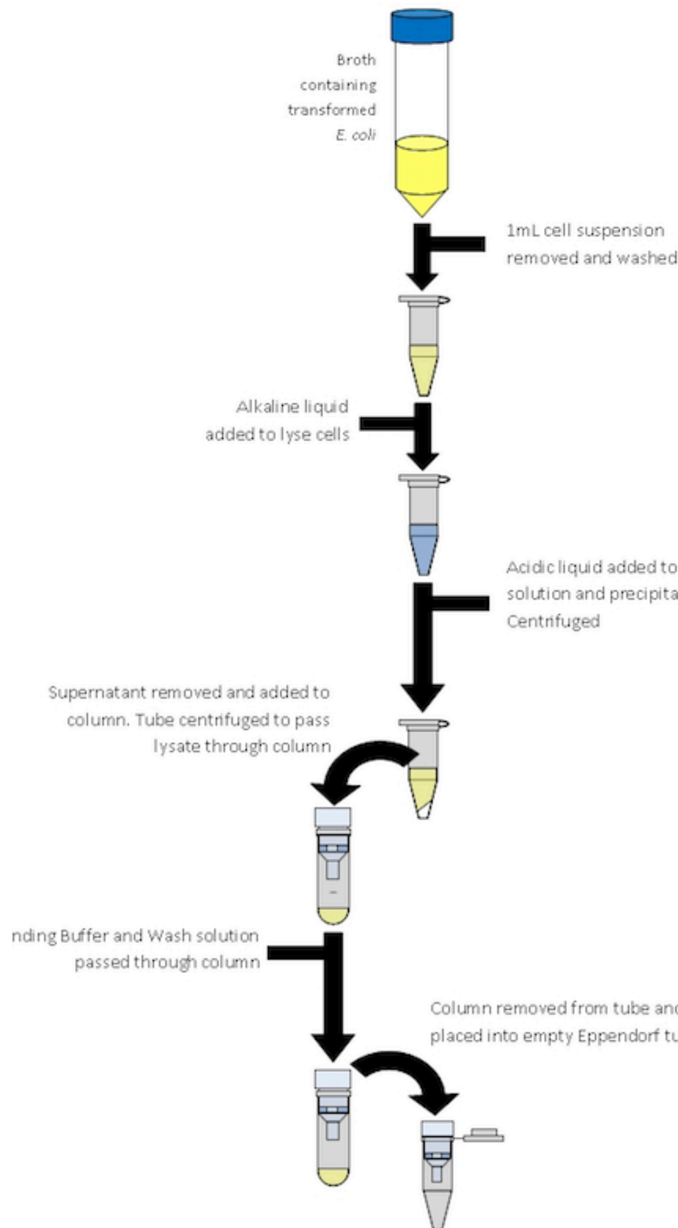
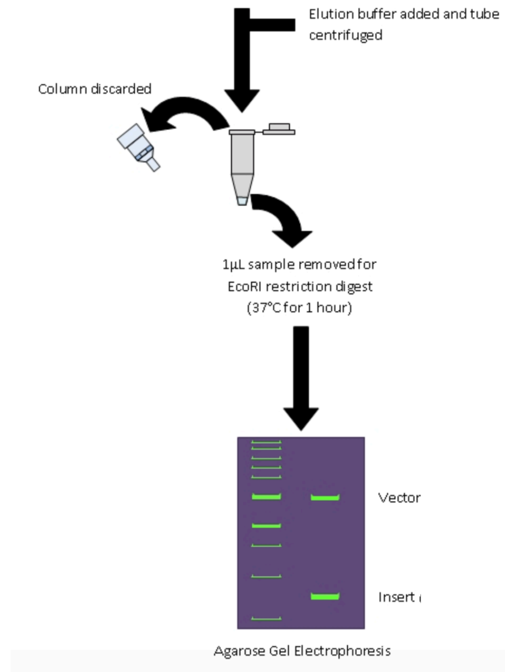
# 2. DNA PREPARATION – Mini prep



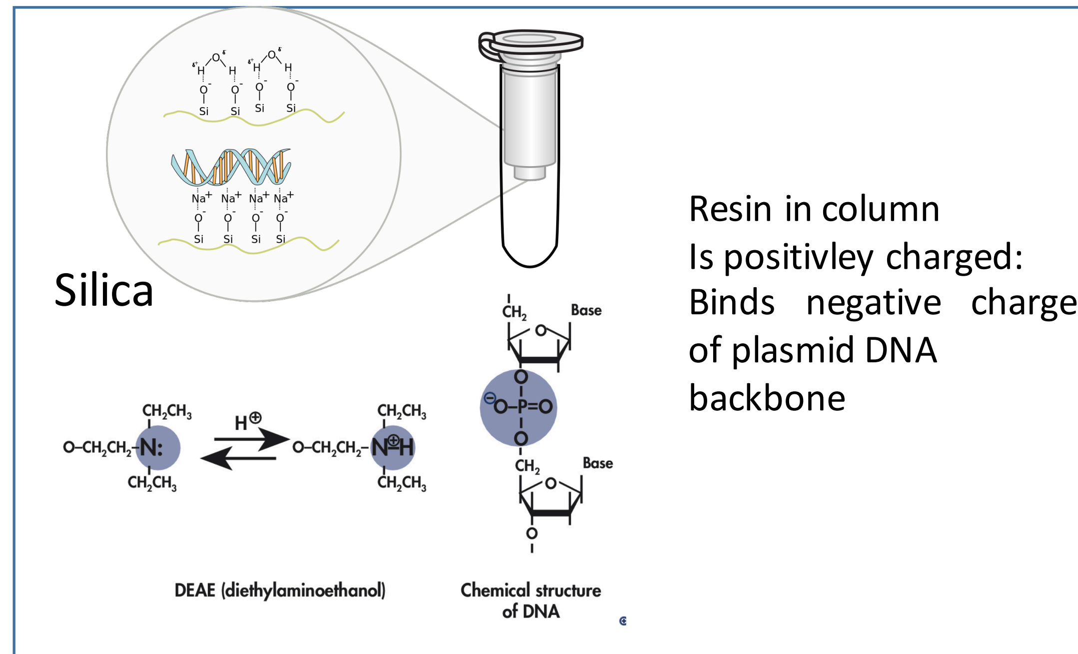

1ml of overnight culture Removed, spinned and supernatant removed. Bacteria pellet resuspendet in buffer that does not kill cells

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)

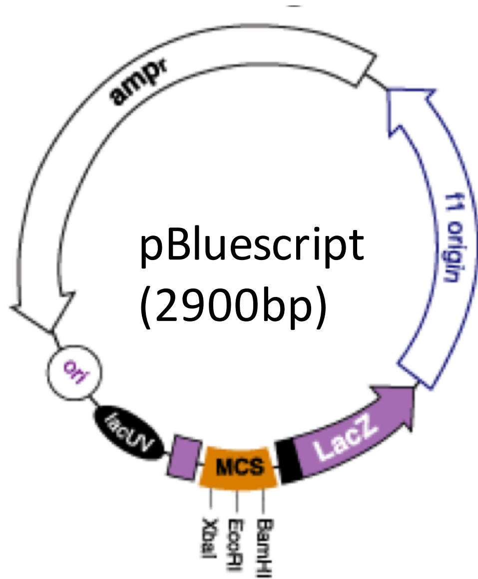



The use of columns Results in very pure plasmid DNA. “**sequence grade**”



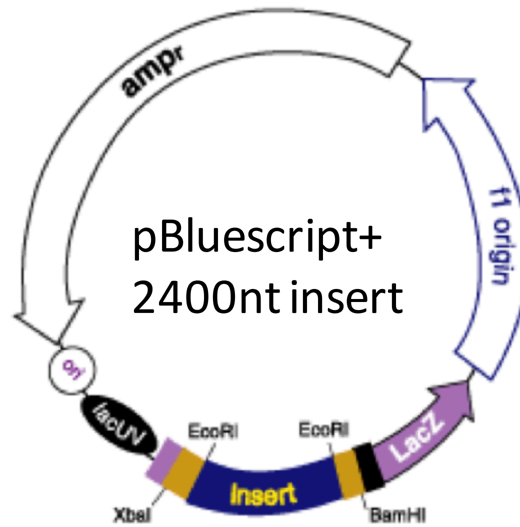

Resin in column Is positivley charged: Binds negative charge of plasmid DNA backbone

# 3. CONTROL DIGEST TO INDETIFY SUCCESSFUL CLONING EVENT



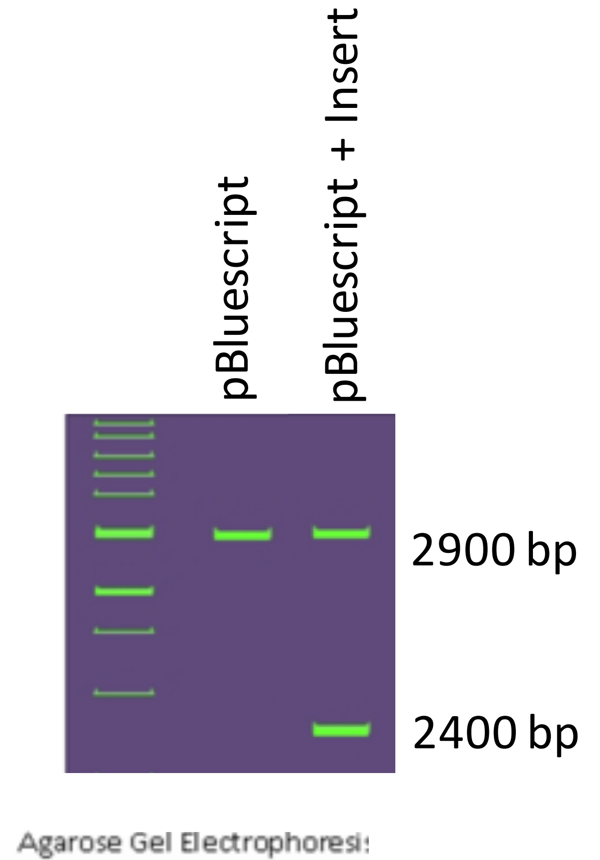

+ EcoRI

2900 bp (linearized)



