DATE
PAGE No.

UNIT- 2

Introduction

Page no. -1 to 20

(a) Study of Cloning Vectors, Restriction Endonucleases and DNA ligase.

(b) Recombinant DNA technology: Application of Genetic Engineering in medicine

(c) Application of rDNA technology and genetic engineering in the production of (i) interferon (ii) Vaccines- Hepatitis B (iii) Hormones- Insulin

(d) Brief Introduction to PCR

Unit-2

(1) Study of Cloning vectors

DATE
PAGE No. 1

→ Vectors are the DNA molecules which can carry a foreign DNA fragment to be cloned. They are self replicating in an appropriate host cell.

Characteristics of an Ideal vector

1) Small in size.
2) have a single restriction endonuclease site.
3) Have an origin of replication (ori).
4) 1-2 genetic markers.

(1) Plasmid → These are extrachromosomal double stranded, circular, self replicating DNA molecules. Almost all the bacteria have plasmids containing a low copy number (1-4 per cell) or a High copy number (10-100 per cell). The size of the plasmid varies from 1 to 500 kilo base pairs. (KbP).

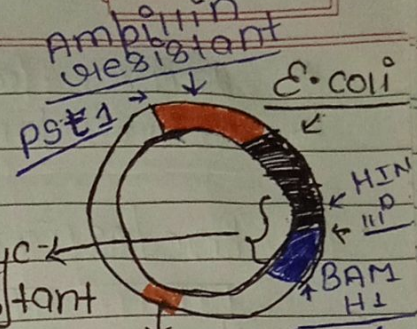
→ PBR322 has a plasmid of E. coli, 4361 BP

Nomenclature of plasmid

PBR322
↓ ↳ Discovered name - Bolivar, Rotriguez
Plasmid

Kb - Kilo base pair
Nucleotide unit

4361 Base pairs



→ Other plasmid vectors i.e
. PBR325, PBR328, PBR329, PUC19.

ex- PBR322 (↑)

(2) Bacteriophages → Virus which can replicate within bacteria. Phage vectors can accept short fragment of foreign D.N.A but they can carry larger segment than Plasmids.
ex- Bacteriophage λ (lambda) virus of E. coli - It can carry 25 Kb

ex:- Phage M13 vector - Single stranded D.N.A phage of E. coli.

(3) Cosmids → It possess both plasmid and bacteriophage characteristics carry 40 Kb.

Artificial Chromosome Vectors :-

1) Human artificial chromosome (HAC) → H. Willard
* Synthetically produced
* Size - 1/10th - 1/5th of human chromosome.
→ gene therapy

2) Yeast artificial chromosome → (M. Olson)

→ Carry large fragment of D.N.A
→ It is large capacity vectors.

(5) Bacteyial artificial chromosome (BAC)

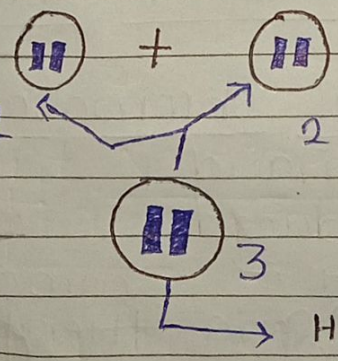
→ It can accept ≈ 300 kb.
→ More Stable than YAC.

4) Shuttle vectoy → This is plasmid ~~vectoy~~ but specially designed to replicate in two host or in E.coli and Streptomyces species.

→ The ori of two Host Combine in one vectoy.

Recombinant D.N.A Technology

→ It is a hybrid D.N.A produced by joining of D.N.A fragments of two different organisms. In 1973 Paul berg, Herbert boyer and Stanley Cohey were found out this technology.



The recombinant D.N.A is an altered sequence of gene. The organism containing R.D.N.A is called recombinant. The technology involved in the production of recombinant or R.D.N.A is called Recombinant DNA technology or gene manipulation or more commonly Genetic Engineering.

