impair alpha LacZ beta-galactosidase forms and converts X-Gal to blue colorant → blue colonies

α –complementation: an efficient system to monitor insert vector ligation

- **The portion of the *lacZ* gene encoding the first 146 amino acids (the α -fragment) of beta-galacosidase protein: located on the plasmid**
- **The omega subunit is encoded *by the bacteria* 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 OF SUCCESSFULL DNA CLONING EVENTS
BLUE-WHITE SELETION

alpha fragment
(pasmid)



encoded by lacZ gene

omega fragment (larger fragment)
(encoded by E-coli laboratory strain)

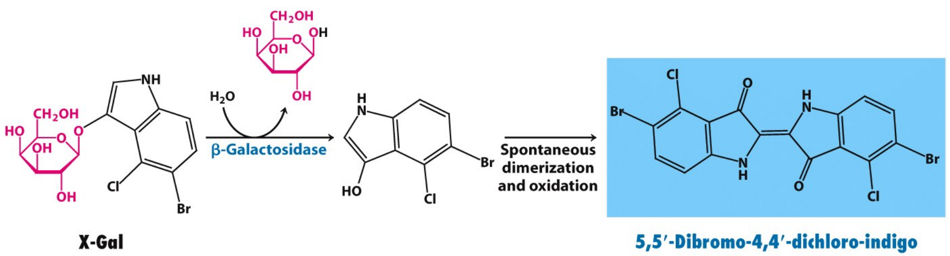


Figure 31.5
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The native E. coli β-galactosidase enzyme can be split in two inactive fragments of different sizes. The smaller fragment, known as the alpha-peptide or enzyme donor, is about 100 amino residues in length and is **inactive** on its own (incapable of hydrolyzing a β-galactosidase substrate). The larger fragment, known as the omega fragment or enzyme acceptor, is about 900 amino residues in length and is also **inactive** on its own. Upon mixing the enzyme donor with the enzyme acceptor, the β-galactosidase enzyme is reconstituted

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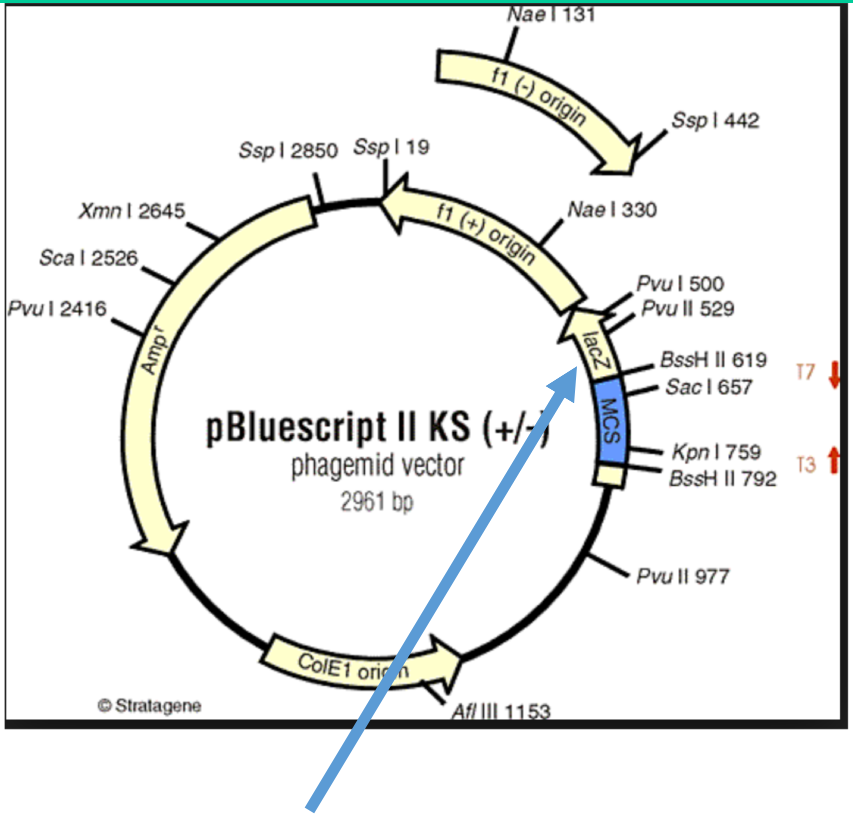
BLUE-WHITE SELETION



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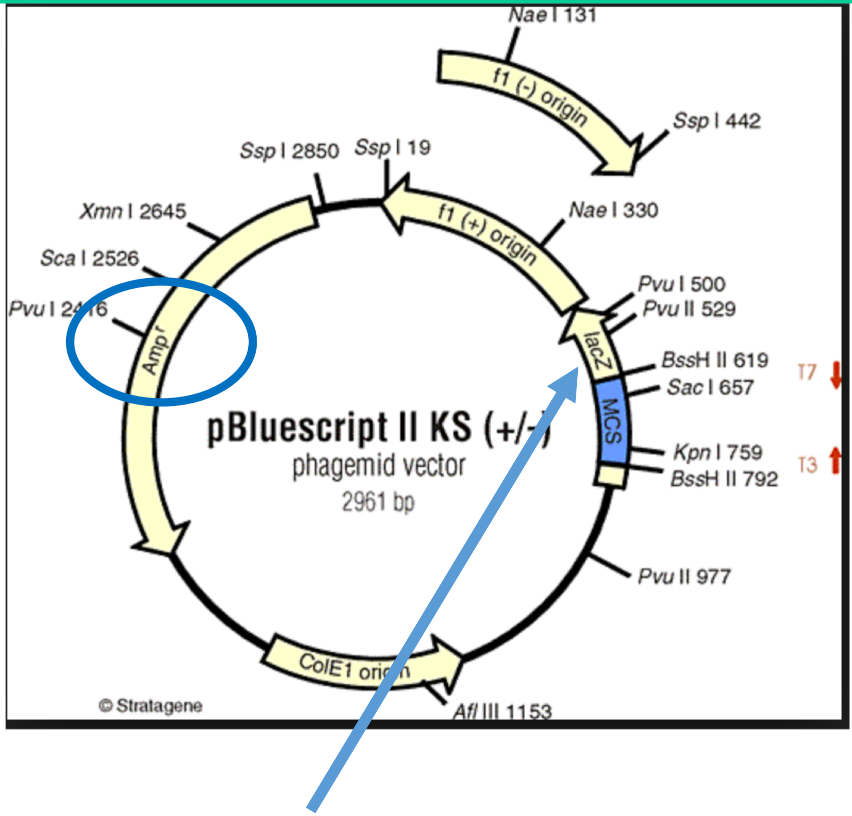
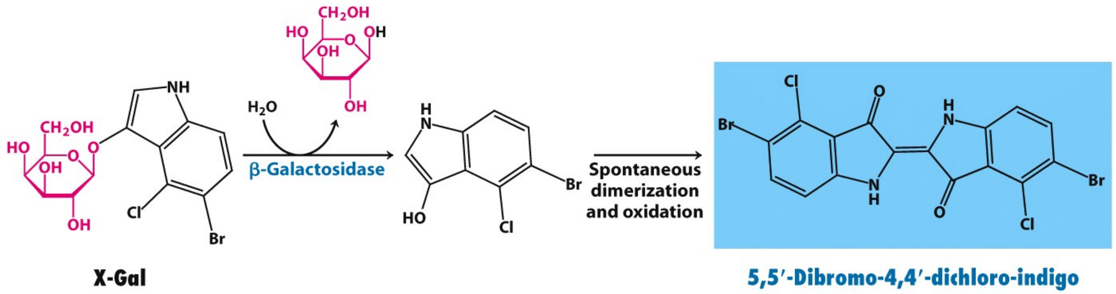
1. EASY IDENTIFICATION SUCCESSFULL DNA CLONING EVENTS
BLUE-WHITE SELETION SCREEN



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

- *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



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

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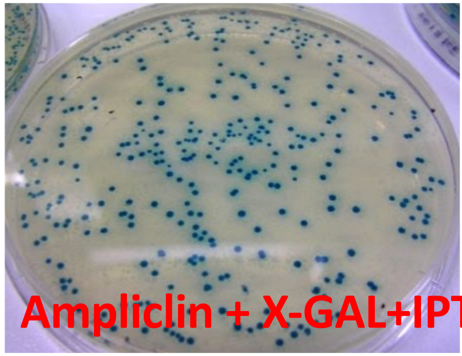
1. EASY IDENTIFICATION SUCCESSFULL DNA CLONING EVENTS
BLUE-WHITE SELETION SCREEN

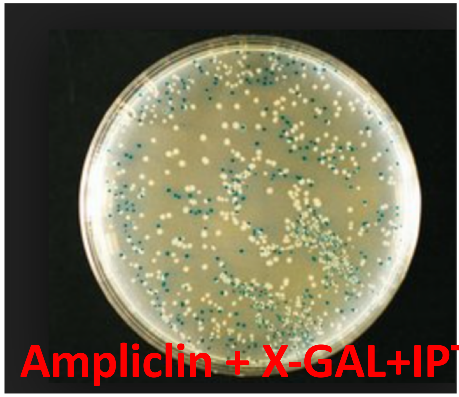
CONTROL LIGATION 1: 50ng plasmid; EcoRI, de-phosph + LIGASE



46 blue colonies

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

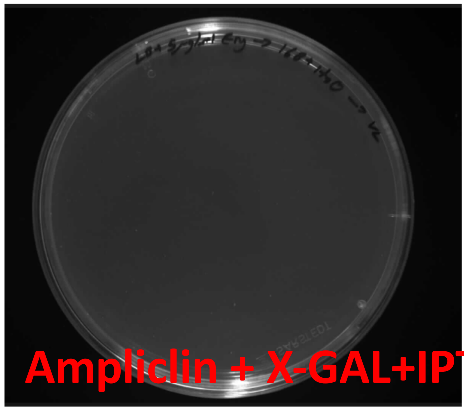
LIGATION : 50ng plasmid; EcoRI, de-phosph + 124ng INSERT + LIGASE



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

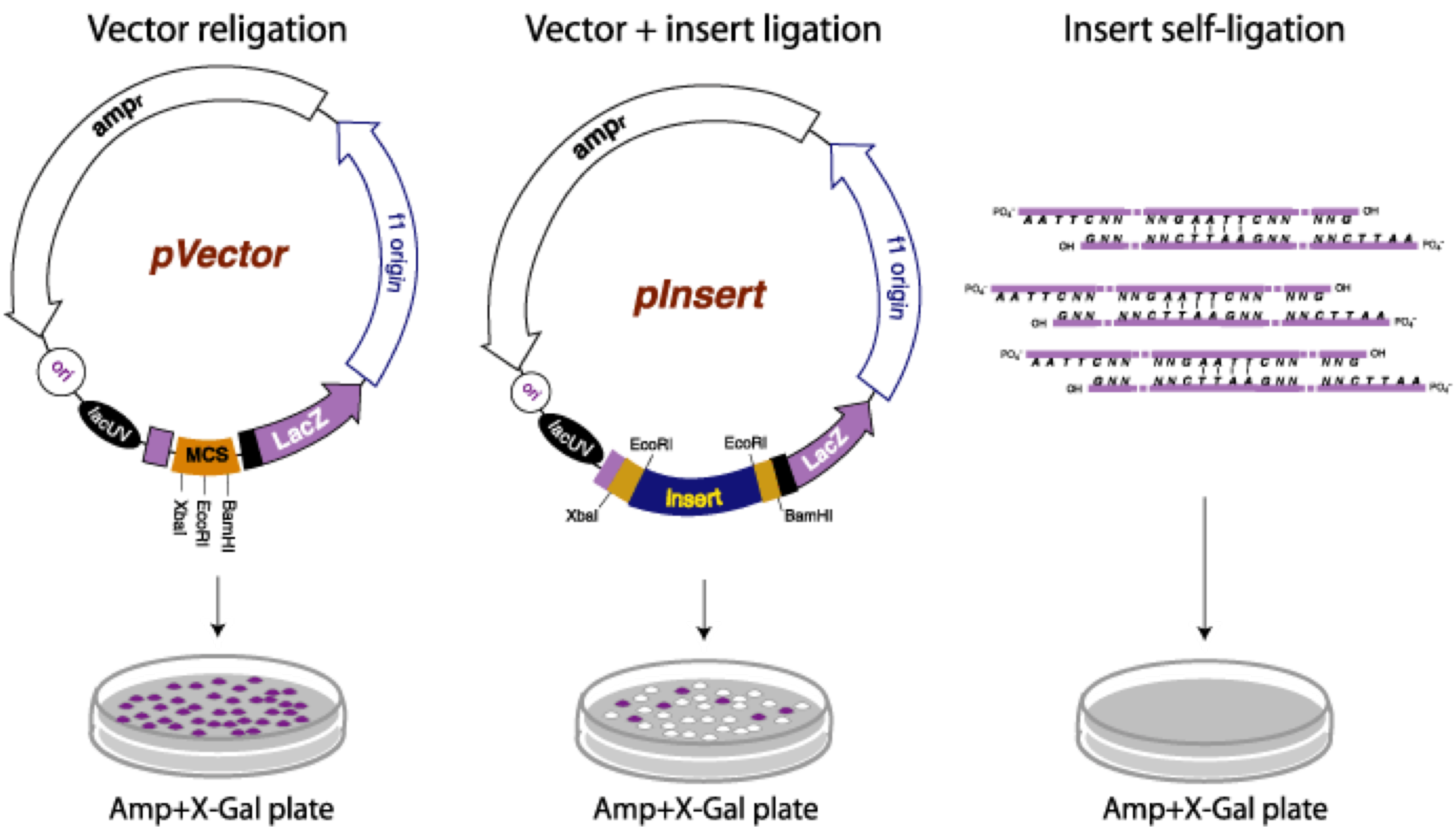


No colonies

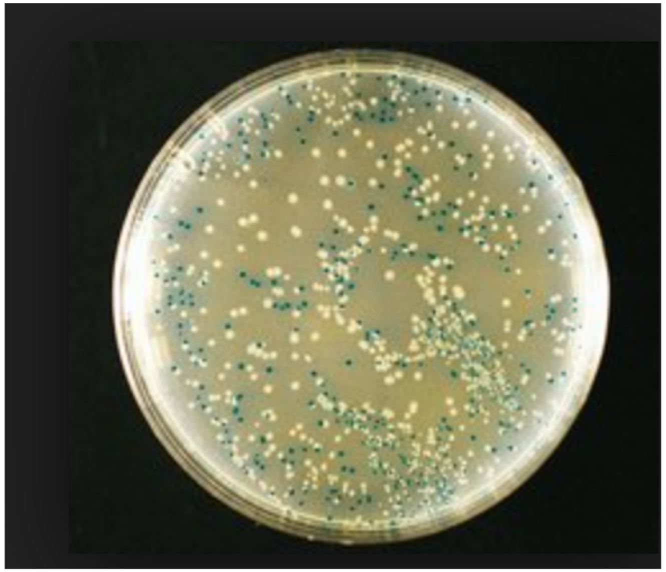
1. **NO COLONIES: no vector backbone present**

For example

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



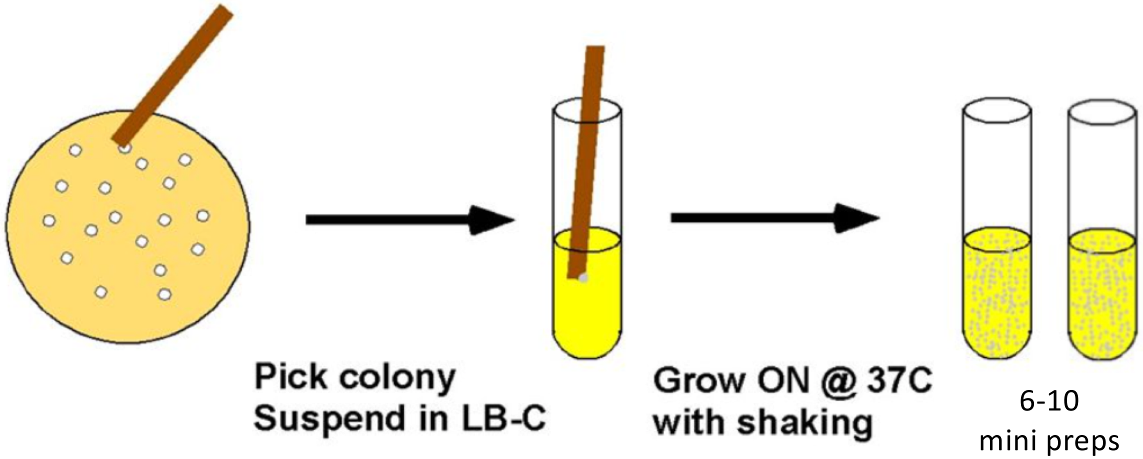
2. DNA PREPARATION AND CONTROL DIGEST



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

Preparation. Grow the bacteria

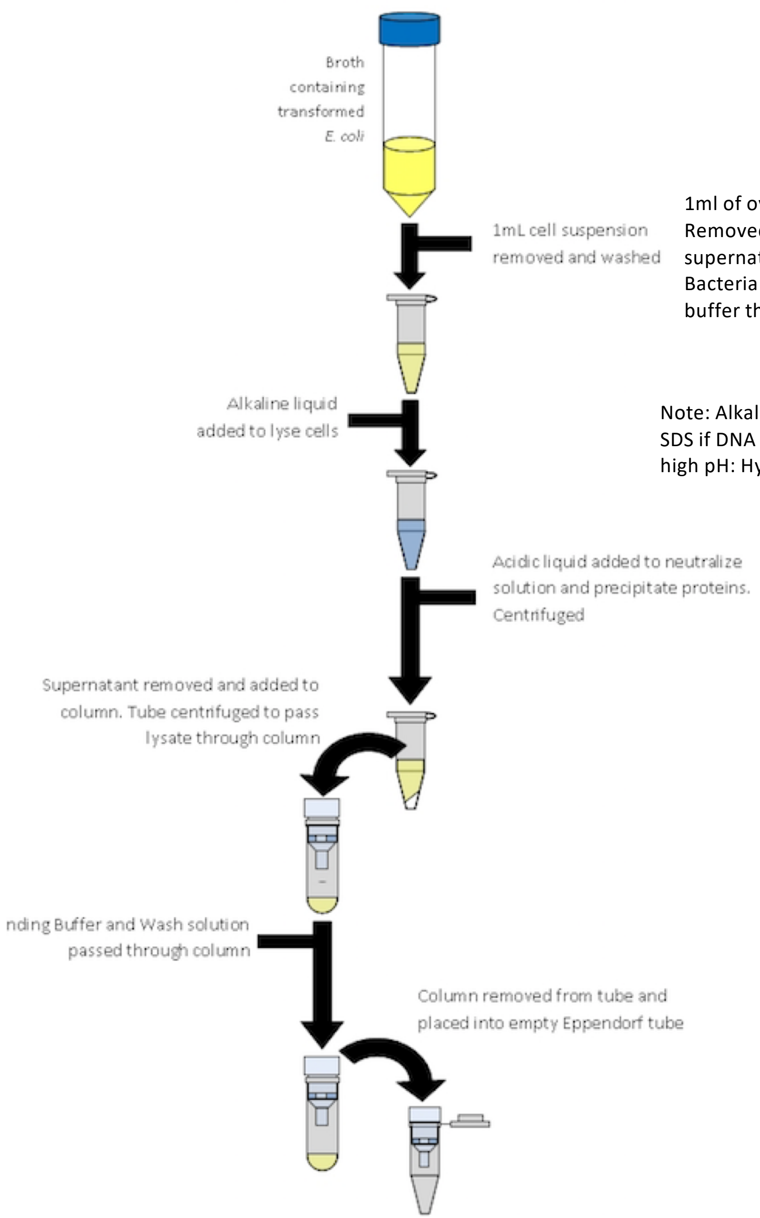
Grow an overnight (ON) culture of the desired bacteria in 2-5 ml of LB medium containing the appropriate antibiotic for plasmid selection. Incubate the cultures at 37°C with vigorous shaking.