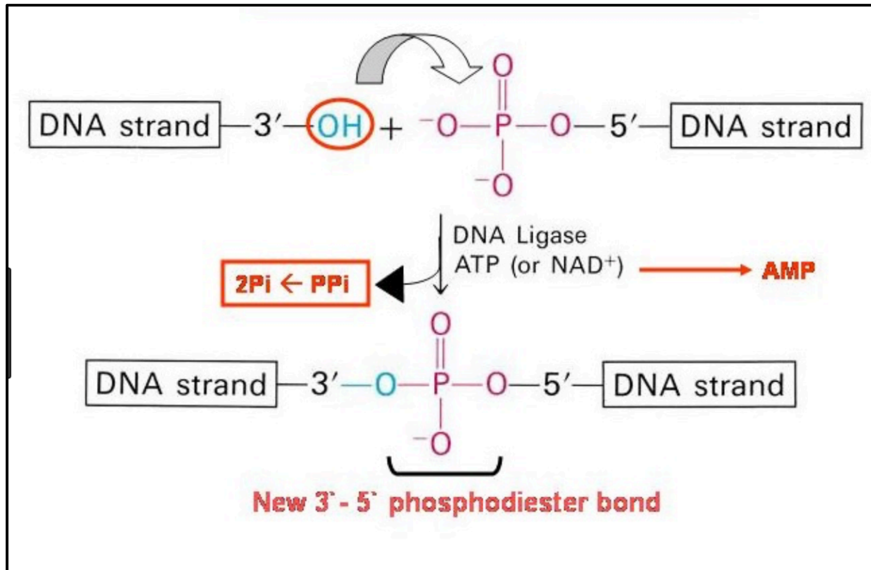

+ EcoRI

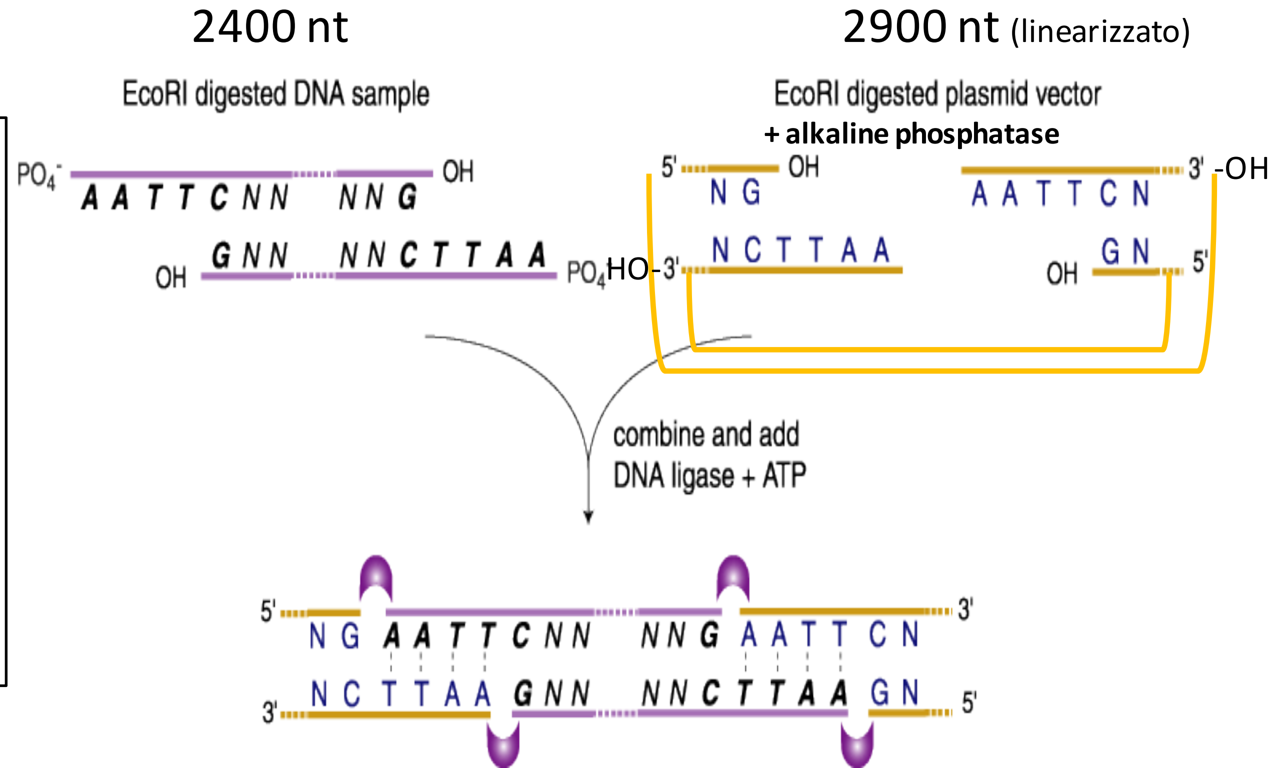
2900 bp
2400 bp




Agarose Gel Electrophoresis

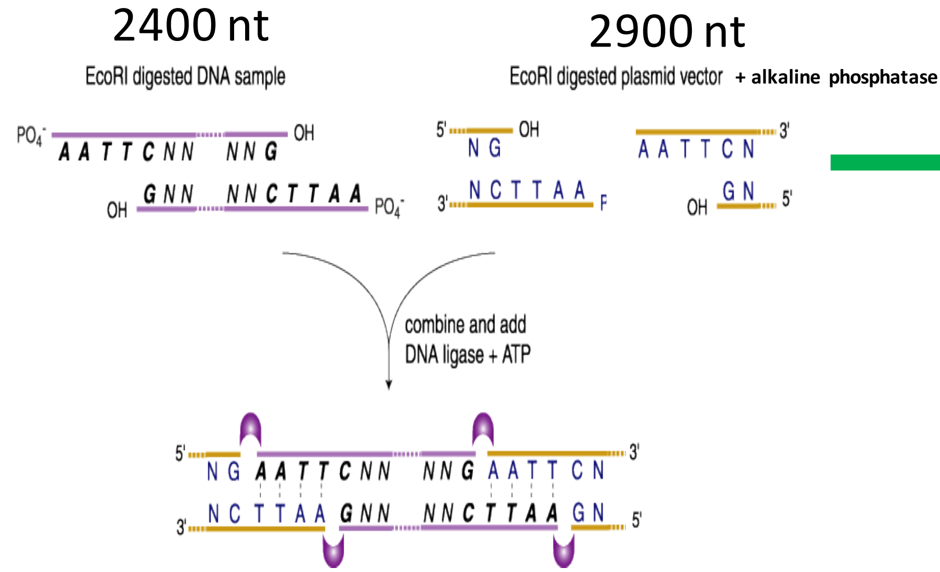
# Ligating 2 fragements with DNA Ligase

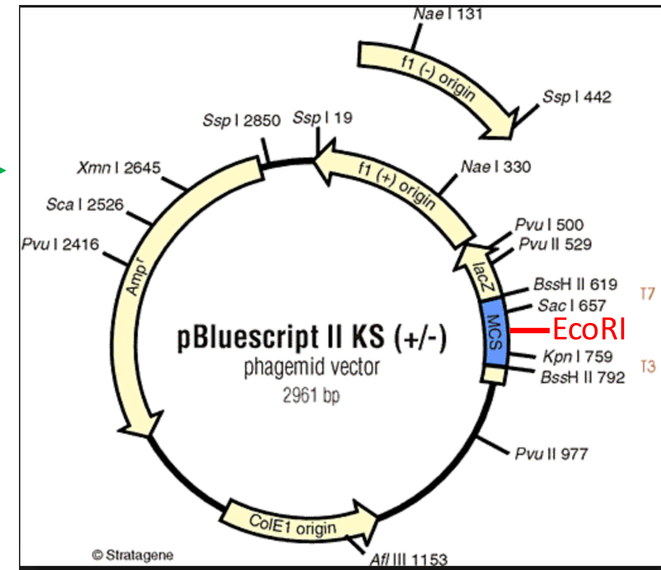


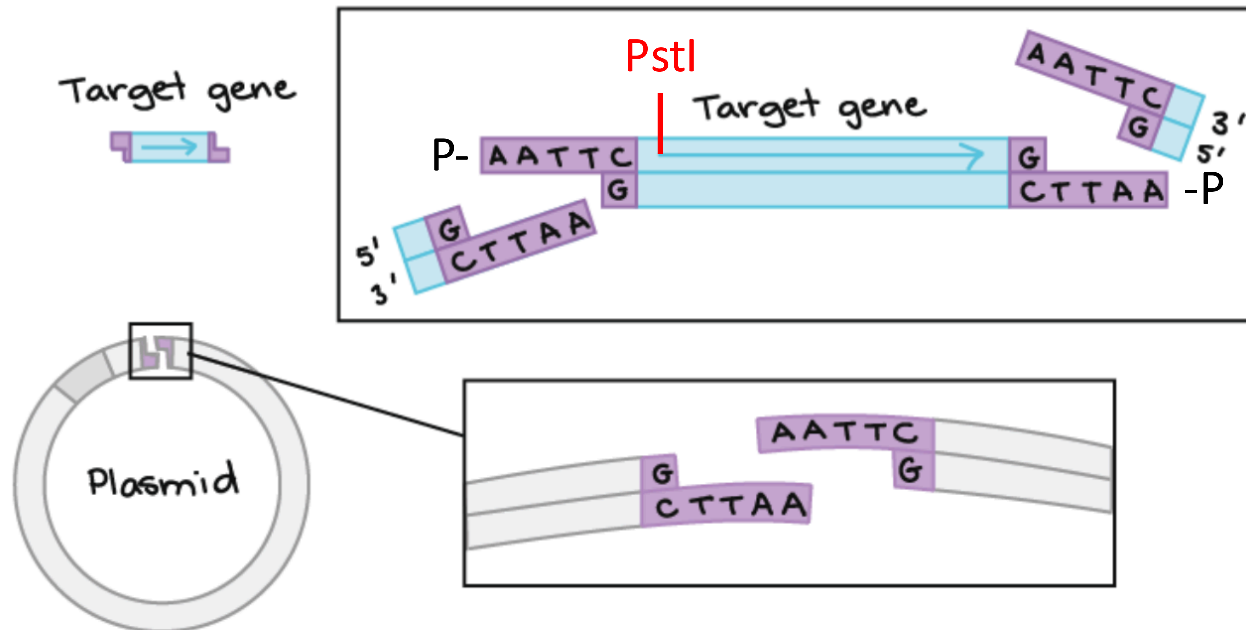




**Attention: All involved overhangs are compatible**
**→ insert can be "ligated" into vector in both orientations**

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

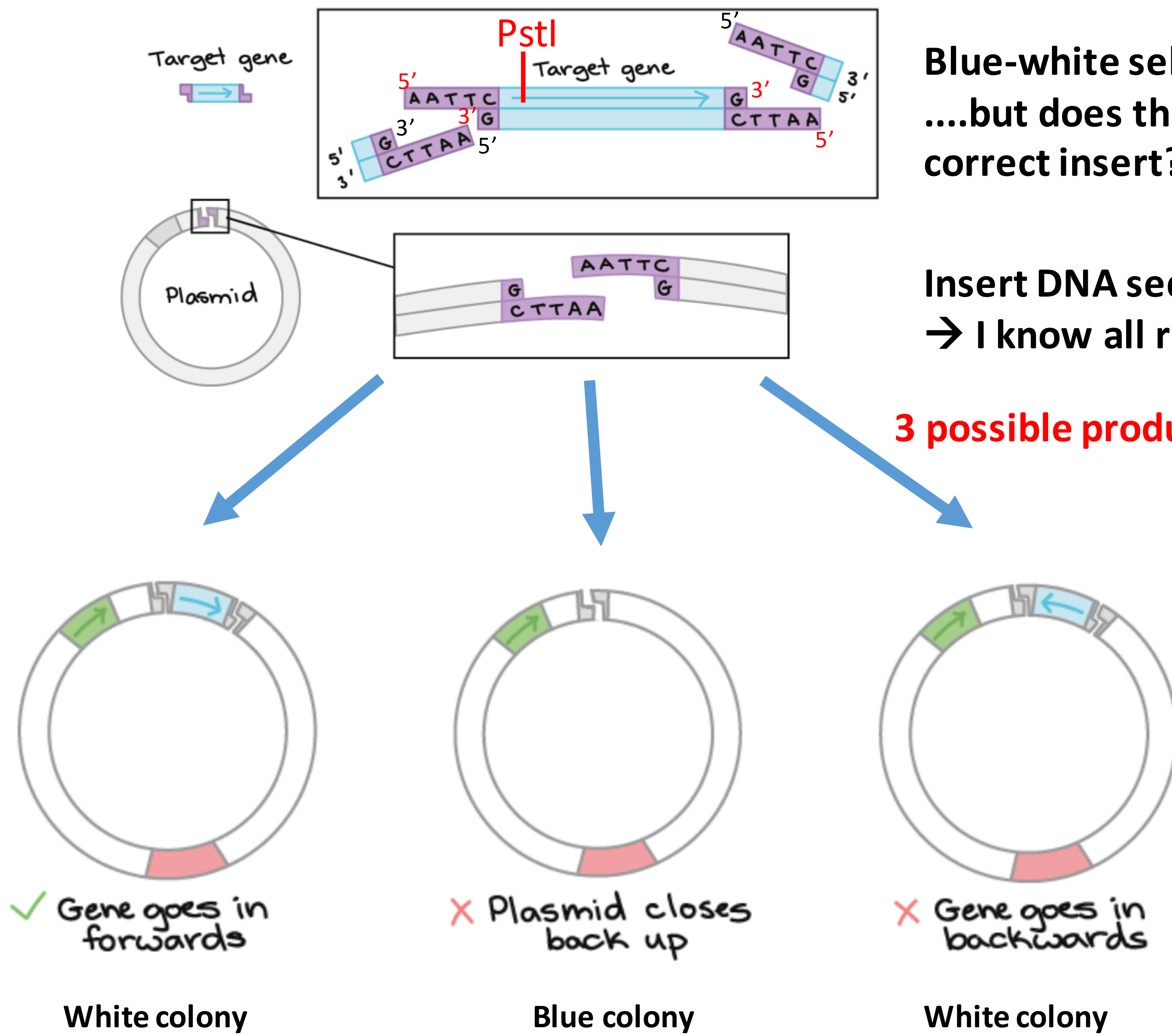