Requirement for Recombinant D.N.A technology

1. Donor D.N.A → It contains required gene sequence.
2. Vectors → They carry or attach with D.N.A donor gene sequence.
3. Restriction Endonuclease → These are biological scissors which cut the D.N.A at a peculiar site
4. D.N.A ligation → That attaching the D.N.A
5. Introduce into host cell : Host cells are those which can carry R.D.N.A Bacteria
6. Expression the gene
7. Extraction of gene product

Application of Genetic Engineering in Medicine

(1) In the production of hormones — Insulin (Humulin), Human growth Hormone (HGH)

(2) In the production of therapeutic agents which are useful for human diseases.

R. product	Trade Name	Application
1. α- Interferon	Intron- A	Hairy cell leukaemia
2. Tissue plasminogen activator	Activase	Myocardial Infarction
3. Factor - VIII	Kogenate	Hemophilia
4. DNase	Pulmozyme	Cystic fibrosis
5. Erythropoietin	Epogen / procrit	Severe anemia with kidney damage.

(3) Recombinant Vaccine:- Different V. Vaccines are developed which categorised into 3 groups:-

(a) Sub-unit recombinant Vaccines → These are the component of pathogenic organisms, include proteins peptides DNA

ex- Hepatitis B vaccine, BCG vaccine, Tuberculosis vaccine.

(b) Attenuated recombinant vaccines - These are genetically modified pathogenic organisms (bacteria / viruses) that are made non-pathogenic and used as vaccines. ex- cholera vaccines, Salmonella species vaccines / leishmania sp. vaccines

(c) vector recombinant vaccines :- These are genetically modified viral vectors that can be used as vaccines against certain pathogens.
ex- vaccinia virus vaccines (for cow-pox, small pox).

(4) In the production of monoclonal antibodies (MAbs) - help for diagnostic - pregnancy, cancers, Hormonal disorders, Myocardial infarction, deep vein thrombosis, atherosclerosis.
for therapeutic :- In the treatment of AIDS, autoimmune disease, Cancers, immune suppression of organ transplantation.

(5) In the diagnosis of disease :- Diagnosis of different disease are done by genetic Engineering methods for ex- Tuberculosis (Tb)

→ A gene from firefly encoding enzyme luciferase is introduced into bacteriophage specific for M. tuberculosis (luciferase reporter phage or Mycophage).

→ When the gene is interact with bacteria so, produce luciferase enzyme. When luciferen and ATP are added to the medium, luciferase cleaves the luciferen and emit light which can be seen luminometer.

(1) Malaria, (2) AIDS (3) Tropical disease as filarial disease, leprosy, Tuberculosis (4) Diagnosis of genetic disorder- cystic fibrosis, sickle cell anaemia etc.

Study of Restriction Endonuclease

Restriction endonuclease are one of the most important group of enzymes that can cut/split at specific types or sites. first discovered in E. coli.

Nomenclature

- first letter:- (italics) → genus name of source.

2nd and 3rd letter - Species name
4th letter - Strain name
Lastly - Roman Numerals - indicating order of discovery.

ex- EcoRI - E- Escherichia
Co - Coli
R - RY13
I - Ist enzyme

→ R.Es act as biochemical knifes. It recognises specific nucleotide sequence and make cut on the plasmid and desired D.N.A. The target site for cutting may vary from one enzyme to another enzyme.

Types of Restriction Endonuclease

There are 4 types which are as follows :-

1. Type - I - It can cleave up to 1000 bp from recognition site.
A single enzyme with 3 subunits for recognition, cleavage and methylation.

