STUDY OF CLONING VECTOR, RESTRICTION ENDONUCLEASE AND DNA LIGASE

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What is Vector?

- A vector is a DNA molecule which is used for transporting exogenous DNA into the host cell.

General characteristics of a vector:

- It should have an Origin of Replication, known as ori, so that the vector is capable of autonomous replication inside the host organism.
- It must possess a compatible restriction site for insertion of DNA molecule.
- A vector should always harbour a selectable marker to screen the recombinant organism. This selectable marker can be an antibiotic resistance gene.
- For easy incorporation into the host machinery, a vector should itself be small in size and be able to integrate large size of the insert.

What is Cloning?

Cloning in biotechnology refers to the process of creating clones of organisms or copies of cells or DNA fragments.

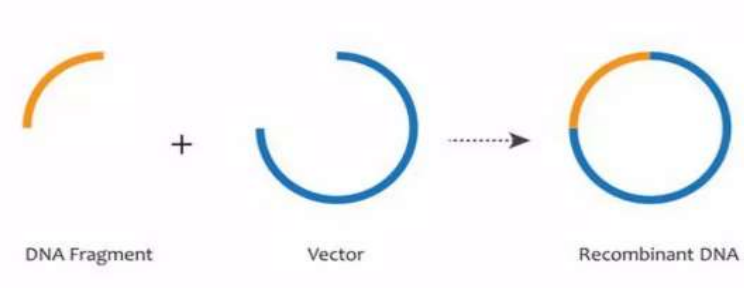
I. CLONING VECTOR

A cloning vector is also a fragment of DNA which is capable of self-replication and stable maintenance inside the host organism. It can be extracted from a virus, plasmid or cells of a higher organism.

The cloning vectors must possess the following general characteristics:

- It should small in size.
- It must have an origin of replication.
- It must also be compatible with the host organism.
- It must possess a restriction site.

- Cloning Vectors are used as the vehicle for transporting foreign genetic material into another cell. This foreign segment of DNA is replicated and expressed using the machinery of the host organism.



- A cloning vector facilitates amplification of a single copy DNA molecule into many copies. Molecular gene cloning is difficult without the use of the cloning vectors.

Types of Cloning Vector

- Plasmids
- Bateriophages
- Phasmids
- Cosmids
- Artificial chromosomes
  - BACs
  - YACs
  - HACs
- Retroviral

Plasmids

Plasmids were the first vectors to be used in gene cloning. They are naturally occurring and autonomously replicating extra-chromosomal double-stranded circular DNA molecules. However, not all plasmids are circular in origin.

- They are present in bacteria, archaea, and eukaryotes.
- The size of plasmids ranges from 1.0 kb to 250 kb.
- DNA insert of up to 10 kb can be cloned in the plasmids.
- The plasmids have high copy number which is useful for production of greater yield of recombinant plasmid for subsequent experiments.
- The low copy number plasmids are exploited under certain conditions like the cloned gene produces the protein which is toxic to the cells.
- Plasmids only encode those proteins which are essential for their ownreplication. These protein-encoding genes are located near the ori.

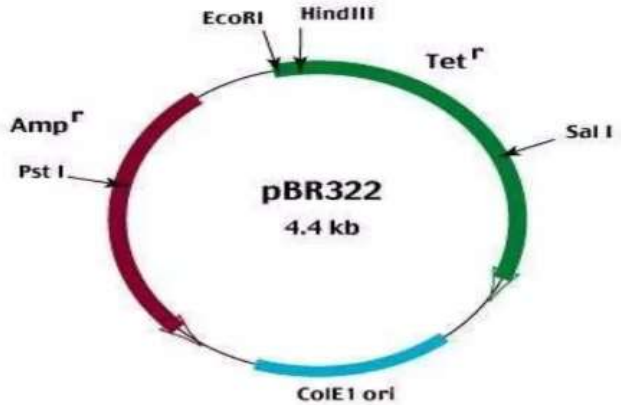
Plasmids

NOMENCLATURE

Nomenclature of plasmid cloning vector:

pBR322 cloning vector has the following elements:

- p= plasmid
- B= Bolivar (name of the scientist)
- R= Rodriguez (name of the scientist)
- 322= number of plasmid discovered in the same lab



