LECTURE 6- ENZYMES IN RDT

Restriction endonuclease. –DNA ligase. –Polynucleotide Kinase. –ALP [removes 5' phosphate from RNA/DNA]. –Terminal transferase. Reverse transcriptase. –Nuclease. –DNA polymerase.
->When ALP is added, the phosphates from the ends of the DNA/RNA strands are removed. –Phosphates are required for phosphodiester bonds.
->If removed, Ligation of the strands are prevented. –Phosphate can be replaced with a labelled phosphate molecule in experiments.
-Terminal transferase: Template independent polymerase enzyme which catalyses addition of deoxynucleotides to 3' end of DNA.
Adds complementary homopolymeric tails to DNA i.e. TTTTTT to the 3' ends
-Reverse transcriptase: Used in cDNA synthesis in RT-PCR. –Enzyme is isolated and purified from retroviruses.
-Nuclease: Enzyme which cleaves the phosphodiester bonds between nucleotide subunits
S1; attacks single strands. –S1 nuclease digestion removes unhybridised RNA/DNA probes [nuclease protection assay].
BAL31- attacks Double stranded DNA, exonuclease degrades both 3' and 5' termini
-DNA Polymerase: (I) Nick translation- DNA label.
Klenow fragment of DNA pol I [large protein fragment made via cleavage of DNA pol I from e.coli by Subtilisin.
(I) Comes from e.coli, function is mainly for Proof reading and DNA repair. –Makes DNA in 5'-3'
DNA labelling: Techniques= Nick Translation. –Random priming. -3' or 5' end labelling. –PCR
Labels used= Radioactive. –Deoxy/Dideoxynucleotides
Non-radioactive methods= DIG. –Flourochromes [cy3/cy5]. –Biotin. –ECL [enhanced chemiluminescence.]
-Nick translation: Requires simultaneous action 2 two enzymes.
DNase I: cleaves or 'nicks' random phosphodiester bonds on both DNA strands.
–In Mg^{2+} presence, DNase I becomes a Single stranded endonuclease.
DNA pol I [from e.coli]: Adds labelled nucleotides to the 3' ends created by DNase I during cleaving.
It also has a 5-3' exonucleolytic activity, removing nucleotides on the 5' of the nick
Simultaneous elimination and polymerisation shifts the nick along the DNA labelling the DNA
-Random Primer: Denaturation and annealing of DNA strand with random primers.
Klenow fragments then polymerise extending the oligodnucleotides with labelled dNTP+s
-Non radioactive/ECL: Double stranded DNA is denatured with boiling water.
HRP [Horse radish peroxidase] added, it is an enzyme with a [+] charge which binds randomly to [-] DNA
Cross-linkage of HRP+ and DNA via tight binding of Glutaraldehyde.
-Flourescent probe: DNA probe is synthesised using modified dNTPs and Flourochrome side group. –Allows for a single probe binding event.
-DNA microarrays: mRNA extracted to determine the gene expression. –Allows you to analyse multiple genes at once.
Involves hybridisation of nucleic acids to oligonucleotide probes [attached to solid support]
->Molecular analysis of DNA: Bioinformatics. –DNA sequencing. –Restriction mapping

-DNA sequencing methods: Manual- Using ssDNA template [Sanger]. –Primer annealing. –Elongation via DNA Pol [Klenow fragment].
Cycle- Automated system, Thermal cycling sequence. –PCR