2. Type - II - Two different enzyme to cleave, to modify the recognition site.

3. Type - III → A single enzyme with 2 subunits from recognition and cleavage site is 24-26 bp from recognition site.

4. IIS → Two different enzyme cleavage site is up to 20 bp from recognition site.

Enzyme/Source	Recognition Sequence	Products
① EcoRI [Escherihia Coli]	5' G↓A-A-T-T-C 3' 3' C-T-T-A-A↑G 5'	A-A-T-T-C-G -G C-T-T-A
② BAMHI [Bacillus amyloliquefaciens]	5' G↓G-A-T-C-C 3' 3' C-C-T-A-G↑G 5'	GA-T-C-C-G -G-C-C-T-A-G
③ Hae III [Haemophillus aegyptus]	Sticky ← [ ] 5' G-G↓C-C 3' 3' C-C↑G-G 5' [ ] → Blunt	-C-C -G-G
④ Hind III [Haemophillus influenzae]	5' A↓A-G-C-T-T 3' 3' T-T-C-G-A↑A 5'	-A -A-G-C-T-T -A-T-T-C-G-A
⑤ Not I [Nocardiodia Otitidis]	5' G-C↓G-G-C-C-G-C 3' 3' C-G-C-C-G-G↑C-G 5'	① G-G-C-C-G-C-C-G ② G-C-C-G-C-C-G-G-

- **Palindromic sites:-** It is a site wherein reading in a certain direction on one strand matches the sequence reading in the opposite direction on the complementary strand.

Recognition sequence:- It is the site where D.N.A is cut by R.Es. Each recognition sequence has palindromic sequence (Sequence on both strand same).

Cleavage pattern:- The cut D.N.A fragments by R.E may generate

- **Sticky End** → This is useful → They have one free site because, that site nucleotide is cut by R.Es
- **Blunt End** → No more use → They do not have one free nucleotide site.

Study of DNA Ligase.

These are D.N.A joining enzymes D.N.A Ligase enzyme joined covalently. They were originally isolated from virus but also occurs in E.Coli and Eukaryotic cells. DNA Ligase actively participated in cellular DNA repair process. The DNA Ligase form the phospho-

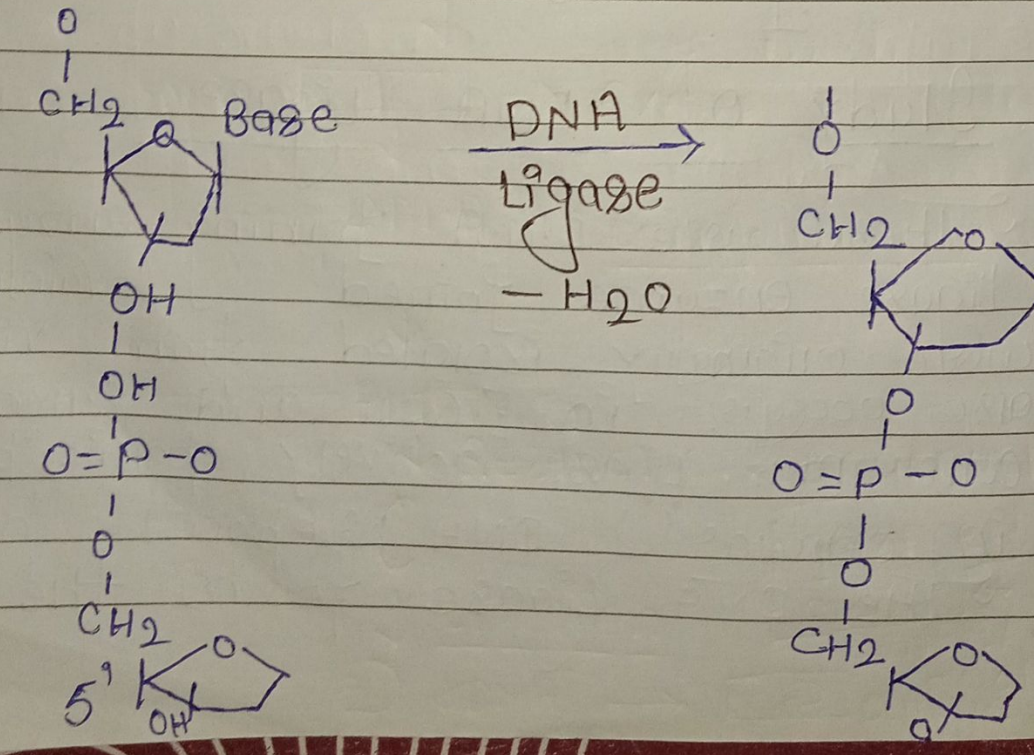
diester bond to Hold the DNA repair process. The DNA - Ligase form strands together in very firmed manner.

