# Free Response Questions – Application Syllabus

**Instructions to students:**

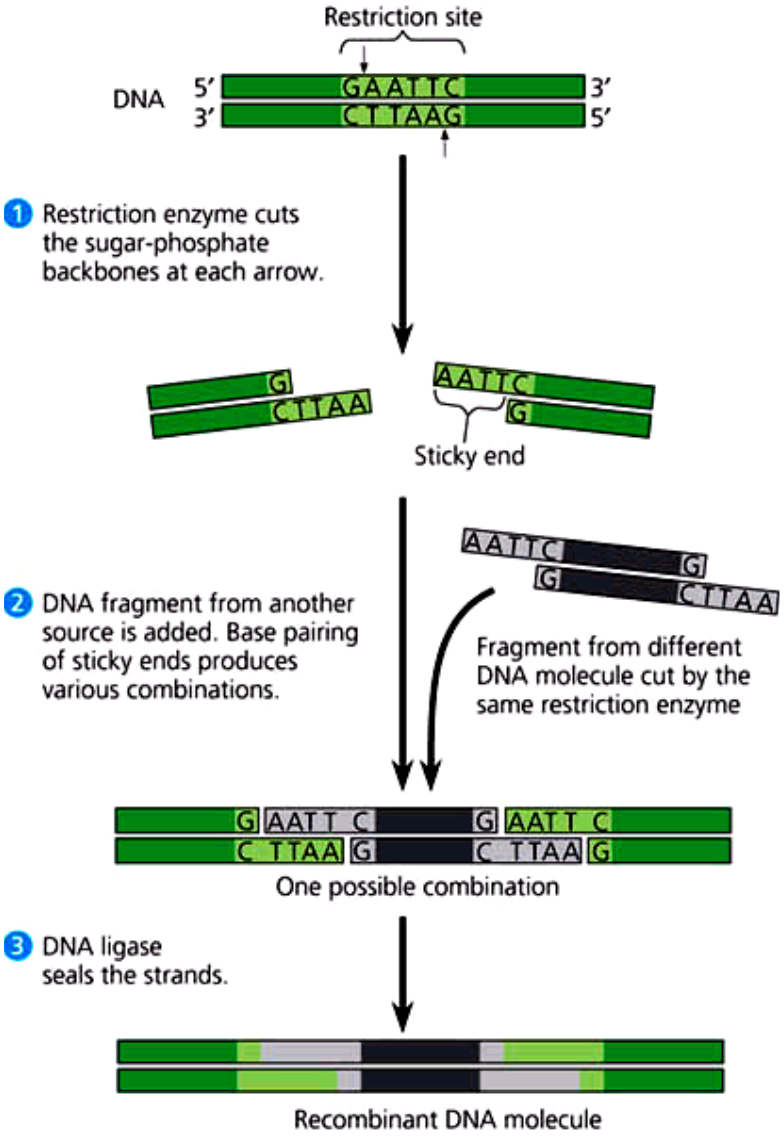
- **Revise** the relevant section/s of your lecture notes, tutorials, tests and exams after looking at each question.
- **Memorize** these relevant section/s before writing your answers. If you forget any point while writing, revise again and re-write the entire answer. Do not cheat by just looking at the notes and adding on the points to your answer – you are merely cheating your own learning!
- The marks allocated for each question is a rough gauge of how many points there should be in your answer – they are not the absolute number of points. Thus, if you have more points than the marks allocated, write everything down. **Highlight / underline all the key words and phrases in your answers.** This set of answers can serve as your revision notes in future, if it is well done.
- Pace your learning. Attempt **10 questions a day, everyday** and you will finish revising and memorizing the Biology syllabus before the end of Term 3!

## Isolating, Cloning and Sequencing DNA

1. Describe the natural function of restriction enzymes and its role in formation of recombinant DNA molecule. [8]

1. Restriction endonucleases / restriction enzymes are naturally found in bacteria;
2. They protect the bacterial cells against foreign DNA from viruses i.e. phages;
3. They work by hydrolysis / breaking of phosphodiester bonds of the foreign DNA;
4. The bacterial DNA is protected by the addition of methyl groups (-CH3) to adenines or cytosines;
5. Restriction enzyme has active site that recognise short specific nucleotide sequence / restriction site that are palindromic;

(b) Explain the formation of recombinant DNA molecule.




1. Recombinant DNA refers to DNA molecules containing DNA from two or more sources.

2. Restriction enzyme cleave the phosphodiester bonds in the sugar-phosphate backbone of both DNA strands at specific restriction sites creating sticky ends;
3. Gene-of-interest and plasmid is cut with the same restriction enzyme;
4. The single-stranded DNA can form hydrogen bonds with complementary sticky ends on the gene of interest by complementary base pairing;
5. DNA ligase catalyzes the formation of phosphodiester bonds between two nucleotides to produce recombinant DNA molecule

OR

6. Restriction enzyme cleave the phosphodiester bonds in the sugar-phosphate backbone of both DNA strands at specific restriction sites creating blunt ends;
7. Gene-of-interest and plasmid is cut with the same restriction enzyme;

8. ~~Linker DNA~~ Nucleotides are ~~is~~ added to 3' ends by **terminal transferases** to form ~~complementary~~ sticky ends / Specific linker DNA are added to blunt ends and cut with another restriction enzyme that create sticky ends;
9. The single-stranded DNA can form hydrogen bonds with complementary sticky ends on the gene of interest by complementary base pairing;
10. DNA ligase catalyzes the formation of phosphodiester bonds between two nucleotides to produce recombinant DNA molecule

Explain how the recombinant plasmid can be "put back" into the bacteria. [2]

- bacterial cells made competent (i.e able to take up DNA) by addition of calcium ions / $CaCl_2$ solution;
- brief heat shock treatment to create transient pores in bacterial cell membrane to allow entry/uptake of DNA; [Reject: electroporation – this is less common for bacteria]

2. [N2014/P3/Q5] Describe and explain the properties of plasmids that allow them to be used as DNA cloning vectors. [7]

1. Small circular DNA: Plasmids are induced to enter host cells easily by transformation;
2. Contain one or more selection marker genes (e.g. two antibiotic resistance genes or one antibiotic resistance gene + lac Z gene).: Allows for production of proteins giving a phenotype used to identify plasmids which have successfully incorporated the gene of interest and cells which had taken up the recombinant plasmid via transformation;
3. Insertional inactivation of one of the selection marker when the foreign gene is inserted is used to identify recombinant cells;
4. Contains unique restriction enzyme recognition sites: Allows cleavage and insertion of foreign genes using restriction enzymes and DNA ligase;
5. Contains own origin of replication: Allows them to replicate autonomously, independent of the bacterial chromosome and allow the binding of DNA polymerase to initiate replication of the plasmid with the gene of interest;
6. Exists in high copy number: Quantity of plasmid DNA that can be purified from each host cell is high / increases number of copy of genes;

3. [N2009/P3/Q4] Distinguish between a genomic DNA and cDNA library. [6]

| ***Features*** | ***Genomic DNA Library*** | ***cDNA Library*** |
|---|---|---|
| 1. Starting material; | Genomic DNA / complete set of genetic material | Mature mRNA present in a specific cell type at a specific stage |
| 2. Nature of genetic material; | Contains all coding and non-coding sequences, including introns, regulatory and intergenic sequences | Contains only coding sequence / exons |
| 3. Types of vectors used; | λ phage, BAC, YAC (spell in full) | Plasmid, λ Phage |

| | | |
|---|---|---|
| 4. Genetic engineering tools to obtain library; | Restriction enzyme to digest gDNA<br>DNA ligase for ligation of fragments to vectors<br>DNA fragments inserted into vectors | Reverse transcriptase to reverse transcribe mRNA to ssDNA<br>Primers to start synthesis of complementary strand<br>DNA polymerase to synthesise complementary strand<br>(RE, DNA ligase & vectors) |
| 5. Purpose; | Study regulatory sequences /<br>Introns + alternative splicing /<br>genes which expression pattern is unclear | Study expression of proteins of a cell type /<br>expression of a protein in different cell types /<br>expression of a protein through different developmental phases |

| Features | gDNA | cDNA |
|---|---|---|
| Starting Material | Genomic DNA:<br>Complete set of genetic material of an organism | Mature messenger RNA<br>Present in a specific cell type at a specific stage |
| Nature of Genetic Material | Contains coding & non-coding seq, e.g.<br>- Regulatory seq<br>- Introns<br>- UTRs<br>- Intergenic seq | Contains only exons |
| Vectors Used | Phage , BAC, YAC | Plasmid, Phage |
| Process | RE : for digestion of gDNA<br>DNA Ligase : for ligation of fragments to vectors<br>DNA fragment inserted into vectors | -RT : reverse transcribe mRNA to ssDNA<br>-Alkalis : to degrade mRNA<br>-Primers : to start syn of complementary strand<br>-DNA Pol : Syn of complementary strand<br>- RE, Ligase & vector |
| Purpose | -Study regulatory seq<br>-Study introns / alternative splicing<br>-Study genes which expression pattern is unclear | -Study expression of proteins of a cell type<br>-Study expression of a protein through different developmental phases<br>-Study expression of a protein in different cell types |

4. [N2014/P3/Q5] Eukaryotic genes cannot be expressed directly in the bacterial plasmid because of differences between prokaryotes and eukaryotes, including the presence of introns.
   Outline these problems and explain how they are overcome in order to allow expression of eukaryotic genes in plasmids within E.coli cells. [7]

**Problem 1:**

1. Eukaryotic genes possesses introns which prokaryotes do not have the cellular machinery for removal of introns; Prokaryotes lack spliceosome;
2. Mature mRNA is extracted and reverse transcriptase is then used to produce complementary DNA (cDNA), which does not contain intron sequence.

**Problem 2:**

3. Most eukaryotic proteins consist of various subunits which are covalently attached via post-translational modification e.g. insulin. / @ chemical modification of protein to be functional
4. Prokaryotes are unable to carry out post-translational modifications as they lack endoplasmic reticulum and Golgi apparatus;
5. Genes of each subunit is cloned into separate bacterial plasmid and transformed into different bacteria cells; / idea of additional step needed to chemically modified proteins after being synthesised.

**Problem 3:**

6. Prokaryotic translation is initiated by addition of N-formyl-methionine, which is the first amino acid added as compared to methionine in eukaryotes, so the protein synthesized may be different;
7. Polypeptides synthesized has to be treated to cleave off N-formyl-methionine and bacterial amino acids as it may result in conformational changes in the protein, rendering it non-functional;

@ -Adding of a prokaryotic/ bacterial promoter at the 5' end
→ to allow for binding of RNA polymerase and sigma factor for transcription initiation;

5. [N2013/P3/Q5] Therapeutic genes can be introduced into stem cells. Discuss why the genes used are more likely to be obtained from a cDNA library, than a genomic DNA library. [7]

1. cDNA is reverse transcribed from the mature mRNA present in a specific cell type at a specific stage;
2. gDNA is the complete set of genetic material found in a nucleus of a species;
3. cDNA contains only coding sequences as the introns would have already been removed during post-transcriptional modification;
4. thus, cDNA can be transcribed and translated into functional proteins without the need for additional modification processes;
5. cDNA library is a smaller library compared to the gDNA library and thus it would be easier to obtain the gene of interest;

6. cDNA gene sequences are smaller and thus it is more likely that the entire sequence can be found in a single clone/ storage vector/ plasmid;
7. gDNA library is very large, thus it is more difficult to locate gene of interest;
8. unlikely for an entire coding region to be found in a single clone/ storage vector/ plasmid;
9. thus additional process of ligation from different fragments stored in different plasmids is required before entire coding sequence is complete;

6. [N2014/P3/Q5] A eukaryotic gene is isolated with blunt ends. Outline the procedures for cloning this gene in a bacterial plasmid. [6]

1. Complementary sticky ends are added via [max 3]

Method 1: Addition of specific linker DNA to both blunt ends of the gene fragment

- Specific linker DNA is a short, synthetic double-stranded oligonucleotide which contains a specific restriction site of a particular restriction enzyme.
- Added to both the blunt ends of the DNA fragment using DNA ligase
- Linker DNA is then further cut by the restriction enzyme which recognises the restriction site to produce sticky ends.

