Biomedical Techniques

Only online module for BMI3702

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Word of welcome

Welcome to the module Biomedical Techniques (BMI3702). We hope that studying this module will be an exciting and memorable learning experience for you.

The module has been especially designed as an introductory course, and we assume that you have little to no prior knowledge of biomedical techniques. If you do, however, have some background knowledge on the subject, the up-to-date information, applications and activities in this module will complement your knowledge.

Purpose of the module

Why is it important to enrol for a module on biomedical techniques?

As a subject, Biomedical Techniques forms one of the most important cornerstones of the health sciences. The purpose of this module is to allow you to gain insights into the practical methods used to identify pathogens for diagnostic purposes, and to use basic as well as more sophisticated techniques to analyse macromolecules such as DNA, RNA and proteins. We use these practical methods to gain insight into the structure and functioning of the healthy (and the unhealthy!) human body.

Outcomes of the module

The aim of this module is to enable you to

- improve your ability to handle information
- conduct independent study and extract information from scientific literature
- appreciate and understand the principles underlying the techniques used in biomedical scientific research
- think critically to solve problems in a familiar setting (context) on the subject
- communicate practical results in a clear and structured way.

Learning time

How much time should you spend on this module?

This module carries 12 credits – it should, therefore, take you approximately 120 hours to successfully complete. This includes reading time, completing activities and assignments, and preparing for the examination. The mind map below gives you an idea of how to spend your time. Remember that this is only an example, the guidelines are not cast in stone. You will have to plan carefully and decide what will work for you, as an individual.

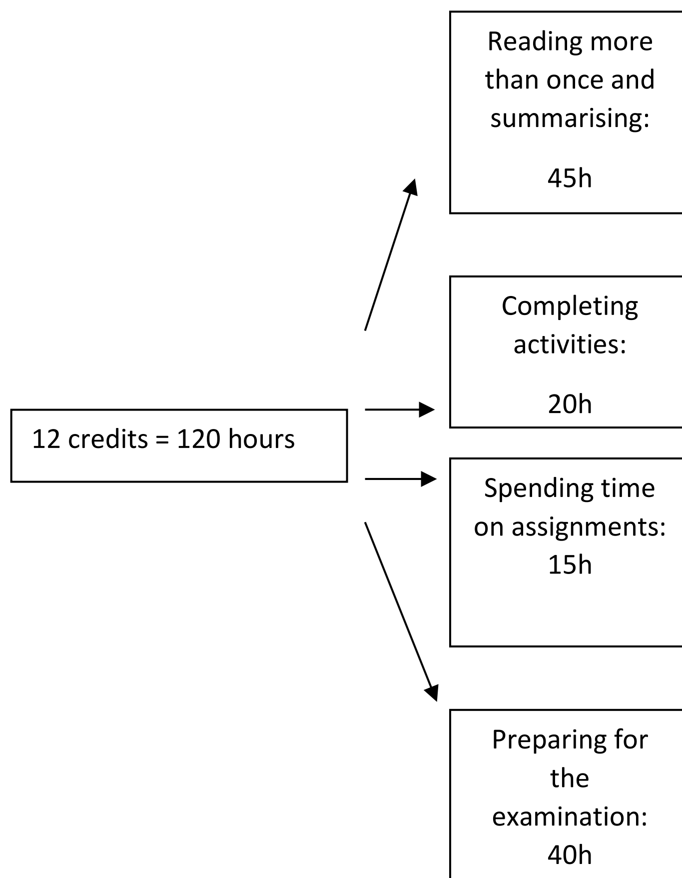

Figure 0.1: Structuring your learning time

Learning approach of this module

Like many of your fellow students who have enrolled for this module, you probably have a full-time job, and will therefore only be able to study part time. Much of what you will achieve, is therefore dictated by your own efforts and commitment. The most successful distance education students are not necessarily the cleverest or the most experienced – rather, the successful students are those who are the most disciplined, organised, willing to reflect critically on their learning, best able to apply theory to practice, and clever in managing their time.

To support you in your efforts, we have designed this module to get you actively involved in the learning process. This means you not only have to read and write, but also have to apply what you have learnt; to reflect on the success or failure of the application and learn from your mistakes. Learning is therefore not simply a theoretical exercise, but a practical, experiential undertaking.