→ The phosphodiester bond is formed between phosphate group of 5' carbon of one deoxyribose with the -OH (Hydroxyl) group at 3' carbon of another - deoxyribose.

- Phage T4 DNA Ligase required ATP as cofactor.
- E.coli DNA Ligase require NAD+ as cofactor

Nitrogen base + Phosphate group = Nucleotide

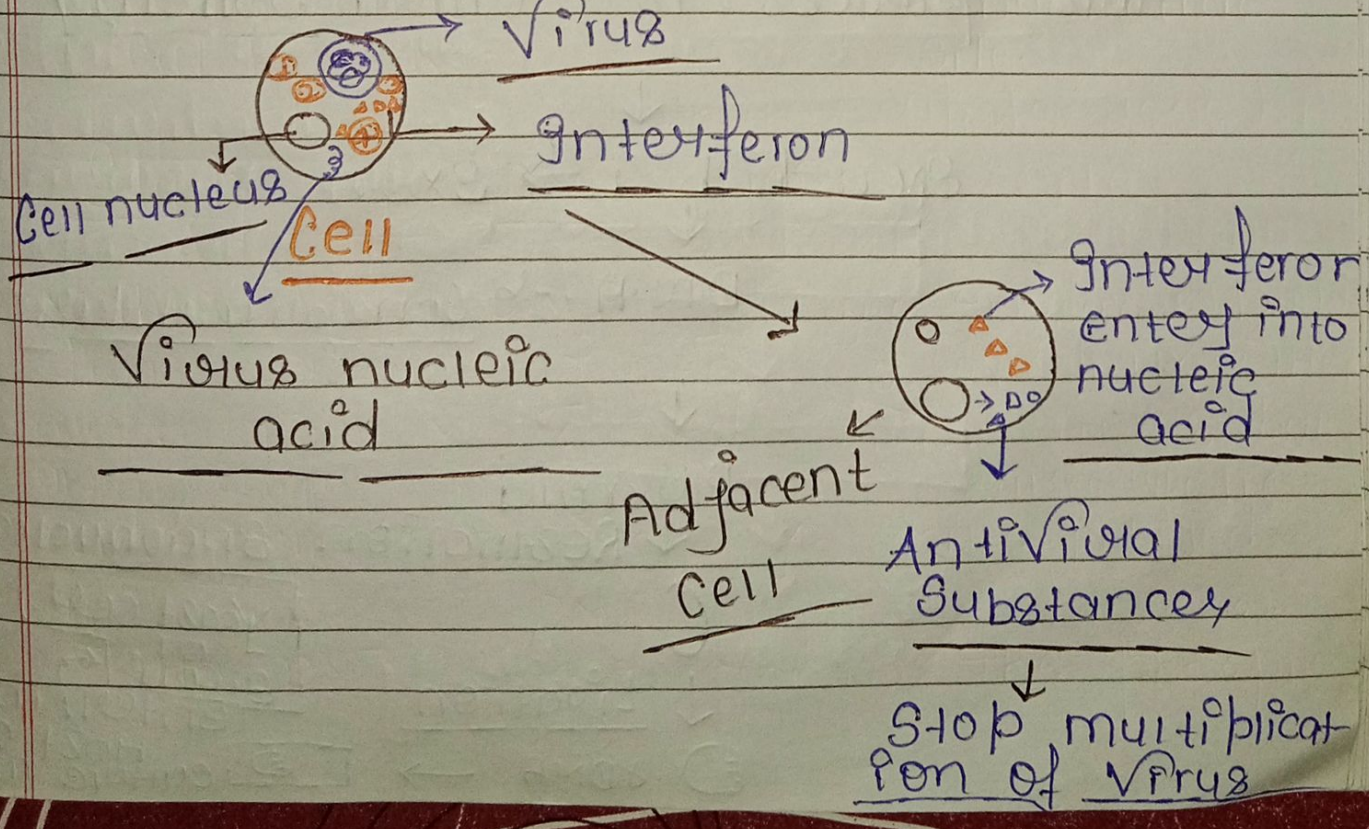
- NAD = Nicotinamide odenine dinucleotide



