ICS 1

Plasmids = Circular, self replicating piece of extra-chromosomal dsDNA molecules found in bacteria (e.g. pBR322)

1. Origin of replication
    - Allows DNA polymerase to bind to origin of replication to initiate replication independently of bacterial chromosomes
    - Creates multiple copies of the plasmid and **gene of interest** within one bacterium
    - High copy number = Number of plasmids found in a single bacterial cell and is determined by strength of origin of replication
        - Increases the likelihood that the recombinant plasmid will be present in all daughter cells upon bacterial division
2. Selectable markers (at least 2)
    - Confer host bacterial cell with selectable phenotypic trait
    - One allows for selection of transformed cells
    - Other allows for selection of transformed cells with recombinant plasmid
    - Example: Ampicillin resistance gene
        - Acts as selectable marker to select for bacteria transformed with plasmid
        - Confers the bacteria ampicillin resistance
        - Transformed bacteria will survive on an ampicillin containing plate while untransformed bacteria will be killed by ampicillin
    - Example: lac Z gene
        - Acts as selectable marker to select for bacteria transformed with recombinant plasmid
        - Codes for β-galactosidase which breaks down colourless X-gal into blue compound
        - When gene of interest is inserted into the plasmid, lac Z gene will be insertionally inactivated
        - Bacteria transformed with the reannealed plasmid will appear blue while bacteria transformed with the recombinant plasmid will appear white
    - Example: Tetracycline resistance gene
        - Acts as selectable marker to select for bacteria transformed with recombinant plasmid
        - Confers tetracycline resistance
        - Gene will be insertionally inactivated when gene of interest is inserted
        - Bacteria transformed with recombinant plasmid will die, bacteria transformed with reannealed plasmid will survive
3. Multiple cloning site/Polylinker within the second selectable marker
    - Contains a variety of restriction sites ⇒ Allows a wide range of different foreign genes to be inserted
    - Insertion of gene into the MCS located within a selectable marker results in **insertional inactivation** and hence, allows for the selection of bacteria transformed with recombinant plasmids
4. Prokaryotic promoter allows prokaryotic RNA polymerase and sigma factors to initiate transcription
5. Gene of interest
    - Inserted in correct orientation downstream of prokaryotic promoter ⇒ Ensure

correct strand is transcribed
    - Introns removed ⇒ Prokaryotes cannot remove introns
6. Small
    - Allows the recombinant plasmid to be easily taken up through transient pores in competent bacteria that have undergone heat shock treatment in $CaCl_2$

Restriction Enzymes

- Cleave phosphodiester bonds that link adjacent nucleotides
- Recognise and bind to specific **palindromic** restriction site where reading both strands in 5' to 3' direction yield the same sequence
- Natural function:
    - Protects bacteria against bacteriophages by destroying foreign DNA molecules carried by bacteriophages
    - Bacterial DNA is protected as it is methylated
- Advantage of restriction enzymes which produce sticky ends
    - Allows cut DNA fragment to anneal to a plasmid which has been cut by the same restriction enzyme
    - By complementary base pairing where hydrogen bonds are formed allowing for greater stability for ligation
    - DNA fragments and plasmids with blund ends cannot anneal and require extra steps such as by adding linkers or using terminal transferase to convert the blunt ends to sticky ends

Human growth hormone gene → Found in **anterior pituitary gland**

General Procedure of Producing Recombinant Bacteria
Preparation of cDNA (Start here for cDNA library)

1. Isolate **total mRNA** from the cells
2. Add oligo-dT primer which will anneal by complementary base pairing to the poly-A tail of the mRNA
3. Use **reverse transcriptase** to add DNA nucleotides to free 3’ OH group of primer to synthesise a cDNA strand using mRNA strand as a template forming a RNA-DNA hybrid
4. **RNase** is added to degrade the mRNA strand of the RNA-DNA hybrid
5. Add **DNA polymerase** to synthesise second cDNA strand complementary to first cDNA strand, producing double stranded cDNA

Isolation of genomic DNA (Start here for genomic library)

1. Extract genomic DNA from any somatic cell
2. Digest the DNA using restriction enzymes
3. Incorporate DNA fragments into plasmids (see below)

Formation of Recombinant Plasmid

1. Generate sticky ends (see below)
2. Mix plasmid with cDNA to allow complementary sticky ends to anneal to one another
3. Add **DNA ligase** to seal the nicks by forming phosphodiester bonds between adjacent nucleotides to form a recombinant plasmid

Generating Sticky Ends
**Using Terminal Transferase (esp for cDNA):

1. Cut plasmid with restriction enzyme that leaves blunt ends
2. Use **terminal transferase** to add **dCTPs** to blunt 3' ends cDNA mixture
3. Use **terminal transferase** to add **dGTPs** to blunt 3' ends of plasmid

Using Restriction Enzymes that leave sticky ends:

1. Cut the DNA and the plasmid with the same restriction enzyme to generate sticky ends

Using Linkers:

1. Add linkers to the blunt ends of the cDNA using DNA ligase
2. Cut the cDNA and the plasmid with the same restriction enzyme to produce complementary sticky ends

Transformation of Bacteria

1. Mix ligation mixture with bacterial cells and $CaCl_2$
2. **Transform** bacteria with recombinant plasmid by applying heat shock treatment to create transient pores on the bacterial cell membranes

Selection of Successfully Transformed Cells (Stop here for cDNA/genomic library)
Using lac Z selectable marker:

1. Grow bacteria on agar plate containing ampicillin, lactose and X-gal to select for bacteria that are transformed
2. Bacteria that possess the plasmid will exhibit antibiotic resistance and will survive in the medium
3. Bacteria that contain the **recombinant** plasmid will appear as **white** colonies while those that contain the **reannealed** plasmid will appear as **blue** colonies as lac Z gene has undergone **insertional inactivation** due to insertion of the gene of interest into the lacZ gene