**Note:**
**5'overhangs of insert and linearized plasmids are compatible; both have been cut with EcoRI.**
**Ligase covalently links both molecules**
**EcoRI sites are reconstiuted and now flank the insert sequence!!!**

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




**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**

**White colony**

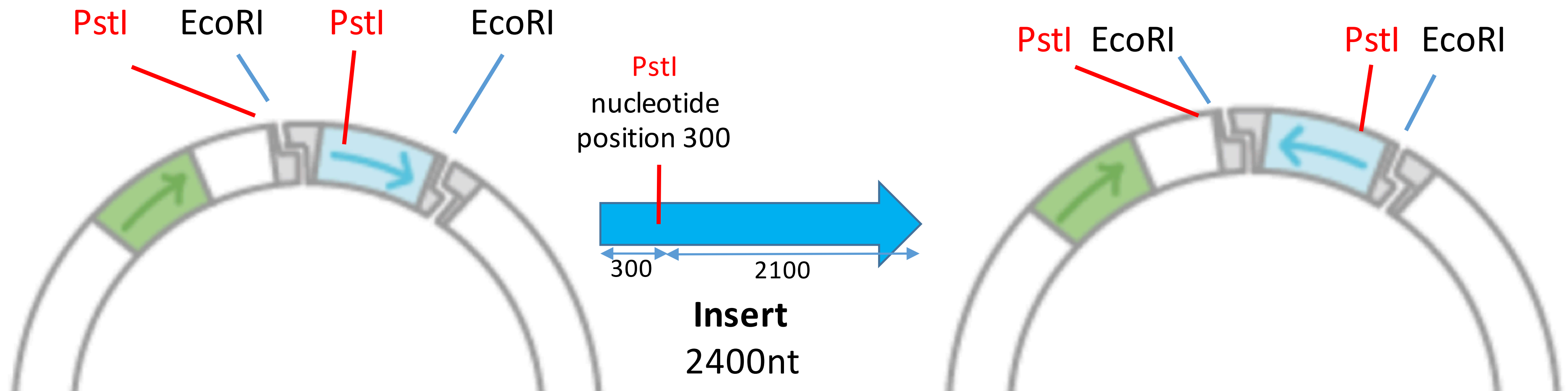
**Blue colony**

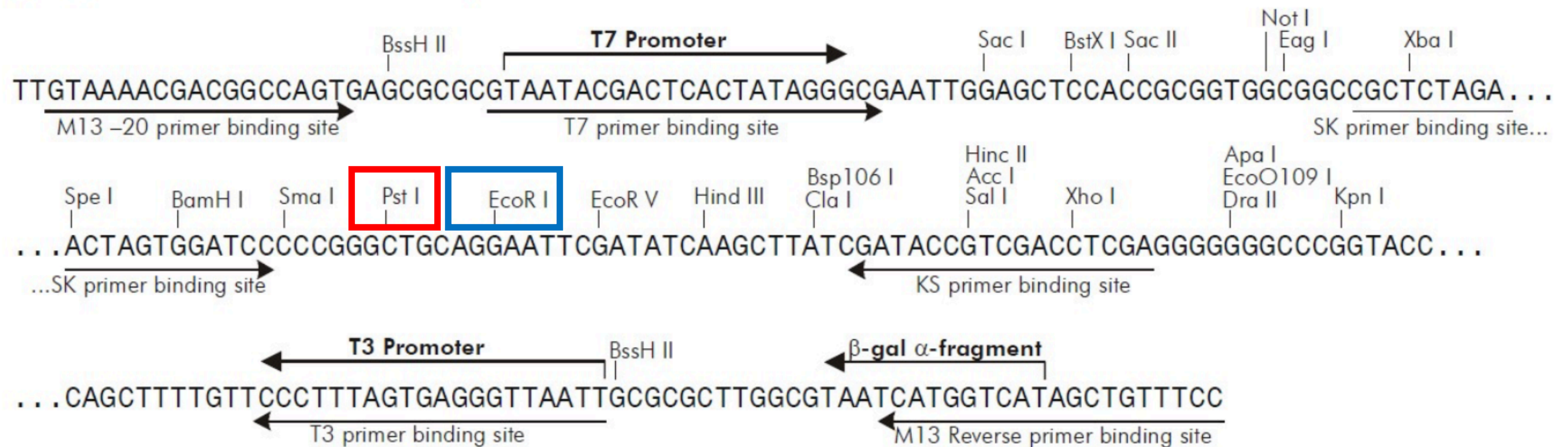
**White colony**

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

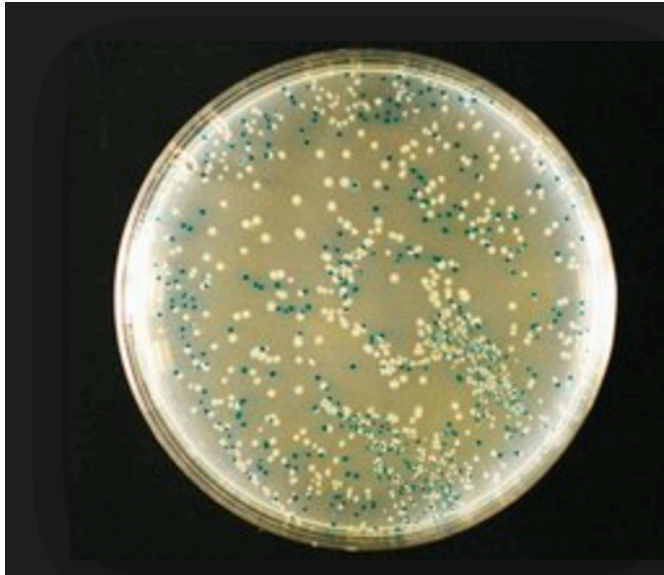
Blue-white screening using for example the pBluescript vector