Interferon

- antiviral substance
- first line defense against viral attack
- Discovered by "Alick Isaccs and Jean Lindemann.
- Interferon are protein in nature and some glycoprotein in nature
- Generally there are three types of interferon
  (a) Interferon-α , (b) Interferon-β
  (c) Interferon-γ

* Interferon words originate from interference, means these protein and glycoprotein (protein + carbonydrate) particles generate interference in viral multiplication.

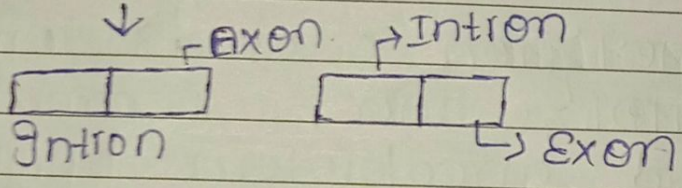


Interferon
↓
m·RNA
↓ Reverse transcription
C·D·N·A
[Complementry DNA]
↓
DNA which develop through exon part
R·N·A

- DNA → RNA → Protein ⇒ Transcription

Reverse transcription

↓



↓

'Intron remove from this part and this process is called Splicing'

↓

Exon → Exon

↓

D·N·A → Complimentary D·N·A

↓

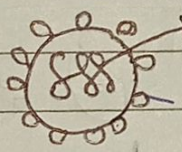
Plasmid
↓ Restriction Endonuclease
C
↓ Ligation
rDNA → [yeast cell or E.coli] enter into Host and culture into

appropriate medium.
↓
Now inf. cells are extracted out from the culture media.

→ Yeast cells are more preferred into as compared to bacterial cell because they have machinery for glycosylation of protein.

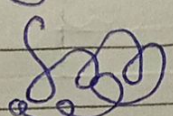
Hepatitis - B

- It primarily affects liver causing chronic Hepatatis, cirrhosis and Liver Cancer.



- 42 nm particle
- It is called dane particle.
- Viral genome - D.N.A
- Surrounding phospholipid envelop carrying surface antigen - HB_3Ag

Viral D.N.A



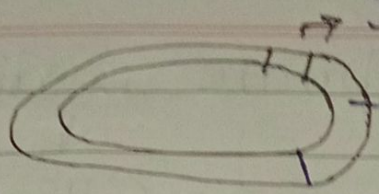
↓ R.E

Required piece of D.N.A

III

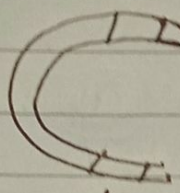
↓ ligation

Steps



PMA56 (Yeast Vector)

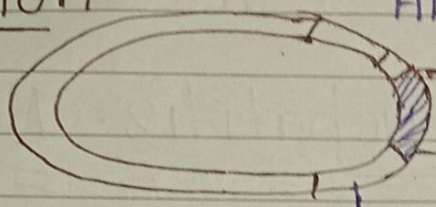
↓ R.E



↓

Ligation

ADH Promoter



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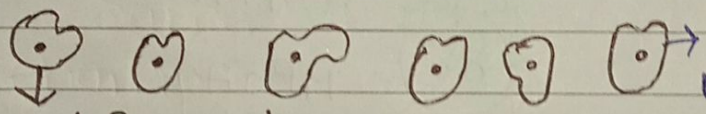
Transfer into Yeast Cell

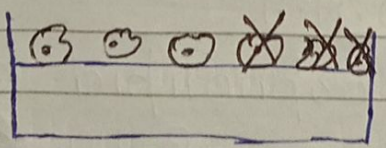


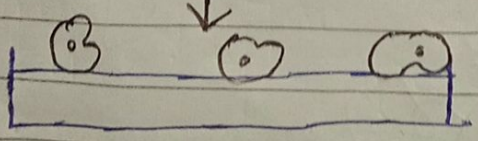
Recombinant host cell

Non recombinant host cell

↓

Grow in Tryptophan free medium in this medium only recombinant cells survive.