**OR**

Method 2: Addition of deoxyribonucleotides by terminal transferase

- Terminal transferases catalyse addition of deoxynucleotides at the 3' ends of a DNA molecule without using a template.
- Cytosine nucleotides can be added to one blunt-ended DNA fragment and guanosine nucleotides can then be added to the plasmid to produce complementary sticky ends.

2. Plasmid is cut with same restriction enzyme as the eukaryotic gene

3. Restriction enzyme hydrolyses the phosphodiester bonds in the sugar-phosphate backbone of both DNA strands at specific restriction sites creating complementary sticky ends;

4. Eukaryotic gene is then mixed with plasmid DNA to allow formation of hydrogen bonds between complementary sticky ends

5. DNA ligase is added to catalyse formation of phosphodiester bonds between the gene fragment and the plasmid, forming recombinant plasmid.

7. [N2009/P3/Q4] Outline the large-scale production of a **named** important protein by genetic engineering. [8]

1. Large amounts of human growth hormone mature mRNA is extracted from the pituitary gland;
2. Reverse transcriptase is then used to produce cDNA and sticky ends are added to cDNA;
3. Vector plasmid containing selection marker genes of ampicillin resistance gene and *lac Z* gene with multiple cloning site can be used;
4. Human growth hormone cDNA and plasmids are cleaved with the same restriction

enzyme to produce complementary sticky ends and ligated using DNA ligase to form recombinant DNA;
5. Recombinant plasmid are introduced into bacterial host, E. coli via transformation;
6. Bacteria are made competent by treating with calcium chloride and mix with recombinant plasmid and heat shock followed by ice / by electroporation;
7. Bacteria are grown in agar plate containing ampicillin, X-gal and IPTG and in nonrecombinant plasmid, lacZ gene remains intact and functional β-galactosidase is synthesized. X-gal will be cleaved by β-galactosidase and a blue colony is observed;
8. Insertional inactivation of lac Z gene occurs in recombinant plasmids where human gene interrupts Lac Z gene / recombinant plasmids containing human gene produce nonfunctional β-galactosidase;
9. White colonies are picked and cultured in fermenters;
10. After translation, the protein is transported to the periplasmic space of bacterial cell due to the signal peptide. The signal peptide is cleaved at the periplasmic space;
11. The cell is placed into a hypotonic solution. This disrupts the outer membrane and releases the protein into the solution;
12. Further purification is carried out to obtain pure and sterile hormone;

Outline 2 important proteins that can be produced by genetic engineering technique (e.g. human growth hormone and insulin).

***Insulin***

1. As *Escherichia coli* is a prokaryote, it is unable to carry our post-transcriptional or post-translational modification;
2. Amino acid sequence of A chain and B chain of insulin are used to synthesize artificial insulin gene / DNA sequence without introns;
3. A strong bacterial promoter is added at the 5' end for each of the DNA sequence of A and B chain.
4. DNA fragments for the A and B chain are amplified by Polymerase Chain Reaction;
5. Genes for A-chain and B-chain are cloned into two separate bacterial plasmids;
6. Vector plasmid must contain selection marker genes (e.g. Ampicillin resistance gene and tetracycline resistance with multiple cloning site / Ampicillin resistance gene and lac Z gene with multiple cloning site);
7. Human insulin gene and plasmids are cleaved with the same restriction enzyme to produce complementary sticky ends;
8. Hydrogen bonds formed between complementary sticky ends and insulin gene and plasmid are joined using DNA ligase to form phosphodiester bonds;

***Transformation***

9. Recombinant plasmid are introduced into bacterial host, E. coli via transformation;
10. Bacteria are made competent by treating with calcium chloride and mix with recombinant plasmid and heat shock followed by ice / by electroporation;
11. Temporary gaps in membrane of *E.coli* appear allows uptake of plasmid DNA from the surrounding by bacteria;

***For selection, write either antibiotic resistance genes or pBluescript***

*Selection using antibiotic resistance genes*

12. Replica plating : Sterile velvet cloth pressed on first (ampicillin) plate transfers bacteria to second (tetracycline) plate, ensuring the same position of bacteria colonies with respect to one another;
13. Insertional inactivation of tetracycline resistance gene occurs in recombinant plasmids where human gene interrupts tetracycline resistance gene / recombinant plasmids containing human gene is not resistant to tetracycline;
14. Colonies which survive ampicillin but not tetracycline are picked and cultured;

**Replica Plating**

The plasmid contains ampicillin-resistance gene as first selectable marker and tetracyclin-resistance gene used as second selectable marker.

Transformed bacterial cells (containing a recombinant or non-recombinant plasmid) are able to survive and grow on nutrient agar with ampicillin.

Restriction enzyme cuts the plasmid at restriction site within the gene coding for tetracycline resistance.

If human insulin gene is inserted into restriction site in the tetracycline-resistance gene, tetracycline-resistance gene will be disrupted / insertional inactivation.

Non-functional gene product produced, bacterial cells unable to survive and grow on agar plate containing tetracycline.

Transformed bacterial cells with recombinant plasmids are present on the ampicillin plate but absent in the tetracycline plate.

*Selection using pBlueScript*

15. Bacteria are grown in agar plate containing ampicillin, X-gal and IPTG and in non-recombinant plasmid, lacZ gene remains intact and functional β-galactosidase is synthesized. X-gal will be cleaved by β-galactosidase and a blue colony is observed;
16. Insertional inactivation of lac Z gene occurs in recombinant plasmids where human gene interrupts Lac Z gene / recombinant plasmids containing human gene produce non-functional β-galactosidase;
17. White colonies are picked and cultured;

***Purification***

18. Bacteria lysed and A-chain or B-chain subunits/polypeptides are harvested and purified separately;
19. Polypeptides treated to cleave off N-formyl-methionine and bacterial amino acids;
20. A-chain and B-chain subunits incubated together under suitable conditions to form disulfide bonds and synthesise functional insulin;

***Human growth hormone***

*21.* As *Escherichia coli* is a prokaryote, it is unable to carry our post-transcriptional or post-translational modification;

22. Eukaryotic genes possess introns which bacterial cells do not have the cellular machinery for splicing of introns.
23. Large amounts of human growth hormone mature mRNA is extracted from the pituitary gland;
24. Reverse transcriptase is then used to produce cDNA and sticky ends are added to cDNA;
25. Vector plasmid must contain selection marker genes (e.g. Ampicillin resistance gene and tetracycline resistance with multiple cloning site / Ampicillin resistance gene and lac Z gene with multiple cloning site);
26. Human growth hormone cDNA and plasmids are cleaved with the same restriction enzyme to produce complementary sticky ends;
27. The cDNA is then joined to the plasmid using DNA ligase, at a site after a bacterial promoter and signal peptide;
28. The recombinant plasmids are transformed into bacterial host cells, *E. coli*. (Refer to transformation;; 2m)
29. Selection of bacterial colonies containing recombinant plasmids; (Refer to Selection;;; 3m)
30. After translation, the protein is transported to the periplasmic space of bacterial cell due to the signal peptide. The signal peptide is cleaved at the periplasmic space;
31. The cell is placed into a hypotonic solution. This disrupts the outer membrane and releases the protein into the solution;
32. Further purification is carried out to obtain pure and sterile hormone;

8. Describe the polymerase chain reaction (PCR) and explain the advantages and limitations of this procedure. [8]

***Description of PCR***

1. PCR is a process used to, amplify DNA / synthesize large amounts of DNA from a minute amount of starting material;
2. Thermostable, DNA polymerase / *Taq* polymerase is used;
3. Two DNA primers / single-stranded DNA molecules which are complementary to the flanking sequence / start and end of the segment to be amplified;
4. Denaturation: heating it to 95$^{o}$C + involves denaturation of double-stranded DNA into single-stranded DNA / breaking of hydrogen bonds between nitrogenous bases of the two strands;
5. Annealing: temperature lowered to 50 – 60$^{o}$C + allows primers to anneal via complementary base pairing due to hydrogen bonding;
6. Elongation: temperature increased to 72$^{o}$C + *Taq* polymerase adds nucleotides to free 3'-OH end of primers using the DNA molecule as a template;
7. Sequential process of denaturation-annealing-elongation is repeated many times / products of the previous reaction are used as reactants in the next cycle;

***Advantages of PCR***

8. Large amounts of DNA can be produced from very minute amount of starting materials;
9. Large amounts of DNA can be produced in a short period of time;
10. Specific sequences of DNA can be amplified by using of specific primers;

***Limitations of PCR (max 2 marks)***

11. Knowledge of the DNA or amino acid sequence of desired gene or protein is needed to synthesise flanking nucleotide primers;

12. Non-target DNA sequence may be amplified instead of the desired sequence as primers are short nucleotide sequences and may not be specific enough;
13. *Taq* polymerase, of bacteria origin, does not perform proofreading, thus, there may be mistakes in complementarity of the nucleotides added;

9. [N2011/3/Q5] Explain how gel electrophoresis is able to separate fragments of DNA [6]
Explain how gel electrophoresis is used to analyse nucleic acids and proteins and to distinguish between two alleles of a gene. [8]

***(a)*** ***T:*** *Explain how / account of the process with reasons* ***T:*** *Gel Electrophoresis*
***F:*** *Separation of DNA fragments*

- Porous structure of agarose gel allows it to act as a molecular sieve;
- The agarose gel is then placed within an electrophoresis chamber filled with buffer solution to prevent changes in pH that arise due to electric field (since charge of DNA dependent on pH);
- Buffer solution provides ions to carry a current so that it can conduct electricity;
- DNA samples are mixed with loading dye to give an indication of the progress of electrophoresis and to allow DNA to sink into the wells during loading of DNA;
- Glycerol is usually added to loading dye as it is dense and allows the DNA to sink into the wells.
- DNA samples are loaded into the wells at the negative electrode;
- Direct current (DC) is applied, DNA is negatively-charged due to its sugar-phosphate backbone and they move towards the positive electrode;
- Separation of DNA fragments is based on size only in an electric field/ the rate at which the DNA fragment travels is inversely proportional to its size;
- Smaller fragments will travel further (@ vice versa)
- Ethidium bromide can be added in the buffer or agarose gel. When viewed under UV radiation, DNA bands fluoresce;
- The DNA ladder comprising a mixture of pre-digested DNA fragments of known size is used to estimate the size of each DNA fragments.

Examiner's comments:

Many candidates demonstrated a clear knowledge of how gel electrophoresis can separate DNA fragments. The roles of the buffer and loading dye were often described in insufficient detail.

Explain how gel electrophoresis is used to analyse DNA.