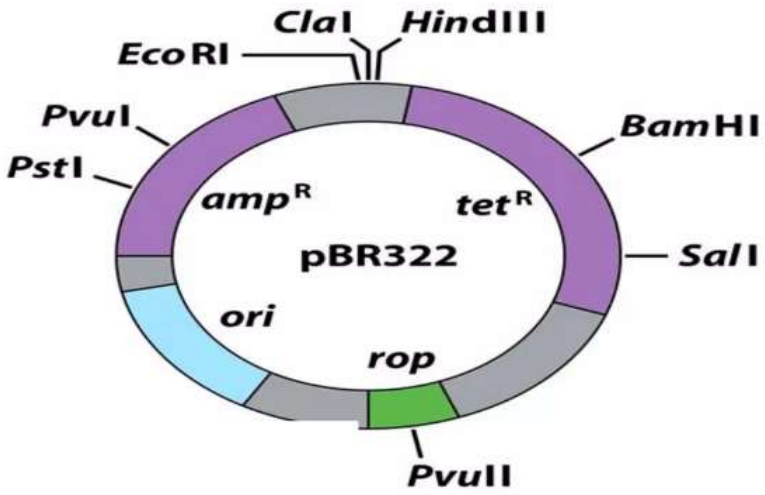
pBR322 plasmid

https://youtu.be/KRpik9mNRm0

Plasmids

- Most commonly used cloning vector is pBR322
- Unique restricted sites *pvu*l and *pst*l-ampiciline, *Bam*HI and *Sal*I-tetracycline.
- *Bam*HI –gene encoding resistance to tetracycline

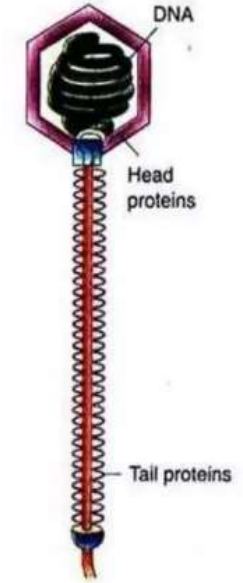
- pBR322 has 4361 base pairs
- Origin of replication (*ori*)
- Antibiotic resistance genes *amp* and *tet*
- *Rop* gene regulates replication for ~20 copies of the plasmid per cell



Bacteriophages

- Bacteriophages or phages are viruses which infect bacterial cells.
- The most common bacteriophages utilized in gene cloning are Phage λ and M13 Phage.
- A maximum of 53 kb DNA can be packaged into the phage.
- If the vector DNA is too small, it cannot be packaged properly into the phage.

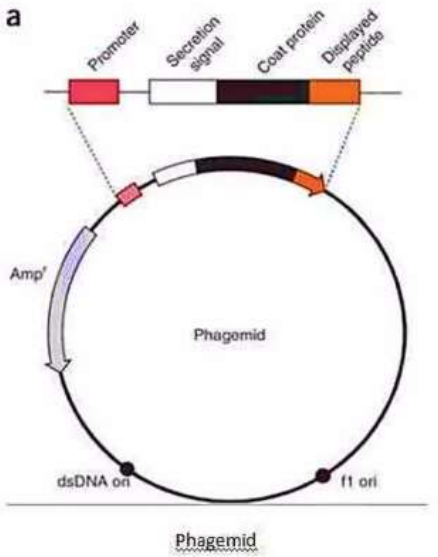
Examples: Phage Lambda, M13 Phage, etc.



Structure of bacteriophage λ

Phasmid

- They are prepared artificially.
- Phasmid contains the F1 origin of replication from F1 phage.
- They are generally used as a cloning vector in combination with M13 phage.
- It replicates as a plasmid and gets packaged in the form of single-stranded DNA in viral particles.