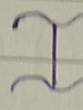


↓

Lyse yeast cell and purify HBSAg

Insulin

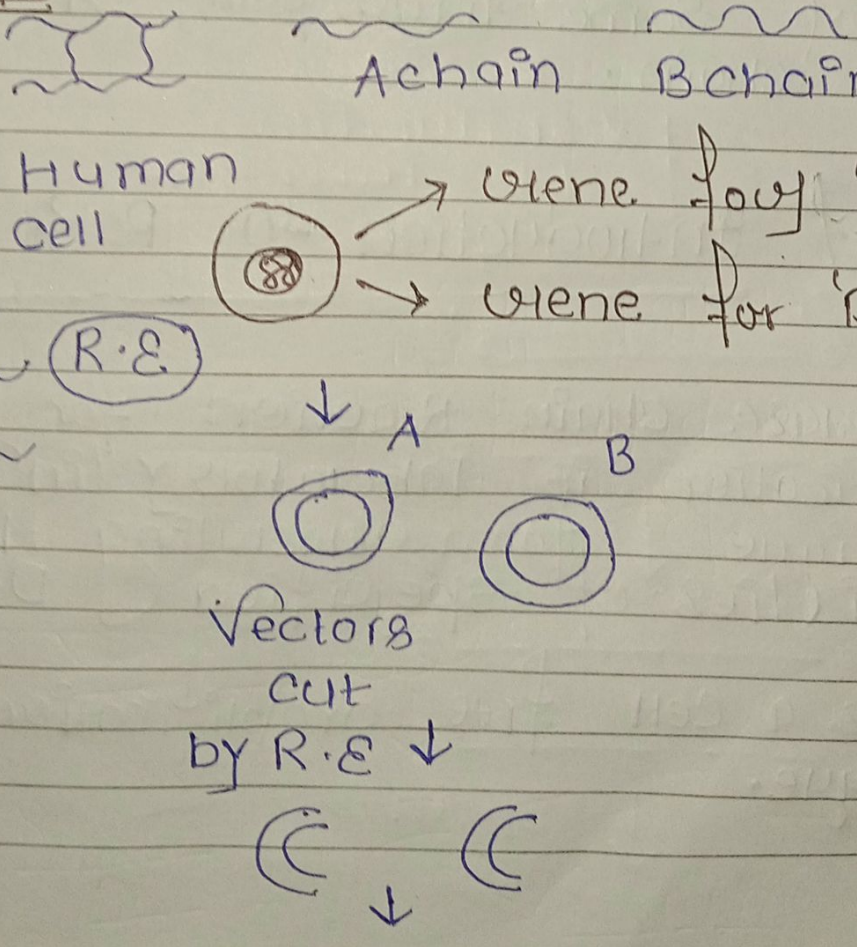
- Insulin has two polypeptide chain
  * (a) A chain (21 amino acid)
    (b) B chain (30 amino acid)