1. Gel electrophoresis separates DNA fragments based on size / number of nucleotides by passing them through a gel in an electric field / direct current;
2. The porous structure of agarose gel allows it to act as a molecular sieve;
3. Rate at which a DNA fragment travels is inversely proportional to its size / Longer DNA fragment moves at a slower rate / Shorter DNA fragment moves at a faster rate;
4. Samples are loaded into wells and gel is orientated such that wells are near to the negative electrode;

5. Since DNA is negatively-charged due to its sugar-phosphate backbone, it will move towards positive electrode;
6. DNA fragments of the same length appear at the same band position;
7. The DNA can be visualised by staining the gel with ethidium bromide and when viewed under UV radiation, DNA bands fluoresce.

10. Outline the process of nucleic acid hybridisation and explain how it can be used to detect and analyse restriction fragment length polymorphism (RFLP). [8]
[N2011/3/Q5] Explain what is meant by restriction fragment length polymorphism (RFLP) and how it can be detected. [6]

(a)

**Nucleic Acid Hybridization Process**

1. **Alkali denaturation** using sodium hydroxide is carried out after gel electrophoresis;
2. causing double-stranded DNA to become single stranded/ denatured by disrupting the hydrogen bonds between complementary nucleotide bases; ® hydrolyse
3. **Fluorescently / radioactively labelled single-stranded DNA probes** are then used to anneal to the ssDNA via complementary base pairing (@ref complementary probes) by forming hydrogen bonds;
4. for visualisation via **autoradiography** (@ref visualisation using X-ray film - the nitrocellulose membrane is exposed to a piece of X-ray film and the band containing the gene of interest will show up as a dark band on the autoradiogram);

**How it is used to detect and analyse RFLP**

5. In RFLP analysis, DNA is first cut/ cleaved by a **restriction enzyme**
6. **Gel electrophoresis** is carried out to separate DNA fragments by size by passing them through a gel in an electric field / direct current;
7. And then transferred onto a nitrocellulose membrane via capillary action during **Southern blotting**
8. Incubating the nitrocellulose membrane in a solution containing the radioactive-labelled DNA probe. The single stranded radioactively labelled DNA probe binds complementarily to the DNA fragment of interest via hydrogen binding;
9. Due mutations which change RE sites, restriction site may be altered such that it is no longer recognised by its restriction enzyme / different tandem repeats/VNTRs/minisatellites between restriction sites, **for different alleles of the same gene loci** give rise to different fragment sizes;
10. Thus bands of various sizes would appear, resulting in a unique / specific band pattern characteristic of each RFLP allele

**Marker's comments:**

Bands =/ Fragments

Focus on the principle of gel electrophoresis rather than just describing the technique in detail.

(b)

[Max 3 – Definition]

- Restriction fragment length polymorphism (RFLP) is a variation in the DNA sequence of a genome.
- It arises due to differences in nucleotide sequences at restriction sites on homologous chromosomes of individuals.
- A restriction enzyme site may be altered such that it is no longer recognised by its restriction enzyme.
- giving rise to different fragment sizes when cut by the same restriction enzyme;
- Restriction fragment pattern is unique and differs between homologous chromosomes of an individual and between individuals such that the number and position of the bands present may differ.
- Reason for polymorphism (point mutations at restriction site, variable number of tandem repeats)

- A RFLP can be in coding or non-coding DNA although studies concentrate on the latter.
    - Why do non-coding DNA sequences tend to have small nucleotide sequences differences between individuals?
    - Since these sequences do not code for any protein or RNA product, mutations occurring here are tolerated and are allowed to accumulate over time.

[Max 3 – Detection of RFLP]

After cutting DNA sample with restriction enzyme, carry out gel electrophoresis to separate fragments followed by Southern blotting and nucleic acid hybridization;

Carry out gel electrophoresis to separate DNA fragments based on size / number of nucleotides by passing them through a gel in an electric field / direct current;

Southern blotting is carried out to transfer the separated DNA fragments onto a nitrocellulose membrane before nucleic hybridisation can be conducted;
First, the double-stranded DNA strands are denatured in sodium hydroxide to from single-stranded DNA;
Next, the bands on the gel are transferred to a nitrocellulose membrane via capillary action;

The nucleic acid hybridization step involves incubating the nitrocellulose membrane in a solution containing the radioactive-labelled DNA probe;
The single stranded radioactively labelled DNA probe binds complementarily to the DNA fragment of interest via hydrogen binding;

In autoradiography, the nitrocellulose membrane is exposed to a piece of X-ray film and the band containing the gene of interest will show up as a dark band on the autoradiogram, resulting in a specific banding pattern;

11. Describe how restriction fragment length polymorphism analysis can be carried out. [15]

12. Explain how RFLP analysis facilitated the process of
    (i) genomic mapping, [6]
    (ii) diseases detection, [4]
    (iii) DNA fingerprinting [4]

    [N2011/3/Q5] Explain how RFLP analysis has helped the process of detecting a named genetic disease. [8]

(a)

(1 max for intro)

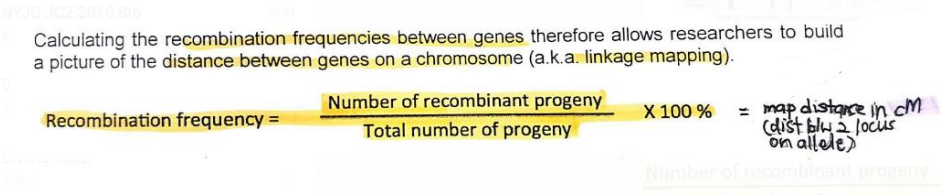
1. Restriction fragment length polymorphism (RFLP) is a variation in the DNA sequence of a genome that can be detected by breaking the DNA fragments with restriction enzymes and analyzing the size of the resulting fragments by gel electrophoresis.
2. Due mutations which change RE sites, restriction site may be altered such that it is no longer recognised by its restriction enzyme / different tandem repeats/VNTRs/minisatellites between restriction sites, **for different alleles of the same gene loci** give rise to **different fragment sizes**;
   - RFLPs at a particular chromosomal locus -> alternative forms (analogous to alleles) that differ in **nucleotide seq** ( due to point mutations at restriction site) or have **variable number of tandem repeats**
   - Restriction fragment pattern is unique and differs between homologous chromosomes of an individual and between individuals such that the number and position of the bands present may differ.
   - RFLP can be detected by cleaving DNA with restriction enzymes and analyzing the size of the resulting DNA fragments by gel electrophoresis and nucleic acid hybridisation;

   *Again remember to STATE CLEARLY the name of the method before jumping in straight to the detailed process.*

3. Genome mapping is the mapping of genes to specific locations on chromosomes within a genome.

4. In mapping a large genome, the first stage is to construct a linkage map of several thousand RFLP genetic markers spaced throughout the chromosomes

5. to determine the order and the relative distances between these markers based on recombination frequencies.




6. The frequency in which two RFLP markers are inherited together is a measure of closeness of the two loci on a chromosome /

   the frequency at which two RFLP markers or a RFLP marker and a certain allele of a gene undergo crossing-over is a measure of the relative distance between the two loci on a chromosome
   - RFLP loci which are heterozygous in individuals may be used as markers in chromosomal mapping by crossing them;

7. 1 % = 1 centiMorgan OR

   increase frequency of inheritance indicates increase closeness of loci / the lower (higher) the frequency of crossing over/ recombination frequency between the two gene loci, the closer ( further apart) the two loci;

8. By performing analysis on many other pairs of RFLP markers and with other cytogenetic studies, the researcher is able to come up with a detailed map of the genome;

9. RFLPs can be mapped against gene loci once many RFLPs have been found/ RFLPs can be used as positions from which genes of interest can be found;

**(Max 2m for e.g.)**

1. RFLP markers are inherited in a Mendelian fashion;
2. RFLP analysis can be performed on RFLP markers located on two different loci of the genome
    1. Genes are linked to RFLPs / can be detected as RFLPs / represented by RFLPs
    2. Parents with known genotype at two RFLP loci are crossed to find recombinant frequency

3. 2 individuals, each being homozygous for each RFLP marker, but have different band patterns for each particular RFLP marker, can be crossed to produce heterozygous offsprings;
4. Heterozygotes are then crossed with one of the homozygous parent
5. offspring can inherit pairs of RFLP markers like the parents or inherit recombinant pairs of RFLP markers;
6. If the 2 gene loci are not linked, number of offspring showing the four phenotypes will be of ratio 1:1:1:1;
7. If the two gene loci are closely linked, the proportion of offspring with recombinant phenotypes will be less than parental phenotypes;

| Type of Chromatid | Alleles |
|---|---|
| Parental | RFLP 1 and 2 |
| Parental | RFLP 3 and 4 |
| Recombinant | RFLP 1 and 4 |
| Recombinant | RFLP 3 and 2 |

When these two flies mate, the frequency of the four possible progenies can be measured. From this information, the genetic distance between the two RFLP loci (upper and lower) can be determined (Fig 18):

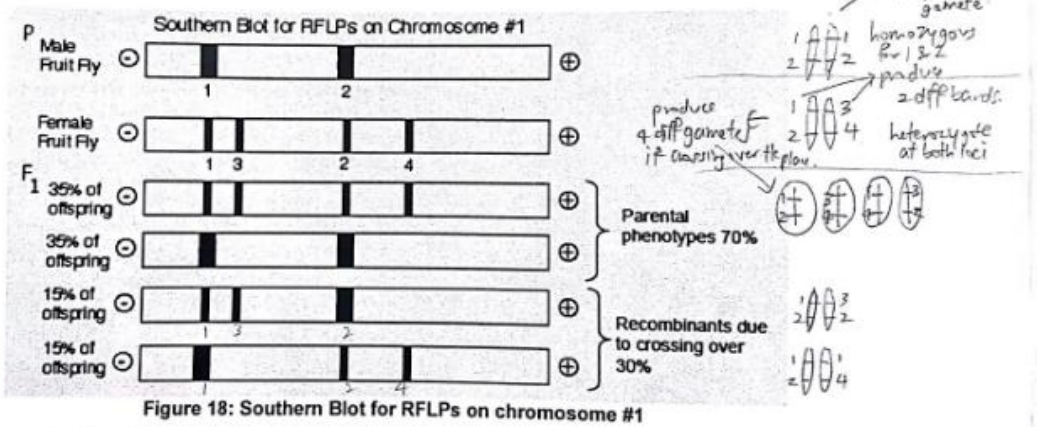


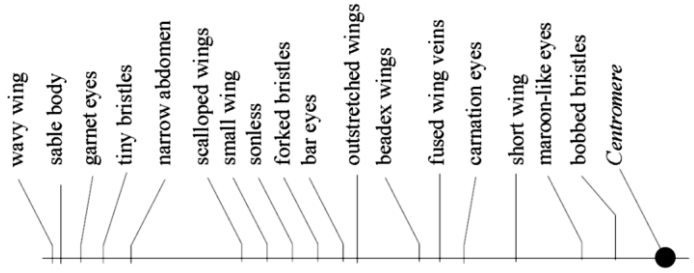

**Figure 18: Southern Blot for RFLPs on chromosome #1**

In this example, 70% of the progeny were produced from parental genotype gametes and 30% were produced by recombinant genotype gametes. Therefore, the recombination frequency is 30%. In terms of distance, this translates to the two RFLP loci being 30 centiMorgans (cM) apart from each other. The position of the two loci can thus been mapped on the chromosome, relative to each other.

Many students mixed up with the other application of RFLP (disease detection), whereby the RFLP locus is tightly linked to the disease allele. In this case however we are looking at linkage between RFLP markers; **how tightly linked** are the markers instead of just whether they are linked (all the genes are linked since we are mapping the genes found on each chromosome)

**What is a chromosome map? [1]**

Before the genome of an organism is sequenced, preliminary mapping is usually first carried out. Shown below is a chromosome map for the *X* chromosome of the fruit fly *Drosophila melanogaster*.



