Section 16.1 – Producing DNA fragments

Recombinant DNA – combined DNA of two different organisms
The process of using DNA technology to make certain proteins is as follows:

1.) Isolation of the DNA fragments that have the gene for the desired protein
2.) Insertion of the DNA fragment into a vector
3.) Transformation of DNA to a suitable host
4.) Identify the host cells that have taken up the gene
5.) Growth/cloning of the population of host cells

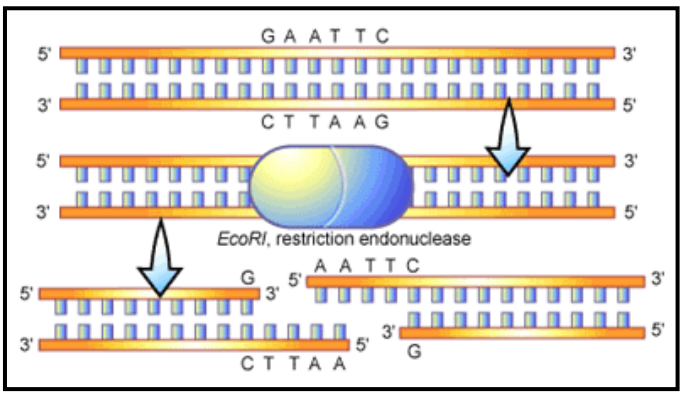
Using reverse transcriptase

Reverse transcriptase catalyses the process of producing DNA from RNA
The process is as follows:

1.) A host cell that already produces the desired protein is selected
2.) Since the cells that produce the protein will have a lot of the relevant mRNA, reverse transcriptase can be used to make DNA from the mRNA already present
3.) Complementary (cDNA) is then produced from complementary nucleotides to that of mRNA
4.) DNA polymerase then builds up the complementary DNA strand to that of the cDNA form in step 4 to form a double helix

Using restriction endonuclease

Restriction endonuclease can cut a double stranded segment of DNA at a specific "recognition sequence"
R.e – can cut either in a straight line to form blunt ends, or in a staggered fashion, forming "sticky ends" which are so called as they have exposed unpaired nucleotides
A recognition sequence is a 6 base palindrome sequence
It is a palindrome sequence since reading the bases from right to left on one strand will produce the same sequence if you read from left to right on the opposite complementary strand



Section 16.2 – In vivo gene cloning – the use of vectors

Obtained DNA fragments must be cloned. This can be done in two ways

1.) In vivo – transferring fragments to a vector (host cell)
2.) In vitro – using polymerase chain reactions

The importance of "sticky ends"

When the same restriction endonuclease is used to cut DNA, all the ends of the fragments will be complementary to one another.
When two sticky ends join up, DNA ligase can be used to join the sugar phosphate backbone

Insertion of DNA fragment into a vector

A vector is used to transport DNA to the host cell
Plasmids are commonly used as a vector
Plasmids often contain the gene for antibiotic resistance
Restriction endonuclease can be used on one of these genes to break the plasmid loop
When the same restriction endonuclease is used to cut the plasmid is the same as that which is used to cut the DNA into fragments, the sticky ends will be complementary
DNA ligase can be used to join the recombinant DNA permanently

Introduction of DNA to host cells

Transformation – involves plasmids and bacterial cells being mixed together in a medium containing calcium ions
Changes in temperature and the addition of calcium ions cause the bacterial cells to become more permeable to plasmids
Not all of the bacteria cells will however take up the recombinant DNA. This is due the plasmid sometimes closing up again before the DNA fragment is incorporated

The process for determining which cells have taken up the recombinant DNA involves the use of antibiotic resistant genes and is as follows:

1.) All bacterial cells are grown in a medium containing the antibiotic, ampicillin
2.) The cells that have taken up the plasmid will have the gene for ampicillin resistance and so will survive whereas the others will die.
3.) This will leave only the bacteria that has taken up the plasmid left

Gene markers

Most gene markers involve using another gene on the plasmid. The second gene is identifiable because:

1.) It may allow the bacteria to be resistant to a certain type of antibiotic
2.) It may cause the bacteria to produce a fluorescent protein that can be easily seen
3.) It may cause an enzyme to be produced that will have noticeable affects

