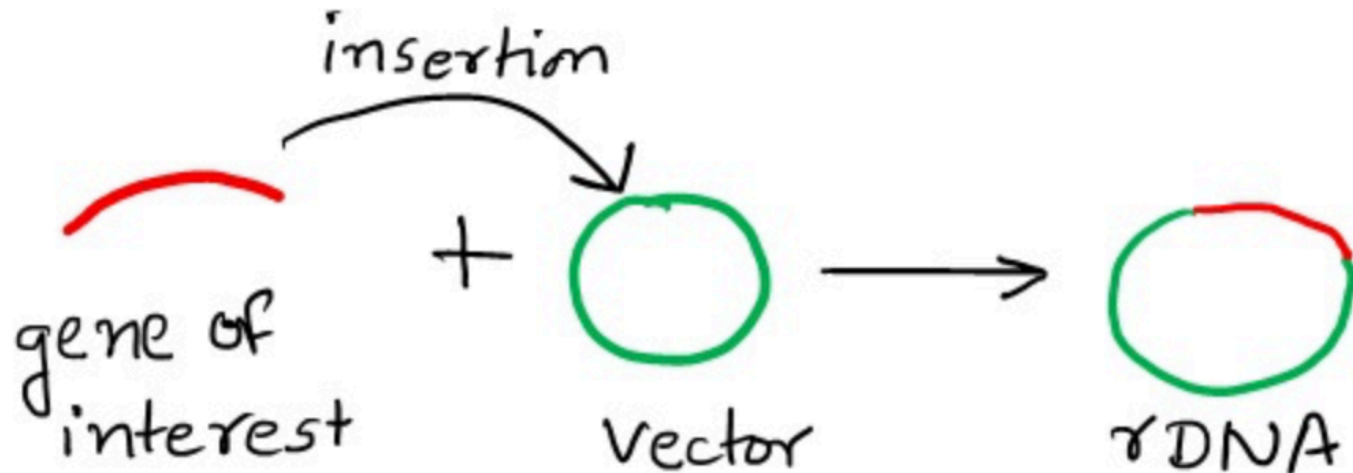


# RECOMBIANANT DNA TECHNOLOGY

## CLONING OF DNA FRAGMENTS INTO PLASMIDS



### DNA RICOMBINANTE:

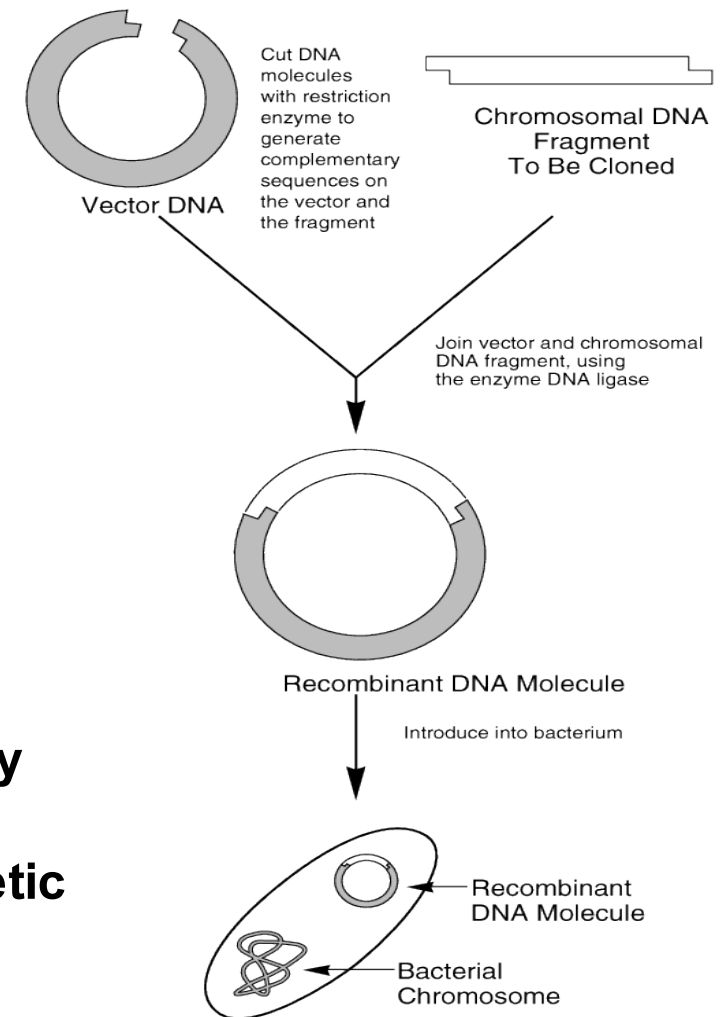
DUE MOLECOLE DI DNA VENGONO UNITE IN PROVETTA E FATTE RIPRODURRE IN LABORATORIO

### DIPENDE DALLA DISPONIBILITÀ DI ENZIMI PURIFICATI:

GLI ENZIMI A DISPOSIZIONE DEI BIOLOGI MOLECOLARI SI DIVIDONO IN 4 CATEGORIE

# 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*

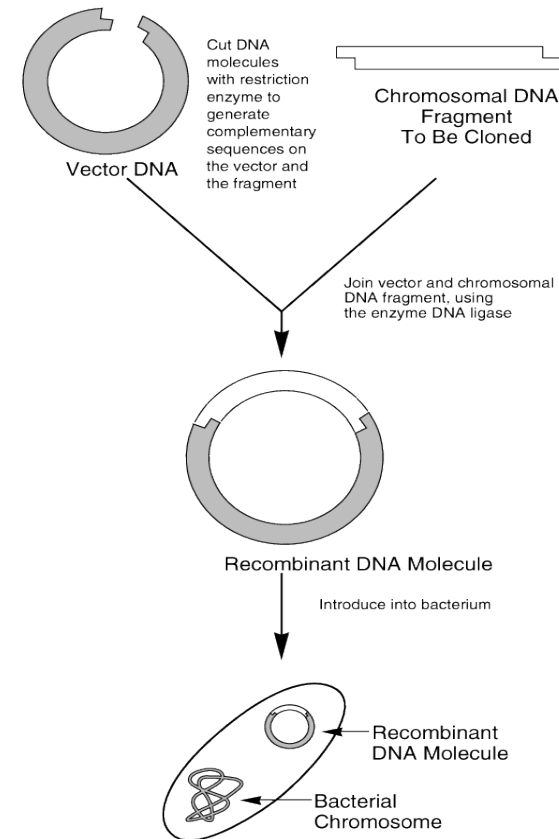


# DNA cloning

- Massive amplification of DNA sequences
- Stable propagation of DNA sequences using E.coli proofreading mechanisms 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*

# DNA cloning

- Gene of interest is cut out with RE
- Host plasmid is cut with same RE
- Gene is inserted into plasmid and ligated with ligase
- Ligation of DNA sample products and plasmid vector.
- Transformation with the ligation products.
- Growth on agar plates with selection for antibiotic resistance.





# Principal enzyme types used to generate recombinant DNA

Nucleasi (per esempio: endonucleasi di restrizione)

DNA polimerasi

Fosfatasi

Ligasi

Enzimi che modificano le estremità

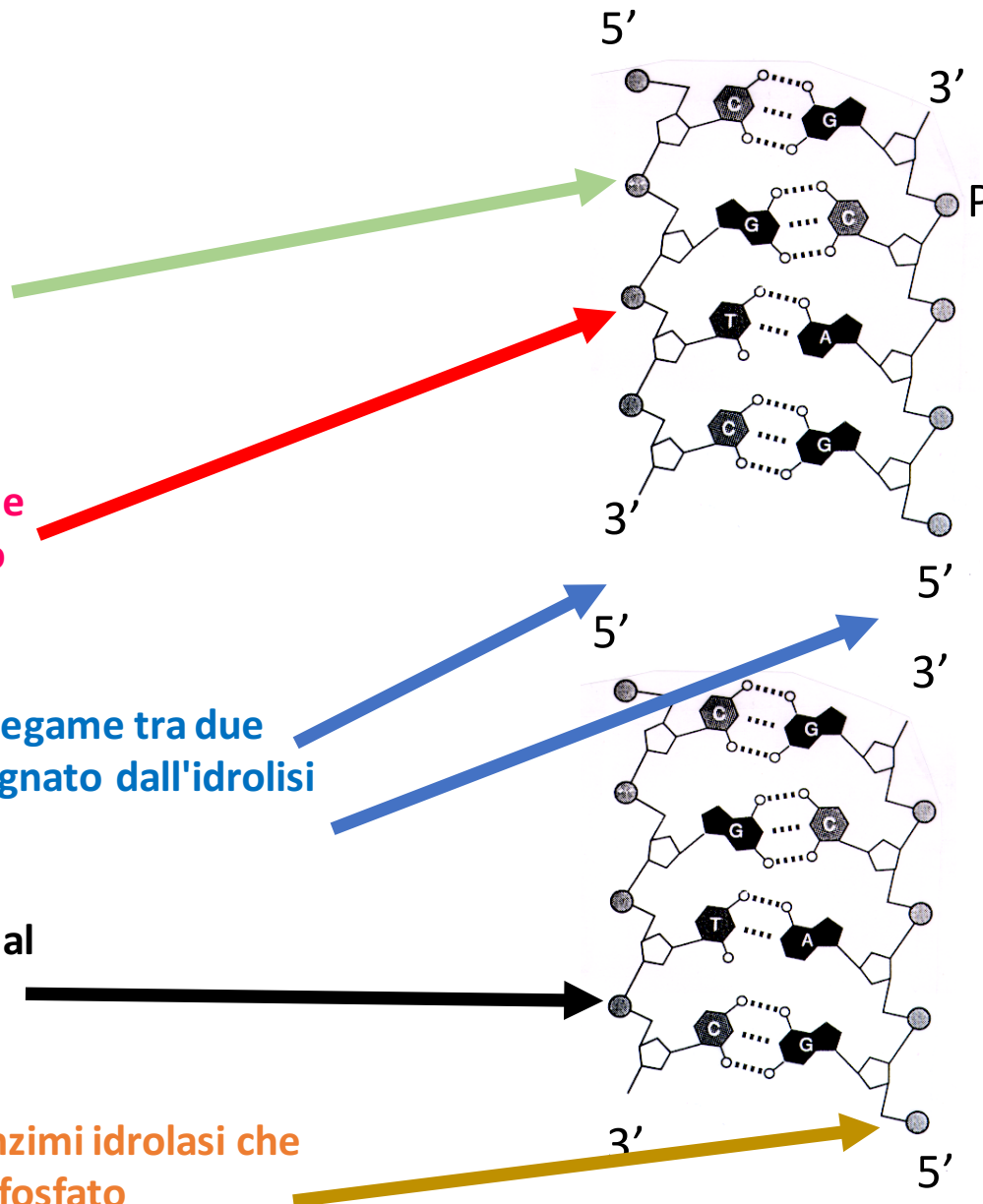
Le esonucleasi rompono il legame al termine dei filament (5' → 3')

Le endonucleasi rompono il legame internamente nel filamento dando prodotti sia 5' sia 3' fosfati

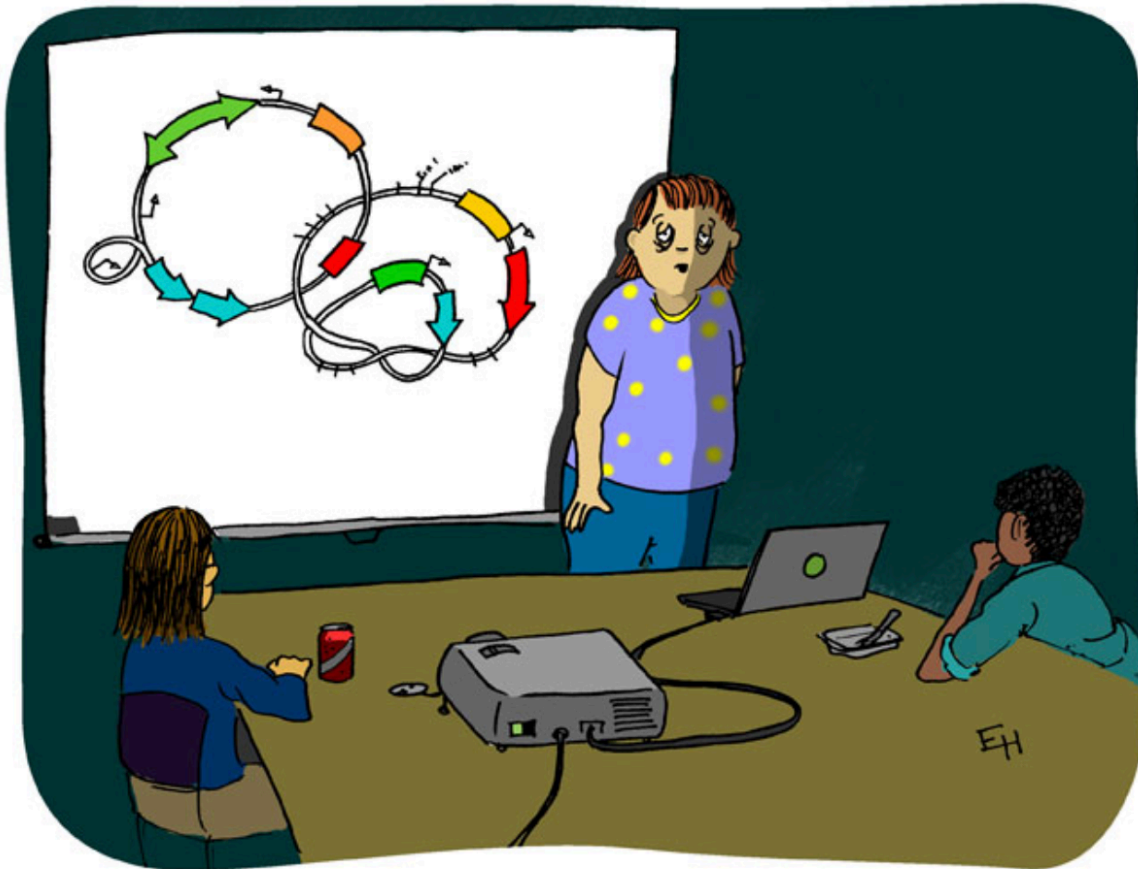
Ligasi: catalizza la formazione del legame tra due molecole di DNA, spesso accompagnato dall'idrolisi di una molecola come ATP

Le esonucleasi rompono il legame al termine dei filament (3' → 5')

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



# DNA cloning

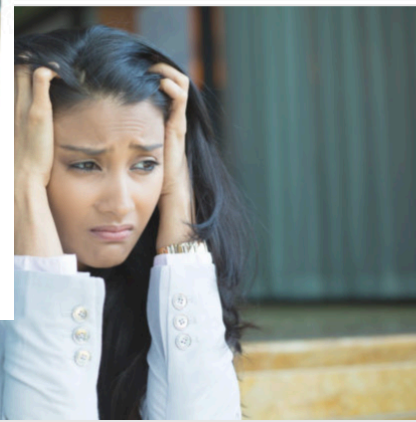


**I wish I could report otherwise,  
but the cloning is not going very well.**

....can be simple....

Or

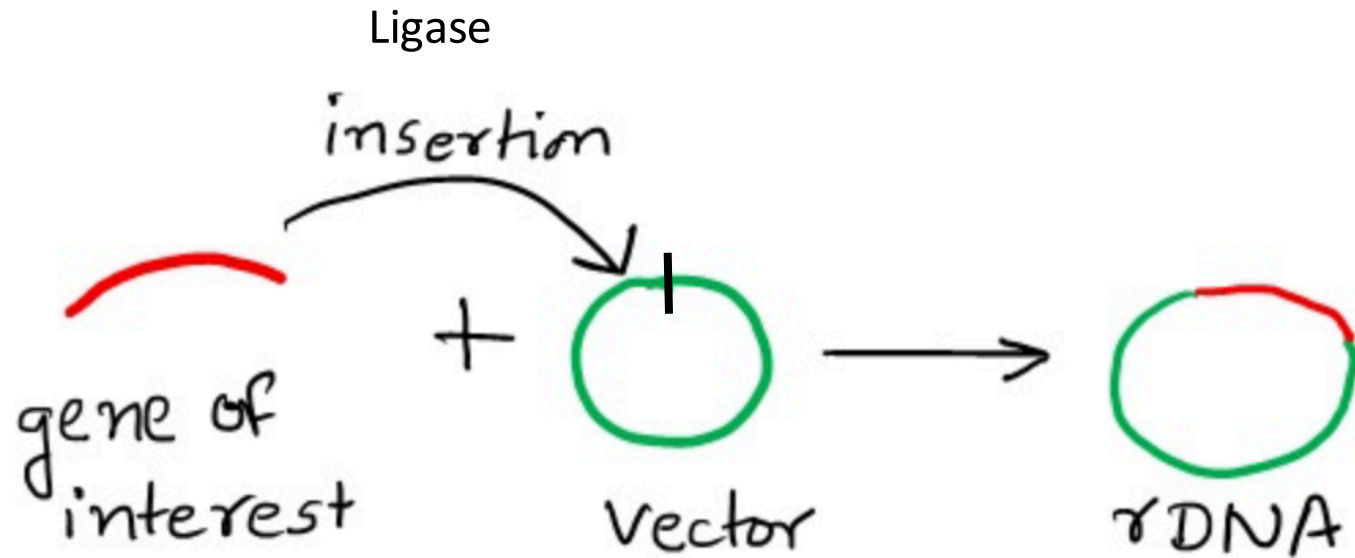
...frustrating...



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.

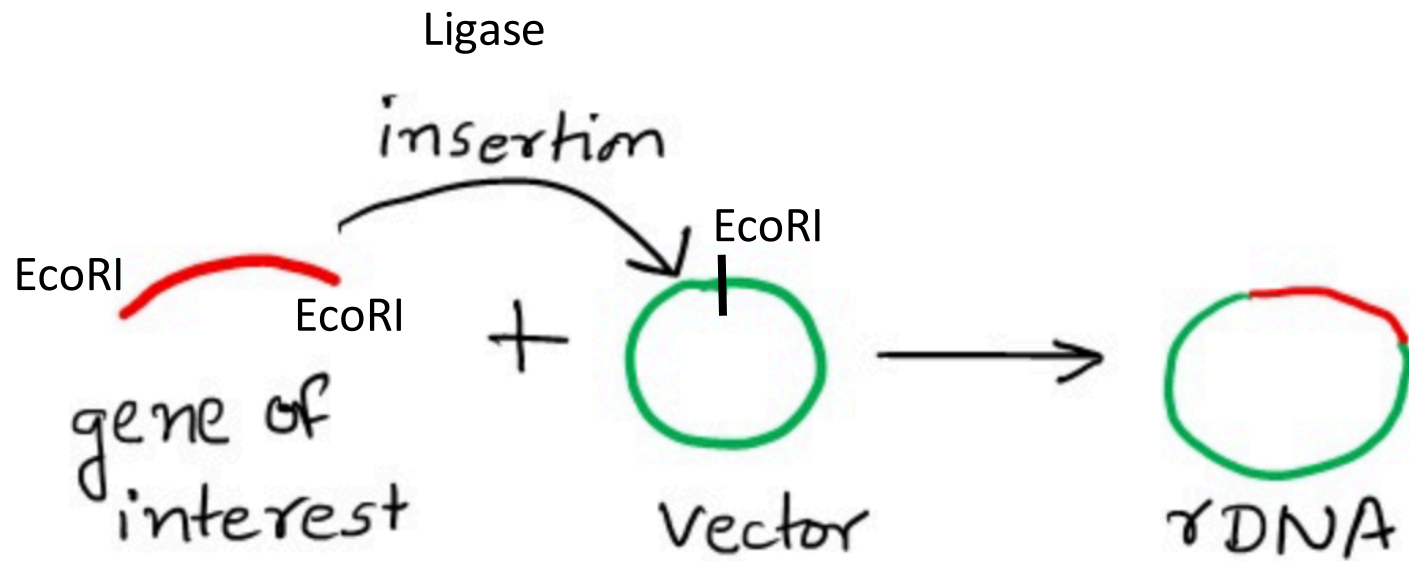
This can trigger what's known as "The 5 Stages of Failed Cloning Grief." As you work through each stage at your own pace, just know that scientists all over the world feel your pain and can empathize with you in this difficult time. [Continue reading](#) →

# Making recombinant DNA

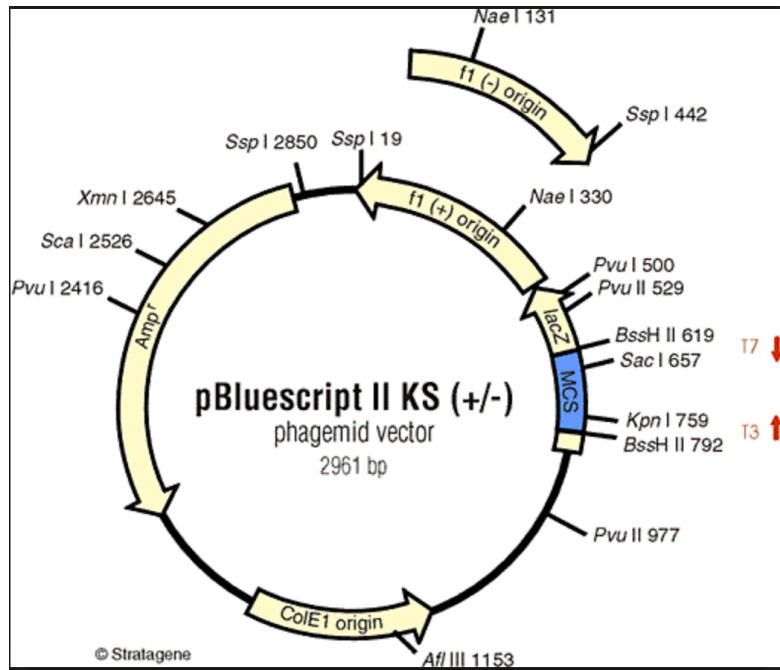


1. Fragment and Insert are cut with 1 (the same) restriction enzymes
2. Fragment and Insert are cut with 2 (the same) restriction enzymes
3. Fragment and Insert are cut with blunting restriction enzymes
4. Overhangs generated after cutting are modified (filled up; or overhang digested)

# Making recombinant DNA



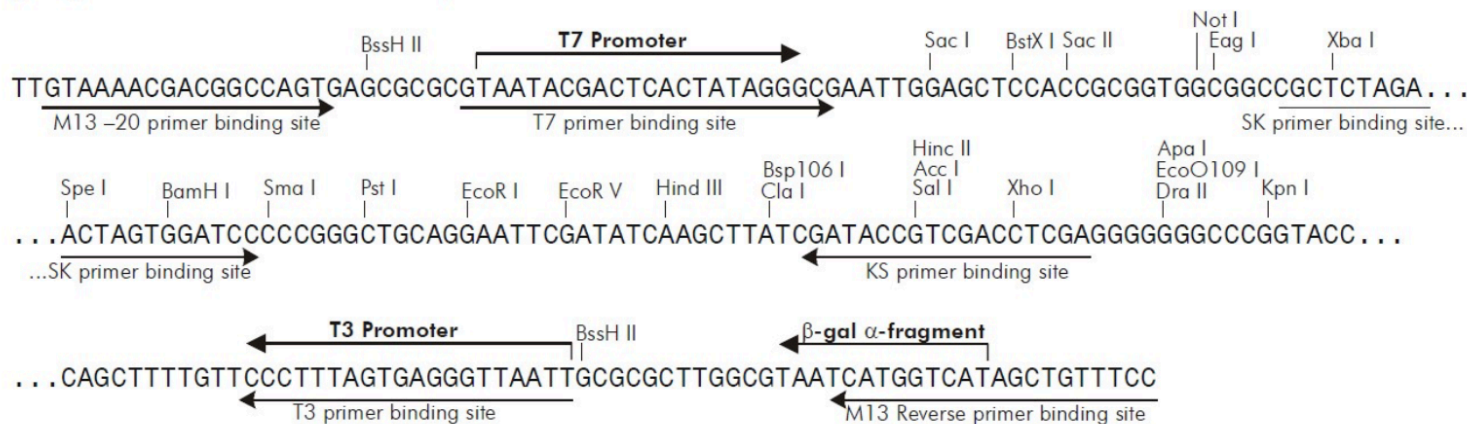
# Making recombinant DNA – Plasmid features



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

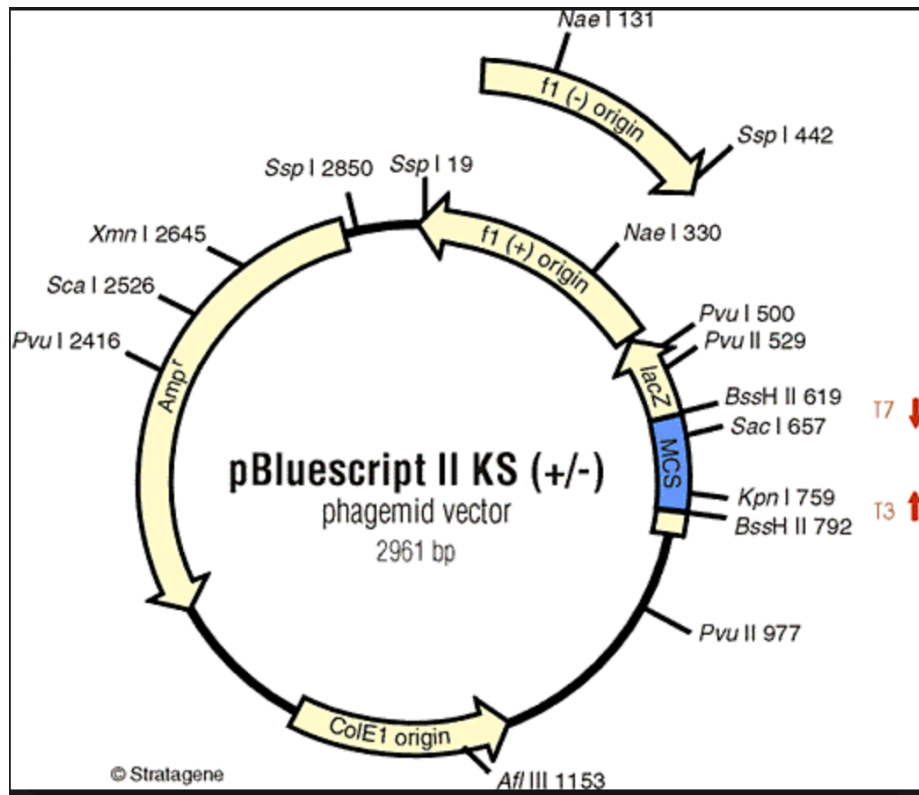
- **oriC**, an origin of replication. Gotta start making new plasmid somewhere.
- a **selectable marker**: This is usually an antibiotic resistance of some sort, to give the bacteria with plasmids a selective advantage in specific media.
- a **multiple cloning site (MCS) inside a scorable marker**. The 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**.