Next day: harvest bacteria by centrifugation and prepare plasmid DNA

DNA PREPARATION – Mini prep

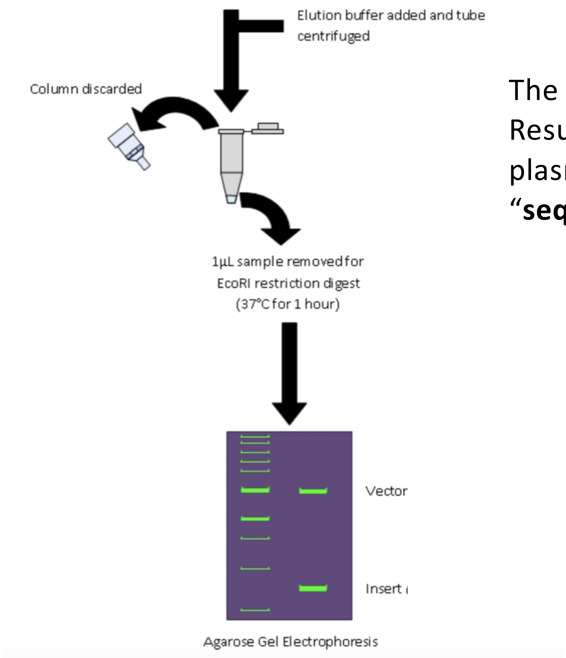
Single Page Protocol for Alkaline Lysis Mini-Plasmid Preparation



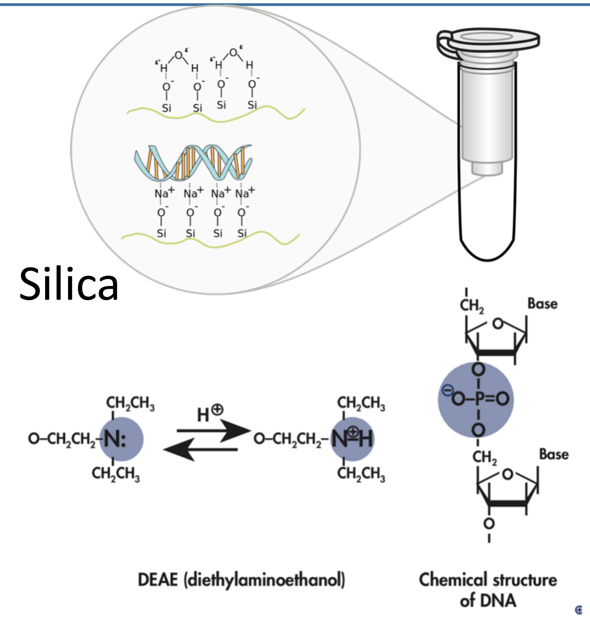
1ml of overnight culture Removed, spinned and supernatant removed. Bacteria pellet resuspendet in buffer that does not kill cells

Note: Alkaline liquid: mix of NaOH and SDS if DNA is too long in solution with high pH: Hydolysis → destroyed

The lysate is neutralized by the addition of acidic potassium acetate; The high salt concentration causes Potassium dodecyl sulfate to precipitate, and the denatured proteins, chromosomal DNA, and cellular debris become trapped in salt–detergent complexes. Plasmid DNA, being smaller and covalently closed, renatures correctly and remains in solution **Centrifugation an high speed** (ca. 13.000 rpm); cell debries and genomic DNA precipitate; small DNA molecules (plasmid remain in supernatant)

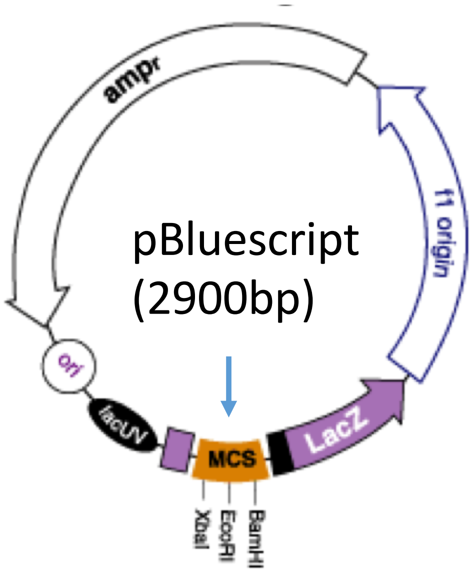


The use of columns Results in very pure plasmid DNA. “**sequence grade**”



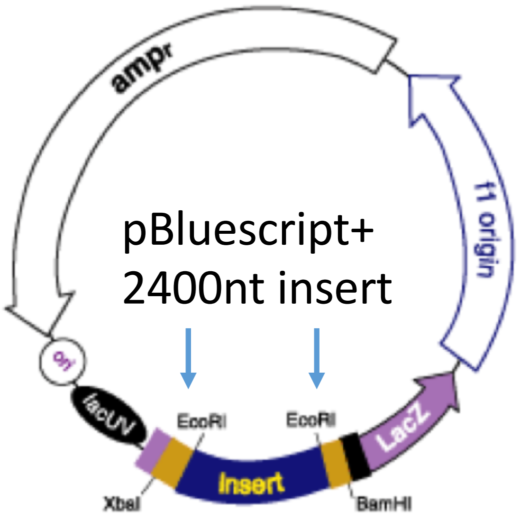
Resin in column Is positivley charged: Binds negative charge of plasmid DNA backbone

CONTROL DIGEST TO INDETIFY SUCCESSFUL CLONING EVENT



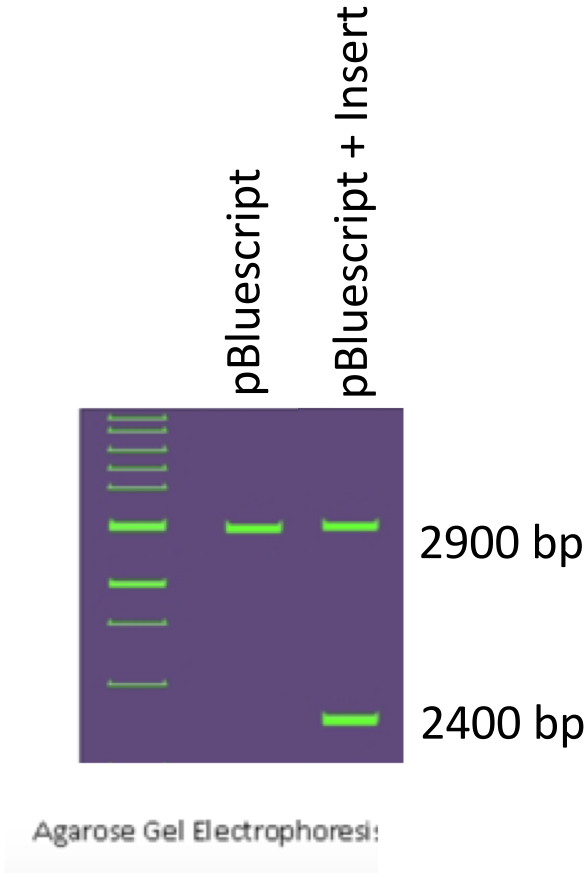
+ EcoRI
(taglia 1x)

2900 bp (linearized)



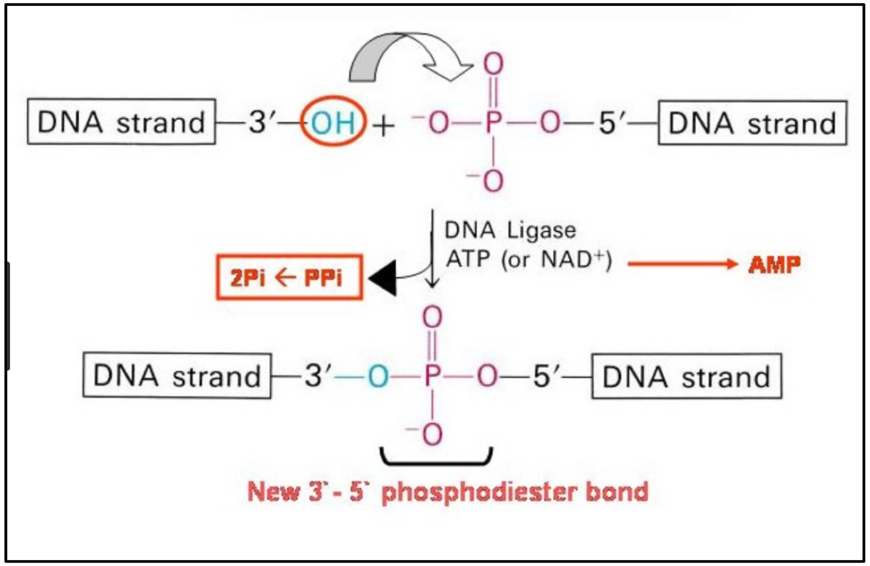
+ EcoRI
(taglia 2x)

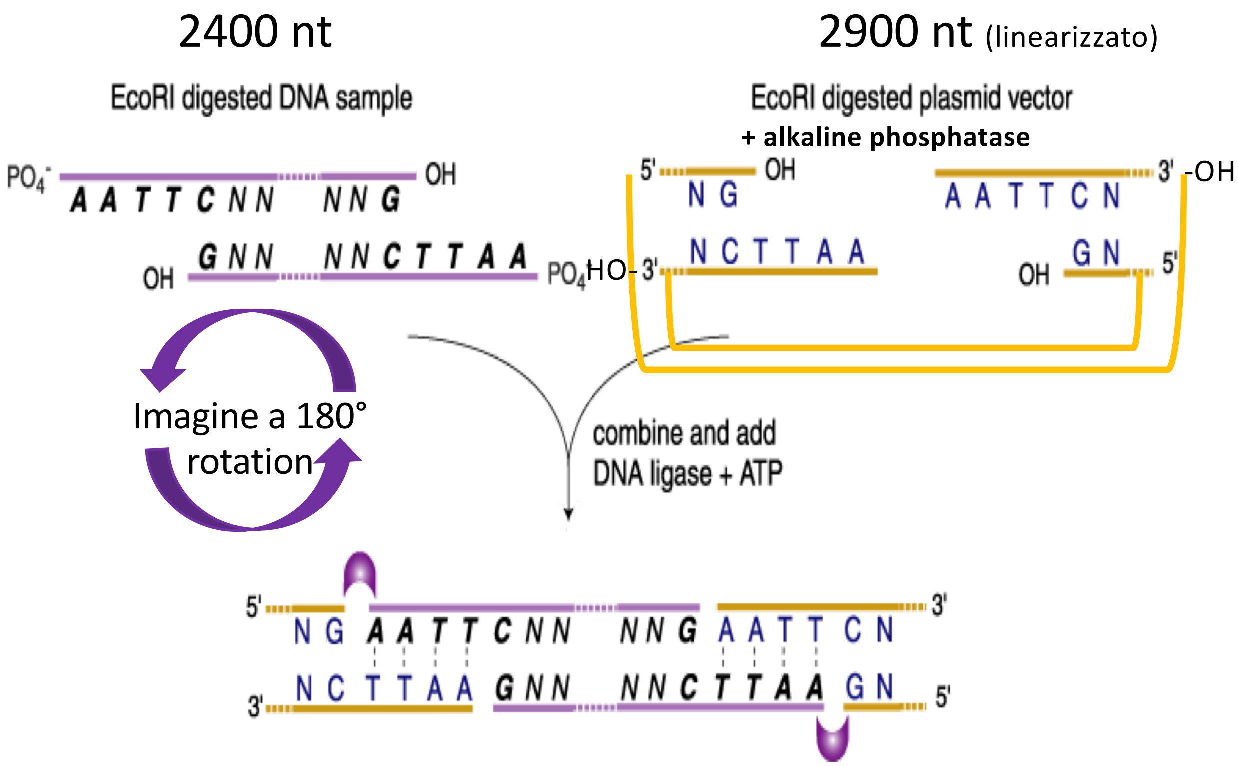
2900 bp
2400 bp



→ **REMEMBER: RESTRICTION MAPPING !!!!**

....but we have to consider something....

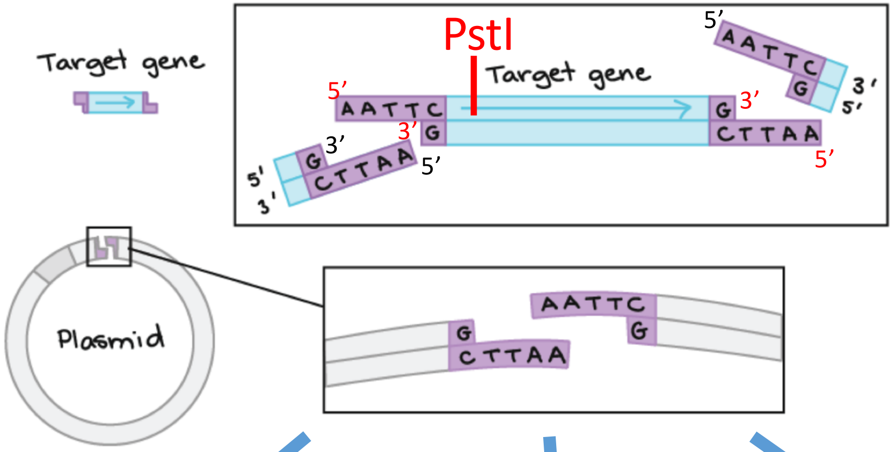




Attention: All involved overhangs are compatible

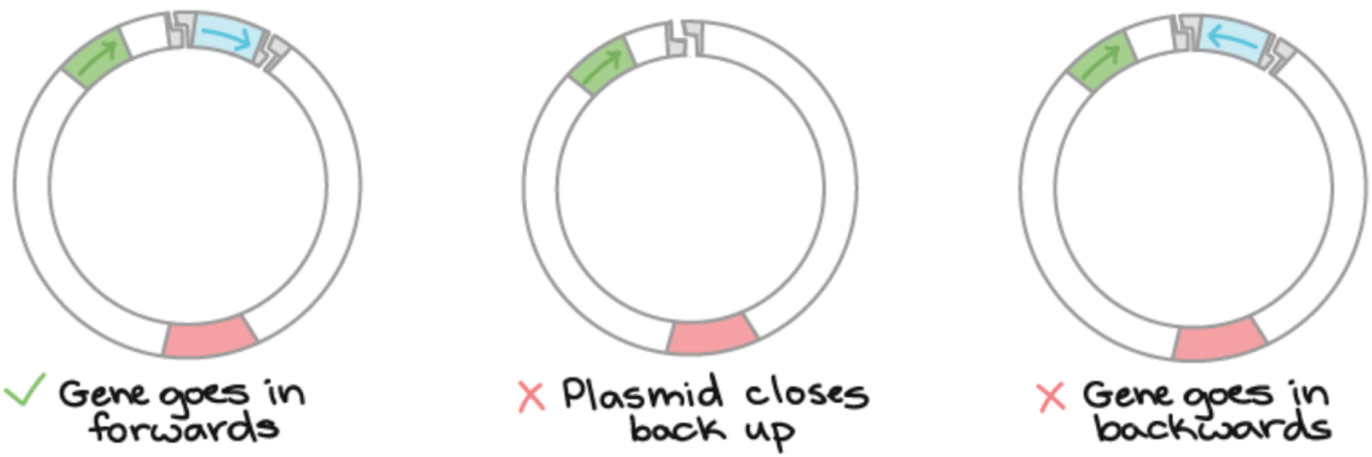
→ insert can be "ligated" into vector in both orientations

DNA PREPARATION AND CONTROL DIGEST – ORIENTATION OF INSERT?



Blue-white selection is OK…
….but how can we control the orientation of the insert???

3 possible products of ligation of vector



White colony

Blue colony

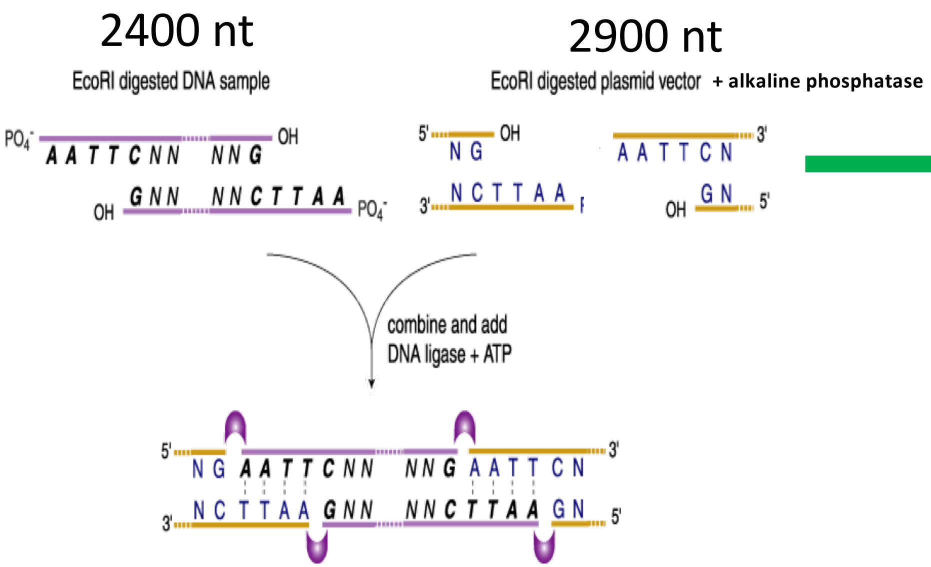
Not efficient dephosphorylation
Contamination with circular plasmid

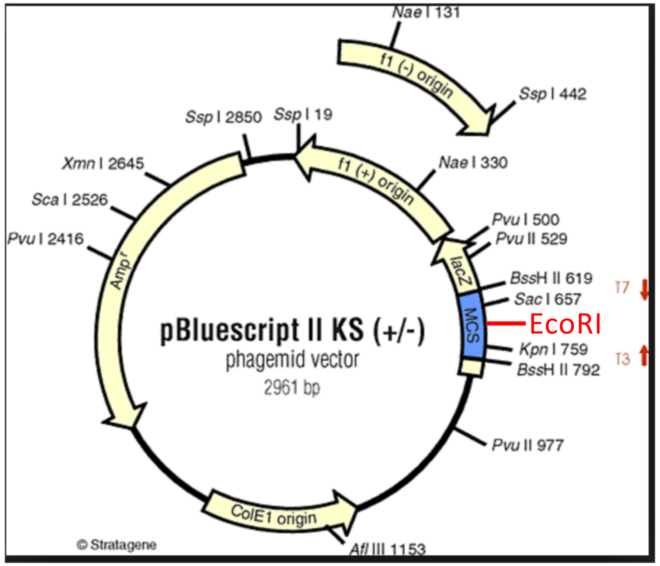
White colony

HOW CAN WE DETERMINE THE ORIENTATION AND IDENTITY OF THE INSERT?