Study materials

There is no prescribed textbook for this module.

You will need

- a workbook in which to can complete your activities, summaries and review questions. Please buy this yourself. It is your book, we do not want to see it;
- access to the internet, in order to download specified research articles.

Try to organise a study group with your fellow students. That will allow you to discuss everything in your workbook, as a group.

Introduction to biomedical techniques

Biomedical science relates to the development and application of new concepts, methods and techniques to solve diagnostic and treatment-related problems in medicine. Most of the experimental techniques and skills that you have acquired and mastered in other laboratory courses will be of great importance in this subject. You will, however, be introduced to new concepts, procedures and instruments that you have not used in chemistry or biology laboratories.

Translational biomedical studies have become increasingly common and very useful in the evidence-based practise of medicine. Many of these biomedical techniques are a result of genetic engineering, and have become increasingly relevant in translational biomedical studies, especially in the fields of cancer, genomics and immunology.

Biomedical techniques have wide clinical applications in diverse fields of medicine, including disease prevention (vaccination), diagnosis and treatment, and toxicology studies, among others.

Learning Unit 1: Basic laboratory and safety procedures

1.1 Introduction

In Learning Unit 1, you will learn about laboratory safety procedures using a variety of techniques and equipment, and will gain the theoretical knowledge needed to prepare buffer solutions. In addition, you will be introduced to the theoretical principles and calculations behind the measurement of weight, volume and pH.

Some chemicals that are used in biomedical science laboratories are potentially harmful. Many procedures involve glassware, open flames and sharp objects that can cause damage if used improperly. This learning unit is aimed at introducing you to those procedures that are of the utmost importance for the safe and successful completion of a biomedical project.

1.2 Learning unit outcomes

On completion of this learning unit, you should be able to:

- Understand laboratory procedures which require a variety of techniques and equipment
- Explain the theoretical knowledge behind buffer preparations
- Apply the theoretical principles and calculations behind the measurement of weight, volume and pH
- Compare different sample materials during collection and preparation

1.3 Safety in the laboratory

1.3.1 Safety first

Below are a few general laboratory practices that can significantly decrease your risks of accidents.

- You need to familiarise yourself with the procedures to follow when working in any laboratory.
- Be familiar with the materials you are working with (e.g., chemical, biological, radioactive). Always refer to written laboratory protocols and review the material safety data sheets for different chemicals. Consider the toxicity of the materials, and the health and safety hazards of each procedure.

- Take advantage of the knowledge and experience of laboratory personnel and the guidelines for using the safety equipment that is available.
- Familiarise yourself with the location of safety equipment and emergency procedures in the area you occupy.
- Always wear appropriate clothing such as long trousers, long-sleeved shirts and closed shoes as well as a laboratory coat, when you are in the laboratory. Open-toed sandals are prohibited; shorts and skirts are not recommended.
- Do not work alone in the laboratory. When conducting hazardous operations, make arrangements to have another person present in the lab.
- Keep the laboratory and work area clean and uncluttered.
- Work with all hazardous chemicals inside a fume hood.
- Never eat, smoke, drink, prepare food or apply cosmetics in the laboratory.
- Do not leave your experiments unattended.
- Prohibit unauthorised individuals from entering the laboratory.

Click on the link below to learn more about how to stay safe in a biomedical science lab.

https://www.wikihow.com/Stay-Safe-in-a-Science-Lab

1.3.2 Material safety data sheets

As mentioned earlier, material safety data sheets are documents that contain information on potential hazards (health, fire, reactivity and environmental) and how to work safely with chemical products. These documents are essential starting points for the development of a complete health and safety programme.

Material safety data sheets also contain information on the use, storage, handling and emergency procedures related to the hazards of working with particular materials. These documents contain much more information on the materials than what you can read on the label. The supplier or manufacturer of the material has prepared the document in such a way so as to inform the user of any and all hazards associated with the product, how to use it safely, what to expect if the recommendations are not followed, how to recognise symptoms of overexposure, and what to do if accidents or incidents occur.

https://www.youtube.com/watch?v=yFHvuDnEEK0

1.3.3 Safe practices in the laboratory

It is easy to overlook some of the potential hazards of working in a biomedical laboratory. Students have the impression that they will be working less with chemicals than with natural biomolecules, and therefore can be less cautious. But this is not true! Many reagents are flammable or toxic, and materials such as fragile glass, sharp objects and potentially infectious biological materials must be used and disposed of with caution. The extensive use of electrical equipment (including hot-plates, stirring motors and high-voltage power supplies for electrophoresis) presents special hazards.

