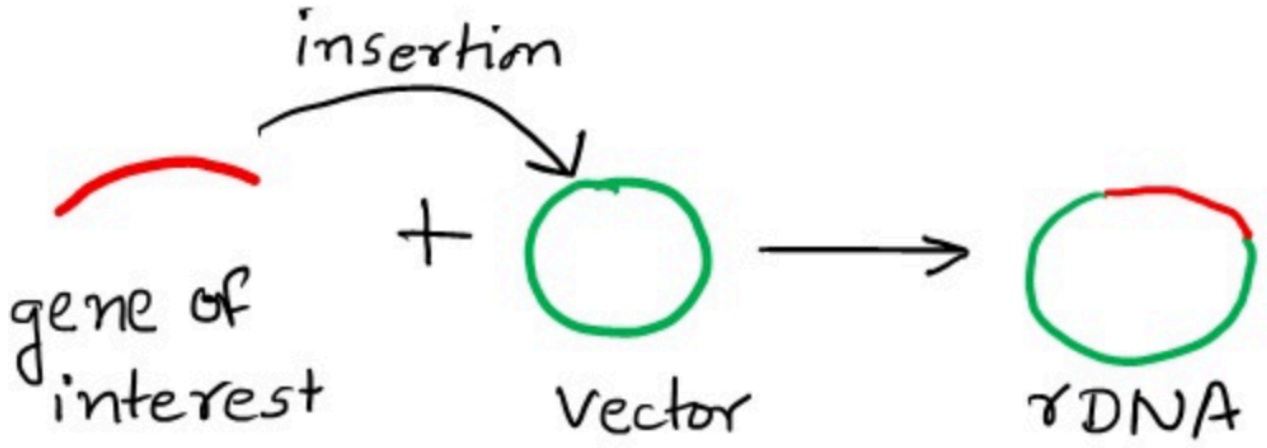
RECOMBIANANT DNA TECHNOLOGY
CLONING OF DNA FRAGMENTS INTO PLASMIDS



DNA RICOMBINANTE:

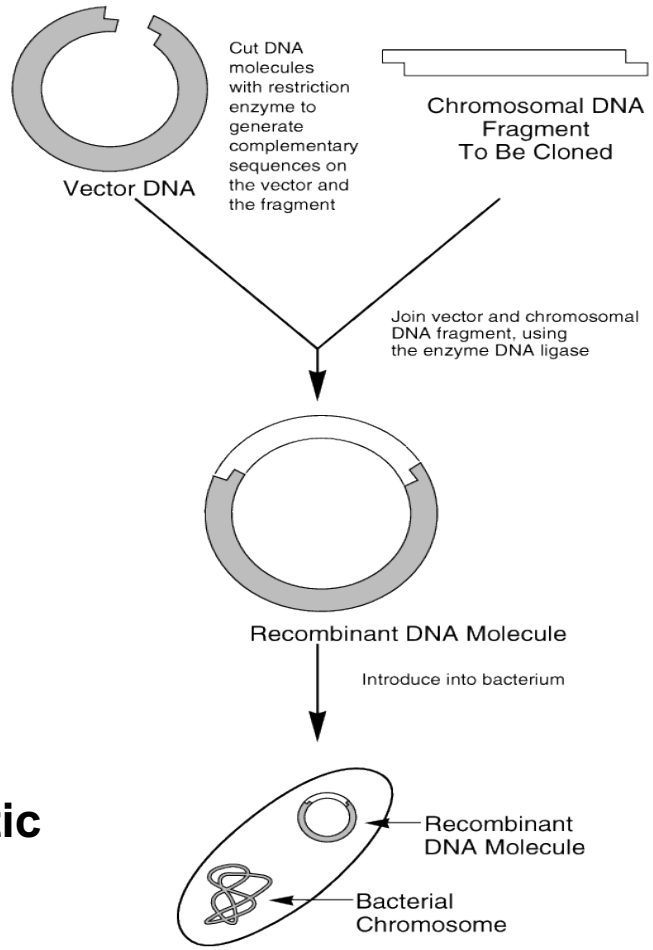
DUE MOLECOLE DI DNA VENGONO UNITE IN PROVETTA E FATTE RIPRODURRE IN LABORATORIO

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***

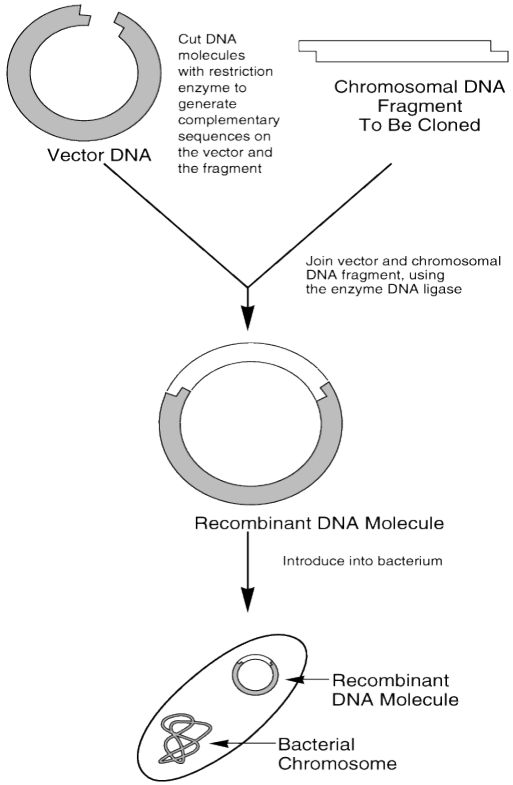


DNA cloning

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

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 recombiant DNA

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

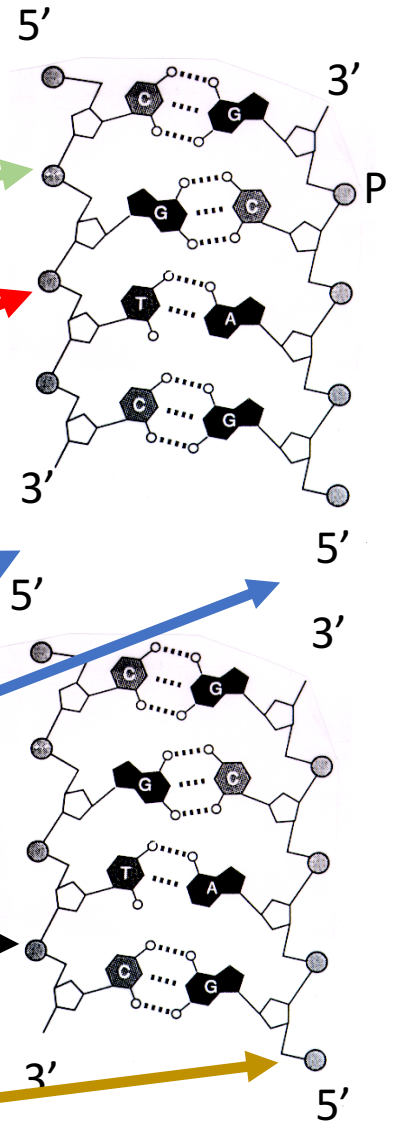
Le esonucleasi rompono il legame al <u>termine</u> dei filament (5’ → 3’)

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

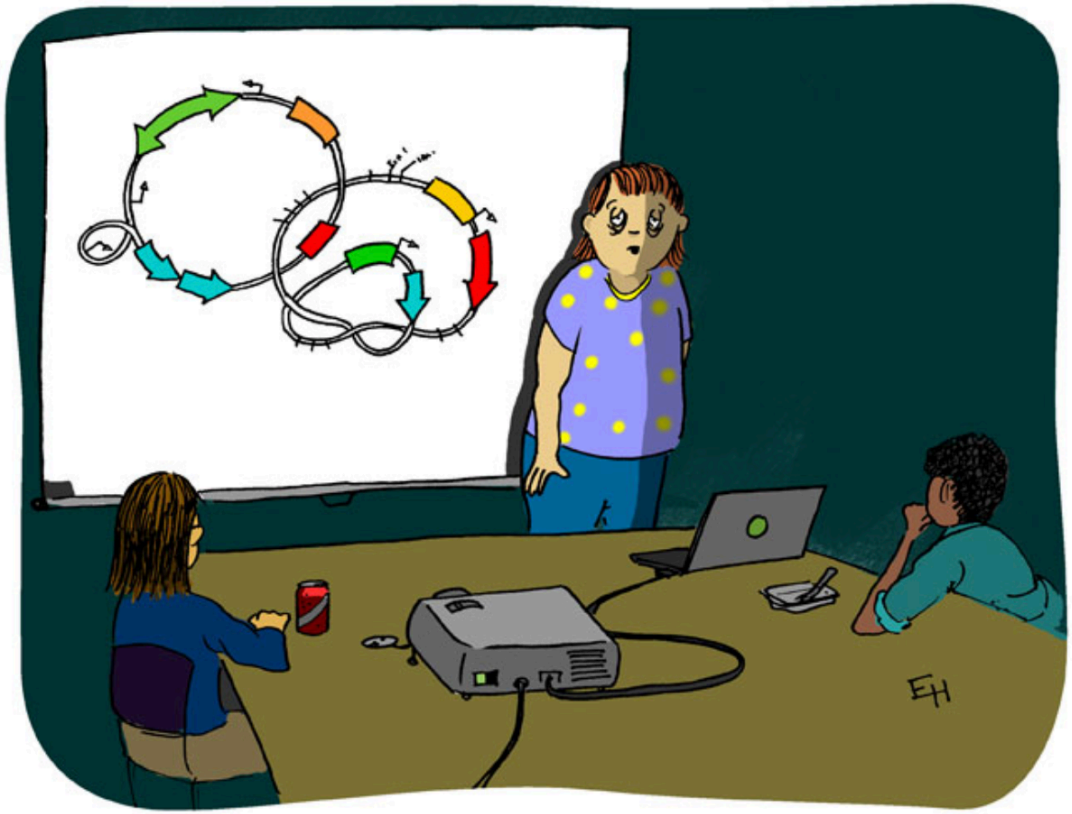
Ligasi: catalizza la formazione del legame tra due molecole di DNA, spesso accompagnato dall'idrolisi di una molecola come ATP

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

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



DNA cloning



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

….can be simple….

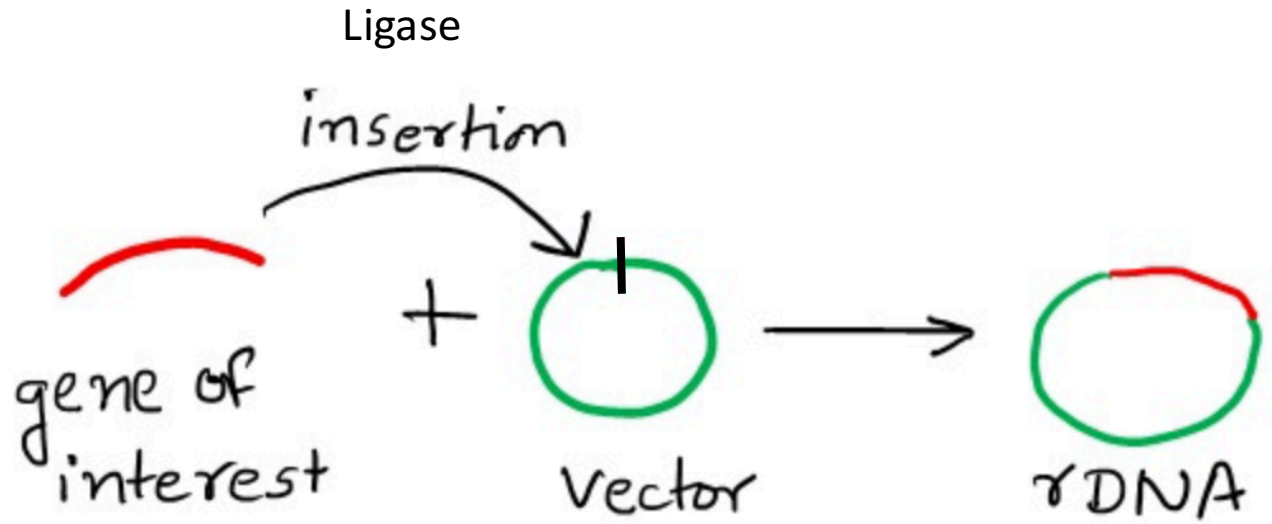
Or

…frustrating…

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

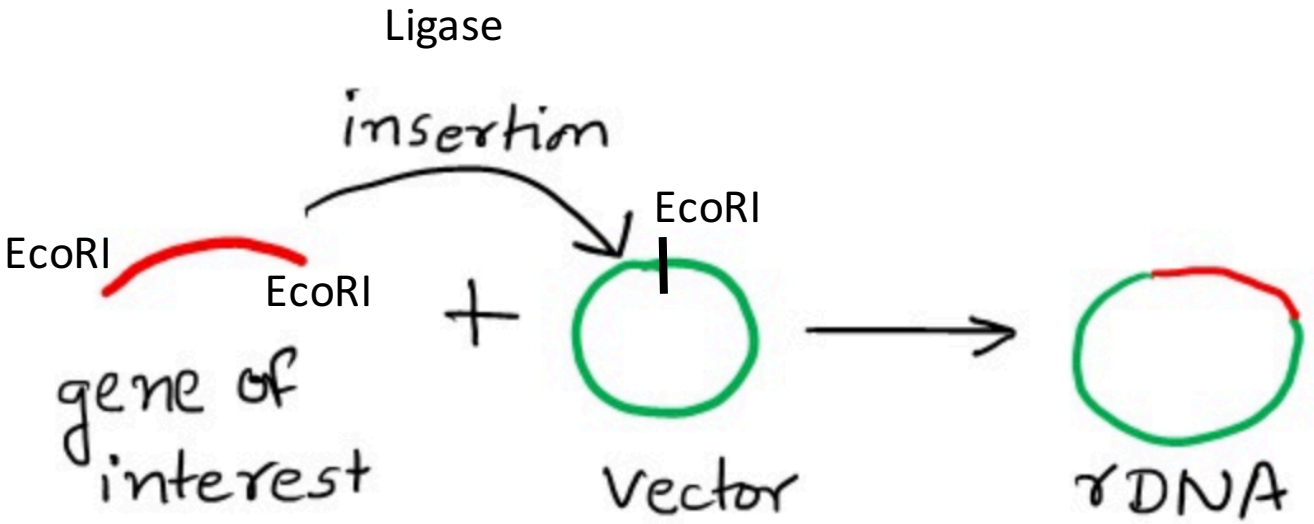
This can trigger what's known as "The 5 Stages of Failed Cloning Grief." As you work through each stage at your own pace, just know that scientists all over the world feel your pain and can empathize with you in this difficult time. Continue reading →

Making recombinant DNA

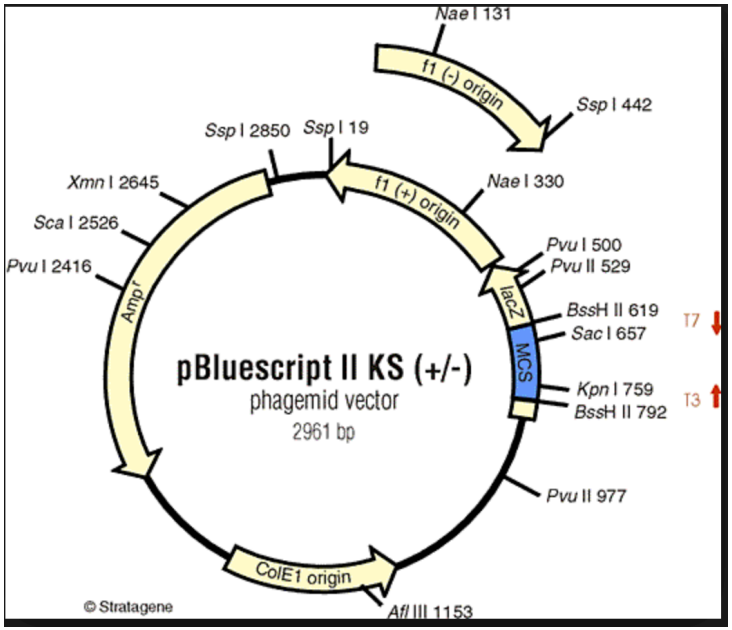


1. **Fragment and Insert are cut with 1 (the same) restriction enzymes**
2. **Fragment and Insert are cut with 2 (the same) restriction enzymes**
3. **Fragennt and Insert are cut with blunting restriction enzymes**
4. **Overhangs generated after cutting are modified (filled up; or overhang digested)**

Making recombinant DNA

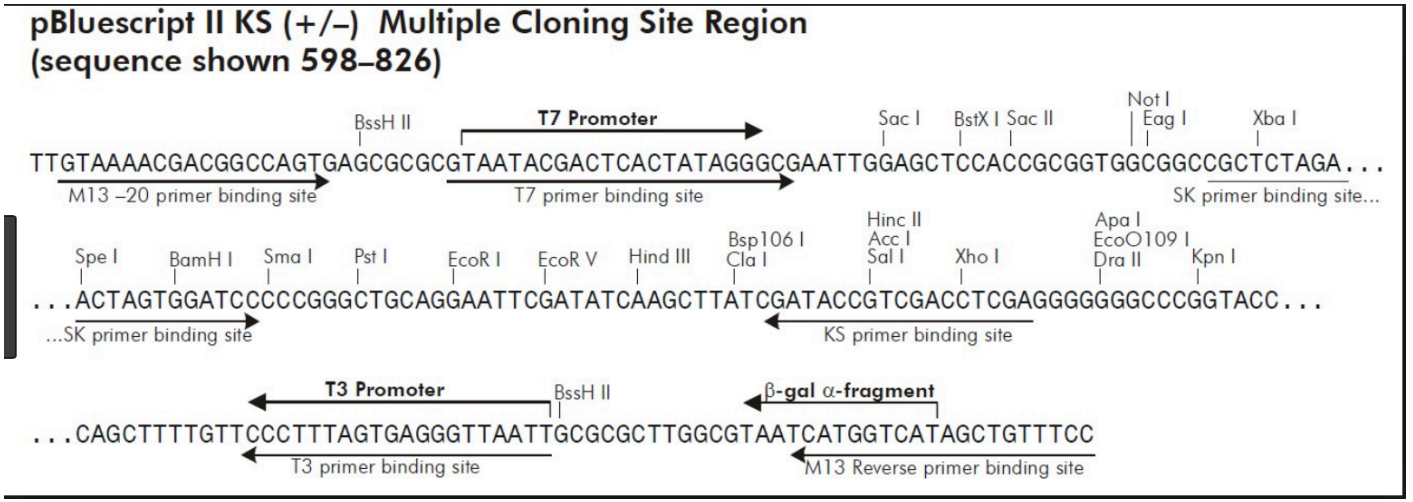


Making recombinant DNA – Plasmid features

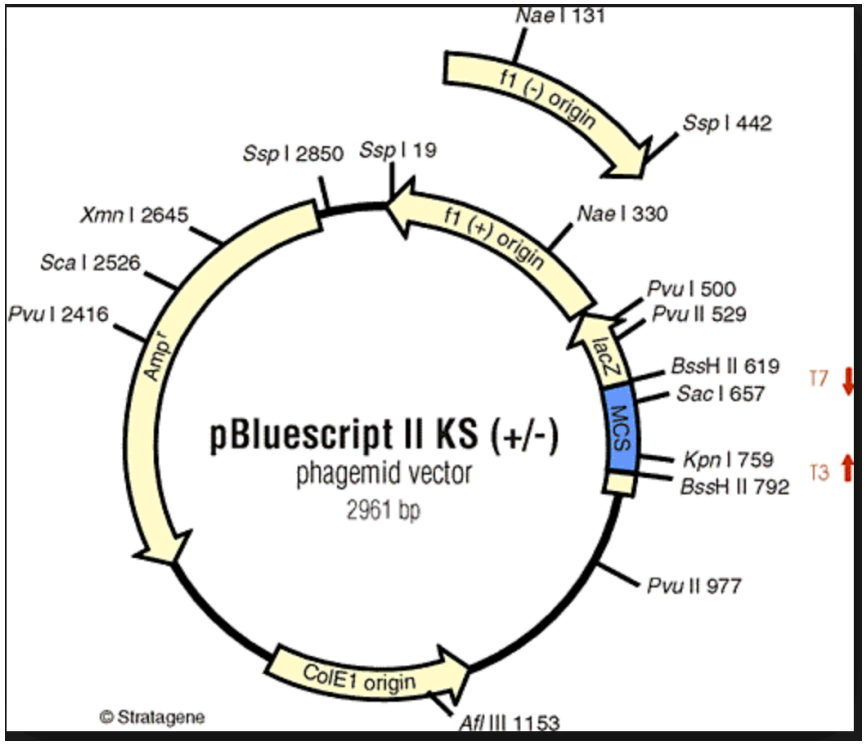


What makes a good plasmid for cloning (generating recombinant DNA)??

- **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.**



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

F1 (-) (+) origin:

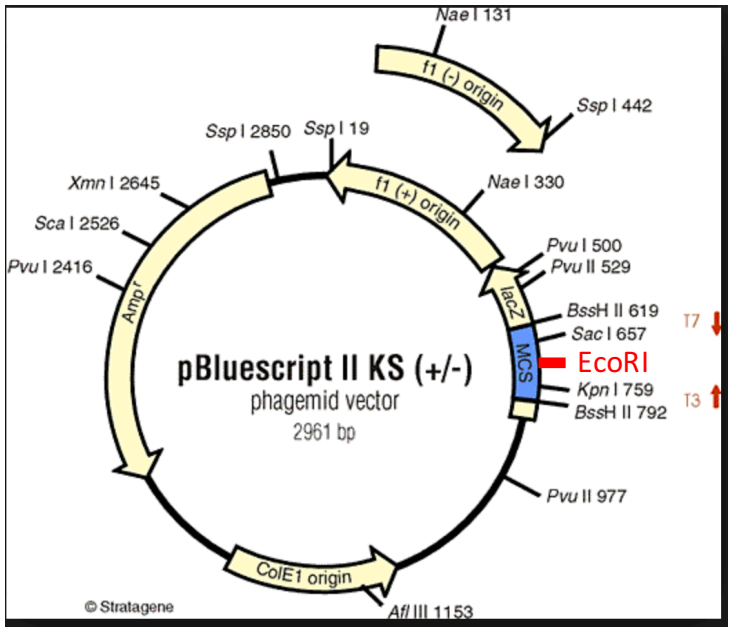
pBluescript II is a phagemid that can be secreted as single-stranded DNA in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous f1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E. 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, fl) will not infect *E. coli* without an F´ episome coding for pili, **it is essential to use XL1-Blue MRF´ or a similar strain containing the F´ episome.**[7,8]

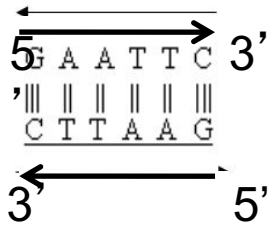
Similarly to a plasmid, a phagemid can be used to clone DNA fragments and be introduced into a bacterial host by a range of techniques, such as transformation and electroporation. However, infection of a bacterial host containing a phagemid with a 'helper' phage, for example VCSM13 or M13K07, provides the necessary viral components to enable single stranded DNA replication and packaging of the phagemid DNA into phage particles. The 'helper' phage infects the bacterial host by first attaching to the host cell's pilus and then, after attachment, transporting the phage genome into the cytoplasm of the host cell. Inside the cell, the phage genome triggers production of single stranded phagemid DNA in the cytoplasm. This phagemid DNA is then packaged into phage particles. The phage particles containing ssDNA are released from the bacterial host cell into the extracellular environment.