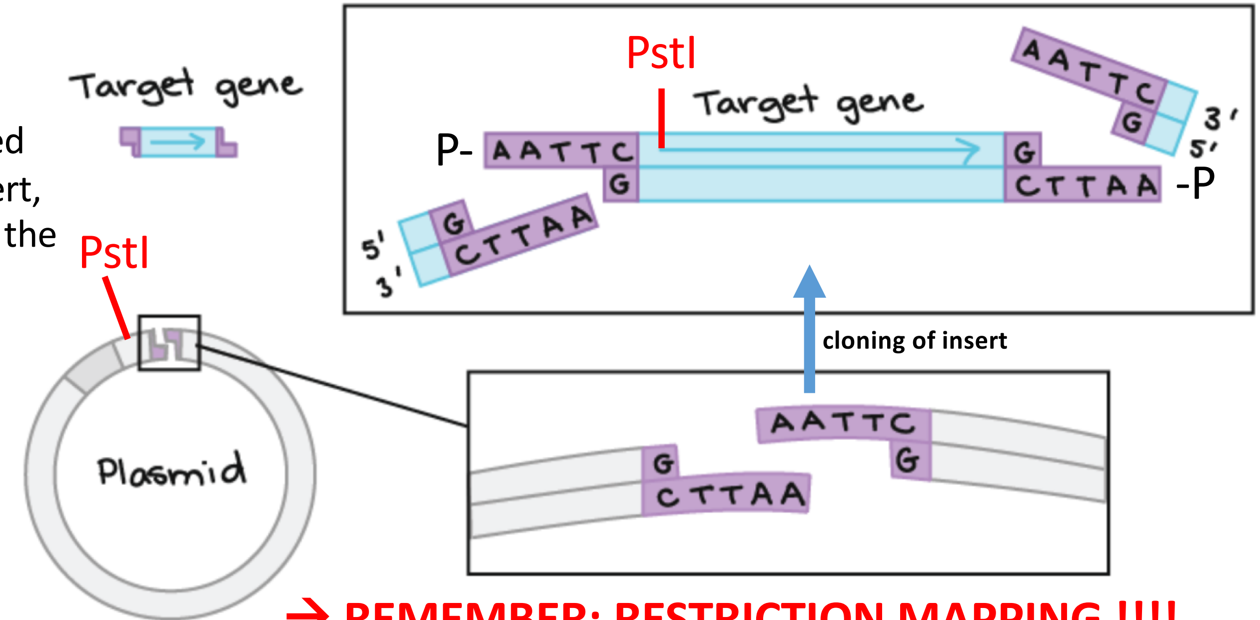
Blue-white screening using for example the pBluescript vector

DNA PREPARATION AND CONTROL DIGEST – ORIENTATION OF INSERT?





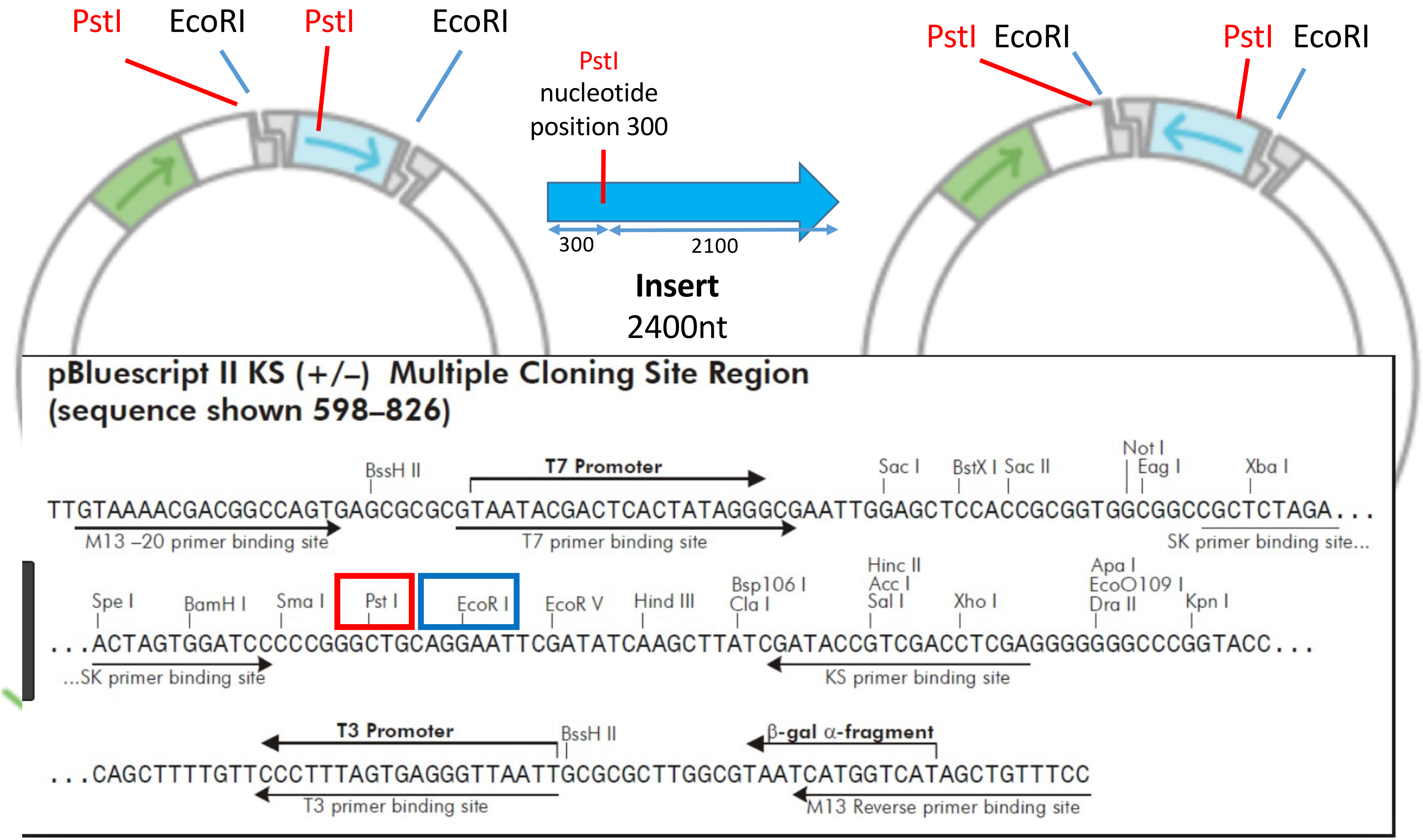
Select resitriction site located "asymetrically" in insert, ...and that is located in the MCS in vector



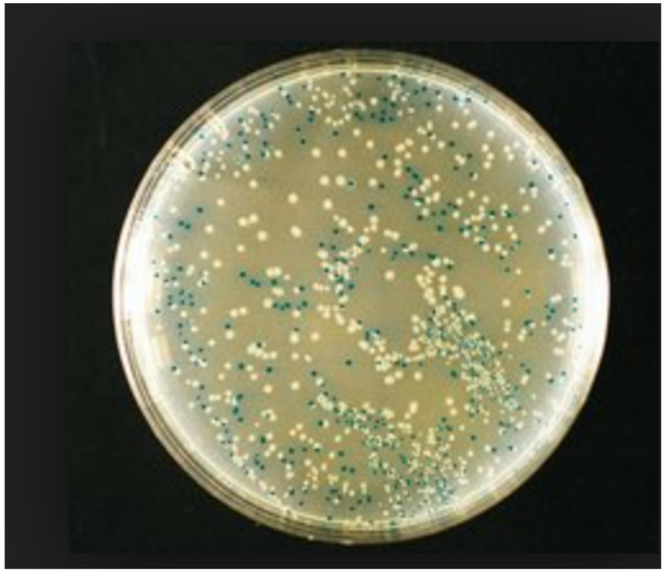
Note:
5'overhangs of insert and linearized plasmids are compatible; both have been cut with EcoRI.
Ligase covalently links both molecules
EcoRI overhangs are reconstiuted resulting 2 EcoRI sits that flank the insert sequence!!!

→ REMEMBER: RESTRICTION MAPPING !!!!

DNA PREPARATION AND CONTROL DIGEST – ORIENTATION OF INSERT?



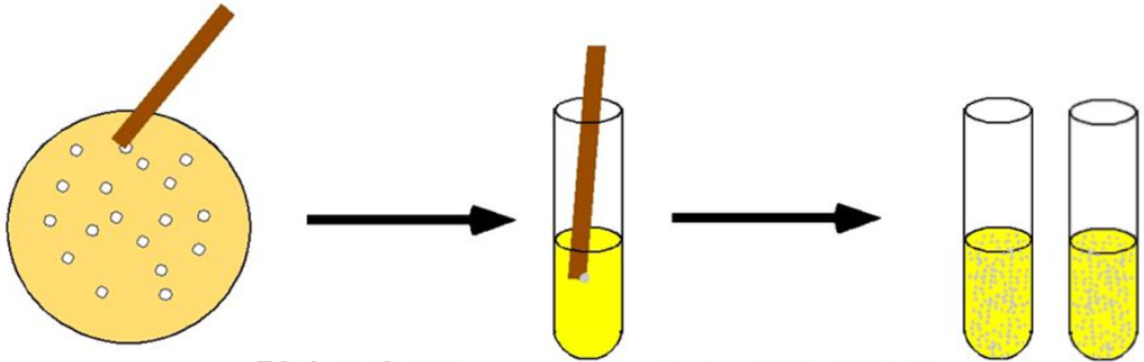
A CONTROL DIGEST IS PERFORMED ON MULTIPLE COLONIES OBAINED FROM CLONING EXPERIMENT (5-10)



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

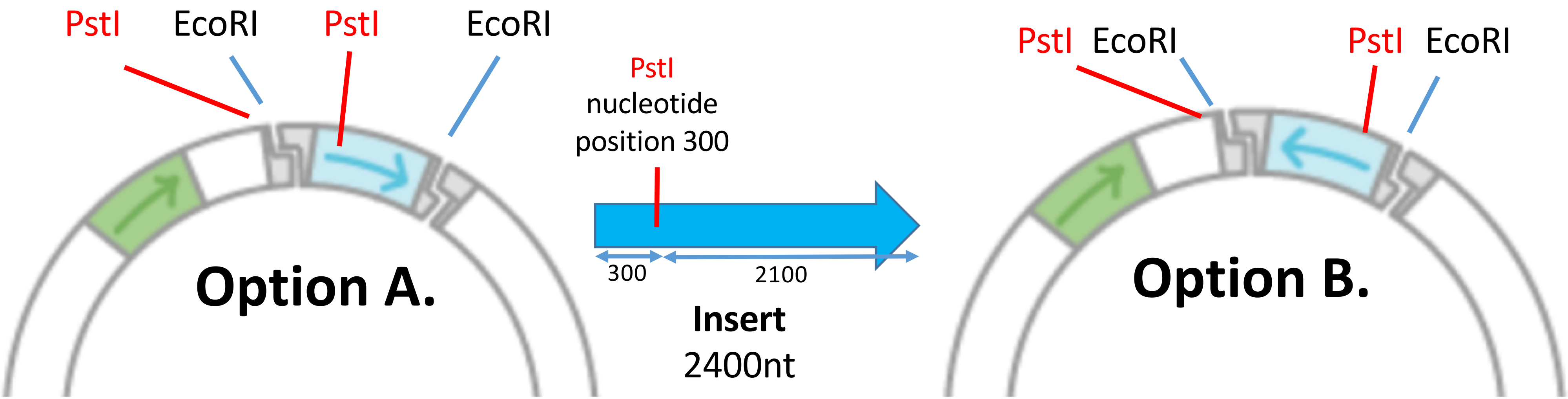
Preparation. Grow the bacteria

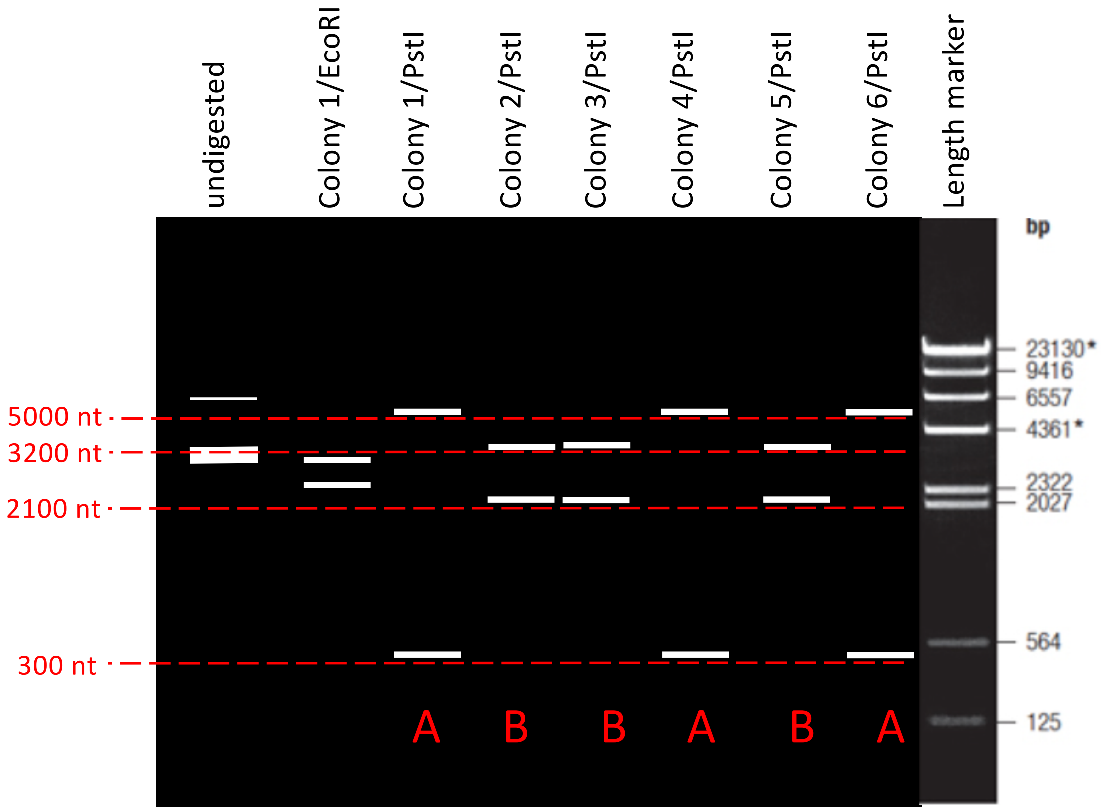
Grow an overnight (ON) culture of the desired bacteria in 2-5 ml of LB medium containing the appropriate antibiotic for plasmid selection. Incubate the cultures at 37°C with vigorous shaking.



Next day: harvest bacteria by centrifugation and prepare plasmid DNA

3. DNA PREPARATION AND CONTROL DIGEST – ORIENTATION OF INSERT?



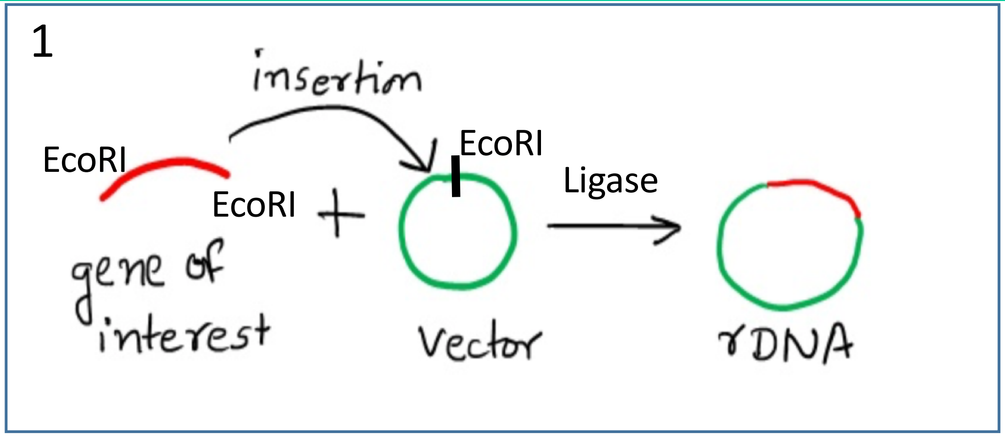


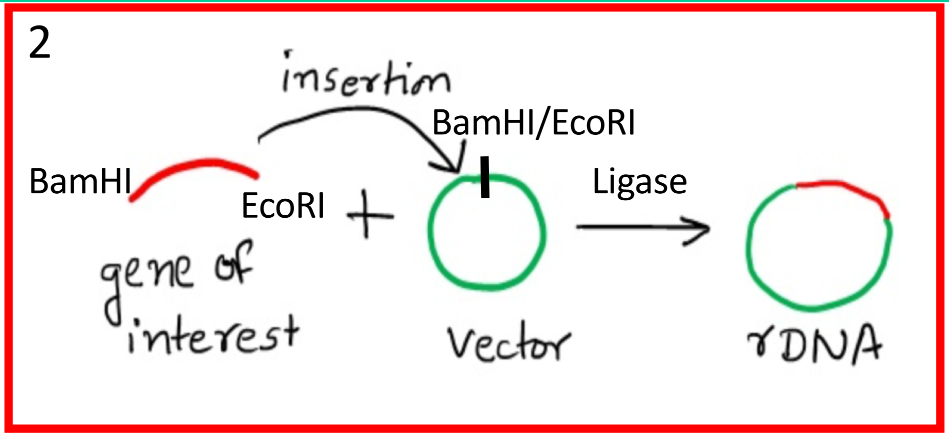
Insert: 2400 nt

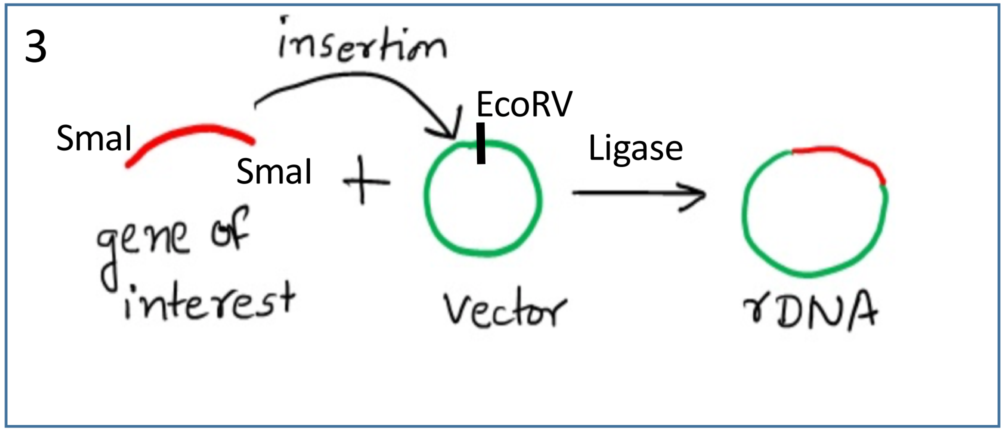
Plasmid: 2900 nt

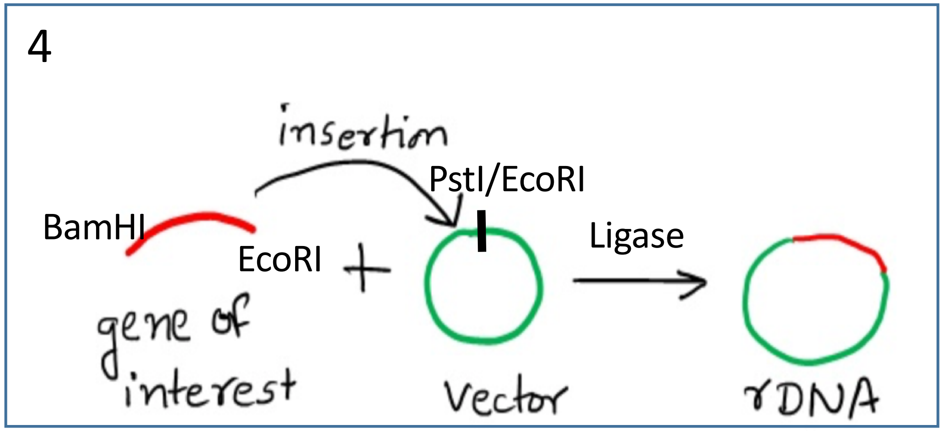
Cut with restriction enzyme that result asymetric digestion products