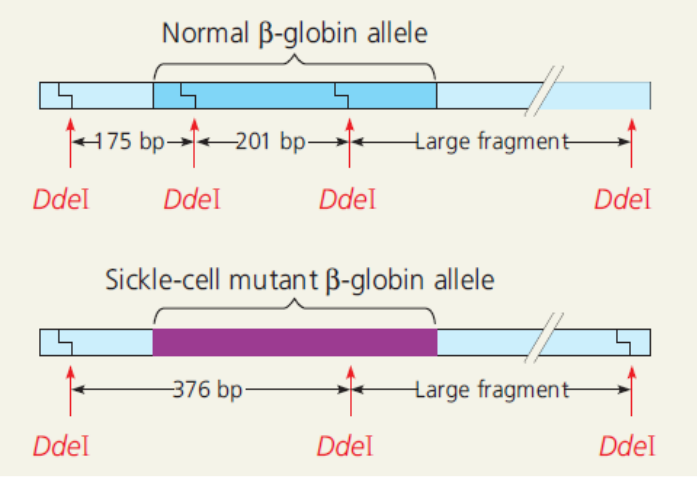

***Limitations of using linkage mapping techniques to map the human genome:***

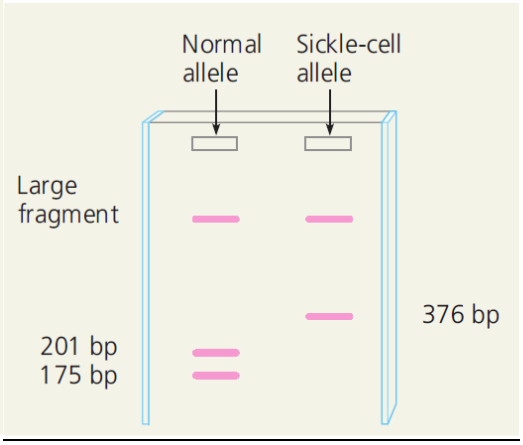
- Linkage mapping requires crossing over to obtain recombinants
  - unethical for human beings
- Linkage mapping requires short generation time in the studied organism
  - human beings's generation time is too long
- Linkage mapping involve observable phenotypes
  - human genome mapping aims to find all phenotypes, including non-observable phenotypes
- Linkage mapping involve heritable diseases
  - human genome mapping aims to find all types of diseases including non-heritable

***Disease detection (in direct detection, single nucleotide substitution is the disease-causing mutation (known) and occurs within the coding region)***

1. Some RFLPs are known to be closely linked to alleles that are related to a particular disease;
2. Example: Sickle-cell anaemia is a genetic disease caused by point mutation of a single base pair in the β-globin gene. A thymine is substituted for an adenine, resulting in a hydrophobic valine being coded for instead of hydrophilic glutamic acid
3. Mutated base pair happens to lie within the restriction site of a restriction enzyme, *Dde*I
4. Normal β-globin allele has four restriction sites for *Dde*I , therefore producing 3 fragments upon digestion: 2 shorter ones (291bp and 175bp) and a much longer one
5. Mutant β-globin allele only has three restriction sites for *Dde*I
6. Produces 2 fragments upon digestion: 1 shorter fragment (376bp) and a much longer one
7. RFLP analysis shows different band patterns formed by normal allele and sickle cell allele; When analysed by gel electrophoresis, the band patterns (number of bands and position of bands) formed by normal allele and sickle cell allele are different.

*Illustrate with diagrams showing position of restriction sites / possible banding patterns




***Dde*I restriction sites in normal and mutant β-globin allele**

Protocol:

-Genomic DNA from an individual with sickle-cell anaemia and a normal individual are digested with *Dde*I/ *Mst*I. Different sized restriction fragments are produced and separated using agarose gel electrophoresis. Southern blot is then carried out using a probe complementary to part of the β-globin gene region.

-Results – presence of a diseased allele in an individual can be seen from characteristic banding pattern in Southern blots.

- In indirect detection, the single nucleotide substitution occurs at regions flanking the gene responsible for the disease and is tightly linked and hence inherited together with the disease-causing mutation.

  e.g. in the disease **phenylketonuria (PKU)**[4], the RFLP is used to detect the disease **from changes in the DNA sequence flanking** the 3' region of the gene phenylalanine hydroxylase (i.e. outside the gene), rather than from changes within the gene itself. Because this RFLP is tightly linked to the mutation within the gene itself, it can be used as a "proxy" to the actual gene mutation that causes the disease (Fig 12).

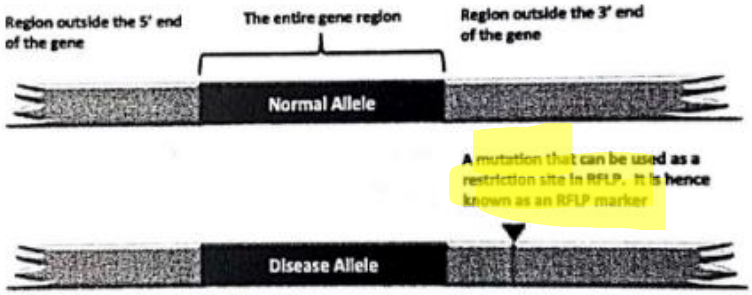



**Figure 12: Indirect detection using RFLP** - In this case the mutation affecting the function of the disease allele is unknown although it is present within the gene. However an RFLP marker (a restriction site that can be distinguished through the process of RFLP) that is outside the disease allele but ***closely linked*** can be used to detect the disease allele indirectly. The diagram above shows a pair of alleles of the same gene. One that is normal and another with a disease-causing mutation.

Note: *Why not just detect the mutation within the gene itself?*

The reason is because at the time the mutation within the gene itself hadn't been discovered yet, so the best alternative was to use the RFLP in the flanking region that is tightly linked to the disease causing mutation. However, when the actual disease causing mutation is discovered, it is better to use that as it is more accurate.

*Detecting* ***short tandem repeats (STRs)***

- Short tandem repeats (STRs) are polymorphic loci that can differ in the number of repeating units.
- It is also possible for a STR locus to be tightly linked with a disease-causing gene locus. Hence, a particular number of repeats at the STR locus could be linked with the presence of the disease-causing mutation (indirect detection – similar to how an SNP can be linked to a disease-causing mutation in PKU).

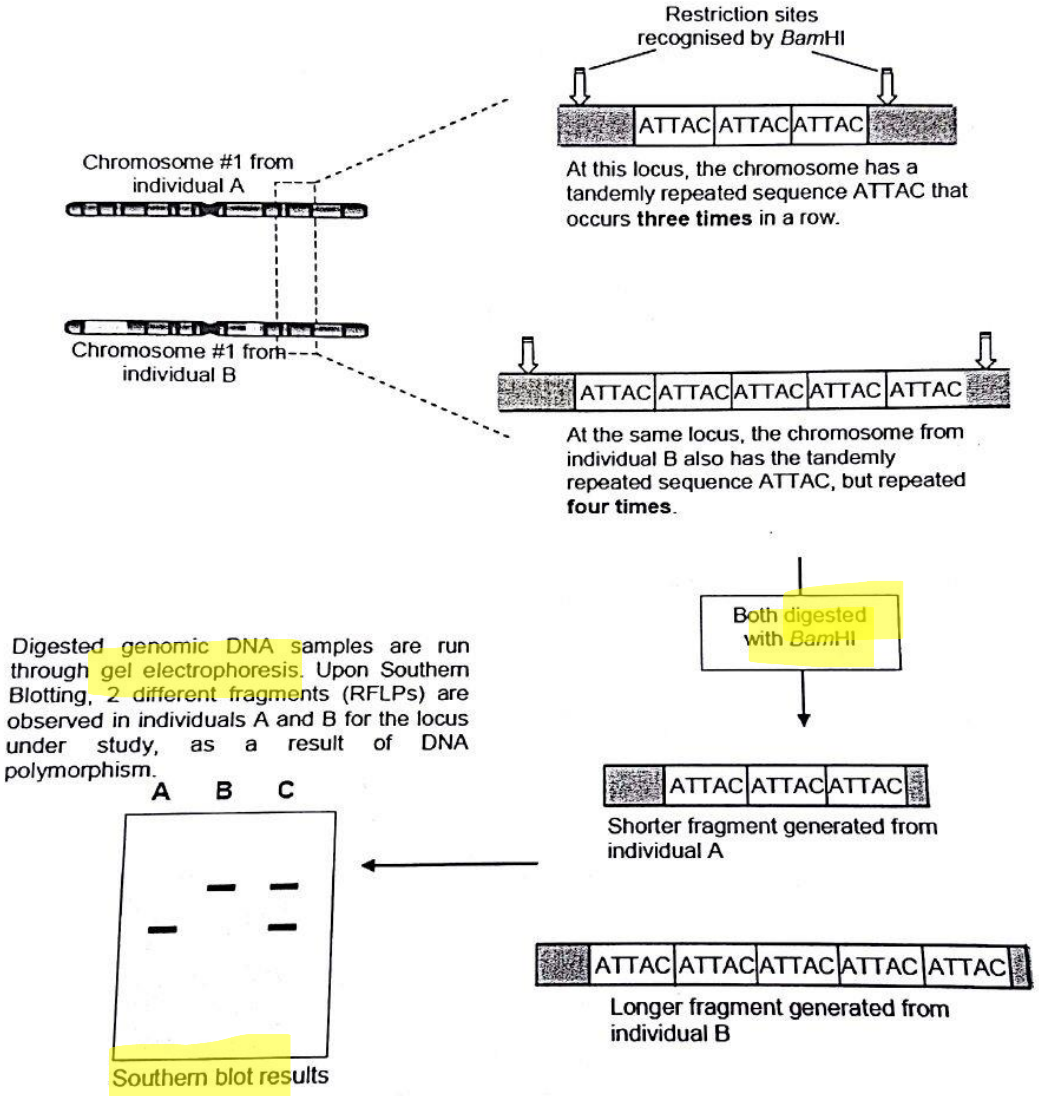
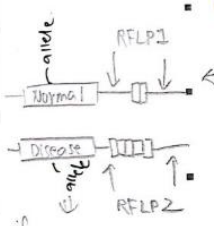


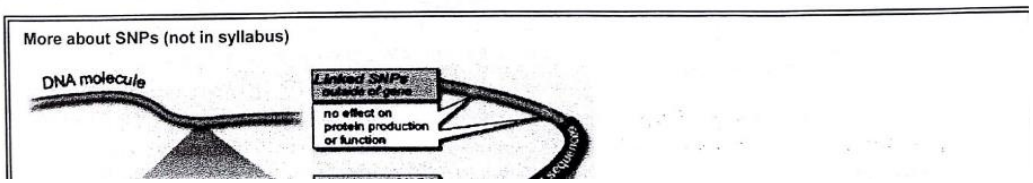

**Figure 14: DNA polymorphism in terms of tandem repeats can result in different restriction fragments.** Genomes from 2 individuals A and B, are cut using *Bam*HI. The 2 individuals differ in the number of tandem repeats (of the sequence ATTAC) at a particular locus on chromosome #1. As a result of the larger number of tandem repeats present, individual B generates a longer restriction fragment which travels a shorter distance down the gel during electrophoresis. The 2 different-sized fragments are detected using the same probe which has a sequence complementary to segments of both fragments e.g. ATTAC.