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



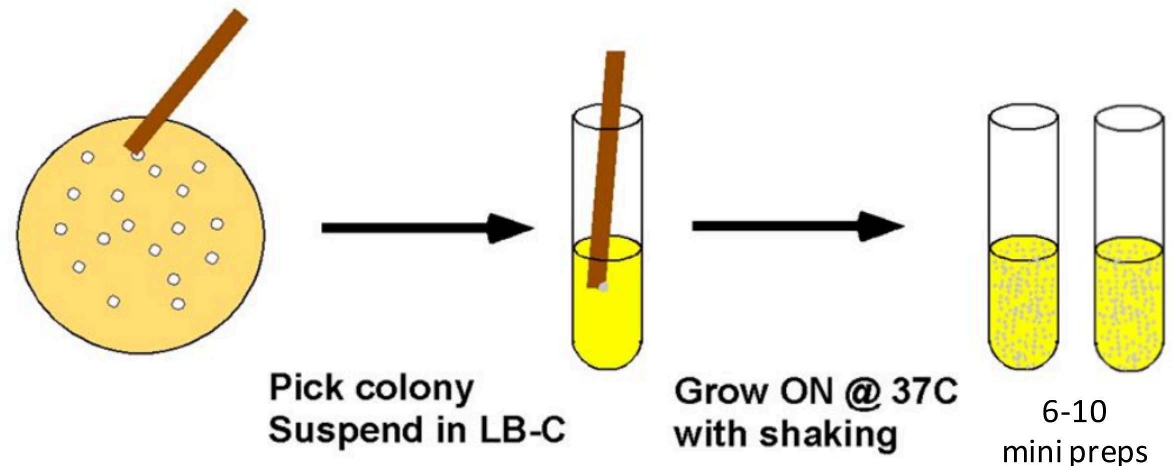
# A CONTROL DIGEST IS PERFORMED ON MULTIPLE COLONIES OBAINED FROM CLONING EXPERIMENT (5-10)



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

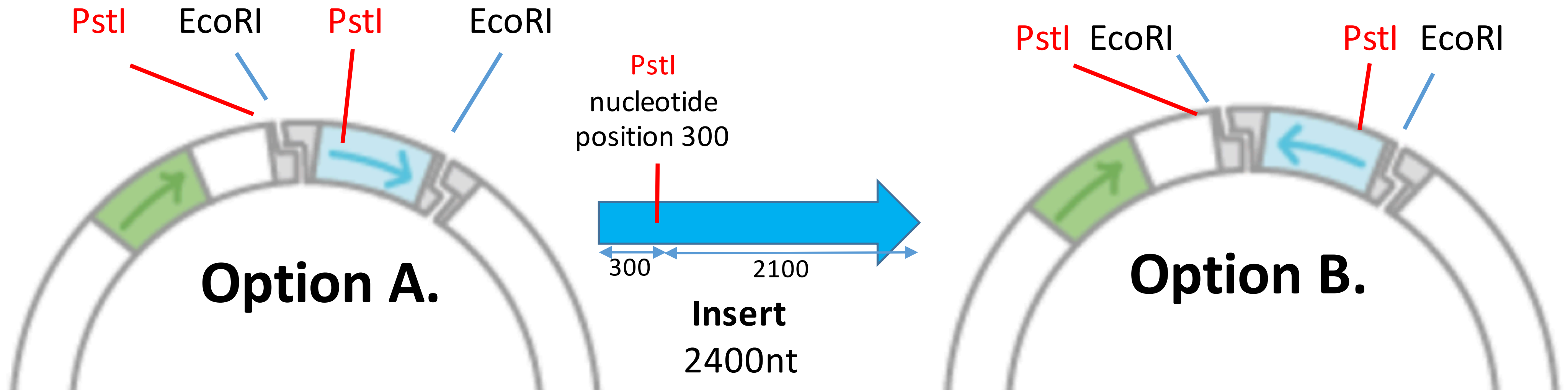
## Preparation. Grow the bacteria

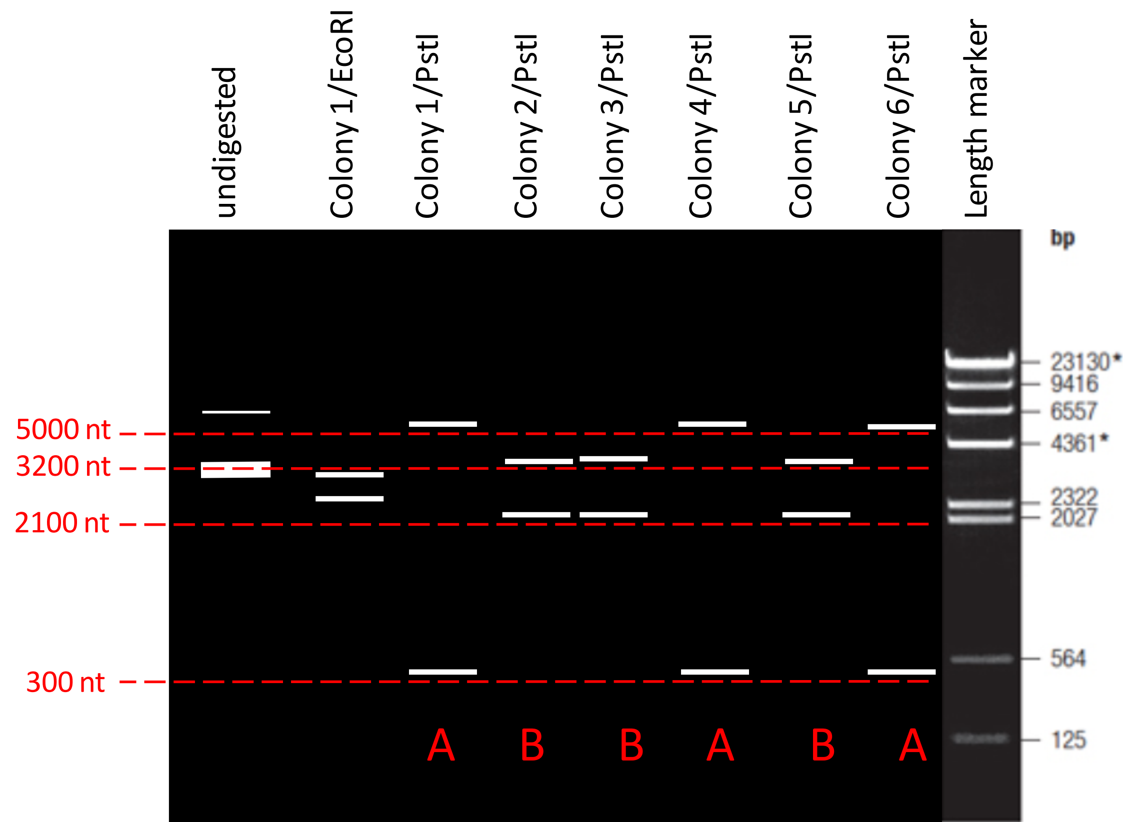
Grow an overnight (ON) culture of the desired bacteria in 2-5 ml of LB medium containing the appropriate antibiotic for plasmid selection. Incubate the cultures at 37°C with vigorous shaking.