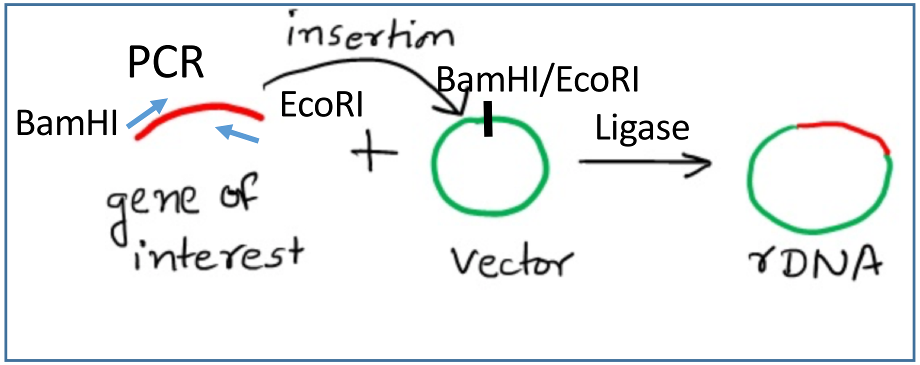
OVERVIEW OVER ON CLONING STRATEGIES





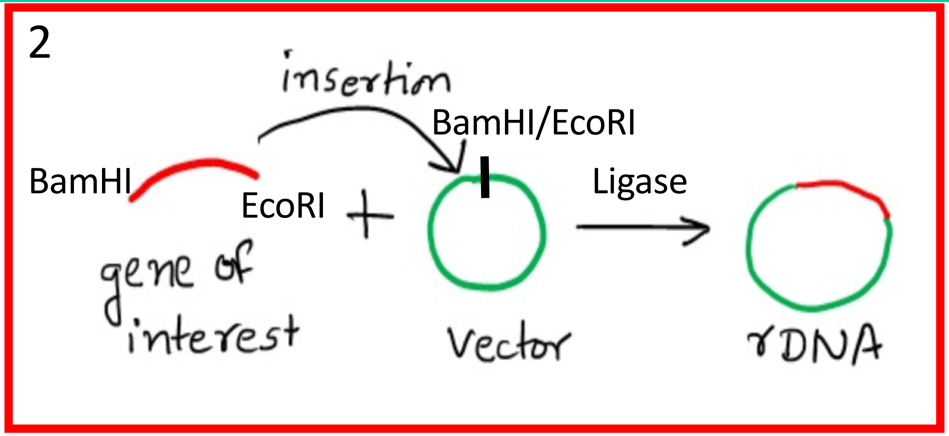






OVERVIEW OVER ON CLONING STRATEGIES

2. Fragment and Insert are cut with 2 (the same) restriction enzymes



DIRECTIONAL CLONING

→ Always preferred cloning strategy

DNA CLONING WITH 2 COHESIVE OVERHANGS

DIRECTIONAL CLONING

→ Generation of cohesive (dirctional) end between insert and vector

→ Always preferred cloning strategy

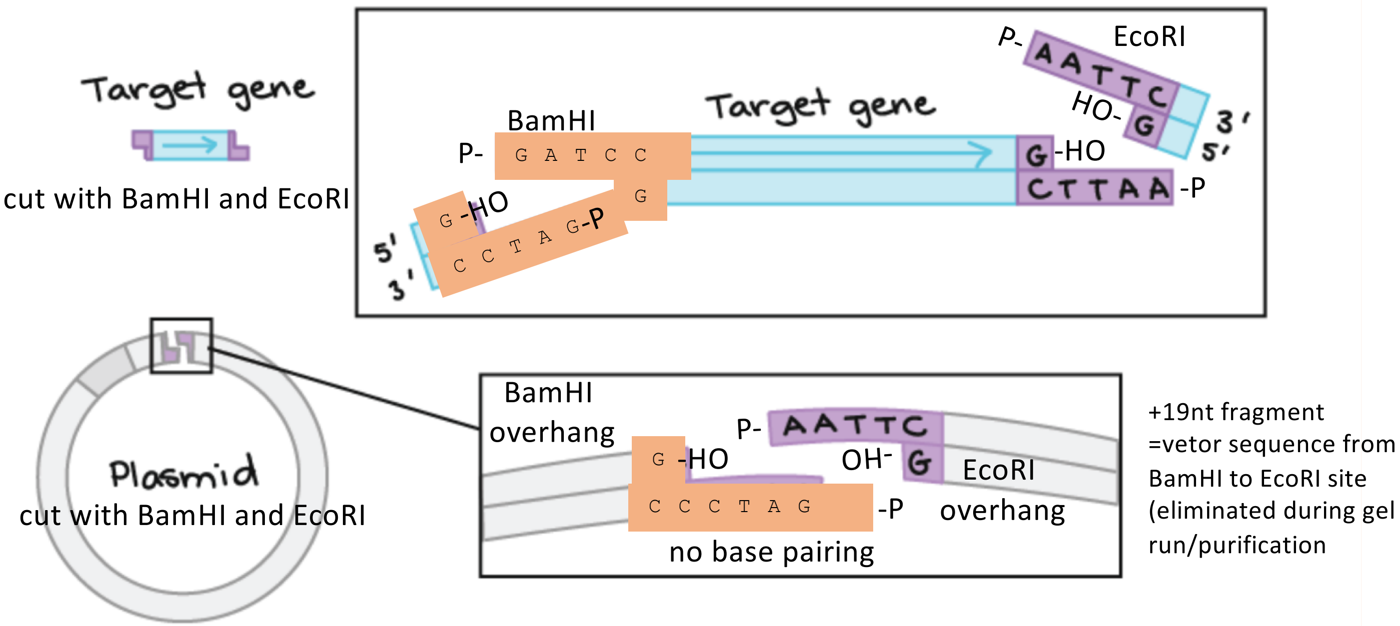
→ No vector dephosphorylation required

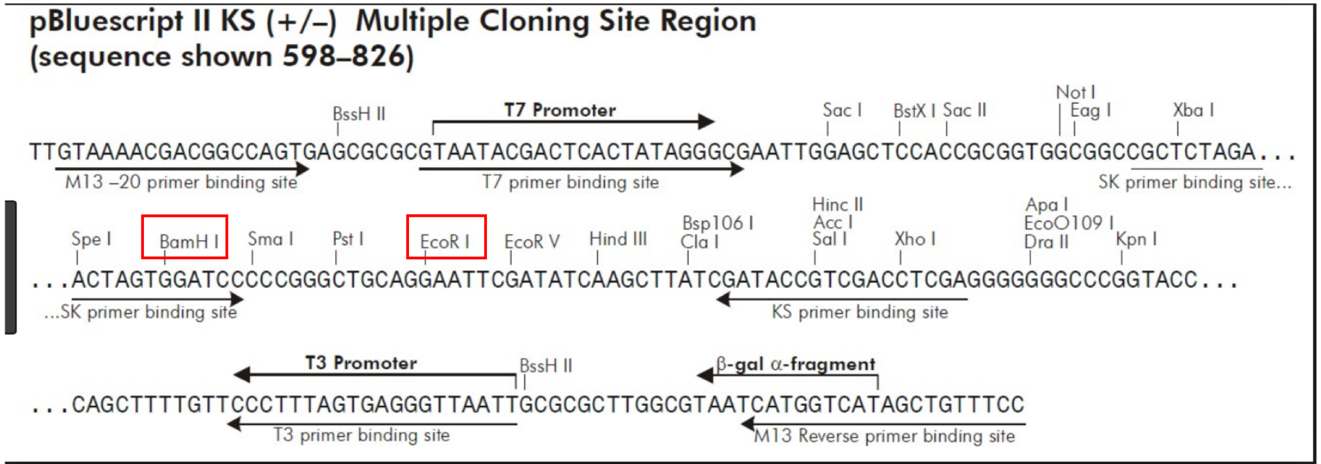
EcoRI: G/AATTC
CTTAA/G

BamHI: G/GATCC
CCTAG/G

INSERT

BACKBONE

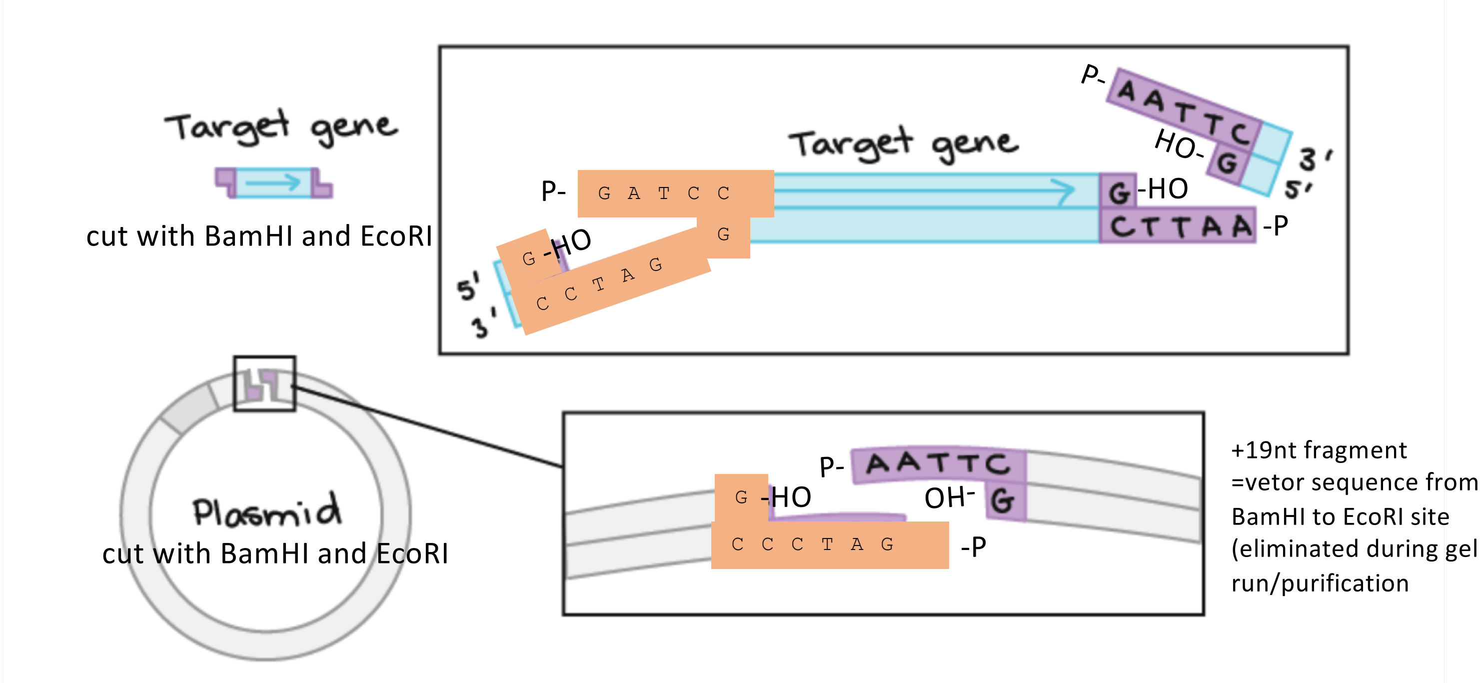




DNA CLONING WITH 2 COHESIVE OVERHANGS

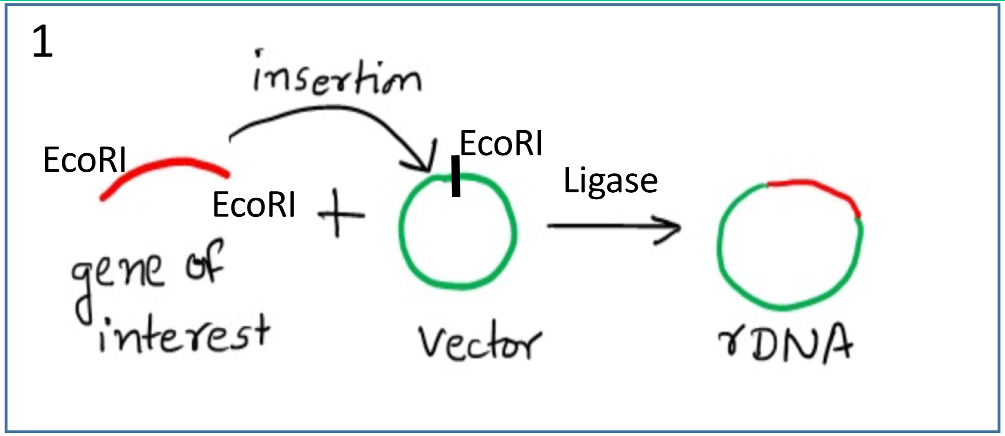
EcoRI: G/AATTC
CTTAA/G

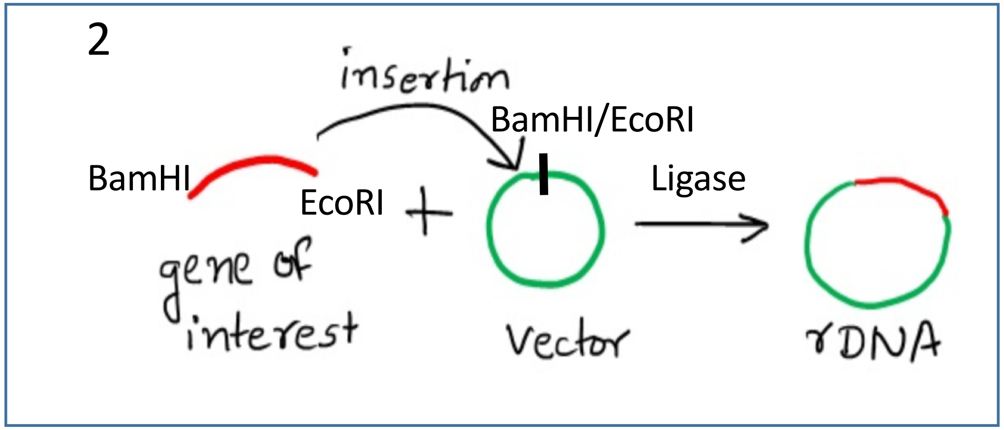
BamHI: G/GATCC
CCTAG/G

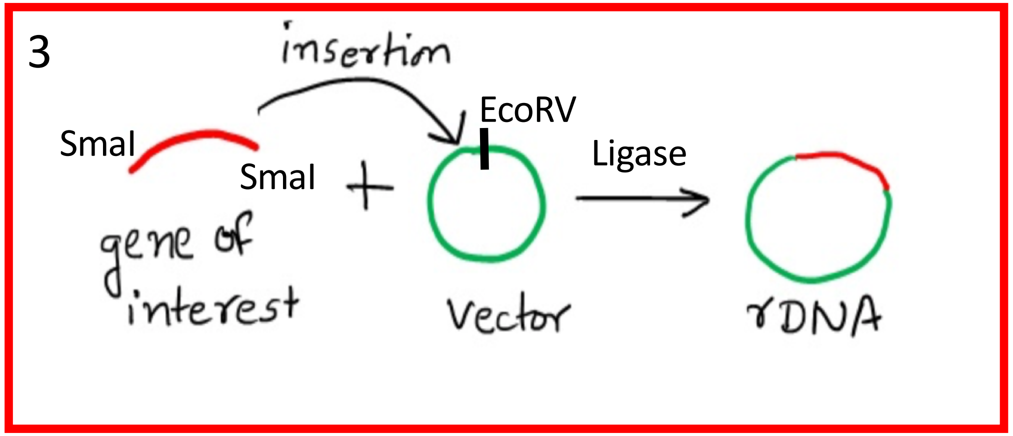


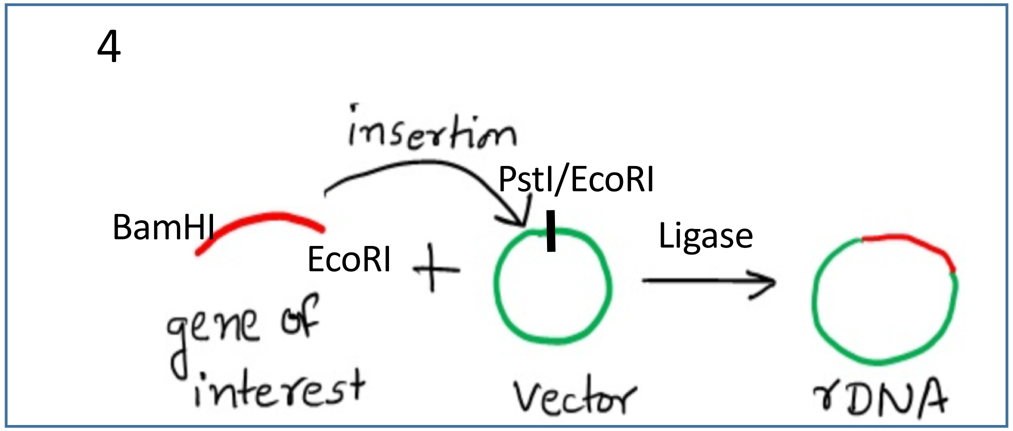
1. EcoRI/BamHI digest to obtain insert
2. EcoRI/BamHI digest to obtain linearized pBluescript
3. Gel run and purification of relevant DNA fragments
4. Set up ligation (plasmid:insert = 1:3)
5. Transform competent bacteria; plate on agar plates + X-GAL, IPTG, ampicillin → pick white colony → make liquid bacterial culture
6. Plasmid preparation and control digest to verify presence of correct insert
7. IMPORTANT: NO ALKALINE PHOSPHATASE REQUIRED → EcoRI and BamHI do not represent cohesive ends!!
8. IMPORTANT: ORIENTATION OF INSERT IS ALWAYS THE SAME!!!