Antibiotic resistant markers

All the bacteria that has survived the first treatment will be resistant to ampicillin, however some may have taken up plasmids that were not altered and so the tetracycline gene will still be functional
Replica plating is used to identify the bacteria that have taken up the new gene and hence are not resistant to tetracycline
This is achieved as follows:
The bacteria that have survived the first treatment, all have the gene for ampicillin resistance
These cells are cultured on agar plates
Each cell on the agar will grow into a colony of identical bacteria
A tiny sample of each colony is placed on the exact same position but on a different plate
The second plate will contain the antibiotic tetracycline
The colonies that are killed by on this plate will be those which contain the modified gene.
Using their exact position, it is therefore possible to deduce the modified bacteria on the first plate as they will be in the same place

Fluorescent markers

The gene GFP produces a green fluorescent protein
The gene to be cloned is placed in the centre of the GFP gene and hence the GFP gene no longer works. So the bacteria that have successfully taken up the plasmid will be those that do not fluoresce
Bacteria can then be viewed under a microscope and those that do not fluoresce are retained. This process is more rapid than using antibiotic resistance

Enzyme markers

The gene lactase turns a particular substrate blue.
By incorporating a desired gene into the middle of the lactase gene, those bacteria that successfully take up the modified plasmid will not have the ability to change the substrates colour therefore they can be indentified

Section 16.3 – In vitro gene cloning – the polymerase chain reaction

Polymerase chair reaction (PCR)

DNA polymerase - an enzyme that joins together nucleotides and does not denature at high temperature

Primers – short sequence of bases complementary to those at one end of a DNA strand

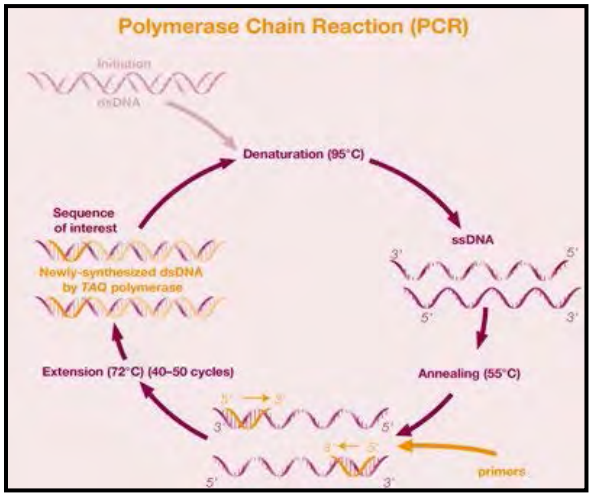
How the PCR creates copies of DNA is as follows:

1.) DNA fragments, primers and DNA polymerase are placed in the vessel of a thermocycler and the high temperature (95^{o}C) causes separation of the DNA strands

2.) The mixture is the cooled 55^{o}C. This causes the primers to anneal to their complementary bases at the end of the DNA strand. This provides a starting sequence for DNA polymerase to start copying DNA. DNA polymerase can only attach nucleotides at the end of a pre-existing chain.

3.) The temperature is then raised to the optimum temperature of DNA polymerase to work. This is 72^{o}C. At this stage, DNA polymerase joins up nucleotides starting at the primer and finishing at the end of the DNA molecule

4.) The cycle is then repeated several times to create more and more copies each time. The amount of DNA double after each cycle



In vitro advantages	In vivo advantages
Very rapid – just small amount of DNA can be copied very quickly in to billions of copies. This can save time in forensic investigations	**Useful in introducing a gene to another organism –** The use of plasmids can be used to introduce genes into other organisms
	Little risk of contamination – The restriction endonuclease cuts at a specific point producing the complementary sticky ends. Contaminant DNA cannot enter the plasmid
Does not require living cells – No complex culturing techniques required, save time and effort	**It is more accurate** – mutations during in vivo cloning are rare. Errors during in vitro are multiplied in subsequent cycles
	Only specific genes are copied – since the gene is cut out, only the required piece of DNA is copied
	Produces useful G.M bacteria - modified bacteria can be used to make useful proteins

Section 16.4 – Use of recombinant DNA technology

Genetic modification

The benefits to humans of genetic modification include:
Increasing the yield from animals or plant crop
Creating more nutrient rich food
Making crops resistant to disease, pests, herbicides and environmental changes
Producing vaccines and medicines

Examples of GM microorganisms

Antibiotics – improvements have been made in the amount of antibiotics produced but has not substantially improved the quality
Hormones – Incorporating the human gene for insulin into bacteria and using this method to produce the hormone is much more affective as it is not rejected by the immune system unlike the previous method which involved extracting insulin from cows and pigs
Enzymes – many enzymes which are used in the food industry are produced by microorganisms. These include protease to tenderise meat, amylase to break down starch during beer production and lipase to improve the flavour of certain cheeses