LECTURE 7- MIGHTY MOUSE, VECTORS AND CLONING

DNA sequencing- the next step: Bioinformatics= assigns the DNA sequence to gene function. –Molecular diagnostics. –Disease mapping.
-Bioinformatics: Computer analysis, provides data on the ORF [coding sequences] & generates restriction site maps.
->Mighty Mouse: PEPCK_C, a crucial gluconeogenic enzyme which catalyses diversion of TCA intermediates towards gluconeogenesis.
-Gene expression: Studied via Northern blot [RNA] analysis. –Western blot used in overexpression studies.
-PEPCK_C involved in Gluconeogenesis in the Liver & White/brown adipose tissue.
->Skeletal muscles were targeted for over expression of PEPCK_C as they do not synthesise their own Glucose.
-Knockout studies: Stop genes from working. –Case study= PKC/HINT1 knockout mice
->PCK/HINT1 gene: Protein Kinase C interacting protein [small]. –Located within the neurons [neuronal processing activity.]
Absence of this gene is associated with Mood dysfunction behaviour, depression and anxiety
=>Hypothesis testing: Create a PCK/HINT1 knockout mouse [lacking gene] and compare to the wild type.
=>How to test: Forced swim test- knockout mice exhibited less immobility than WT mice

Obtaining large quantities of a particular DNA fragment- Use PCR or cell based cloning techniques.
-Recombinant DNA cloning: DNA fragment for cloning enzymatically inserted into a Plasmid vector.
Incubate e.coli cells with the plasmid vector in $CaCl_2$ presence & culture on nutrient agar containing Ampicillin.
Cells which have incorporated plasmid survives, ones than do not die.
Cells replicate the plasmid and begin multiplying. –Colony of cells now contain copies of recombinant plasmid.
->Choice of Vectors: Features- multiple cloning sites, a replication of origin, and selectable markers [i.e. Amp gene]- easier to grow and detect.
Types- Plasmid pBR322, pUC. –Viral [Lambda vector]. –Hybrid= Cosmids [COS site of phage λ + plasmid] i.e. Phagemid.
Artificial chromosomes. –Specialised vectors aka Expression vectors [engineered to produce a gene product]
->Colour selection/screening: LacZ gene. –β Galactosidase.
Mutant LacZ gene + absence of β Galactosidase. -> White colonies
β Gal + Xgal [colourless] -> Blue colonies.

-Protein and its mutant separates in Reverse phase chromatography but not in ion exchange chromatography.
=> Mutation type- Polar uncharged to Hydrophobic residue. –If proteins are not separated in Ion exchange, then mutation has not altered charge

LECTURE 15-16: PROTEOMICS

Proteome: A set of proteins expressed in a particular cell at a particular time in a set of specific conditions [differentiation, disease, drugs]
-Single gene can produce many proteins via Alternative splicing and Post-translational modifications [phosphorylation]
->Average: 1 gene= 40 proteins. –High throughput techniques; analyses large number of proteins in a single experiment.
=>2D PAGE: Identifies proteome components. –Protein Microarrays: Identifies protein interactions.
Proteomic analysis: Comparison between a diseased person and health person with 2D PAGE pattern allows disease biomarker identification
-Cancer biomarkers found via Proteomics.
->Proteins can be extracted from gel spots and identified via: Edman Degradation. –Mass spec.
=>Edman Degradation; protein sequencing- Use of chemicals to remove amino residues step by step from N terminus to obtain small fragment
Sequence <20 amino residues analysed on a database
=->Reaction: Coupling [PITC] reacts with the amino group of polypeptide to give PTC. ->Excess PTC washed with benzene
Cleavage- Dried PTC is treated with anhydrous acid cleaving the molecule.
Conversion- Cleaved product= unstable, therefore it is heat treated in HCl. –Then identified via HPLC using UV absorbance.
-Doing the experiment 20 times means 20 amino sequences.
==>Some proteins require cleavage before sequencing- Edman degradation requires free amino groups at the N terminal.
Some proteins have modified N-terminus therefore needed to be cleaved into Peptides
Chemical cleavage; Cyanogen bromide. –Enzymatic cleavage: Trypsin
-Protein Microarray: Microscope slide with microscopic spots each containing a type of protein.
Determination- Calmodulin labelled with green fluorescence is applied and binded to some proteins
-Mass Spec: Once proteins are extracted from the gel spots on 2D PAGE, mass spec can identify them.
Proteins are ionised. -> Accelerated in a electric field. –Acceleration depends on mass & charge of protein
->Proteins must be digested first into peptides to remove complications of Post-translational modifications and hydrophobicity.
Both make it harder to analyse in Mass spec, plus protein sequences in database are translated from cDNA.
->MALDI/ ESI is used to ionise proteins and peptides to prevent fragmentation.
=>MALDI: Mix protein with organic compound [matrix] -> Deposit the mixture onto a plate then dry to produce a film with protein+matrix
Use a laser, produces an energy pulse of a specific frequency for that matrix.
Energy is absorbed into the Matrix -> Transferred to the protein becoming Ionised -> Protein enters the gas phase.
Ions analysed with a TOF [time of flight] analyser- lighter ions reach the detector first.
=->Mass determination: M/Z -> peak at 5700 with a charge of +1. -> M= 1x 5700 = 5700, then do 5700 -1 (Z) = 5699

