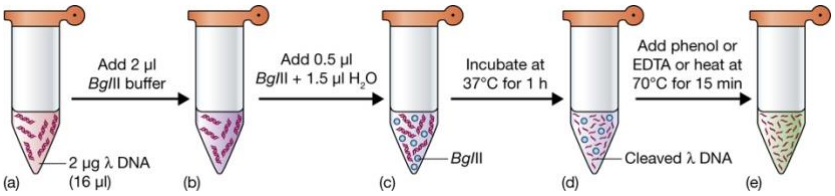
- **Phosphorus-32 ^{32}P**
  - Half-life – **14.3 days**.
  - Radiation – **beta** (**high energy** negatively charged electrons).
  - Range – **790cm** in air.
  - Detection – **Geiger-Muller**.
  - Shielding – **1cm Perspex**.
  - Uses – **labelling nucleic acids** e.g. DNA fingerprinting.
- **Sulphur-35 ^{35}S**
  - Half-life – **87.4 days**.
  - Radiation – **beta** (**low energy** electrons).
  - Range – **26cm** in air.
  - Detection – **Geiger-Muller**.
  - Shielding – **1cm Perspex**.
  - Uses – **labelling proteins** (methionine), **DNA sequencing** (S substitutes O in phosphate – used instead of radioactive phosphorus as it's lower energy so tighter band on X-ray film, and safer).
- **Tritium ^{3}H**
  - Half-life – **12.4 years**.
  - Radiation – **beta** (**very low energy** electrons).
  - Range – **6mm** in air.
  - Detection – **swabs and liquid scintillation** (too low for Geiger counter, hard to detect so have to swab for it).
  - Shielding – none (can be absorbed through skin, danger of contamination.
  - Uses – **labelling sugars**.
- **Iodine-125 ^{125}I**
  - Half-life – **59.6 days**.
  - Radiation – **gamma, X-rays**.
  - Range – long way.
  - Detection – **scintillation counting**, **thyroid scans** (gets concentrated there so check there for contamination).
  - Shielding – **lead**.
  - Uses – **labelling peptides** (once labelled not dangerous).

Genetic Modification - John Beeching

Introduction to genetic modification

- Gene cloning – **the purification, isolation and amplification of nucleic acid molecules in a cloning vector or via the polymerase chain reaction**, depends largely on the exploitation of prokaryotic biology (especially *Escherichia coli*, which we know more about than probably any other organism).
- **Basic cloning** involves: insertion of DNA fragment into **vector** to form recombinant molecule; **transport** of recombinant molecule into a bacterial cell; **multiplication** of cell and recombinant molecule; production of bacterial **colonies** containing recombinant molecule, each colony is a **clone** of identical host cells containing the recombinant molecule.
- **Polymerase chain reaction (PCR)** achieves exponential amplification of its target sequence.
  - Requires: a thermostable DNA polymerase, **Taq**; short DNA **primers** that can anneal to sequences that flank the gene of interest; a **thermocycler**, computer controlled heating block that can cycle through precise temperature changes.
  - **Heats** DNA to **break hydrogen** bonds and **separate strands**, **anneals primers** by complementary base pairing using **Taq**, **DNA polymerase** uses primer to **extend strand**, amount of DNA doubles through each cycle.
- **Why gene cloning**?
  - Provides a pure sample of the gene of interest – the raw material for the study of gene structure and function.
  - Enables genes to be modified and introduced into organisms (the same or different).
  - Enables genes and genomes to be sequenced.
- **Cloning fragments** – cloning permits the purification of individual nucleic acid fragments (can be genes or other molecules of interest), which can be used for further analysis or manipulation.

- **Using restriction enzymes** – reaction is carried out in a **microfuge tube** using sterile components, requires an appropriate **buffer** for the enzyme, usually incubated at 37°C for 1 hour for digestion to go to completion, enzyme is inactivated (by EDTA, heat or phenol).