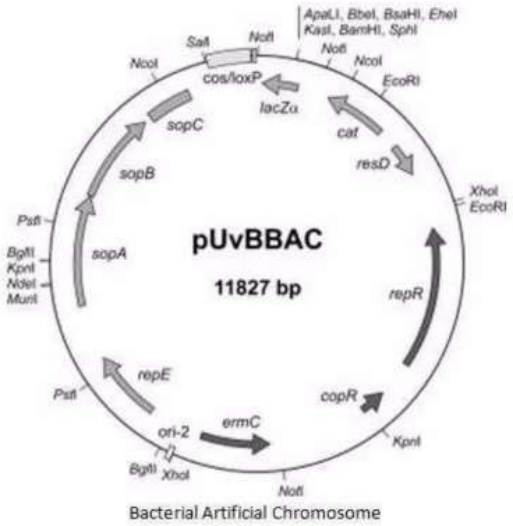


Cosmids

- Cosmids are plasmids.
- They are capable of incorporating the bacteriophage λ DNA segment. This DNA segment contains cohesive terminal sites (cos sites).
- Cos sites are necessary for efficient packaging of DNA into λ phage particles.
- Large DNA fragments of size varying from 25 to 45 kb can be cloned.
- They are also packaged into λ This permits the foreign DNA fragment or genes to be introduced into the host organism by the mechanism of transduction.

Bacterial Artificial Chromosomes

- Bacterial artificial chromosomes are similar to E. coli plasmid vectors.
- They contain ori and genes which encode ori binding proteins. These proteins are critical for BAC replication.
- It is derived from naturally occurring F' plasmid.
- The DNA insert size varies between 150 to 350 kb.



Bacterial Artificial Chromosome

Yeast Artificial Chromosomes

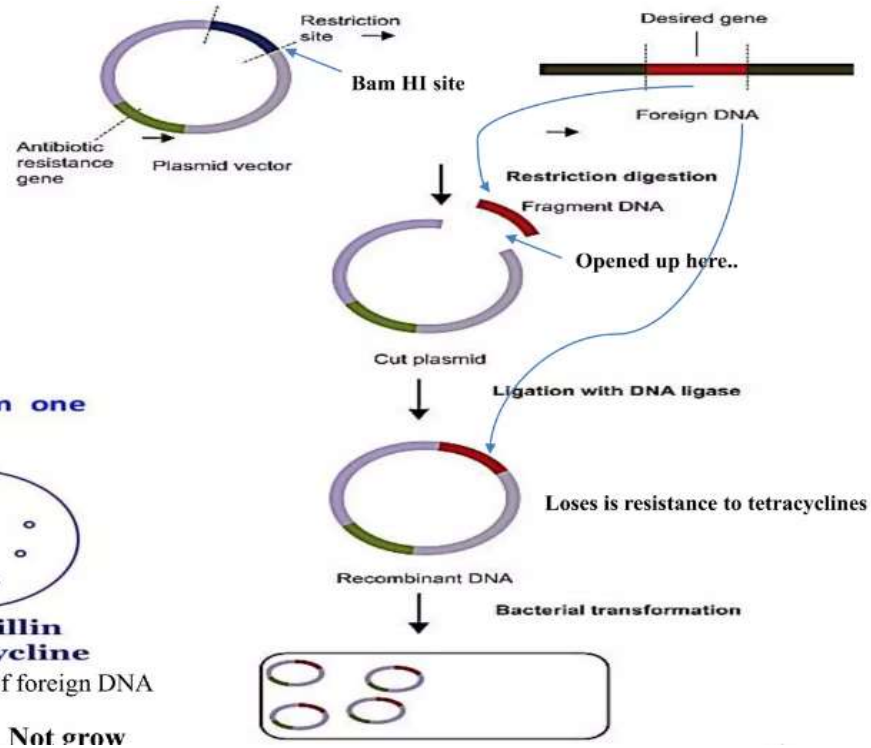
- A large DNA insert of up to 200 kb can be cloned.
- They are used for cloning inside eukaryotic cells.These act as eukaryotic chromosomes inside the host eukaryotic cell.
- It possesses the yeast telomere at each end.
- A yeast centromere sequence (CEN) is present which allows proper segregation during meiosis.
- The ori is bacterial in origin.
- Both yeast and bacterial cells can be used as hosts.