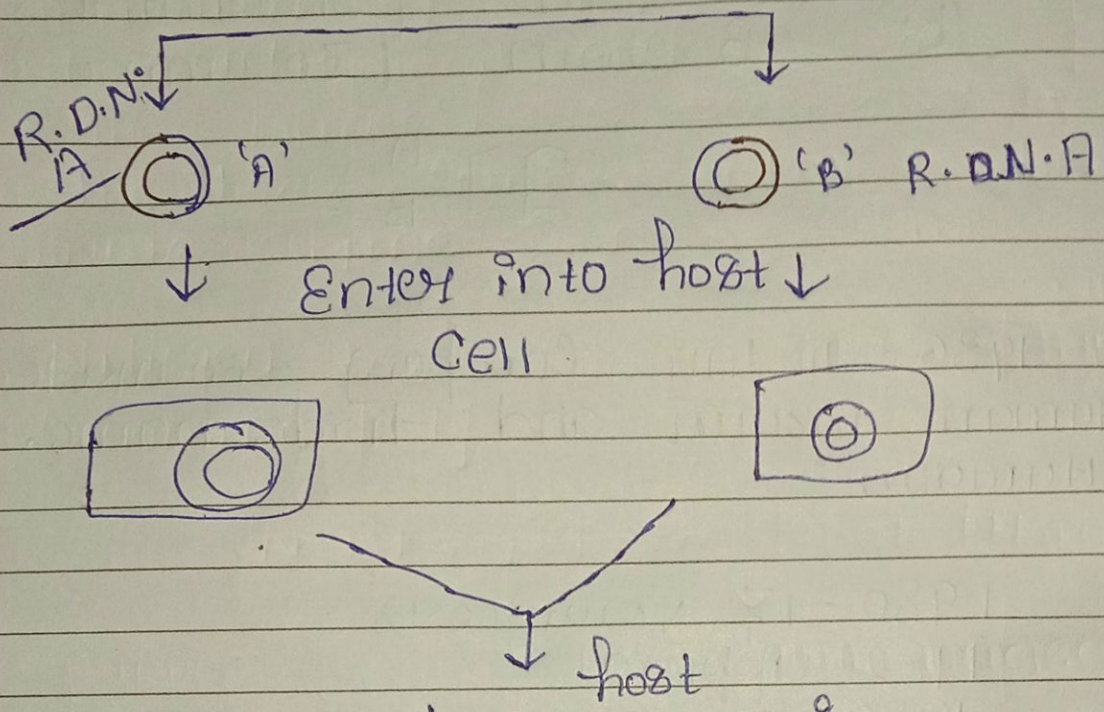
→ In 1986 Eli lilly Company marketed Human insulin and trade name, "Humulin"

→ 1980 - 17 Volunteers
→ 1982 - approval
→ 1986 - Marketed

Steps



↓ Ligation with A and B Gene Separately



Culture these cells in a nutrient medium containing Lactose and after Same time purify 'A' and B chain.

Brief Introduction to PCR

→ Polymerase Chain Reaction for gene amplification is laboratory (in vitro) technique for generating large quantities of specified D.N.A.

→ PCR is a cell free amplification technique.

* It generate billions of copies from very very small quantity of DNA in few hours.

* Requirement for PCR

1) A target D.N.A (100 - 35000 bP)

2) Two primers (around 17-30 nucleotides in length).

3) four types of deoxyribosenucleotide in length)

4) Heat Stable D.N.A polymerase (can withstand up to 95°C -> Taq D.N.A polymerase [Thermus aquaticus]

Pfu - II - [Pyrococcus furiosus]
Vent - II - [Thermococcus litoralis]

Steps

(1) Denaturation -> The mixture Heated up 95° C for 1-2 min.

(2) Renaturation -> The temp. is slowly down up to 55°C that permit annealing of primer to the Complementary sequence.

Time - 1 min. High Conc^n of primer

ensure the annealing.

(3) Synthesis / Primer extension → The initiation of D.N.A synthesis occur at 3'OH (Hydroxyl) end of each primer. The primer are exhausted by joining the bases, complementry to D.N.A Strands. Time - 2 min.