- **Agarose gel electrophoresis** – invaluable tool for analysing, mapping and purifying DNA molecules, agarose is a highly pure form of agar, use to form a transparent gel matrix which can then be used to separate DNA molecules (like the products of restriction digests).
    - **Preparing an agarose gel** – agarose and buffer are heated until the agarose dissolves, cooled to around 50°C, add ethidium bromide, pour onto glass plate gel former, comb produces wells.
    - **Preparing for DNA electrophoresis** – the gel formers and comb are removed from the gel which is then placed in the electrophoresis tank and covered with the electrophoresis buffer, the digested DNA is mixed with a loading buffer which contains a dye (for visibility) and sucrose or glycerol (to make the sample heavier than the buffer so it sinks to the bottom of the well).
    - **Gel electrophoresis of DNA** – DNA moves **from the cathode towards the anode**, the gel matrix separates the DNA according to size, smaller fragments move further so it produces a **size fractionation**.
    - **Visualising DNA fragments** – DNA fragments are stained with ethidium bromide (EtBr) either during or after electrophoresis, then gel is placed on UV transilluminator, DNA bands fluoresce and can be photographed, can detect >10 ng DNA.
    - **Sizing DNA on gel** – fragments of DNA with known molecular weight are run on the gel in order to determine the size of unknown fragments, sizing can be done visually or more accurately by plotting a graph of size against distance travelled and using that to calculate the size of the unknowns (plot log of size of DNA for better graph).
- **Restriction mapping** – perform single and double RE digests on DNA (with one and then two Res), electrophorese them in parallel, accurately measure size of fragments, work out relative positions on DNA molecule.
    - **Restriction maps** can be useful for cloning particular genes or comparing molecules from different sources (whether they're the same), can be useful for identifying particular regions or combinations of enzymes for cloning genes of interest.
- DNA digest – agarose gels can also be used to isolate particular restriction fragments of DNA which can then be used for cloning or other purposes
- **Ligation – construction of a recombinant molecule**
    - Gene is inserted into a cloning vector by the action of **T4 DNA ligase** which originally comes from bacteriophage T4 where its function is to repair nicks in DNA.
    - **Action of DNA ligase** – it will join DNA molecules together (blunt ends can be joined but the process is not efficient as there is no base pairing, compatible sticky ends are easily joined), reaction requires **ATP**.
    - **Inter- and intra-molecular association** – in ligation you have no control over which complementary sticky ends join, so the insert and the vector may ligate with themselves rather than form the desired recombinant molecule.
    - **Alkaline phosphatase** – removes 5' terminal phosphates from the plasmid to prevent self-ligation, the DNA of interest can still be ligated as it has the 5' phosphates and the nicks will be repaired when the recombinant molecule is reintroduced into *E. coli*.

Plasmid Cloning Vectors

- Cloning vectors – used for cloning fragments of DNA, derived from naturally occurring plasmids or bacteriophage, have been modified to make them more convenient and versatile, derivatives of *E. coli* as well as its plasmids and bacteriophage are the principle tools of the genetic manipulator (although other bacteria and organisms can be used), other prokaryotes and eukaryotes can be used as well.
- **Cloning can provide many mg of a selected DNA molecule** – start with single *E. coli* cell containing a recombinant DNA molecule, produce a bacterial colony on petri dish, use colony to inoculate a liquid culture, grow overnight, purify recombinant DNA (note: the polymerase chain reaction can play a similar role).