## pBluescript II KS (+/-) Multiple Cloning Site Region (sequence shown 598–826)





# Making recombinant DNA – Plasmid features



Amp<sup>R</sup>: Ampicillin resistance

ColE1 origin: origin of replication in bacteria

MCS: multiple cloning site

Lac Z: beta galactosidase: cleaves 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 sequencing using primers; same for M13)

F1 (-) (+) origin:

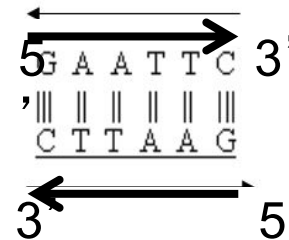
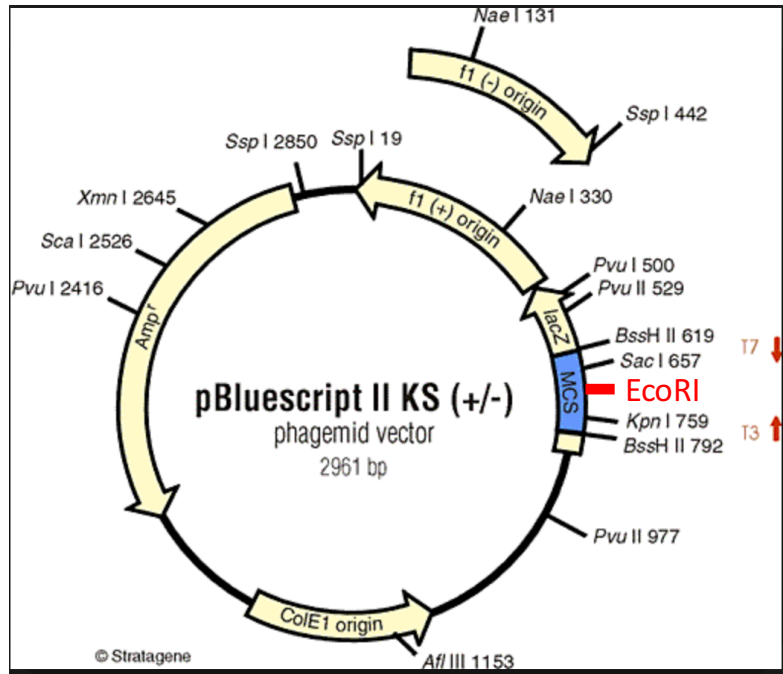
pBluescript II is a phagemid that can be secreted as single-stranded DNA in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous **f1** phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E. coli* with the F<sup>+</sup> 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, f1) 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.**<sup>7,8</sup>

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.

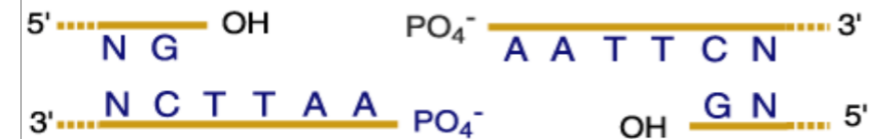
## Ampicillin

- 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<sup>r</sup>**) 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<sup>r</sup> 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



EcoRI  
Digest



**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

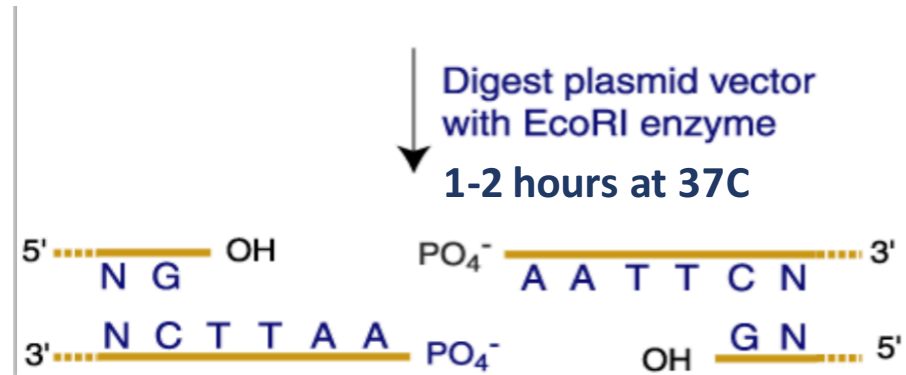
**EcoRI:** 20Units/ul:

**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 recommended: = 1ug DNA + 5-10 units (you are never sure about “real” activity of enzyme – storage – handling, etc)

# 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

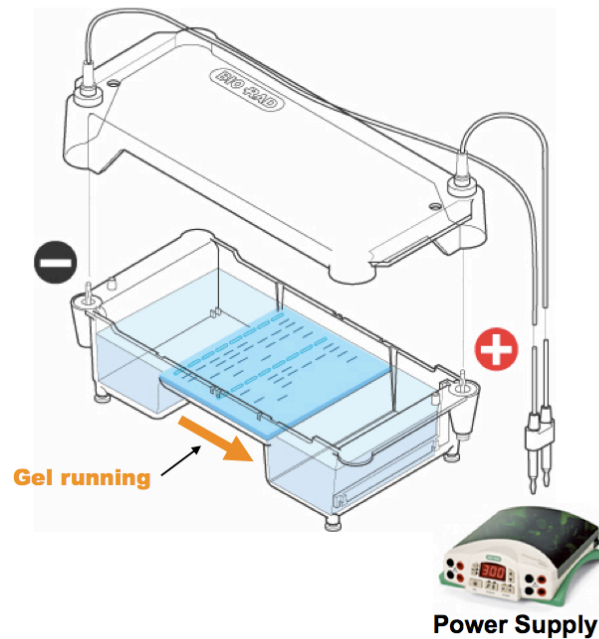




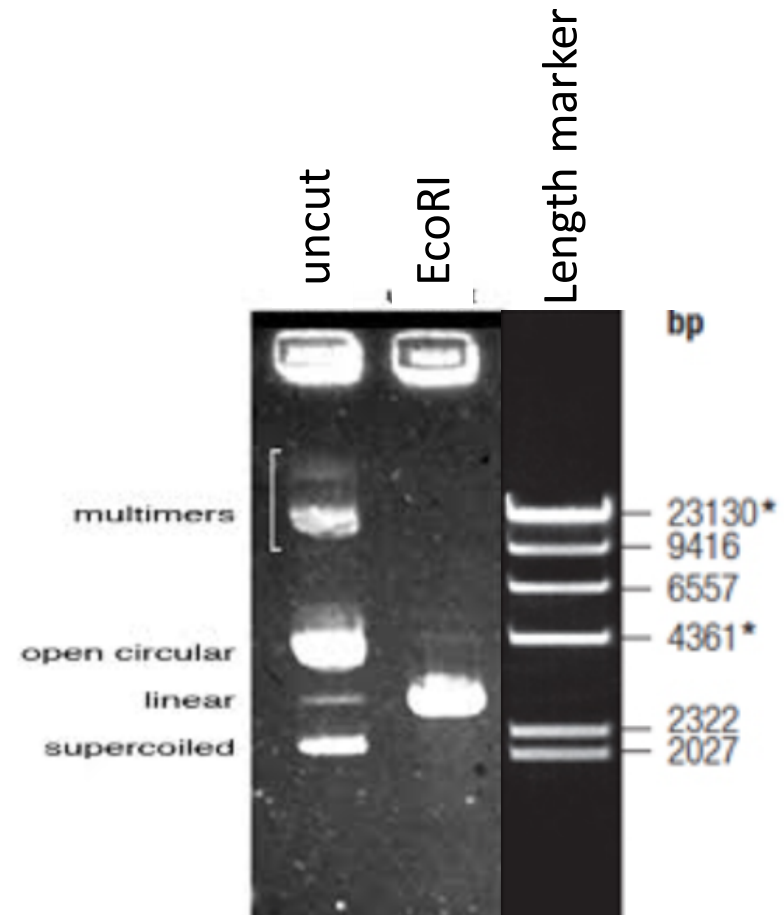
# Making recombinant DNA – Plasmid features

## 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)



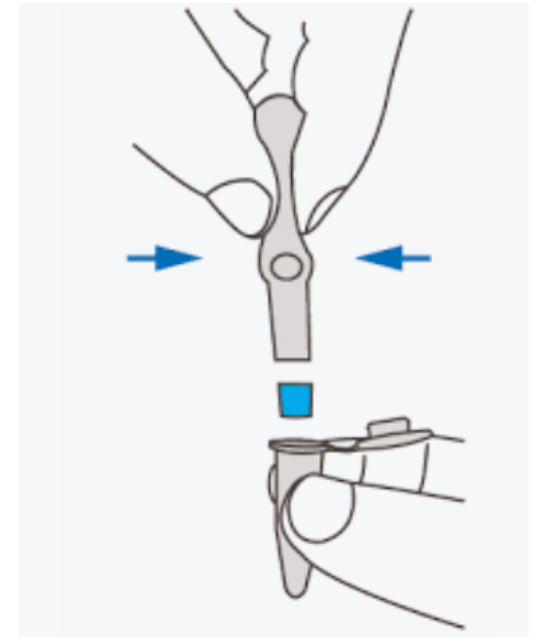
Length marker: mix of DNA  
fragments with defined  
length

Linearized pBS: 2900 nt

# Making recombinant DNA – Plasmid features

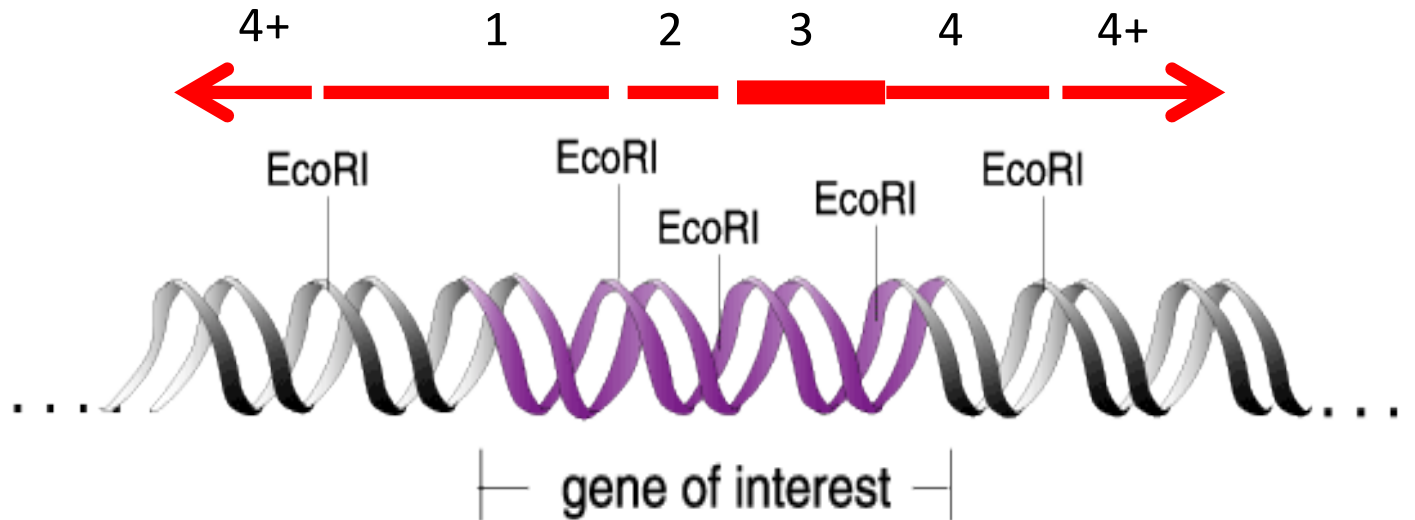


Cut out band from gel using a scalpel blade

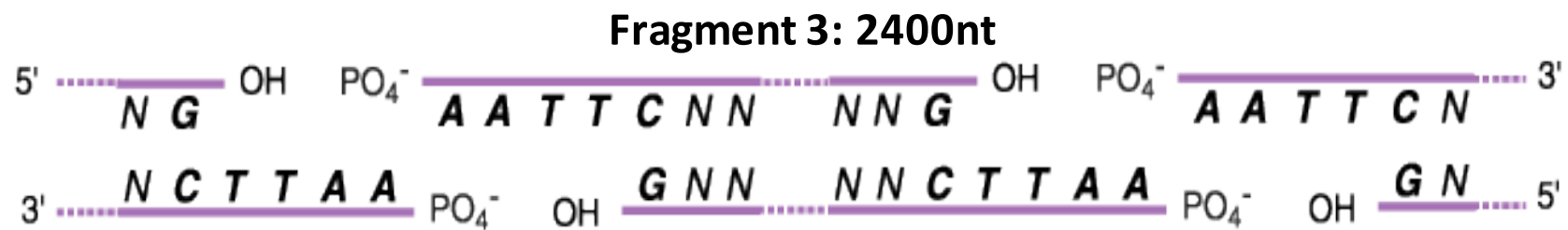


Purify DNA and eliminate agarose  
Determine concentration of purified plasmid DNA (ca. 50% loss of starting material)

# Preparing the insert

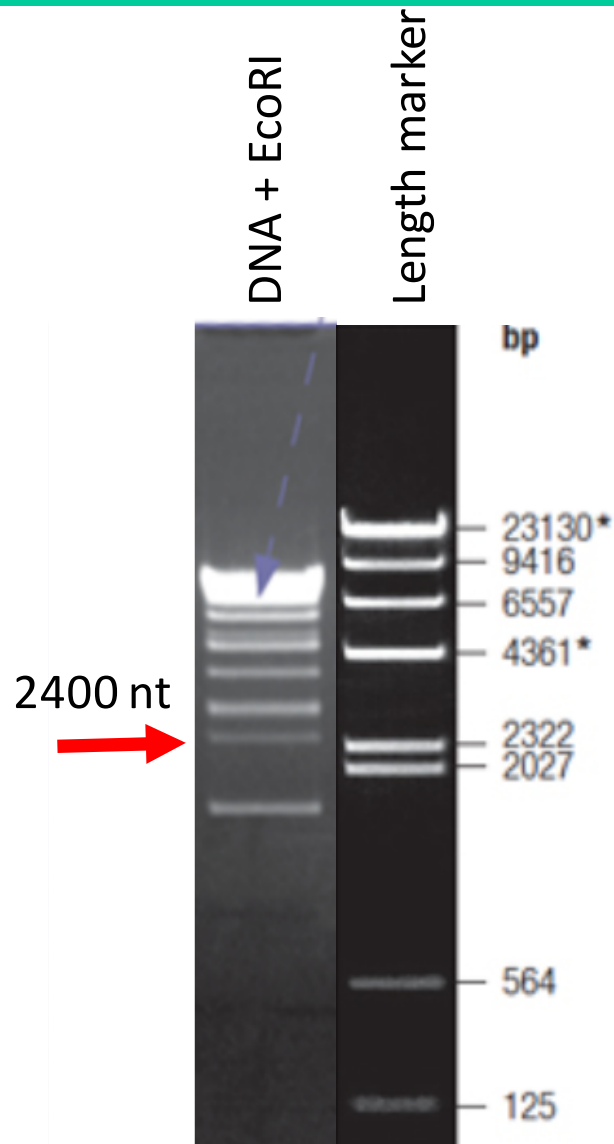


Digest DNA sample  
with EcoRI enzyme

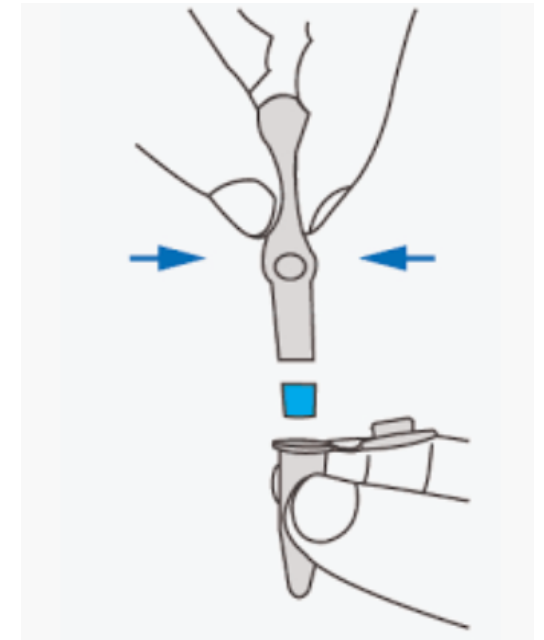


Defined size

# Preparing the insert

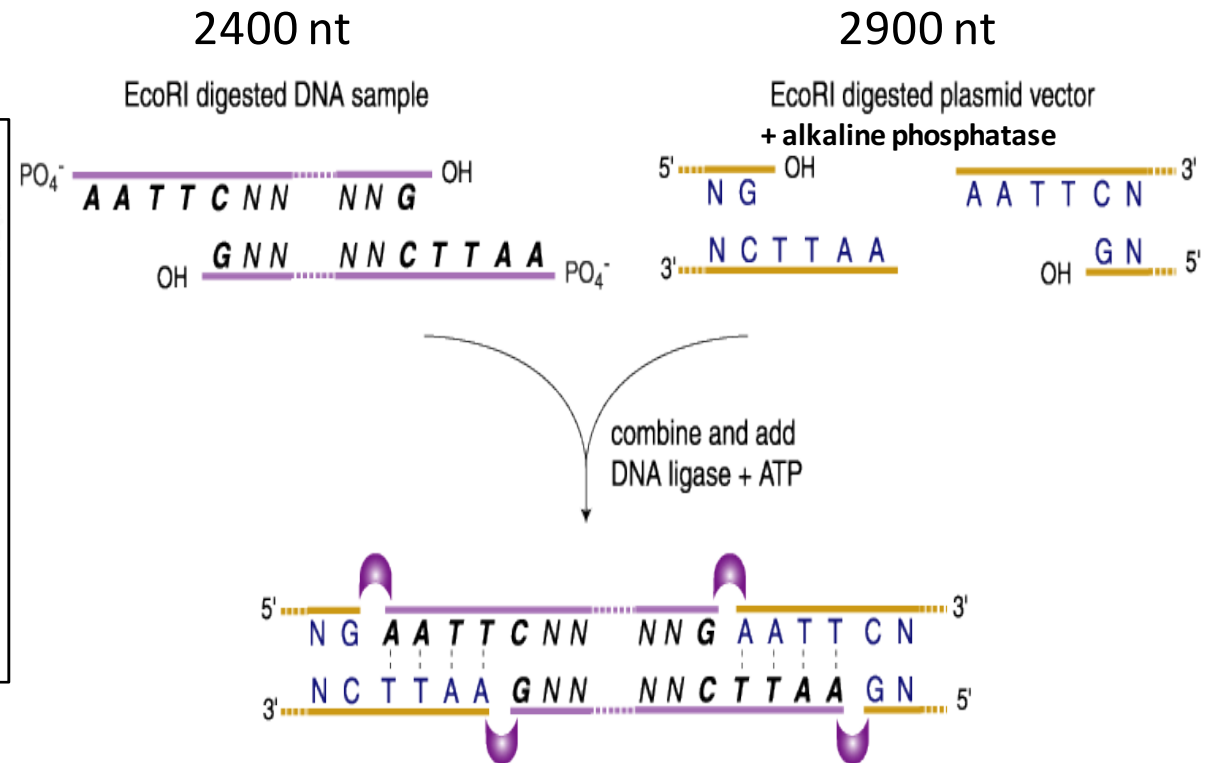
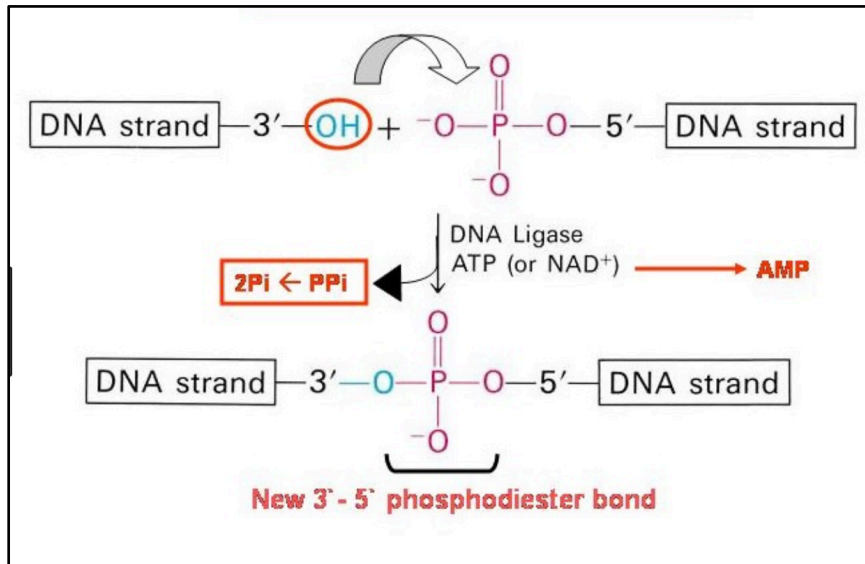