Always bear in mind that the proper disposal of all waste chemicals, unwanted biological samples, disposable tubes, tips, gloves, sharp objects and infectious agents is essential not only to maintain safe laboratory working conditions, but also to protect the general public and your local environment.

Activity 1.1

1. Why is it essential that all students working in the laboratory take precautions against potential hazards?
2. What do you think is the most appropriate way of communicating the importance of practising safe science?
3. Do you think rules around laboratory safety and the handling of chemicals are designed to impede productivity? Why?
4. What is the purpose of laboratory safety and rules for handling chemicals?

1.4 Buffer preparations

Biological processes in a cell mostly take place in a water-based environment. Water is an amphoteric substance; it may serve as a proton donor (acid) or proton acceptor (base). Equation 1.1 illustrates the ionic equilibrium of water.

$$H_2O_{(l)} \rightleftharpoons H^+_{(aq)} + OH^-_{(aq)}$$ ………………………….. ……………equation 1.1

Acidic and basic molecules, when dissolved in water in a biological cell or test tube, react with either H^+ or OH^- to shift the equilibrium of the equation given above, and this results in a pH change to the solution.

Biological pH is maintained at a constant value by natural buffers. When biological processes are studied, *in vitro*, artificial media must be prepared that mimic the cell's natural environment. Because biochemical reactions are dependent on pH, it is important to accurately determine the hydrogen ion concentration.

Always bear in mind that an inaccurate pH measurement or poor choice of buffer can lead to failure in a biomedical laboratory. You should therefore familiarise yourself with several aspects of pH measurements, electrodes and buffers.

1.4.1 Measurement of pH

During your first-year chemistry module, the term "pH" is defined as a measure of the hydrogen ion concentration of a solution. You were taught that solutions with a high concentration of hydrogen ions have a low pH and solutions with low concentrations of H^+ ions have a high pH. This may have seemed like a confusing way in which to express these relationships, and it was – until you understood what pH stands for.

A pH measurement is usually taken by immersing a glass or plastic combination electrode in a solution and reading the pH directly from a meter. A pH meter is standardised with buffer solutions of a known pH, before a measurement of an unknown solution is taken. Remember to always have a pH instrument on standby when the electrode is not in a solution.

Click on the link below to learn more about how to calibrate and use a pH meter.

http://www.wikihow.com/Calibrate-and-Use-a-pH-Meter

1.4.2 Measurement of pH is always susceptible to experimental errors

Some common problems include the following:

The sodium error – this becomes significant at very high pH values (0.1 M Na^+ may decrease the measured pH by 0.4 to 0.5 unit), artificially high pH values can be reported at high sodium levels. To minimise this effect, a pH electrode with a low sodium error can be used, measuring above pH 12.

Concentration effect – the pH of a solution varies with the concentration of buffer ions or other salts in the solution. This is because the pH of a solution depends on the activity of an ionic species, not on the concentration.

Temperature effect – the pH of a buffer solution is influenced by temperature. This effect is due to a temperature-dependent change in the dissociation constant (pK_a) of ions in the solution. The pH of the commonly used buffer Tris is greatly affected by temperature changes, with a ΔpKa/C° of -0.031. This means that a pH 6.0 Tris buffer made up at 4 ℃ will have a pH of 4.95 at 37 ℃.

The best way to avoid this type of problem is to prepare the buffer solution at the temperature at which it will be used, and to standardise the electrode with buffers at the same temperature as the solution you wish to measure.

1.4.3 Biochemical buffers

Buffer ions are used to maintain solutions at constant pH values. How a buffer is selected for use in an experimental process is crucial. The most effective buffering system contains equal concentrations of the acid, HA, and its conjugate base, A^-. The effective pH range for a buffer = pKa ±1.