Retroviral Vectors

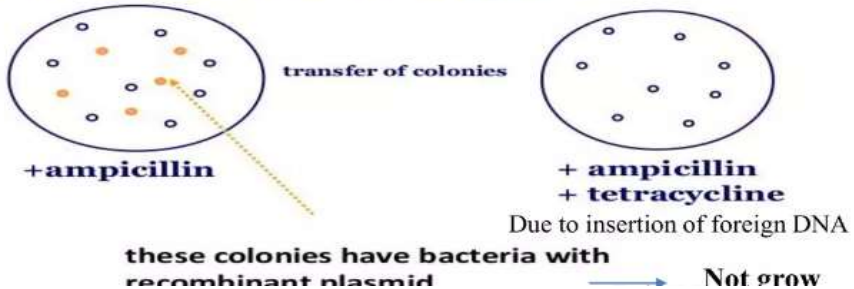
- Retroviruses are the virus with RNA as the genetic material.
- Retroviral vectors are used for introduction of novel or manipulated genes into the animal or human cells.
- The viral RNA is converted into DNA with the help of reverse transcriptase and henceforth, efficiently integrated into the host cell.
- Any gene of interest can be introduced into the retroviral genome. This gene of interest can then integrate into host cell chromosome and reside there.

Steps Involve in Cloning

- isolation of foreign DNA.
- pBR322 is Opened.
- Foreign DNA is ligated
- Recombinant plasmid losses its resistance to tetracycline .



Replica plating: transfer of the colonies from one plate to another using absorbent pad or Velvet



Due to insertion of foreign DNA

these colonies have bacteria with recombinant plasmid → Not grow

Restriction Endonucleases

Restriction Enzymes

- Bacterial enzymes that cut DNA molecules only at restriction sites.
- Categorized into two groups based on type of cut
  - Cuts with sticky ends
  - Cuts with blunt ends
- Named after the organism from which they were derived
  - *Eco*RI from *Escherichia coli*