Cut out band from gel using a scalpel blade



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

# Ligating 2 fragments with DNA Ligase

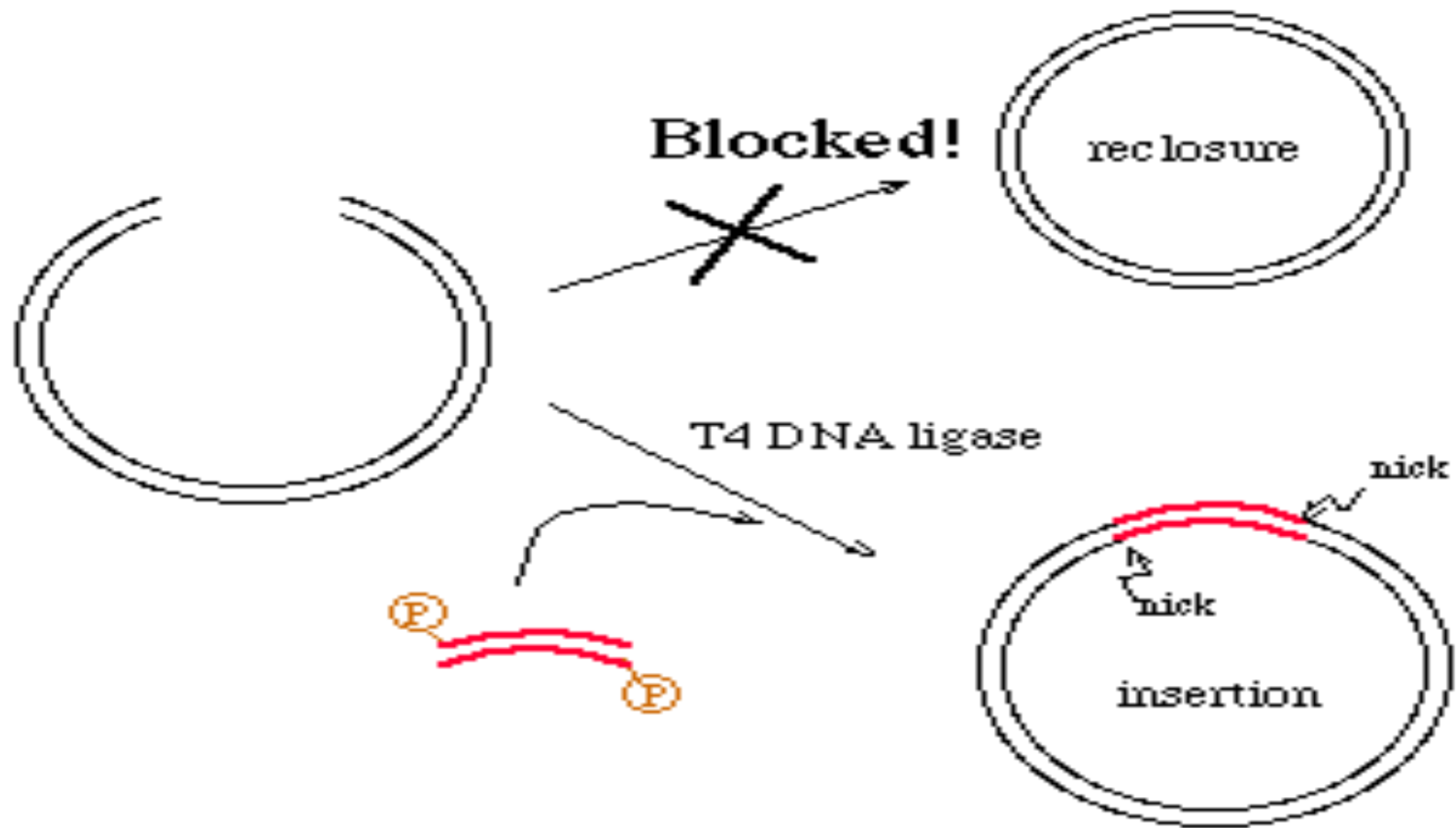


Sticky-end Ligations: 50 ng linearized plasmid + 3 fold molar excess of insert (=124 ng)

**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!!!

## Ligating 2 fragments with DNA Ligase



NICKS ARE TOLERATED – REPAIRED BY BACTERIA

## **REAL SETTING: USE CONTROLS!!**

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

**LIGATION 2: 50ng plasmid; EcoRI, de-phosph + 124ng INSERT + LIGASE**

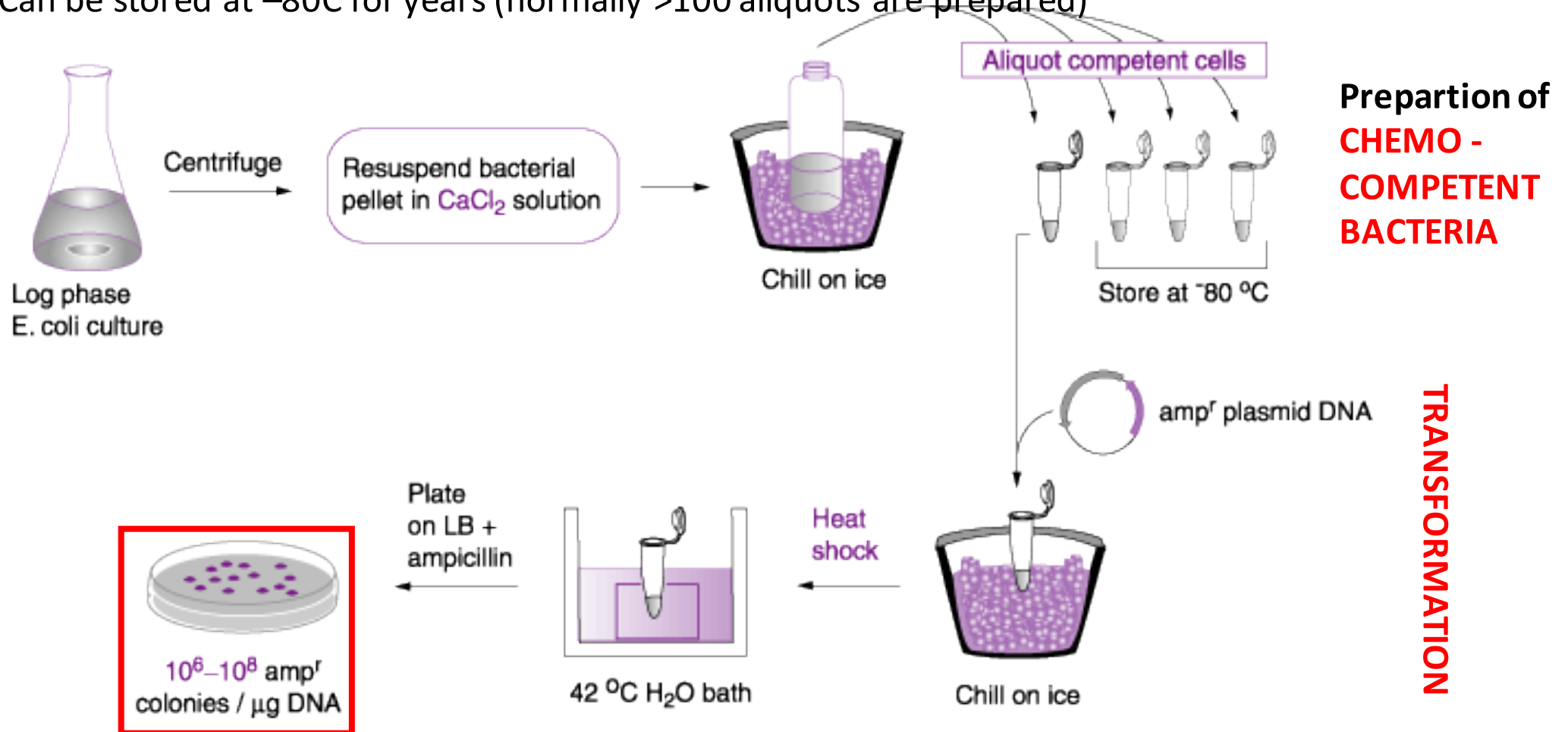
**Ev. LIGATION 3: 124ng INSERT + LIGASE**

# TRANSFORMATION: Insertion of ligated productis into bacteria

CaCl<sub>2</sub> and cold environment makes membrane permeable without killing the cells

= **CHEMOCOMPETENT BACTERIA**

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



Compent bacteria are put on ice until bacteria are thawed; add ligation product; induce heat shock (42C); DNA can enter the bacteria;  
add liquid media to allow bacteria to recover; plate on media plate containing ampicillin

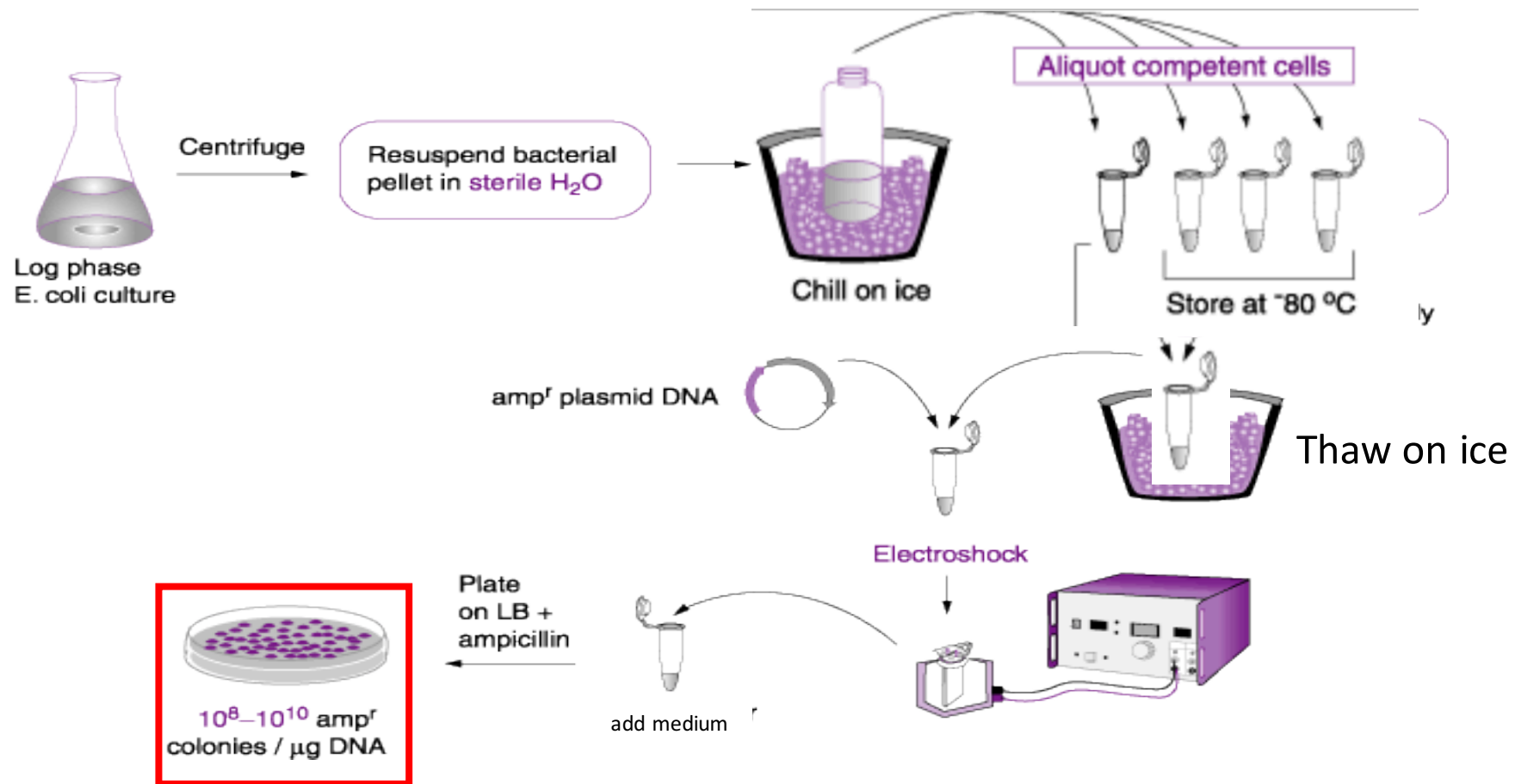


# TRANSFORMATION: Insertion of ligated productis into bacteria

H<sub>2</sub>O and cold environment makes membrane permeable without killing the cells

= **ELECTROCOMPETENT BACTERIA**

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



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

# EVALUATING THE SUCCESS OF DNA CLONING EVENTS

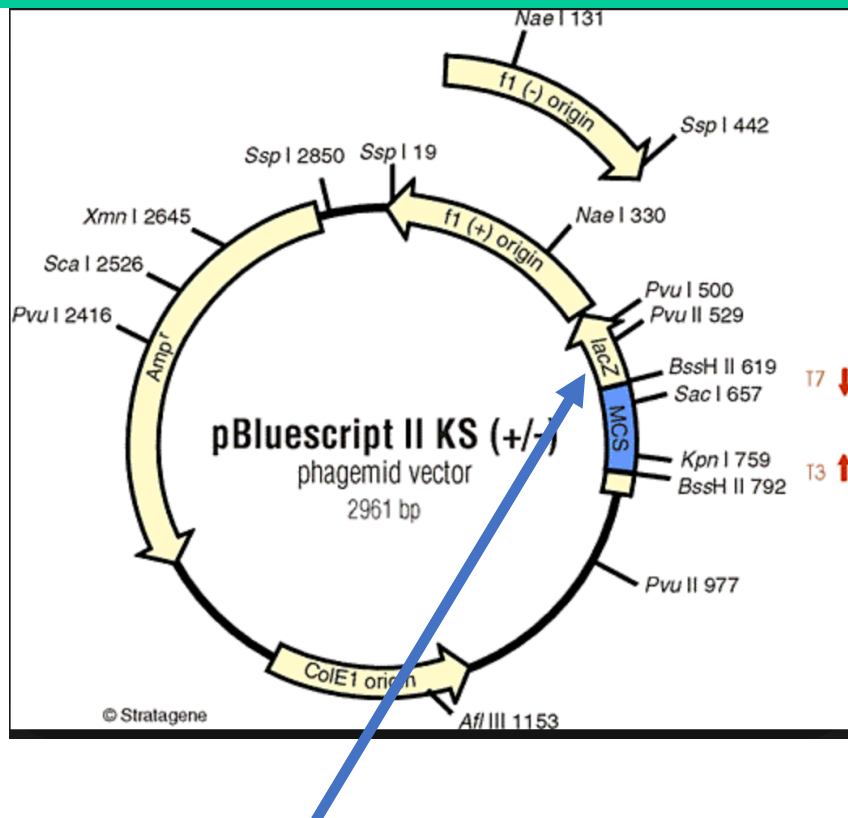
**LIGATION 1: 50ng plasmid; EcoRI, de-phosph + LIGASE**

**LIGATION 2: 50ng plasmid; EcoRI, de-phosph + 124ng INSERT + LIGASE**

**Ev. LIGATION 3: 124ng INSERT + LIGASE**

# 1. EASY IDENTIFICATION SUCCESSFULL DNA CLONING EVENTS

## BLUE-WHITE SELECTION SCREEN



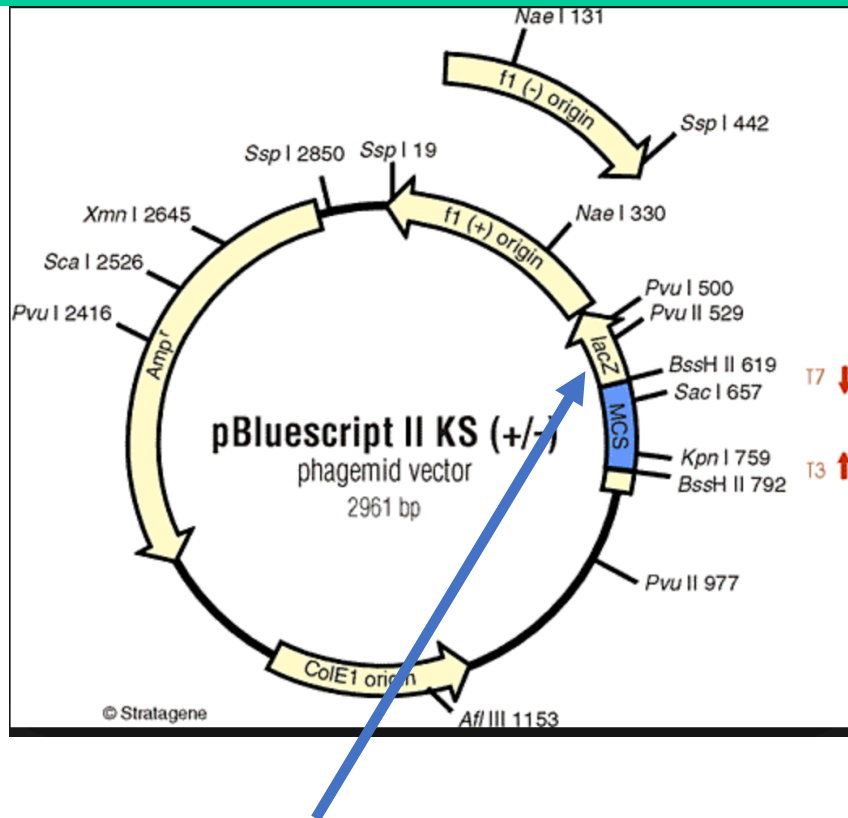
### **α-complementation**

- 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.

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

# 1. EASY IDENTIFICATION SUCCESSFUL DNA CLONING EVENTS

## BLUE-WHITE SELECTION SCREEN



### ....some things to consider

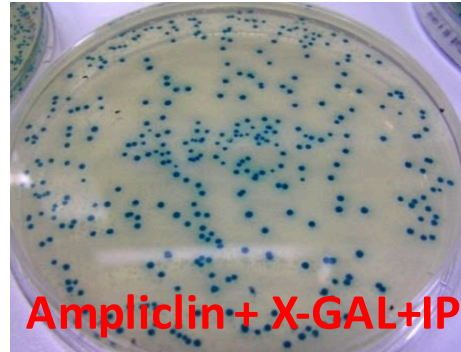
- *lacZ* gene not expressed constitutively
- X-gal does not activate gene expression
- must use IPTG as inducer
- (isopropyl- $\beta$ -D-thio-galactoside)
- small inframe insertions may not inactivate  $\alpha$  peptide
- still get blue colonies (often lighter – less activity)