Ampicillin

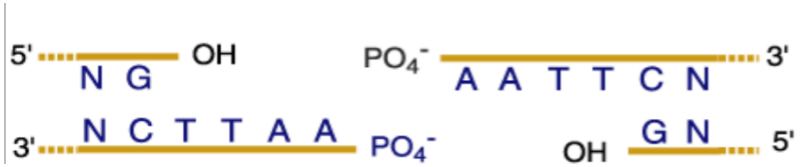
- Ampicillin binds to and inhibits a number of enzymes in the bacterial membrane that are involved in the synthesis of the ***gram negative*** cell wall.
  - Therefore, proper cell replication cannot occur in the presence of ampicillin.
- The ampicillin resistance gene (***amp^r***) codes for an enzyme (***b-lactamase***) that is secreted into the periplasmic space of the bacterium where it catalyzes hydrolysis of the b-lactam ring of the ampicillin.
  - Thus, the gene product of the amp^r gene **destroys the antibiotic**.
- Over time the ampicillin in a culture medium or petri plate may be substantially destroyed by b-lactamase.
  - When this occurs, cell populations can arise which have "lost" the plasmid.

Making recombinant DNA – Plasmid features





EcoRI
Digest



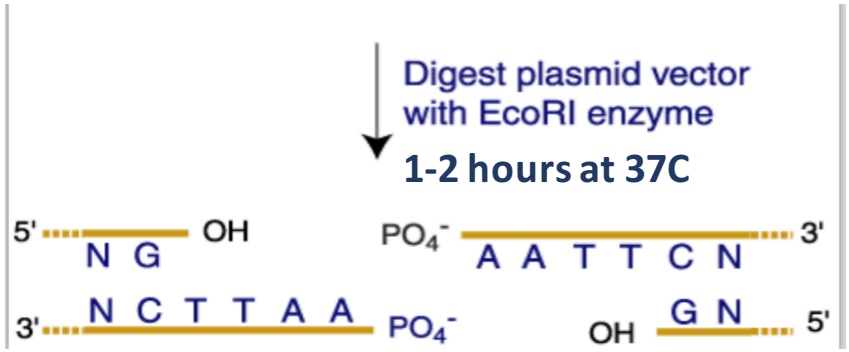
Linearized pBS with 5'overhangs

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

Making recombinant DNA – Plasmid features

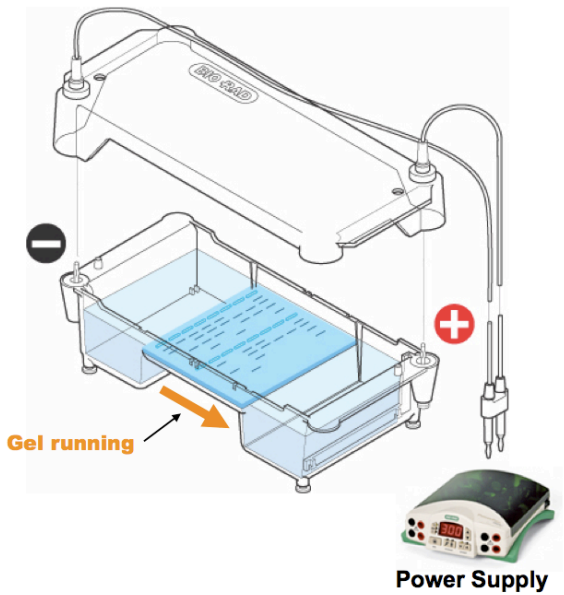
- Alkaline phosphatase removes 5' phosphate groups from DNA and RNA. It will also remove phosphates from nucleotides and proteins. These enzymes are most active at alkaline pH.
- In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby greatly facilitates ligation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



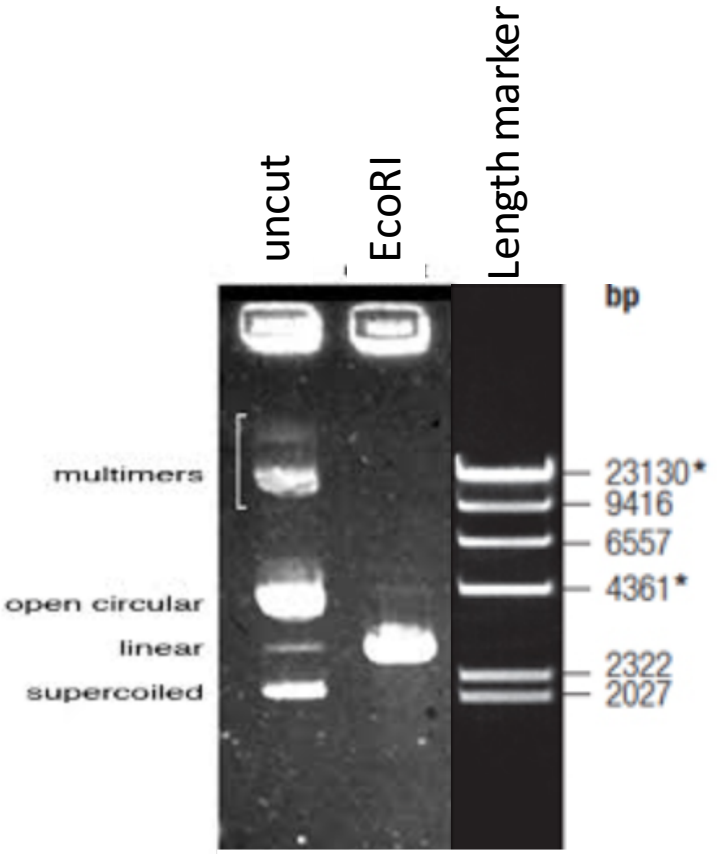
Making recombinant DNA – Plasmid features

Agarose
Electrophoresis
Running

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



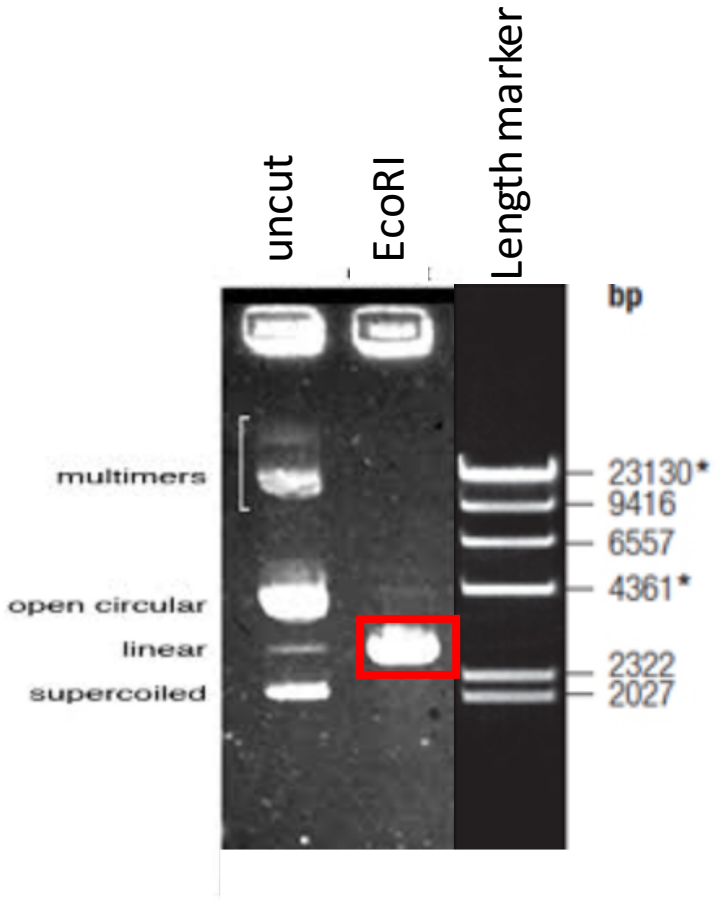
Digest run on agarose gel
(gel contains Ethidiumbromide; or samples contains DNA colouring agent)

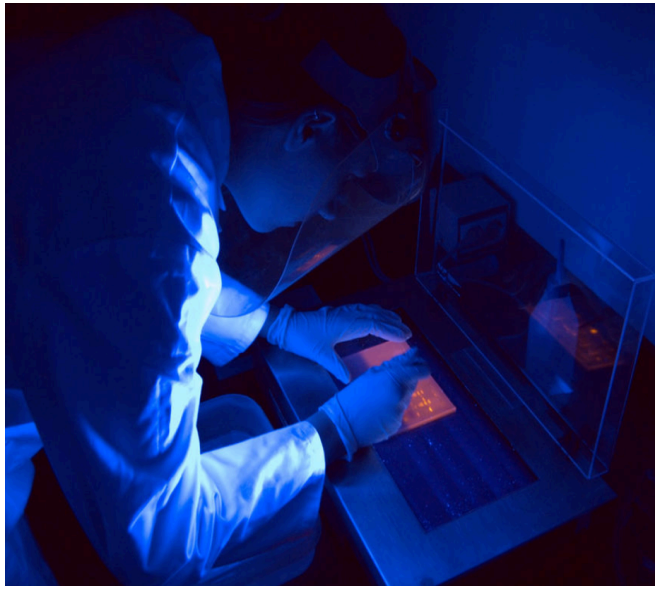


Length marker: mix of DNA fragments with defined length

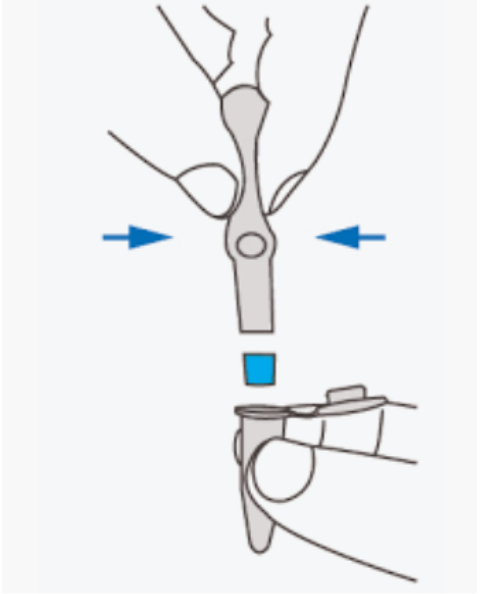
Linearized pBS: 2900 nt

Making recombinant DNA – Plasmid features



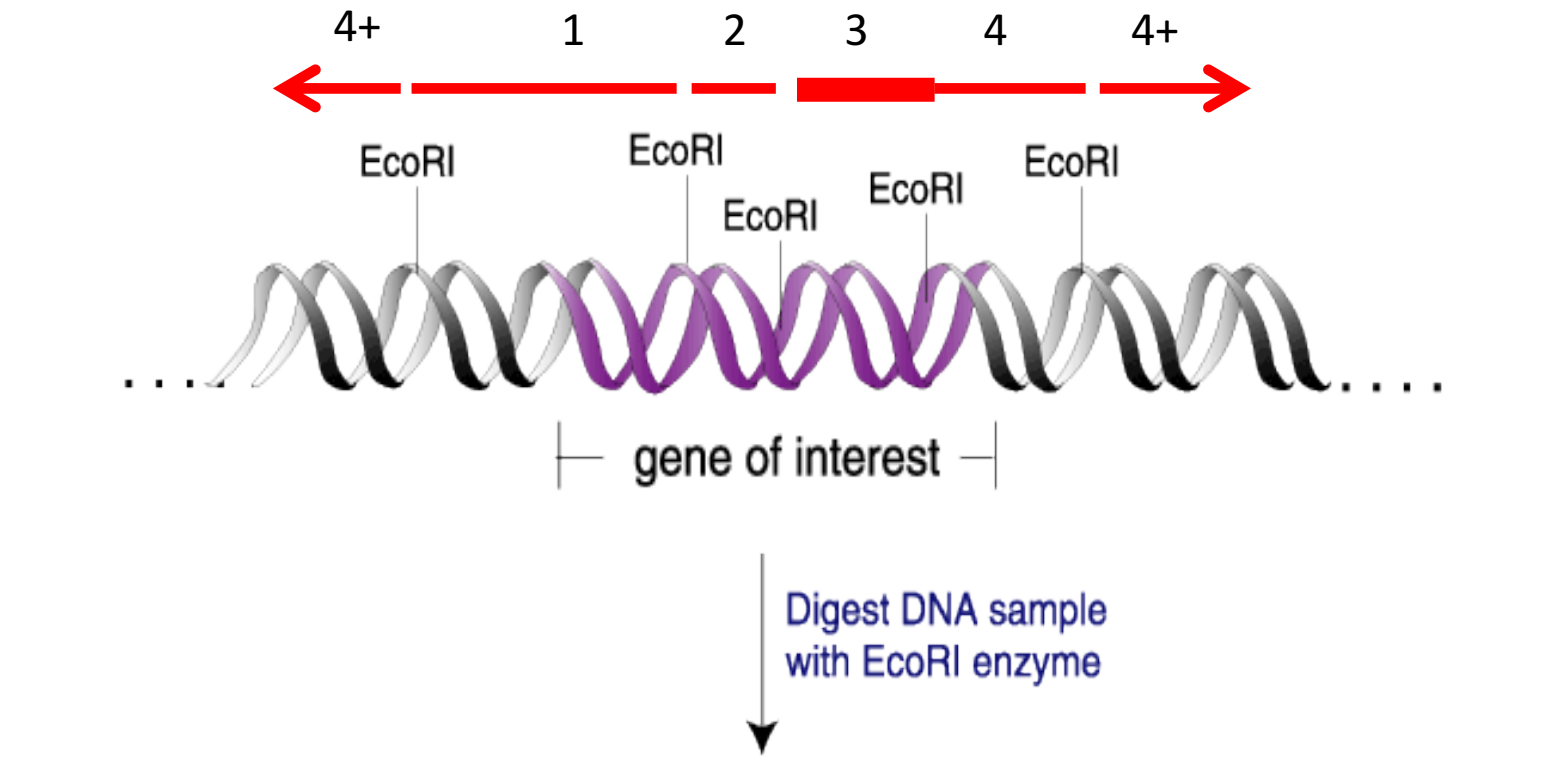


Cut out band from gel using a scalpel blade



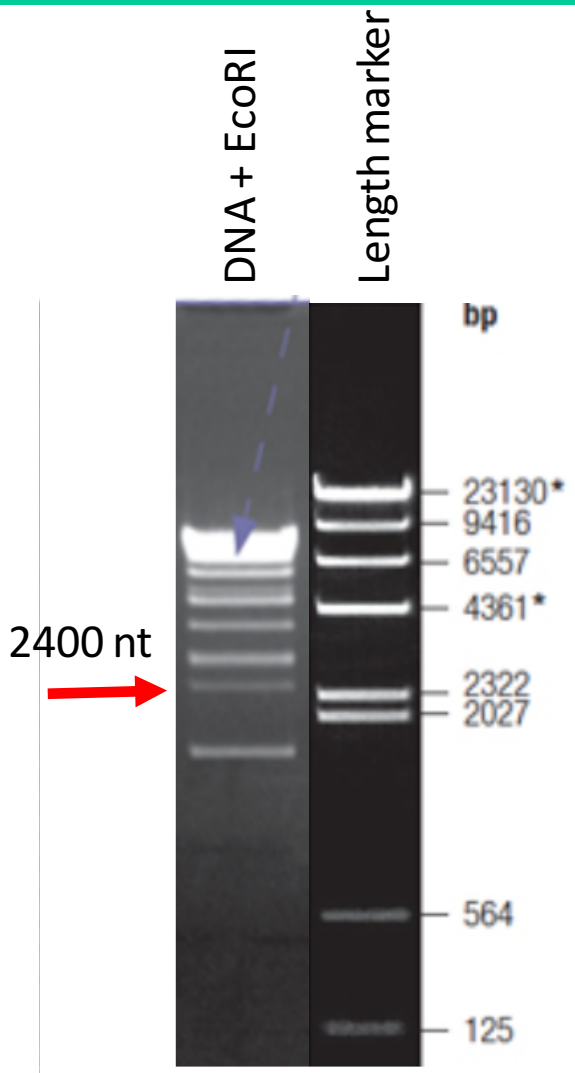
Purify DNA and eliminate agarose
Determine concentration of purified plasmid DNA (ca. 50% loss of starting material)

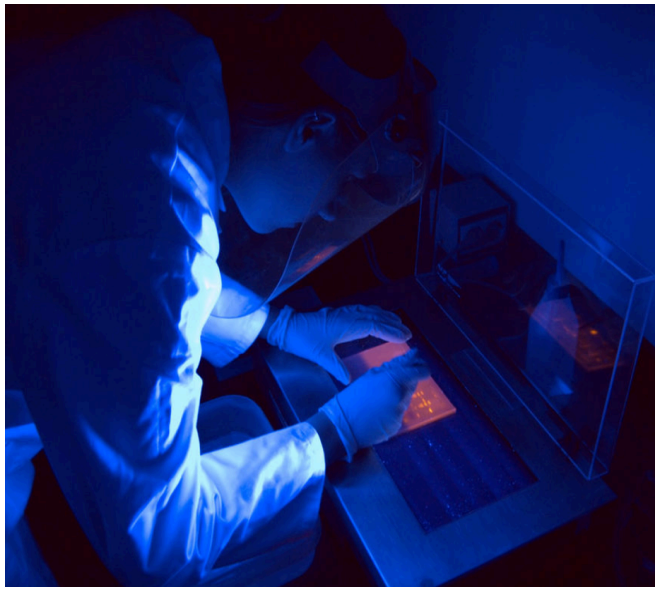
Preparing the insert



Defined size

Preparing the insert





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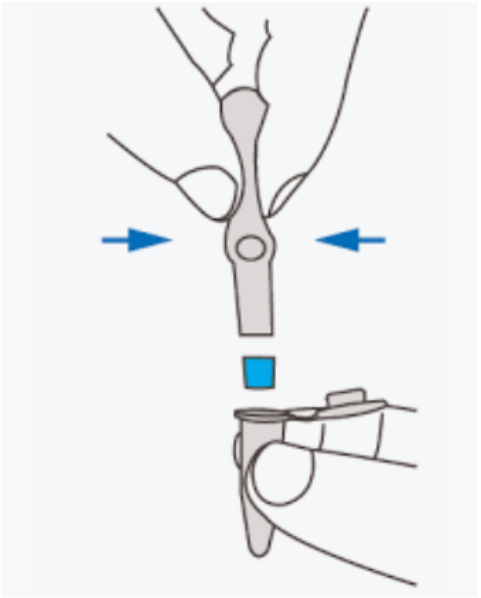
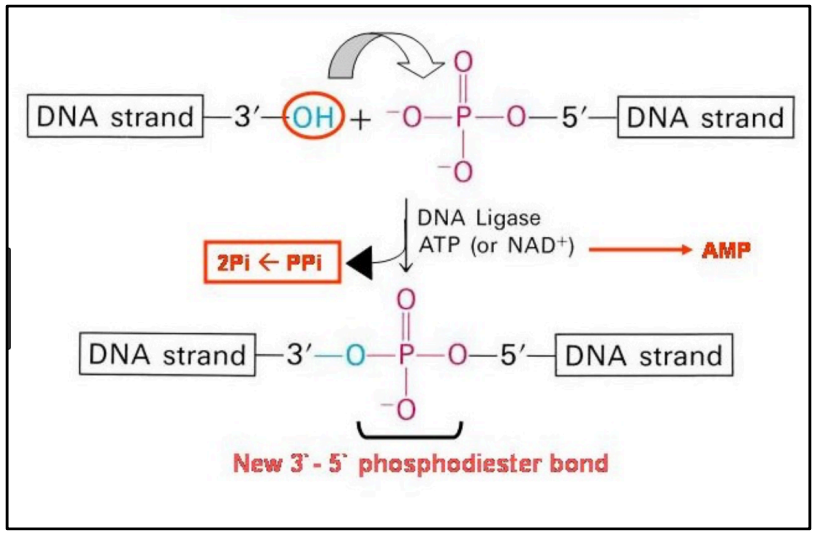
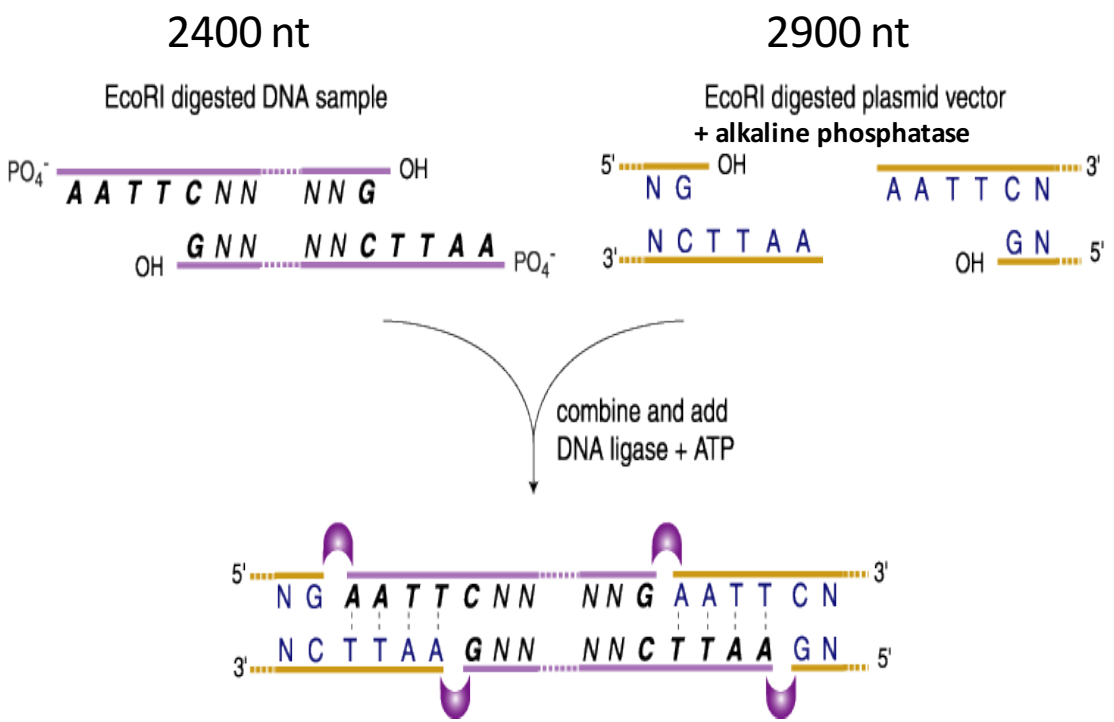