Examples of genetically modified

GM tomatoes – a gene that produces a complementary mRNA molecule to the mRNA that causes tomatoes to soften is added to the tomato DNA. The two mRNA stands combine once formed and thus the corresponding protein/enzyme that causes softening is not produced as it cannot be translated.
Pest resistant crops – some crops can be modified so that they produce a toxin harmful to pest that feed on it.
Plants that produce plastics – possible source of plastics in the future

Examples of genetically modified animals

Production of growth hormones
Resistance to disease thus making animals more economically feasible
Anti-thrombin is a protein that slows blood clotting, inserting the gene for this protein alongside the genes for proteins found in goats milk causes goats to the produce the anti-thrombin gene in their milk which can be used in medicine
The process is as follows:

1. Mature eggs are removed from female goats and fertilised with sperm
2. The gene for anti-thrombin found in humans is added to the DNA of the fertilised egg alongside the genes for other milk proteins
3. The modified egg is them transplant into a female goat
4. The resulting goats with anti-thrombin gene are cross bred to give a heard that produces rich anti-thrombin milk
5. The anti-thrombin is extracted and purified and given to humans as medical treatment

Section 16.5 - Gene therapy

Gene therapy - replacing defective genes with with those cloned from a healthy individual

Cystic fibrosis

Deletion mutation on recessive allele that causes the loss of an amino acid in a protein.
The gene affects the cystic fibrosis trans-membrane-conductance regulator (cftr) which is used for transporting chloride ions across the epithelial membrane
The effect of this is that less chloride ions are transported out of the cell, so less water moves out also by osmosis
Epithelial membranes with the defective gene become defective and the mucus produced is very thick and difficult to move
Symptoms of cystic fibrosis include:
Mucus congestion in lungs so greater risk of infection since mucus traps pathogens which are not removed
Less efficient gas exchange
Thick mucus accumulates in pancreatic ducts which prevents enzymes produced by the pancreas reaching the duodenum. This leads to fibrous cysts
Accumulation of mucus in sperm ducts may cause infertility

Treatment using gene therapy

Gene replacement - replacing a defective gene with a normal gene
Gene supplementation - adding copies of the healthy gene alongside the defective gene. The copies are dominant alleles and so the recessive allele which is defective has little/no affect
Depending on which yep of cell is being treated their are two different types of methods of gene therapy:
Germ-line gene therapy - replacing the defective gene whilst inside the fertilised egg. All daughter cell will therefore also have the healthy gene. This is a permanent solution but raises ethical questions
Somatic-cell gene therapy - targets only the affected tissues so is not present in gametes and cannot be passed on to offspring. Since the cells are constantly dying and are needed to be replaced, the treatment is not permanent and must be repeated.

Delivering cloned CFTR genes

Using a harmless virus

Adenoviruses cause colds by I netting their DNA into epithelial cells of the lungs
They can therefore be used as vectors to transfer a normal CFTR gene
This is done as follows:
The virus is made harmless by interfering with a gene involved in their replication
The Adenoviruses are grown in epithelial cells in a laboratory along with plasmids with the normal CFTR gene incorporated in them
The CFTR gene becomes incorporated into the DNA of the virus

The virus is taken up through the nostrils of a patient
The adenovirus then inject DNA into the epithelial cells of the lungs alongside the normal CFTR gene.

Wrapping the gene in lipid molecules

By "wrapping" genes in lipid molecules, they can then pass through the phospholipid bilateral of a plasma membrane.
The process is carried out as follows:
CFTR genes are isolated from healthy human tissue and are inserted into a plasmid that is then taken up by a bacterial cell. Gene markers are used to indemnify the bacteria with the healthy gene
The bacterial cells then multiply and so clone the plasmid and therefore also the gene
The plasmid are then isolated from the bacteria and are wrapped up in a lipid soluble molecule forming a liposome
The liposomes with the gene are sprayed into the nostrils of patients and are drawn down into the lungs
The liposome then enters the epithelial cells of the lungs causing the correct protein to be made

The previous two methods are sometimes not effective because:
Adenoviruses may cause infection
Patients may develop immunity
The liposome aerosols may not be fine enough to pass through the bronchi
Even when the gene is supplied to the epithelial cells, the protein is not always expressed.