Next day: harvest bacteria by centrifugation and prepare plasmid DNA

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

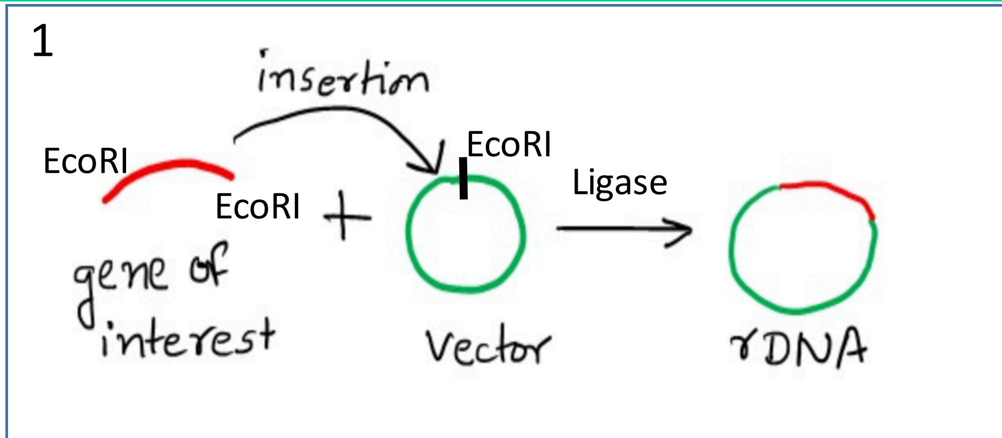


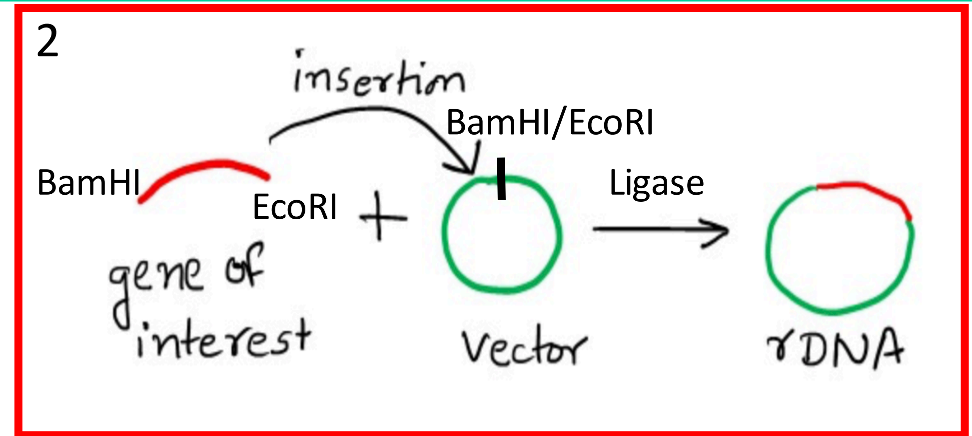



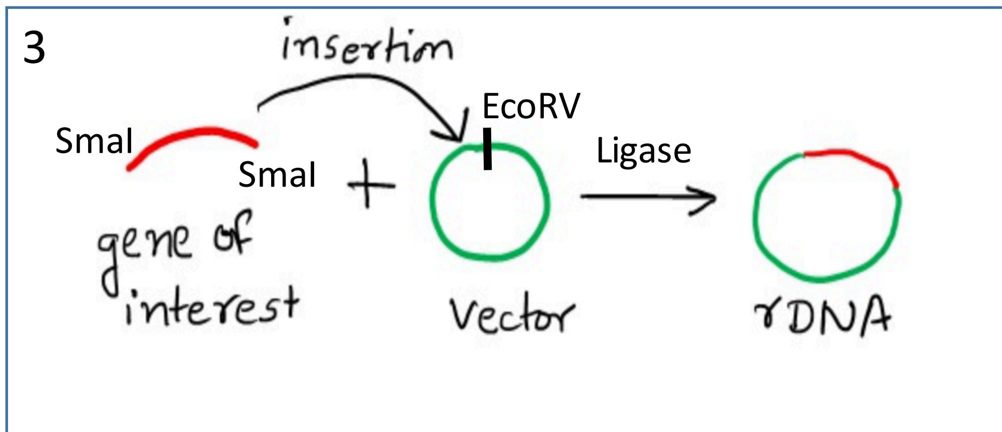

Insert: 2400 nt
Plasmid: 2900 nt

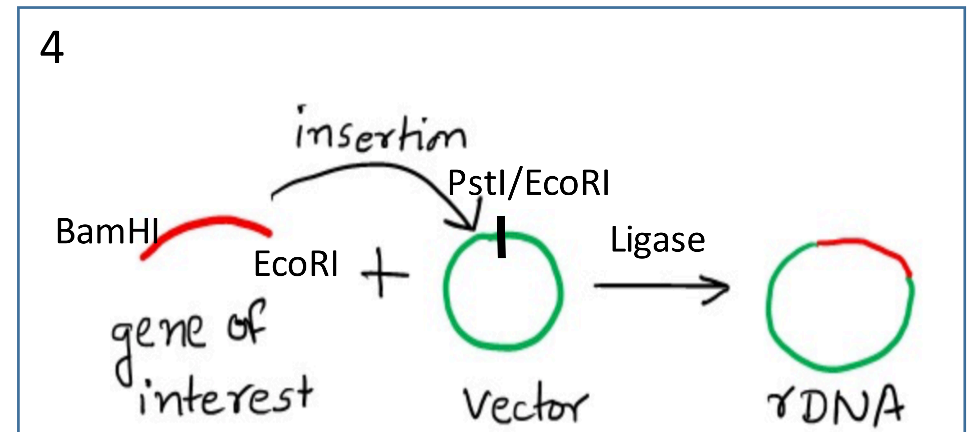
**Cut with restriction enzyme that result asymetric digestion products**