Purify DNA and eliminate agarose
Determine concentration of purified plasmid DNA (ca. 50% loss of starting material)

Ligating 2 fragements with DNA Ligase



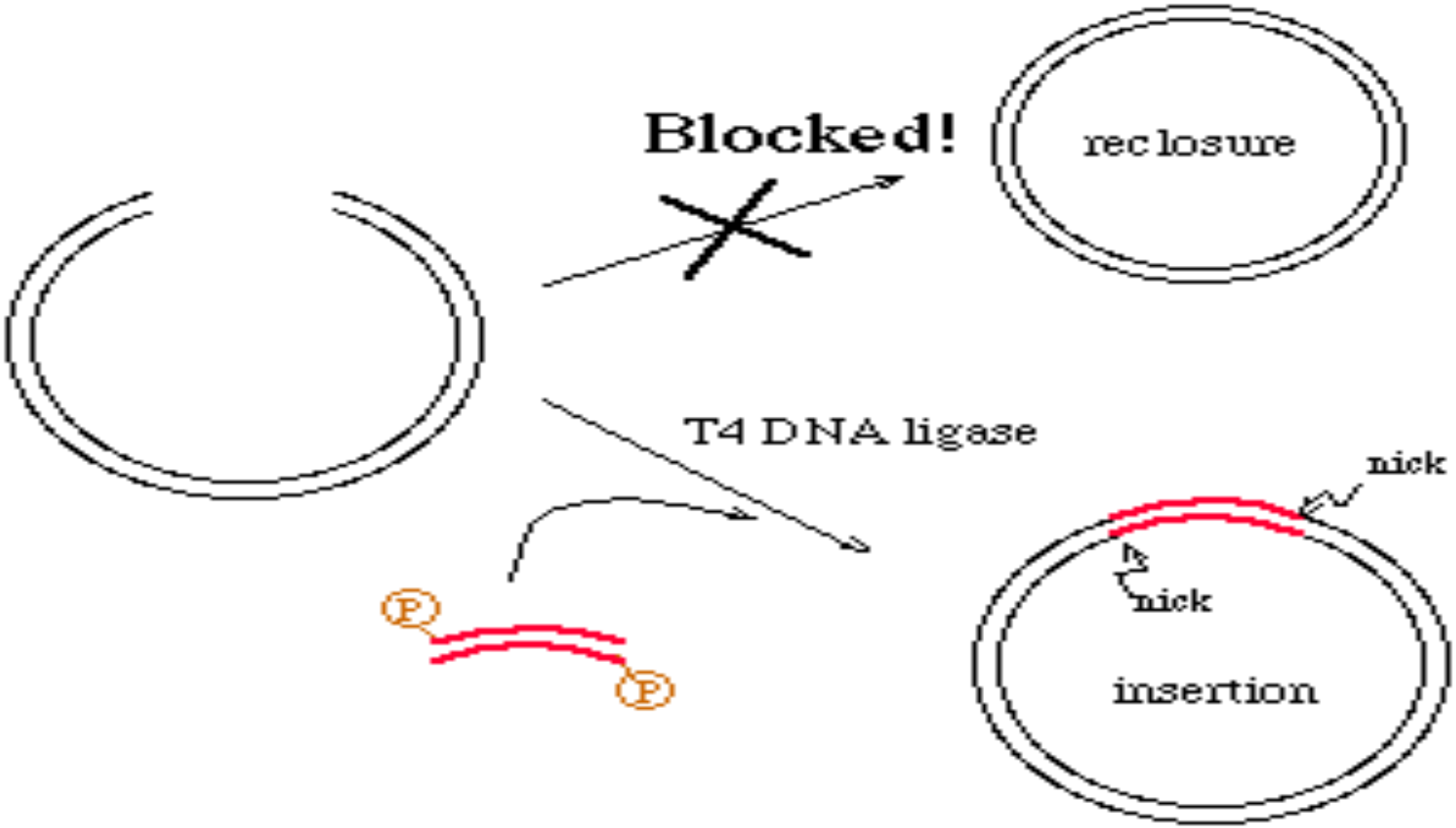


Sticky-end Ligations: 50 ng linearized plasmid + 3 fold molar excess of insert (=124 ng)

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

2400 nt fragment was NOT dephosphorylated → Ligation between 5'Phosphate + 3'OH of linearized plasmid is possible!!!

Ligating 2 fragements with DNA Ligase



NICKS ARE TOLERATED – REPAIRED BY BACTERIA

REAL SETTING: USE CONTROLS!!

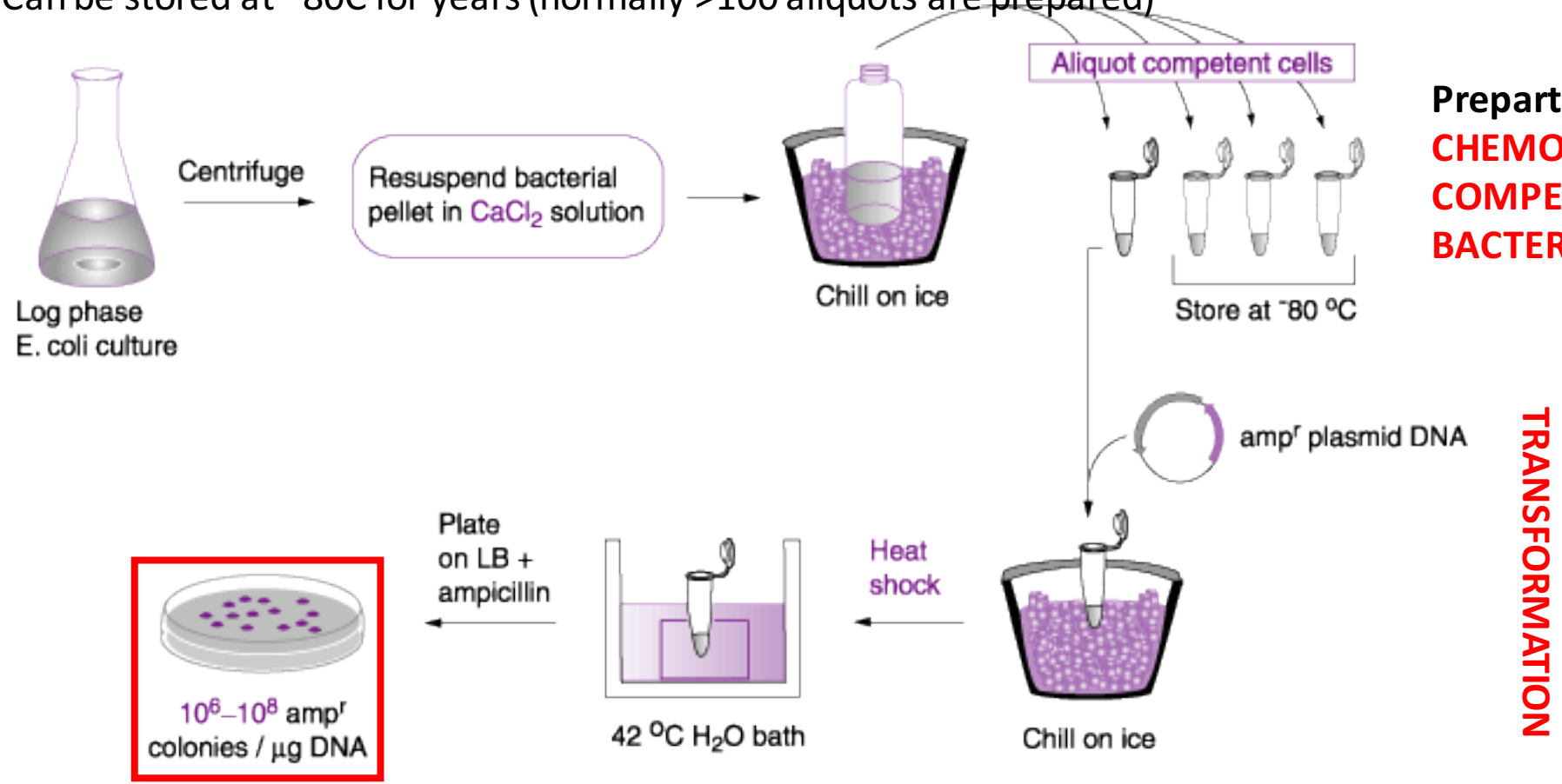
LIGATION 1: 50ng plasmid; EcoRI, de-phosph + LIGASE

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

Ev. LIGATION 3: 124ng INSERT + LIGASE

TRANSFORMATION: Insertion of ligated productis into bacteria

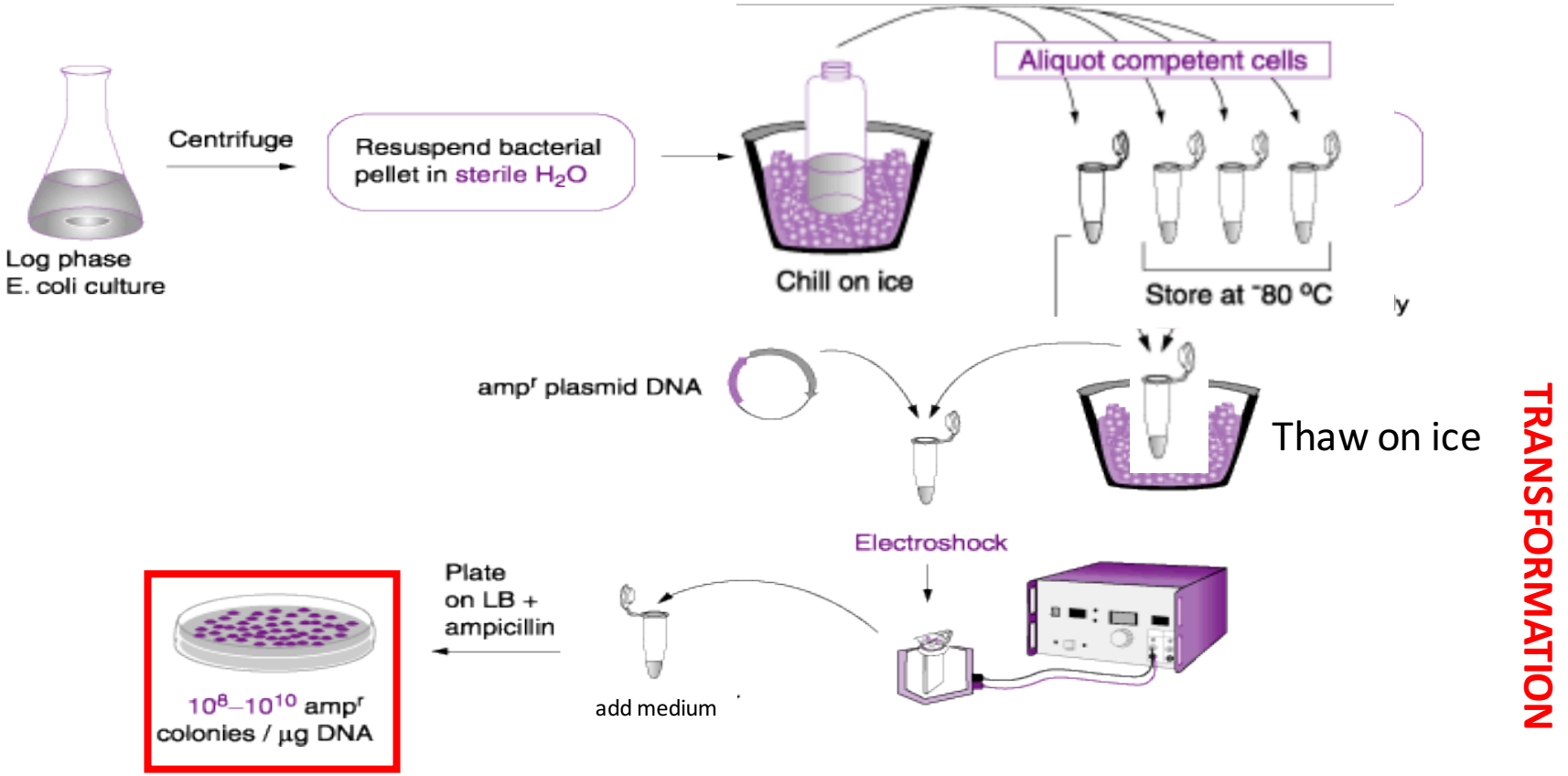
$CaCl_2$ and cold environment makes membrane permeable without killing the cells
= **CHEMOCOMPETENT BACTERIA**
(Can be stored at –80C for years (normally >100 aliquots are prepared)



Compent bacteria are put on ice until bacteria are thawn; 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 amplicilin

TRANSFORMATION: Insertion of ligated productis into bacteria

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



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

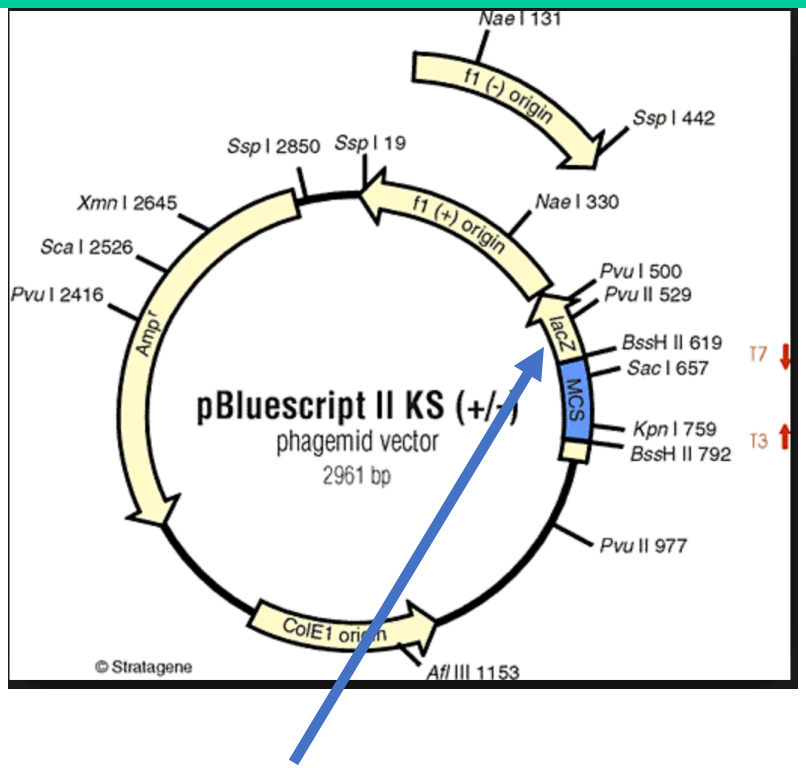
EVALUATING THE SUCCESS OF DNA CLONING EVENTS

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 SELETION SCREEN

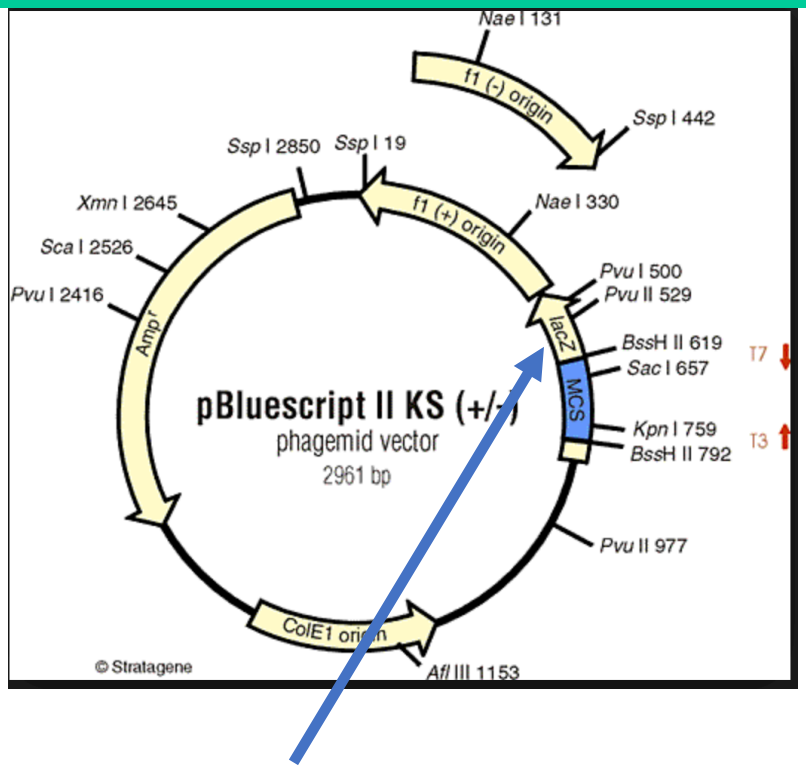


LacZ open reading frame:
MCS does not impair alpha LacZ
beta-galactosidase forms and converts X-Gal to blue colorant → blue colonies

α -complementation

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

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



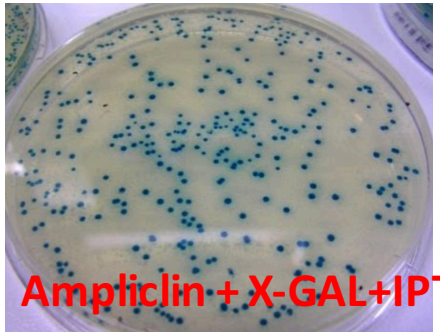
LacZ open reading frame:
MCS does not impair alpha LacZ
beta-galactosidase forms and converts X-Gal to blue colorant → blue colonies

….some things to consider

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

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

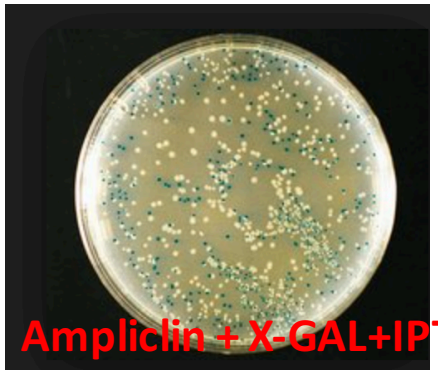
LIGATION 1: 50ng plasmid; EcoRI, de-phosph + LIGASE



46 blue colonies

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

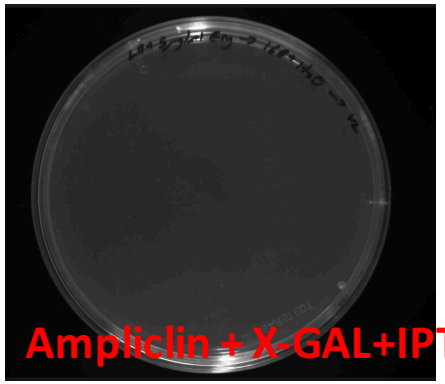
LIGATION 2: 50ng plasmid; EcoRI, de-phosph + 124ng INSERT + LIGASE