5. *Advantages:*
   - *Fast - it is not necessary to actually sequence the DNA of multiple individuals to find SNPs. Restriction digest is sufficient.*
   - *Specific - RFLP serve as specific genetic markers to diagnose genetic disorders.*

6. Limitations of RFLP in disease detection:
   - More than one mutation may cause a disease (e.g. cystic fibrosis). In such cases, a negative test for 1 point mutation does not necessarily rule out presence of mutations at other loci that may also cause the disease;
   - A cross over event may occur between adjacent disease-associated SNPs and the disease-causing mutation within an allele. In the case of indirect detection of a disease the marker becomes less reliable;
   - A disease could be caused by multiple gene interactions; No gene has yet been discovered for a disease or that the gene has yet to be sequenced for molecular tests to be available.




if crossing over occurs, disease allele is linked to RFLP1 instead of RFLP2; will it be able to detect (false negatives)



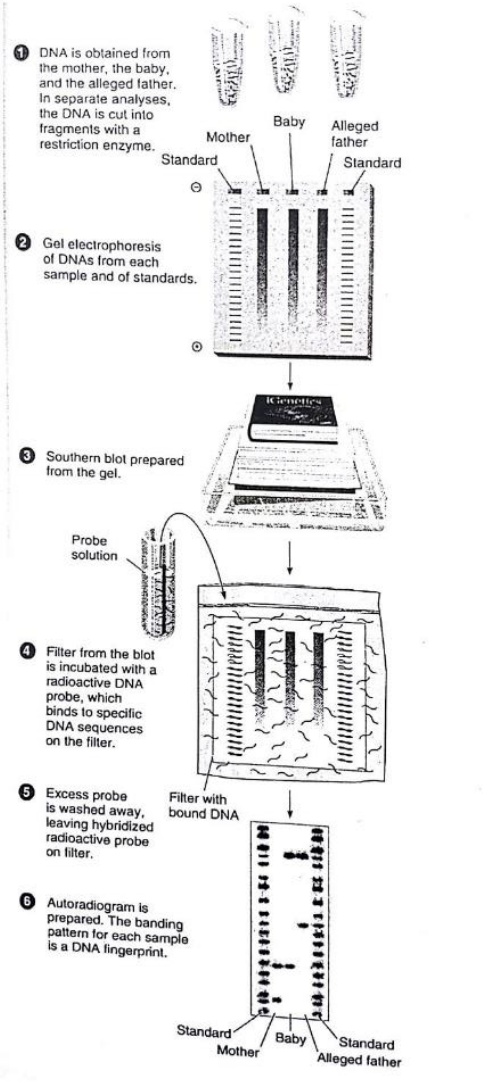

***DNA fingerprinting***

1. Polymorphism is usually due to variable number of tandem repeats (VNTR) present in intergenic regions of the genome;
2. **Probes for STRs** (microsatellites 2-6 bp seq repeated 15-100x) **or VNTRs** (minisatellites 20-100 bp seq repeated up to thousands x) are used. A restriction enzyme that **cuts on either side of a STR or VNTR locus** is required.
   a. These tandem repeats of a particular DNA seq occur in **varying numbers in different individuals –** generate a **unique genetic profile/ DNA fingerprint for each individual**
   b. Why do non-coding DNA sequences tend to have small nucleotide sequences differences (**DNA polymorphisms** – either in **nucleotide sequence** or in **numbers** of tandemly repeated nucleotide units) between individuals?
      Since these sequences do not code for any protein or RNA product, mutations occurring here are tolerated and are allowed to accumulate over time.
3. The number and position of the bands observed by RFLP analysis is referred to as the "DNA fingerprint" of the individual;
4. Example: In forensic science, any small amount of DNA left by a culprit in the crime scene can be used as evidence for guilt;
5. Example: In paternal testing, parents of a child can be identified by comparing the DNA fingerprints of the parents and the child as a child's band patterns should be a combination of his parents' band patterns;
6. Example: DNA fingerprinting is used to identify the deceased after a mass disaster by comparing DNA fingerprint of the deceased with those obtained from close relatives;

Steps for process of DNA/ genetic fingerprinting:

7. Extract DNA from a sample of ____
8. DNA sequences may contain different restriction sites due to mutations, giving rise to different fragment sizes and different number of fragments when cut by the same restriction enzyme;

9. Carry out gel electrophoresis to separate DNA fragments based on size / number of nucleotides by passing them through a gel in an electric field / direct current + bands contain fragments of same length / size;
10. Southern blotting is carried out to transfer the separated DNA fragments onto a nitrocellulose membrane before nucleic hybridisation can be conducted;
11. First, the double-stranded DNA strands are denatured in sodium hydroxide to from single-stranded DNA;
12. Next, the bands on the gel are transferred to a nitrocellulose membrane via capillary action;
13. Nucleic Acid Hybridisation step involves incubating the nitrocellulose membrane in a solution containing the radioactive-labelled DNA probe;
14. In autoradiography, the gel is exposed to a piece of photographic film and the band containing the gene of interest will show up as a dark band on the autoradiogram. Bands are visualised using autoradiography and appear with specific banding patterns; The number and position of the bands observed by RFLP analysis is referred to as the "DNA fingerprint" of the individual;
15. Different tandem repeats/VNTRs/minisatellites between different alleles of the same gene loci give rise to different fragment sizes



(b)

- Sickle-cell anaemia is a genetic disease caused by point mutation of a single base pair in the β-globin gene. A thymine is substituted for an adenine, resulting in a hydrophobic valine being coded for instead of hydrophilic glutamic acid
- Mutated base pair happens to lie within the restriction site of a restriction enzyme, *Dde*I
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  *Illustrate with diagrams showing position of restriction sites / possible banding patterns

13. [N2008/P3/Q4] Discuss the aims/ goals of the human genome project. [6]

1. identify all the approximately 20,000-25,000 genes in human DNA by 2003;
2. determine the sequences of the 3 billion chemical base pairs that make up human DNA;
3. store this information in databases and make it freely and totally accessible;
4. improve tools for data analysis, especially for sequencing and comparison of genomes of other organisms;
5. transfer related technologies to the private sector and improve training and manpower;
6. address the ethical, legal, and social issues that may arise from the project;

*(Common mistake: Students incorrectly gave examples of the uses of knowledge gained from the human genome project rather than selecting the goals.)*

14. [N2008/P3/Q4] Discuss the benefits of the human genome project. [8]

***(Min 2 points from each category + elaboration / describe one example!)***

7. Molecular Medicine:
   - Improved diagnosis of disease + **elaborate (physical map)** /
   - **Earlier detection** of genetic predispositions to disease + **elaborate (markers)**/
     - Many diseases like Alzheimer disease, familial breast cancer, cystic fibrosis etc. can be detected at an early stage. Early detection opens the door for early treatment.
   - Rational drug design **+** allows scientists to design drugs to target a specific gene/protein /
   - Gene therapy and control systems for drugs **+**
     - identification of genes associated with human genetic diseases and a better understanding of the genetic basis of disease may increase the potential to treat genetic and acquired diseases by gene therapy;

- Gene therapy allows **defective genes** to be either **replaced** by normal functional genes or supplemented with other genes to prevent a disease taking its course /
- Pharmacogenomics "custom drugs";
- The HGP allows scientists/doctors to know which genes/alleles affect an individual's response to a drug and how these genetic differences affect the way the individual reacts to a drug
- This may eventually allow doctors to prescribe the right/effective drug in the right dosage for each individual
- It is also theoretically possible to design drugs based on molecular information that would work better and cause fewer side effects
- Many customised drugs work by targeting specific proteins within our body. Knowledge of the gene sequence that codes for these specific proteins allows us to deduce three-dimensional conformation of these proteins and therefore design drugs that are would target these proteins

8. DNA Forensics (Identification)
   - Identify potential suspects whose DNA may match evidence left at crime scenes **+** elaborate on DNA fingerprinting using 13 short tandem repeat loci to develop DNA profile/
   - Exonerate persons wrongly accused of crimes **+** elaborate using DNA fingerprinting/
   - Identify crime and catastrophe victims **+** elaborate using DNA fingerprinting/
   - Establish paternity and other family relationships **+** elaborate using DNA fingerprinting **+** elaborate using DNA fingerprinting/
   - Identify endangered and protected species as an aid to wildlife officials (could be used for prosecuting poachers) **+** elaborate using DNA fingerprinting/
   - Detect bacteria and other organisms that may pollute air, water, soil, and food **+** elaborate/
   - Match organ donors with recipients in transplant programs **+** the more genetically similar the recipient is to the donor, the greater the compatibility of the donor organ/
   - Determine pedigree for seed or livestock breeds **+** elaborate/
   - Authenticate consumables such as caviar and wine **+** elaborate;
9. Risk Assessment
   - Assess health damage and risks caused by radiation exposure, including low-dose exposures + elaborate (genetic differences make some people more susceptible and others more resistant to such agents) /
   - Assess health damage and risks caused by exposure to mutagenic chemicals and cancer-causing toxins + elaborate (genetic differences make some people more susceptible and others more resistant to such agents) /
   - Reduce the likelihood of heritable mutations **+** elaborate;
10. Bioarchaeology, Anthropology, Evolution, and Human Migration
    - Study evolution through germline mutations in lineages/
    - Study migration of different population groups based on female genetic inheritance (elaborate on mitochondria DNA) /
    - Study mutations on the Y chromosome to trace lineage and migration of males/

- Compare breakpoints in the evolution of mutations with ages of populations and historical events
- Comparative genomics between humans and other organisms such as mice already has led to similar genes associated with diseases and traits / HGP data allows Comparative studies with other model organisms, so that disease development can be investigated. + E.g. of model org. This helps researchers to gain more insights about homologous genes, encoding proteins with similar functions in the human genome.

11. Energy and Environmental Applications
    - Use microbial genomics research to create new energy sources (biofuels) + E.g. New energy-related biotechnologies as photosynthetic systems, microbial systems that function in extreme environments, and organisms that can metabolize readily available renewable resources and waste material with equal facility /
    - Use microbial genomics research to develop environmental monitoring techniques to detect pollutants + New genetic techniques now allow us to establish more precisely the diversity of microorganisms and identify those critical to maintaining or restoring the function and integrity of large and small ecosystems. This knowledge also can be useful in monitoring and predicting environmental change /
    - Use microbial genomics research for safe, efficient environmental remediation **+** elaborate/
    - Use microbial genomics research for carbon sequestration **+** elaborate;
12. Agriculture, Livestock Breeding, and Bioprocessing
    - Disease-, insect-, and drought-resistant crops + mention of insertion of genes;
    - Healthier, more productive, disease-resistant farm animals;
    - More nutritious produce;
    - Biopesticides;
    - Bioengineered seeds to grow insect- and drought-resistant crops that require little or no pesticide → Increase outputs and reduce waste
    - Edible vaccines incorporated into food products;
    - New environmental cleanup uses for plants like tobacco;

15. [N2008/P3/Q4] Discuss the ethical concerns that have arisen about the human genome project. [6]

1. **Fairness in the use of genetic information** by insurers, employers, courts, schools, adoption agencies, and the military, among others.

Insurers, employers, courts, schools, adoption agencies, and the military, among others may have access to personal genetic information and may use such information in an ethically questionable way;

e.g. **(use any example below after stating the above statement):**

- Insurers - raising premiums based on genetic predisposition to disease (insurers).
- Employers – hiring and promoting based on genetic superiority rather than on merit
- Courts – judge a defendant based on a genetic predisposition to violence rather than the severity of the crime itself

2. **Privacy and confidentiality of genetic information;**

e.g. **(use any example below after stating the above statement):**

- Risk profiles for diseases may be accessible to insurers, employers, schools etc / individual's privacy infringed. May be used in discriminatory manner
- Genetic information of individuals becoming private intellectual property of biotechnology companies. Commercialization of related technologies derived from such research, but possibly no reciprocal direct benefit for individuals contributing their genetic information to the research.