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

# 1. EASY IDENTIFICATION SUCCESSFULL DNA CLONING EVENTS

## BLUE-WHITE SELECTION SCREEN

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

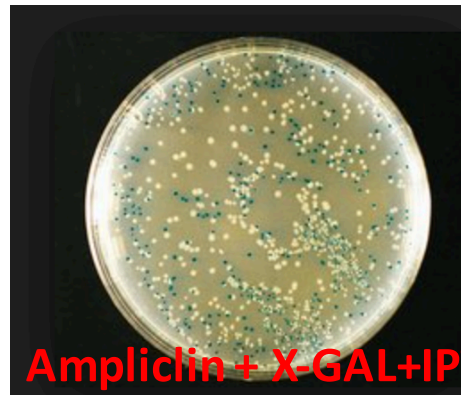


46 blue colonies

**Ampliclin + X-GAL+IPTG**

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

**LIGATION 2: 50ng  
plasmid; EcoRI,  
de-phosph +  
124ng INSERT +  
LIGASE**

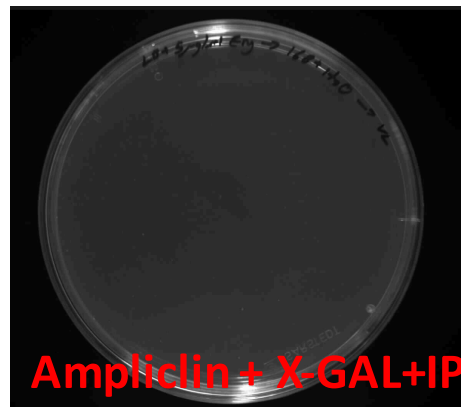


200 white colonies  
40 blue colonies

**Ampliclin + X-GAL+IPTG**

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

**Ev. LIGATION 3:  
124ng INSERT +  
LIGASE**



No colonies

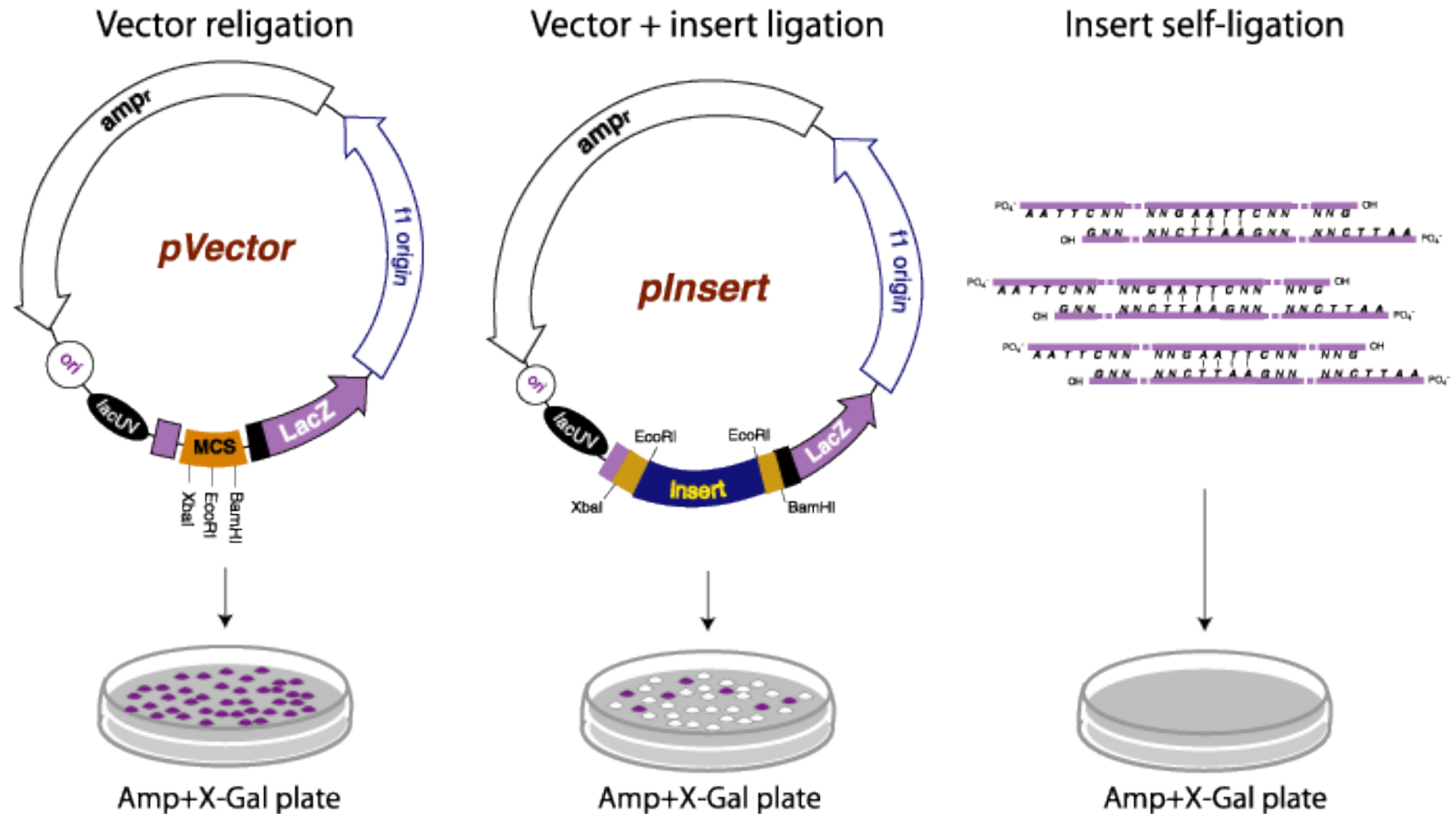
**Ampliclin + X-GAL+IPTG**

1. **NO COLONIES**

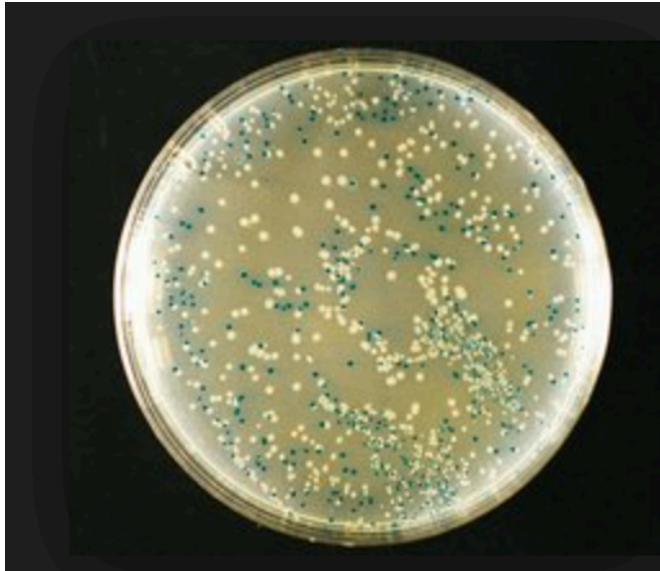
For example

# 1. EASY IDENTIFICATION SUCCESSFUL DNA CLONING EVENTS

## BLUE-WHITE SELECTION SCREEN



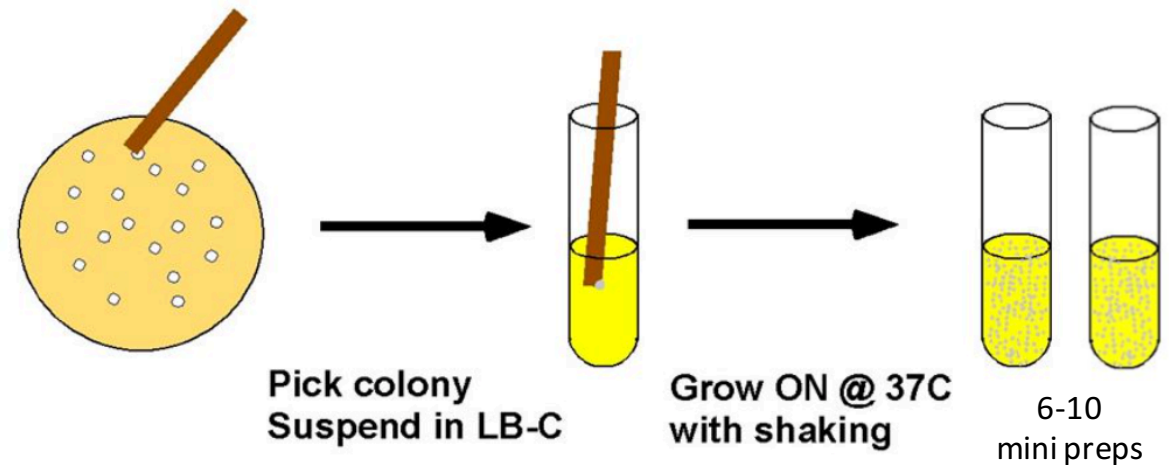
## 2. DNA PREPARATION AND CONTROL DIGEST



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.

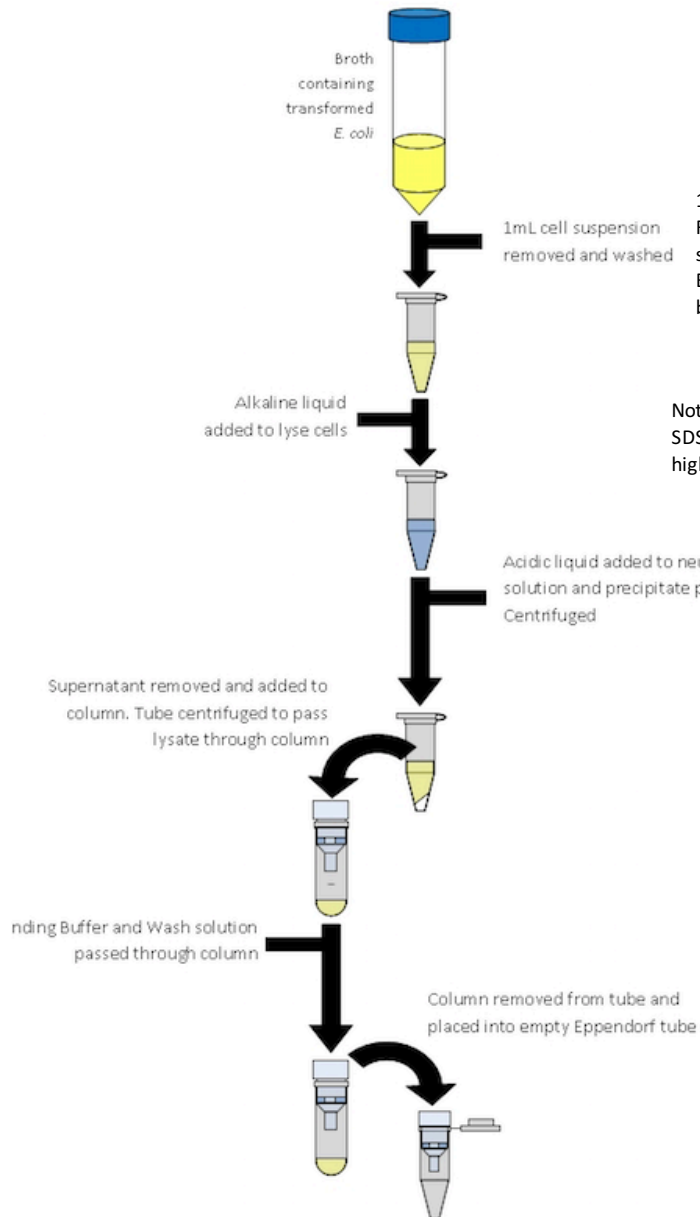


Next day: harvest bacteria by centrifugation and prepare plasmid DNA



## 2. DNA PREPARATION AND CONTROL DIGEST

### Single Page Protocol for Alkaline Lysis Mini-Plasmid Preparation

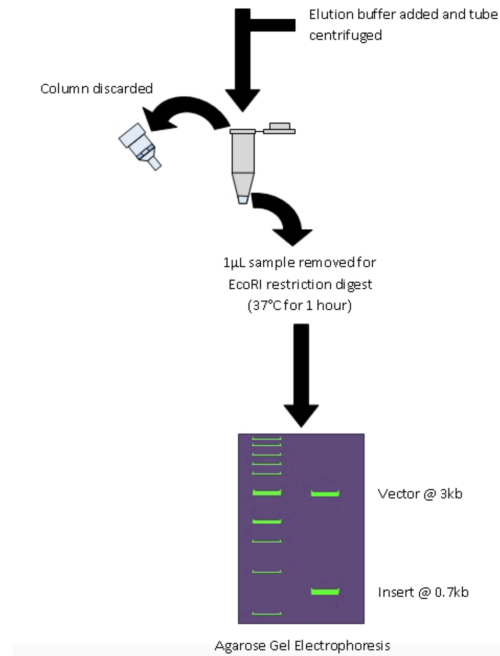


1ml of overnight culture Removed, spinned and supernatant removed. Bacteria pellet resuspended in buffer that does not kill cells

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

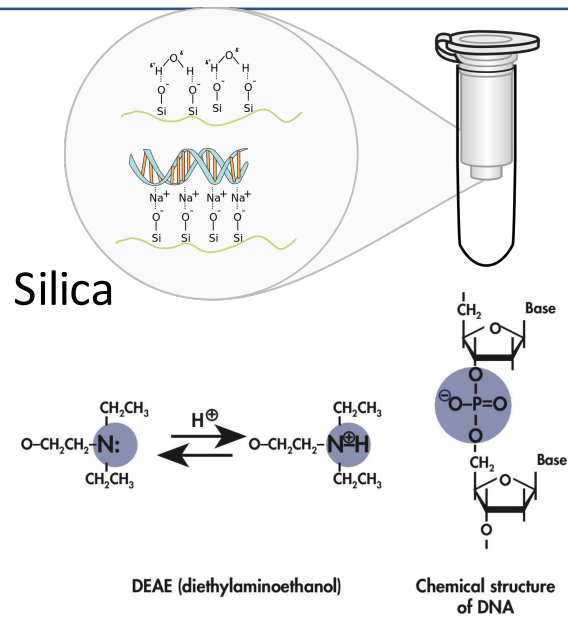
The lysate is neutralized by the addition of acidic potassium acetate; The high salt concentration causes Potassium dodecyl sulfate to precipitate, and the denatured proteins, chromosomal DNA, and cellular debris become trapped in salt-detergent complexes. Plasmid DNA, being smaller and covalently closed, renatures correctly and remains in solution

**Centrifugation at high speed** (ca. 13,000 rpm); cell debris and genomic DNA precipitate; small DNA molecules (plasmid) remain in supernatant)



The use of columns Results in very pure plasmid DNA. "sequence grade"

### Silica



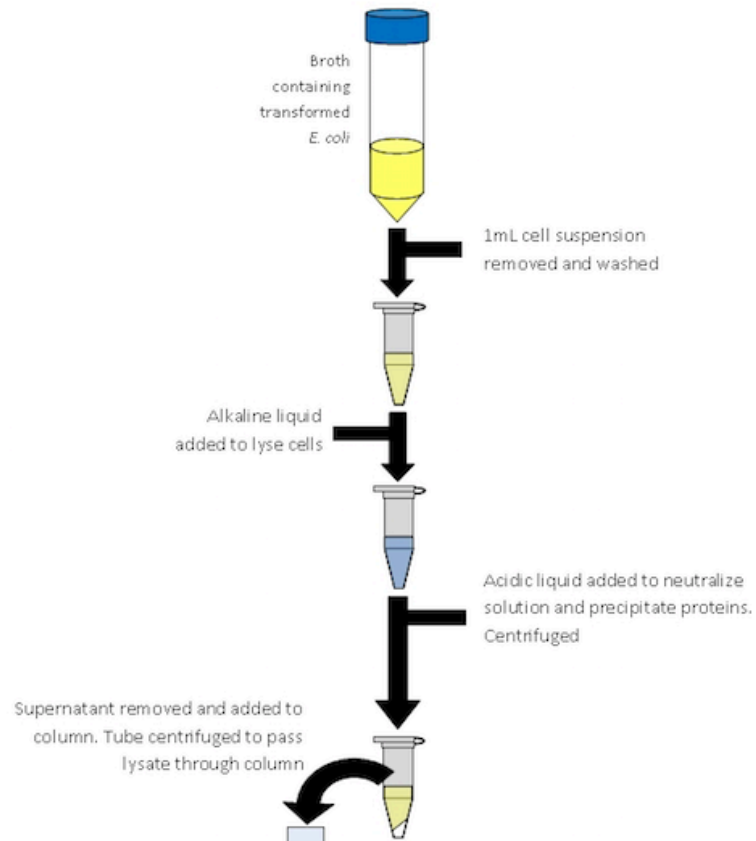
Resin in column Is positively charged: Binds negative charge of plasmid DNA backbone



## 2. DNA PREPARATION AND CONTROL DIGEST

### Alternative method without columns

#### Single Page Protocol for Alkaline Lysis Mini-Plasmid Preparation



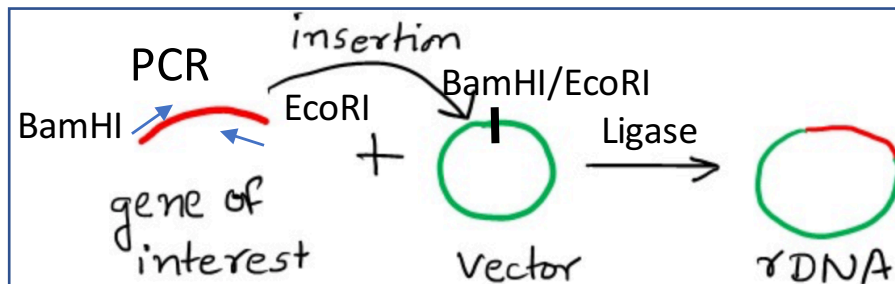
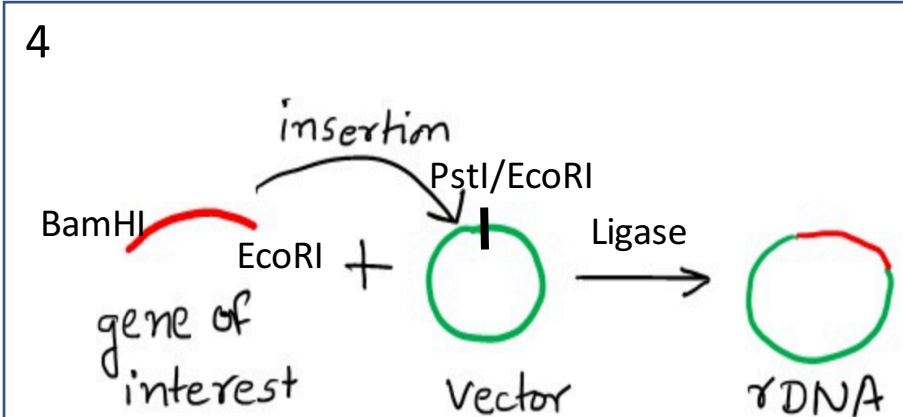
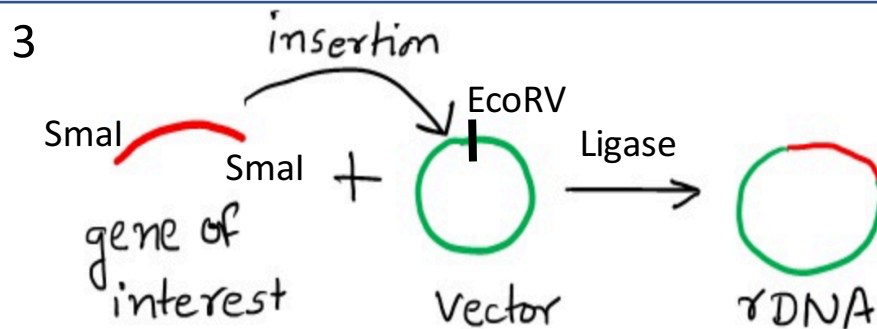
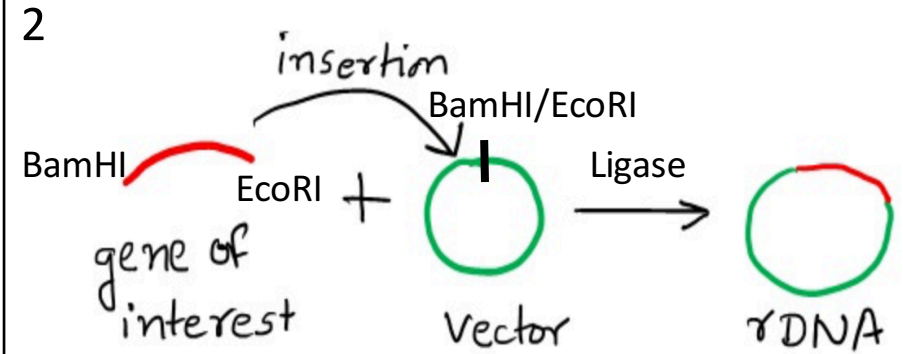
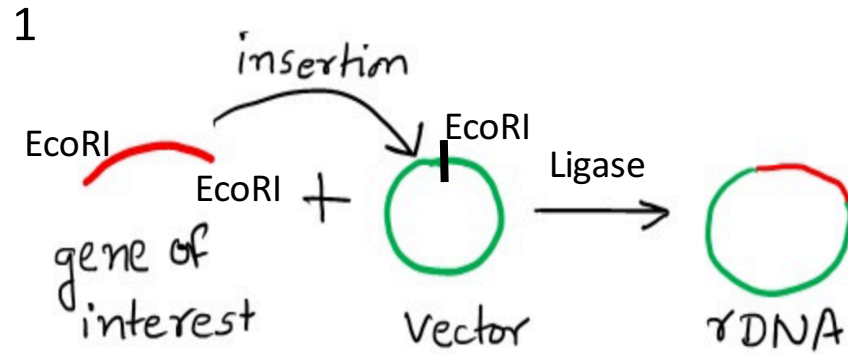
- put supernatant in new tube
- add salt (final 0,5M NaCl)
- add Isopropanol
- put at -20C for 1 hour
- centrifuge
- plasmid DNA will precipitate



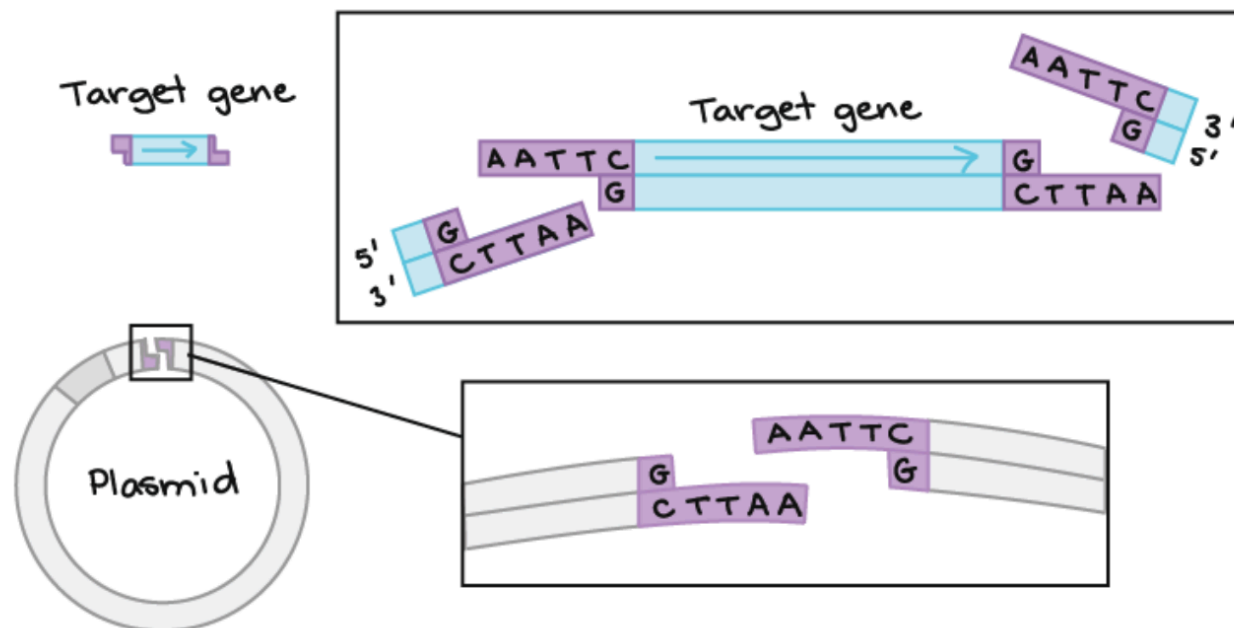
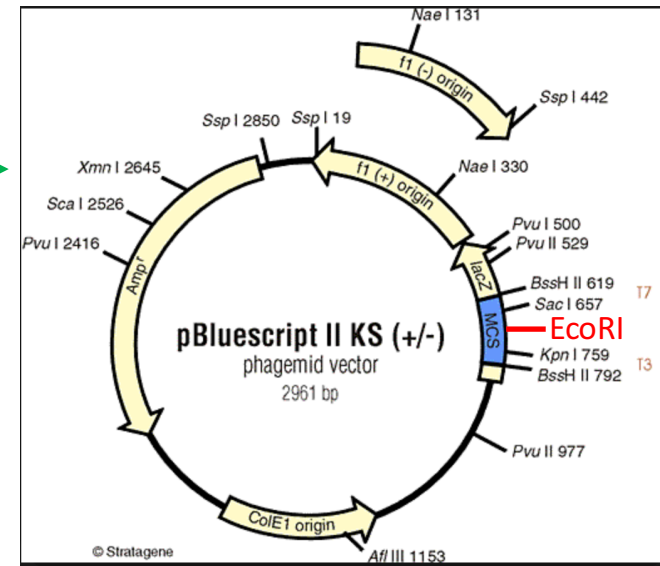
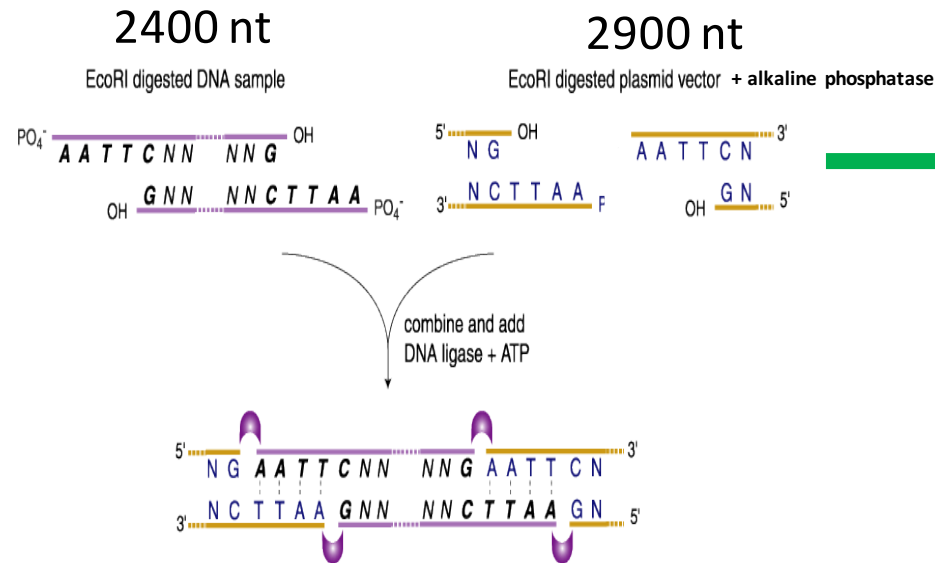
Plasmid is not very clean; sufficient for digestion with restriction enzymes; not usable for DNA sequencing  
**"not sequencing grade"**

- Much cheaper; you can test many colonies for correctness of plasmid
- Takes some more time

### 3. OVERVIEW OVER OTHER CLONING STRATEGIES

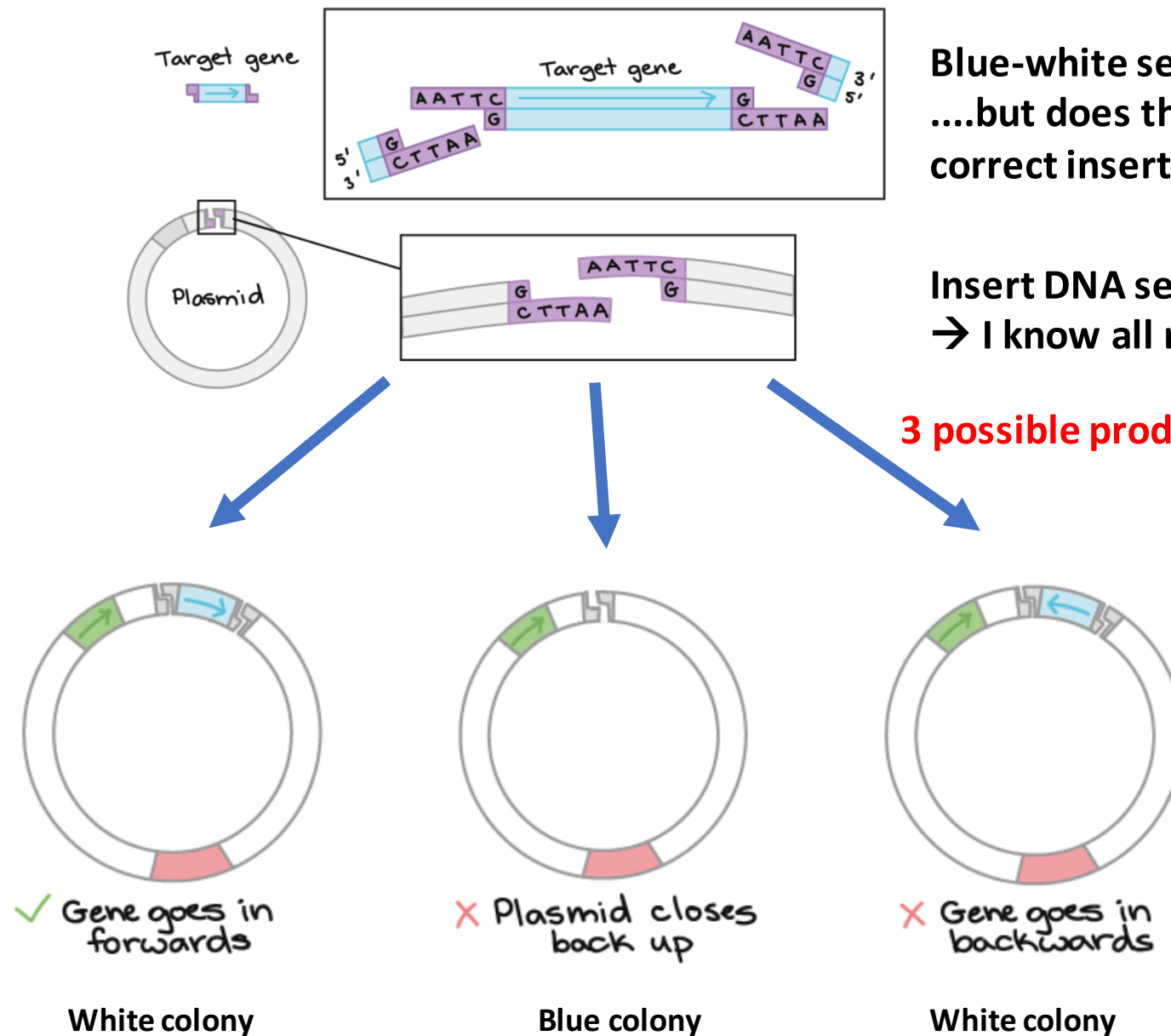


## 2. DNA PREPARATION AND CONTROL DIGEST



**Note:**  
5' overhangs of insert and linearized plasmids are compatible; both have been cut with EcoRI. Ligase covalently links both molecules. EcoRI sites are reconstituted and now flank the insert sequence!!!