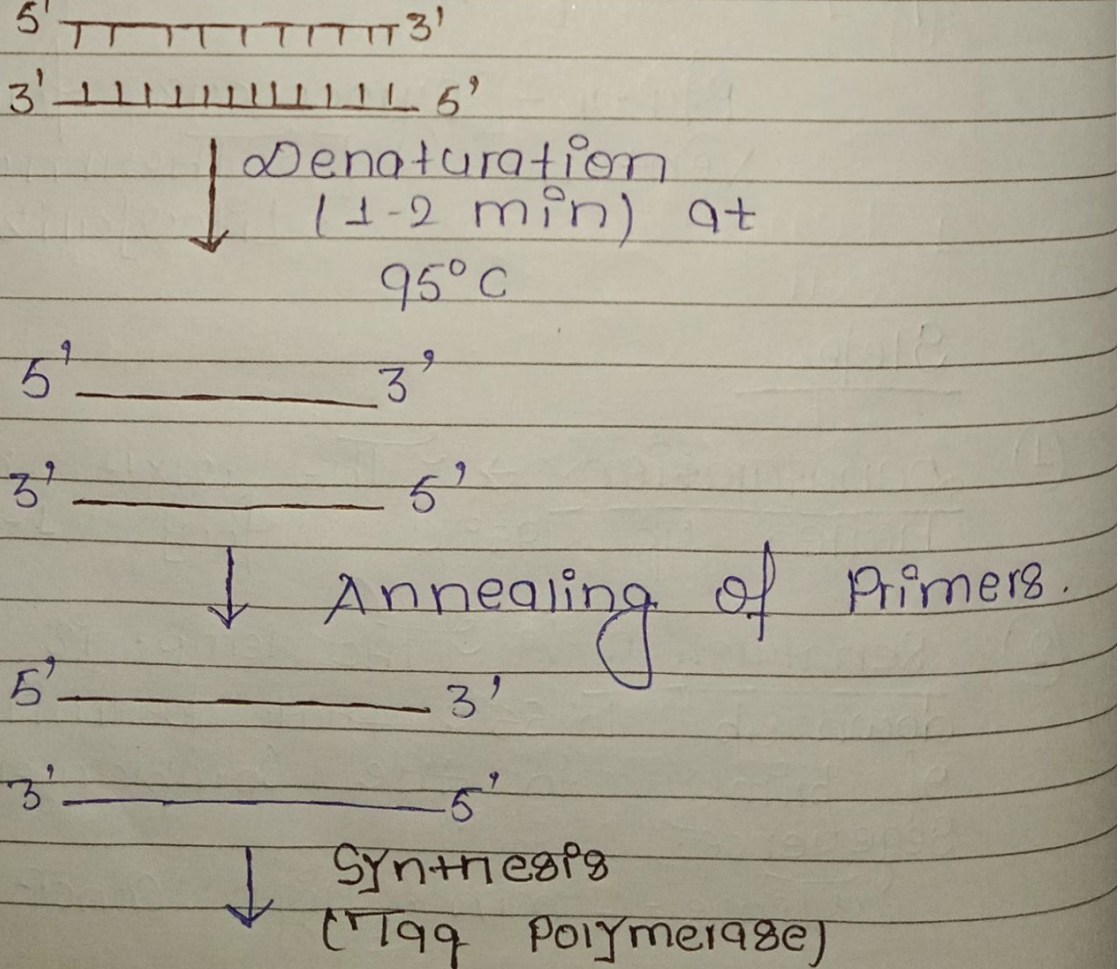
- Nitrogen base - Adenine (A), Thymine (T) Guanine [G], cytosine (C)

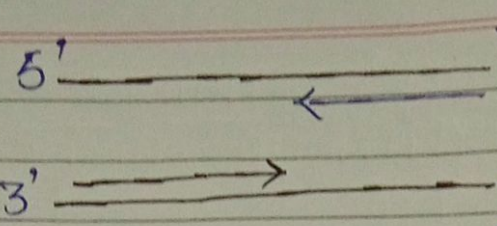
N.B + Sugar + Phosphate (H_3PO_4)

↓

Deoxyribonucleotide.

Steps





Triphosphate

- DATP → Deoxyribonucleotide adenine
- DCTP → " " Cytosine "
- DTTP → " " Thymine "
- DUTP → " " Uracil "

Application of PCR

1. PCR in forensic medicine
2. " " DNA Sequencing
3. In diagnosis of inherited dose. ie Thalassemia, Sickle cell anaemia etc.
4. In diagnosis of Cancer - Several virally induced cancer (e.g cervical cancer - caused by papiloma virus) Can be detected by PCR.
5. PCR in sex determination of Embryos.
6. PCR in comparative Studies of Genome.

The End