1.4.4 Selection of a biochemical buffer

Virtually all biochemical investigations must be carried out in a buffered aqueous solution. The natural environment of biomolecules and cellular organelles is under strict pH control. When these components are extracted from cells, they are most stable if maintained in their normal pH range (6–8). However, an artificial buffer system is the best substitute for the natural cell milieu. Although most biochemical solutions require buffer systems to be effective in the pH range 6–8, there is occasionally a need for buffering over the pH range 2–12.

1.2 Activity

1. Discuss the advantages and disadvantages of commonly used buffers.

Feedback

The most commonly used buffers in biochemical investigations include phosphate, zwitterionic, carboxylic acid, borate and amino acid buffers.

1.4.4 Buffer dilutions

Buffers of many different concentrations and volumes are required. It is usually not practical, economical or convenient to prepare all the necessary buffers from scratch. It is, however, possible to have available a few concentrated stock buffer solutions that may be diluted to produce a new buffer solution of the desired concentration and quantity.

Note that the dilution of a concentrated stock buffer solution with purified water should not change its pH. Dilution decreases the actual concentrations of the acceptor and donor species, but their ratio does not change. Click on the links below to learn how dilutions are made from stock solutions.

https://www.youtube.com/watch?v=vHx4nqRdpMg

https://www.youtube.com/watch?v=C_Nu0CvC97k

Learning Unit 2: Immunochemical techniques

2.1 Introduction

Learning Unit 2 focuses on identifying and using diagnostic chemical techniques to determine the response of an antibody to a specific antigen. Immunochemical techniques are based on the reaction of an antigen with an antibody – or, more precisely, the reaction of an antigenic determinant with the binding site of the antibody. Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies, and are thus employed to detect or quantify either antigens or antibodies.

2.2 Learning unit outcomes

On completion of this learning unit, you should be able to:

- Define immunochemical techniques
- Explain the characteristic features and roles of antigen–antibody reactions
- Describe and distinguish the types of immunochemical techniques

2.3 Factors controlling immunochemical techniques

Not all techniques can be used to identify a specific antigen or antibody. To use immunochemical techniques, several controlling criteria are appropriate:

- Experimental conditions – the nature and place of work, type of sample collected
- Nature of reagents – the quality is studied, standardised and analysed
- Sensitivity and selectivity of technique to the particular sample

2.4 Characteristics and role of immunochemical techniques

Immunochemical methods are processes which utilise the highly specific affinity of an antibody for its antigen. They can detect the distribution of a given protein or antigen in tissues or cells. The methods used for immunochemical analysis are called immunochemical

techniques; they are highly important in a diagnostic and clinical context, as even normal cells with many proteins are altered in a diseased state (e.g., cancer).

2.4.1 Characteristics of immunochemical techniques

- Simple, rapid and robust
- Highly sensitive
- Easily automated – applicable to regular clinical diagnostic laboratories
- Do not require extensive and easily destructible sample preparation
- Do not require expensive instrumentation
- Mostly based on simple photo-, fluoro- and luminometric detection
- Measurements may be either qualitative or quantitative

2.4.2 Roles of immunochemical techniques

The characteristic features of these techniques are helpful for

- determining the function of newly identified or novel proteins
- analysing the importance of uncharacterised proteins in their natural environment
- determining species or tissues in which the proteins or residues are expressed
- identifying the cell type or sub-cellular compartment in which the protein can be found
- detecting whether there is any variation in protein expression during the development of the organism
- giving information that may be useful for diagnosis and treatment, e.g., some alteration in the normal expression pattern of a particular protein may indicate a disease state.

2.4.3 Methods of analysis

All immunochemical methods are based on the highly specific and sensitive reaction between an antigen and an antibody.

The measured strength of the binding is known as its affinity, and it is usually expressed in terms of the concentration of an antibody–antigen complex measured at equilibrium. It is measured using the quantitative precipitin curve (the basis for many immunochemical techniques) proposed by Heidelberger and Kendall in 1934.

Quantitative precipitin curve – it describes the relationship between the antigen concentration and the amount of precipitate for a constant quantity of an antibody.