## 2. DNA PREPARATION AND CONTROL DIGEST



Blue-white selection is OK...  
....but does the plasmid really has the correct insert???

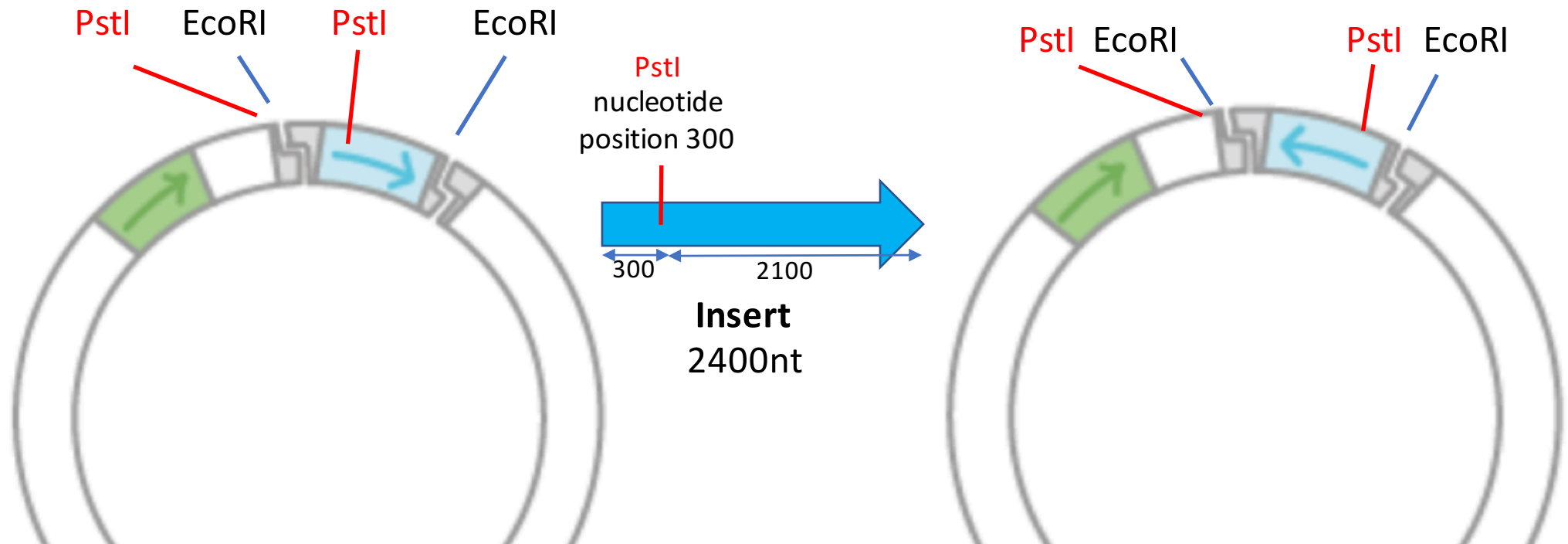
Insert DNA sequence is known  
→ I know all restriction sites

3 possible products of ligation

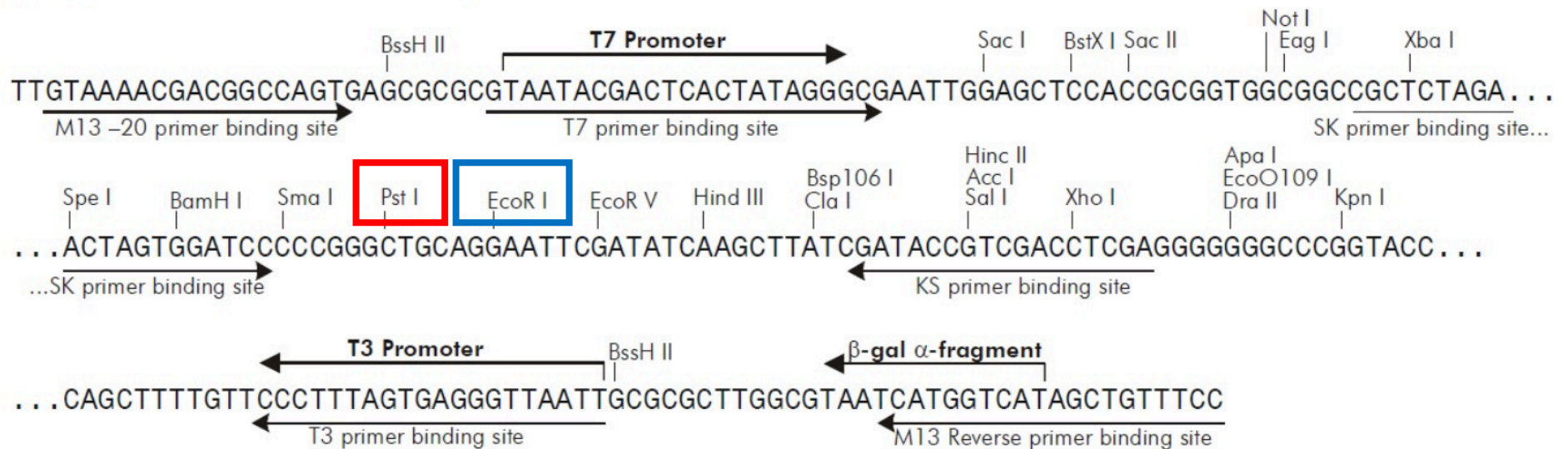
**HOW CAN WE  
DETERMINE THE  
ORIENTATION AND  
IDENTITY OF THE  
INSERT?**

Blue-white screening  
using for example the  
pBluescript vector

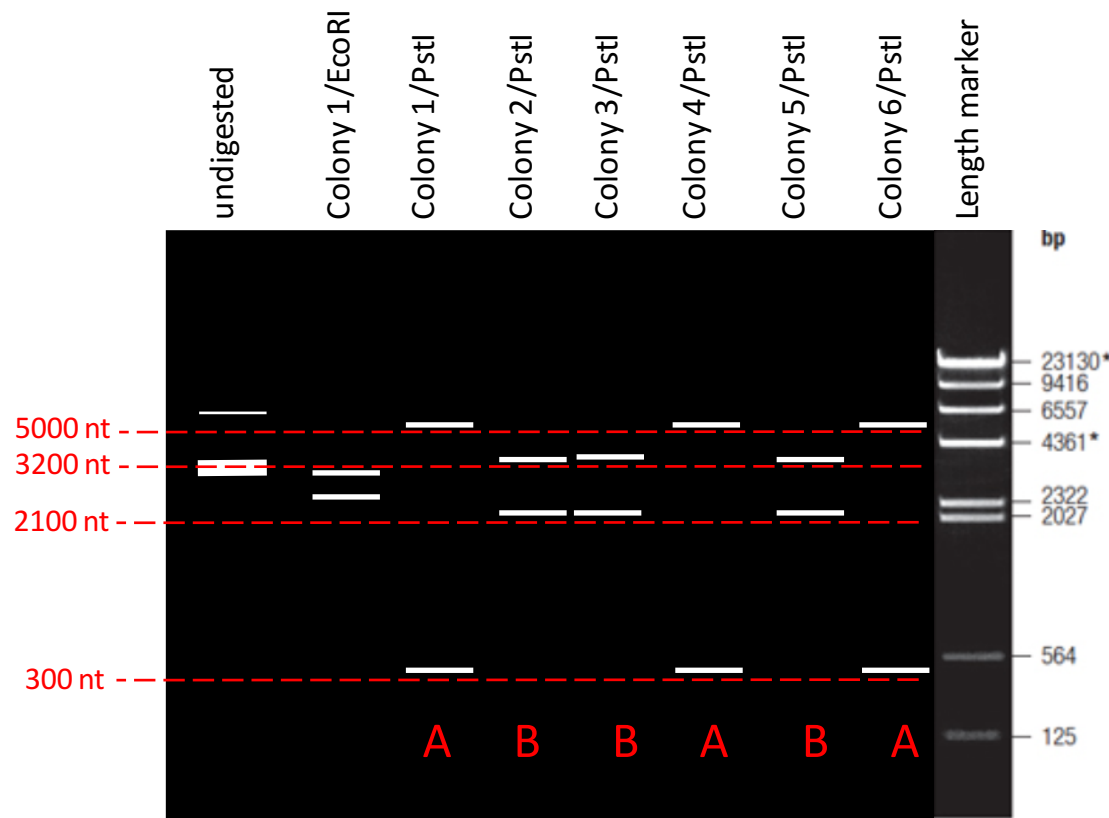
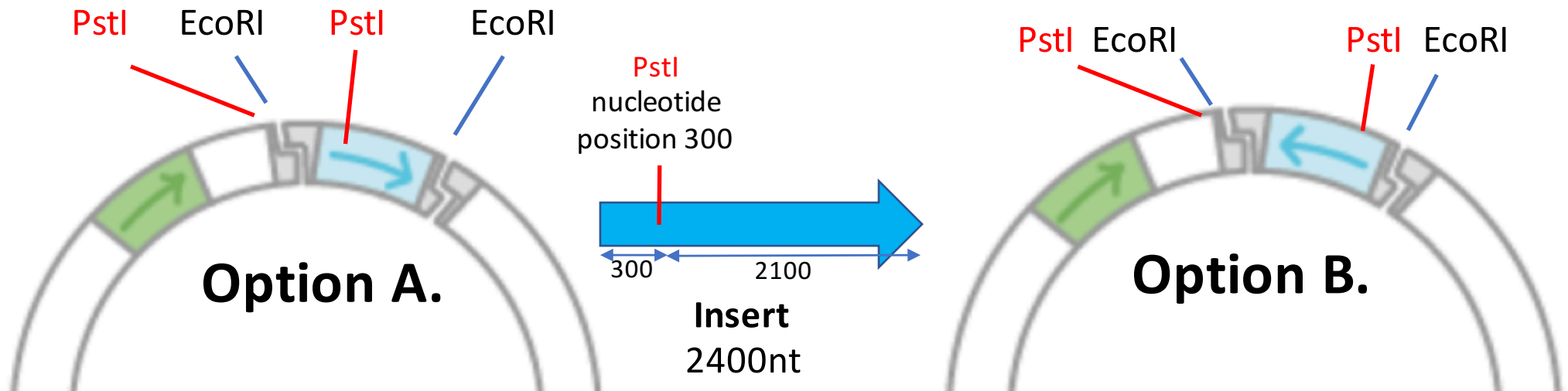
## 2. DNA PREPARATION AND CONTROL DIGEST



### pBluescript II KS (+/-) Multiple Cloning Site Region (sequence shown 598–826)



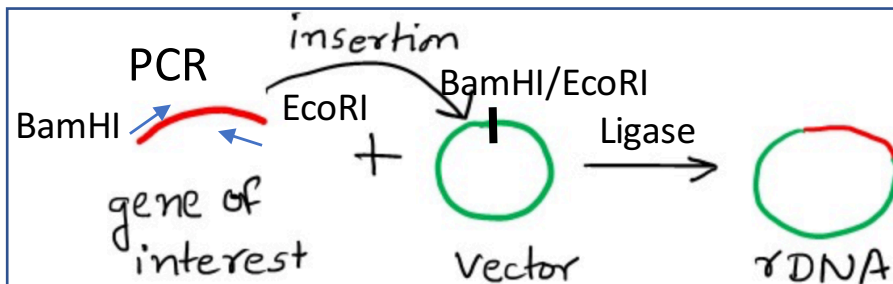
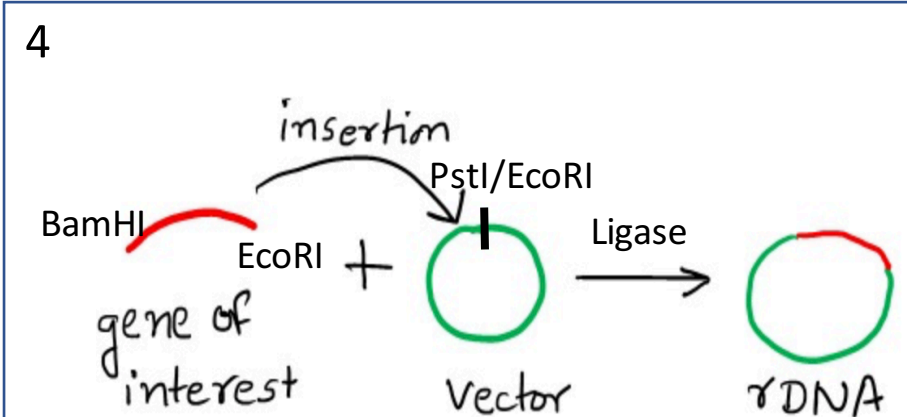
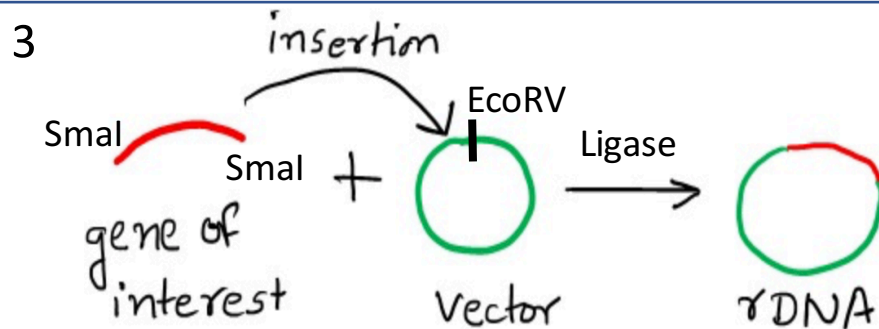
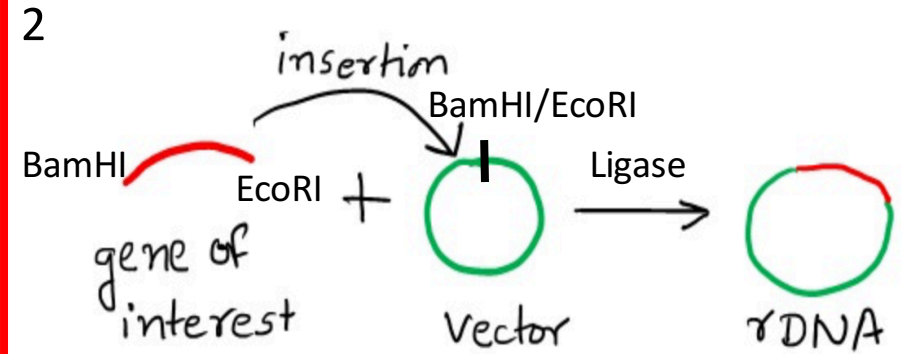
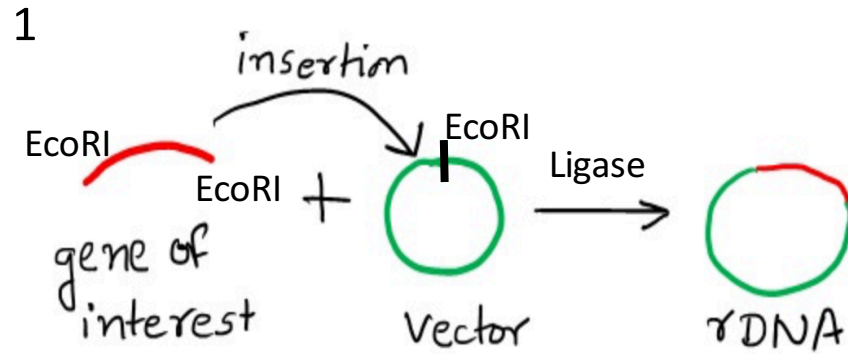
## 2. DNA PREPARATION AND CONTROL DIGEST



Insert: 2400 nt  
Plasmid: 2900 nt

Cut with restriction enzyme  
that result asymmetric  
digestion products

### 3. OVERVIEW OVER OTHER CLONING STRATEGIES





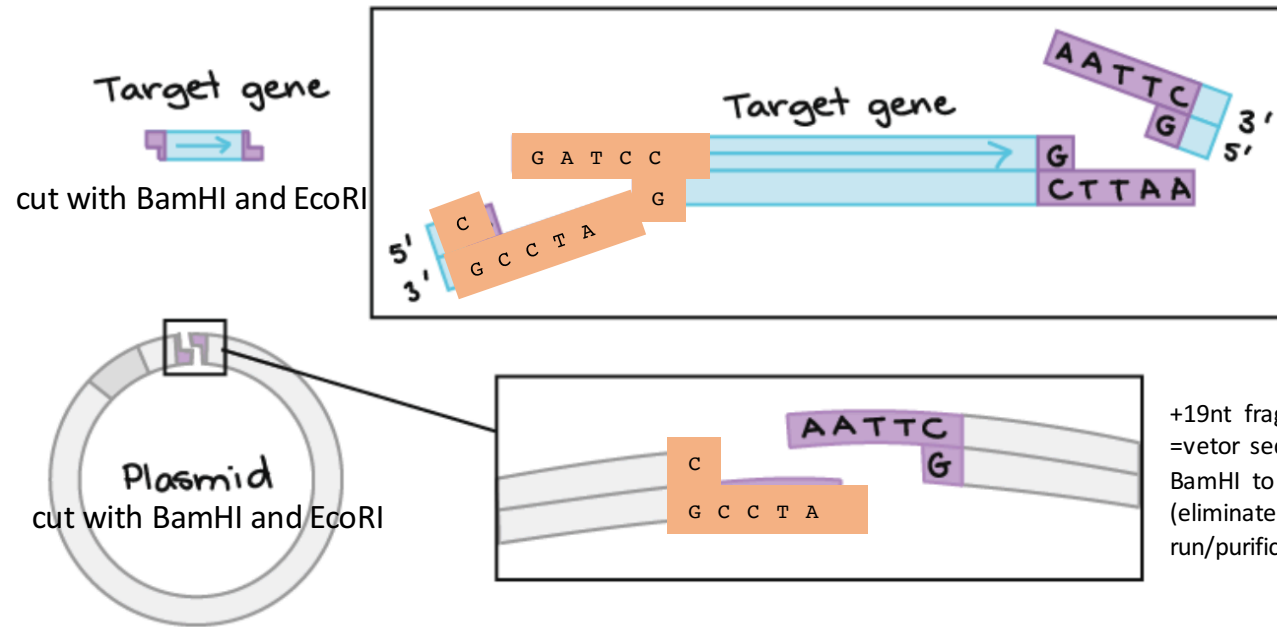
## 3.2. DNA CLONING WITH 2 COHESIVE OVERHANGS

