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Course Code: BMI3702

Assignment Number: 864389

QUESTION 1

Describe in detail the steps involved in eukaryotic DNA isolation and precautions you would take to prevent shearing of the eukaryotic DNA.

DNA extraction is the procedure used to isolate DNA from the nucleus of cells. The three major steps involve DNA isolation are lysis (dissociation of the cell membrane and release of DNA), precipitation and purification.

Cell lysis: The cell and nucleus are broken open to release the DNA inside. Unlike prokaryotic tissue, eukaryotic (plant and animal) tissue must be ground up first before lysis in a process called homogenization (mechanical disruption) to increase the surface area for cell lysis. During lysis, an enzyme such as lysozyme or is used to free the DNA. Detergents such as SDS are used to break down the lipids in the cell membrane and to denature deoxyribonucleases and other proteins (Boyer, 2000).

Precipitation: before precipitation, the solution is deproteinized by adding an enzyme such as proteinase K to degrade DNA-associated proteins and other cellular proteins. A high concentration of salt (NaCl) is added to ensure complete dissociation of the DNA-protein complex and removing bound cationic amines. This neutralizes the negative charge on the DNA molecules, making them more stable and less water soluble (DNA is soluble in water but insoluble in the presence of salt and alcohol). Precipitation separates DNA from cellular debris. During precipitation an alcohol such as ethanol is added to the DNA sample and causes the DNA to precipitate out of the aqueous solution.

Purification: after the DNA has been separated from the aqueous phase, it can be thoroughly rinsed in 70% ethanol to remove residual salts. At this point the purified DNA is then resuspended in a low ionic concentration buffer ideally at pH 8.0 for easy handling and storage. If a highly purified state of DNA is required, several deproteinization and precipitation steps may be carried out (Boyer, 2000; Quick & Loman, 2018).

To prevent DNA from shearing, extra care must be taken especially when choosing the methods for lysis step of extraction. Some of the mechanical disruption methods can be too rough on the DNA. For example, mechanical

method of bead-beating is very effective at releasing DNA from cells but also causes a lot of shearing due to its vigorous nature. This makes it a highly unsuitable method for isolating high molecular weight DNA. One way of preventing shearing would therefore be to choose gentler lysis methods and steer clear of methods that are rough on the DNA. One should also avoid vigorous stirring and vortexing of the lysate as this causes shearing (Quick & Loman, 2018).

QUESTION 2

Discuss in detail the theory and application of southern and western blotting

Named after Edwin Southern who developed the procedure, Southern blotting is a technique which enables the detection of DNA fragments/sequences from a mixture of DNA molecules. Southern blotting is a process which involves separating DNA fragments by electrophoresis, transferring the separated DNA fragments from agarose gels to a cellulose-nitrate (filter) membrane and the subsequent fragment detection by probe hybridization using a labelled probe specific for the gene of interest (Willey *et al.,* 2014). Extracted and purified DNA is cut into fragments using a suitable restriction enzyme, and the resultant fragments are amplified by PCR. The fragments are then separated by gel electrophoresis, then the SDS gel is soaked in alkali to denature the double-stranded DNA and separate its strands. The separated DNA strands are then transferred and bound to a nylon membrane by the process of blotting. The DNA of interest is then identified by bathing the filter in a solution containing a radioactive probe. The probe, which contains complementary sequences to the DNA of interest, then binds with complementary DNA on the membrane. The DNA to which the probe hydrogen bonds is now radioactive and can be detected by autoradiography (Willey *et al.,* 2014).

Western blotting, also known as protein immunoblotting, is a technique used to detect specific proteins in a given sample of cell lysates, tissue homogenate or extract. The process involves gel electrophoresis of a purified protein sample followed by transfer of the separated proteins to sheets of either nitrocellulose, nylon or polyvinyl-difluoride (PVDF), and the subsequent visualisation of protein

bands by treating the membrane sheets with solutions containing enzyme-tagged antibodies (Boyer, 2000; Willey *et al.,* 2014). Extracted protein is separated based on molecular weight through gel electrophoresis (SDS-PAGE is usually preferred). The separated proteins are blotted onto a matrix membrane (usually nitrocellulose) and protein binding sites on the membrane that are not occupied by blotted proteins are blocked using a solution of bovine serum albumin to prevent the detection proteins from binding to the membrane and interfering with the detection process. The blotted membrane is then incubated with a labelled antibody specific to the protein of interest (primary antibody) and it forms an antigen-antibody complex. The blot is further incubated with a secondary antibody which is enzyme-labelled in a process much like indirect ELISA. Unbound antibody is washed off, and the bound antibodies are visualised by a coloured band produced by conversion of substrate by the enzyme. The coloured band indicates the position of the protein of interest (Boyer, 2000).

According to Boyer (2000), blotting techniques have many applications, including mapping the genes responsible for inherited diseases using restriction fragment length polymorphisms (RFLPs), screening collections of cloned DNA libraries. Southern blotting is used in RFLP analysis and to perform DNA fingerprinting for analysis of biological material in criminal identification and forensics, paternity and maternity testing, and personal/victim identification. It is also used in prognosis of cancer and prenatal diagnosis of diseases caused by genetic defects by identifying mutations, deletions and gene rearrangements in DNA sequences. Western blotting is used for diagnosis of diseases as it can show strain-specific immune responses to microorganisms, making it an important diagnostic indicator of a recent infection with a particular strain of microorganism (Willey *et al.,* 2014). It can also be used for prognostic implications with severe infectious diseases. The Western blot technique is often used as a confirmatory/definitive test for a positive ELISA result to confirm the presence of an antibody in diagnostics as it provides better specificity than ELISA does. For example, western blotting is the confirmatory test for HIV. It is used as a definitive test for Creutzfeldt-Jakob disease (CJD), Lyme disease, Hepatitis B and Herpes. The western blot is also used to detect defective

proteins for example, in diagnosis of prion diseases such as CJD (Online Biology Notes, 2017a; Online Biology Notes, 2017b; Willey *et al.,* 2014).