3. **Psychological impact and stigmatization due to an individual's genetic differences;**

e.g. **(use any example below after stating the above statement):**

- Personal genetic information may affect an individual and society's perceptions of that individual in a very negative way. Individual may withdraw from society or society may outcast that individual depending on a person's genetic predisposition to illness, violence, life span, intellect etc.
- Genomic information may members of minority communities negatively again based on genetic predisposition of individuals belonging to a particular race. Such information may further serve to strengthen negative perceptions of minority races.

4. Reproductive issues including **adequate informed consent** for complex and potentially controversial procedures / use of genetic information in **reproductive decision making** / reproductive rights;

e.g. **(use any example below after stating the above statement):**

- There is a chance that fetal genetic testing may not be reliable in instances involving complex diseases where genetic predisposition and actual occurrence of a disease may not correlate so parents may abort healthy fetuses unknowingly
- There may be elements of society that may practice eugenics based on access to genetic information

5. Clinical issues including the **education** of doctors and other health service providers, patients, and the general public in **genetic capabilities, scientific limitations, and social risks / implementation of standards and quality-control measures in testing procedures**;

e.g. **(use any example below after stating the above statement):**

- Proper **counselling** must be done in conjunction with risk profiling and where there is **no treatment** for certain diseases**.** The public may be unprepared for such information and that it is irresponsible to provide it without an expert to help put it in context.

6. **Uncertainties associated with gene tests** for susceptibilities and complex conditions (e.g., heart disease) linked to multiple genes and gene-environment interactions;

7. Conceptual and philosophical implications regarding **human responsibility, free-will versus genetic determinism** / concepts of health and disease;

e.g. **(use any example below after stating the above statement):**

- Knowledge of whether genes alone affect behaviour and human physiology are not well studied and with so many genes and the complexity of multigenetic interactions, we may

not fully understand such issues enough to determine behaviour and physiology based on just the genetic code.

8. Health and environmental issues concerning genetically modified foods (GM) and microbes;

9. **Commercialization** of products including **property rights** (patents, copyrights, and trade secrets) and accessibility of data and materials;

e.g. **(use any example below after stating the above statement):**
It may be argued as to who owns genes and other pieces of DNA. If commercialization of genetic info is not controlled by the state, companies may patent DNA sequences and limit their accessibility and development into useful products as it may be costly to experiment and share DNA technologies.

Check out this web as a start: https://www.ndsu.edu/pubweb/~mcclean/plsc431/students98/saha.htm

**Markers' comments:**

Some students wrote more than 1 point for each category. For the purpose of 'A' Levels, you can write MAX 2 points for each category together with elaboration. This time round, only 1 point per category is accepted due to overall 6 marks (3 marks for benefits, 3 marks for ethical concern), therefore the teacher wanted the students to recall 3 categories, hence 1 mark MAX per category. Most students attained at least 4 marks for this part.

**Applications of Molecular and Cell Biology**

1. Explain the unique features of stem cells and the normal functions of stem cells in a living organism. [8]

Unique features

1. Stem cells are unspecialized, capable of dividing by mitosis and renewing themselves for long periods and can differentiate to give rise to specialized cell types;
    a. Unspecialised/undifferentiated cells with no tissue specific structures/do not carry out any specific function;
    b. Can undergo mitosis/divide indefinitely and undergo self-renewal to renew pool of stem cells;
    c. They have the ability to differentiate to produce specialized cells upon receiving appropriate molecular signals;
2. Zygotic stem cells are totipotent. These cells can differentiate into any cell types to form whole organisms, and so are also pluripotent and multipotent;
3. Embryonic stem cells are pluripotent. These cells can differentiate into almost any cell type to form any organ or type of cell and so are not totipotent but are multipotent;
4. Blood stem cells are multipotent. These cells can differentiate into a limited range of cell type and so are not pluripotent or totipotent

Normal functions

1. Embryonic stem cells are derived from the inner cell mass of blastocysts;
2. Pluripotent - able to give rise to almost all cell types;

3. To give rise three primary germ layers; ectoderm, endoderm and mesoderm / to the multiple specialised cell types / tissues / organs in the developing foetus;
4. Blood stem cells are found in the bone marrow;
5. Multipotent - able to give rise to different types of blood cells e.g. red blood cells, white blood cells and platelets;
6. maintain the steady state functioning of a cell by generating replacements ® repair for cells lost through disease, tissue injury or normal wear-and-tear;

2. [2011 RI Prelim] Explain the differences between stem cells and cancer cells. [3]

a. Unlike stem cells, cancer cells do not differentiate and are unresponsive to molecular signals;
b. Cancer cells divide indefinitely while stem cell division is determined by molecular signals [that either stimulates cell division or stop it altogether];
c. Cancer cells experience no contact inhibition and is invasive while stem cells experience contact inhibition;
d. Cancer cells metastasize (dislodge from original tumour and form secondary tumours) while stem cells remain in tissue of origin;

3. [N2013/P3/Q5] Describe features of zygotic stem cells and embryonic stem cells that distinguish them from each other. [5]

| Features | Zygotic SC | Embryonic stem cells | mks |
|---|---|---|---|
| potency | totipotent | pluripotent; | 1 |
| | These cells can differentiate into any cell types to form whole organisms, and so are also pluripotent and multipotent; | These cells can differentiate into almost any cell type to form any organ or type of cell and so are not totipotent but are multipotent; | 2 |
| source of cell | single cell formed from the fusion between a sperm and an egg cell; | cells obtained from the inner cell mass blastocyst formed a week after fertilisation; | 2 |

4. [N2013/P3/Q5] Describe the features of blood stem cells and explain their normal functions. [8]

1. type of adult stem cell;
2. found in bone marrow of long bones;
3. capable of dividing and renewing themselves for long periods;
4. unspecialized, does not have tissue specific structures for them to perform specialized functions;
5. can give rise to specialized cell types;
6. multipotent, can differentiate into a limited range of cell type, usually of a closely related family of cells;
7. e.g. red blood cells, white blood cells, platelets;
8. primary function is to maintain the steady state of functioning of a cell by generating replacement for cells lost through disease, tissue injury or normal wear-and-tear;

5. [N2007/P3/Q4] With reference to genetic diseases

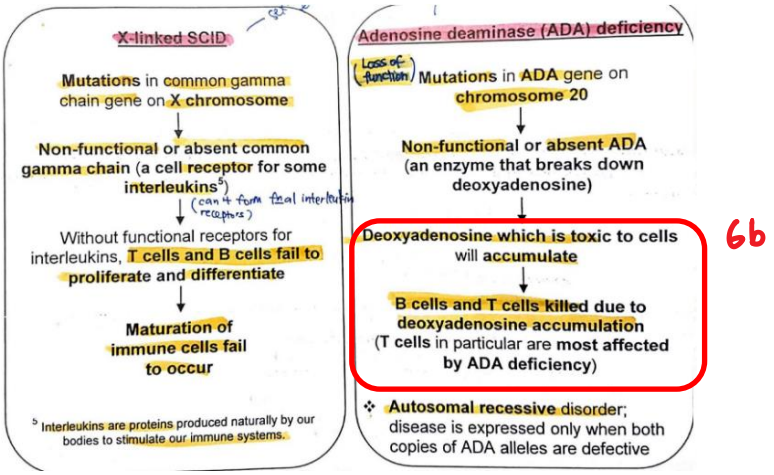
a. Describe SCID (severe combined immunodeficiency) and cystic fibrosis. [8]

***Severe combined immunodeficiency (SCID)*** (4 marks max)

1. **X-linked SCID** is a sex-linked condition caused by a (loss-of-function mutation) mutation of interleukin-2 receptor gamma gene found on the X chromosome;
2. X-linked SCID affect more males than females;
3. Without functional interleukin receptors, immune cells cannot be stimulated to develop and differentiate into T and B lymphocytes. The immune system is compromised (antibodies are not produced against foreign antigens) + susceptible to opportunistic infections;
4. **Adenosine deaminase (ADA) deficient SCID** is an autosomal recessive disorder caused by a (loss-of-function mutation) mutation in in ADA gene on chromosome 20;
5. ADA is necessary for the breakdown of purines and the deficiency in ADA causes accumulation of dATP;

6a 6. The activity of ribonucleotide reductase/ ribonucleoside diphosphate reductase, an enzyme that reduces ribonucleotides to generate deoxyribonucleotides (dNTP) is inhibited.

7. Without functional ribonucleotide reductase, lymphocyte proliferation is inhibited and the immune system is compromised (antibodies are not produced against foreign antigens) + susceptible to opportunistic infections;




6b

***Cystic Fibrosis (CF)*** (4 marks max)

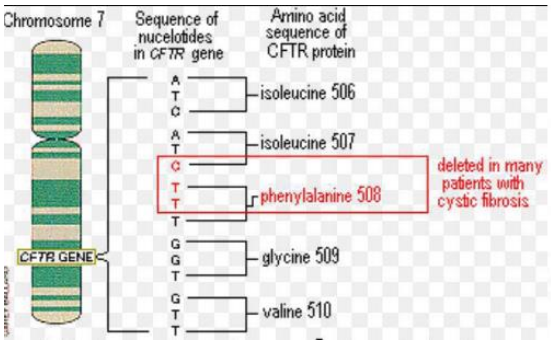
8. CF is an autosomal recessive condition caused by a mutation of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene on chromosome 7;
9. Deletion of 3 consecutive nucleotides ~~/ a codon~~ which codes for phenylalanine of CFTR protein;
10. CFTR protein which functions as a chloride ion channel is no longer able to transport chloride ions out of the cells;

*(FYI: Cl- not transported out of epithelial cells -> Na+ retained too (due to charge inbalance) -> water potential lower in cell -> water retained in cell -> mucus undiluted -> thick & cannot flow -> congestion*

➔ *mucus remains too long in respiratory tract making it conducive for bacteria growth -> lung infection -> severe breathing difficulty.*

➔ *Pancreatic duct choked by thick mucus preventing release of enzymes -> indigestion & thick mucu layer in intestines reduce absorption of digested food)*

11. Causing thick sticky mucus accumulating on surface of epithelial cells of lungs / pancreas / reproductive system;




*FYI: CFTR is an ATP-gated anion channel. ATP binding to CFTR causes a conformational change that allows the channel to open. Cl- moves across by passive diffusion.*

b. Explain gene therapy treatment, using both viral and non-viral delivery systems. [6]

**1** Gene therapy is the technique for introducing normal / functioning allele of a gene; ® therapeutic gene / healthy gene

**2** New allele generates functional protein products ;

**3** Restores target cells to normal state / correct function of cell or phenotype ;

*Viral delivery system (ex vivo* approach*):*

**4** In SCID, retroviruses (e.g. lentivirus) are genetically engineered to carry normal / functioning ADA allele [Reject: gene];

- Attenuated to remove disease-causing genes

**5** Virus infect and integrate allele into target cells / T lymphocytes extracted from the patients suffering from ADA-deficient SCID;

**6** The cells are grown in a culture medium and verified for expression of ADA transgene and the genetically modified cells were then transferred back to the patients;

OR (continue from point 4 after producing recombinant retrovirus) *(for frq of more marks)*