Treatment for severe combines immunodeficiency

Severe combined immunodeficiency means that sufferers do not show a cell-mediated response nor are they able to produce antibodies
Individuals with the defective gene cannot produce the enzyme that would destroy toxins that kill white blood cells
Attempts to cure the disorder with gene therapy include:
The healthy ADA gene is isolated from human tissue using restriction endonuclease
The gene is inserted into a retrovirus
The virus is grown in a lab so the gene is copied
The retroviruses are mixed with the patients T cells
The DNA is injected into the T cells by the virus, thus providing the genetic code to make the enzyme.

Since T cells only live for 6 - 12 months the process has to be repeated
By treating bone marrow stem cells with the gene which divides to produce T cells, there is a constant supply of the Heath ADA gene and therefore

Section 16.6 – Locating and sequencing genes

DNA probes

A DNA probe is a small section of DNA that has an identifiable label attached to it
The probes are normally either radioactively labelled or are fluorescently labelled
DNA probes identify genes as follows:
The probe will be made of a complementary nucleotide sequence; this will allow the position of a gene to be identified
The DNA being tested will have its strands separated
The strands are mixed with the probe, which will bind to specific part of the strand. This is called DNA hybridisation

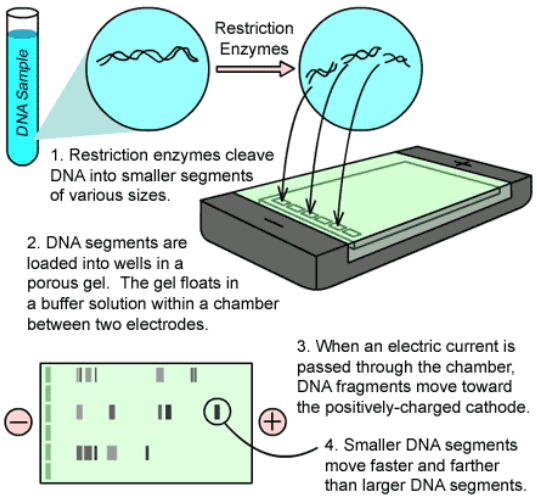
DNA sequencing

Used to identify the sequence of bases in the gene that is being located
The sanger method involves using modified nucleotides than cannot bind to one another and thus terminate the sequence
The process is as follows:
Four test tubes are set up; each of which will contain single stranded fragments of the DNA to be studied, a mixture of normal nucleotides, a small quantity of one of the modified nucleotides, a primer that is labelled with a DNA probe and lastly DNA polymerase which will catalyse the DNA synthesis

Since the nucleotides (either normal or modified) which join to the template DNA strand is random, chains of varying length will be made up depending on when the modified nucleotide has joined on
For the test tube that contains modified adenine, all the complementary DNA strands that are made up will all end in the adenine nucleotide but will be of varying lenth

Gel electrophoresis

This technique is used to separate the DNA fragments in order of length
This is process involves placing DNA fragments on to an agar gel, and applying a voltage across it. Since the gel has resistance, the larger fragments will be made to move more slowly that the smaller ones.
Once the fragments are separated out, a photographic film is placed over the agar gel
The radioactive label will cause the film to change colour where the particular fragment is situated on the gel



Gel electrophoresis will only be used for relatively short fragments of DNA, genes must therefore normally be cut first by restriction endonuclease. This is called restriction mapping

Restriction mapping

Restriction mapping involves cutting DNA at various different recognition sites
Fragments are then separated and identified with gel electrophoresis
When a plasmid is cut by R.E only one strand of DNA is produced. Because of this combinations of R.E are used to cut the plasmid into two fragments.
The size of the fragments produced depends on which restriction endonuclease are used

Automation of DNA sequencing and restriction mapping

Most DNA sequencing is carried out by machines
Fluorescently labelled dyes are used by computerised systems rather than radioactively labelled ones
Each modified nucleotide has a colour associated with it so that the whole process can be carried out in one test tube
PCR cycles are used to speed up the process
The electrophoresis is carried out in a single narrow capillary gel and the results are scanned by lasers and interpreted by computer software

Section 16.7 - Screening for clinically important genes

Screening is used to determine the probability of a couple having offspring with a genetic condition
Gene screening can be used to detect oncogenes
When both alleles of the oncogene in an individual have mutated, a cancer may form. Some people already have one mutated oncogene that they have inherited and so are at greater risk of developing cancer

There are 9 main stages of the process of gene screening:

- DNA sequencing is used to determine the nucleotide sequence on the mutated gene and is stored in a genetic library
- A fragment with a complementary sequence of nucleotide bases to the mutant gene is produced
- The fragment is turned into a DNA probe by radioactively labelling it
- PCR is used to create multiple copies of the probe
- The probe is added to a mixture of single stranded pieces of DNA from the patient being tested
- If the person has the genetic condition the probe will bind to the specific region on the DNA molecule
- The combined fragments are now distinguishable from the other pieces of DNA
- If complementary fragments are present, the DNA probe will be taken up and the x-ray film will be exposed
- If complementary fragments are not produced, the probe will not be taken up and the x-ray film will not be exposed

Genetic Counselling

Examines family history of certain diseases
A counsellor can advise a couple on the what the emotion, economically, medical and social issues that arise from having offspring that suffer from a certain genetic condition
Screening can help detect oncogene mutations. From this, a counsellor can advise the best treatment plan that would give the patient the best chance of survival

Section 16.8 – Genetic fingerprinting

Genetic fingerprinting

The genome of any organisms contains many repetitive, non-coding DNA bases
The repetitive sequences contained in introns are called core sequences
In every individual length and patterns of the core sequences is unique (except in identical twins)
The more closely related two individuals, the more similarities between core sequences
The five main stages of genetic fingerprinting are:
Extraction, Digestion, Separation, hybridisation and development

Extraction

DNA is extracted from sample cells and copied using PCR

Digestion

Specific restriction endonuclease enzymes are chosen that will cut close to the core sequences without altering them

Separation

Gel electrophoresis is used to separate the fragments by size
The gel is immersed in alkali to separate the double strands of DNA
Each single strand is transferred by southern blotting onto a nylon membrane
Southern blotting is achieved as follows:
A nylon membrane is laid over the gel
Absorbent paper is them placed over the nylon membrane. The liquid containing the DNA is soaked up by capillary action
This transfers the DNA fragments to the nylon membrane in exactly the same position as they were in the gel
Ultraviolet light then fixes the DNA to the membrane

Hybridisation

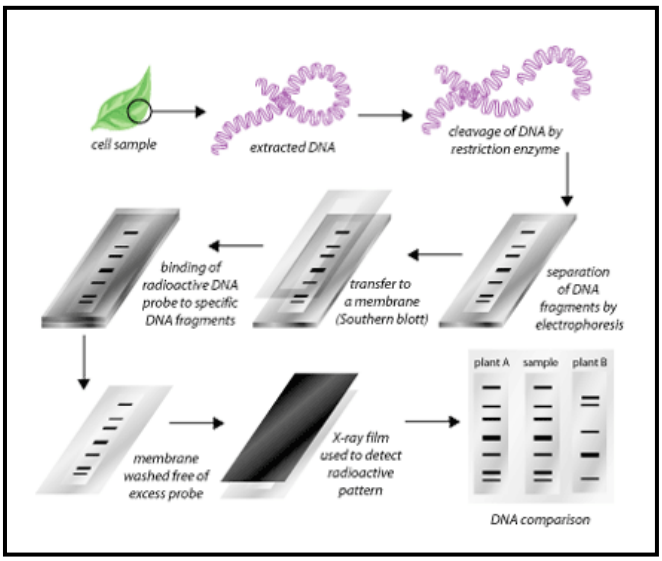
DNA probes complementary to the core sequences are added. They bind to the DNA under specific conditions (temp., pH and light). The various probes bind to different core sequences

Development

X – Ray film is now put over the nylon membrane. The radiation from the probes allows the position of the fragments after electrophoresis to be seen. The pattern of the bands is unique to every individual (except identical twins)

Summary

Extraction – DNA is extracted from the sample
Digestion – Restriction endonuclease cuts the DNA into fragments
Separation – Fragments are separated using gel electrophoresis
The fragments are then transferred from the gel to a nylon membrane by southern blotting
Hybridisation – DNA probes are used to label the fragments by binding to complementary core sequences
Development – Membrane with radioactively labelled DNA is added to x – ray film
X – ray film reveals dark bands corresponding to the position of DNA fragments after gel electrophoresis.



Interpreting the results

An automatic scanning machine can calculate the length of the DNA fragments. This is done using results from known lengths of DNA
The odds are calculated for somebody else having the same pattern
The closer the match, the higher the chance of the DNA coming from the person being checked

Uses of DNA fingerprinting

Since half the DNA of an individual comes from their mother and the other half from their father, each band on a DNA fingerprint should be found on either the mother or fathers DNA fingerprint also
This can be used to test for paternity
Genetic diversity can also be assessed using genetic fingerprinting
When members of the same population have similar genetic finger prints, the population will have little genetic diversity, hence a smaller gene pool