QUESTION 3

Assume that a centrifuge is operating at 43,000 rpm. What is the relative centrifugal force at a distance from the central axis of 6cm

$$\text{RCF} = (1.119 \times 10^{-5})\ (\text{rpm})^2\ (\text{r})$$
$$= (1.119 \times 10^{-5})\ (43{,}000)^2(6)$$
$$= 124{,}141.86 \times \text{g}$$
$$= 124{,}142 \times \text{g}$$

QUESTION 4

Explain the purpose of each chemical reagents that are used for PAGE

a) Acrylamide- it is a water-soluble monomer which polymerizes upon addition of water to form a highly cross-linked matrix through which the proteins migrate. Through this process, pore size can be regulated hence allowing smaller molecules to be examined better. The gel is synthetic, thermo-stable, and strong; hence it can withstand high voltage gradients.
b) TEMED- it is a free radical stabilizer and is an essential catalyst for polymerization of polyacrylamide.
c) Coomassie blue dye- is the most popular protein stain, which anionic and non-specifically binds to proteins. It is used to stain protein samples after electrophoretic separation in polyacrylamide gel.
d) Bromophenol blue – it is used as a tracking dye which helps track the progress of proteins through the gel.
e) Sodium dodecyl sulfate- is used to help identify and isolate protein molecules. It is a powerful negatively charged detergent that acts as a surfactant which binds to hydrophobic regions of the protein molecules and denatures them (causes them to unfold and become released from their associations with other proteins or lipid molecules). The individual protein molecules are then rendered freely soluble in the detergent solution.

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QUESTION 5

5.1 Why can you not use a glass cuvette for absorbance measurement in the UV spectral ranges?

Optical glass shows absorbance throughout the visible and IR regions that extend from 340nm to 2500nm, covering most organic and inorganic species. However, glass absorbs strongly the UV light, and the application of glass cuvettes for wavelengths that are below 340nm is not recommended. Instead a cuvette made of quartz is better suited for UV spectral ranges as quartz can cover wavelengths from 200 nm. Glass cuvettes are therefore not used for absorbance measurement in UV spectral ranges because glass does not allow the transmission of UV light (Boyer, 2000).

5.2 Why must a cuvette with four translucent sides be used for fluorescence?

A spectrofluorometer contains a light source, two monochromators, a sample holder, and a detector. Fluorescent light is directed by an optical system to the excitation monochromator, and the exciting light is passed into the sample chamber which contains a fluorescence cuvette with dissolved sample. Because of the geometry of the optical system, a typical fused absorption cuvette with two opaque sides cannot be used. Instead, special fluorescence cuvettes with four translucent quartz or glass sides must be used (Boyer, 2000). This is because fluorescing light is measured at right angles to the light irradiating the sample (beam path), hence a cuvette with four translucent sides is used to limit contributions to the spectroscopic reading from the beam itself.

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QUESTION 6

Describe the basis for separating proteins using:

a) Ion exchange chromatography

Proteins are separated from one another based upon the difference in their charge. The column is packed with a stationary phase of synthetic resin which is tagged with ionic functional groups. Solute molecules of opposite charge to the resin will bind tightly but reversibly to the stationary phase. The greater the charge, the stronger the interaction. This technique can be used for separation of proteins, peptides, and other charged biomolecules (Rodwell *et al.,* 2015).

b) Affinity chromatography

The separation of molecules is based on biological interactions. An insoluble stationary phase is covalently linked to appropriate ligand molecules (enzymes, antibiotics or metal ions). Only macromolecules that recognize and bind to the immobilized ligand are retarded in their movement on the column., and non-bonding molecules are washed through the column. This technique can be applied to isolation and purification of almost all biological molecules such as nucleic acids and enzymes.

c) Gel filtration chromatography

Also known as gel exclusion chromatography, it achieves separation of molecules according to molecular weight. A solution containing solutes of different molecular weight is passed through a column. Large solute molecules larger than the small pores on the stationary phase cannot enter the interior hence elute fast. Small molecules which can enter the pores will take the most time and intermediate molecules will take time in between the large and small molecules. It is therefore of major importance in the purification of proteins, nucleic acids, enzymes and other biomolecules (Boyer, 2000).

d) Gas chromatography

This is based primarily on partitioning processes involving two different phases, a mobile phase of gas, and a stationary phase of either liquid or solid state. The sample to be analysed is vaporised into gaseous phase and passed through the stationary phase. The chemical constituents will distribute themselves between the mobile phase and the stationary phase on solid support. This technique is used to detect compounds like fatty acids and essential organic solvents (Boyer, 2000).

e) Size-exclusion chromatography

This technique separates molecules based on their difference in size. It is usually applied to large molecules such as proteins and industrial polymers. Proteins of different sizes move through the column at different rates and are therefore separated accordingly. Size-exclusion chromatography is a widely used technique for the purification and analysis of proteins, polysaccharides and nucleic acids (Boyer, 2000; Rodwell *et al.,* 2015).

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RESULTS

Total = 71 / 75 (95%)