200 white 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)**

Ev. LIGATION 3: 124ng INSERT + LIGASE

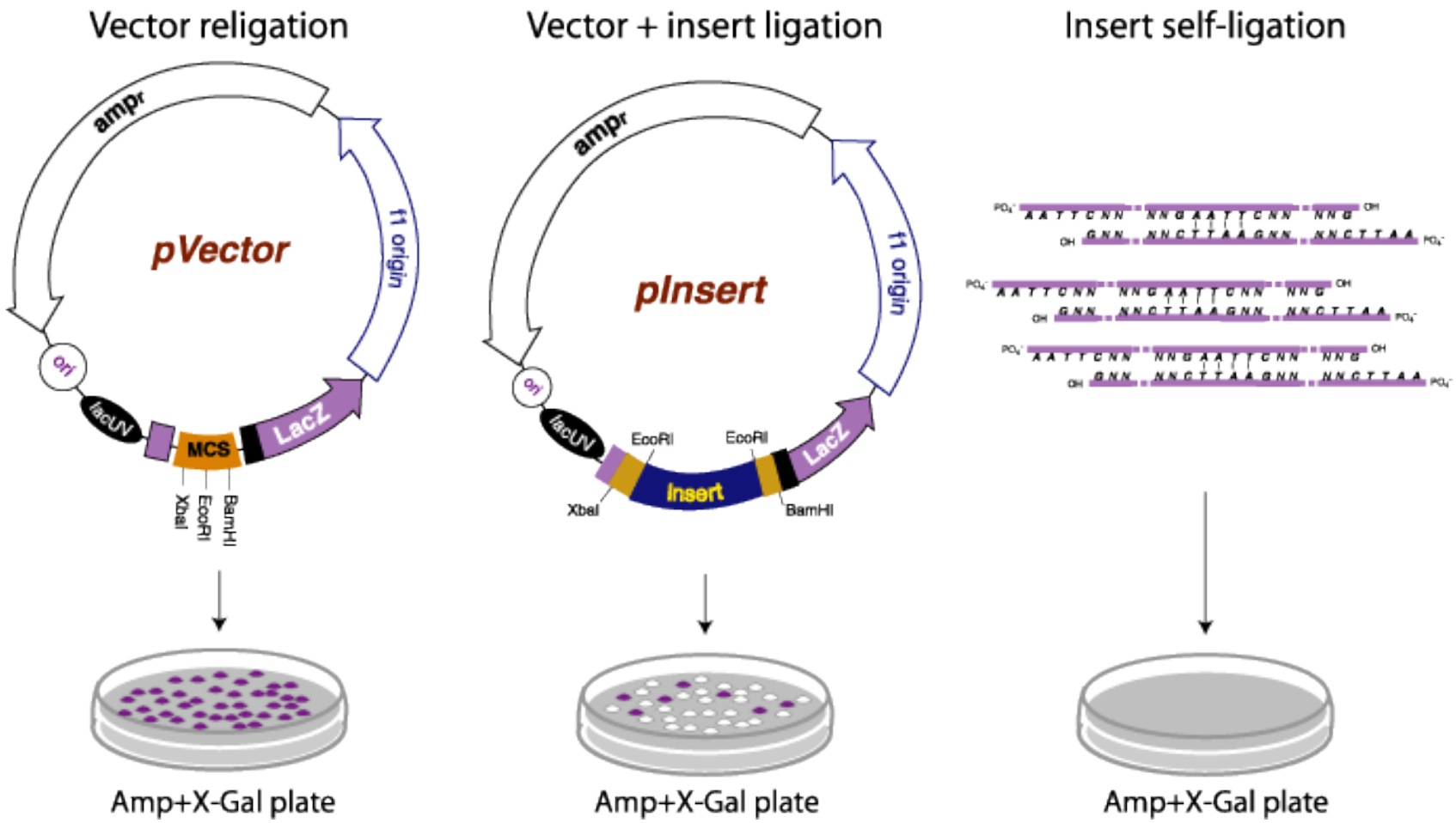


No colonies

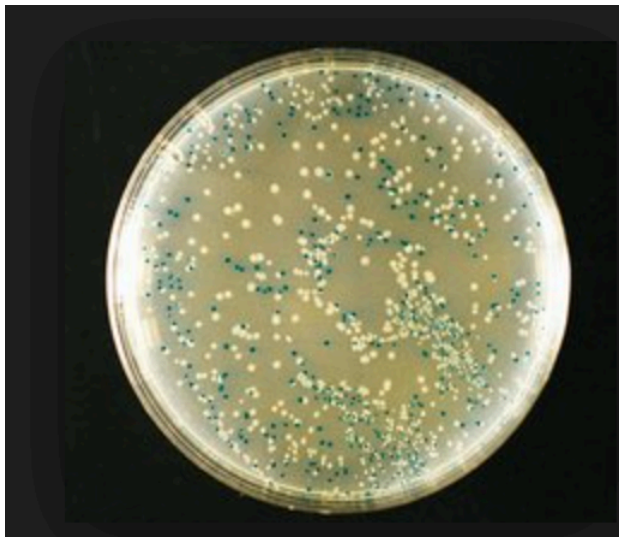
1. **NO COLONIES**

For example

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



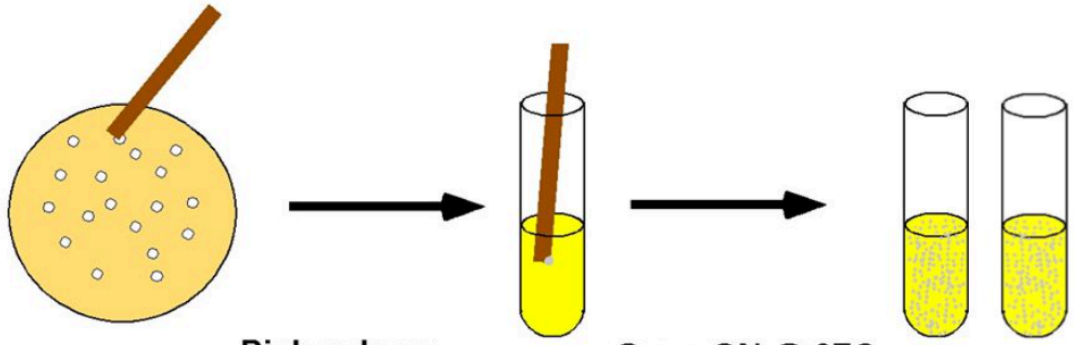
2. DNA PREPARATION AND CONTROL DIGEST



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

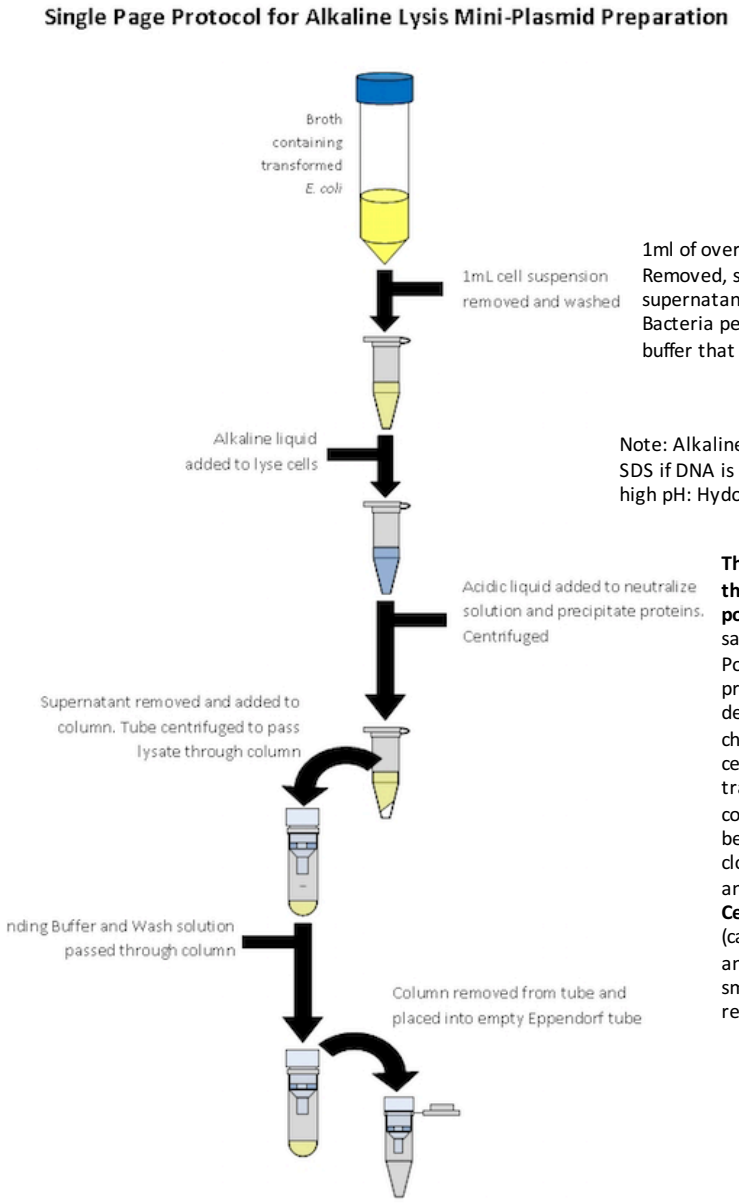
Preparation. Grow the bacteria

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

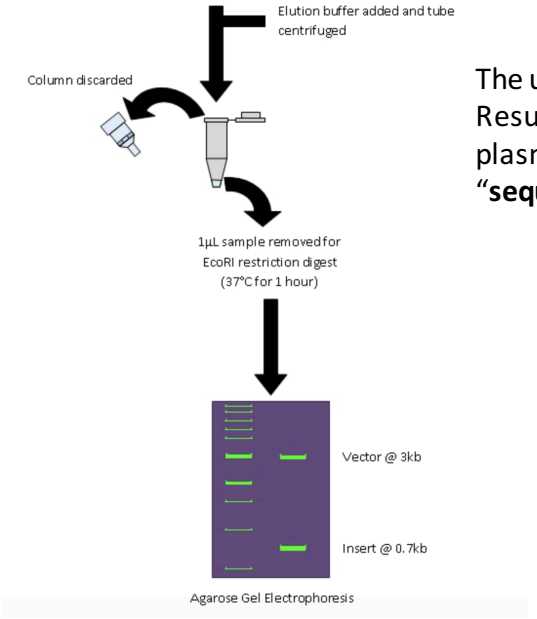
2. DNA PREPARATION AND CONTROL DIGEST



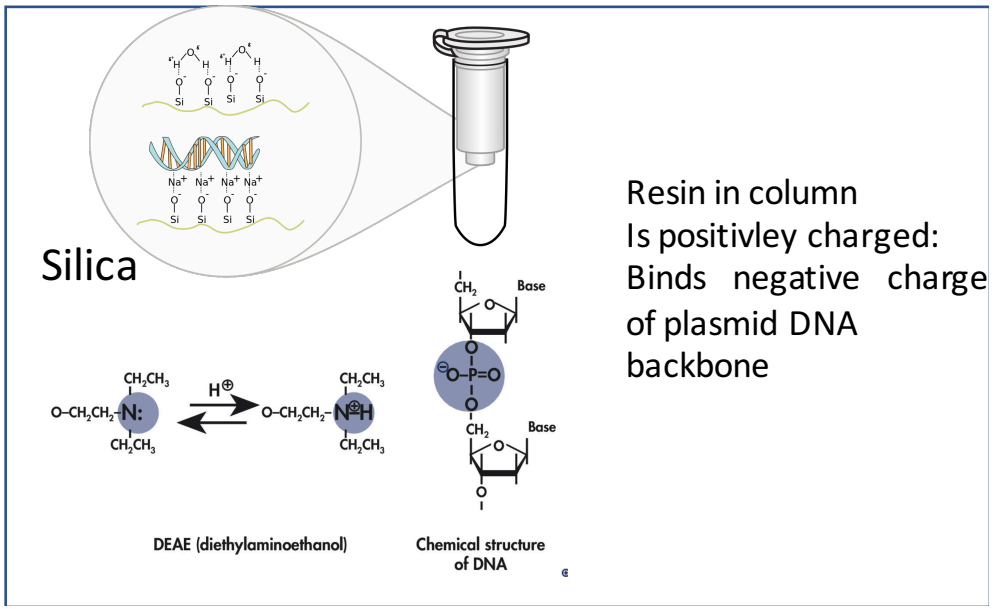
1ml of overnight culture Removed, spinned and supernatant removed. Bacteria pellet resuspendet in buffer that does not kill cells

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)



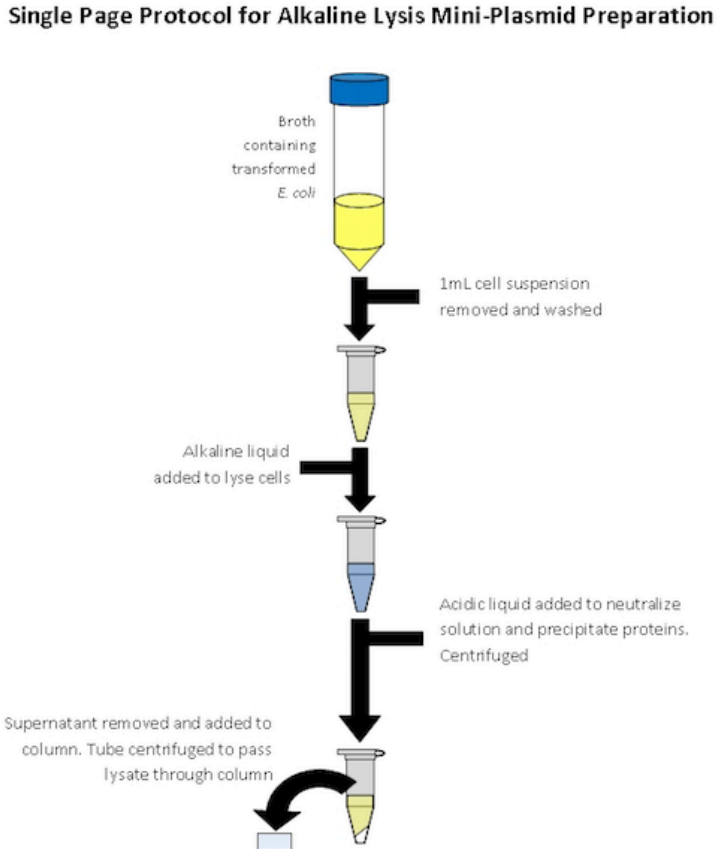
The use of columns Results in very pure plasmid DNA. "**sequence grade**"



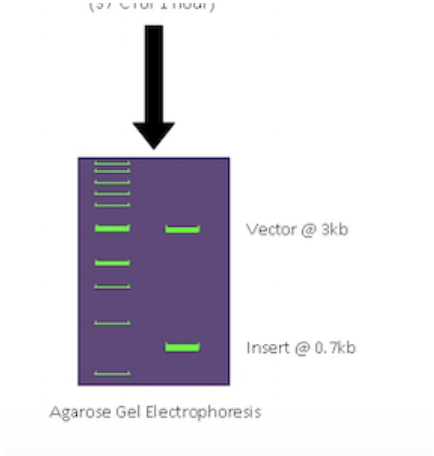
Resin in column Is positivley charged: Binds negative charge of plasmid DNA backbone

2. DNA PREPARATION AND CONTROL DIGEST

Alternative method without columns



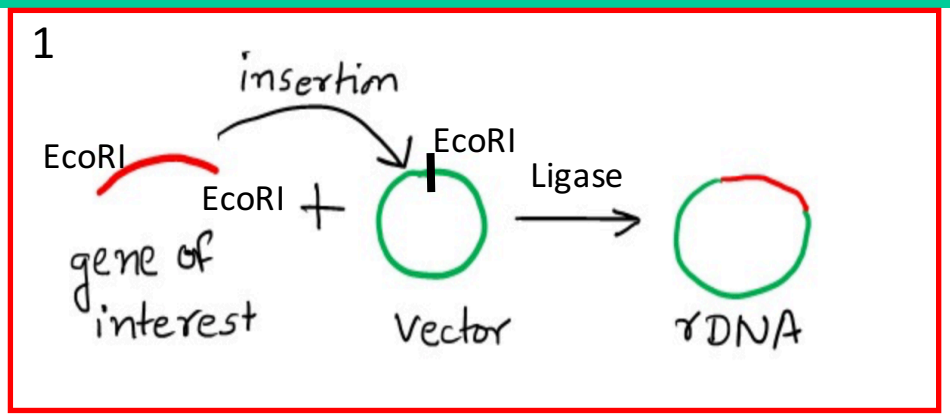
- put supernatant in new tube
- add salt (final 0,5M NaCl)
- add Isopropanol
- put at -20C for 1 hour
- centrifuge
- plasmid DNA will preciptate

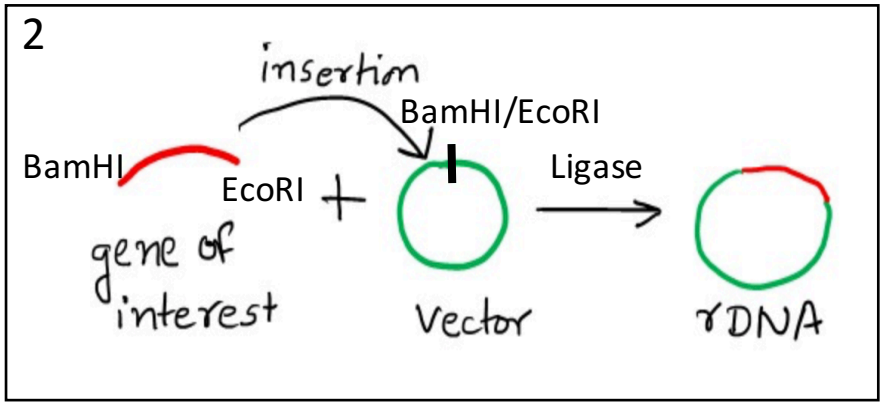


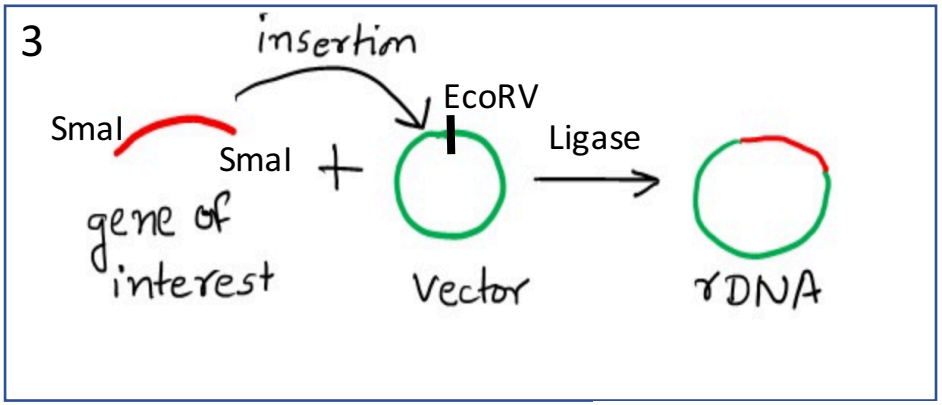
Plasimid is not very clean; sufficent for digestion with restriction enzyemes; not usable for DNA sequencing
"**not sequencing grade**"

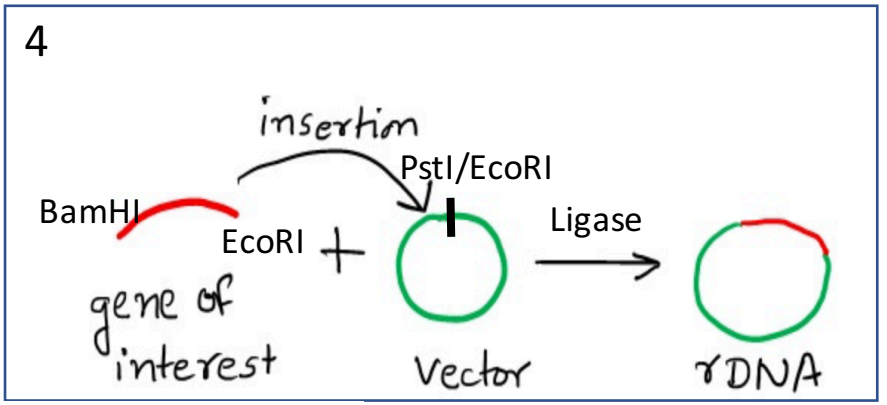
→ **Much cheaper; you can test many colonies for correctness of plasmid**
→ **Takes some more time**

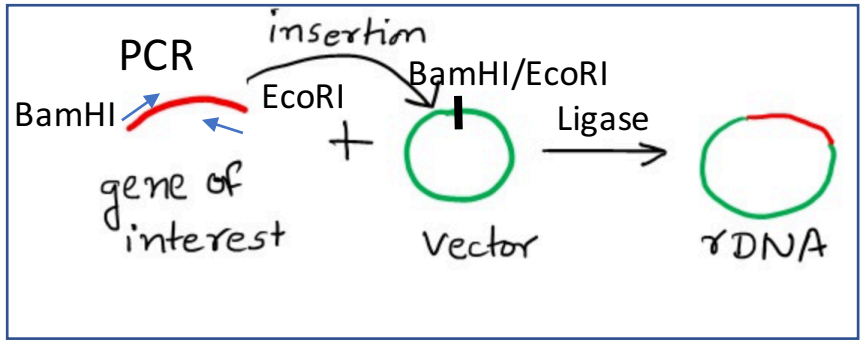
3. OVERVIEW OVER OTHER CLONING STRATEGIES