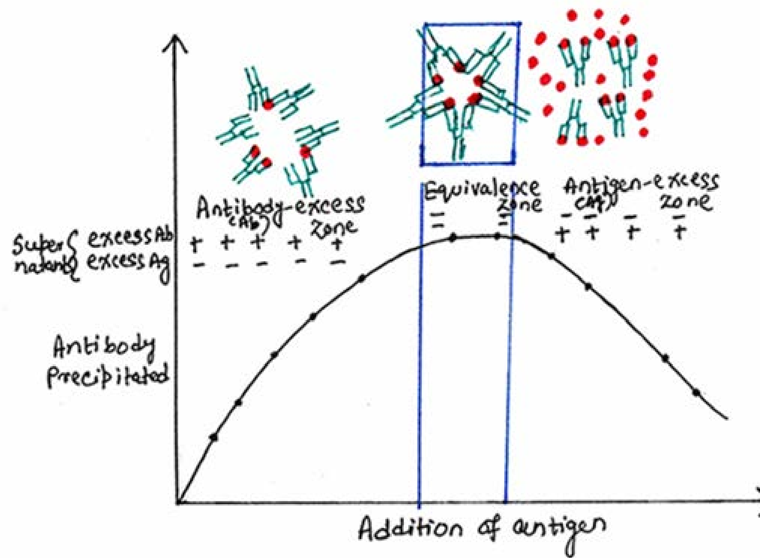

Figure 2.1: The three zones of the precipitin curve

Source:
http://www.discoverbiotech.com/wiki//wiki/Main/Interactions+between+antigen+and+antibody;jsessionid=ACCEB79ED9605CDEFD3FADE643B195BF (site no longer active)

The zones of the precipitin curve are the following:

Antibody excess zone – first phase, where less antigen is present in the sample.

Equivalence zone – both antigen and antibody are cross-linked to form a precipitate; no free antigen or antigen is present.

Antigen excess zone – the amount of precipitate reduces due to the high antigen concentration.

The precipitin curve forms the basis of most of the immunochemical techniques that can be performed in clinical laboratories.

Activity 2.1

Complete the following:

1. _____cells release antibodies against the introduction of a foreign substance into the body.
2. The measure of the strength of the binding is called _____.
3. _____ methods detect or quantify either antigens or antibodies.
4. A/an ______is a foreign substance capable of inducing the production of antibodies.

Answers

1. B-cells
2. Affinity
3. Immunochemical
4. Antigen

2.4.4 Types of antibody used

Virtually all immunochemical techniques rely on the use of antibodies, and their effectiveness is dependent on the quality of the antibody or antibodies employed. The nature of the antibody affects both the specificity of the methods (i.e., the ability to discriminate between the desired analyte and other substances present) and the sensitivity of the procedure (i.e., its ability to detect/measure a low concentration of the analyte). Although some antibodies which can be useful in immunochemical methods occur naturally, it is normally necessary to stimulate their production by immunising animals.

Monoclonal antibodies – these are products of a single clone of plasma cells derived from B-lymphocytes, prepared in the laboratory by hybridoma technology, based on cellular fusion of tumour (myeloma) cells with the splenic lymphocytes of immunised mice. Monoclonal antibodies are directed against a single epitope; and are all identical copies of an immunoglobulin molecule with the same primary structure and specificity as the antigen binding site. They typically display excellent specificity, but have a poor ability to precipitate the antigen.

Polyclonal (conventional) antibodies – these are prepared by immunising animals (rabbits, goats or sheep) with the antigen. The blood serum of the immunised animal, which contains antibodies against the antigen used, is called the **antiserum**. If one antigen (e.g. one protein) is used for immunisation, **monospecific antibodies** (antiserum) result. However, as every epitope stimulates a different clone of the B-cells, and complex antigens bear several epitopes, the antiserum contains a mixture of monoclonal antibodies which differ in their affinity and specificity towards particular epitopes on the antigen used for immunisation.

2.4.5 Categorisation of techniques

Based on the type of reaction performed, and the reagents and samples used, the techniques used are categorised as follows:

Particle methods – where the antigen–antibody interaction is observed – include:

- Agglutination
- Immunoprecipitation
- Immunoelectrophoresis
- Immunofixation
- Immunoturbidimetry
- Immunonephlometry

Label methods – either the antigen or the antibody is labelled, and through the label concentration, the antigen–antibody reaction is observed. These include:

- Immunoassay
- Competitive binding

Other methods – these include immunofluoroscence, immunoelectron microscopy, etc.