EcoRI: G/AATTC  
CTTAA/G

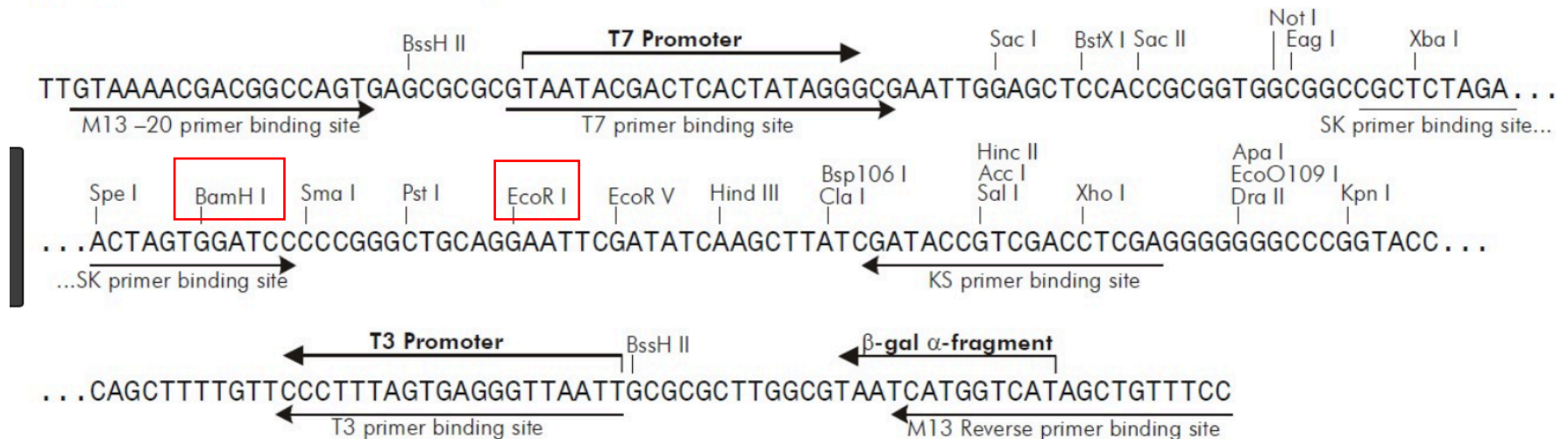
BamHI: G/GATCC  
CCTAG/G

### DIRECTIONAL CLONING

→ Always preferred cloning strategy



### pBluescript II KS (+/-) Multiple Cloning Site Region (sequence shown 598–826)

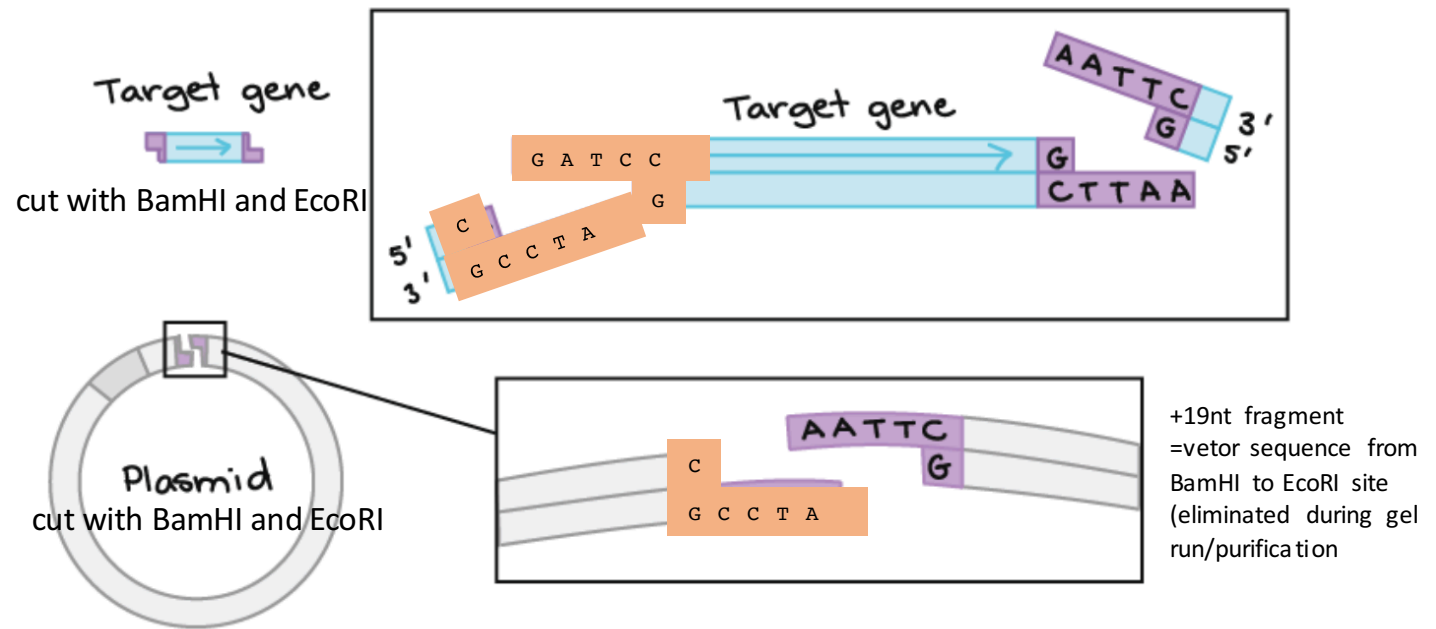




## 3.2. DNA CLONING WITH 2 COHESIVE OVERHANGS

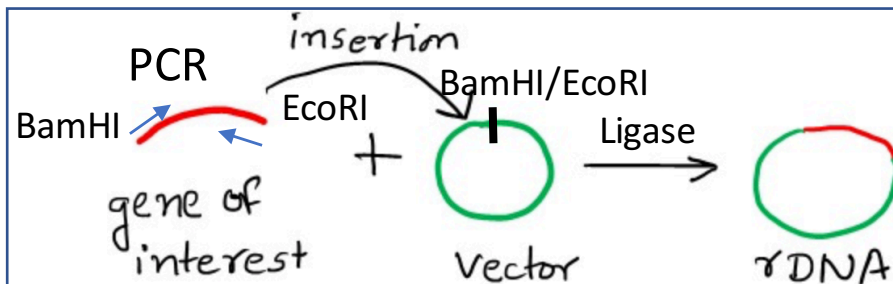
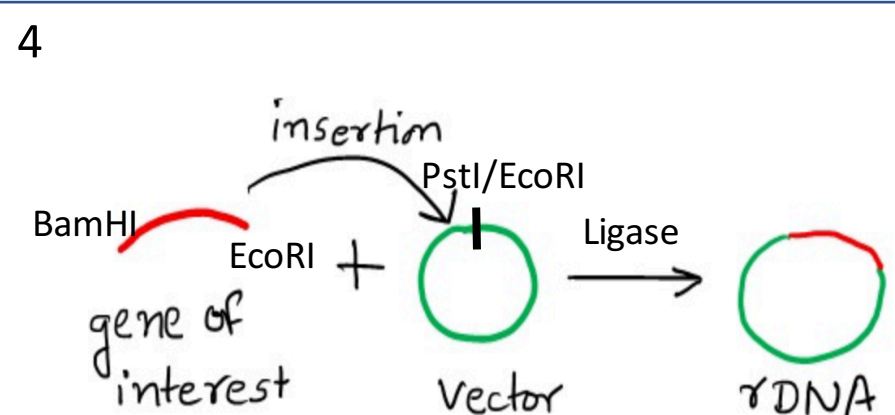
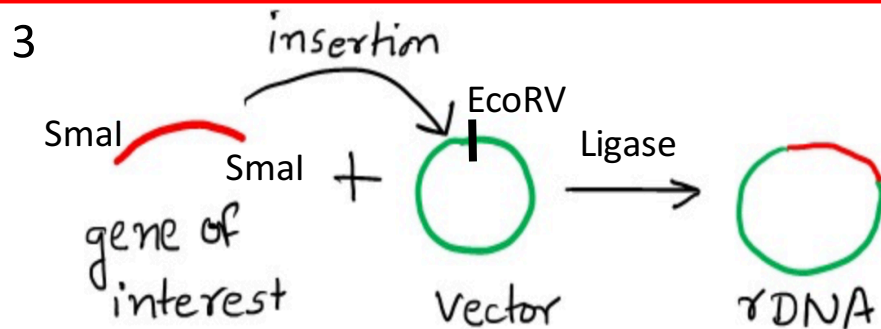
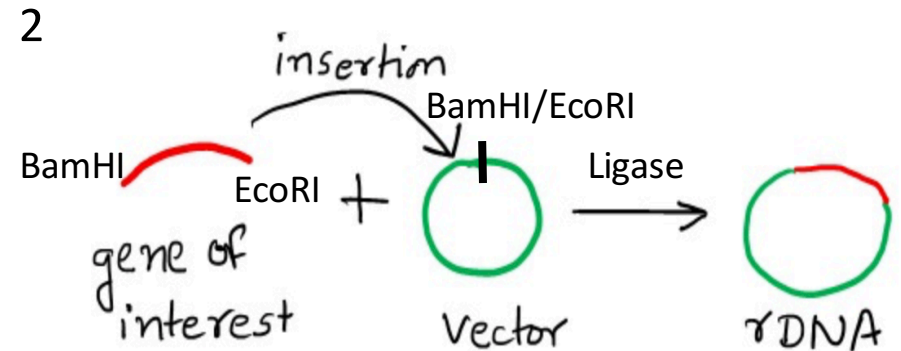
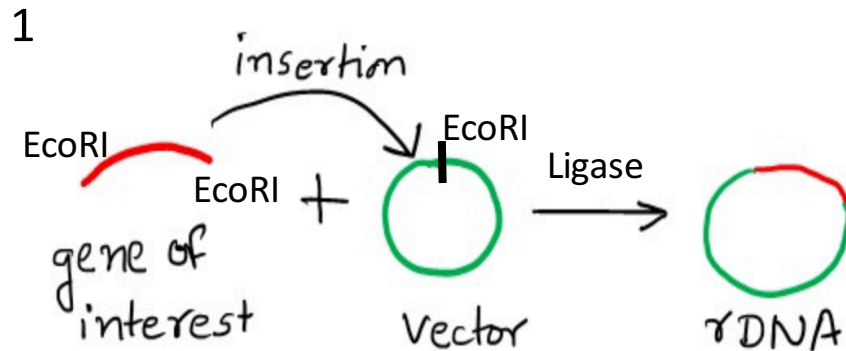
EcoRI: G/AATTC  
CTTAA/G

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))
5. Transform competent bacteria
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!!!

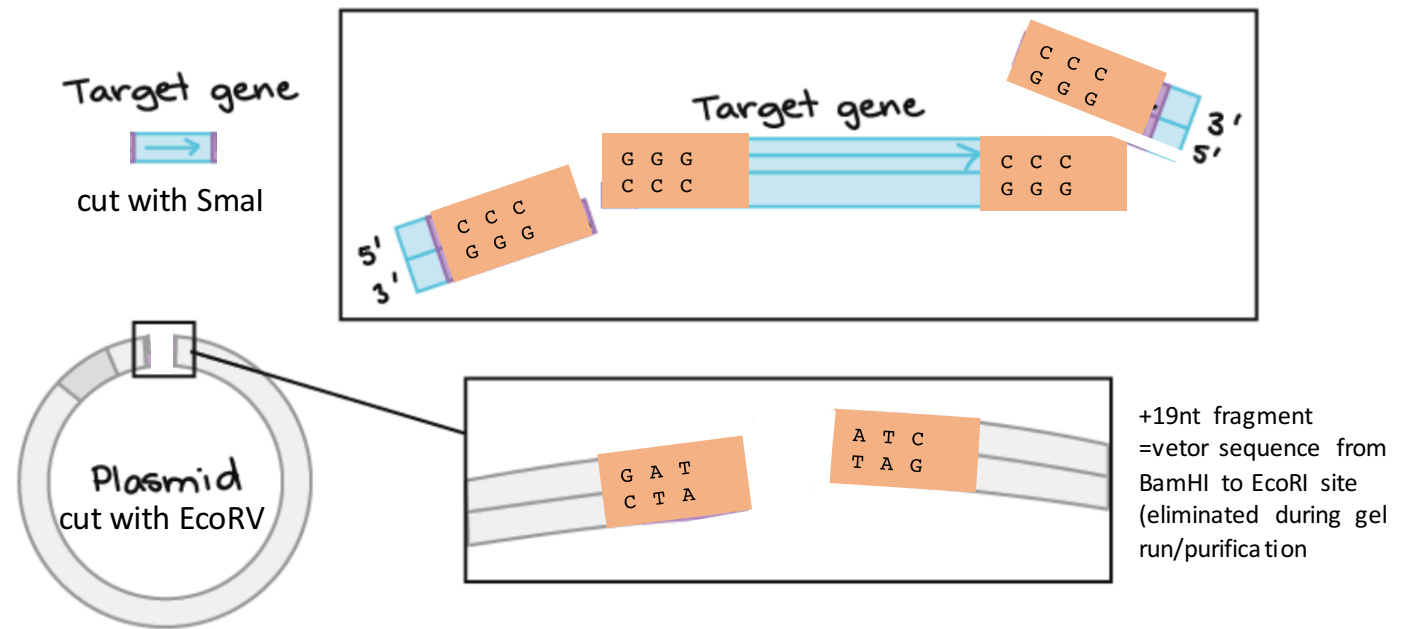
### 3. OVERVIEW OVER OTHER CLONING STRATEGIES



### 3.3. DNA CLONING WITH BLUNT ENDS

SmaI: CCC/GGG  
GGG/CCC

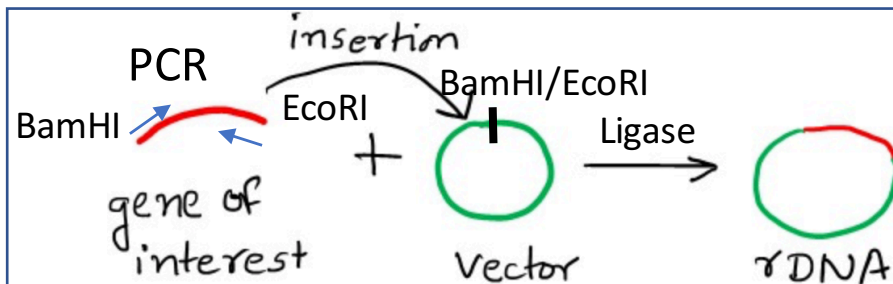
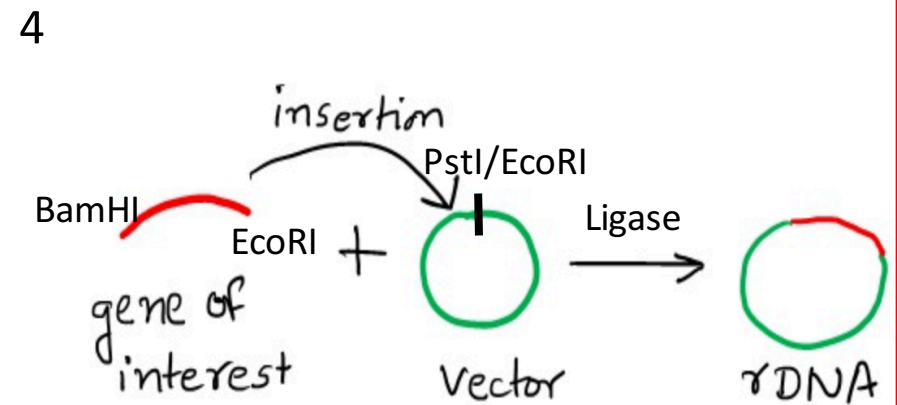
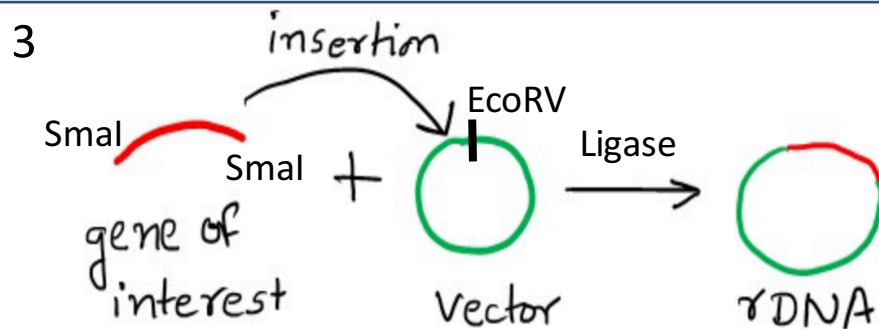
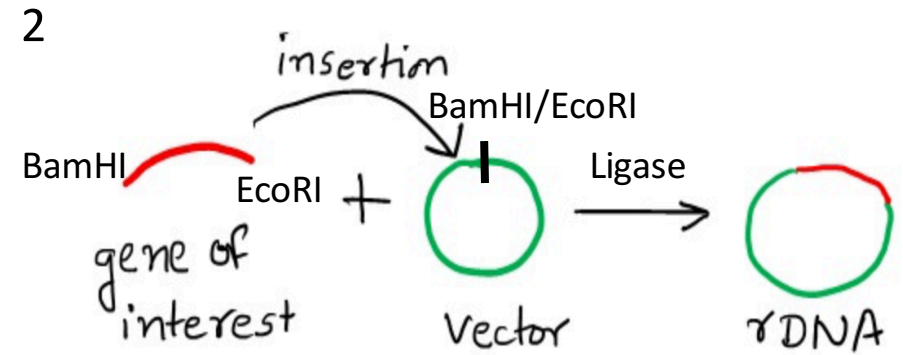
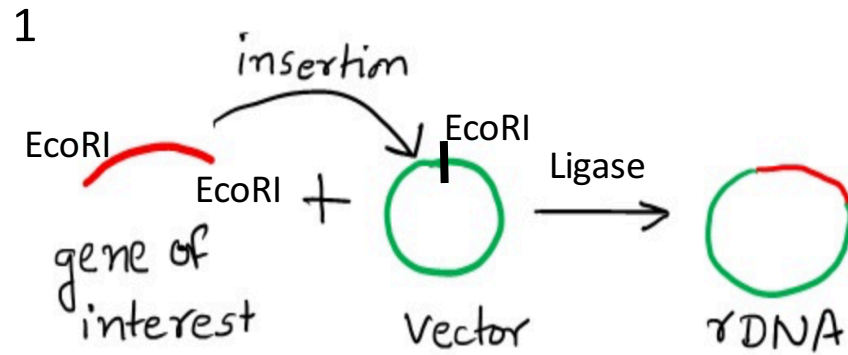
EcoRV: GAT/ATC  
CTA/TAG



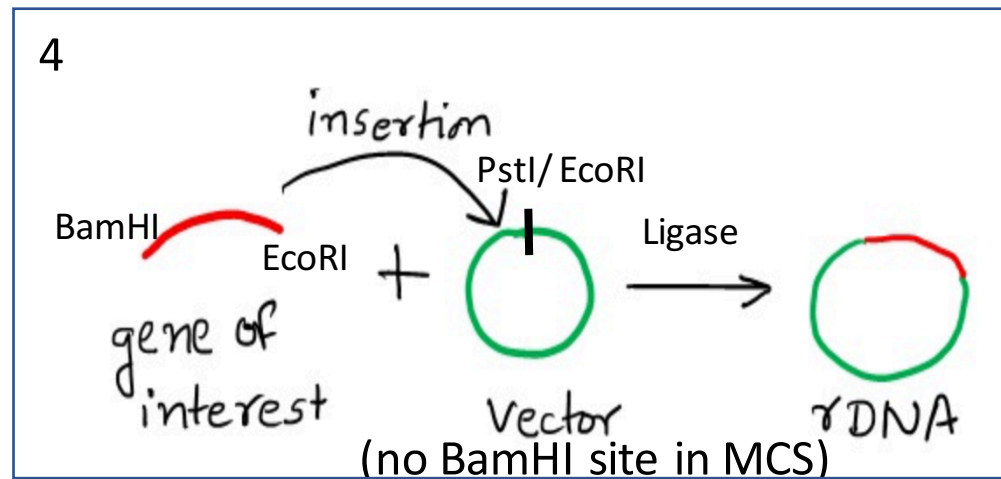
1. SmaI digest to obtain insert
2. EcoRV digest + alkaline phosphatase treatment to obtain linearized pBluescript (that cannot re-ligate)
3. Gel run and purification of relevant DNA fragments
4. Set up ligation (plasmid:insert = 1:3 (5))
5. Transform competent bacteria
6. Plasmid preparation and control 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

GATGGG ——— CCCATC  
CTACCC ——— GGGTAG

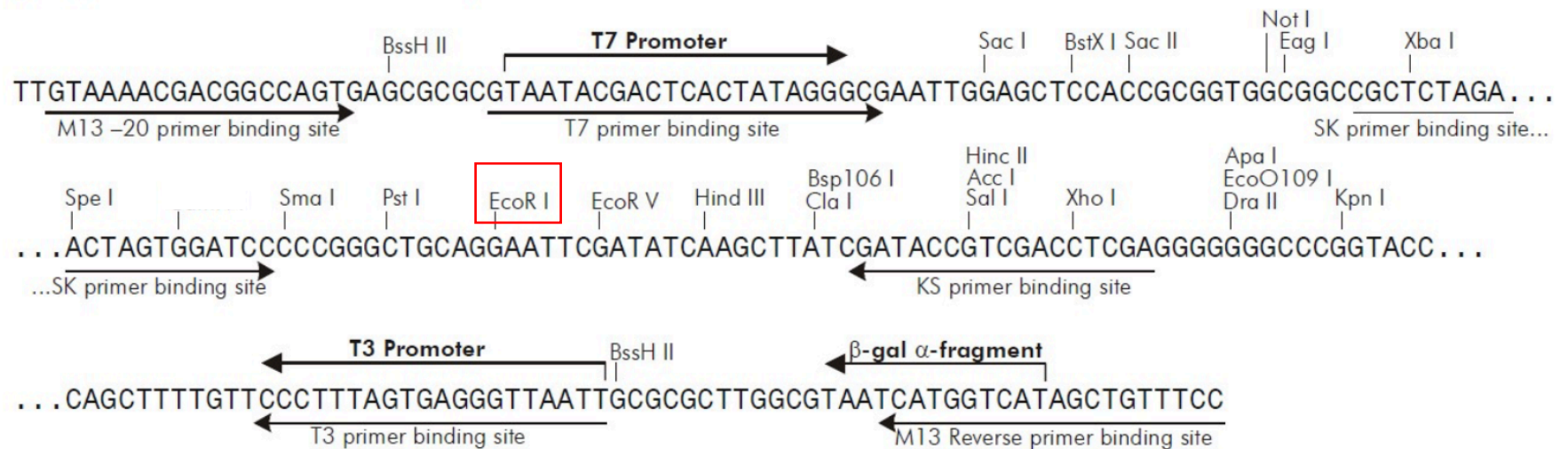
### 3. OVERVIEW OVER OTHER CLONING STRATEGIES