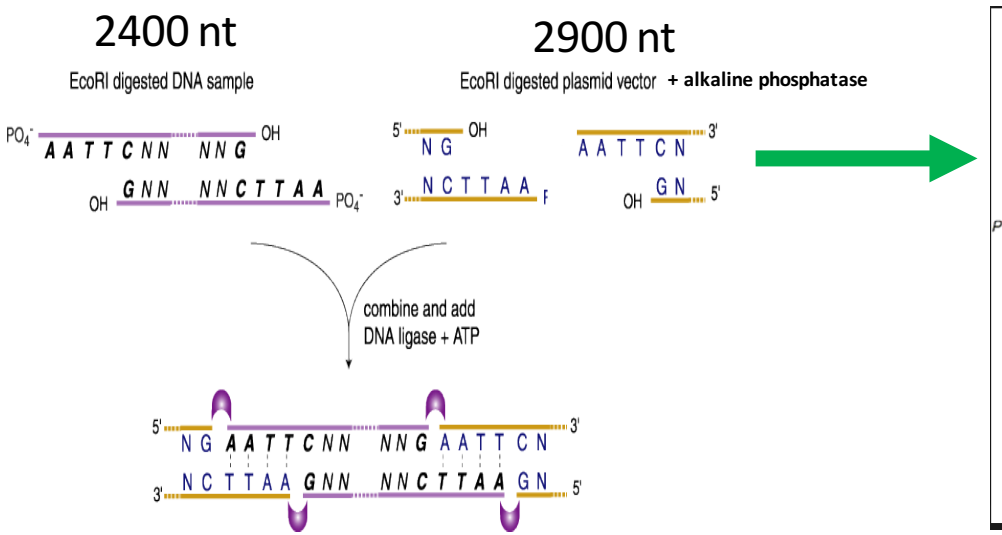


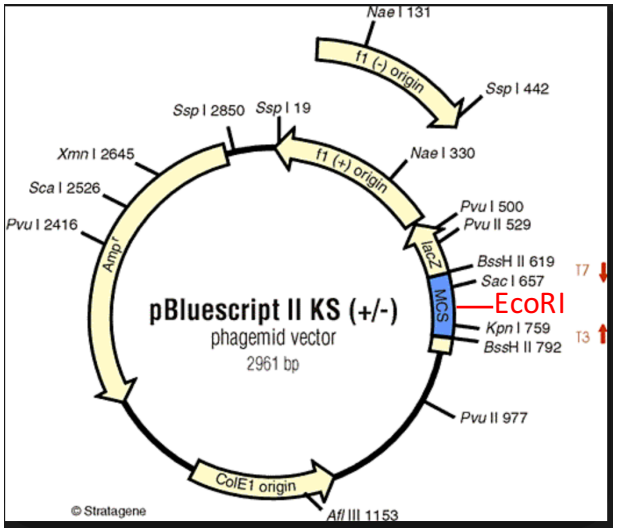


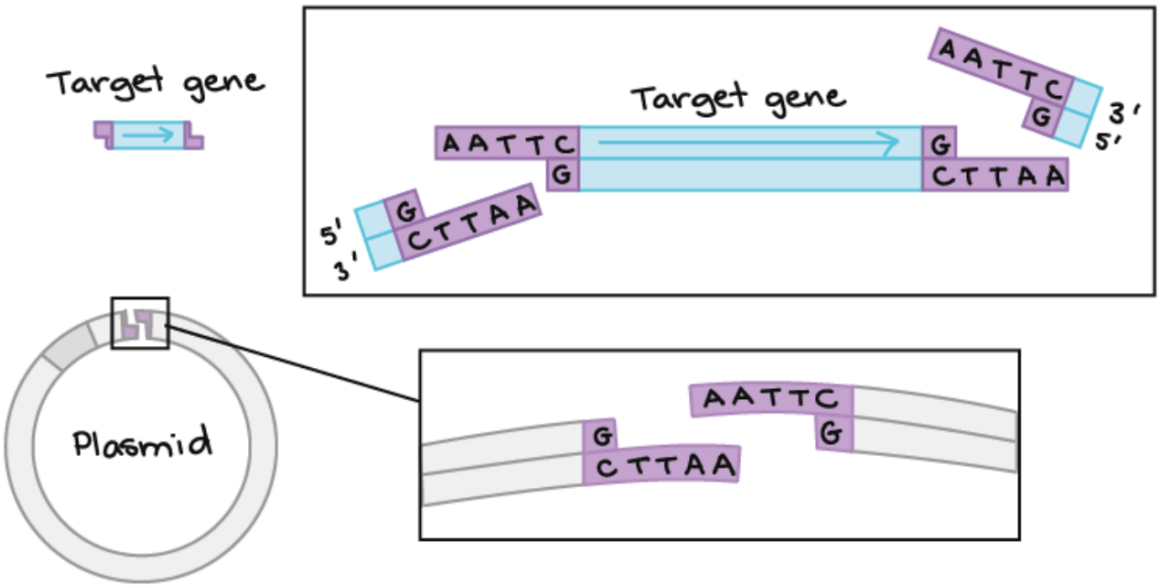




2. DNA PREPARATION AND CONTROL DIGEST

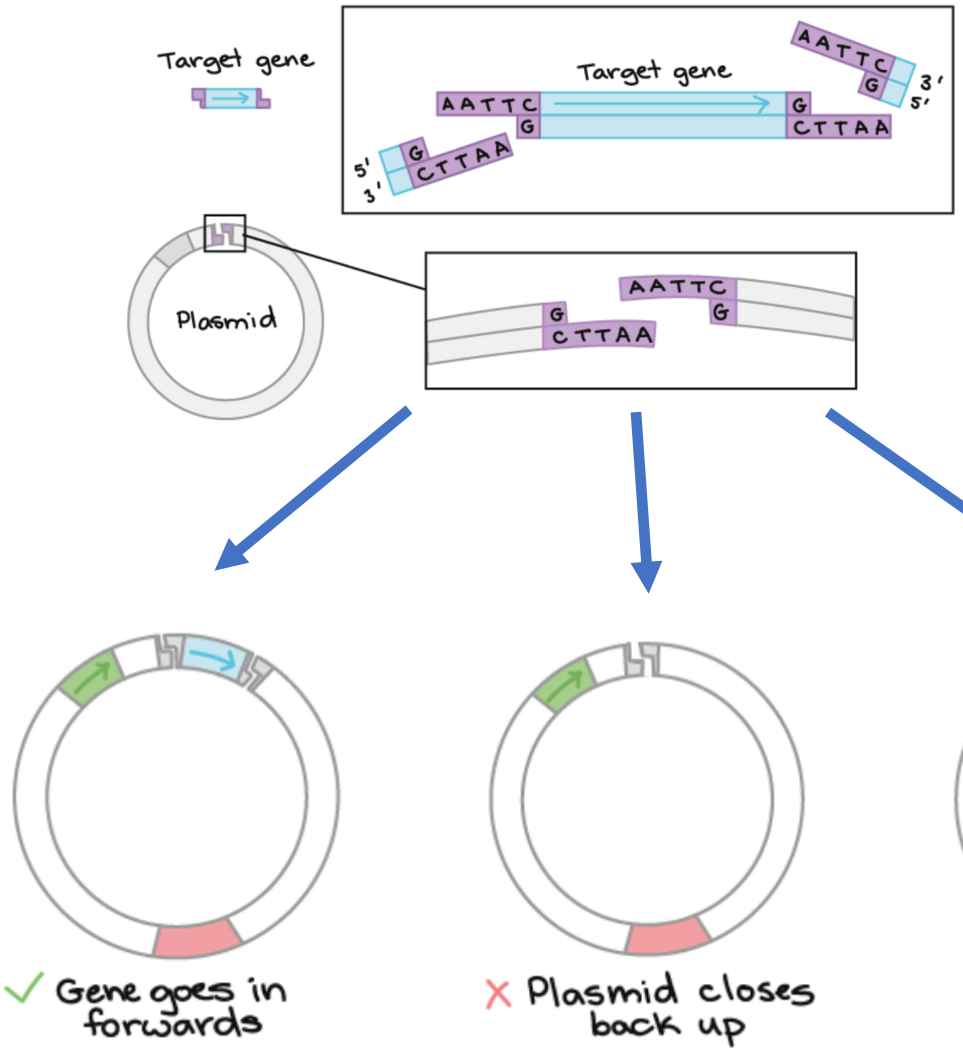






Note:
5'overhangs of insert and linearized plasmids are compatible; both have been cut with EcoRI. Ligase covalently links both molecules EcoRI sites are reconstiuted and now flank the insert sequence!!!

2. DNA PREPARATION AND CONTROL DIGEST



White colony **Blue colony** **White colony**

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

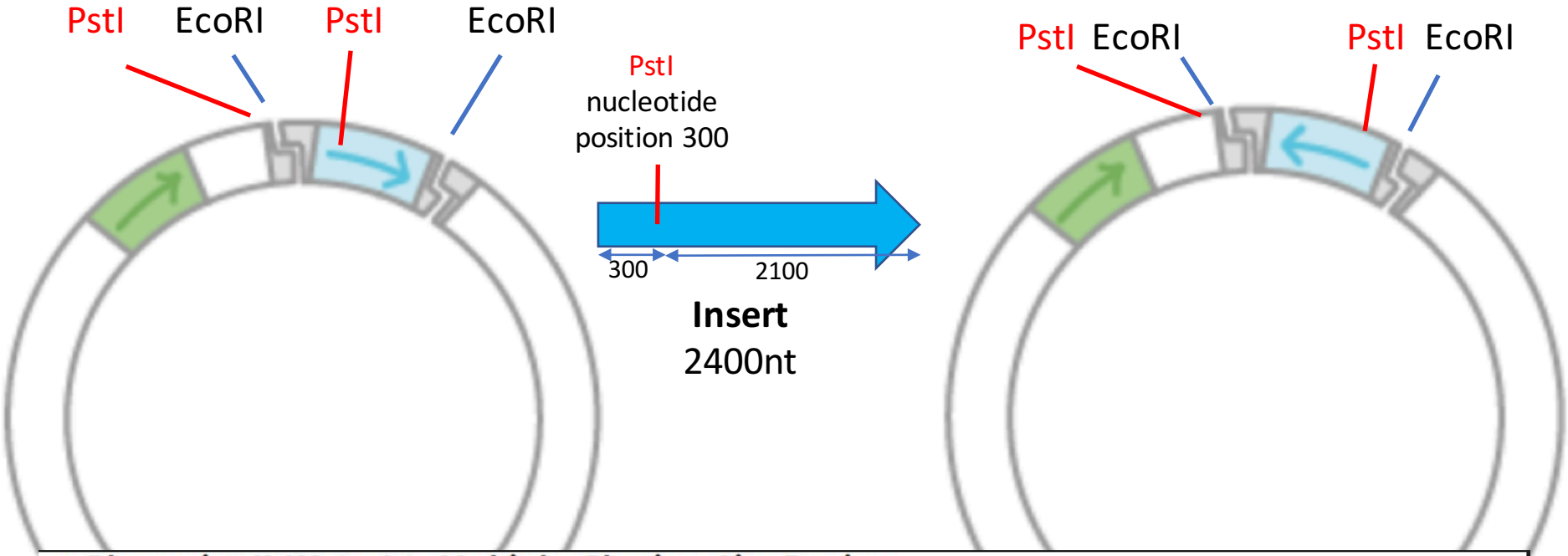
Insert DNA sequence is known
→ I know all restriction sites

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)

BssH II — T7 Promoter — Sac I — BstX I Sac II — Not I Eag I — Xba I

TTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGA...

M13 –20 primer binding site — T7 primer binding site — SK primer binding site...

Spe I — BamH I — Sma I — Pst I — EcoR I — EcoR V — Hind III — Bsp106 I Cla I — Hinc II Acc I Sal I — Xho I — Apa I EcoO109 I Dra II — Kpn I

...ACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACC...

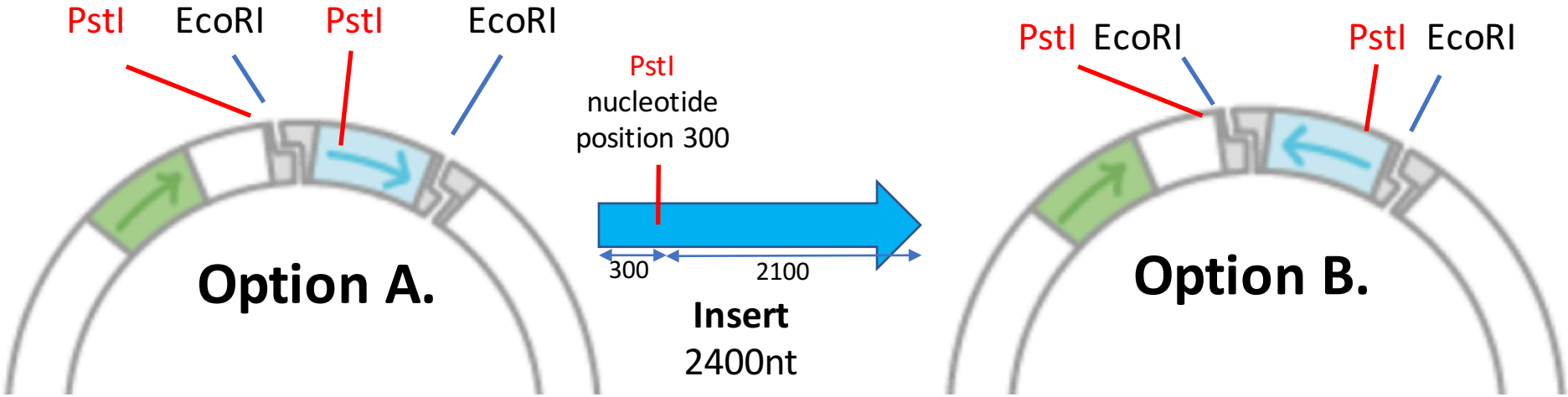
...SK primer binding site — KS primer binding site

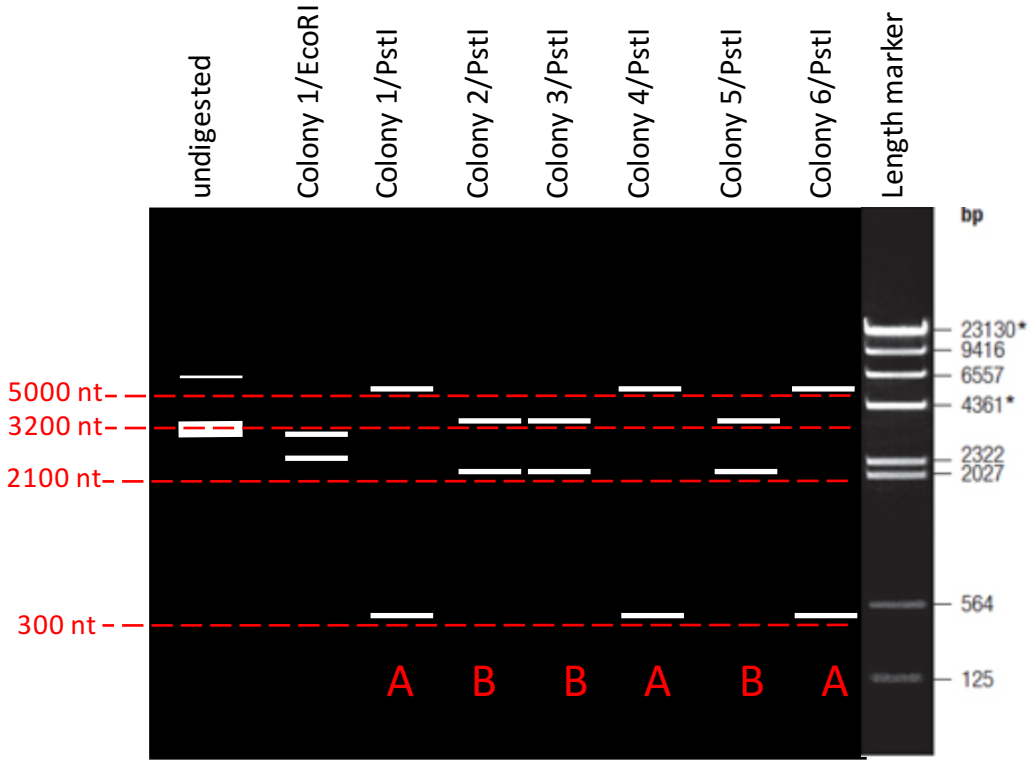
T3 Promoter — BssH II — β-gal α-fragment

...CAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCC

T3 primer binding site — M13 Reverse primer binding site

2. DNA PREPARATION AND CONTROL DIGEST

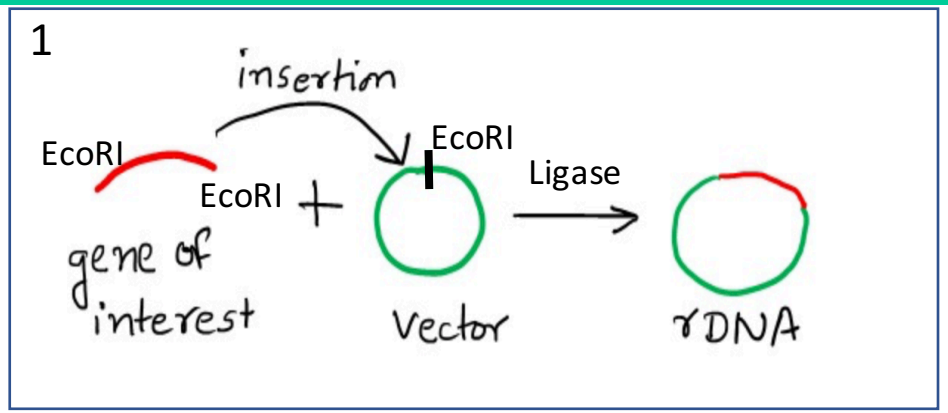


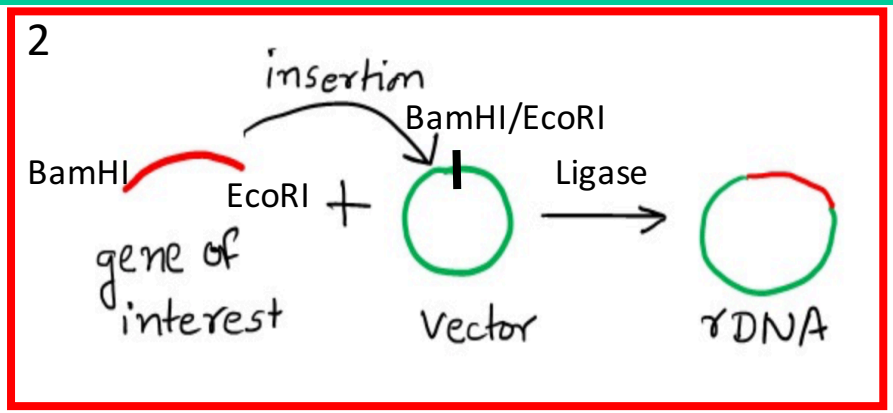


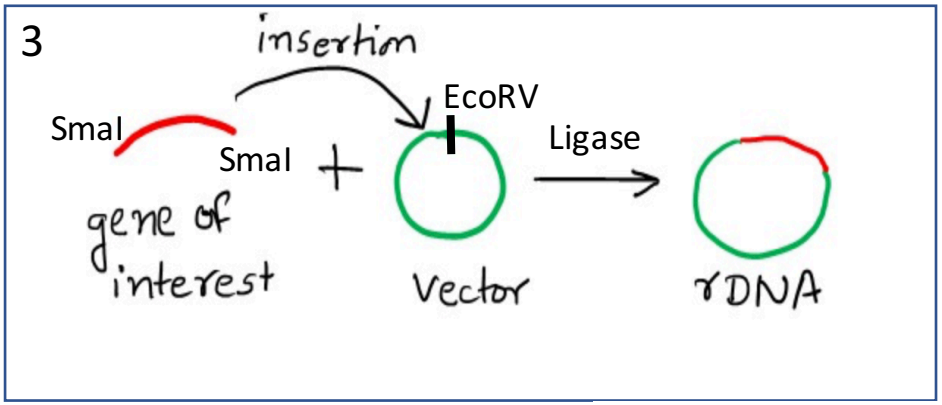
Insert: 2400 nt
Plasmid: 2900 nt

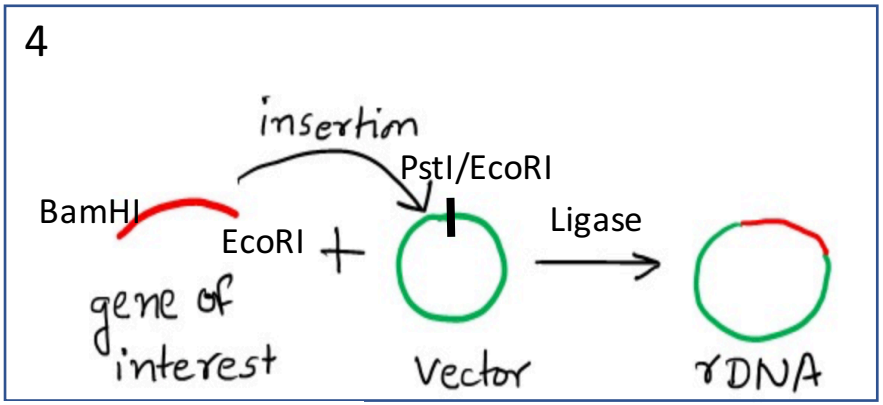
Cut with restriction enzyme that result asymetric digestion products

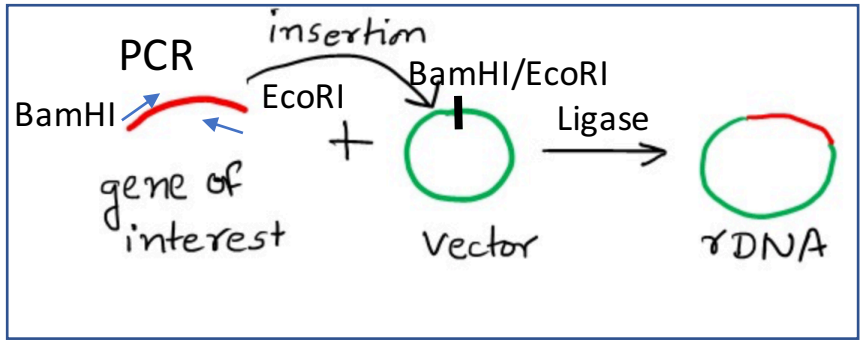
3. OVERVIEW OVER OTHER CLONING STRATEGIES











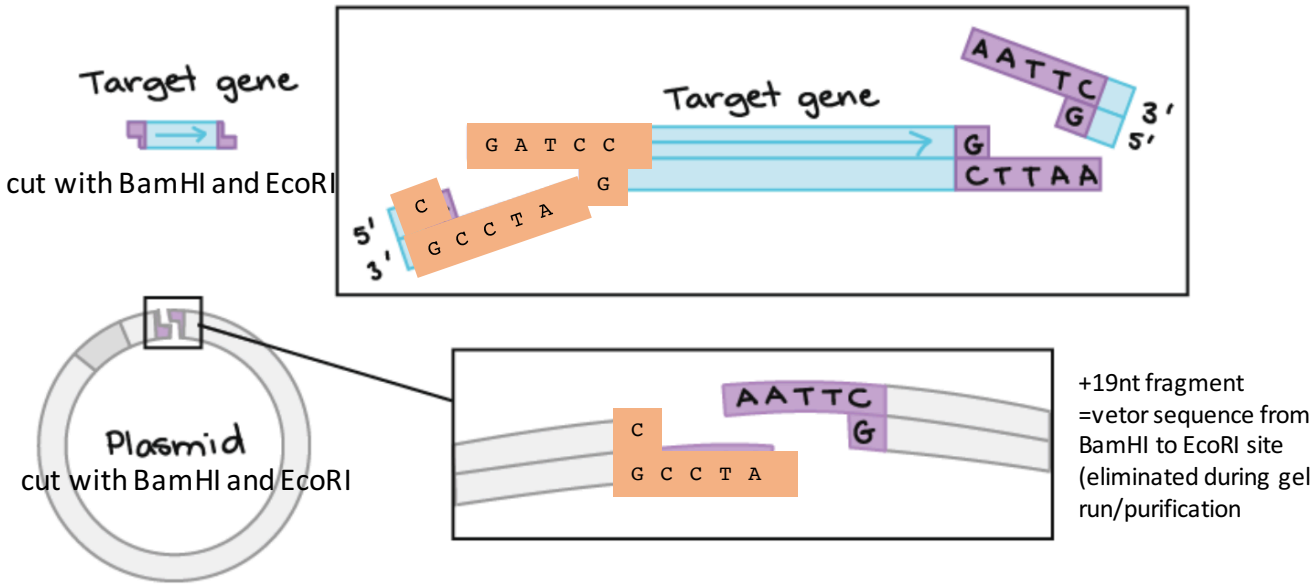
3.2. DNA CLONING WITH 2 COHESIVE OVERHANGS