Clone identification

- The problem - how to identify your clone from amongst the other colonies or plaques, particularly difficult when dealing with nucleic acids from eukaryotes (with prokaryotes it is sometimes more straightforward as their genomes are smaller and because the cloning is carried out in *E. coli*, a prokaryote, you can often get prokaryotic genes to express which can help with identification.
- **Direct selection** – set up so only the desired clone can survive, selection occurs at the plating out stage (only possible in a few circumstances), works with some bacterial genes but not eukaryotic genes because the genes can be expressed in *E. coli*.
    - E.g. you want to clone *kan*^{R} gene, clone all *Eco*RI fragments, plate out on kanamycin agar, only *kan*^{R} recombinants can grow
    - **Marker rescue** (variant of direct selection): use a mutant deficient in the gene you want to clone (auxotrophic mutant), idea is the gene you insert with substitute/complement the mutation – rescuing the genetic marker, works best for bacterial genes in metabolic pathways as enzymes are often essential for growth on particular substrates (so without they die).
    - E.g. if *E. coli* has *trpA*$^-$ mutation can't synthesise tryptophan and requires it in medium to grow, so after marker rescue only *trpA*$^+$ recombinants can grow on minimal medium – **complementation**.
- **Clone identification** – 'shotgun' cloning of all or most genes in a cell, identification through analysis of individual clones to identify correct one.
- **'Magic pointers'** - search the literature to see if "your" gene has already been cloned from **another organism** in another lab, due to descent from a common ancestor there is some degree of sequence similarity between the same genes from different organisms, the more closely related the organisms the better as the sequences will be more similar, ask for a clone of the heterologous gene, perform **nucleic acid hybridisation.**
    - **Nucleic acid hybridisation** – heat will separate (**denature**) the strands of the double helix to give single-stranded DNA molecules, on cooling single stranded DNA will attempt to base-pair together (**hybridise**), degree of hybridisation depends on the extent of complementary similarity between the two strands, measured by determining the energy required to re-separate the strands (**stringency**) which depends on **temperature** & **salt concentration**, although there are usually differences between the same gene from different organisms there is often sufficient similarity of sequence for them to find each other and hybridise (called **heterologous probing**).
    - **Heterologous probing** – use gene from organism A to identify the same gene in a library (cDNA or genomic) from organism B: immobilise library B on a nylon membrane and denature DNA to separate strands, make gene from A radioactive and denature gene probe, hybridise gene A (probe) with library B, wash off non-specifically bound probe (less than 80% complementary), expose membrane to X-ray film and hybridising clones give black spots, pick out correct clone from library B.
    - **Library screeingng: preparation of library** – library (bacterial colonies or phage plaques) is transferred to nylon membrane, DNA is **denatured and immobilised** on membrane, stick to nylon in same position as on petri dish.
    - **Library screening: hybridisation** - **probe** gene is made radioactive (^{32}P) and denatured, **hybridised** to library, wash to remove non-specifically bound probe, control **stringency** through temperature & salt concentration, exposed to X-ray film (**autoradiograph**), hybridise duplicate membranes to eliminate false positives (usually find spot then repeat with small surrounding area at lower concentration to get better separation between plaques).
    - **Screening libraries** – **heterologous probing** can be used to identify similar sequences between and within species.
- **Radioactive labelling of DNA to make a probe** - generally radio-labelled using a radioactive isotope of phosphorus (32-P) as part of the phosphate part of the base that makes up the DNA (generally alpha phosphate as beta and gamma are lost during formation of the phosphodiester bond).
    - **Random priming** – denature DNA, anneal random hexamer oligonucleotides (act as primers for Klenow fragments of DNA Polymerase I), add Klenow polymerase with dNTPs and labelled dNTP, synthesise labelled DNA, denature probe, hybridise.
- **Identification of a 'new' clone** - you cannot always use someone else's gene (becoming rarer), usually interested in a gene because you are also interested in the corresponding protein/enzyme so purify protein to homogeneity, use protein to provide amino acid sequence data (synthesise an oligonucleotide) or raise antibody, both can be used to identify "your" clone.
    - **Amino acid sequence for oligonucleotide synthesis** – determine amino acid sequence of part of your protein (often N-terminus), use genetic code to "**reverse translate**" amino acids to nucleotides,

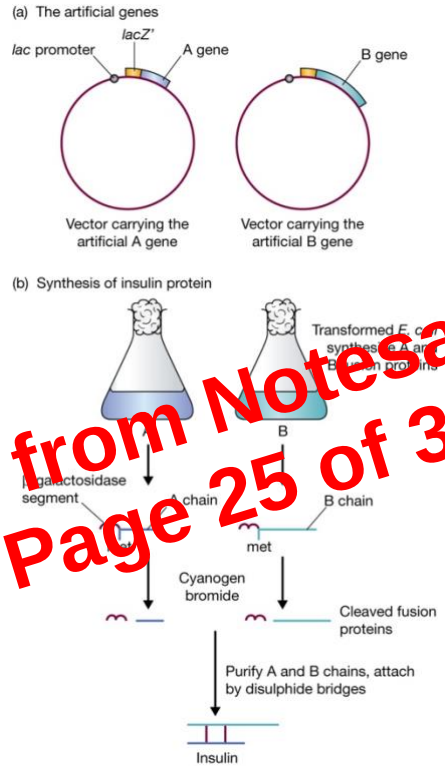
    - Only restriction fragments with **complementary** sequences to probe will hybridise and **'light up'** on autoradiograph, so **changes in band pattern** in test DNA (a RFLP) will be visible, indicating it possessed **mutations with respect to control** DNA.
- **Genetic diversity** – RFLP data can be scored for presence or absence of bands, measure of similarity calculated, trees plotted showing similarity (can compare diversity within and between populations of same species, if comparing different species can be used for phylogenetics).