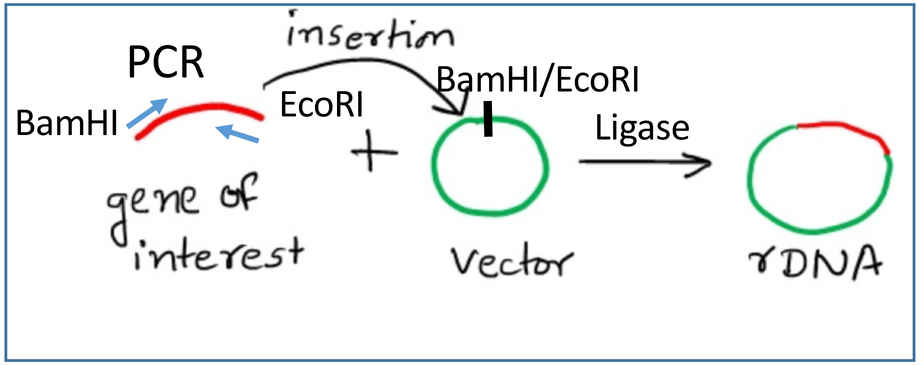
OVERVIEW OVER OTHER CLONING STRATEGIES



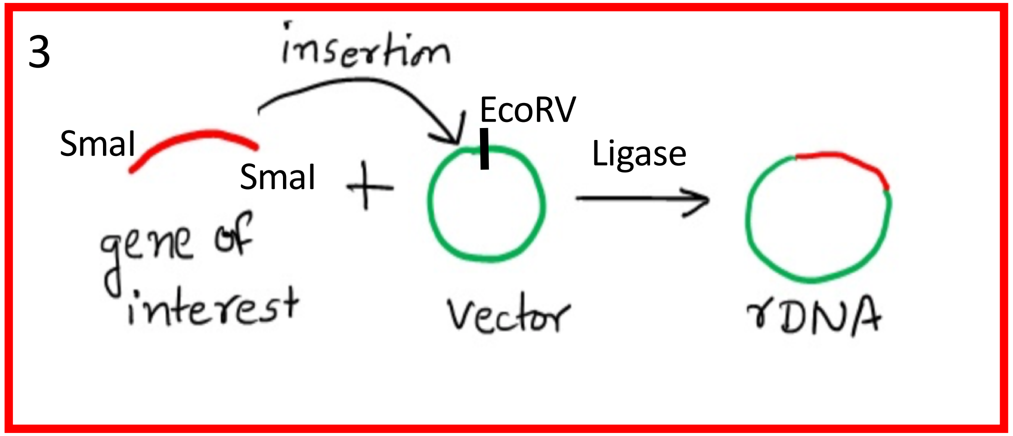








3. Fragment and insert are cut with enzymes that give blunt ends



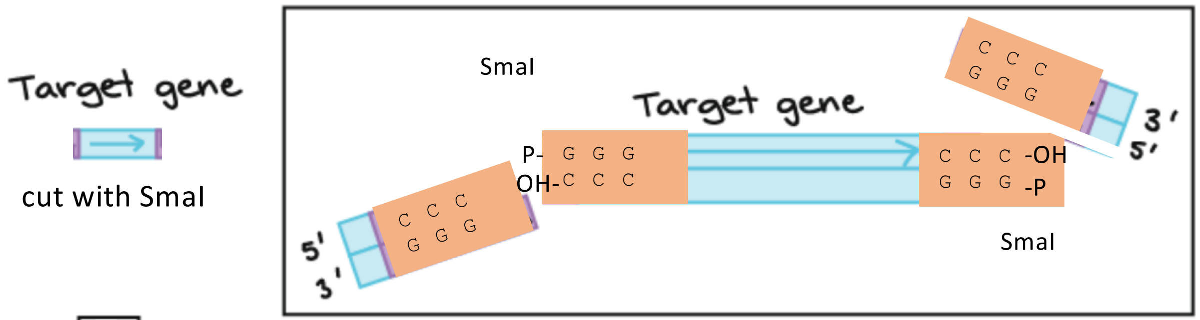
DNA CLONING WITH BLUNT ENDS

SmaI: CCC/GGG
GGG/CCC

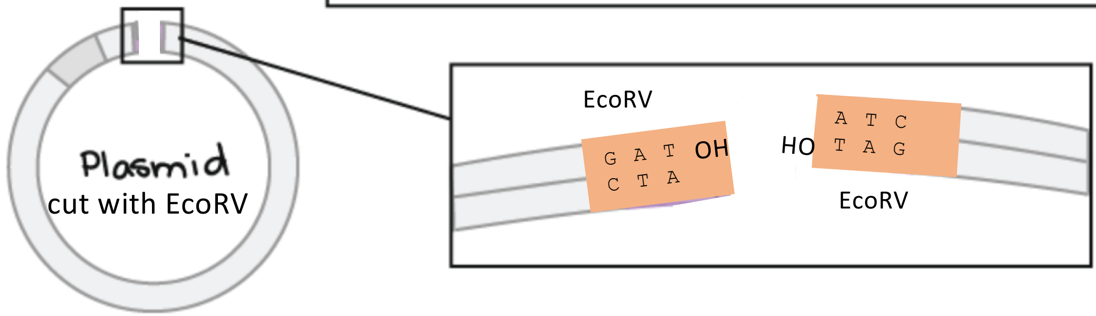
EcoRV: GAT/ATC
CTA/TAG

→ Blunt ends in vector require dephosphorylation

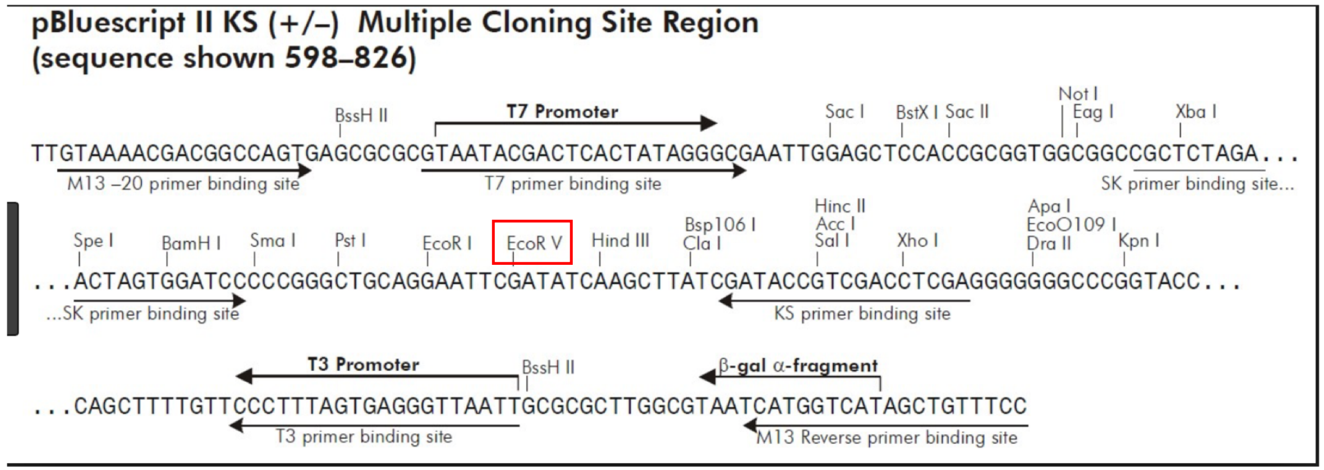
INSERT



BACKBONE



+Linearized with EcoRV and dephosphorylation using alkaline phosphatase



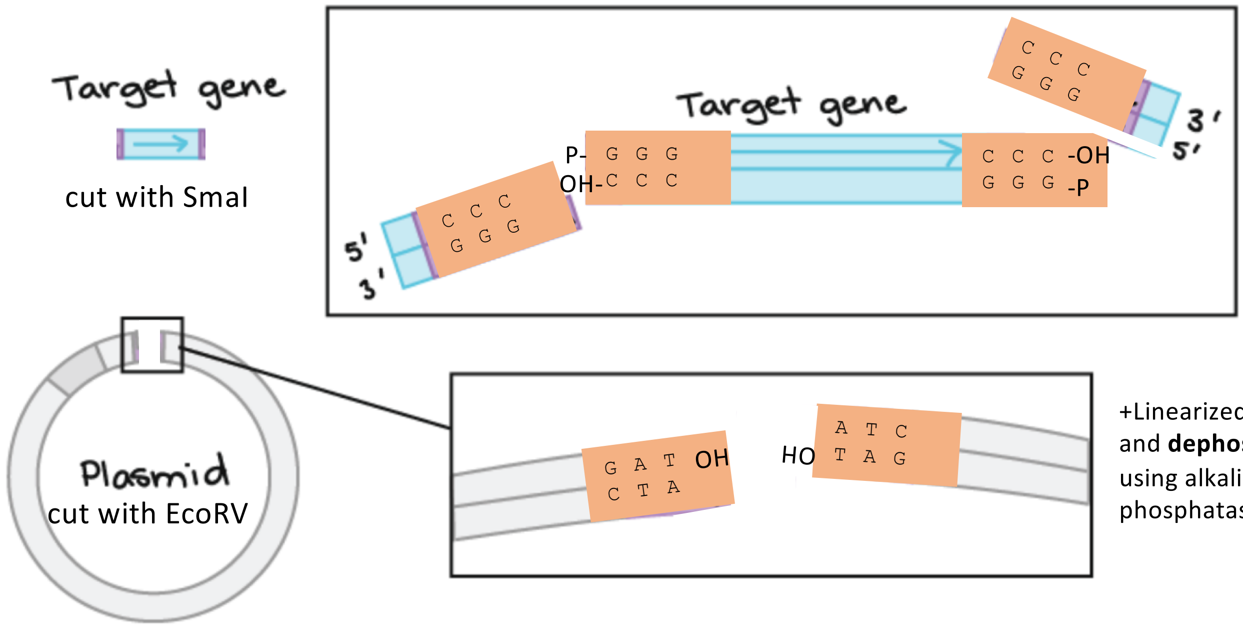
DNA CLONING WITH BLUNT ENDS

SmaI: CCC/GGG
GGG/CCC

EcoRV: GAT/ATC
CTA/TAG

INSERT

BACKBONE



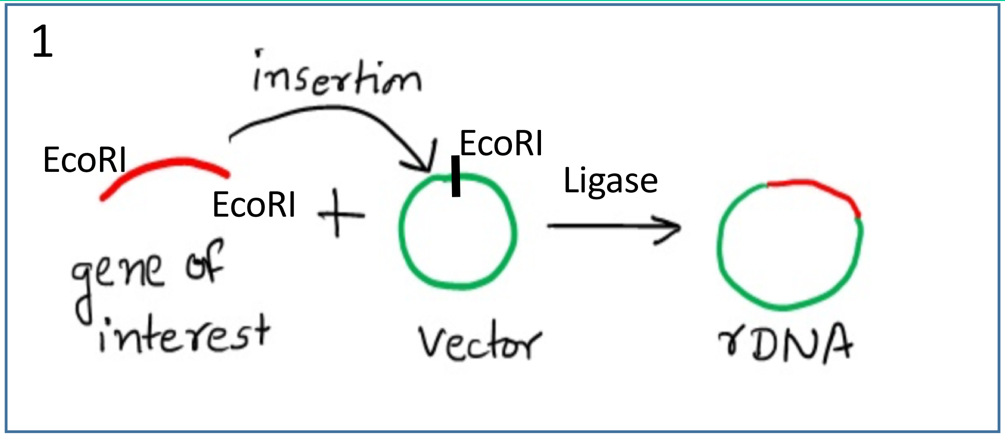
+Linearized with EcoRV and **dephosphorylation** using alkaline phosphatase

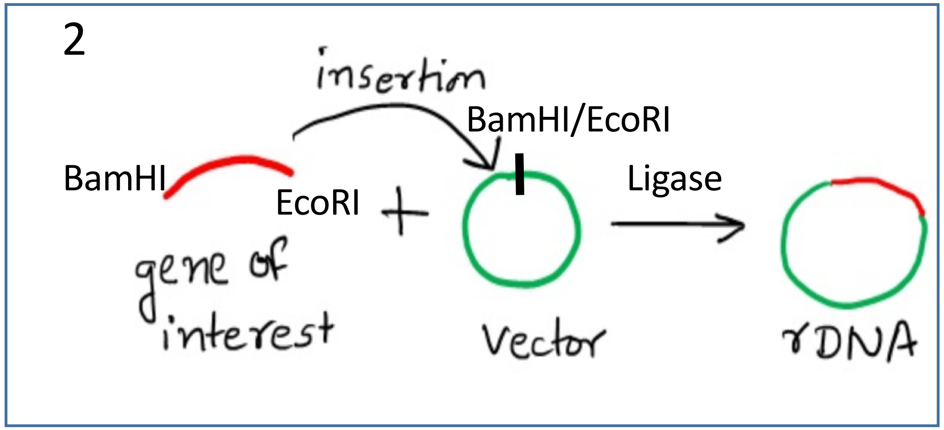
SITES AFTER LIGATION

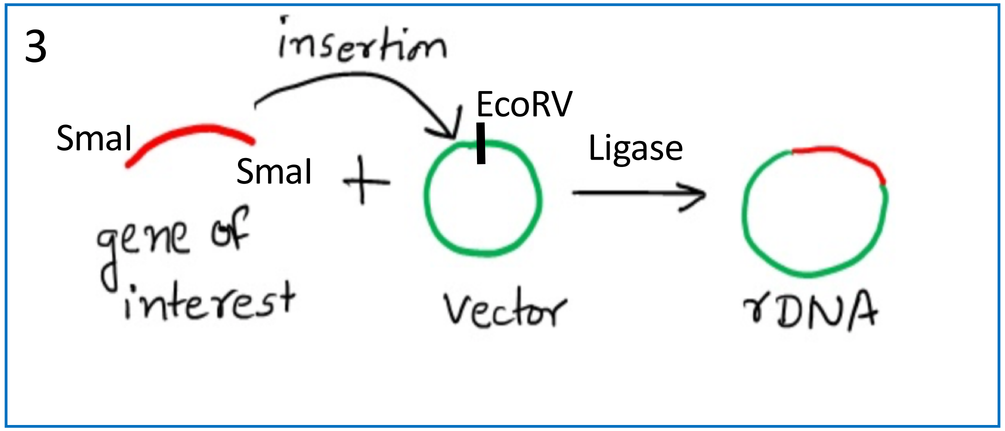
Backbone	INSERT	Backbone
GATGGG	———	CCCATC
CTACCC	———	GGGTAG

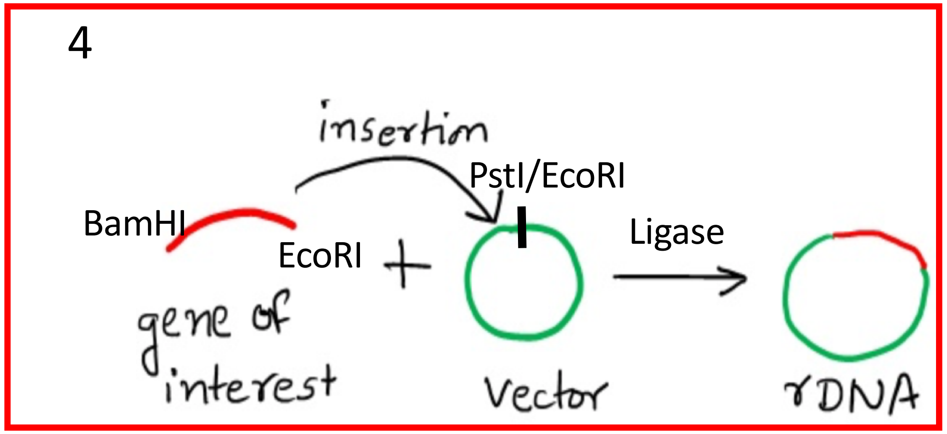
1. SmaI digest to obtain insert
2. EcoRV digest + alkaline phosphatase treatment to obtain linearized pBluescript (that connot re-ligate)
3. Gel run and purification of relevant DNA fragments
4. Set up ligation (plasmid:insert = 1:3 (5))
5. Transform competent bacteria; plate on agar plates + ampicillin → pick colony → make liquid bacterial culture
6. Plasmid preparation and control digest to verify presence of correct insert → insert can be inserted in both orientations!!
7. IMPORTANT: SmaI sites are fused to EcoRV site ➔ cannot be cleaved by SmaI or EcoRV
8. Chose resitrction enzymes for control digest that allow to identify orientation of insert.

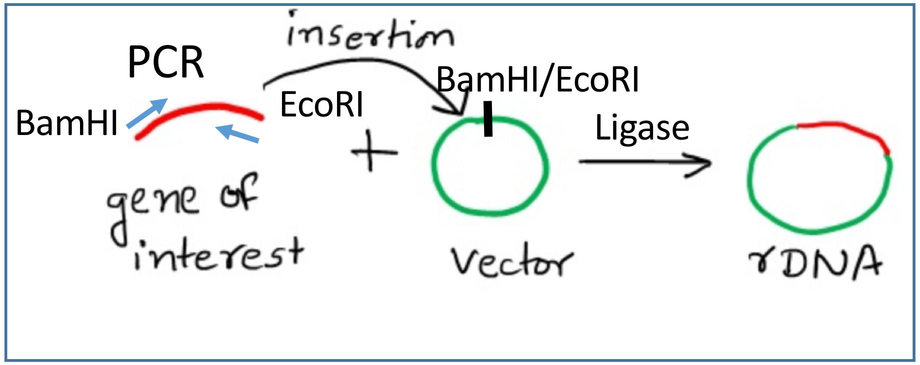
OVERVIEW OVER OTHER CLONING STRATEGIES





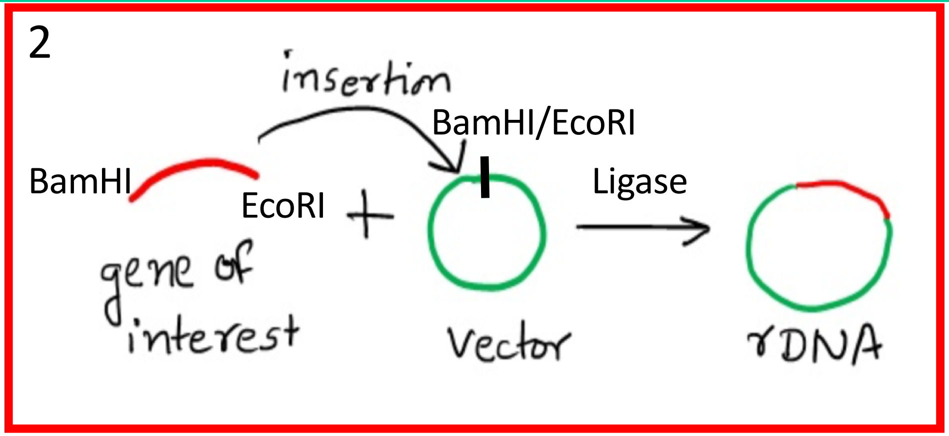






3. Fragment and Insert are cut with enzymes that do NOT have cohesive ends

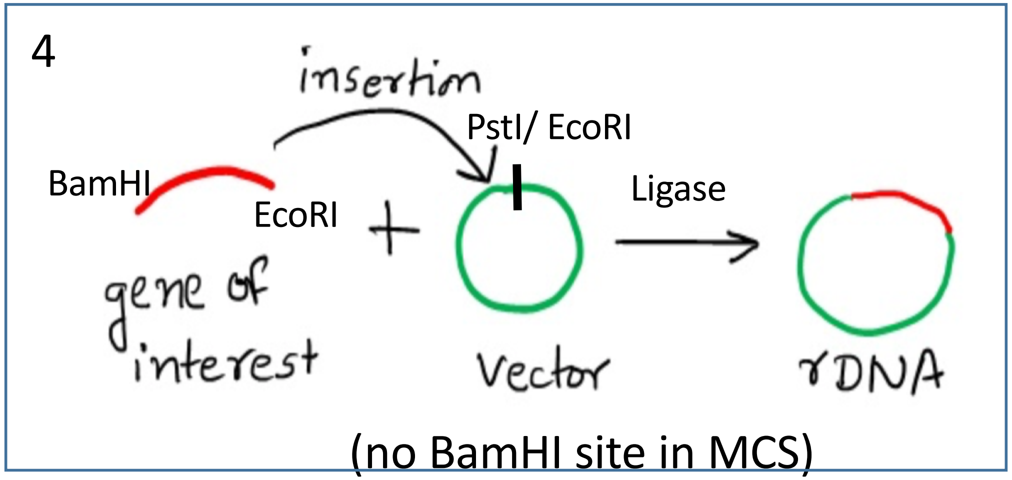
→ Extensive modification of termini
→ Strategy avoided, if possible