**7** Recombinant retrovirus is used to infect the haematopoietic stem cells from the bone marrow previously removed from the SCID patient *in vitro*

**8** Retrovirus enters the cell and releases the normal allele which is then reverse transcribed to double-stranded cDNA by reverse transcriptase. Double-stranded cDNA is integrated into host cell genome using integrase

**9** Cells are grown in culture to screen for recombinant cells and proper gene expression, tumour formation, presence of infectious virus before re-introduction back into SCID patients

**10** Since the haematopoietic stem cells have the ability to self-renew and proliferate ensuring a constant supply of new replacement cells with the ability to produce ADA, effects of treatment are permanent

**11** The stem cells also develop/ differentiate into functioning T & B lymphocytes

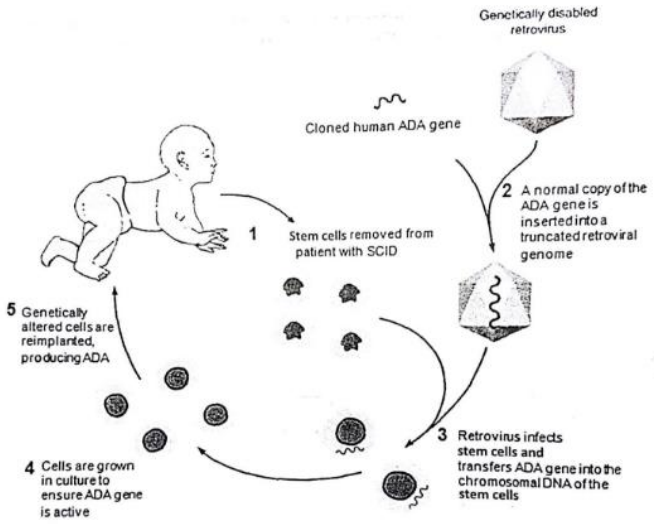



Figure 6: Treatment of SCID using retroviruses in gene therapy

***Why is the ex vivo approach adopted instead of the in vivo approach for viral-mediated gene delivery system to treat SCIDs?***

a. Target cells are easily removed
b. *Ex vivo* approach used because it allows specific screening of target haematopoietic stem cells;
c. Allows monitoring of gene expression before reintroduction as success rate is very low;
d. Safer because can monitor for cancer cells because retroviruses cause insertional mutagenesis which can knock out tumour suppressor genes or activate proto-oncogenes;
e. You don't introduce retrovirus directly into patient – already immunocompromised;

*Non Viral delivery system (in vivo* approach)*:*

**12** In CFTR, normal CFTR allele complex with cationic lipids / liposome to form lipoplex.
- Liposome is an artificial phospholipid bilayer with an aqueous core;
- Phospholipids complex with cDNA of a functional copy of allele of CFTR gene;

**13** Delivered via aerosol spray to reach the respiratory tract ;

**14** Liposome with allele taken in by lung epithelial cells by endocytosis ;
- Interaction of lipoplex with cell membrane of lung epithelial cells results in endocytosis of the lipoplex forming an endosome;

OR liposome fuses with the membrane of the lung epithelial cells and releases DNA into the cell -> lipofection

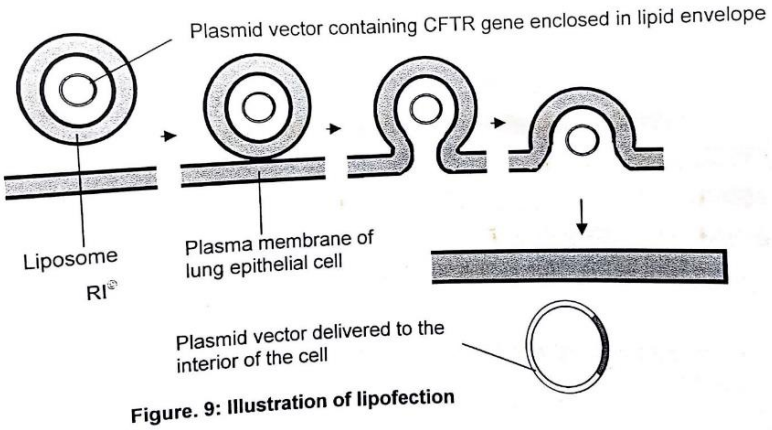
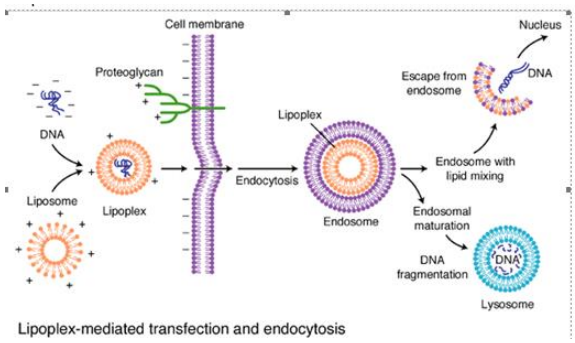



**Figure. 9: Illustration of lipofection**

**15** Normal allele is released into the cytoplasm of the lung epithelial cells. Then normal allele **may** enter nucleus and insert into nuclear genome ; ->CFTR gene may be transcribed and translated




Lipoplex-mediated transfection and endocytosis

**16** Synthesis of functional CFTR protein (incorporated into plasma membrane) allows chloride ions to be transported out of cells, thereby reducing symptoms ;

***(FYI: The in vivo approach targets somatic cells. Stem cell option is not viable.***

***Why is ex vivo somatic stem cell gene therapy not viable?***

***Ans: Identifying, extracting and re-implanting stem cells from the lung epithelium is not possible currently.)***

Alternative viral delivery system for Cystic Fibrosis - Adenovirus

- **cause throat infections** but **not human malignancies** (cancer) because it can express its genes without necessarily inserting its genes into the genome of the host
- as a safety precaution, the virus was modified to **remove genes that cause disease, and allow virus to replicate.** This allows the **accommodation of foreign gene** (need to make room for foreign gene since viral capsid has limited space)
- modified adenovirus used as a 'vector' (carrier of a foreign gene within its own genetic material)
- can effectively infect cells and transfer its genetic material into cell.

6. Describe a type of genetic disease that can be treated with gene therapy. [7] [N2015/P3/Q5] Describe the cause and symptoms of the genetic disease cystic fibrosis/ SCID. [7]
7. [N2015/P3/Q5] Explain how a disease such as cystic fibrosis can be treated by gene therapy, using **non-viral** delivery systems. [5]

Explain how a disease such as SCID can be treated by gene therapy, using **viral** delivery systems. [5]

8. [N2007/P3/Q4] Discuss the factors that keep gene therapy from becoming an effective treatment for genetic diseases. [6]

**1** Gene therapy is short-lived and requires frequent / repeated treatments; (CF)
- Functional copy of gene not integrated into genome and thus will be degraded
- GM cells have short lifespan (means that new cells need to be constantly transformed)
  1. Normal CFTR allele is introduced into lung epithelial cells, and thus when the cells die, the introduced allele is lost together with the cell

**2** It is difficult to ensure DNA is integrated into target cell genome;

**3** Incorrect insertion of normal gene into tumour-suppressor gene may cause cancer / inserted into control elements which affect gene expression, triggering cancer;
- Insertional mutagenesis caused by retroviruses may cause inactivation of tumour suppressor genes and/or activation of proto-oncogenes, leading potentially to cancer;

**4** Immune response may be triggered as a foreign viral vector is introduced;
- immune response against the viral vector such as allergies, inflammatory and toxicity responses [give one example];

**5** Difficult to find a vector for large gene / virus may regain / develop virulence/ may recover its ability to cause disease once in host;

**6** If virus vector triggers the immune response the first time, the immune system will mount a stronger and faster response the second time rendering the therapy ineffective subsequently;

**7** Cannot find a suitable viral vector due to viruses' host specificity

**8** Problem with controlling the activity of gene expression;

**9** Difficult to treat genetic diseases caused by combined effects of many defective genes / multi-gene disease; Difficult to introduce many genes at the same time ;
- (e.g. Alzheimer's disease, diabetes etc)
- Difficult to treat with gene therapy which currently treats only single gene disorders;

**10** Inability to target multiple diseased sites; (CF)
- Gene therapy (for CF) using aerosol spray to targets respiratory tissues but are not able to treat other diseased sites like pancreas ;

**11** Problem with dealing with dominant condition / non dividing cells ;

**12** Reference to problems with liposomes (toxicity and low efficiency)

Reject: Ethical and economic ideas

9. Discuss the social and ethical considerations for the use of gene therapy. [5]

1. There is the fear of genetic discrimination by society because gene therapy could be used for treatment of diseases or enhancement of desired traits not associated with disease;
   a. Ethical concerns in pursuing genetic enhancement including increased social inequality and a lowered tolerance for human diversity
2. As gene therapy treatment is exorbitantly expensive, there may be discrimination against the poor who cannot afford the treatment;
3. Religious group may view gene therapy as tampering with nature and going against the natural way of life;

4. Arguments against germline gene therapy:
    a. It is difficult to follow patients in long-term clinical research. Gene therapy patients would need to be under surveillance for decades to monitor the long-term effects of the therapy on future generations.
    b. Point 1
    c. Because germ-line gene therapy involves research on early embryos and affects their offspring, it essentially creates generations of unconsenting research subjects

(Not for this qns) Arguments in favor of gene therapy:

- Potential for treating desperately ill patients or preventing the onset of terrible illness. Where conventional treatment has failed, gene therapy may offer the only effective way to treat such patients.
- Somatic-cell gene therapy corrects or alleviates the genetic defect present in the individual alone, without impacting the genetic information transmitted to the next generation.
- Arguments for germline gene therapy:
    d. It offers a true cure, and not simply palliative or symptomatic treatment
    e. It may be the only effective way of addressing some genetic diseases, e.g. for diseases that affect an extensive or inaccessible area of the body like the nervous system such as neurodegenerative diseases. T least the next generation need not suffer from the same fate as the parent.
    f. By preventing the transmission of disease genes, the expense and risk of somatic-cell therapy for multiple generations are avoided.
    g. Prospective parents at risk for transmitting serious genetic diseases have an option of having normal children where previously they would simply refrain from having children for fear of passing on the disease.

[N2015/P3/Q5] Describe **four** problems that may be associated with using a **viral vector** to introduce an allele into the cells of a person with a genetic disease. For **each** problem, explain the related ethical issues that should be considered. [8]

10. Discuss the process by which plants are cloned. [6]

Discuss cloning in plants in terms of plant tissue culture techniques. (Gene cloning in plants using Agrobacterium spp. and gene gun is not required.)

1. Plant tissue culture is a technique of propagating plants under sterile conditions;
2. Sterile Murashige and Skoog medium contains sucrose, amino acids, inorganic ions and plant hormones needed for plant growth;
3. and antibiotics or herbicides for selection of successfully transformed cell;
4. An explant (e.g. a small section of the shoot, root or leaf) is selected to grow because they contain meristematic cells which are totipotent and able to divide and differentiate into any other type of cell;
5. The explant is surfaced sterilized using alcohol and grown under light and in sterile conditions to prevent contamination;
6. A callus, containing a mass of unorganized and undifferentiated cells is formed by mitosis;
7. As the callus increase in size, pieces of the callus is sliced off and grown on new medium;
8. By adjusting the concentration of auxin and cytokinin in the growth medium, the cells in the callus can be induced to differentiate into roots and shoots respectively;

9. Plantlets containing both roots and shoots are removed from agar medium and transplanted to sterile soil for further growth;

[N2012/P3/Q5] Describe how plant tissue culture is used to clone plant cells. Explain the scientific reasons for each step in the process. [8]

**QUESTION 5**

a. Describe how plant tissue culture is used to clone plant cells. Explain the scientific reasons for each step in the process. (8m)

**Stage 1: Establishment of aseptic culture**

An **explant** is the part of a plant (tissue / organ) which is selected to grow or culture. A healthy / disease-free plant is selected. A small section of the shoot, root or leaf will usually be used because they contain **meristematic cells which are able to divide and differentiate into any other type of cell.**

**Stage 2: Multiplication & Axillary shoot enhancement**

In this step, we aim is to obtain maximum number of tissue pieces / clumps for generation of future plantlets.