## 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS



### pBluescript II KS (+/-) Multiple Cloning Site Region (sequence shown 598–826)



### 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

BamHI: G/GATCC  
CCTAG/G

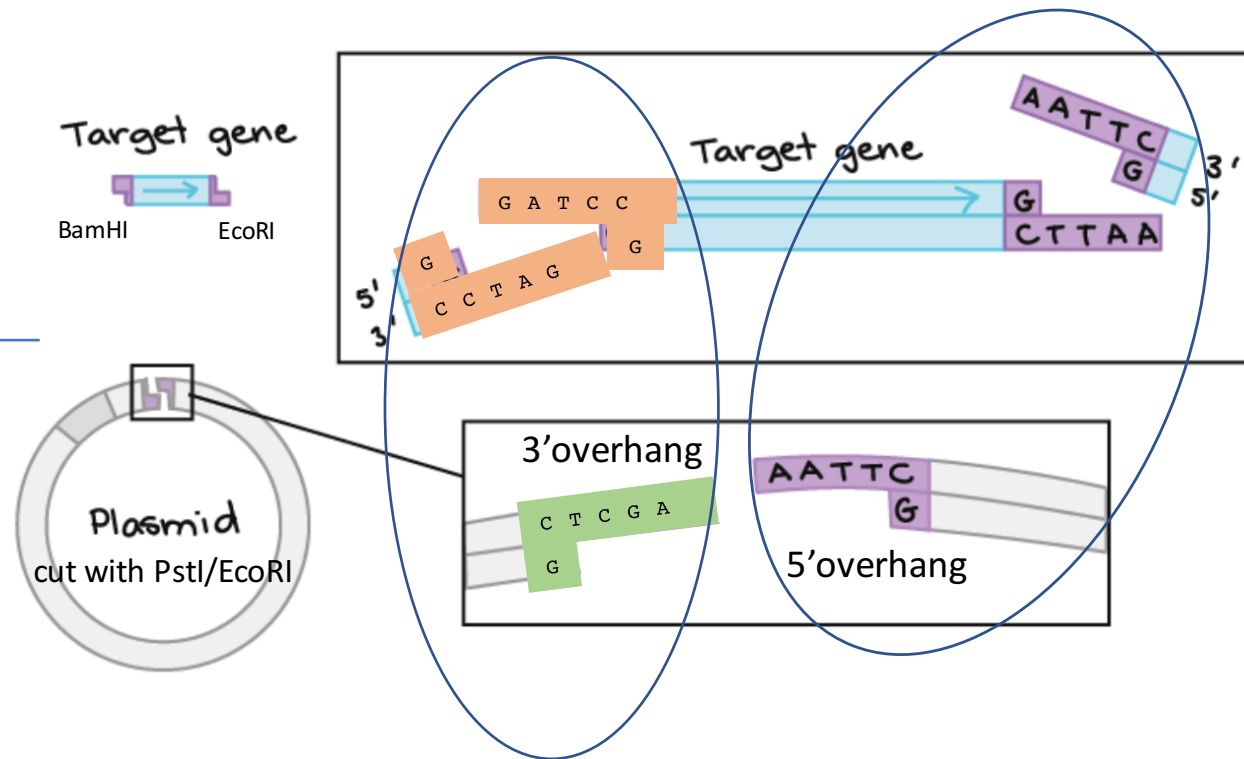
EcoRI: G/AATTC  
CTTAA/G

INSERT

PstI: CTCGA/G  
G/AGCTC

EcoRI: G/AATTC  
CTTAA/G

VECTOR



**NOT COMPATIBLE**

**COMPATIBLE**

**→ make blunt**

→ Modification of 5' overhang of EcoRI site → convert overhang to blunt end

→ Modification of 3' overhang of PstI site → convert overhang to blunt end

→ ➔ Blunt – Blunt AND EcoRI – EcoRI ligation

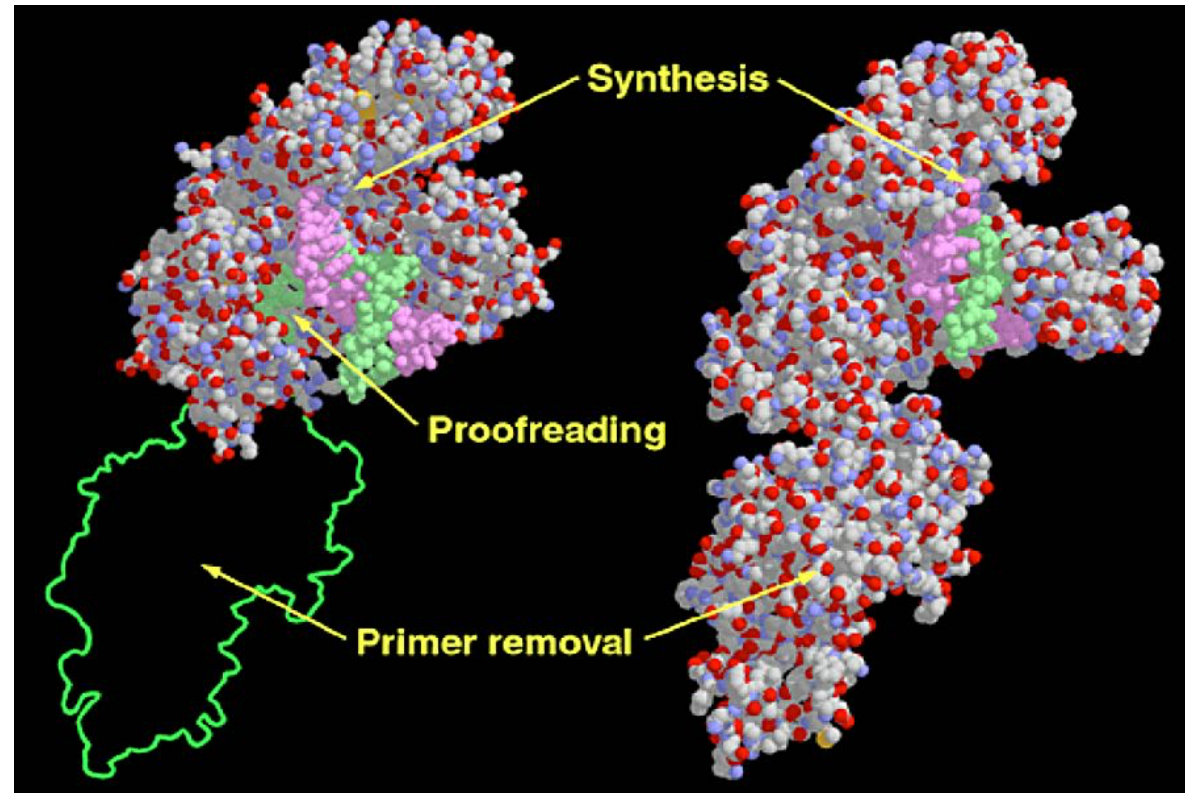


## 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

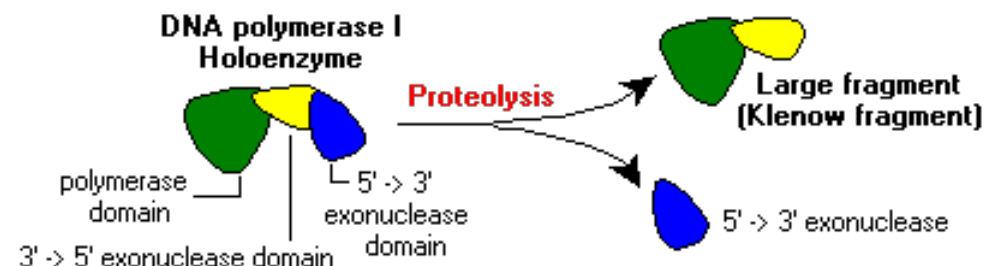
### The Klenow fragment

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.

**It retains the 5' → 3' polymerase activity and the 3' → 5' exonuclease activity** for removal of preexisting nucleotides and proofreading, but **loses 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).



- Synthesis of double-stranded DNA from single-stranded templates
- Filling in receded 3' ends of DNA fragments to make 5' overhang blunt
- Digesting away protruding 3' overhang
- Preparation of radioactive DNA probes

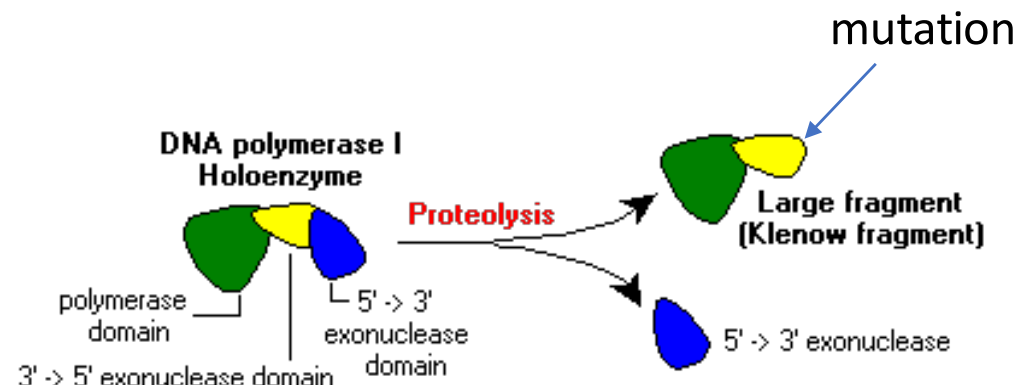




## 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

### The Exo- Klenow fragment

Just as the  $5' \rightarrow 3'$  exonuclease activity of DNA polymerase I from E.coli can be undesirable, **the  $3' \rightarrow 5'$  exonuclease activity of Klenow fragment can also be undesirable for certain applications.** This problem can be overcome by **introducing mutations in the gene that encodes Klenow.** This results in forms of the enzyme being expressed that retain  $5' \rightarrow 3'$  polymerase activity, but lack any exonuclease activity ( $5' \rightarrow 3'$  or  $3' \rightarrow 5'$ ). This form of the enzyme is called the exo- Klenow fragment. The exo-Klenow fragment is used in some fluorescent labeling reactions for microarray, and also in dA and dT tailing, an important step in the process of ligating DNA adapters to DNA fragments, frequently used in preparing DNA libraries for Next-Gen sequencing.



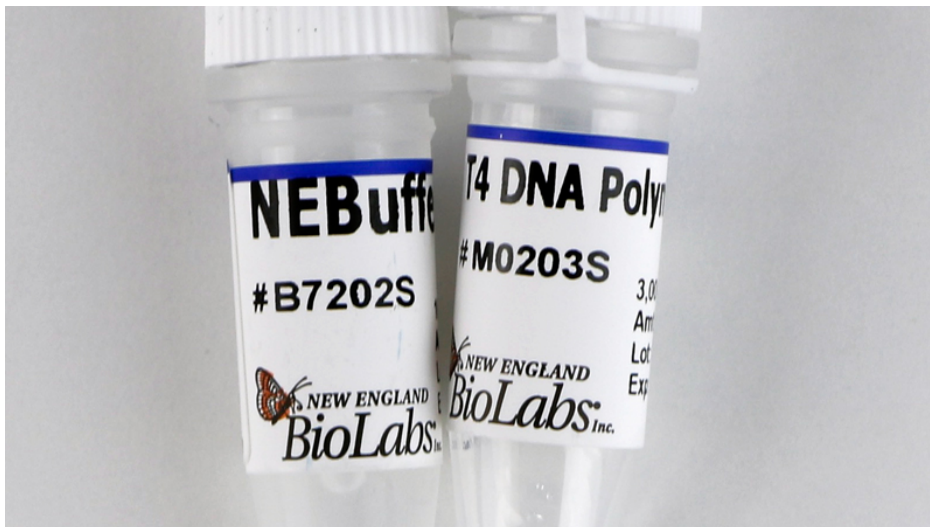
## 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

### The T4 DNA Polymerase

T4 DNA Polymerase catalyzes the synthesis of DNA in the 5' → 3' direction and requires the presence of template and primer. Encoded by T4 phage

- Gap filling (no strand displacement activity)
- Removal of 3' overhangs or fill-in of 5' overhangs to form blunt ends
- Lacks 5' → 3' exonuclease activity
- Probe labeling using replacement synthesis

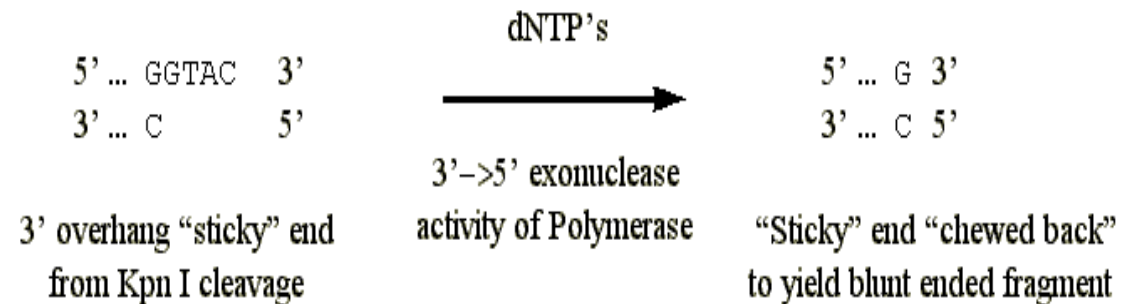
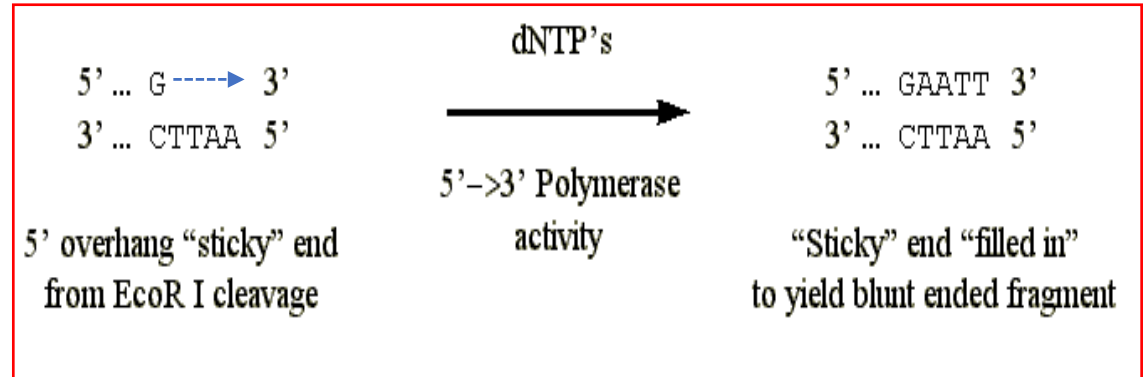
Single-strand deletion subcloning



## 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

### Converting a 5' overhang to blunt end

- Both **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 activity; exonuclease activity will take over
- → degradation of plasmid/insert

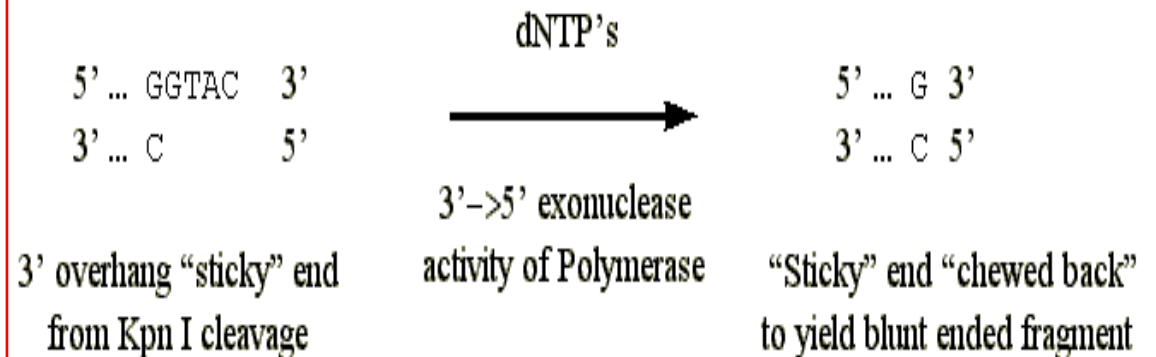
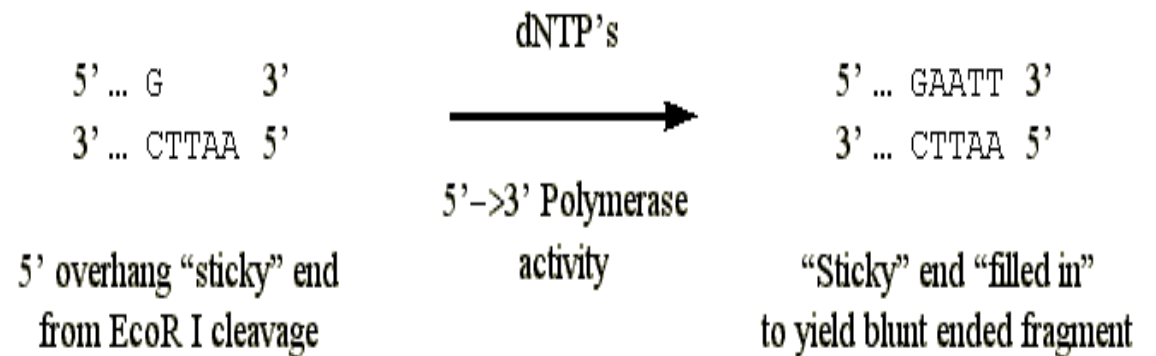
Exo- Klenow fragment is safer in use!

## 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

### Converting a 3' overhang to a blunt end

- T4 DNA polymerase has a 3'→5' exonuclease activity
- In the presence of excess dNTPs will convert a 3' protruding end to a blunt end
- Important 3' exonuclease and 5'→3' DNA polymerase reaction are competing
- Ligation can now proceed

Note: also Klenow fragment has 3'→5' exonuclease activity



IMPORTANT FOR 3'overhang BLUNTING:

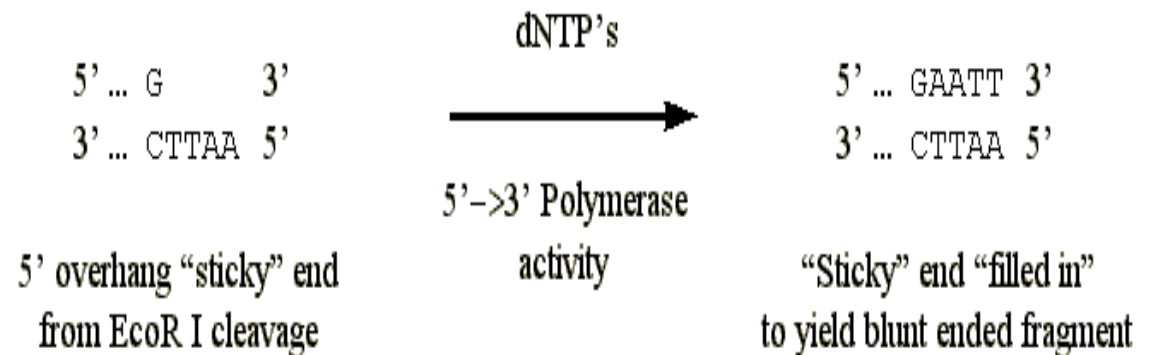
dNTPs need to be present in abundance (and be of good quality!)

- If dNTPs are used up by DNA polymerase activity; exonuclease activity will take over
- → degradation of plasmid/insert

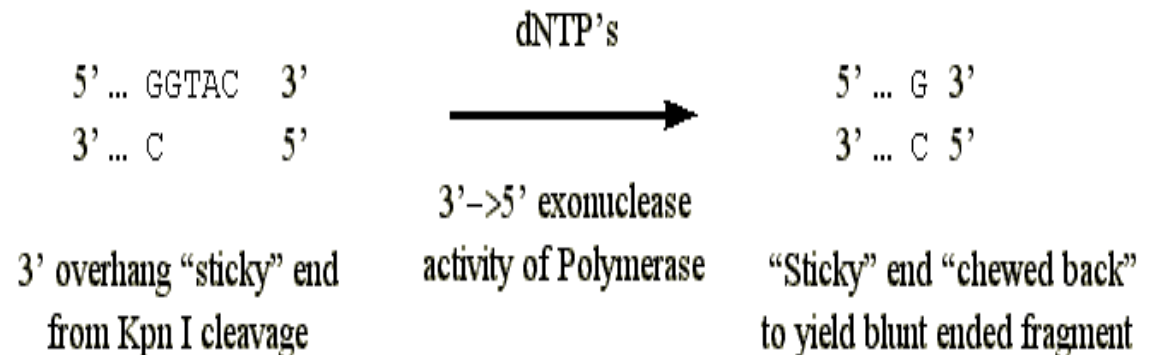
### 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

Laboratory reality → prepared use of enzymes

**Exo-Klenow fragment**  
**5' overhang fill-up**



**T4 DNA polymerase**  
**3' overhang removal**



# 3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

BamHI: G/GATCC  
CCTAG/G

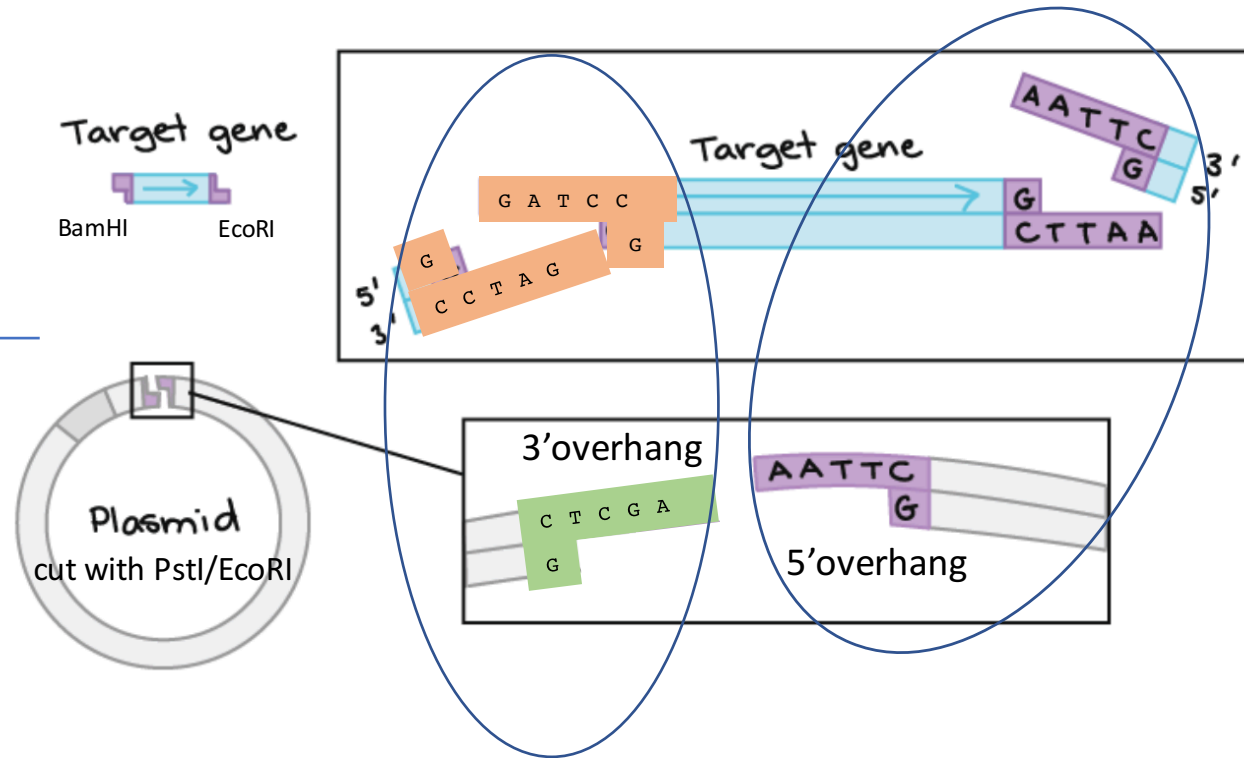
EcoRI: G/AATTC  
CTTAA/G

INSERT

PstI: CTCGA/G  
G/AGCTC

EcoRI: G/AATTC  
CTTAA/G

VECTOR



## HOW TO DO?

### Vector:

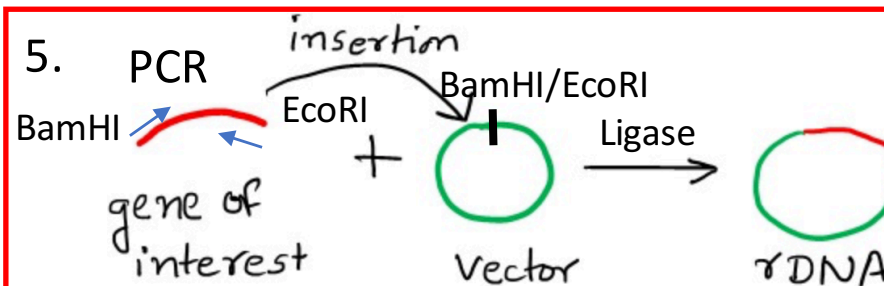
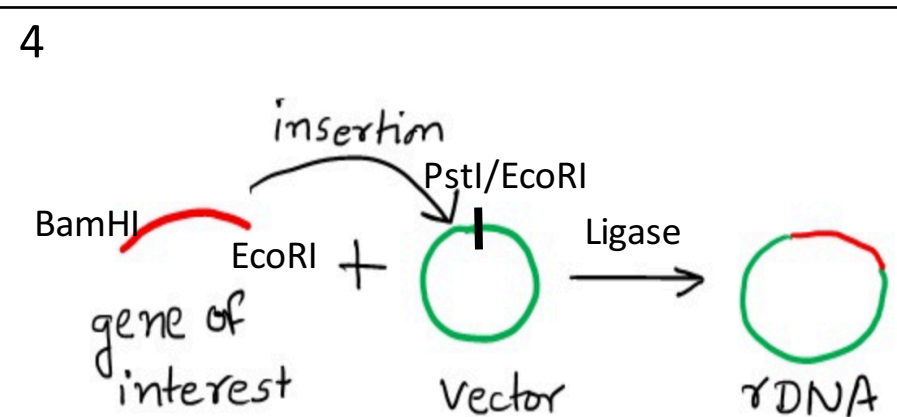
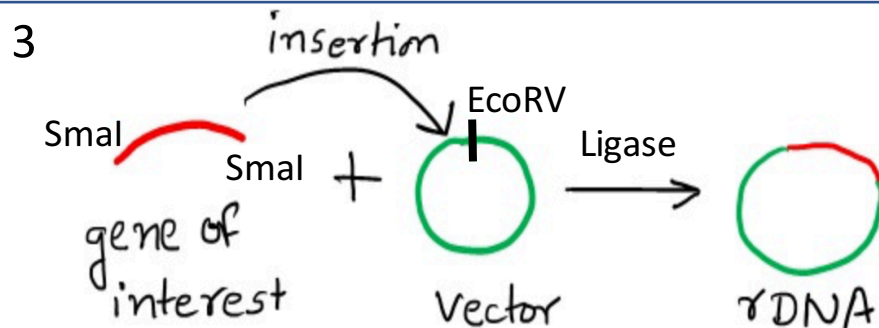
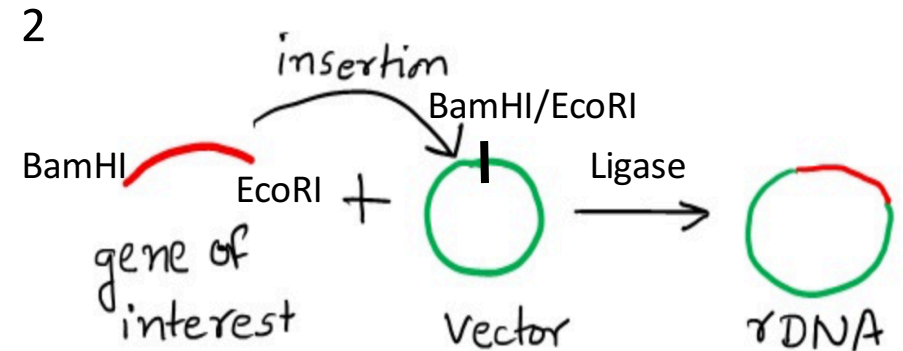
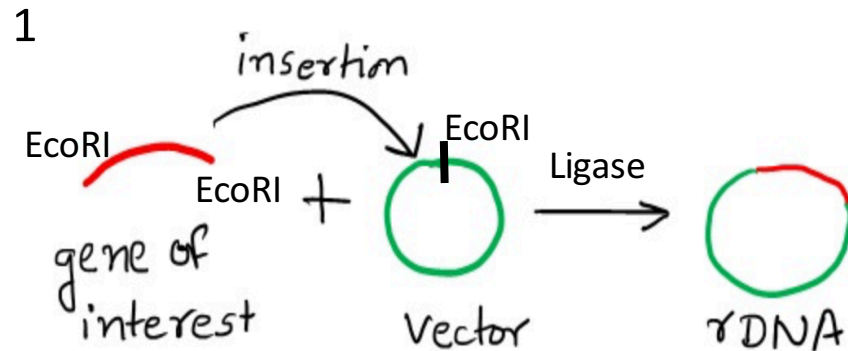
1. Cut PstI
2. Make T4 Polymerase reaction → blunting of 3'overhang
3. Run DNA on agarose gel
4. Cut band and purify linearized DNA
5. Cut DNA with EcoRI
6. Run DNA on agarose gel
7. Cut correct band and purify DNA, determine concentration