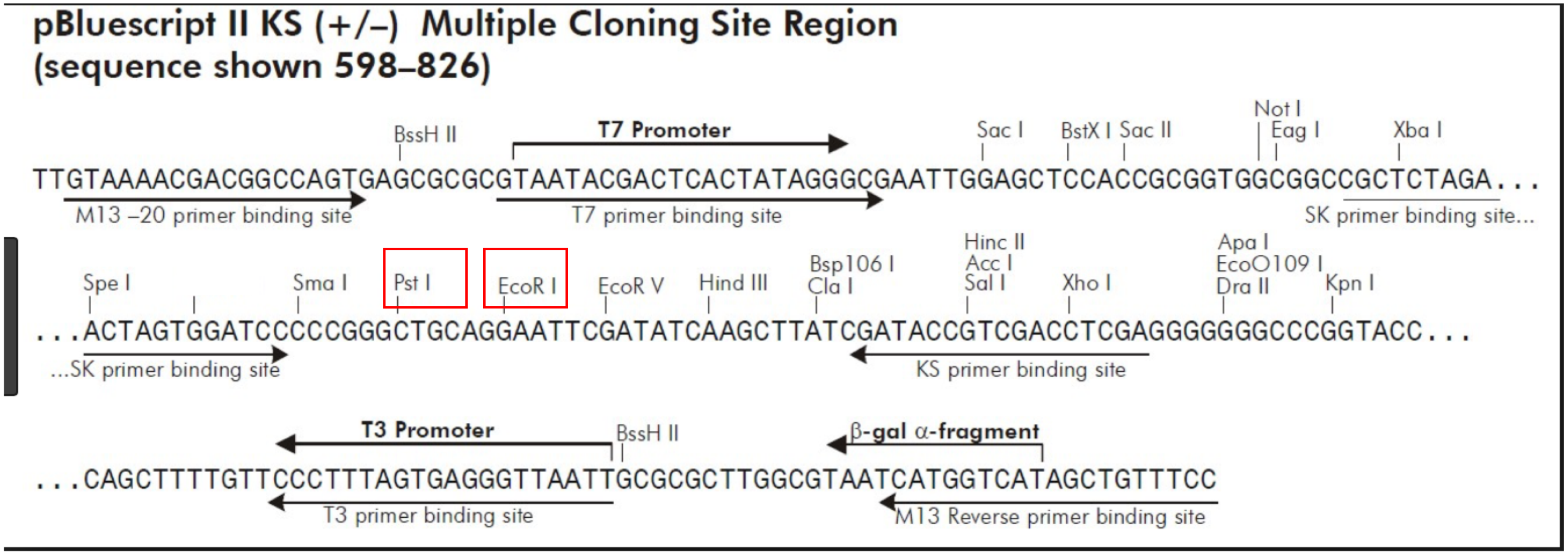
DNA CLONING WITH MODIFICATION OF OVERHANGS

Situation:

I need to clone a DNA seqeunce located between BamHI and EcoRI
Vector does have a EcoRI site, but does not have a BamHI site



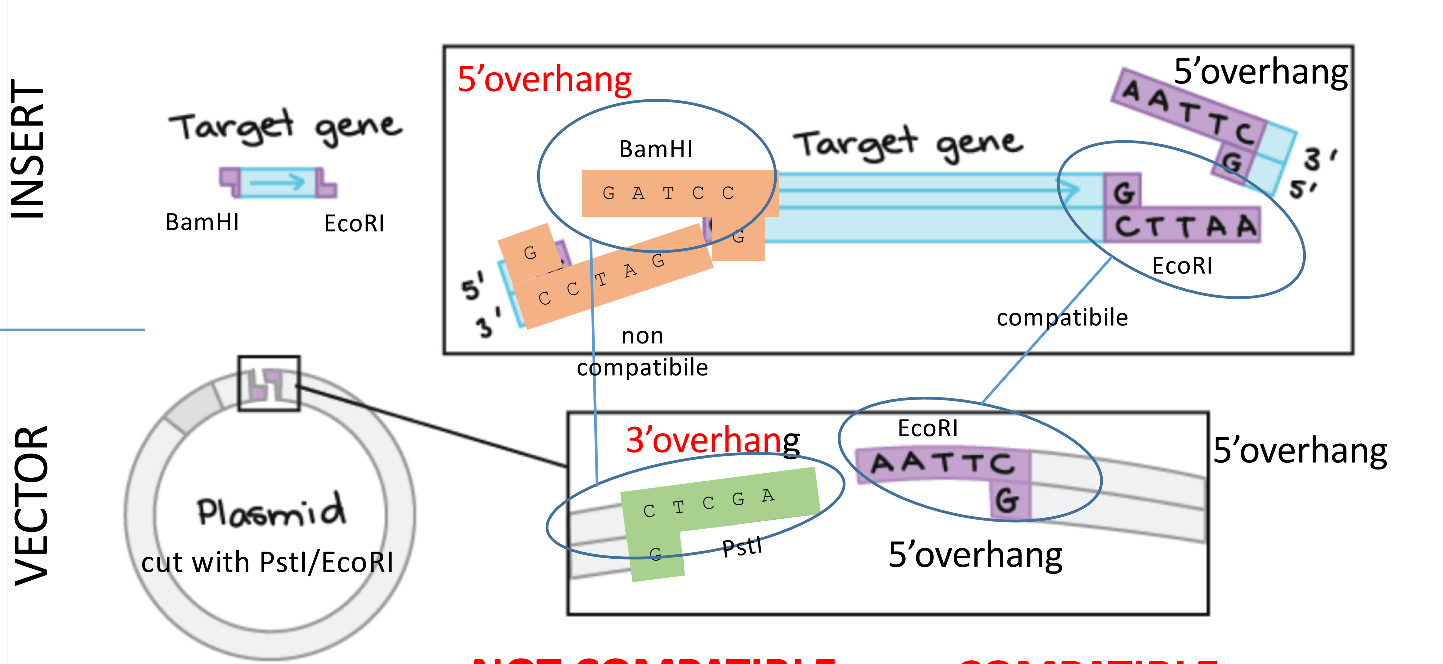
Lets assume: BamHI is not present in pBluescript



DNA CLONING WITH MODIFICATION OF OVERHANGS

BamHI:	G/GATCC CCTAG/G
EcoRI:	G/AATTC CTTAA/G

PstI:	CTCGA/G G/AGCTC
EcoRI:	G/AATTC CTTAA/G



NOT COMPATIBLE
→ make blunt

COMPATIBLE

→ INSERT Modification of 5'-overhang of BamHI site → convert overhang to blunt end
→ VECTOR: Modifcation of 3'-overhang of PstI site → convert overhang to blunt end

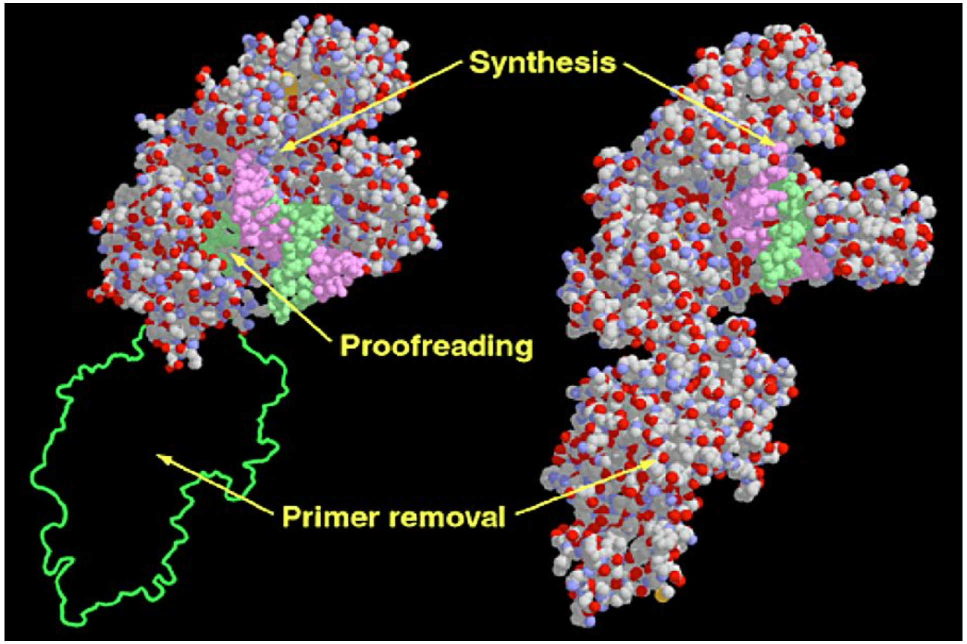
→ ➔ Blunt – Blunt AND EcoRI – EcoRI ligation

HOW CAN WE «BLUNT» 3' or 5' overhangs in linear DNA molecules??

DNA CLONING WITH MODIFICATION OF OVERHANGS

DNA Polymerase I (E.coli)

- **5' → 3' polymerase activity**
- **3’ → 5’ exonuclease activity**
- **5' → 3' exonuclease activity**



The Klenow fragment

The Klenow fragment is a large protein fragment produced when **DNA polymerase I from E. coli** is enzymatically cleaved by the protease subtilisin. First reported in 1970.

Retains the 5' → 3' polymerase activity for DNA sythesis

Retains the 3’ → 5’ exonuclease activity: proofreading,

Deletion of its 5' → 3' exonuclease activity.
The other smaller fragment formed when DNA polymerase I from E. coli is cleaved by subtilisin retains the 5' → 3' exonuclease activity but does not have the other two activities exhibited by the Klenow fragment (i.e. 5' → 3' polymerase activity, and 3' → 5' exonuclease activity).

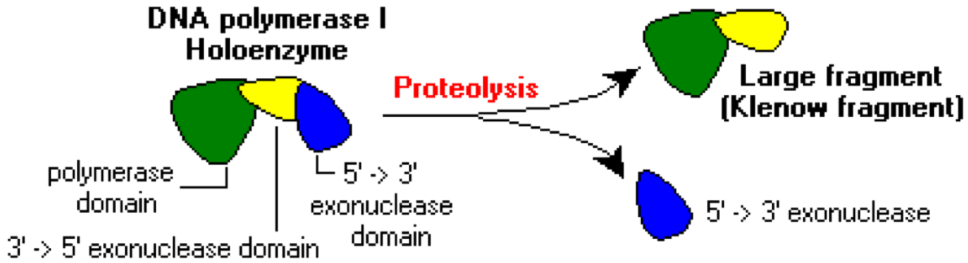
Available as recombinant protein

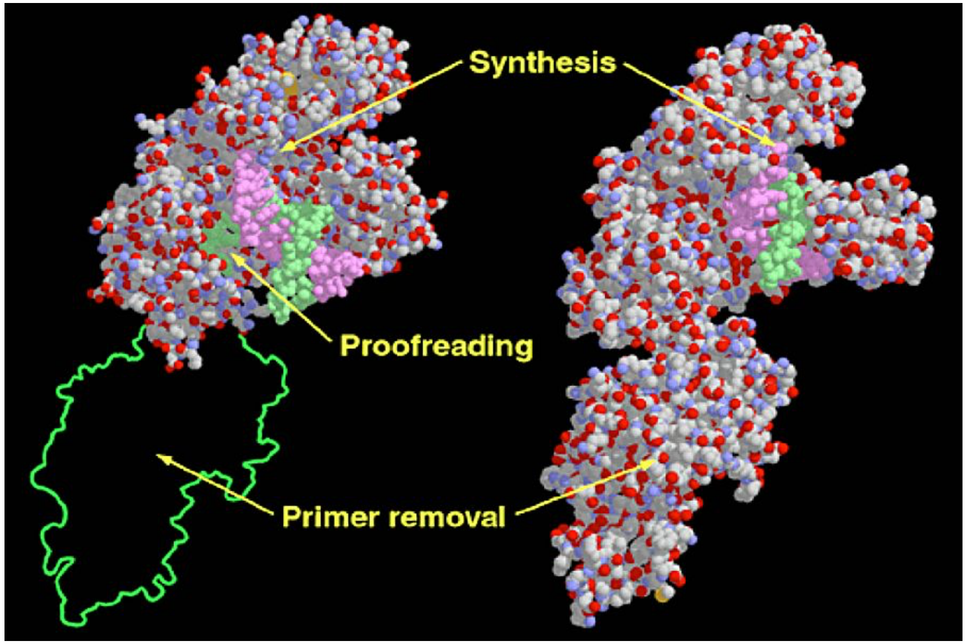
→ Synthesis of double-stranded DNA from single-stranded templates (when primed)
→ Filling in single stranded 5' overhangs, generating blunt ended terminus
→ Digesting away protruding 3' overhang, generting blunt ended terminus
→ Preparation of radioactive DNA probes

DNA CLONING WITH MODIFICATION OF OVERHANGS

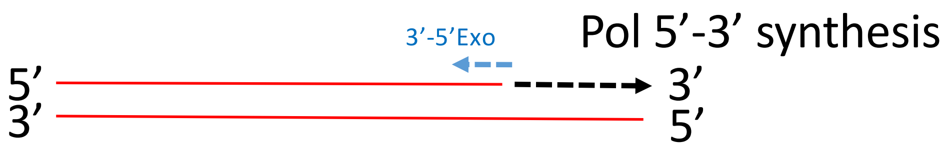
The Klenow fragment

- 5' → 3' polymerase activity
- 3’ → 5’ exonuclease activity



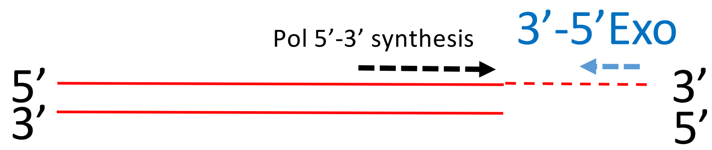


5’ overhang



Klenow:
- in presence of dNTP: DNA synthesis → **blunt end formation**
- in absence of dNTP only 3-’5’ exonuclease activity present → strand degradation

3’ overhang



Klenow:
- 3’-5’ exonucleares degrades ssDNA from 3’ end; when 5’overhang forms: polymerase activity is stronger → **blunt end formation**
- in absence of dNTP: only 3-’5’ exonuclease activity

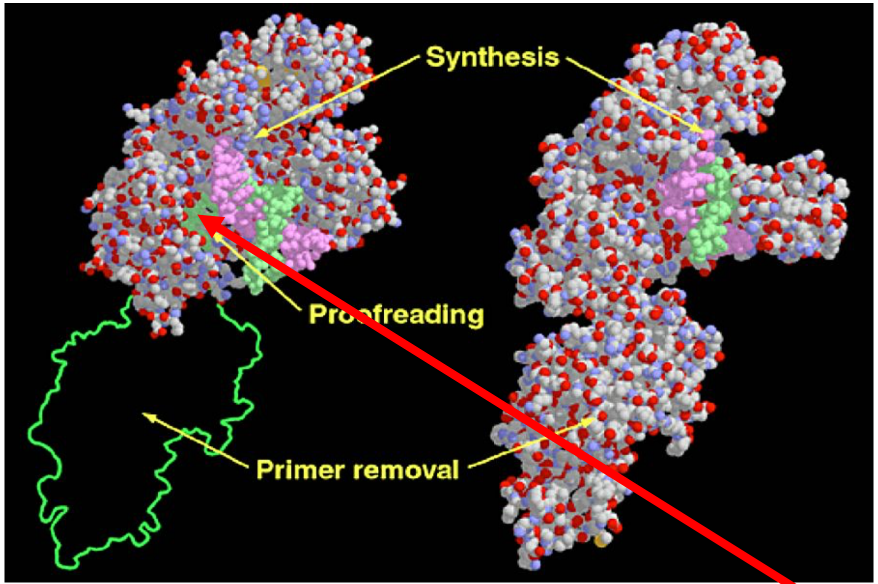
DNA CLONING WITH MODIFICATION OF OVERHANGS

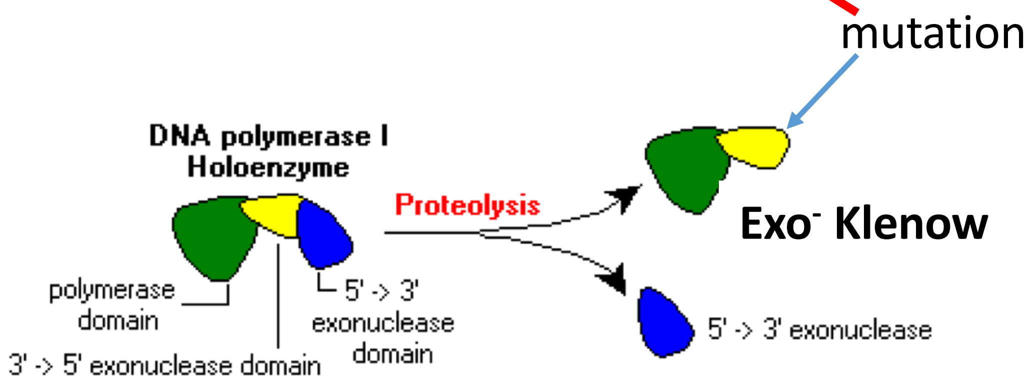
The Exo⁻ Klenow fragment

Just as the 5' → 3' exonuclease activity of DNA polymerase I from E.coli can be undesirable, **the 3' → 5' exonuclease activity of Klenow fragment can also be undesirable for certain applications.**

This problem can be overcome by **introducing mutations in the 3’-5’ exonuclease domain of the Klenow fragment. This results in the formation of an enzyme that retains 5' → 3' polymerase activity, but lacks all exonuclease activity (5' → 3' or 3' → 5').**

This form of the enzyme is called the **exo⁻ Klenow** fragment. The exo-Klenow fragment is used in some fluorescent labeling reactions for microarray, processing of overhangs and also in dA and dT tailing, an important step in the process of ligating DNA adapters to DNA fragments, frequently used in prepararing DNA libraries for Next-Gen sequencing.