Sequencing genes and genomes

- The finer structure of the genes - cloning and PCR are useful for isolating specific DNA sequences, restriction mapping is useful for sub-cloning fragments, RFLPs are useful for determining gene and genetic diversity.
- DNA sequencing: **chain termination** (Sanger) – exploits M13, but can also be used with plasmids and PCR products; **chemical degradation** (Maxam & Gilbert); there are automated methods based on chain termination.
- The phosphodiester bond - chain termination DNA sequencing relies on the fact that during the synthesis of a DNA strand the formation of the phosphodiester bond between the growing strand and the incoming nucleotide requires a 3' hydroxyl group as well as the 5' tri-phosphate.
- **Dideoxynucleotides** – dideoxy NTPs lack 3' hydroxyl group, once incorporated into a growing chain the ddNTP cannot form another phosphodiester bond because **no 3' hydroxyl** so the chain terminates.
- **Chain termination sequencing:**
    - DNA polymerase requires: single stranded template; primer base-paired to template; supply of dNTPs (dATP, dCTP, dGTP & dTTP).
    - In sequencing, also a very small amount of ddNTPs (ddATP, ddCTP, ddGTP & ddTTP), each labelled with a different fluorescent marker.
    - When ddNTP incorporated, the chain stops.
    - End up with four families of molecules that that stopped at a particular ddNTP.
- **Reading the sequence** – **polyacrylamide gel electrophoresis** can **separate** single-stranded DNA fragments that are **one nucleotide different in size** (so all possible different fragments), as fragments pass the detector the four different fluorescent **markers are detected** and the data feeds to the computer, sequence available as nucleotide letters or graphically.
- **Location of primer determines sequence read**: **universal primer** to vector DNA sequence flanking DNA insert is often used, can't sequence long bits of DNA so use universal primer to get as far as possible with confidence then use **internal primers** based on sequence obtained can be synthesised, total sequence is built up from these internal sequences, best to sequence genes in **both directions** for added confidence.
- **Shotgun sequencing** – random fragments sequenced, overlaps identified and used to build up **contiguous genome sequence** (contig).
    - DNA fragmented (sonicated), separated on agarose gel, fragments 1.6–2.0 kb collected, clone library created, end sequences of clones obtained, overlapping sequenced identified to obtain contigs, gaps closed between contigs through directed approach involving hybridisation to identify adjacent clones followed by sequencing.
    - **Problems** – computational aspects complex (best for smaller genomes like bacteria), identical repeated sequences can mess up data.
- **Clone contig** (more expensive and time consuming but better for larger genomes) – overlapping clones identified and map generated prior to sequencing (order them).
    - Use cloning vectors capable of taking very large fragments (e.g. bacterial artificial chromosomes – BACs), individual fragments are sequenced by shotgun approach, genome sequence built up step by step, physical or genetic map of genome helps greatly
    - **How to order BAC clones** (chromosome walking) – one clone (A1) is used as a probe to the whole BAC library, the clones A1 hybridises with (B4, I1) must overlap with it, repeat using B4 and I1 to determine further overlapping clones, build up **clone** contig, then these clones are each sequenced by shotgun method.
- **Post genomics** – after sequence is available genes and their funcitons can be identified, need sophisticated software which can learn.
    - **Genome annotation** – genes and regulatory sequences identified and annotated, existence of other genome sequences of related species helps.
    - **Identifying open reading frames** – 6 possible, start with initiation codon and end with stop codon in the same reading frame so search for these with more than 100 codons to find potential genes, in prokaryotes genes are densely packed and longer ORFs are usually genuine.

- **Pharmed animals** – proteins have been produced in the blood of transgenic animals and the eggs of transgenic sheep but the most successful approach has been in farm animals where the clones' gene is driven by the animals **β-lactoglobin gene** which is active in the **mammary gland** so the protein can be harvested from the milk (cows produce 8,000 litres per year – 40 – 80 kg protein).
- **Pharmed plants** – plant cell culture well established, plants grown in field (rice, tobacco, maize, sugarcane), can use strong organ-specific promoter (seed of bean, potato tuber) so easy to harvest protein when produced, proteins have been produced for pharmaceuticals (interleukin, antibodies, vaccines), there are ethical issues like suffering in animals and the environmental impact of GM plants.

- **Recombinant pharmaceuticals** – many human disorders due to imbalance of proteins (absence or malfunction) which can be corrected by administering human protein, e.g. insulin controls blood glucose levels and pig insulin can be used but there are slight differences so human insulin would be best but it needs post-transcriptional processing.
- **Recombinant insulin** – can be synthesised in *E. coli*, clone A and B chain gene fragments into separate inducible vectors, synthesise **fusion proteins** and purify, **cleave off β-galactosidase segment** with **cyanogen bromide** which cleaves at methionine, purify A and B chains, join by disulphide bridge formation (not very efficient), alternatively the synthesis of proinsulin alone can help form disulphide bonds followed by proteolytic cleavage of C chain.