  - *Bam*HI from *Bacillus amyloliquefaciens*
- Protect bacteria from bacteriophage infection
  - Restricts viral replication

Classes of Restriction Endonucleases

- Type I
  - Cuts the DNA on both strands but at a non- specific location at varying distances from the particular sequence that is recognized by the restriction enzyme
  - Therefore random/imprecise cuts
  - Not very useful for rDNA applications
- Type II
  - Cuts both strands of DNA within the particular sequence recognized by the restriction enzyme
  - Used widely for molecular biology procedures
  - DNA sequence = symmetrical

EcoRI

- Reads the same in the 5’ ➧ 3’ direction on both strands = Palindromic Sequence
- Some enzymes generate “blunt ends” (cut in middle)
- Others generate “sticky ends” (staggered cuts)
  - H-bonding possible with complementary tails
  - DNA ligase covalently links the two fragments together by forming phosphodiester bonds of the phosphate-sugar backbones

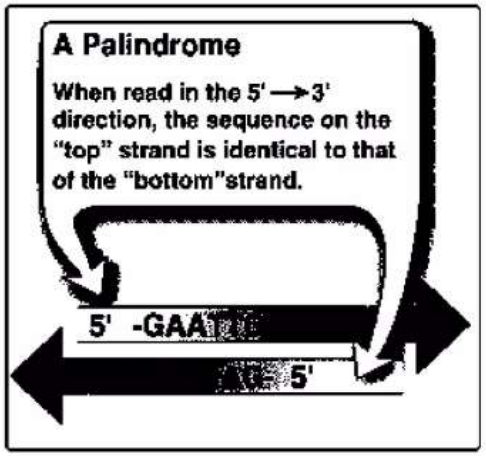
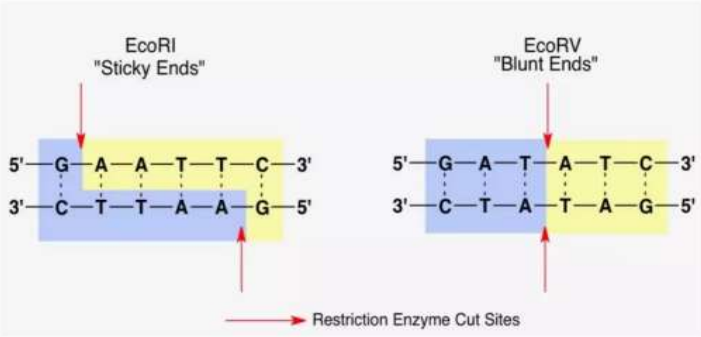
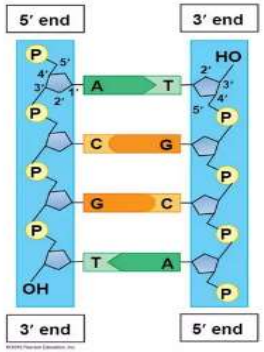
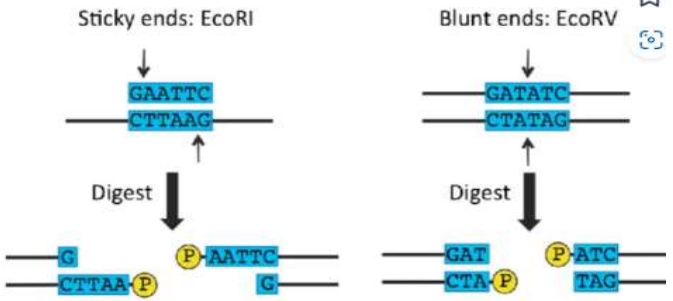
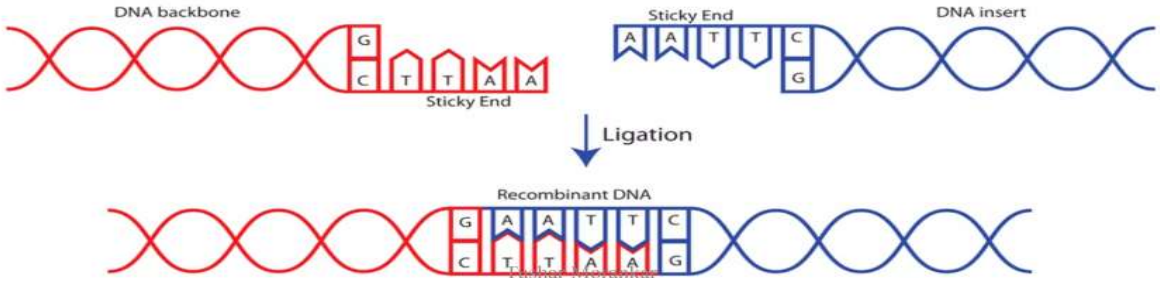