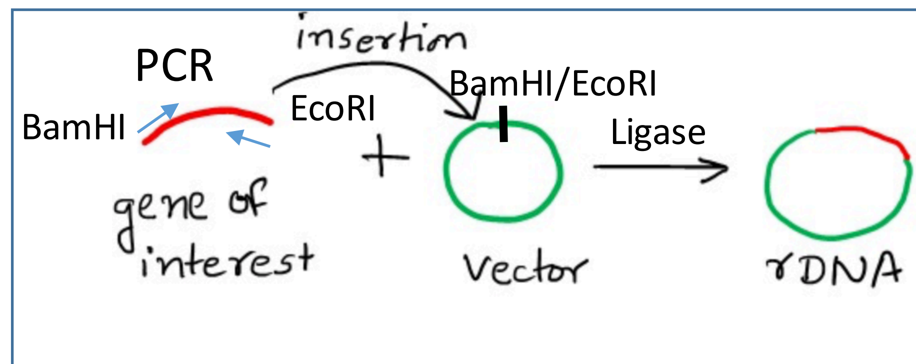
# OVERVIEW OVER ON CLONING STRATEGIES



# DNA CLONING WITH 2 COHESIVE OVERHANGS

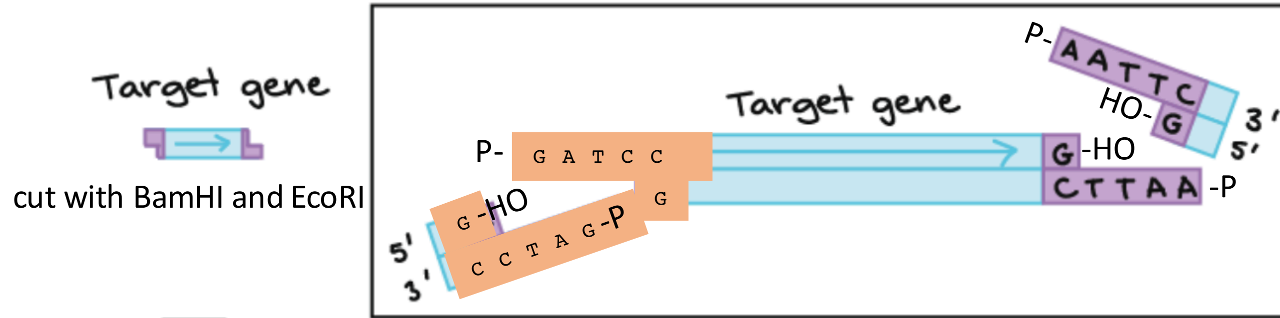
EcoRI: G/AATTC
CTTAA/G

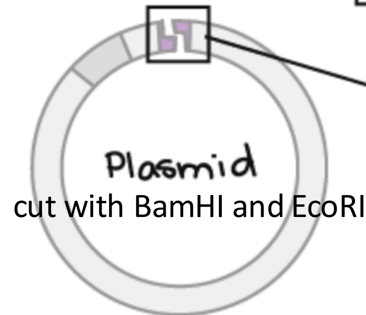
BamHI: G/GATCC
CCTAG/G

**DIRECTIONAL CLONING**
→ Always preferred cloning strategy

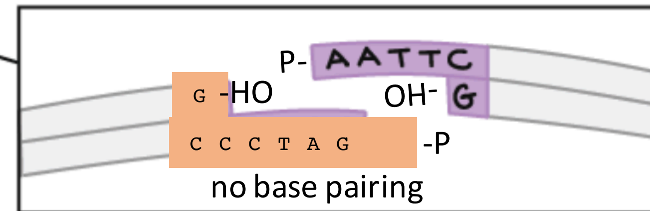
Target gene

cut with BamHI and EcoRI

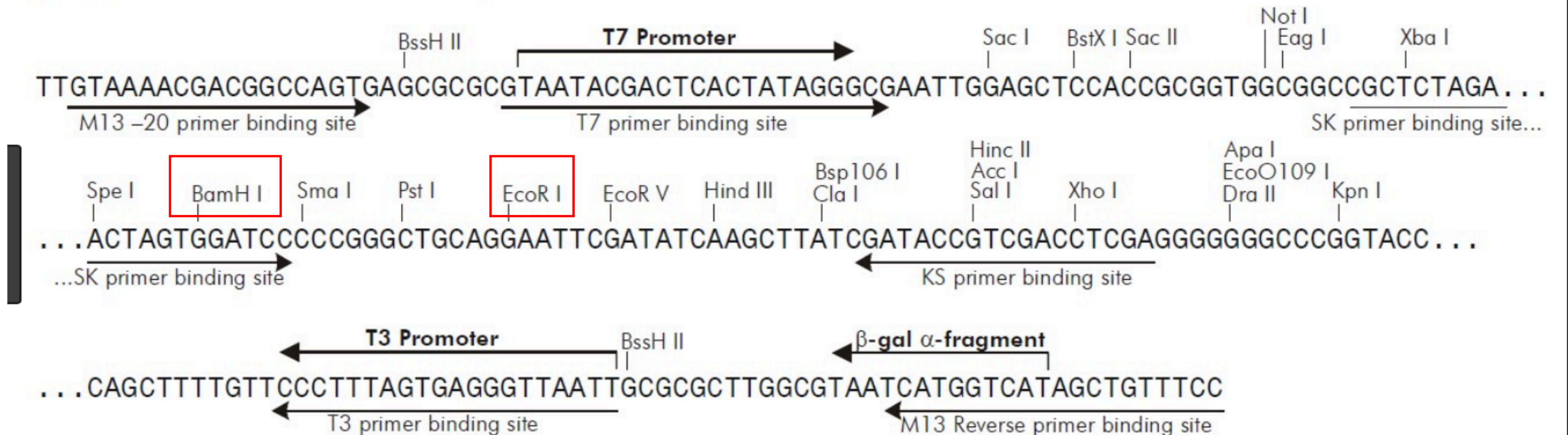



cut with BamHI and EcoRI



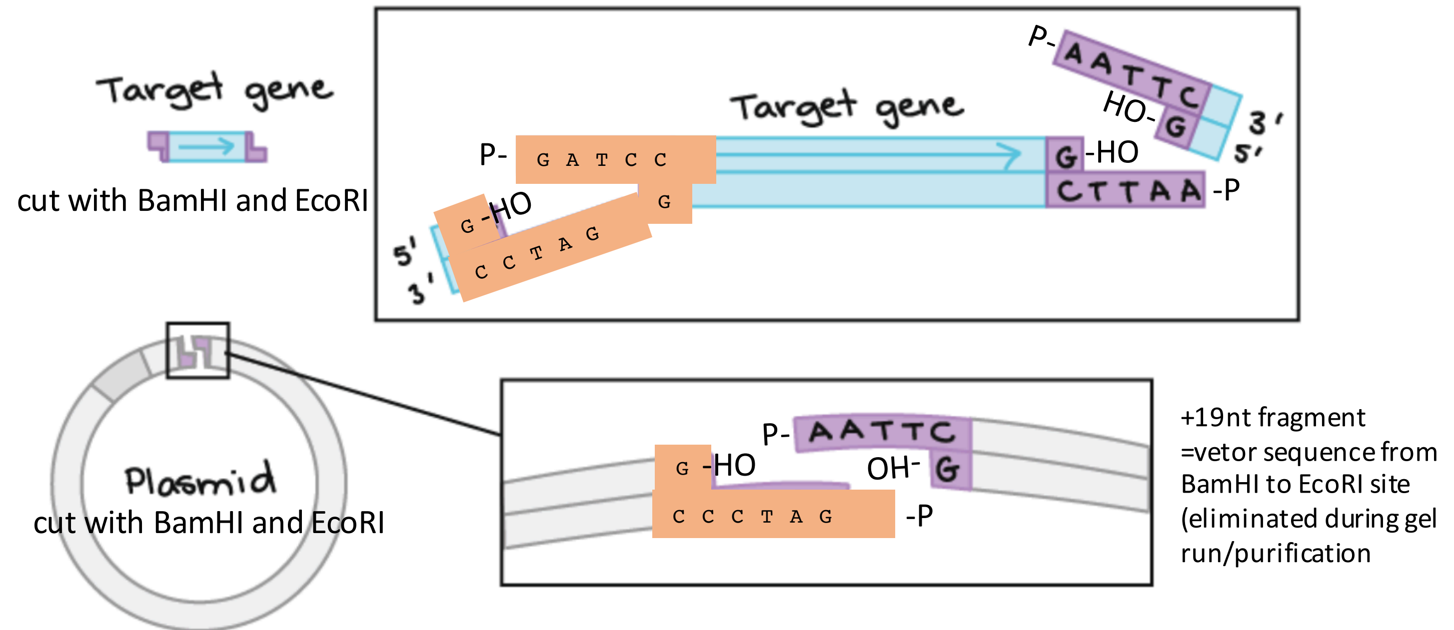

+19nt fragment =vetor sequence from BamHI to EcoRI site (eliminated during gel run/purification



# DNA CLONING WITH 2 COHESIVE OVERHANGS

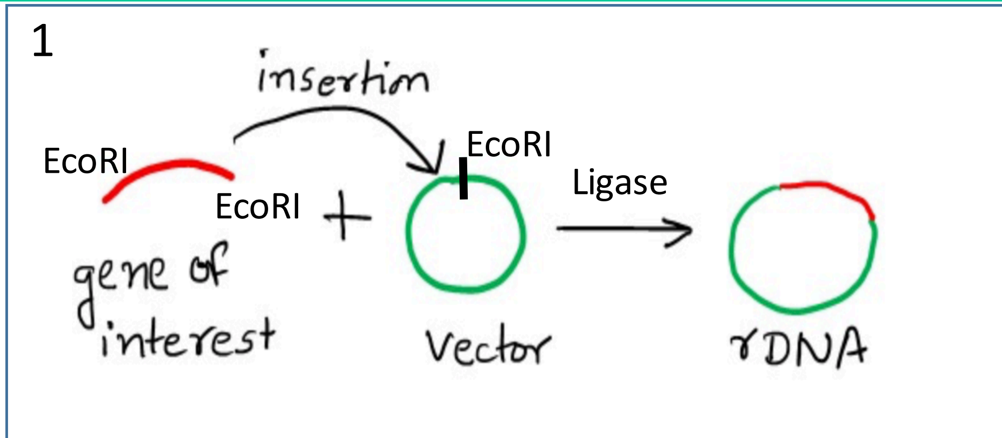
EcoRI: G/AATTC
CTTAA/G

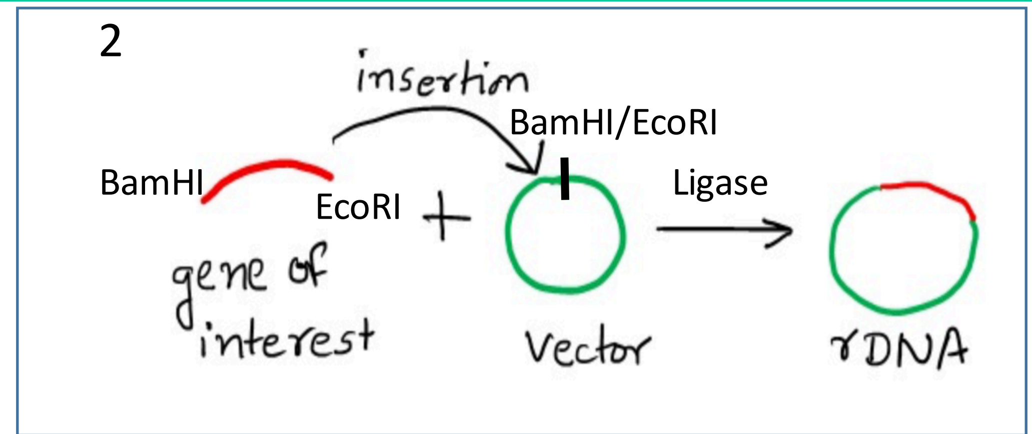
BamHI: G/GATCC
CCTAG/G

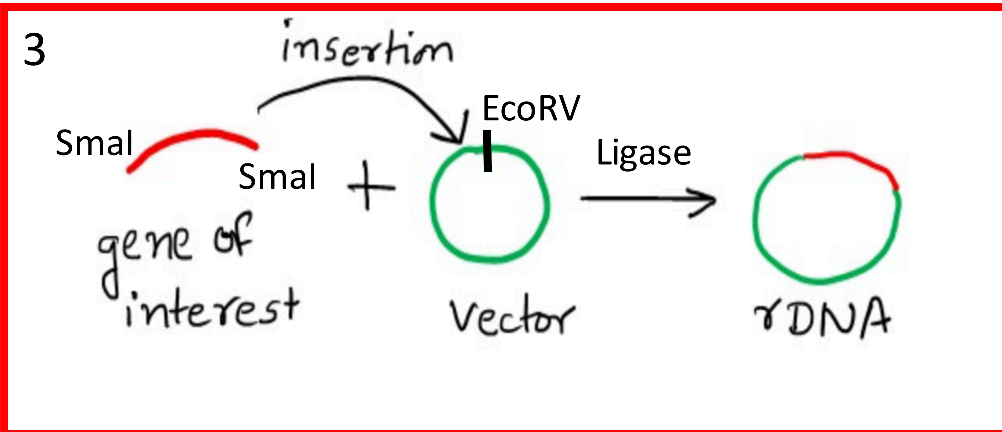


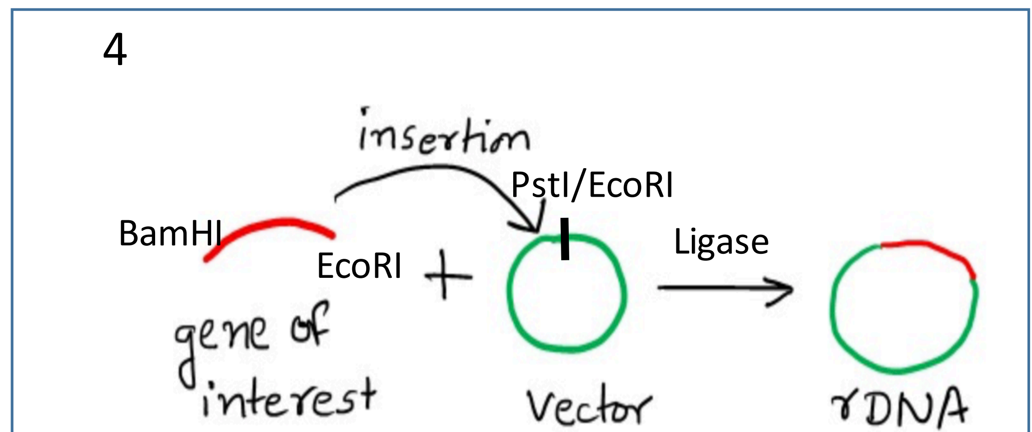

1. EcoRI/BamHI digest to obtain insert
2. EcoRI/BamHI digest to obtain linearized pBluescript
3. Gel run and purification of relevant DNA fragments
4. Set up ligation (plasmid:insert = 1:3)
5. Transform competent bacteria; plate on agar plates + X-GAL, IPTG, ampicillin → pick white colony → make liquid 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!!!

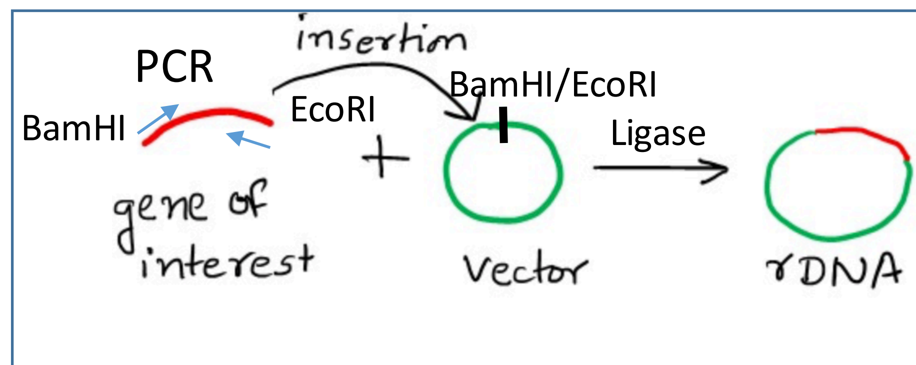
# OVERVIEW OVER OTHER CLONING STRATEGIES