Using tetracycline resistance gene selectable marker:

1. Plate bacteria in an agar plate containing ampicillin to select for bacteria that are transformed
2. Bacteria that possess the plasmid will exhibit antibiotic resistance and will survive in the medium
3. Carry out **replica plating** for second antibiotic, selection for transformed bacteria that contain recombinant plasmid
4. Compare the tetracycline plate with the ampicillin plate
5. Bacteria with recombinant plasmid will not survive on agar plate containing tetracycline as tetracycline resistance gene has undergone **insertional inactivation** due to insertion of the gene of interest but will survive on ampicillin plate

Colony Hybridisation/Probing (if PCR not used)

1. Probing conducted to identify bacterial colonies with recombinant plasmid containing gene of interest
2. **Nitrocellulose membrane** is placed on the surface of the agar plate to replicate the bacterial colonies
3. Membrane is treated with **NaOH** to lyse the cells and denature the double-stranded DNA to single-stranded DNA which will bind to the membrane
4. Membrane washed with saline to remove cell debris
5. Bake the membrane for a few minutes at 80°C to fix DNA to membrane
6. Membrane is incubated with a solution of specific radioactively-labelled DNA probes which will anneal to the complementary DNA sequences on the gene of interest (**hybridisation**)
7. Wash off excess probes
8. Place nitrocellulose membrane on X-ray film, radioactive areas will blacken the film (**autoradiography**)
9. Compare blackened areas on X-ray film with agar plate to identify the bacterial colonies transformed with plasmids containing the gene of interest

Large Scale Production

1. Proper nutrients and optimum conditions → Colonies containing HGH cDNA grown in large quantities in bioreactors
2. Bacteria induced to produce large amounts of HGH
3. Extract, purify and package the protein produced
4. (For insulin only) Enzymatically cleave off chain C using protease and chemically combine chains A and B via disulfide bridges to form functional insulin

Why use cDNA

- Bacteria do not have enzymes for splicing out introns

- cDNA produced from mature mRNA strands hence, transcription of gene will produce mRNA without intron sequences
- Yeast can be used in place of bacteria as it is a eukaryote and thus has the enzymes to carry out post-transcriptional splicing of pre-mRNA to remove introns

Advantages of using bacteria for large scale production of proteins

- Small genomes and relatively easy to manipulate genetically
- High replication/growth rate ⇒ Allows for mass production of proteins
- Takes up relatively small space
- Can be cultured easily in any part of the world ⇒ Proteins can be more easily obtained
- Avoids risks of infection/disease and problems of allergic effects compared to proteins extracted from animals
- Avoids ethical and religious objections compared to proteins extracted from animals

Genomic Library
Definition:

- Collection of clones made from a set of randomly generated overlapping DNA fragments representing the entire genome of an organism
- Includes non-coding sequences and coding sequences
- Each DNA fragment is cloned into a vector molecule

Advantages:

1. Contains all genes of an organism
2. Allows for study of introns/regulatory sequences associated with genes
3. Allows differences in nucleotide sequences in diseased and healthy people to be compared

Disadvantages:

1. Eukaryotic genomes are very large ⇒ Library will contain many fragments ⇒ Takes long time to screen
2. Gene of interest may not be intact as restriction enzymes used may cut the gene of interest internally
3. Contains introns ⇒ Correct polypeptide cannot be expressed in bacteria due to lack of post-transcriptional mechanisms

cDNA library
Definition:

- Collection of cDNA fragments which are generated in vitro using the mature mRNA isolated from a particular cell type as a template and are inserted into a vector molecule
- Represents all mRNA present in a particular tissue

Advantages:

1. Contains only coding sequences ⇒ Smaller number of clones ⇒ Easier to screen
2. Lacks introns ⇒ Can be expressed in prokaryotes
3. cDNA obtained from reverse transcription of mRNA ⇒ Gene of interest remains intact
4. Smaller in size ⇒ Easier to manipulate and insert into vectors
5. Only contains genes expressed in the tissue source ⇒ Easier to create

Disadvantages:

1. Only genes that are expressed in a particular tissue at a particular developmental stage can be harnessed ⇒ Does not represent the entire genome

	Genomic library	**cDNA library**
Content	Contains entire DNA content of an organism Contains all coding and non-coding sequences	Contains entire protein-encoding DNA content of the source tissue Only contains exon sequences which code for amino acids
Starting genetic material to be isolated	Chromosomal DNA	Total mRNA
Place to obtain starting material	Can be from any cell/tissue	Must be isolated from a single cell/tissue where the particular protein is likely to be produced in large quantities
Key enzyme(s) involved before cloning into vector	Restriction enzymes	Reverse transcriptase, RNase, DNA polymerase

Size of library	Larger number of different types of clones	Smaller numbers of different types of clones in the library
Intactness of genes	A gene may be cut in between when there is a restriction site within the sequence	Intact genes are obtained
Sequences which may be studied	Used for studying introns or regulatory sequences associated with a gene	Used for studying the exact coding sequence of the gene
Patterns of gene expression	Cannot be used for studying physiological/developmental-based changes in gene expression	Can be used for tracing changes in patterns of gene expression under different developmental/physiological conditions
Screening of a gene	Can be used for the screening, isolation and characterisation of a gene when the cell type in which it is expressed is currently unknown	It can only be used when the cell type in which the gene is expressed is known
Frequency of fragment inserted into vector	Equal representation of fragments	Unequal proportions of different fragments as there were unequal levels of different mRNAs isolated