Figure 33.2 Recognition sequence of restriction endonuclease EcoRI shows two-fold rotational symmetry.


EcoRI
"Sticky Ends"
EcoRV
"Blunt Ends"
5'—G—A—A—T—T—C—3'
3'—C—T—T—A—A—G—5'
5'—G—A—T—A—T—C—3'
3'—C—T—A—T—A—G—5'
Restriction Enzyme Cut Sites


5′ end
3′ end
HO
OH
3′ end
5′ end


Sticky ends: EcoRI
GAATTC
CTTAAG
Digest
G
CTTAA
AATTC
G
Blunt ends: EcoRV
GATATC
CTATAG
Digest
GAT
CTA
ATC
TAG

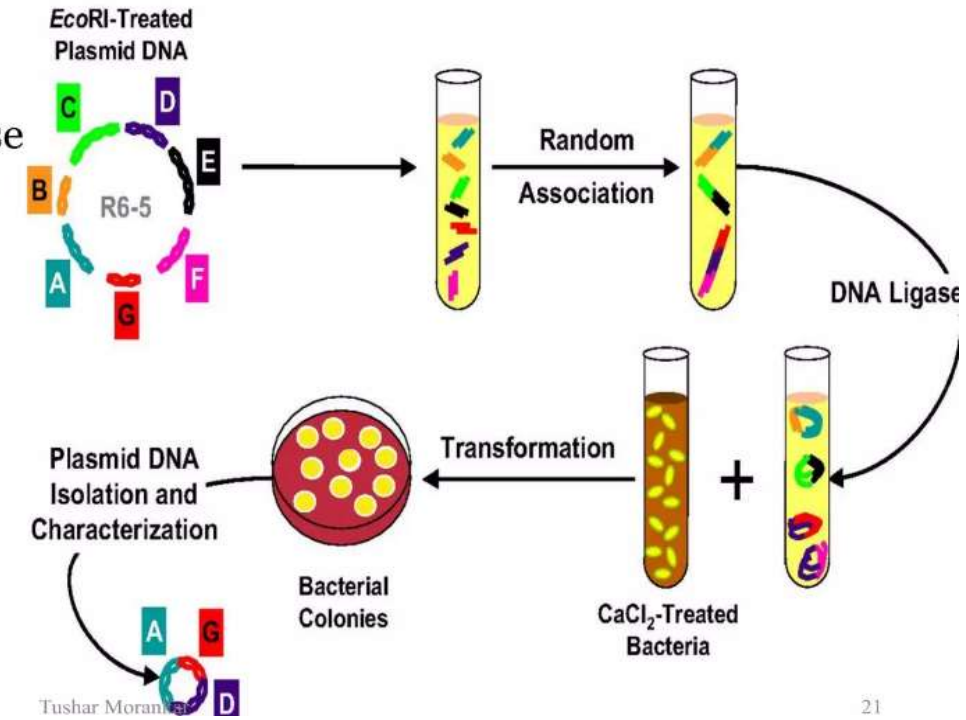

DNA backbone
Sticky End
Sticky End
DNA insert
Ligation
Recombinant DNA

DNA Ligase

- DNA ligase catalyses the formation of phosphodiester bond between the 5'-phosphate of one strand of DNA or RNA and the 3'-hydroxyl of another.
- This enzyme is used to covalently link or ligate fragments of DNA together
- The DNA ligase used in molecular cloning differ in their abilities to ligate noncanonical substrate,such as blunt ended duplexeDNA:RNA hybrid or ssDNAs.

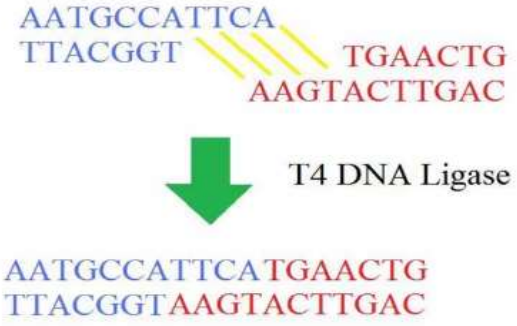
Types of DNA Ligase

1. Bacteriophage T_4 DNA ligase
2. *E.coli* DNA Ligase
3. Taq DNA Ligase
4. T_4 DNA Ligase



Bacteriophage T4 DNA Ligase

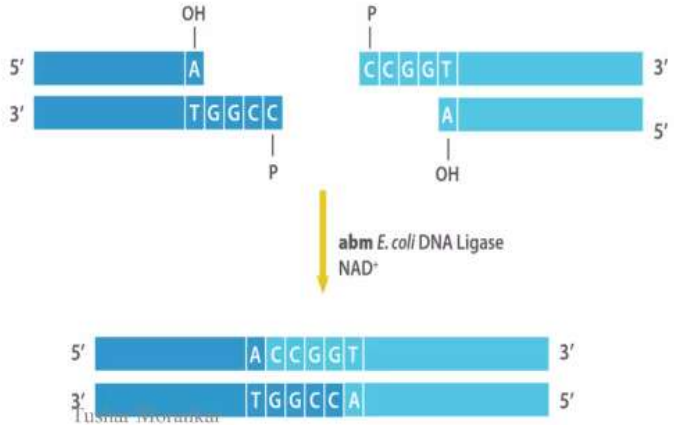
- The most widely used DNA ligase is derived from the T4 bacteriophage.



- It is a monomeric polypeptide
- MW 68KDa is encoded by bacteriophage gene30.
- It has broder specificity and repairs single strended Nicks in duplex DNA, RNA or DNA:RNA hybrids.

E.Coli DNA Ligase

- It is derived from E.coli cell and requires NAD+ as cofacter.
- It is a monomeric enzyme of MW 74KDa which catalyzes the formation of the phosphodiester bond in duplex DNA containing cohesive ends.
- This enzyme has narrower substrate specificity, making it a useful tool in specific application.



Taq DNA Ligase (NAD+)

The gene encoding thermostable ligases have been identified from several thermophilic bacteria. Several of this ligase have been cloned and expressed to high levels in *E.coli*

- It is uses in the detection of mutation as thermostable DNA ligase retain their activities after exposure to higher temp for multiple rounds
- it is uses in DNA amplificaton reaction to detect mutation in mammalian DNA.

T4 RNA Ligase

- T_4 RNA ligase is the only phage RNA ligase that has been extensively characterized and used in genetic engineering.
- This enzyme catalyzed the phosphodiester bond formation of RNA molecule with hydrolysis of ATP to PPI
- It is monomeric enzyme with 373 deduced amino acid residues is a product of the T4 gene 63