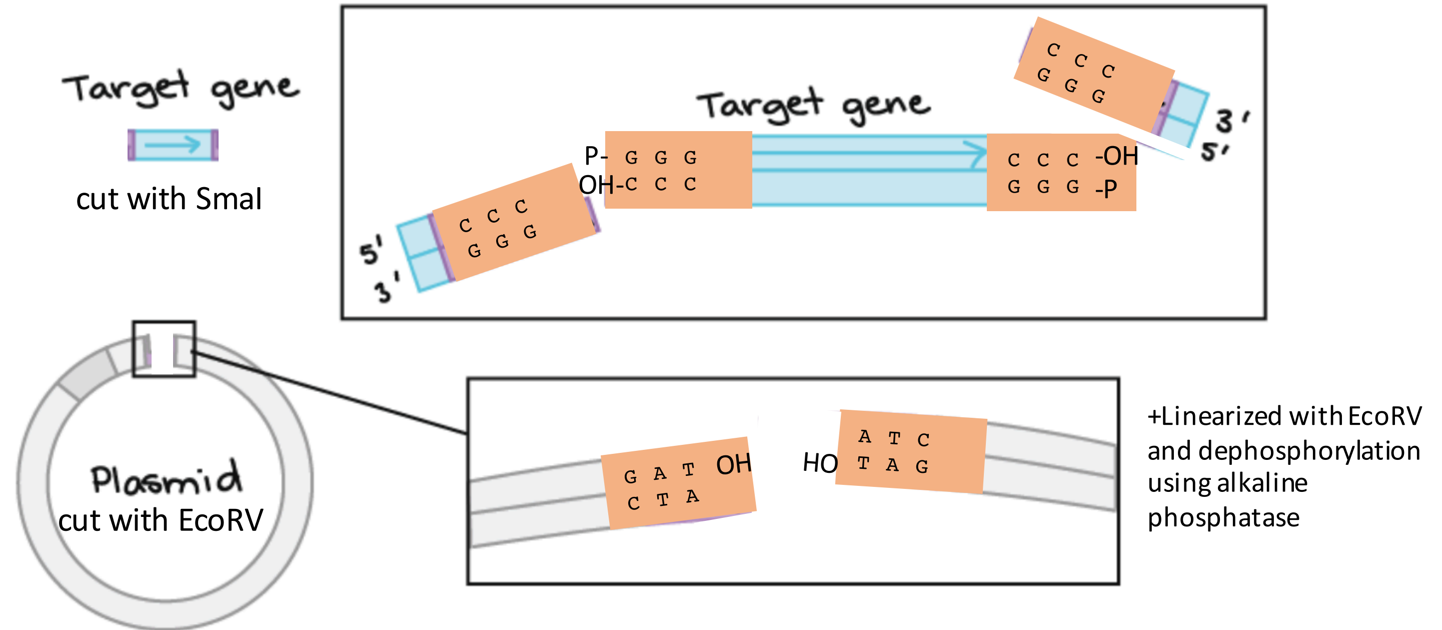



# DNA CLONING WITH BLUNT ENDS

SmaI: CCC/GGG
GGG/CCC

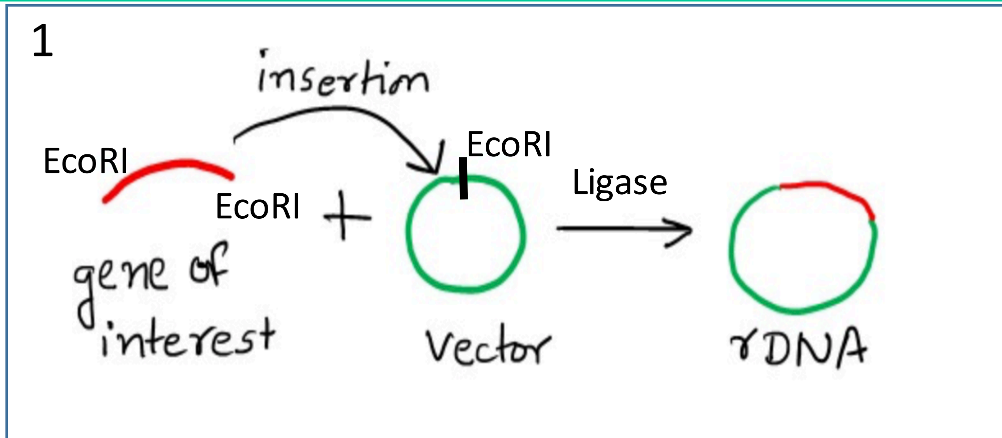
EcoRV: GAT/ATC
CTA/TAG

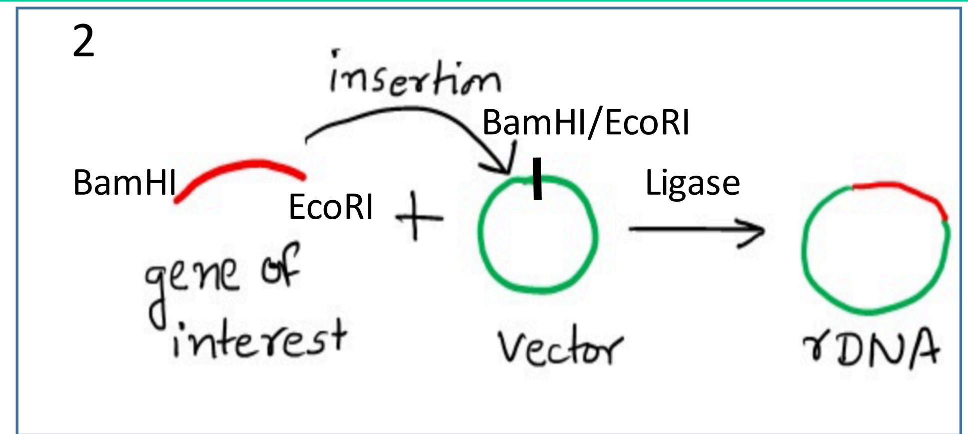


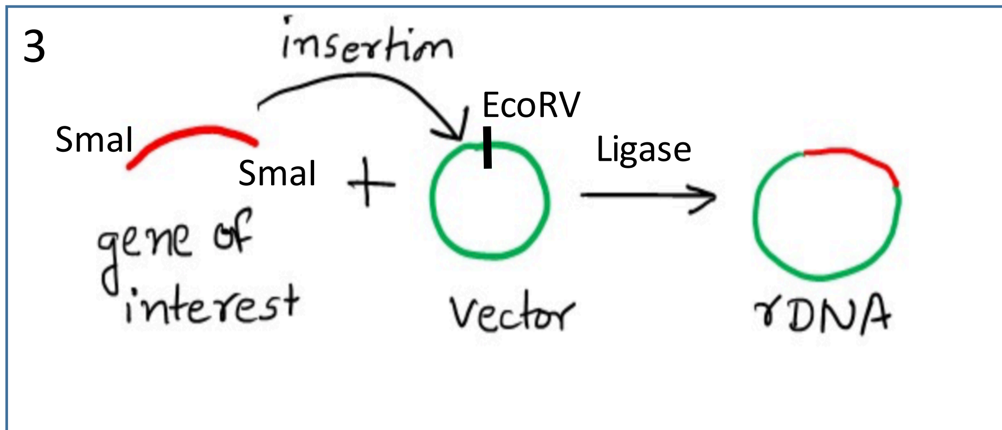

1. SmaI digest to obtain insert
2. EcoRV digest + alkaline phosphatase treatment to obtain linearized pBluescript (that connot re-ligate)
3. Gel run and purification of relevant DNA fragments
4. Set up ligation (plasmid:insert = 1:3 (5))
5. Transform competent bacteria; plate on agar plates + ampicillin → 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.

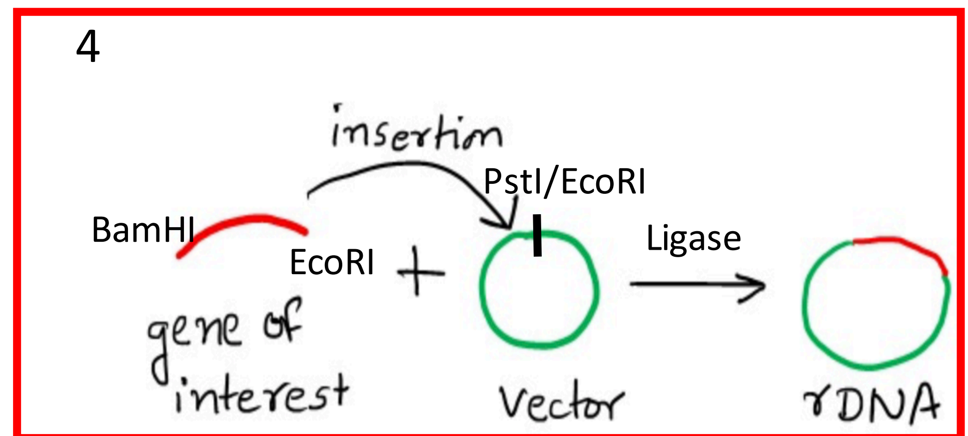
GATGGG ——— CCCATC
CTACCC ——— GGGTAG

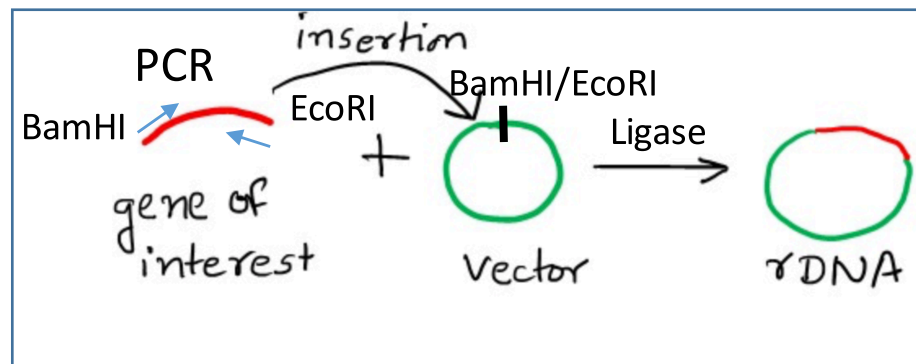
# OVERVIEW OVER OTHER CLONING STRATEGIES