- Human proteins cloned and synthesised in bacteria or eukaryotic cells:

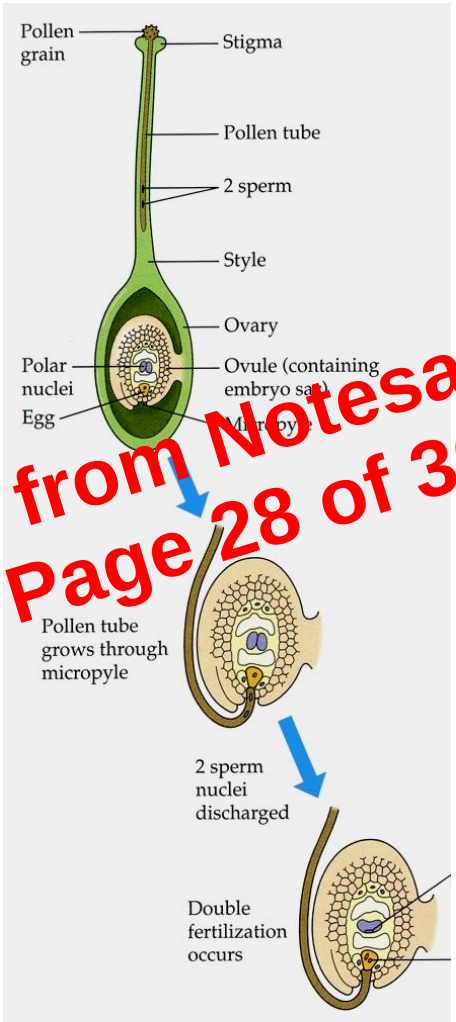
Protein	**Used in treatment of**
Deoxyribonuclease	Cystic fibrosis
Epidermal growth factor	Ulcers
Factor VIII	Haemophilia
Follicle stimulating hormone	Infertility treatment
Insulin	Diabetes
Interferon-α	Leukaemia and other cancers
Interferon-β	Cancers, AIDS
Relaxin	Used during childbirth
Somatostatin	Growth disorders
Somatotrophin	Growth disorders
Tissue plasminogen activator	Heart attack

- **Recombinant vaccines** – usually use inactivated infectious agent to stimulate body to produce antibodies but inactivation must be 100%, using purified viral proteins can also elicit an immune response, cloned genes

monocots only used during germination so we get calories from it in cereal crops), so plant embryos are simple (**cotyledons** – embryonic leaves, first to appear; and **axis** – linear arrangement of different groupings of cells forming first organs of plants); organs are generated by two **meristems** (shoot and root) – vegetative organs, leaves, and then reproductive organs develop so meristems have to be **reprogrammed** to change the phases of development.

- **Cell movement and planes of division:** plant cells have cell walls which cement them in place so the **cells cannot move or migrate during embryonic development** (pollen is moved by other organisms), so development of plant form is dictated by **division planes** and **expansion of immobilised cells** – **anticlinal** divisions are with cell plate at right angle to surface, **periclinal** divisions are with cell plate parallel to surface; altering division planes allows building of depth and in three dimensions.
- **Totipotency:** plants can **regenerate from numerous vegetative** (somatic) **parts**, there are very few stem cells in animals and most can't regenerate body parts; fully differentiated plant cells can differentiate and then re-differentiate into a whole new plant – because not **sessile** (can't react to predators) so meristems allow to regenerate damaged tissues and can keep going for a very long time (Bristlecone pine, *Pinus longaeva*, is 5000 years old).

- **Embryogenesis:**
  - **Pollination/fertilisation –** double fertilisation event.



Fertilised central cell – endosperm.

Fertilised egg – embryo.

  - **Embryo/endosperm**: two-celled proembryo has a small cell at the top and a larger cell at the bottom → globular → heart – start to see pattern (4-5 days in Arabidopsis) → walking-stick – basic structures become visible (9 days in Arabidopsis) → endosperm broken down and nutrients are transferred into embryo.
  - **First zygotic division**: **asymmetric** physically and a molecular level, and mutants which block asymmetry develop differently; produces **small apical cell** (embryo) and **large basal cell** (suspensor) which both have different fates (what the cell will normally become), cell fate is a component of position.
  - **How cells learn their fate:** specific cell types develop in specific places, position and identity (structure and function) are linked, cells within the globular embryo are already different when DNA is looked at.