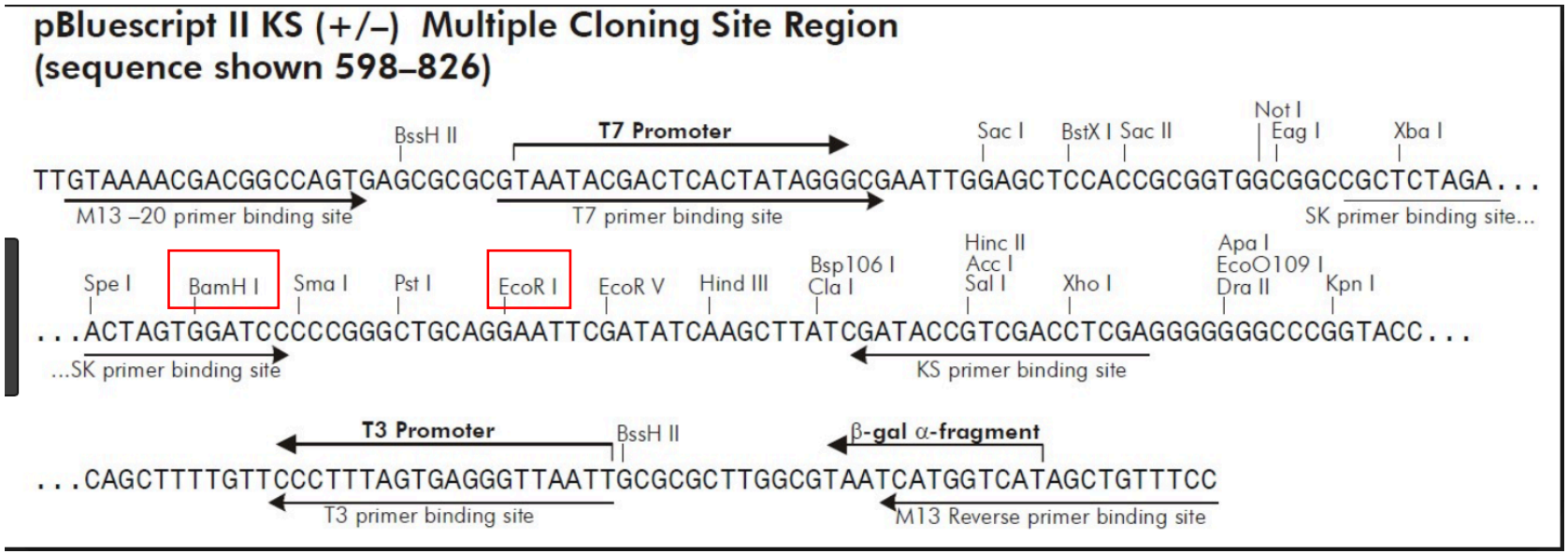
EcoRI: G/AATTC
CTTAA/G

BamHI: G/GATCC
CCTAG/G

DIRECTIONAL CLONING

→ Always preferred cloning strategy





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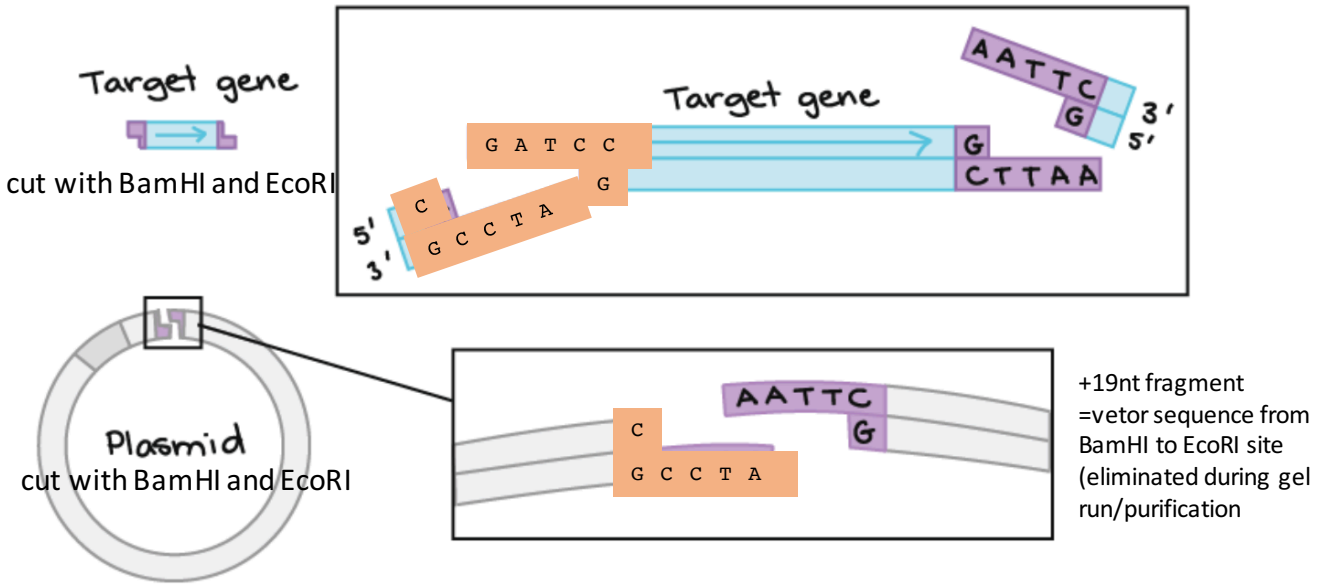
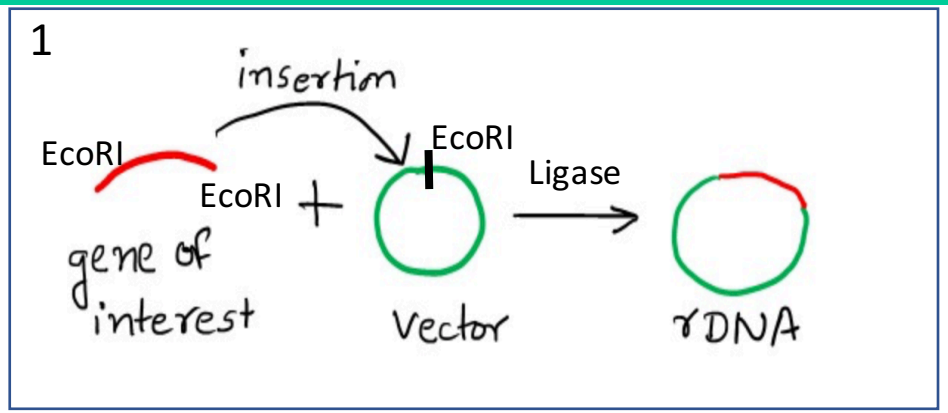
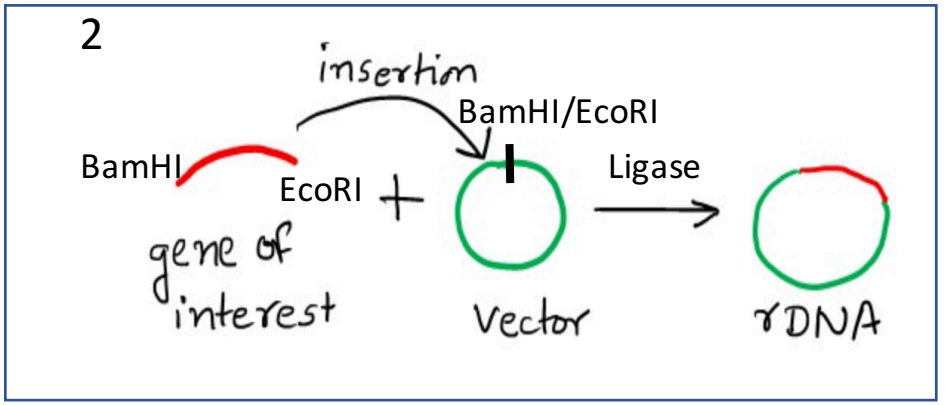


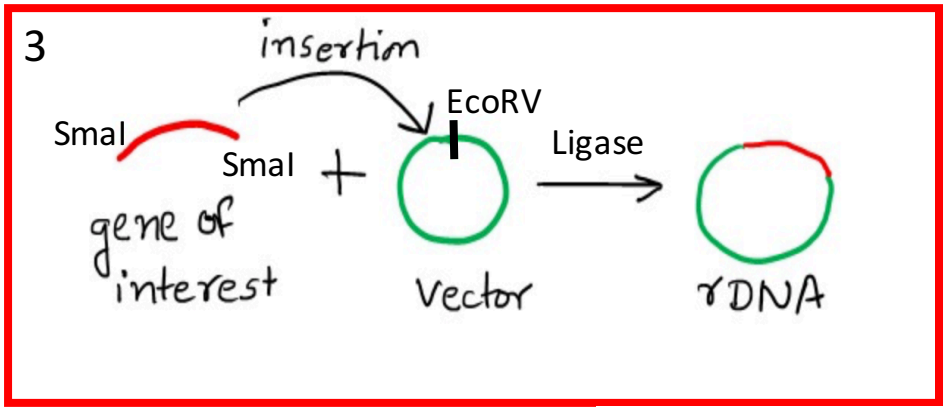
1. EcoRI/BamHI digest to obtain insert
2. EcoRI/BamHI digest to obtain linearized pBluescript
3. Gel run and purification of relevant DNA fragments
4. Set up ligation (plasmid:insert = 1:3(5))
5. Transform competent bacteria
6. Plasmid preparation and control digest to verify presence of correct insert

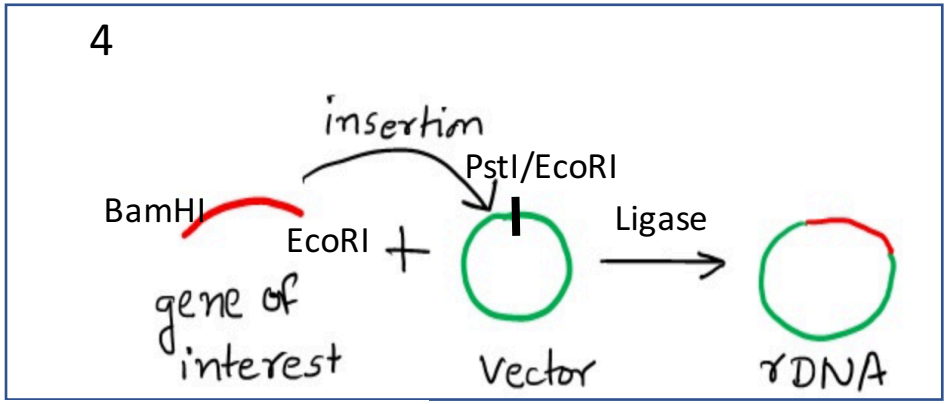
7. IMPORTANT: NO ALKALINE PHOSPHATASE REQUIRED → EcoRI and BamHI do not represent cohesive ends!!
8. IMPORTANT: ORIENTATION OF INSERT IS ALWAYS THE SAME!!!

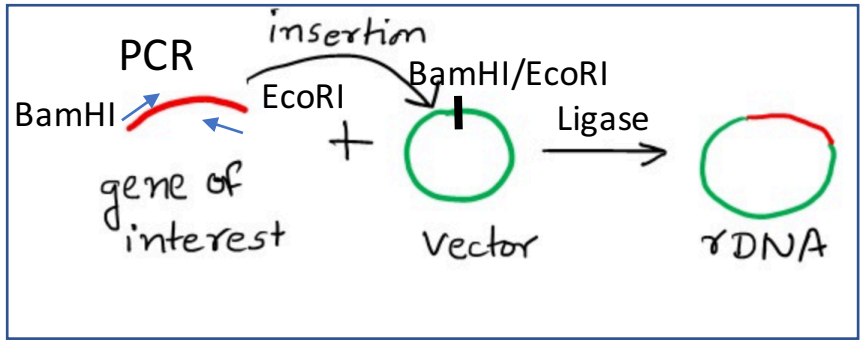
3. OVERVIEW OVER OTHER CLONING STRATEGIES







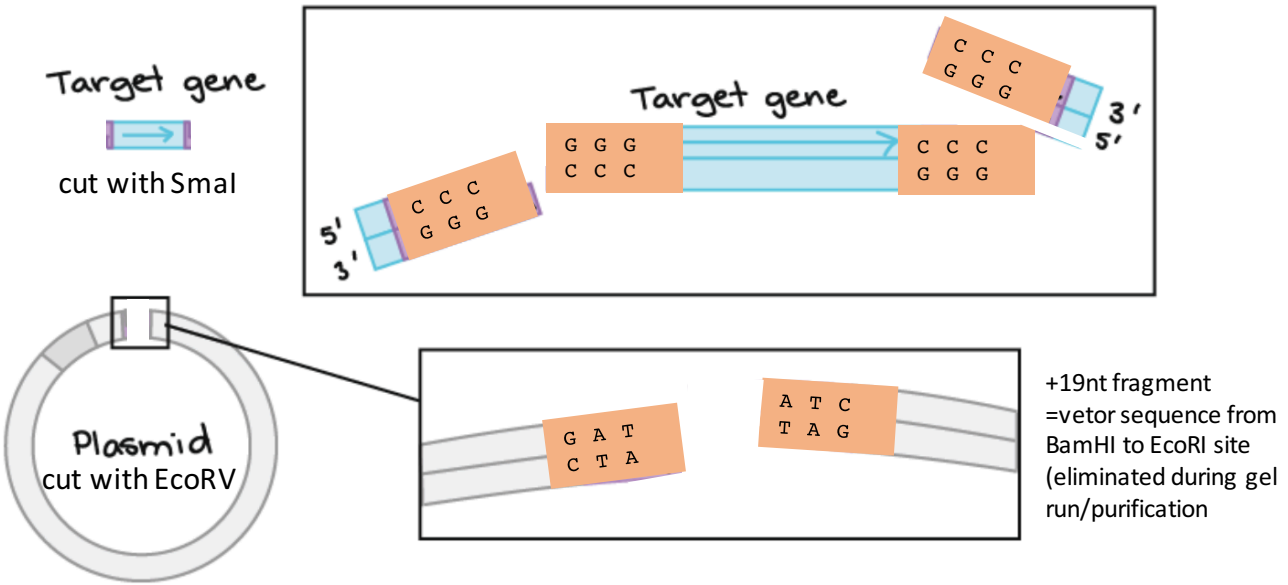




3.3. DNA CLONING WITH BLUNT ENDS

SmaI: CCC/GGG
GGG/CCC

EcoRV: GAT/ATC
CTA/TAG

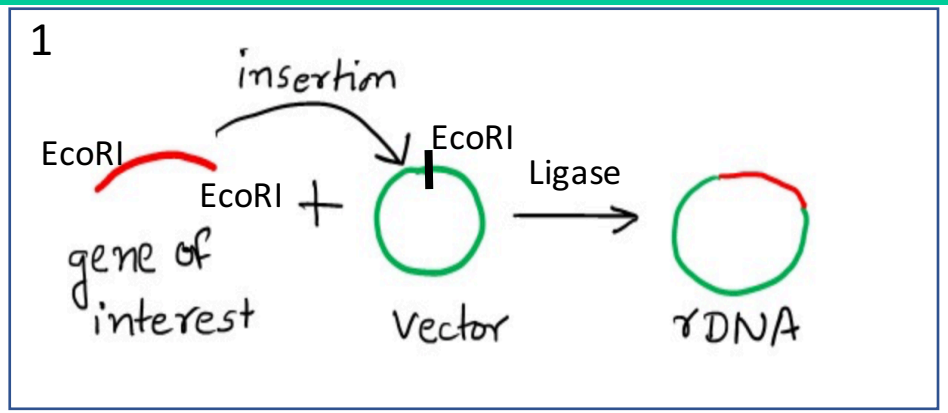


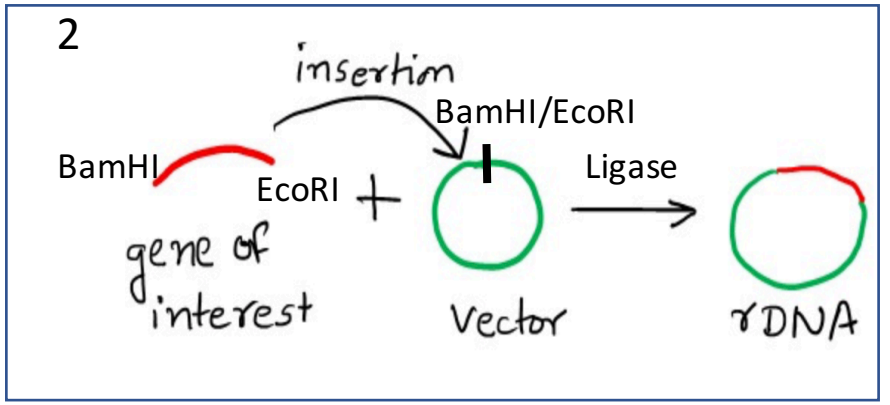
1. SmaI digest to obtain insert
2. EcoRV digest + alkaline phosphatase treatment to obtain linearized pBluescript (that connot re-ligate)
3. Gel run and purification of relevant DNA fragments
4. Set up ligation (plasmid:insert = 1:3 (5))
5. Transform competent bacteria
6. Plasmid preparation and control digest to verify presence of correct insert → insert can be inserted in both orientations!!

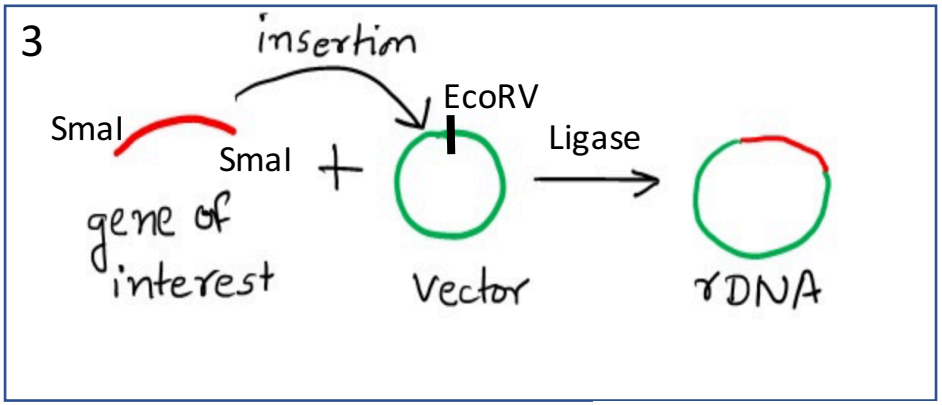
7. IMPORTANT: SmaI sites are fused to EcoRV site ➔ cannot be cleaved by SmaI or EcoRV

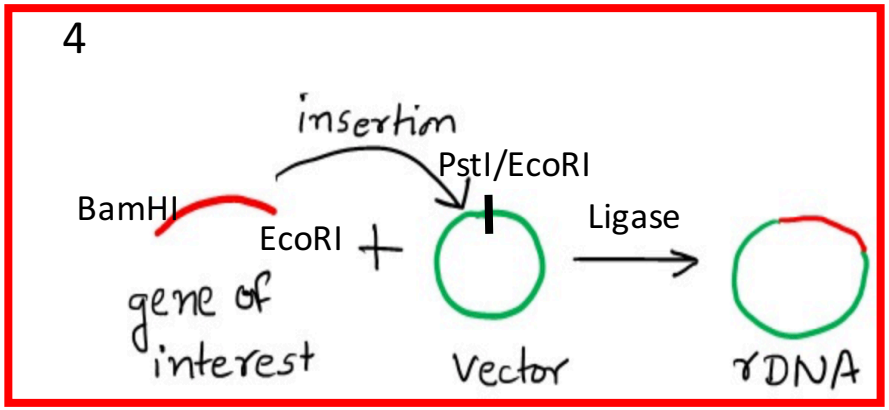
GATGGG —— CCCATC
CTACCC —— GGGTAG

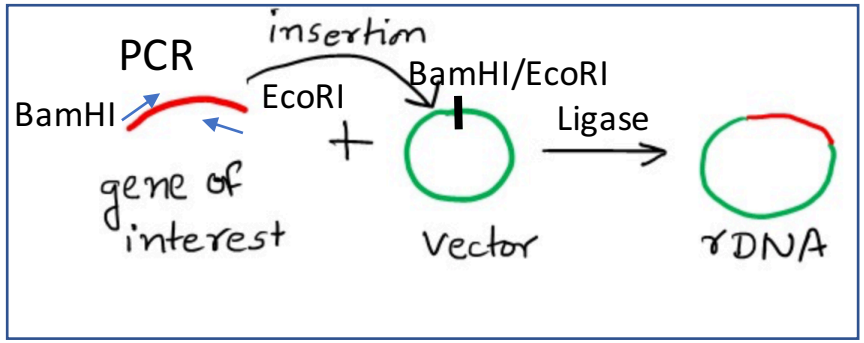
3. OVERVIEW OVER OTHER CLONING STRATEGIES