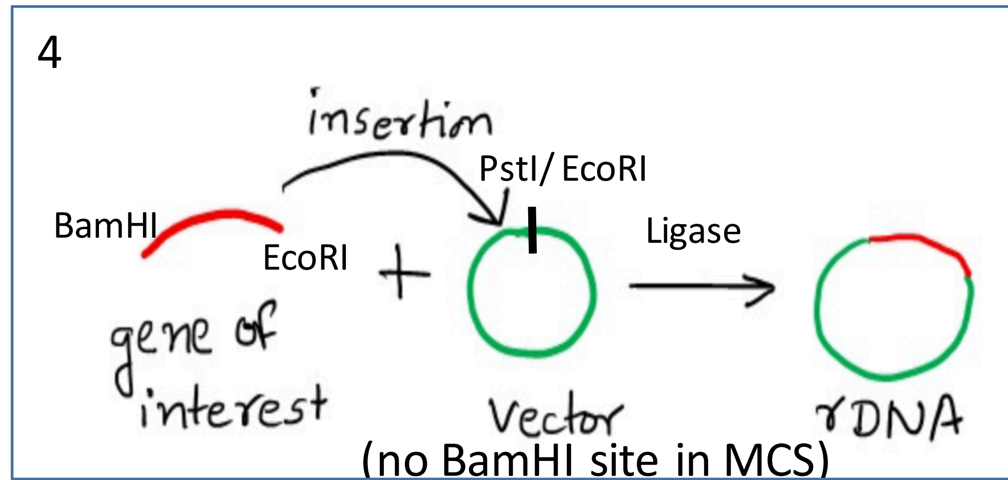



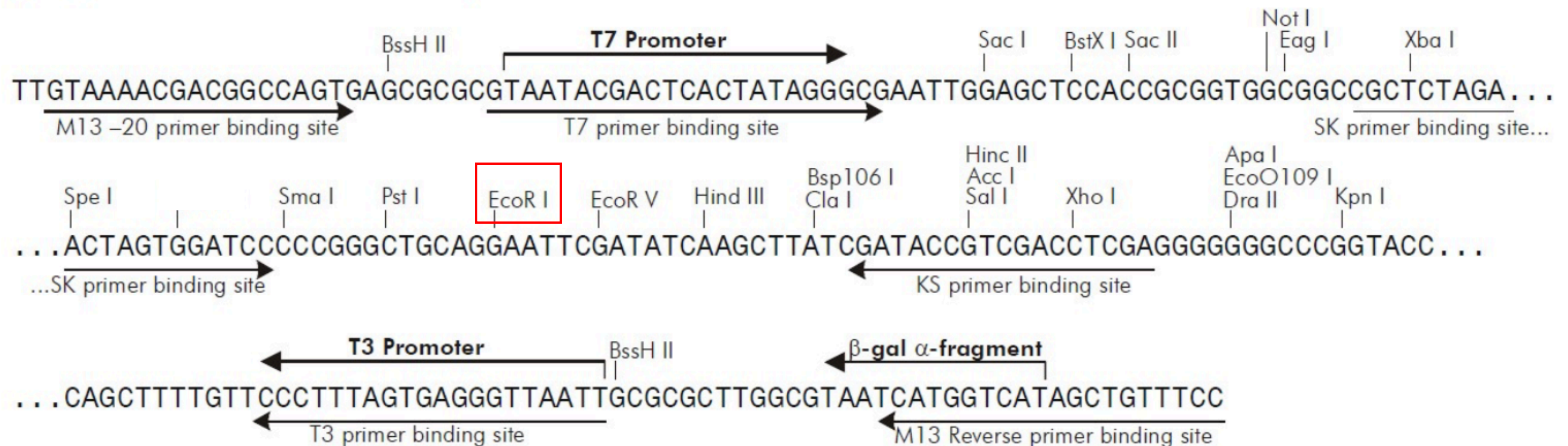



# DNA CLONING WITH MODIFICATION OF OVERHANGS




Lets assume: BamHI is not present in pBS



# DNA CLONING WITH MODIFICATION OF OVERHANGS

INSERT

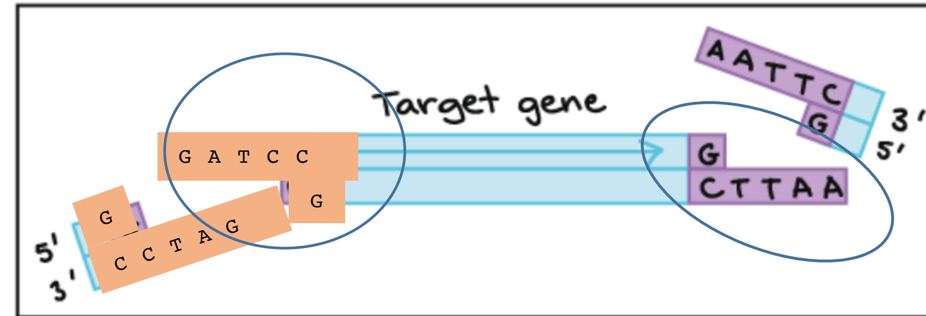
BamHI: G/GATCC
CCTAG/G

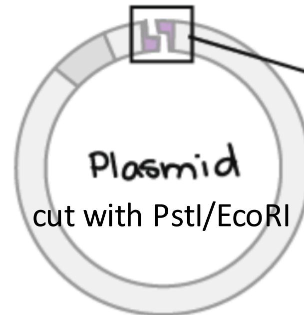
EcoRI: G/AATTC
CTTAA/G

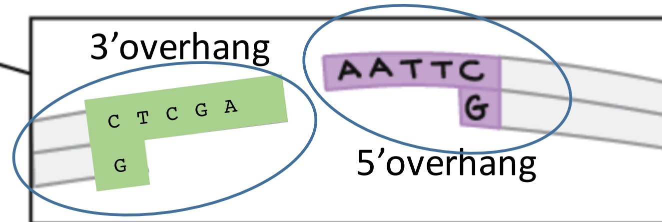
VECTOR

PstI: CTCGA/G
G/AGCTC

EcoRI: G/AATTC
CTTAA/G




**NOT COMPATIBLE**
**→ make blunt**

**COMPATIBLE**

→ Modification of 5'overhang of BamHI site → convert overhang to blunt end
→ Modifcation of 3'overhang of PstI site → convert overhang to blunt end

→ ➔ Blunt – Blunt AND EcoRI – EcoRI ligation

# DNA CLONING WITH MODIFICATION OF OVERHANGS

## DNA Polymerase I (E.Coli)

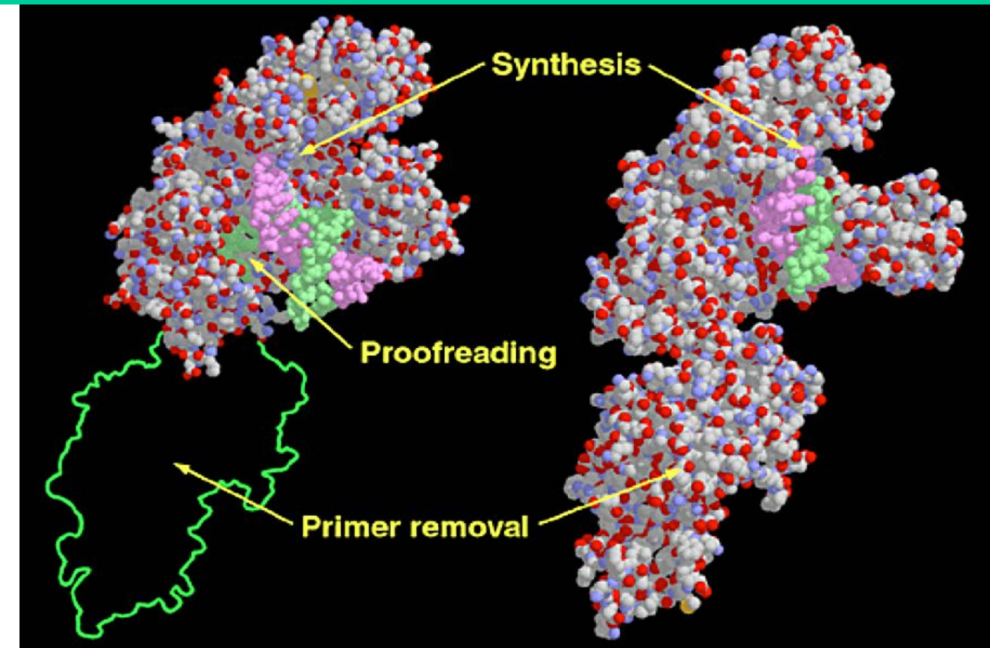
- **5' → 3' polymerase activity**
- **3’ → 5’ exonuclease activity**
- **5' → 3' exonuclease activity**

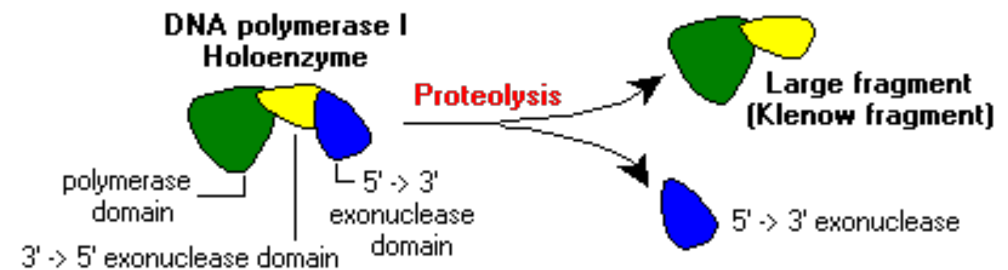
## The Klenow fragment

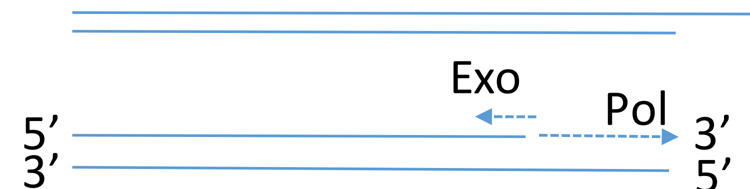
The Klenow fragment is a large protein fragment produced when **DNA polymerase I from E. coli** is enzymatically cleaved by the protease subtilisin. First reported in 1970.

**It retains the 5' → 3' polymerase activity and the 3’ → 5’ exonuclease activity** for removal of precoding 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




**Klenow**: in presence of dNTP: synthesis
In absence of dNTP: 3→’5’ exonuclease activty