Cells of an explant divide by **mitosis** to form a **callus**, which is **a mass of unorganized and undifferentiated cells**. As the callus increase in size, pieces of the callus is sliced off and grown on fresh medium in another vessel in a process known as sub-culturing. Therefore a callus can be subcultured to become many calli.

**Plant growth regulators** are chemical substances produced by plants and they direct growth and differentiation of plants. In the medium, these hormones **direct growth and differentiation of plant tissue.**

For the formation of callus, **equal ratio of auxin and cytokinin** are added to the nutrient medium. intermediate

By adjusting the concentration of auxin and cytokinin in the growth medium, the cells in the callus can be induced to differentiate into roots and shoots. At this stage, **shoot formation** is stimulated.

**Stage 3: Rooting shoots (shoots obtained from stage 2 culture)**

The shoots are re-cultured into a growth medium with **auxin > cytokinin** to induce **root formation**, prior to their transfer to soil.

**Stage 4: Acclimatisation (Transfer to natural environment / soil)**

Plantlets containing both roots and shoots are removed from agar medium, agar washed away from roots and **transplanted to sterile soil for further growth in a sheltered environment.** Eventually the young plants can be re-planted in an open uncontrolled environment. This allow plantlets to gradually get used to external conditions away from *in vitro* environment where they were previously grown in.

*in vitro* environment, they rely mostly on chemical/ / organic sources of energy (sucrose) and it requires a source of carbon other than $CO_2$ (unable to produce carbon); chemoheterotrophs. Therefore it is not photosynthetically competent. By placing in external environment, they will gradually become photoautotrophs (derive energy from light, carbon from $CO_2$).

**To obtain a callus culture, suitable explants must first be chosen. Suggest a suitable explant and explain your choice. [2]**

a Root tips/ shoots tips/ cambium/ bud/ hypocotyls
b Contains meristematic tissue which are fast-growing / tend to be disease-free

**Outline the steps in obtaining a callus culture from an explant. [3]**

a Surface-sterilize explants by immersing in a solution of sodium hypochlorite/bleach/chlorox
b Cut explants into small fragments
c Transfer to culture vessels containing nutrient agar media [reject: agarose]
d with intermediate auxin: cytokinin for inducing callus formation
e Seal and incubate in sterile containers

**State two advantages of using callus cells in producing genetically modified crops. [2]**

a Modified callus cells may be sub-cultured to obtain high multiplication rates
a2 / high reproductive capacity or high fecundity – get many plants within short time
a3/ genetic uniform – desirable traits can be pass down the progeny
a4/ idea of controlled microenvironment vs natural environment for plant growth - with examples
a5/ reasons for getting disease-free plants – benefits like high yield and robust, healthy plants
b Undifferentiated modified callus cells may be induced to form plantlets or embryoids
c Callus cells can be easily screened for special characteristics
d Easy to obtain protoplasts to insert foreign DNA

11. [N2010/P3/Q4] Discuss the role that cloning in plants could play in such crop improvements. Include, in your discussion, any advantages or disadvantages of such cloning. [8]

- Genetic modification is possible, so transgenic plants can be developed;
- Cells or explants could be genetically engineered to take up gene-of-interest via gene gun / Agrobacterium;
- Cells are able to divide through mitosis to form genetically identical cells and a callus can be formed;
- rapid, large scale production of genetically identical plants, all with the desired phenotypes, would be advantageous.
- A larger number of plants can be produced / mass production is possible, thus lowers production costs;
- Plantlets are relatively small, so requires less space ;
- Sterile environment ensures that new plants are disease-free, so yield is higher ;
- Not limited by seasonal changes / external conditions / weather, so yield is higher ;
- Cultures are easy to transport / ref. air freight thus lowers transport costs; / large numbers of small containers of plants could be transported more cheaply than adult plants;
- Genetically identical plants may be at a selective disadvantage especially when the selection pressure changes (e.g. bacterial / fungal infection);
- All plants will not be able to survive as there is no genetic variation within the population;

12. [N2012/P3/Q5] Discuss the social implications of genetically modified crop plants. [6]

### Transfer of antibiotic resistance markers

While there is no evidence to show that any of the transgenes found in genetically modified foods are injurious to humans, opponents of genetic engineering and GMOs have focused on the potential threat to human health. It is questioned whether the **plasmid vectors on which antibiotic resistance genes are inserted as markers, may be accidentally transferred from transgenic plants to pathogenic microorganisms which may result in increase in resistance to clinically important antibiotics.**

### Probability of introducing novel allergens

GM food might cause allergies. **People with allergies could suffer reactions after unwittingly consuming genetically modified foods containing proteins introduced from sources they are allergic to.** Some consumers are worried that introduced gene, toxic against pests could also cause the plant to alter the pollen of the plant, thereby affecting the health of humans already prone to pollen allergies.

**Possibility of GM food being toxic or carcinogenic**

Many consumers are wary of GM food, fearing that introduced genes could prove harmful to human health. E.g. if transgenes can cause a plant to produce toxins at higher levels than are present naturally, they may be toxic or carcinogenic when consumed on a long term basis.

**Monopolistic behaviour of biotechnology companies**

Seed companies have traditionally sought to control their product by limiting farmers from saving seeds for future planting. In 1998, a U.S. patent was granted for producing plants with sterile seed called the "technology protection system". The **terminator gene is likely to be bred into many GMO seeds, causing second generation seeds to be sterile and farmers have no choice but to purchase a new batch of GMO seeds.** Country farmers will become dependent on biotechnology companies and may be driven further into poverty without resources to purchase new seed each year.

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Scientists have raised concerns about innovations in research that are not shared. Companies charge steep prices for the GMO seeds produced to recoup their investment. It raised fears that world food production may be dominated by a few large biotechnology companies.

**Increasing dependence of developing nations on industrialized nations**

The potential of using GMO for better yields and enhanced nutritional value can possibly alleviate problems of food shortage and malnutrition in developing nations. However in developing nations, farmers have agricultural practices of saving seeds from one harvest for the next. The increase in dependence for GMO seeds on large biotechnology companies highlights **a need to ensure that developments in GM technologies do not benefit rich countries at the expense of the poor.**

**Impact on international trade**

Europe has been much more hesitant than the United States in accepting GM products in processed food. European Union regulations require labelling of GM food products because of their strong belief that consumers have a right to know how their food is produced. The response of European nations and individual governments towards US GM food has become a major trade issue between the United States and these countries.

Concern: Resistance genes may be passed onto weed relatives through cross-pollination/ horizontal gene transfer [Reject: gene transfer]
Solution: Create a large buffer zone / isolation distance by planting unrelated crops
OR
Concern: Intense use of same herbicide will eventually select for resistant weeds, for which Round-Up™ will no longer be effective
Solution: Alternate use of Round-Up™ with other types of herbicides
OR
Concern: Forced dependence of farmers on Round-Up Ready™ soybeans as they are not allowed to replant seeds from their crops
Solution: Allow farmers to replant seeds

OR
Concern: Allows excessive use of Round-Up™ herbicide, causing runoffs which are toxic to fish and amphibians
Solution: Promote responsible use of herbicide among farmers

13. [N2010/P3/Q4] Explain the significance of genetic engineering in improving the quality and yield of crop plants and animals in solving the demand for food in the world. [12 (6m – quality; 6m – yield)]

Details required
- a named crop,
- the source of the gene,
- the gene product in the crop,
- the problem and the solution.

(i) Quality
***Golden rice***
1. "Golden" rice is a transgenic plant that produces yellow rice grains containing beta-carotene, which human body uses to make vitamin A;
2. Three beta-carotene biosynthesis genes (phytoene synthase from daffodil, crt1 and lycopene cylase) are transformed into rice;
3. This prevent vitamin A deficiency in world's population that depends on rice as a staple food, improving quality and nutritional value of crops;

***Flavr Savr Tomato***
4. Transgenic plants contain an antisense polygalacturonase mRNA
5. Polygalacturonase is one of the many genes expressed during ripening of tomatoes and results in softening of the hard flesh.
6. This results in the formation of mRNA-antisense RNA hybrid, which prevents the polygalacturonase mRNA from being translated and the enzyme will not be synthesized.
7. It ensures tomatoes are not too ripe and soft when it reaches the consumer in the market place.

(ii) yield
***Pest-resistant transgenic plants***
1. Transgenic plants containing the *Bacillus thuringiensis (*Bt) gene will produce the protoxin which is ingested by the insect;
2. *Bacillus thuringiensis (*Bt) gene is from bacteria *Bacillus thuringiensis*;
3. Protoxin is cleaved in the insect gut and the active Bt toxin is released;
4. Protein binds to cell membranes and causes them to be permeable causing gut cells of insect to lyse, eventually killing insects;
5. Pest-resistant crops have reduced the need for pesticides, improving quality and yield of crop;

***Genetically modified Atlantic salmon***
6. Eel pout's anti freeze protein (AFP) gene promoter fused to growth hormone cDNA from Chinook salmon is injected into salmon eggs;
7. In cold climates, anti freeze promoter is active and growth hormone is actively expressed
8. Transgenic salmon grow up to ten times faster than non-transgenic salmon, improving yield;

14. Discuss the ethical and social implications of genetically modified organisms. [6]

***Social implications***

1. Transfer of antibiotic resistance markers to pathogenic microorganisms which may result in increase in resistance to clinically important antibiotics;
2. Probability of introducing novel allergens as GM foods may contain proteins introduced from sources people are allergic to;
3. Possibility of GM food being toxic or carcinogenic as they may cause over-expression of other proteins;
4. Monopolistic behaviour of biotechnology companies as terminator gene is likely to be inserted into many GMO seeds, causing second generation seeds to be sterile;
5. Scientists have raised concerns about innovations in research that are not shared, raising fears that world food production may be dominated by a few large biotechnology companies;
6. Increasing dependence of developing nations on industrialized nations;
7. Impact on international trade as Europe has been much more hesitant than the United States in accepting GM products in processed food;

***Ethical issues***

8. Tampering with nature as it is going against the natural way of life;
9. Lack of mandatory food labelling in some countries;
10. Religious groups are concerned that GM foods might contain genes from animals prohibited by their religion;

15. [N2012/P3/Q5] Comment on the statement 'Genetically modified crop plants and animals should be treated as new species'. [6]

ref to BSC's definition: interbreeding, production of fertile, viable offspring;
ref to the limitation of using BSC to classify GMO because GMOs have different genetic makeup from the original organism but yet are able to interbreed and thus produce fertile and viable offspring;
ref to definition of a GMO (i.e. A **genetically modified organism** is an organism that has its genetic material altered through the use of **recombinant DNA technology**. This technology involves the combination of DNA molecules from different sources into one molecule to create a new set of genes.)
ref to the use of other species concept (e.g. ESC, MSC) to determine species status;
ref to using molecular methods/ molecular homology to determine how different GMOs are to their natural counterparts;