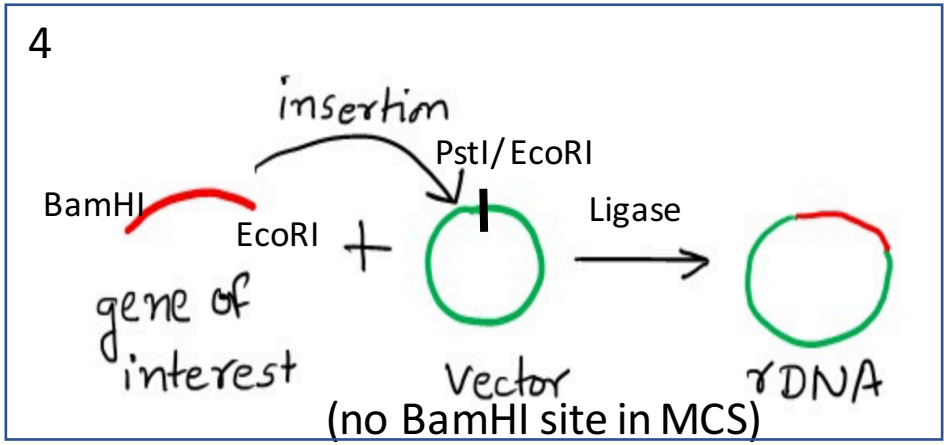






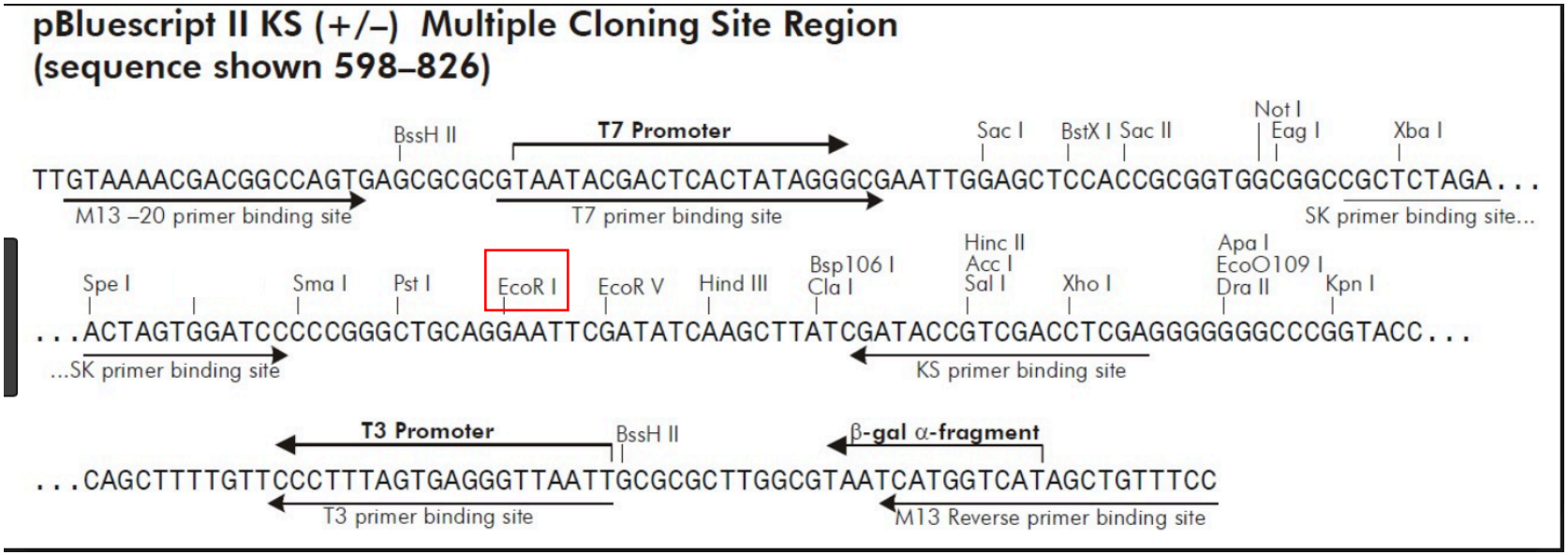


3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS



(no BamHI site in MCS)

Lets assume: BamHI is not present in pBS



3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

INSERT

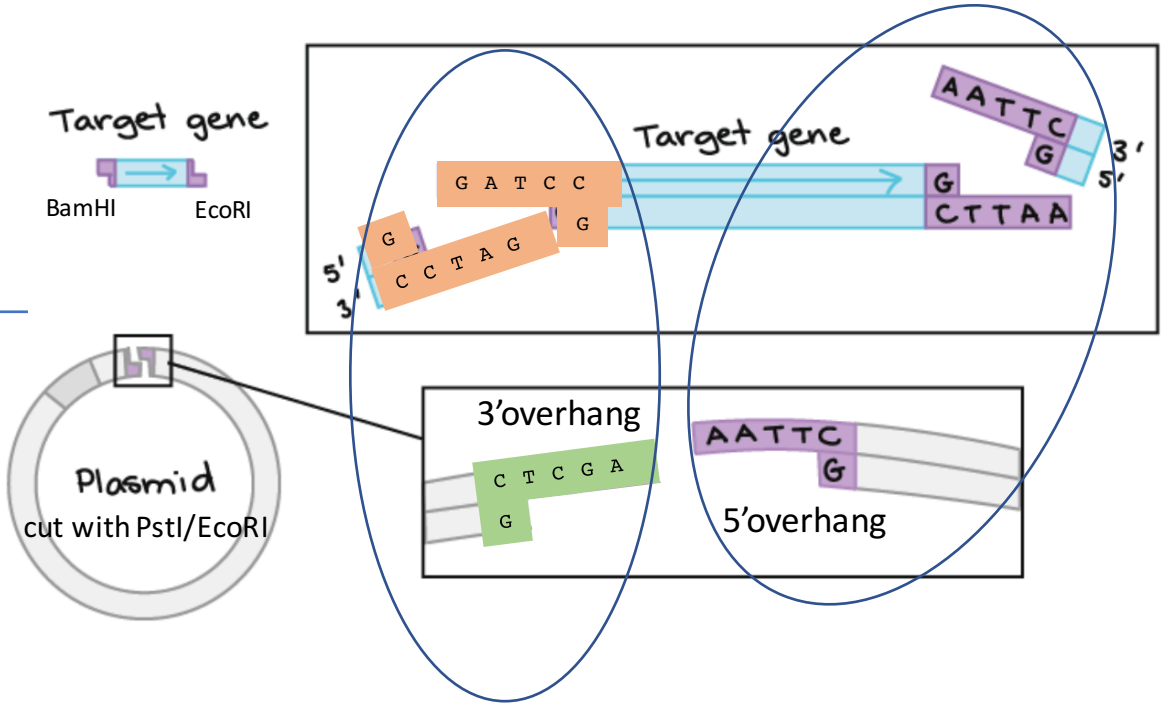
BamHI: G/GATCC
CCTAG/G

EcoRI: G/AATTC
CTTAA/G

VECTOR

PstI: CTCGA/G
G/AGCTC

EcoRI: G/AATTC
CTTAA/G



NOT COMPATIBLE COMPATIBLE

→ make blunt

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

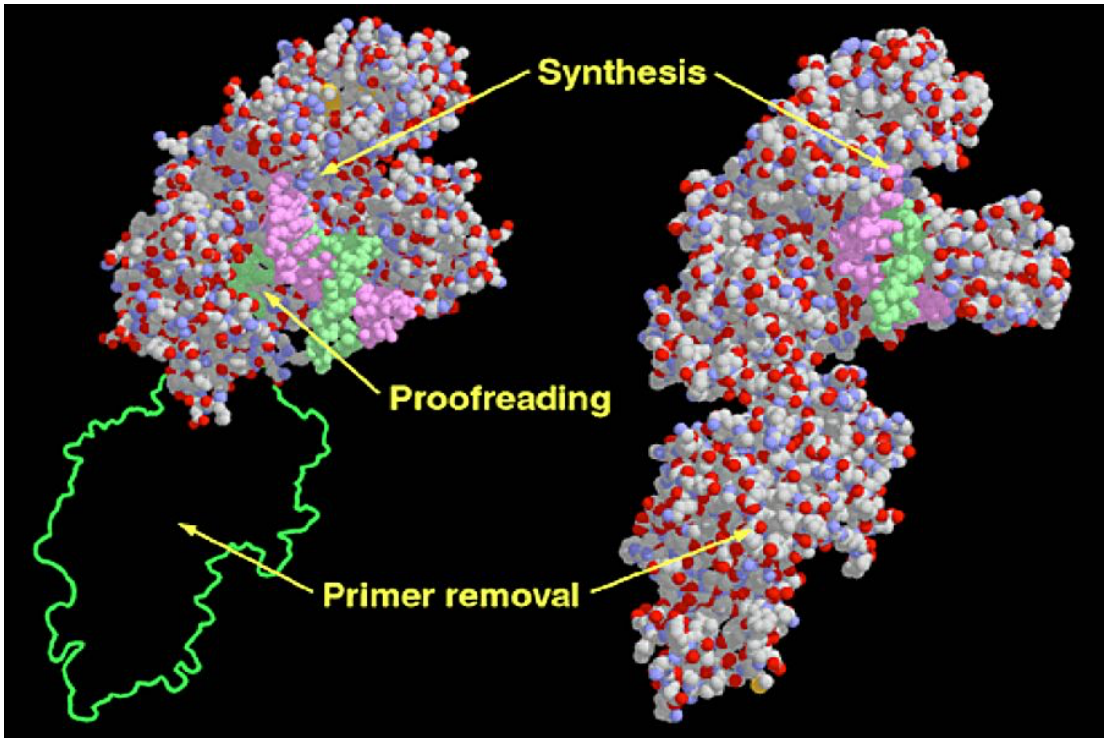
→ Modifcation of 3'overhang of PstI site → convert overhang to blunt end

→ ➔ Blunt – Blunt AND EcoRI – EcoRI ligation

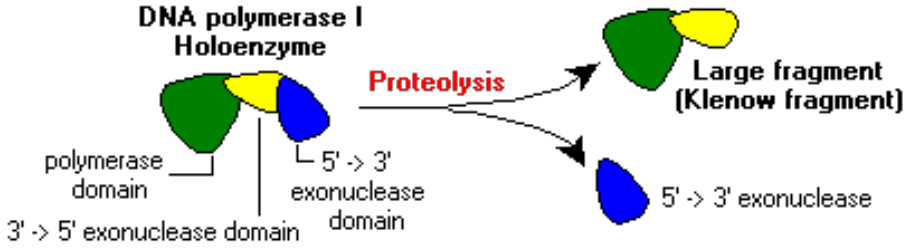
3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

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 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).



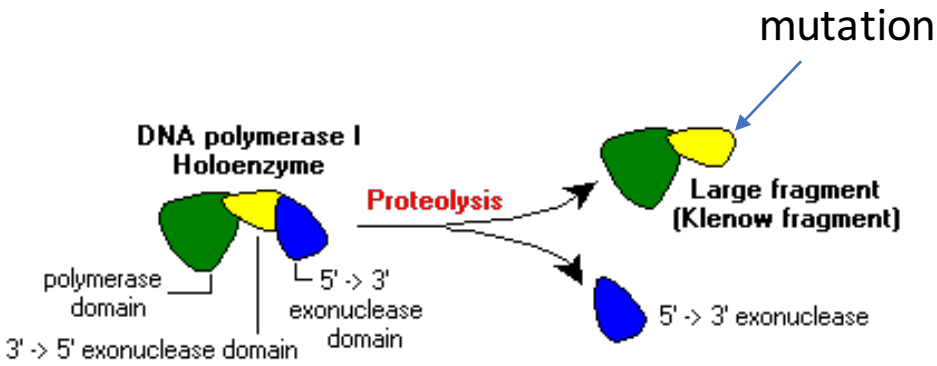
- → Synthesis of double-stranded DNA from single-stranded templates
- → Filling in receded 3' ends of DNA fragments to make 5' overhang blunt
- → Digesting away protruding 3' overhang
- → Preparation of radioactive DNA probes



3.4 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 gene that encodes Klenow. This results in forms of the enzyme being expressed that retain 5' → 3' polymerase activity, but lack any 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, 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.



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. Encodend by T4 phage

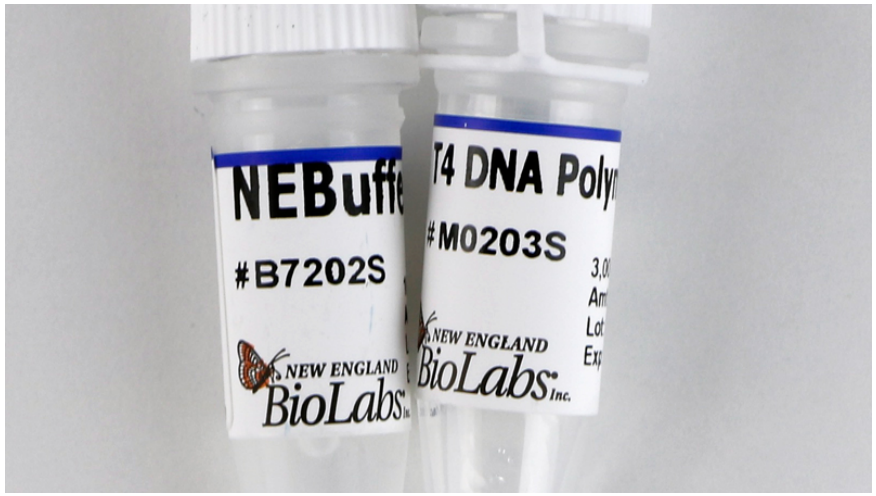
→Gap filling (no strand displacement activity)

→Removal of 3’ overhangs or fill-in of 5’ overhangs to form blunt ends

→ Lacks 5’ —> 3’ exonuclease activity

→ Probe labeling using replacement synthesis

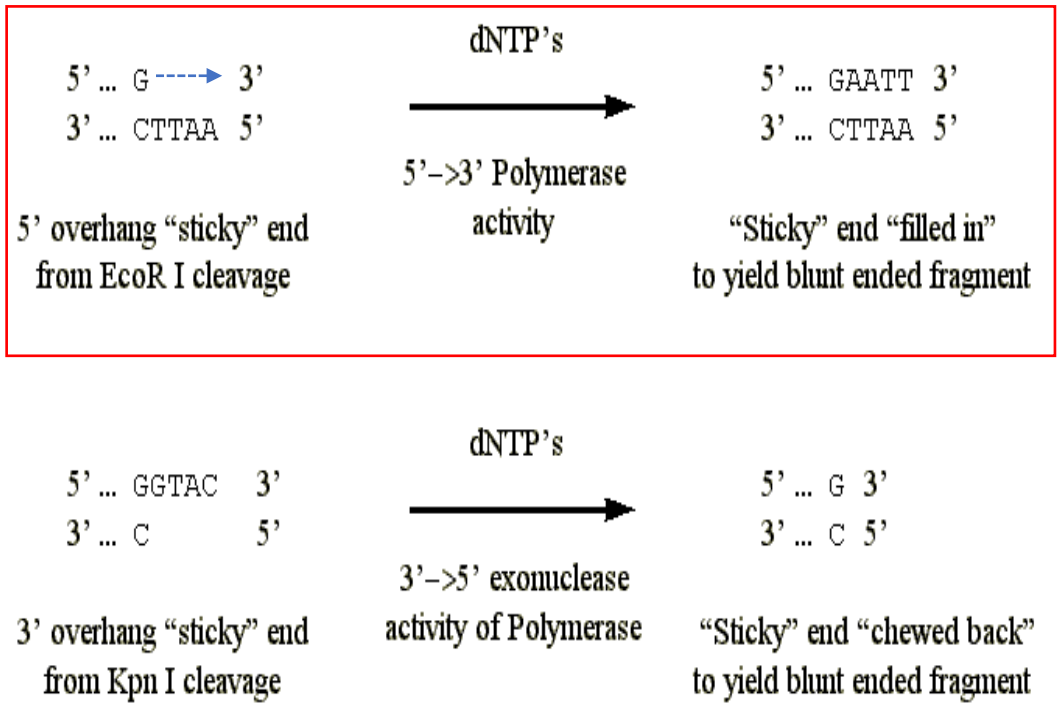
Singe-strand deletion subcloning



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 actiyivity; exonuclease activity will take over
→ → degradation of plasmid/insert

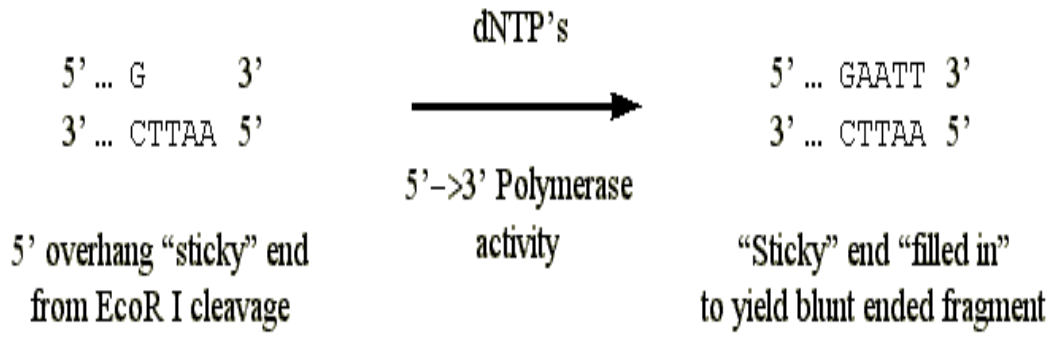
Exo- Klenow fragment is safer in use!

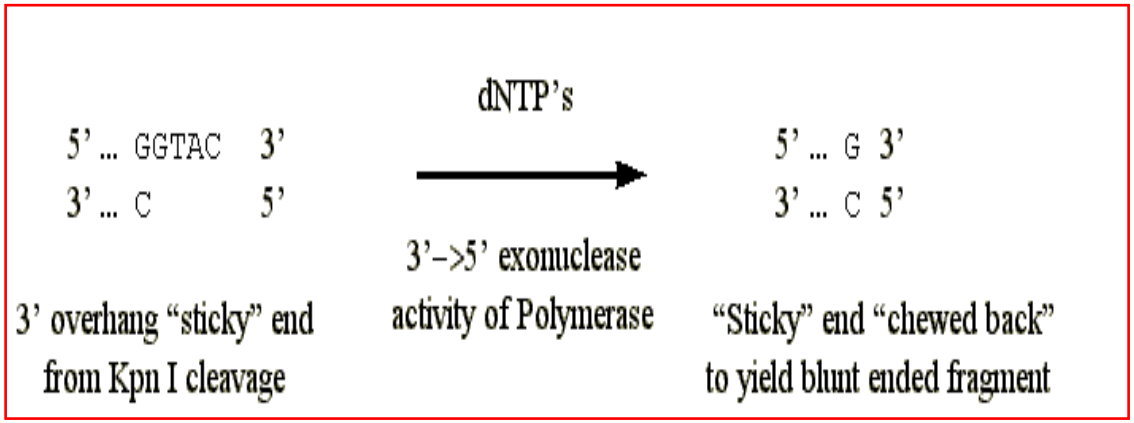
3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

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 know 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!)

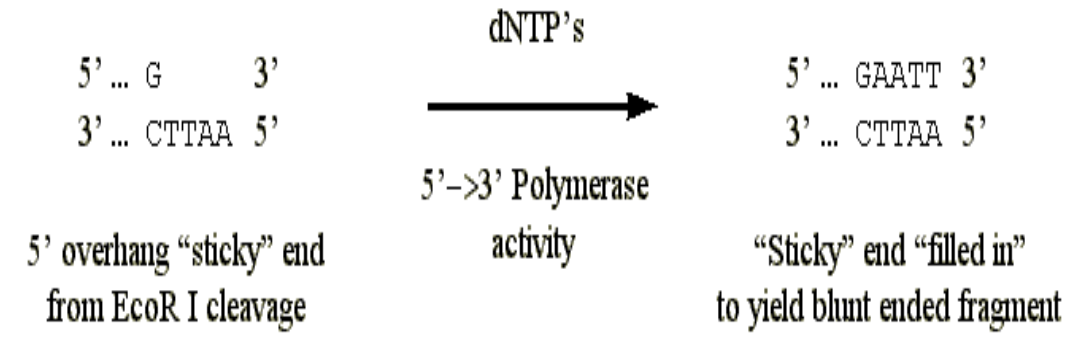
→ If dNTPs are used up by DNA polymerase activity; exonuclease activity will take over

→ → degradation of plasmid/insert

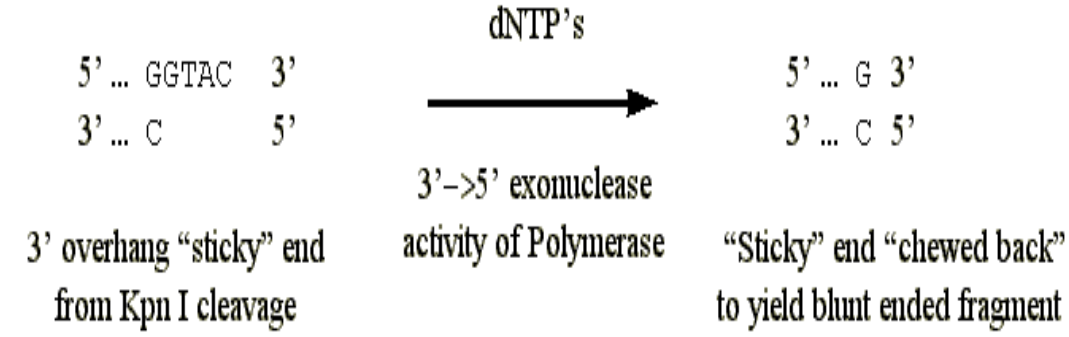
3.4 DNA CLONING WITH MODIFICATION OF OVERHANGS

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

INSERT

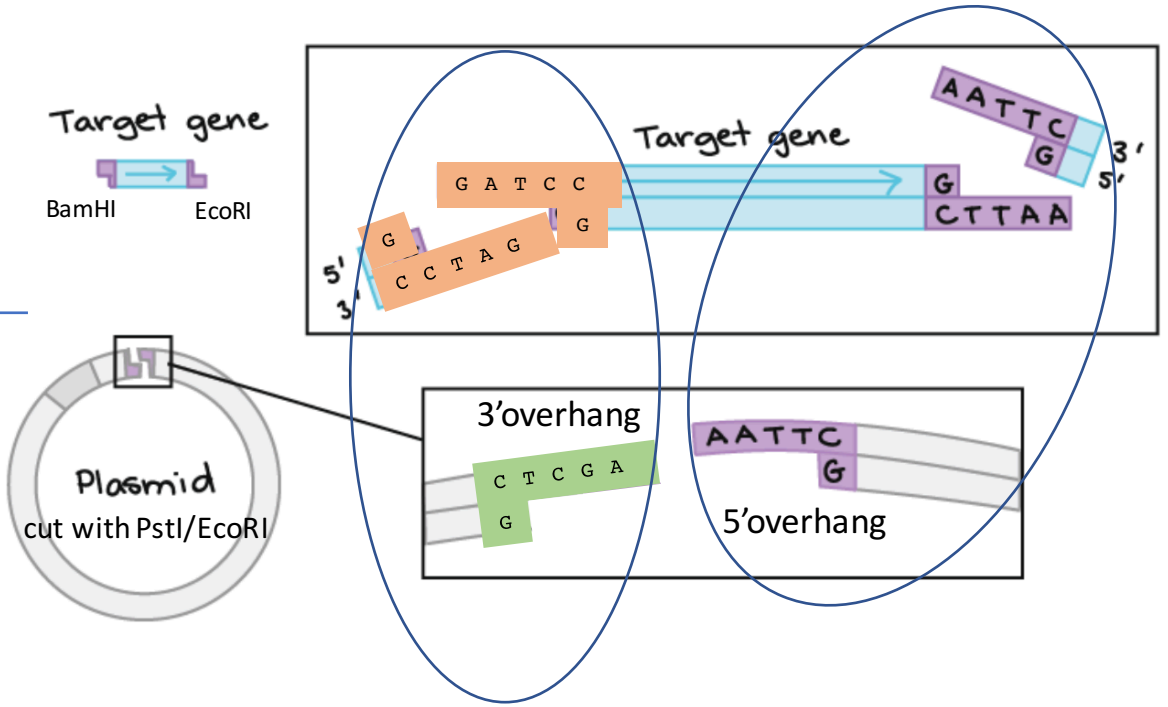
BamHI: G/GATCC
CCTAG/G

EcoRI: G/AATTC
CTTAA/G

VECTOR

PstI: CTCGA/G
G/AGCTC

EcoRI: G/AATTC
CTTAA/G



HOW TO DO?