2.4.5.1 Agglutination

This process involves the formation of a clumping of cells. It occurs due to a reaction of the antibody to a particulate antigen.

The following tests may be performed:

- *Qualitative test* – to identify the presence of an antigen or antibody.
- *Quantitative test* – to measure the level of antibodies to particulate antigens; serial dilutions of the sample are used.

There are many variations of the agglutination method:

- *Direct* – specific antibodies directly agglutinate antigen present on the cell surface.
- *Indirect* (reverse agglutination) – uses particles already coated with antigens (antibodies) to determine the antibody (antigen) in a given sample. For instance a pregnancy test, which uses hormone secretion (human chorionic gonadotropin), or the diagnosis of rheumatoid arthritis.
- *Agglutination inhibition* – where competitive binding of the antigen occurs, determine the concentration of soluble antigen in the sample.
- *Haemagglutination* – involves reactions using red blood cells; the detection of diseases and other viruses, blood typing, etc.

2.4.5.2 Immunoprecipitation

Immunoprecipitation methods include flocculation and precipitation reactions. When a solution of an antigen is mixed with its corresponding antibody under suitable conditions, the reactants form flocculating or precipitating aggregates. They can be assessed visually by the formation of a precipitin line at the region of equivalence, where equivalent amounts of antigens and antibodies are present.

It may be either:

- *Simple* – reaction of one antigen and a precipitin line antibody
- *Complex* – when many unrelated reactants are used

2.4.5.3 Immunoelectrophoresis

Immunoelectrophoresis is a qualitative technique that combines of two methods, one after another:

- *Gel electrophoresis* – the separation of components by charge
- *Immunodiffusion* – diffusion of both the antibody and antigen toward each other, to produce a precipitin line

It is a strictly qualitatively technique to detect antibodies and antigens in the sample. However, a quantitative technique also exists, called rocket immunoelectrophoresis, which measures the antigen level in a sample. Another variation, called counter-current immunoelectrophoresis, is similar to double immunodiffusion.

2.4.5.4 Immunofixation

Immunofixation is used to detect and identify antibodies in serum, urine and other bodily fluids. The principle, which is similar to immunoelectrophoresis, involves two stages:

- *Electrophoresis* – the separation of antibody proteins
- *Immunoprecipitation* – separation with specific antibodies.

This is easy to perform, more sensitive and easily evaluated in clinical laboratories, and is used for the detection for cancerous myeloma through specific antibodies.

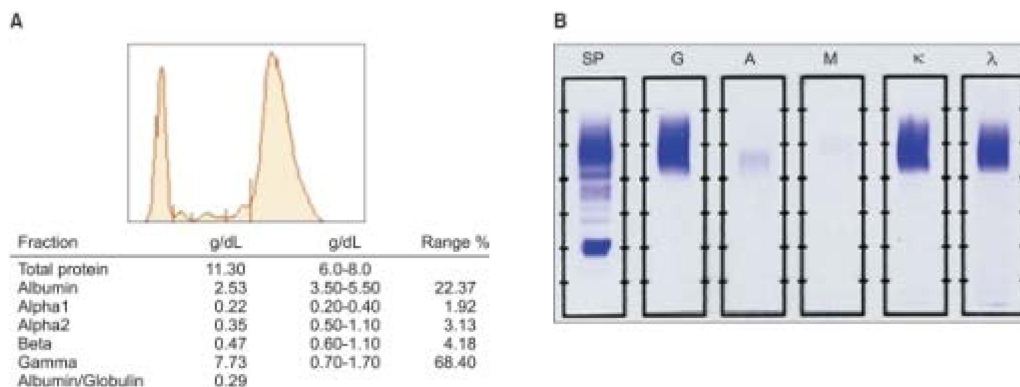

Figure 2.2: Immunofixation – serum-electrophoresis with biclonal gammopathy

Source:
https://upload.wikimedia.org/wikipedia/commons/d/de/Electrophoresis_of_MM_with_biclonal_gammopathy.png

2.4.5.5 Immunoturbidimetry

The precipitin line or curve that forms in a gel can also be formed in a solution. When an antigen solution is combined with a specific antibody solution, the clumping formed results in a cloudy appearance. This is called turbidity; it works according to the principle of immunoturbidimetry. It is measured based on the intensity/amount of light that passes through the sample, measured using an instrument called a spectrophotometer.