Exclusive Pol 5’-3’ synthesis

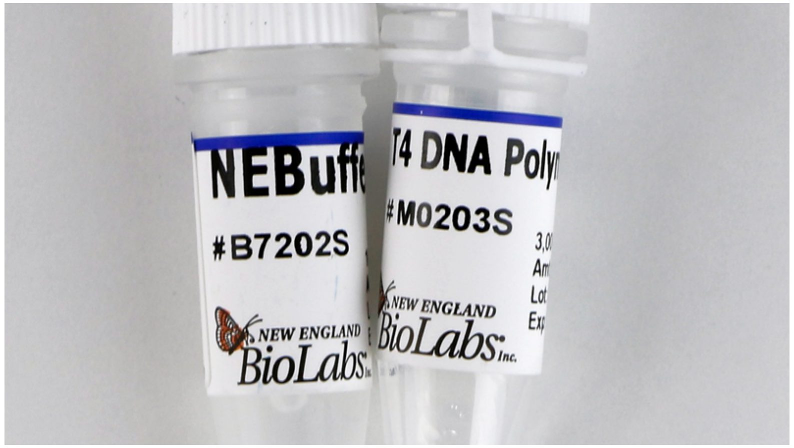
NO exonuclease activity

DNA CLONING WITH MODIFICATION OF OVERHANGS

The T4 DNA Polymerase
Encodend by T4 phage:

T4 DNA Polymerase catalyzes the synthesis of DNA in the 5´→ 3´ direction and requires the presence of template and primer.
→ contains 3’→5’ exonuclease activity
→ no 5’ —> 3’ exonuclease activity

Applications:
→Gap filling (no strand displacement activity) of 5’ overhangs to form blunt ends
→ Removal of 3’ overhangs



DNA CLONING WITH MODIFICATION OF OVERHANGS

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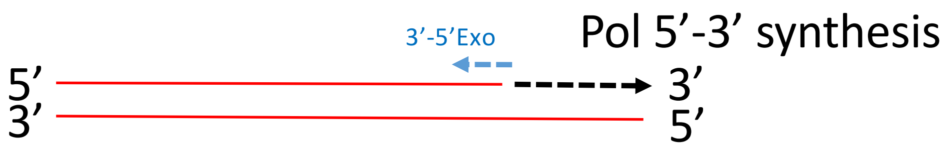
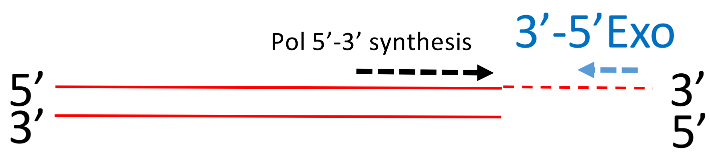
5' overhang



T4 DNA polymerase:
- in presence of dNTP: DNA synthesis → **blunt end formation**
- in absence of dNTP only 3-'5' exonuclease activity present → strand degradation

3' overhang

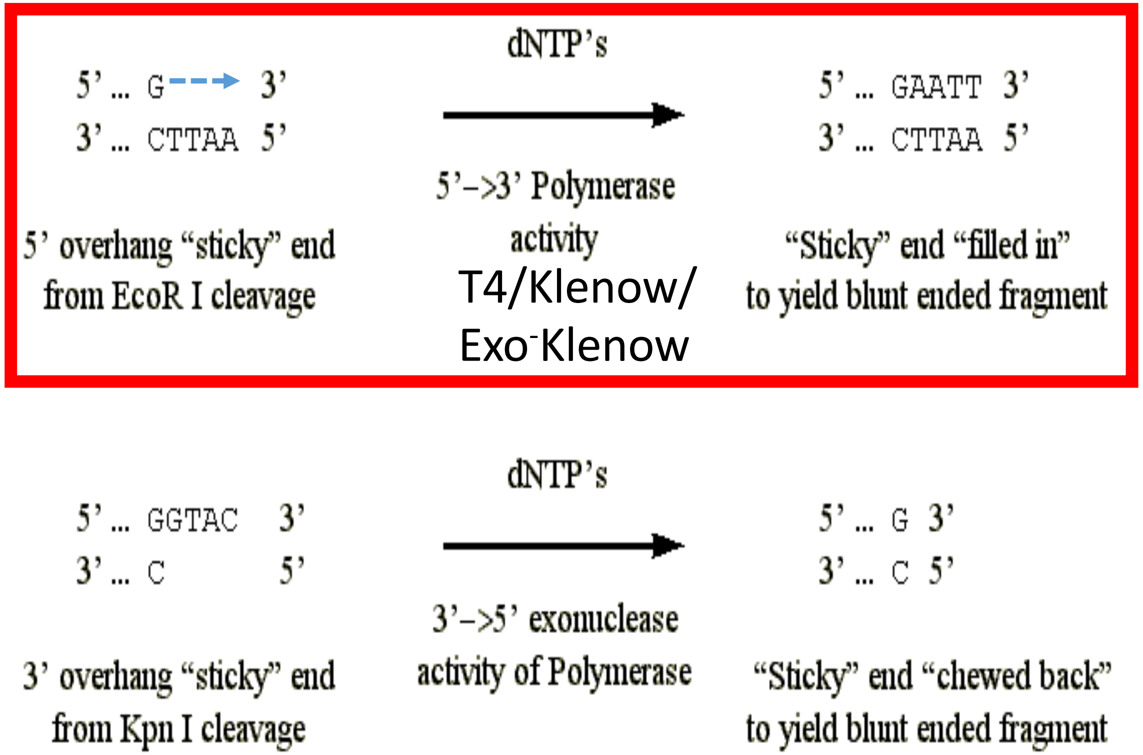


T4 DNA polymerase:
- 3'-5' exonucleares degrades ssDNA from 3' end; when 5'overhang forms: polymerase activity is stronger → **blunt end formation**
- in absence of dNTP: only 3-'5' exonuclease activity

DNA CLONING WITH MODIFICATION OF OVERHANGS

Converting a 5’ overhang to blunt end

- Both (**exo**⁻) **Klenow, Klenow** and **T4 DNA polymerase** can be used to fill in 5’ protruding ends with dNTPs
- Polymerase activity: 5’ → 3’
- Used in joining DNA fragments with incompatible ends
- Once the ends have been blunted, ligation can proceed



IMPORTANT FOR KLENOW and T4 DNA POLYMERASE REACTION:

dNTPs need to be present in abundance (and be of good quality!)

→ If dNTPs are used up by DNA polymerase; exonuclease activity will take over

→ → degradation of plasmid/insert

Exo- Klenow fragment is safer in use!

DNA CLONING WITH MODIFICATION OF OVERHANGS

Converting a 3’ overhang to a blunt end

5’ ... G 3’
3’ ... CTTAA 5’

5’ overhang “sticky” end
from EcoR I cleavage

dNTP’s
→
5’–>3’ Polymerase
activity

5’ ... GAATT 3’
3’ ... CTTAA 5’

“Sticky” end “filled in”
to yield blunt ended fragment

- T4 DNA polymerase/Klenow fragment have a 3’→5’ exonuclease activity
- In the presence of excess dNTPs enzymes will convert a 3’ protruding end to a blunt end
- Important 3’exonuclease and 5’→3’ DNA polymerase reaction are competing
- Ligation can know proceed

5’ ... GGTAC 3’
3’ ... C 5’

3’ overhang “sticky” end
from Kpn I cleavage

dNTP’s
→
3’–>5’ exonuclease
activity of Polymerase
T4/Klenow

5’ ... G 3’
3’ ... C 5’

“Sticky” end “chewed back”
to yield blunt ended fragment

IMPORTANT FOR 3’overhang BLUNTING:

dNTPs need to be present in abundance (and be of good quality!)

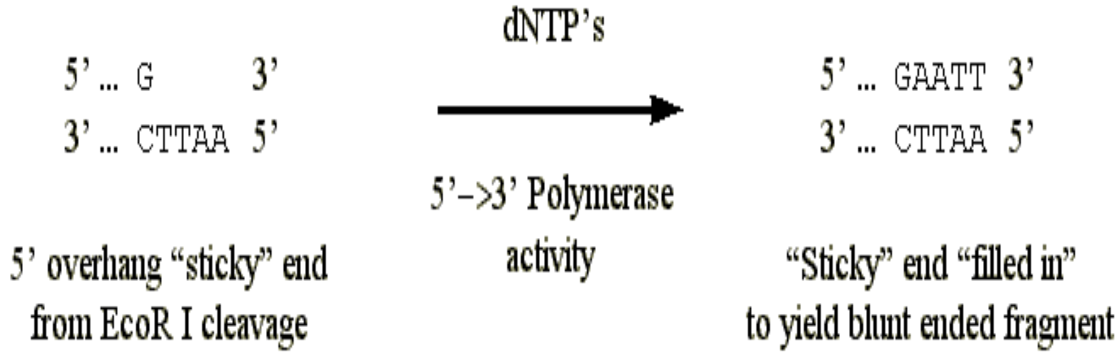
→ If dNTPs are used up by DNA polymerase activity; exonuclease activity will take over

→ → degradation of plasmid/insert

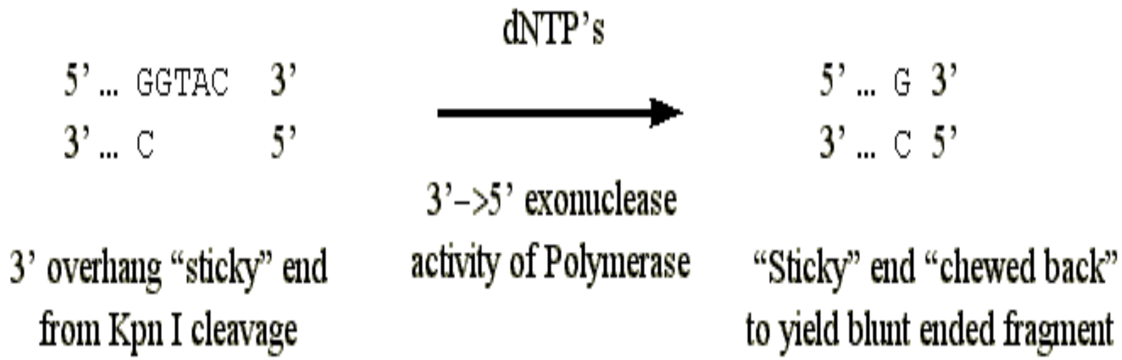
DNA CLONING WITH MODIFICATION OF OVERHANGS

Laboratory realty → ideal use of enzymes

Exo$^-$Klenow fragment
5’ overhang fill-up
→ Proofreading function



T4 DNA polymerase
3’ overhang removal



Note: some researchers use T4 polymerase for 5’-overhang blunting AND 3’-overhang blunting.
Why: only one enzyme; used frequently (always updated on enzyme activity), T4 is stabile, cost extensive; you can blunt a fragment that has 3’ and 5’ overhang in single reaction

DNA CLONING WITH MODIFICATION OF OVERHANGS

5'OH

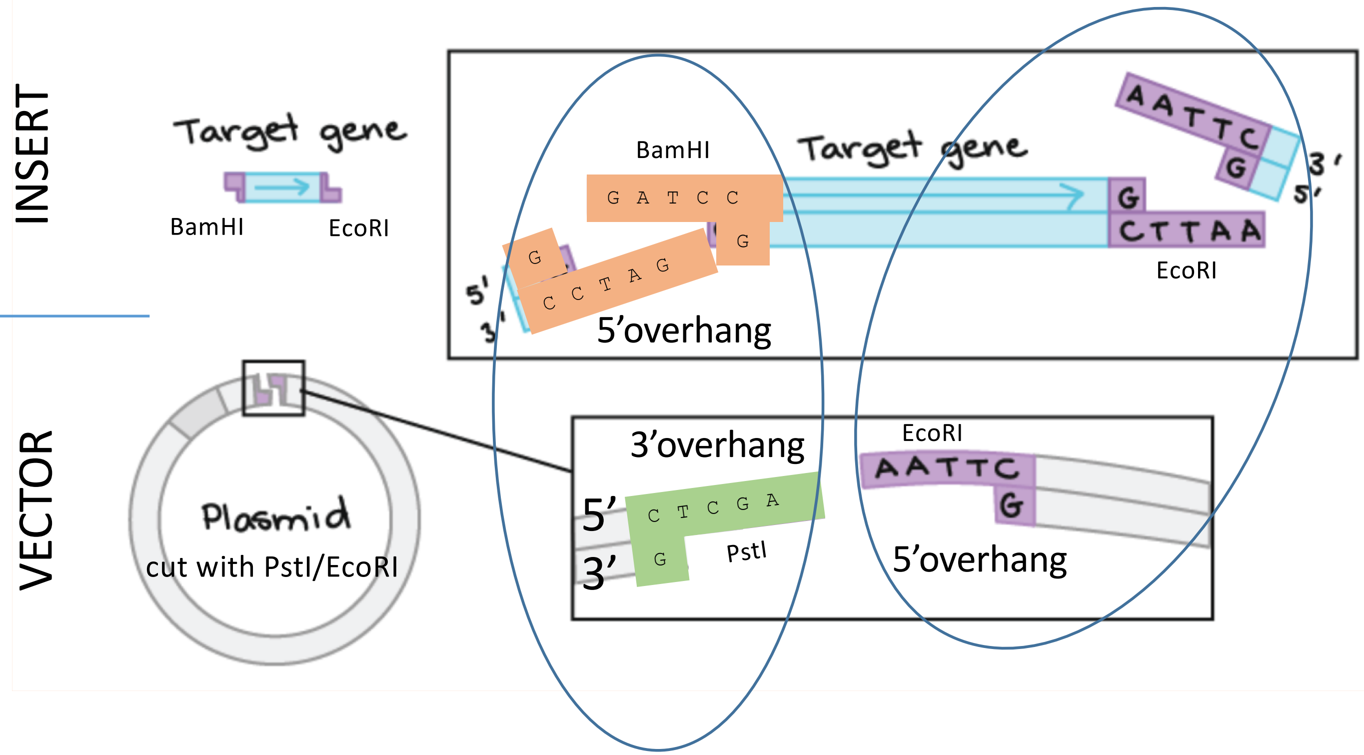
BamHI: G/GATCC
CCTAG/G

EcoRI: G/AATTC
CTTAA/G

3'OH

PstI: CTCGA/G
G/AGCTC

EcoRI: G/AATTC
CTTAA/G



HOW TO DO?

Vector:

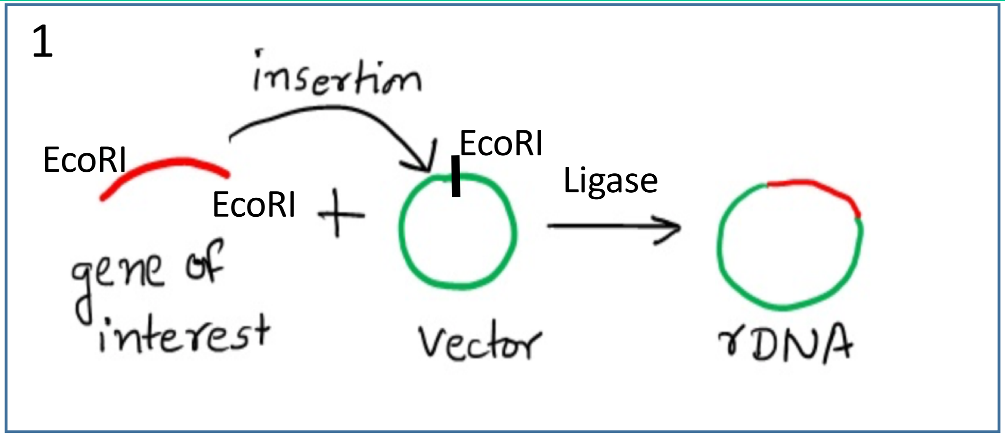
1. Cut PstI
2. Make T4 Polymerase reaction → blunting of 3'overhang
3. Purify DNA from enzymatic reaction (for example column)
4. Cut DNA with EcoRI
5. Run DNA on agarose gel
6. Cut correct band and purify DNA, determine concentration

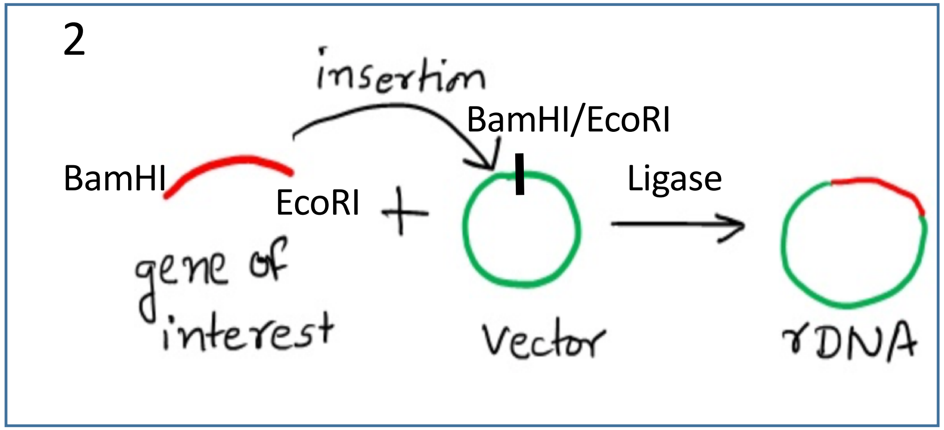
Insert:

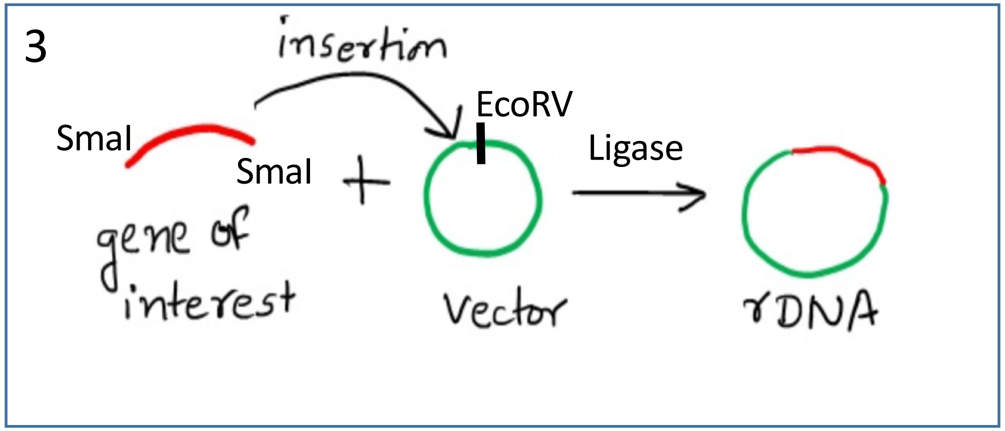
1. Cut DNA with BamHI
2. Make Exo$^-$ Klenow reaction → blunting of 5'overhang
3. Purify DNA from enzymatic reaction (for example column)
4. Cut DNA with EcoRI
5. Run DNA on agarose gel
6. Cut correct band and purify DNA; determine concentration

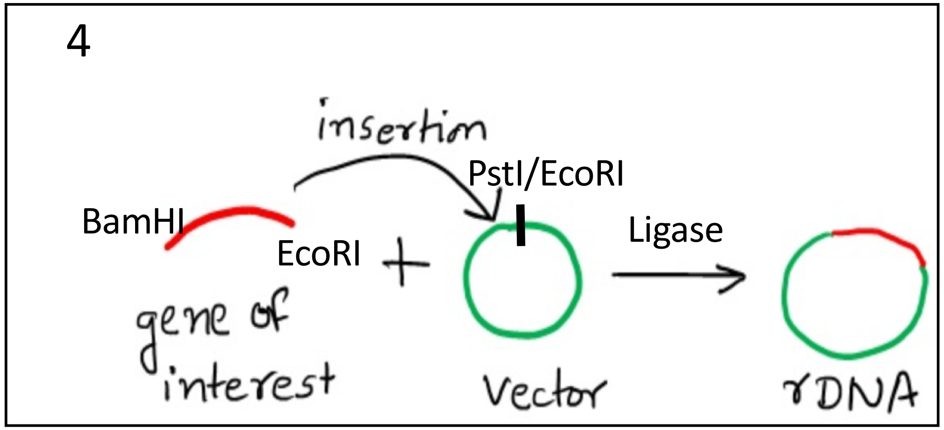
7. Setup ligation (Blunt- Blunt; EcoRI – EcoRI)