=>ESI: Electrospray Ionisation- Droplet molecules spray from a Capillary at high current. –Droplets contain sample + solvent with charges (H+)
Droplets begin to slowly lose solvent molecules in mixture giving sample ions with multiple charges.
Quadruple analyser used: Ions accelerated with Quadruple [constant oscillating electric field applied]
Ions of different M/Z are selected to pass through detector.

LECTURE 16-17: PROTEIN STRUCTURE & FUNCTION OF SERINE PROTEASES

Proteolysis= Hydrolysis of peptide bonds in proteins. –Protein + Water ---[protease]---> Peptide1 + Peptide 2. –Split into 2 domains.
-Roles: Digestion of proteins from food. –Cleavage of signalling sequences after the protein has reached its destination. –Cyclin degradation.
Conversion of precursor proteins into their final form [Proinsulin -> Insulin].
-Serine proteases: Similar structure but different substrate specificity. –Both have Alpha helices & Beta sheets
Chymotryspin: Digests bonds after aromatic residues [Trp, Phe, Tyr] & those with long hydrophobic chains [Met, Leu].
Trypsin: Digest bonds before basic residues [Lys, arg] except if it follows Proline
->Active site shape is different but the cleavage part is the same. –C=O-N- is cut.
Zymogens: Inactive precursor form of the enzymes. –Serine proteases are made like this [Trypsinogen -> Trypsin, Plasminogen -> Plasmin]
Stored in Zymogen granules within the Pancreas and only secreted into SI and are then activate
Proteases are required to cleave Zymogens to form active Proteases. –Process= irreversible. –Inhibitors required to stop Proteolysis
Inhibitor: Pancreatic Trypsin inhibitor- binds strongly to the active site of Trypsin. –However it is cleaved off very slowly [1 year]
=>Defective inhibition of Elastase: α1-Antitrypsin [AAT] = Elastase inhibitor. –Low/defective AAT binding means excessive Elastase in the body.
Elastase excessively destroys walls of the lungs making them less elastic.
Not enough Elastin to help Alveoli during expiration, air is trapped meaning less O2 in the blood.
Emphysaema: Smoke oxidises Methionine 358 in AAT- residue essential for elastase binding.
Hereditary- Z mutation of Glu342 -> Lys, slows AAT secretion
-Chymotrypsin activation: Chymotrypsinogen [inactive] –[Trypsin]---> π-Chymotrypsin [active] --> α-Chymotrypsin [active]
The result of cleavage causes conformational change forming specific active sites for aromatic & long hydrophobics
Serine: Contains a Catalytic triad- Ser195, His57, Asp102 form the active site. –They are H bonded via Imizadole rings between them.
A substrate binding to Serine will have the aromatic ring attached to the binding pocket, and the Cleavage site positioned above Ser-195
Ser-195 transfers H+ to His-57 via help of Asp102. –Serines O2 bonds with Carbonyl Carbon forming a tetrahedral transition state.
H+ is transferred from His57 to the substrate. –Peptide bond is then cleaved & the C-terminal peptide is released.
Water molecule enters the active site and H bonds with His57. –H2O transfers H+ to His & OH- to substrate forming 2nd Transition state.
H+ transferred back from His57 to Ser195 & N terminal peptide is released.
->Protein is digested into 4 peptides for Mass spectrometry via Trypsin, 3 Molecules of Water therefore needed for digestion of each protein