### Insert:

1. Cut DNA with BamHI
2. Make Exo- Klenow reaction → blunting of 5'overhang
3. Run DNA on agarose gel
4. Cut band and purify linearized DNA
5. Cut DNA with EcoRI
6. Run DNA on agarose gel
7. Cut correct band and purify DNA; determine concentration

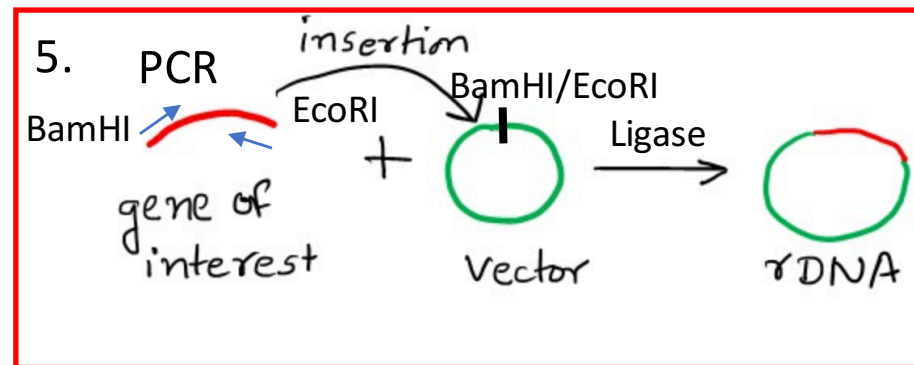
8. Setup ligation (Blunt- Blunt; EcoRI – EcoRI)

### 3. OVERVIEW OVER OTHER CLONING STRATEGIES





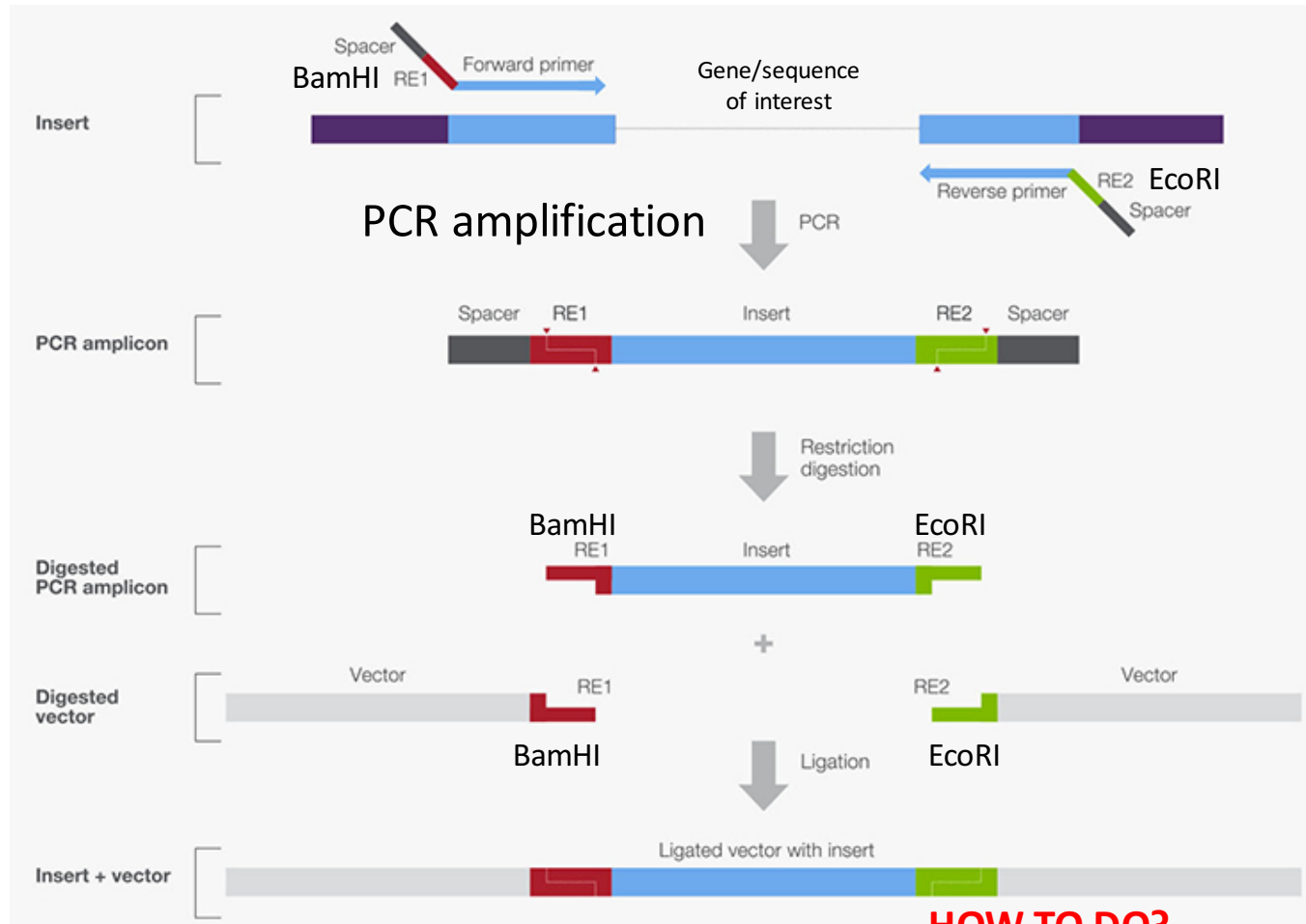
## 3.5 PCR CLONING



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

# 3.5 PCR CLONING

## 1. Classic PCR cloning



Forward primer

5'-CGC**GGATCC**xxxxxxxxxxxxxxxxxxxx-3'

Spacer **BamHI** Sequence pairing with  
-5 nucleotides) Sequence of interest  
(min. 18 nucleotides)

Reverse primer

3'-xxxxxxxxxxxxxxxxxxxx**GAATTC**CGC-5'

Sequence pairing with **EcoRI** Spacer  
Sequence of interest 3-5 nucleotides  
(min. 18 nucleotides)

### HOW TO DO?

1. Design + synthesize 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**

## 3.5 PCR CLONING

### 1. Classic PCR cloning

#### WHY IS A SPACER NEEDED???

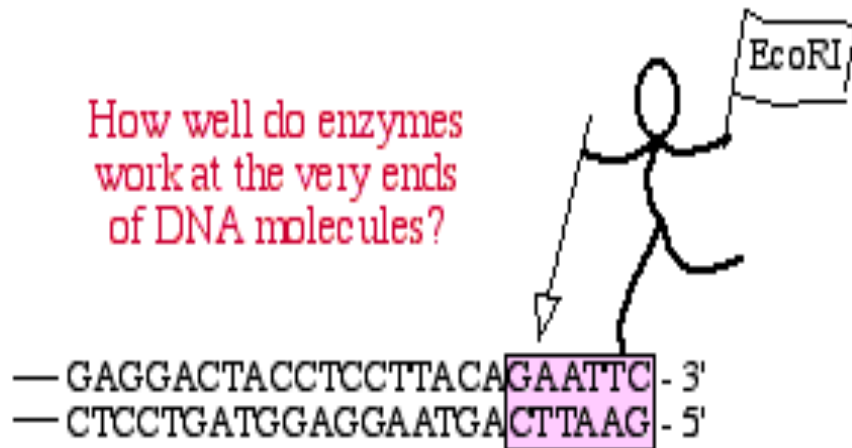
Forward primer

5' - CGC**GGATCC**xxxxxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxxxxxCTTAAGGCG

3' - GCGCCTAGGxxxxxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxxxxx**GAATTC**CGC-5'

Reverse primer

How well do enzymes  
work at the very ends  
of DNA molecules?

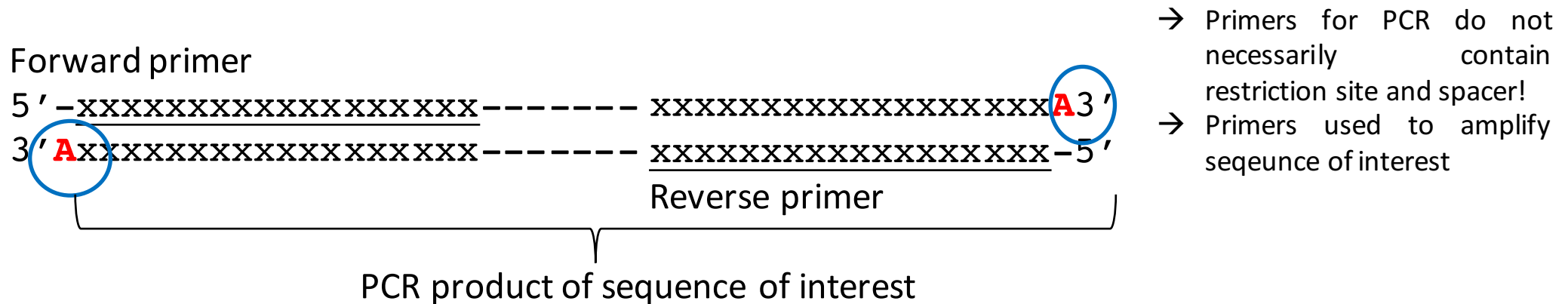


- Many restriction enzymes work poorly on DNA termini
- Catalogues of enzymes provide anecdotal data on the efficiency of enzymes trying to work at the ends of DNA molecules.
- Generally, enzymes work better if they have a couple of extra nucleotides at the end - they don't do very well if they are perched on the end of a molecule.
- Some additional nucleotides are better for interaction with DNA

## 3.5 PCR CLONING

### 2. TA-cloning

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



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

Taq polymerases are the most frequently used polymerases for PCR

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 may be 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**

## 3.5 PCR CLONING

### 3. TA-Cloning

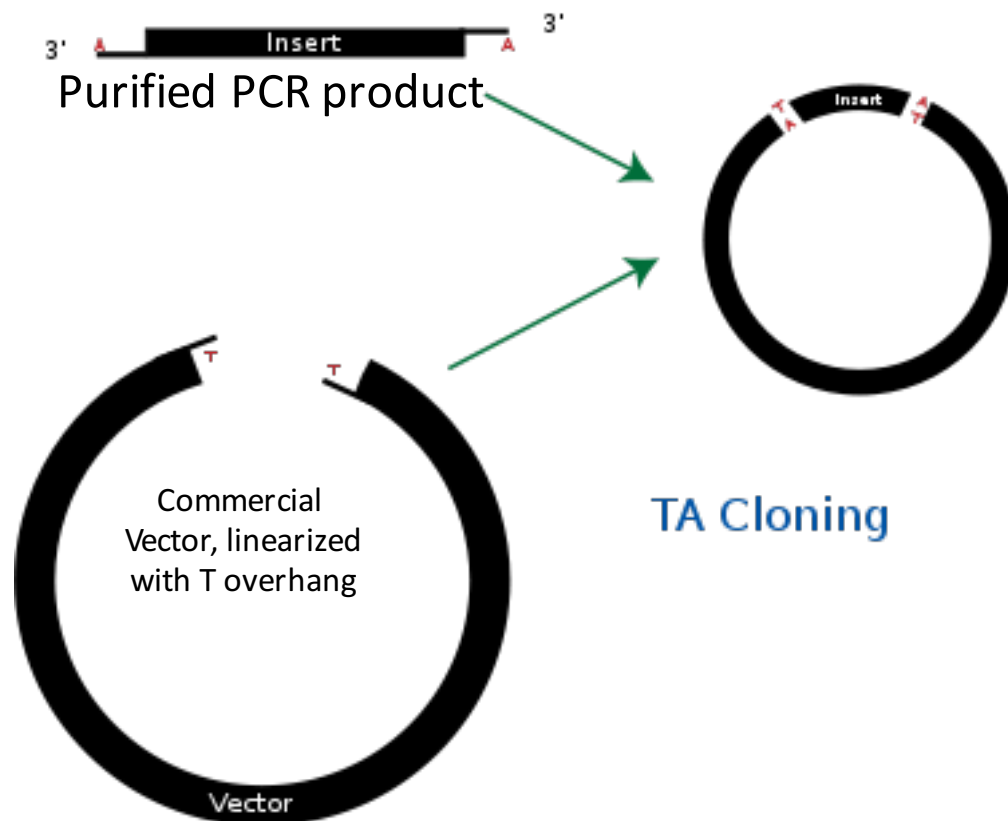
**ATTENTION: Taq polymerases produce PCR products with A on 3'ends**

Forward primer

5' -CGC**GGATCC**XXXXXXXXXXXXXXXXXXXX--XXXXXXXXXXXXXXXXXXXXCTTAAGGCG**A**3'

3' **A**AGCGCCTAGGXXXXXXXXXXXXXXXXXXXX--XXXXXXXXXXXXXXXXXXXX**GAA**TTC CGC-5'

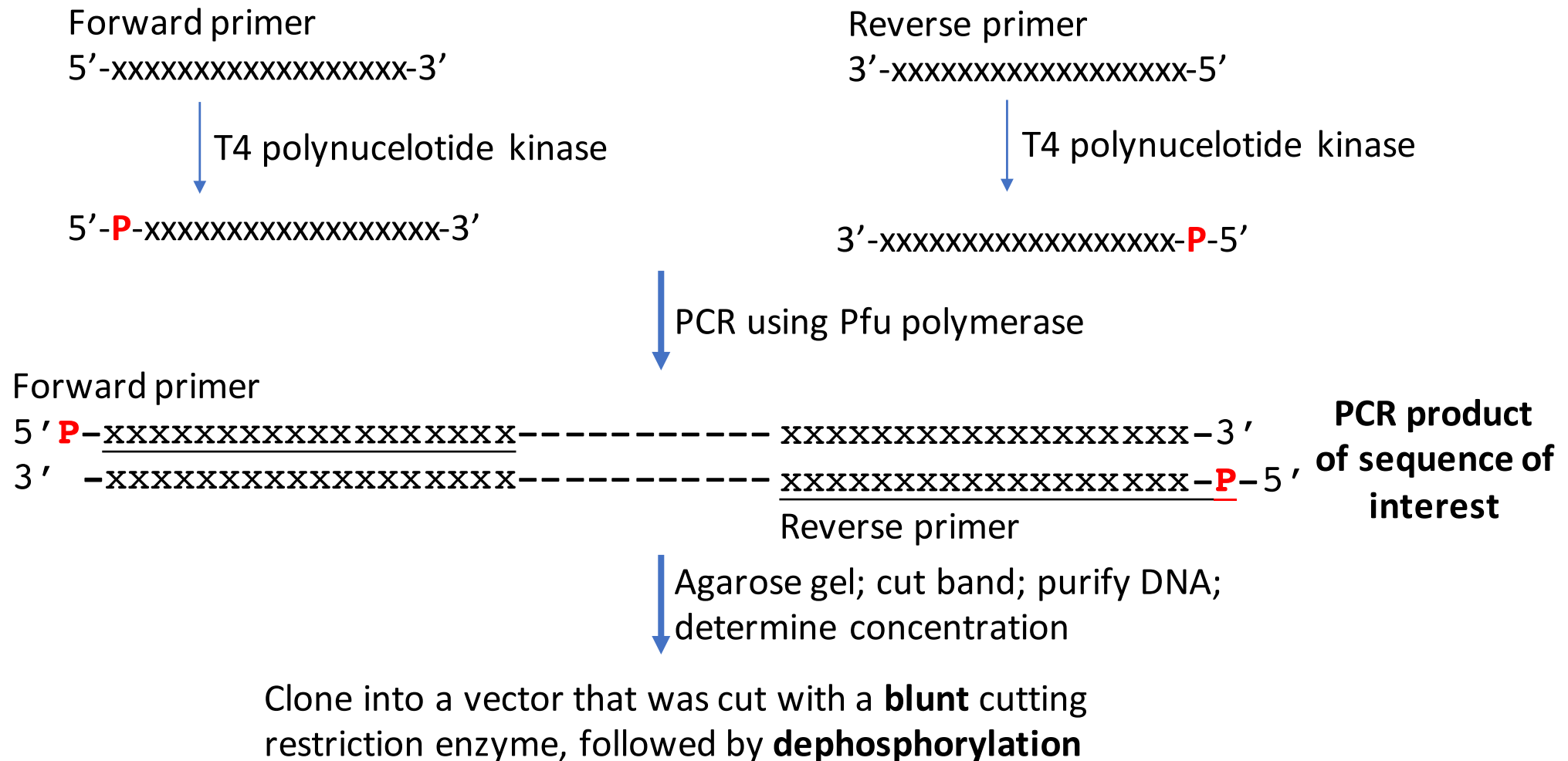
Reverse primer



## 3.5 PCR CLONING

### 2. Blunt end cloning using PCR

**ATTENTION: Other polymerases do not add A on 3' end: for example Pfu polymerase**



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

## 3.5 PCR CLONING

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

	5'→3' Exonuclease	3'→5' Exonuclease	Error Rate( $\times 10^{-6}$ ) <sup>a</sup>	Strand Displacement	Nick Translation	Thermal Stability	K <sub>m</sub> dNTPs	K <sub>m</sub> DNA <sup>d</sup>	Extend RNA Primer	Extension from Nick	Primary Applications
<i>Bst</i> DNA Polymerase, Full Length	+	–		_r	+	+			+	+	Labeling, 2nd Strand Synthesis
<i>Bst</i> DNA Polymerase, Large Fragment	–	–		++++	–	+			+	+	Strand Displacement Applications, isothermal amplification
<i>Bsu</i> DNA Polymerase, Large Fragment	–	–		++	–	–			+	+	Labeling, 2nd Strand Synthesis, Strand Displacement
Crimson <i>Taq</i> DNA Polymerase	+	–	285	_r	+	++			–	+	PCR (routine)
Deep Vent <sub>R</sub> <sup>TM</sup> DNA Polymerase	–	+++		++	–	++++	50 $\mu$ M <sup>e</sup>	0.01 nM <sup>e</sup>	–	+	PCR (high-fidelity)
Deep Vent <sub>R</sub> <sup>TM</sup> (exo–) DNA Polymerase	–	–		+++	–	++++			–	+	PCR (long)
<i>E. coli</i> DNA Polymerase I	+	++	g <sup>h</sup>	_r	+	–	1-2 $\mu$ M <sup>f</sup>	5 nM <sup>f</sup>	+	+	Nick Translation
Klenow Fragment (3'→5' exo–)	–	–	100 <sup>o</sup>	+++	–	–			+	+	Labeling
DNA Polymerase I, Large (Klenow) Fragment	–	++	18 <sup>o</sup>	++	–	–	2 $\mu$ M <sup>g</sup>		+	+	Polishing Ends
LongAmp® <i>Taq</i> DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (routine, long)
LongAmp® Hot Start <i>Taq</i> DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (hot start, long)
M-MuLV Reverse Transcriptase	–	–		+++	–	–	18 $\mu$ M <sup>s</sup>				cDNA Synthesis



## 3.5 PCR CLONING

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

OneTaq® DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (routine, difficult)
OneTaq® Hot Start DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (hot start, routine, difficult)
phi29 DNA Polymerase	–	++++		+++++	–	–	0.5 $\mu\text{M}^q$		+	+	Strand Displacement Applications
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 <sup>h</sup>	–	–	–	2 $\mu\text{M}^n$		+	–	Polishing Ends, 2nd Strand Synthesis
T7 DNA Polymerase (unmodified)	–	++++	15 <sup>b</sup>	–	–	–	18 $\mu\text{M}^k$	18 nM <sup>k</sup>	+	–	Site Directed Mutagenesis
Taq DNA Polymerase with Standard Taq Buffer	+	–	285 <sup>c</sup>	_r	+	++	13 $\mu\text{M}^e$	2 nM <sup>e</sup>	–	+	PCR (routine)
Therminator™ DNA Polymerase	–	–		+	–	++++			+	+	Chain Terminator Applications
VentR® DNA Polymerase	–	++	57 <sup>b</sup>	++ <sup>e</sup>	–	+++	60 $\mu\text{M}^e$	0.1 nM <sup>e</sup>	–	+	PCR (routine, high-fidelity)
VentR® (exo–) DNA Polymerase	–	–	190 <sup>b</sup>	+++ <sup>e</sup>	–	+++	40 $\mu\text{M}^e$	0.1 nM <sup>e</sup>	–	+	PCR, Sequencing

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

## 3.5 PCR CLONING

### 4. Characteristics 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%
<i>Pyrococcus furiosus</i> DNA polymerase	8.4%	25.2%
<i>Taq</i> 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!**

→ Keep PCR cycles at the lowest minimum possible