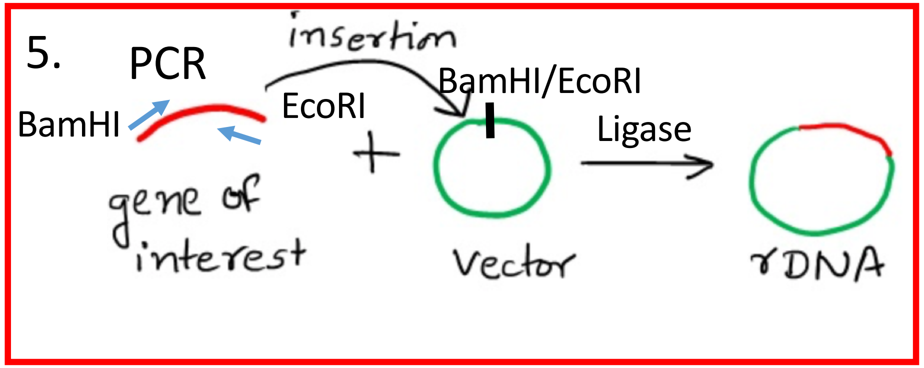
OVERVIEW OVER OTHER CLONING STRATEGIES



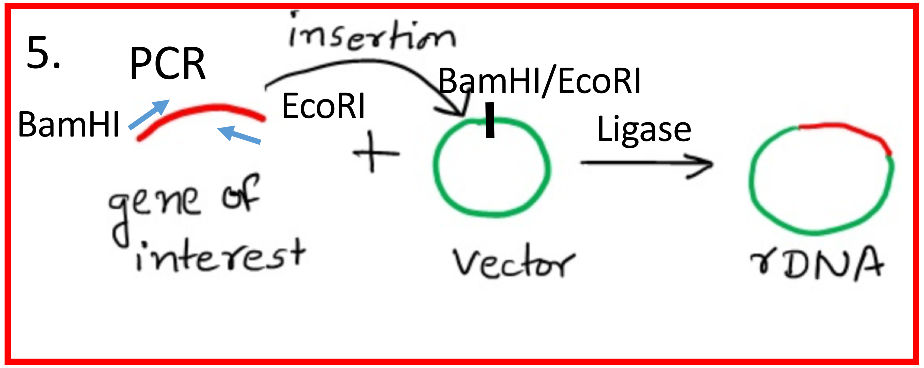








PCR CLONING



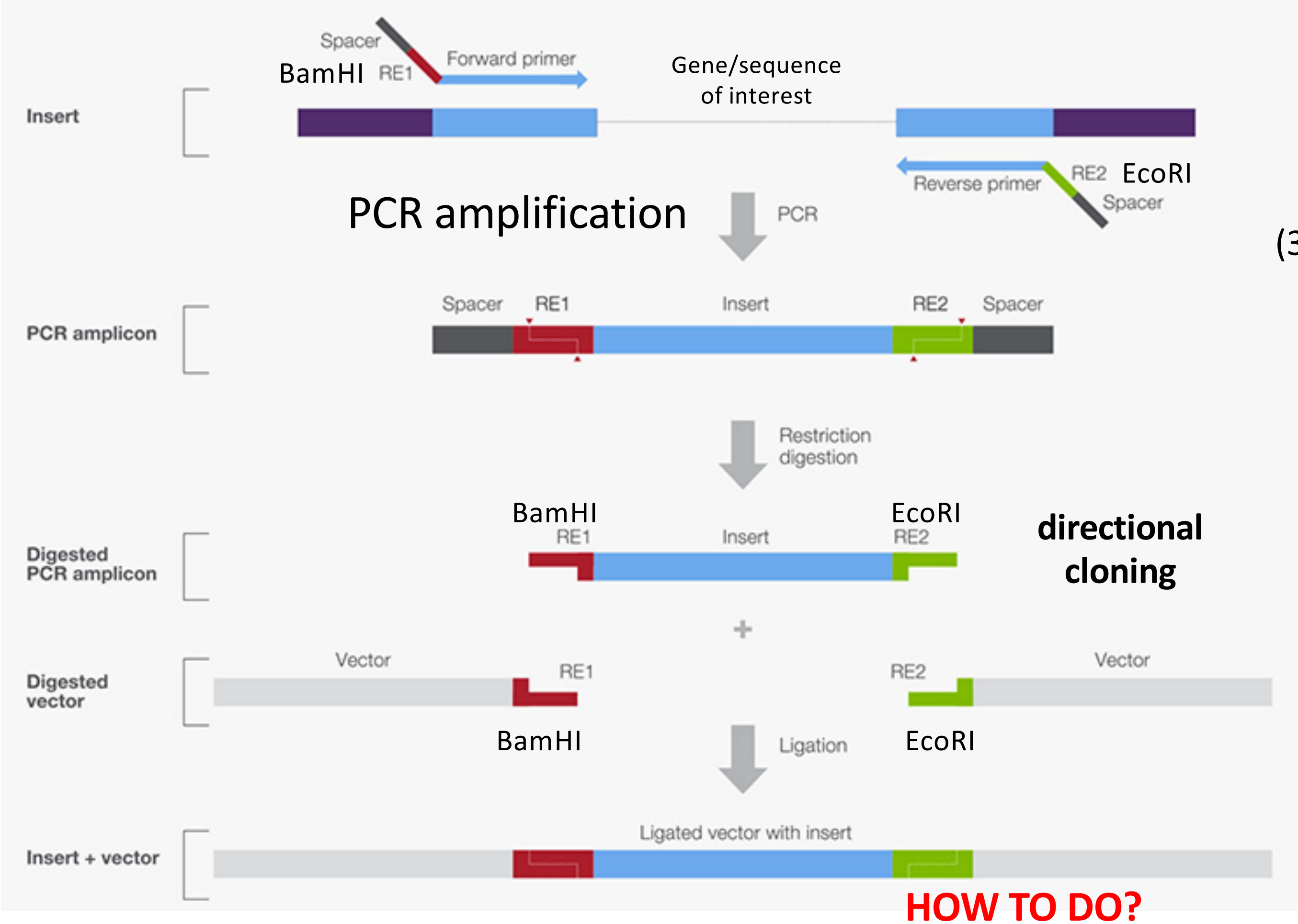
1. DNA of interest is amplified by PCR. PCR oligos contain sequence for restriction enzyme

2. PCR generates dsDNA that can be cut with restriction enzyme

3. Fragment cloned into vector

PCR CLONING

1. Classic PCR cloning



Efficient strategy because directional cloning cen be used

Forward primer

5'-CGCGGATCCxxxxxxxxxxxxxxxxxx-3'

Spacer (3-5 nucelotides) | BamHI | Sequence pairing with Sequence of interest (min. 18 nucelotides)

Reverse primer

3'-xxxxxxxxxxxxxxxxxxCTTAAGCGC-5'

Sequence pairing with Sequence of interest (min. 18 nucelotides) | EcoRI | Spacer 3-5 nucelotides

Attention: synthesized primers do not contain 5'P

HOW TO DO?

1. **Design + synthezie oligos**
2. **Make PCR**
3. **Purify PCR product (agarose gel or columns)**
4. **Cut PCR product with BamHI and EcoRI**
5. **Run agarose gel; cut out band; purify DNA; determine concentration**
6. **Setup ligation with vector linearized by EcoRI/BamHI**

PCR CLONING

1. Classic PCR cloning

WHY IS A SPACER NEEDED???

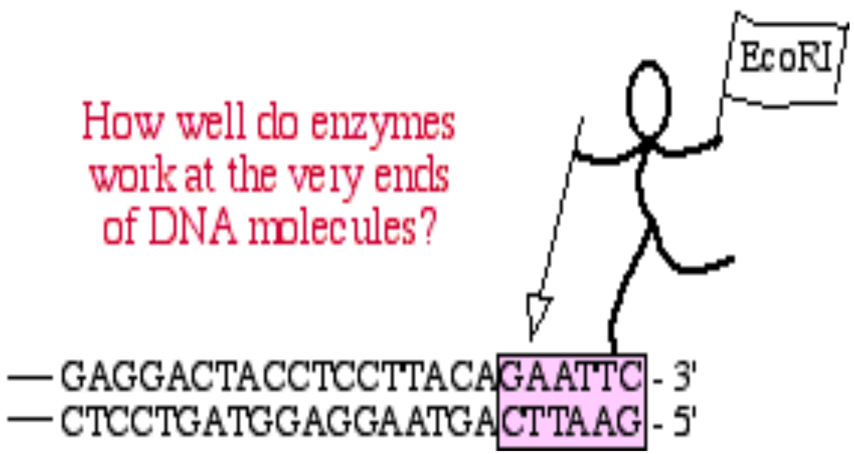
Forward primer

```
5'-CGCGGATCCxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxGAATTGGCG
3'-GCGCCTAGGxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxCTTAAGCGC-5'
```

BamHI

Reverse primer

EcoRI



→ Many resitriction enzymes work poorly on DNA termini

→ Catalogues of enzymes provide data on the cutting efficiency of enzymesat the end of DNA molecules.

→ Generally, enzymes work better if they have a couple of extra nucleotides at the end – improved interaction with DNA

PCR CLONING

1. Classic PCR cloning

WHY IS A SPACER NEEDED???

Forward primer

```
5'-CGCGGATCCxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxGAATTGGCG
3'-GCGCCTAGGxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxCTTAAGCGC-5'
```

Reverse primer

PCR, EcoRI digest
Agarose Gel-electophoresis
Fragment purification

```
5'P-GATCCxxxxxxxxxxxxxxxxxx-- xxxxxxxxxxxxxxxxxxG-3'OH
   3'OH-Gxxxxxxxxxxxxxxxxxx-- xxxxxxxxxxxxxxxxxxCTTAA-5'P
```

Digest with EcoRI creates 5'overhang with terminal 5'P
Ready for cloning

PCR CLONING

2. TA-cloning – primers to not necessarily need to contain restriction site

ATTENTION: Taq polymerases produce PCR products with A on 3'ends

Forward primer

```
5'-xxxxxxxxxxxxxxxxxxx-------xxxxxxxxxxxxxxxxxxxA3'
3'Axxxxxxxxxxxxxxxxxxx-------xxxxxxxxxxxxxxxxxxx-5'
```

Reverse primer

PCR product of sequence of interest

- → Primers for PCR do not necessarily contain restriction site and spacer!
- → Primers used to amplify seqeunce of interest

Taq polymerase is a thermostable DNA polymerase named after the thermophilic bacterium <u>Thermus aquaticus</u> from which it was originally isolated.

Taq polymerases are the most frequently used polymerases for PCR
Taq holds *<u>terminal transferase activity</u>* adding dATP at 3' termini
Taq DNA polymerase catalyzes the non-template directed addition of an adenine residue to the 3´-end of both strands of DNA molecules → blunt cloning not possible

This is useful in TA cloning, whereby a cloning vector (such as a plasmid) that has a T (thymine) 3' overhang is used, which complements with the A overhang of the PCR product, thus enabling ligation of the PCR product into the plasmid vector. = **TA cloning**

ATTENTION: Taq does not have 3'-5' exonuclease activity → no proofreading

PCR CLONING

3. TA-Cloning

ATTENTION: Taq polymerases produce PCR products with A on 3'ends

Forward primer

```
5'-xxxxxxxxxxxxxxxxxx-------xxxxxxxxxxxxxxxxxxA3'
3'Axxxxxxxxxxxxxxxxxx-------xxxxxxxxxxxxxxxxxx-5'
```

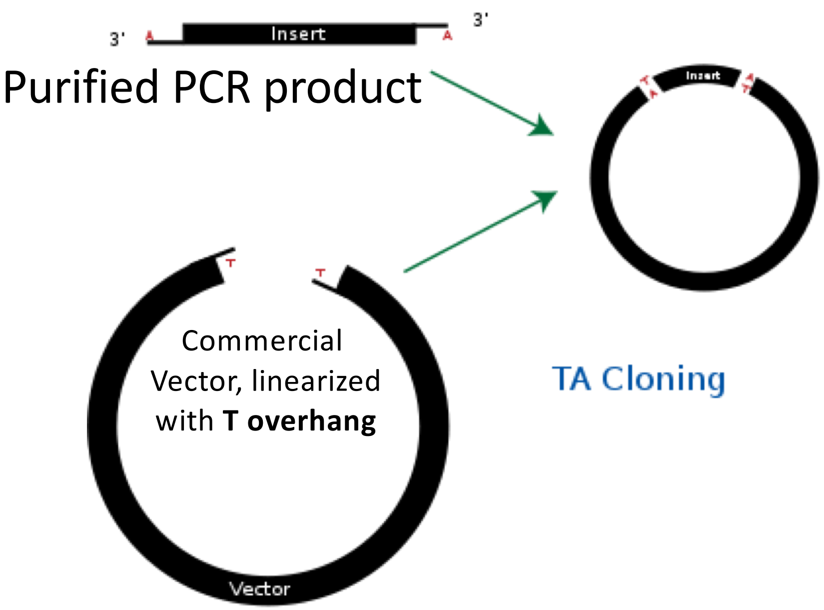
Reverse primer

OR ALSO

Forward primer

```
5'-CGCGGATCCxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxGAATTCGCGA3'
3'AGCGCCTAGGxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxCTTAAGCGC-5'
```

Reverse primer



PCR CLONING

2. Blunt end cloning using PCR

ATTENTION: Other polymerasese <u>do not add</u> A on 3’end: for example:
Pfu polymerase creates blunt PCR products

Synthesized primers do not contain 5’P

Forward primer
5’-xxxxxxxxxxxxxxxxxx-3’

↓ T4 polynucelotide kinase +rATP

5’-**P**-xxxxxxxxxxxxxxxxxx-3’

Reverse primer
3’-xxxxxxxxxxxxxxxxxxx-5’

↓ T4 polynucelotide kinase+rATP

3’-xxxxxxxxxxxxxxxxxxx-**P**-5’

↓ PCR using Pfu polymerase

Forward primer
```
5’P-xxxxxxxxxxxxxxxxxxx-----------Xxxxxxxxxxxxxx-----→OH-3’
3’OH<--xxxxxxxxxxxxxxxx-----------xxxxxxxxxxxxxxxxxxx-P-5’
```
Reverse primer

BLUNT
PCR product of sequence of interest contains a 5’P for ligation

↓ Agarose gel; cut band; purify DNA; determine concentration

Clone into a vector that was cut with a **blunt** cutting restriction enzyme, followed by **dephosphorylation**

Pfu DNA polymerase is an enzyme found in the hyperthermophilic archaeon *Pyrococcus furiosus*

ATTENTION: Pfu has 3’-5’ exonuclease activity → proofreading

PCR CLONING

4. Characteristics of DNA polymerases that can be used for DNA cloning

	5′–>3′ Exonuclease	3′–>5′ Exonuclease	Error Rate($x10^{-6}$)[a]	Strand Displacement	Nick Translation	Thermal Stability	K_m dNTPs	K_m DNA[d]	Extend RNA Primer	Extension from Nick	Primary Applications
Bst DNA Polymerase, Full Length	+	–		_r	+	+			+	+	Labeling, 2nd Strand Synthesis
Bst DNA Polymerase, Large Fragment	–	–		++++	–	+			+	+	Strand Displacement Applications, isothermal amplification
Bsu DNA Polymerase, Large Fragment	–	–		++	–	–			+	+	Labeling, 2nd Strand Synthesis, Strand Displacement
Crimson *Taq* DNA Polymerase	+	–	285	_r	+	++			–	+	PCR (routine)
Deep Vent$_R$™ DNA Polymerase	–	+++		++	–	++++	50 μM[e]	0.01 nM[e]	–	+	PCR (high-fidelity)
Deep Vent$_R$™ (exo–) DNA Polymerase	–	–		+++	–	++++			–	+	PCR (long)
E. coli DNA Polymerase I	+	++	9[h]	_r	+	–	1-2 μM[f]	5 nM[f]	+	+	Nick Translation
Klenow Fragment (3′→5′ exo-)	–	–	100[o]	+++	–	–			+	+	Labeling
DNA Polymerase I, Large (Klenow) Fragment	–	++	18[o]	++	–	–	2 μM[g]		+	+	Polishing Ends
LongAmp® *Taq* DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (routine, long)
LongAmp® Hot Start *Taq* DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (hot start, long)
M-MuLV Reverse Transcriptase	–	–		+++	–	–	18 μM[s]				cDNA Synthesis

PCR CLONING

4. Characteristics of DNA polymerases that can be used for DNA cloning

Taq derivative	One*Taq*® DNA Polymerase	+	++	~140	–[r]	+	++			–	+	PCR (routine, difficult)
	One*Taq*® Hot Start DNA Polymerase	+	++	~140	–[r]	+	++			–	+	PCR (hot start, routine, difficult)
	phi29 DNA Polymerase	–	++++		+++++	–	–	0.5 µM[q]		+	+	Strand Displacement Applications
Pfu derivative	Phusion® Hot Start Flex DNA Polymerase*	–	++++	<0.44	–	–	+++			–	–	PCR (high-fidelity, long)
	Phusion® High-Fidelity DNA Polymerase*	–	++++	<0.44	–	–	+++			–	–	PCR (high-fidelity, long, hot start)
	Q5® + Q5® Hot Start DNA Polymerase	–	++++	<0.44	–	–	+++			–	–	PCR (high-fidelity)
	Sulfolobus DNA Polymerase IV	–	–		–	–	+					DNA Synthesis Across Template Lesions
	T4 DNA Polymerase	–	++++	<1[h]	–	–	–	2 µM[n]		+	–	Polishing Ends, 2nd Strand Synthesis
	T7 DNA Polymerase (unmodified)	–	++++	15[b]	–	–	–	18 µM[k]	18 nM[k]	+	–	Site Directed Mutagenesis
Classic Taq	*Taq* DNA Polymerase with Standard *Taq* Buffer	+	–	285[c]	–[r]	+	++	13 µM[e]	2 nM[e]	–	+	PCR (routine)
	Therminator™ DNA Polymerase	–	–		+	–	++++			+	+	Chain Terminator Applications
	Vent$_R$® DNA Polymerase	–	++	57[b]	++[e]	–	+++	60 µM[e]	0.1 nM[e]	–	+	PCR (routine, high-fidelity)
	Vent$_R$® (exo–) DNA Polymerase	–	–	190[b]	+++[e]	–	+++	40 µM[e]	0.1 nM[e]	–	+	PCR, Sequencing

Phusion Polymerase: trade name for Pfu polymerase that had been engeneered to have improved function

PCR CLONING

4. Characeristics of DNA polymerases that can be used for DNA cloning

What percent of the product molecules contain an error after PCR (30 cycles) with different polymerases?

Polymerase	1 kb template	3 kb template
Phusion High-Fidelity DNA Polymerases (HF Buffer)	1.32%	3.96%
Phusion High-Fidelity DNA Polymerases (GC Buffer)	2.85%	8.55%
Pyrococcus furiosus DNA polymerase	8.4%	25.2%
Taq DNA polymerase	68.4%	205.2%

The table above demonstrates the low error rate of Phusion DNA Polymerase. After 30 cycles of PCR amplifying a 3 kb template, only 3.96 % of the product DNA molecules contain 1 (nucleotide) error each. This means that 96.04 % of the product molecules are entirely error-free. In contrast, after the same PCR protocol performed with *Taq* DNA polymerase, every product molecule contains an average of 2 errors.

PCR is error prone!

→ When maintainig DNA seuquence is central, use Pfu type polymerases and keep PCR cycles at the lowest mininum possible