Vector:

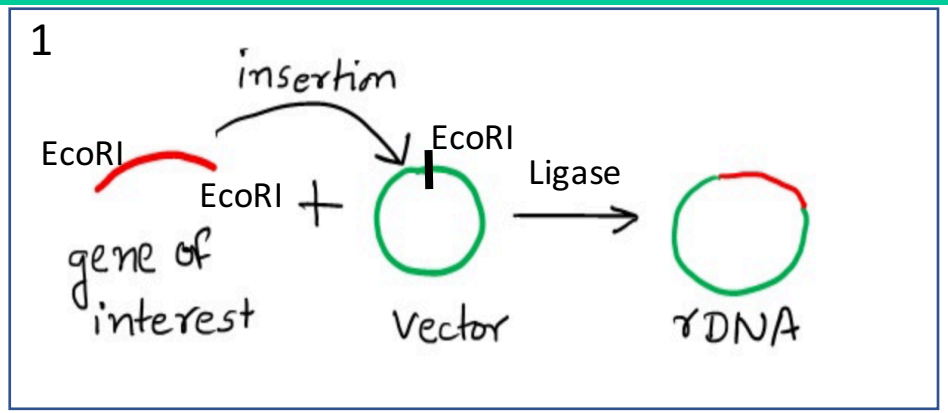
1. Cut PstI
2. Make T4 Polymerase reaction → blunting of 3'overhang
3. Run DNA on agarose gel
4. Cut band and purify linearized DNA
5. Cut DNA with EcoRI
6. Run DNA on agarose gel
7. Cut correct band and purify DNA, determine concentration

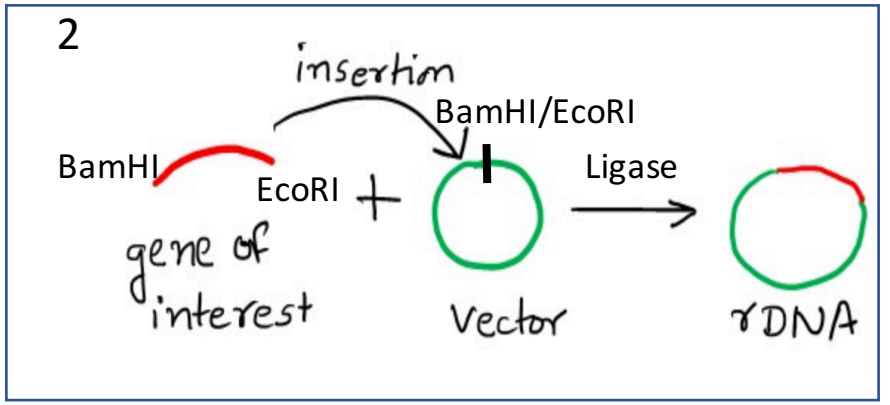
Insert:

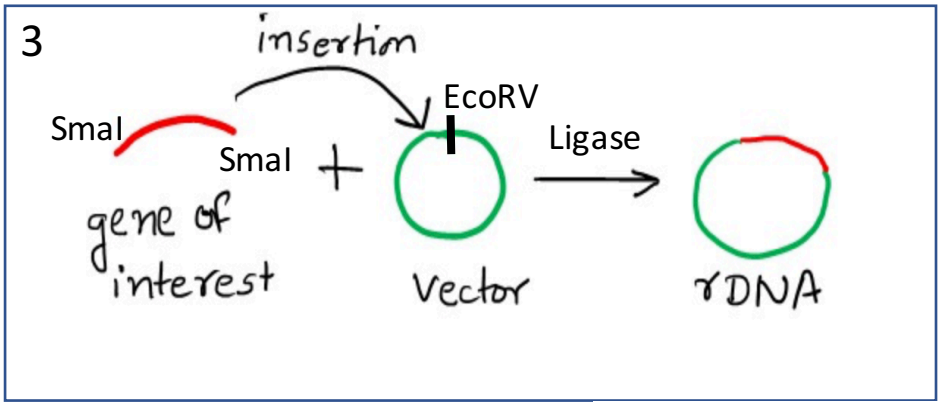
1. Cut DNA with BamHI
2. Make Exo- Klenow reaction → blunting of 5'overhang
3. Run DNA on agarose gel
4. Cut band and purify linearized DNA
5. Cut DNA with EcoRI
6. Run DNA on agarose gel
7. Cut correct band and purify DNA; determine concentration

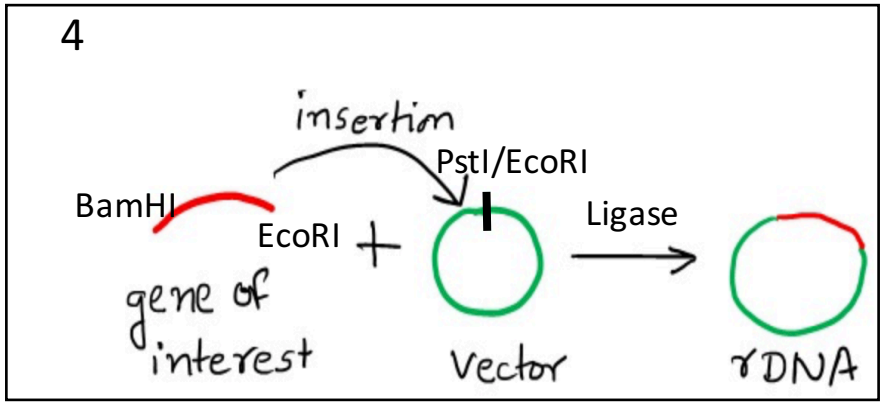
8. Setup ligation (Blunt- Blunt; EcoRI – EcoRI)

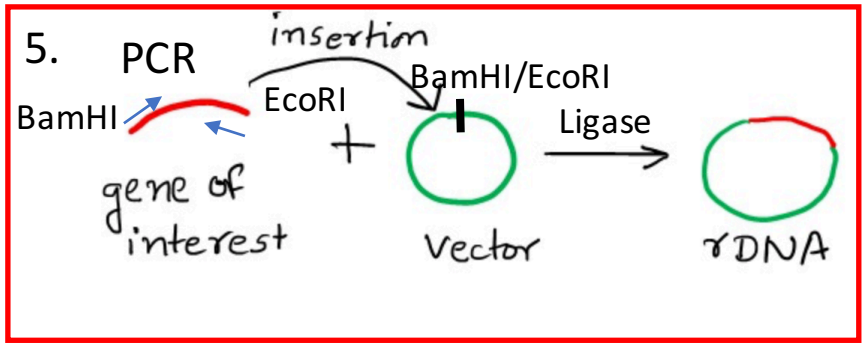
3. OVERVIEW OVER OTHER CLONING STRATEGIES



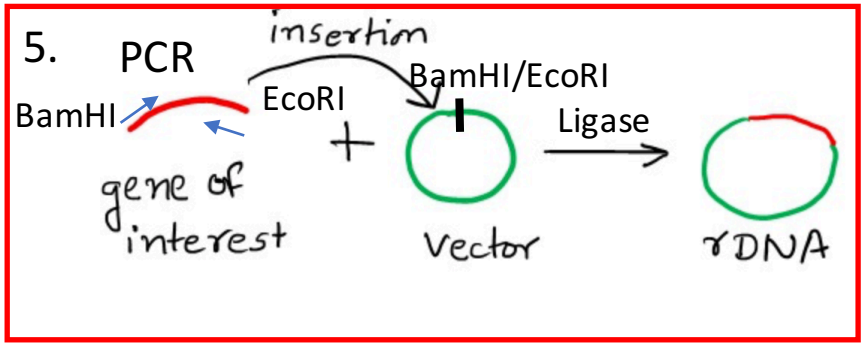








3.5 PCR CLONING



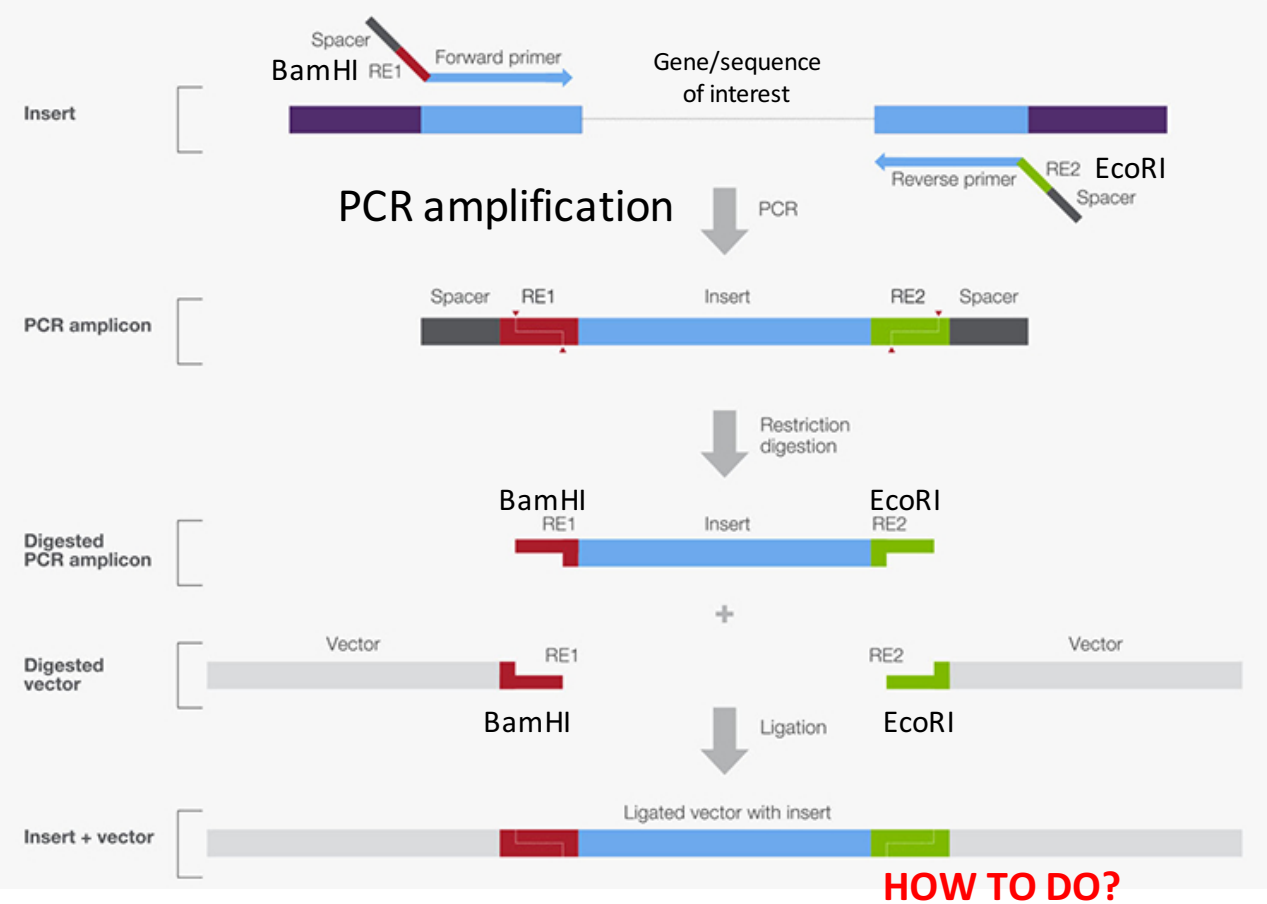
1. DNA of interest is apmplified by PCR. PCR oligos contain seqeunce for restriction enzyme

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'-CGCGGATCCxxxxxxxxxxxxxxxxx-3'

Spacer BamHI (3-5 nucelotides)

Sequence pairing with Sequence of interest (min. 18 nucelotides)

Reverse primer

3'-xxxxxxxxxxxxxxxxxGAATTCCGC-5'

Sequence pairing with Sequence of interest (min. 18 nucelotides)

EcoRI

Spacer 3-5 nucelotides

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**

3.5 PCR CLONING

1. Classic PCR cloning

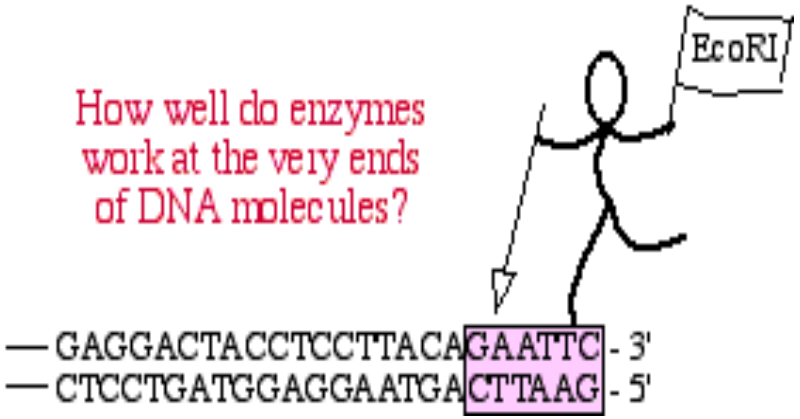
WHY IS A SPACER NEEDED???

Forward primer

5′–CGCGGATCCxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxCTTAAGGCG

3′–GCGCCTAGGxxxxxxxxxxxxxxxxxx--xxxxxxxxxxxxxxxxxxGAATTCCGC–5′

Reverse primer



→ Many resitrictioenzymes 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.

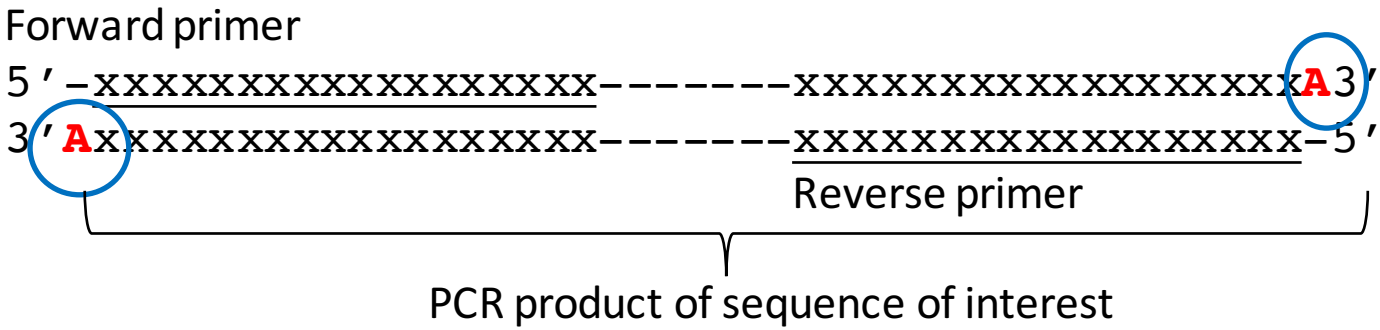
→ Generally, enzymes work better if they have a couple of extra nucleotides at the end - they don't do very well if they are perched on the end of a molecule.

→ Some additional nuceotides are better for interaction with DNA

3.5 PCR CLONING

2. TA-cloning

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



→ 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 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

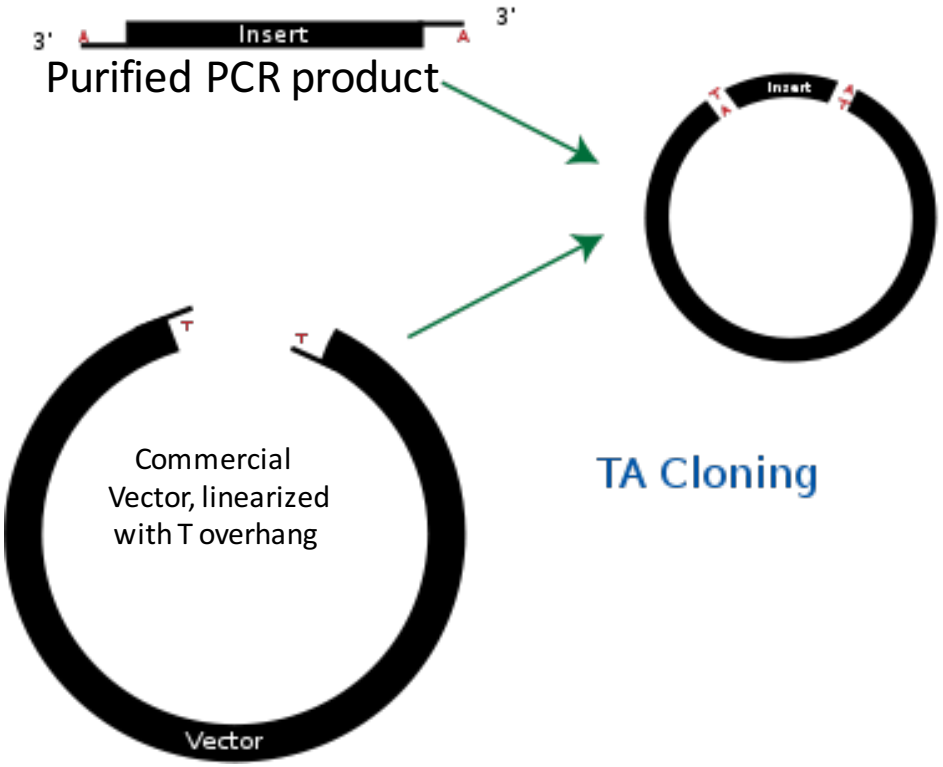
ATTENTION: Taq polymerases produce PCR products with A on 3'ends

Forward primer

5′–CGCGGATCCxxxxxxxxxxxxxxxxxx––xxxxxxxxxxxxxxxxxxCTTAAGGCGA3′

3′AGCGCCTAGGxxxxxxxxxxxxxxxxxx––xxxxxxxxxxxxxxxxxxGAATTCCGC–5′

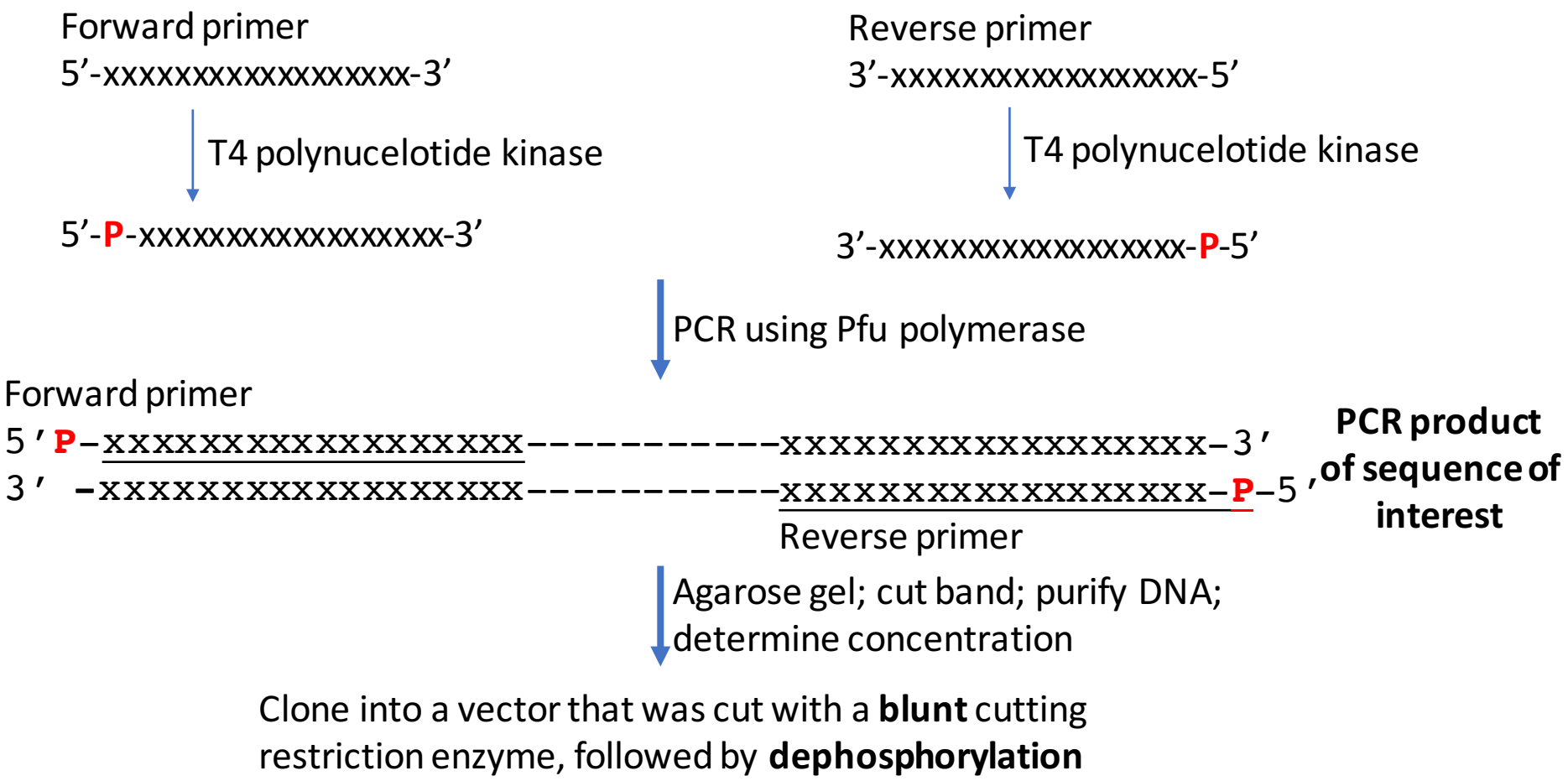
Reverse primer



3.5 PCR CLONING

2. Blunt end cloning using PCR

ATTENTION: Other polymerasese 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. Characeristics 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

3.5 PCR CLONING

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

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
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
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

3.5 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!

→ Keep PCR cycles at the lowest mininum possible