2.4.5.6 Immunonephelometry

Immunonephelometry works on a principle similar to turbidimetry, but this method measures the intensity of scattered light directly from the sample. Using laser light, it is measured using a nephelometer. Both methods are fast, but are more expensive. Increased sensitivity of the antigen and antibody can be obtained by automating these techniques.

2.4.5.7 Immunoassay

The immunoassay technique works on the principle of labelling either the antigen or antibody before the reaction. This increases the sensitivity of detection in the experiment, making it more sensitive than the formation of an immunoprecipitate.

The label can indicate:

- Radioisotope – called Radio Immunoassay (RIA)
- Enzyme – reaction known as Enzyme Immunoassay (EIA); it also includes
- ELISA (Enzyme-Linked Immuno Sorbent Assay)
- Fluorescent substance
- Chemiluminescent substance

RIA – first immunoassay technique, based on the measurement of radioactivity associated with antigen–antibody complexes.

ELISA – safer and more efficient method, based on the measurement of an enzyme action associated with antigen–antibody complexes. Most commonly used enzymes are biotin, alkaline phosphatase, etc.

2.4.5.8 Competitive binding

Another method with which to measure the amounts of antigen is competitive binding; a technique that works on the following principles:

- The antibody is first incubated in a solution which contains the antigen
- This mixture is added to antigen coated reaction well

The more antigens that are present in the sample, the fewer the antibodies that will bind to the coated antigen. When the labelled antibody is added to this, we can measure the amount of antigen present in the sample.

2.4.4.9 Other immunochemical techniques

- **Immunofluorescence** – an antibody, labelled with a fluorescent molecule (fluorescein or rhodamine or one of many other fluorescent dyes) is used to detect the presence of an antigen in or on a cell or tissue, by the fluorescence emitted by the bound antibody
- **Immunoelectron microscopy** – uses the specificity of the antibody to view tissues and their components through a microscope
- **Immunostaining** – the use of an antibody labelled with an enzyme or a dye, to detect a specific protein in a sample. The most important staining method is the **Western Blot**, which includes three processes:
  - Electrophoretic separation of the proteins in the sample
  - Immunoblotting – the transfer of proteins from a gel to a membrane
  - Immunodetection – identification using a labelled antibody

2.5 Detection

To detect and diagnose diseases and certain bodily conditions, several fast immunochemical techniques can be used, including:

- Detection of *Salmonella typhi* (causing typhoid) in blood
- Detection of narcotics in urine and saliva
- Detection of protein hormones like insulin, thyroxine, etc.
- Pregnancy test, for the level of human choriogonadotropic hormone (hCGH)

Activity 2.2

1. Give the characteristics of immunochemical techniques.
2. Compare immunoprecipitation and immunoelectrophoresis.
3. Explain the importance of immunoassay and describe the various types.
4. Enumerate the differences between immunoturbidimetry and immunonephlometry.

Learning Unit 3: Centrifugation techniques

3.1 Introduction

Learning Unit 3 presents the basic principles of centrifugation, the instrumentation used in centrifugation and the application thereof. Centrifugation is a process used to separate or concentrate materials suspended in a liquid medium. This method, which separates molecules based on their sedimentation rate within a centrifugal field, uses the centrifugal force to effect the sedimentation of molecules. It is also used to measure the physical properties (such as molecular weight, density and shape) of molecules. If centrifugation is used to separate one type of material from others, it is termed **preparative centrifugation**. On the other hand, if it is used to measure the physical properties of macromolecules, then it is termed **analytical centrifugation**. Click on the links below for more information on these techniques.

https://www.youtube.com/watch?v=9CYaPLIX4VM

https://www.youtube.com/watch?v=OvnaH_uNRbs

Centrifuges have many applications, but they are primarily used for the preparation of biological samples and for analysing the physical properties of a biological sample at a high rate of speed, by subjecting it to an intense force.

Preparative procedures can be applied to the separation or purification of biological samples including cells, organelles, macromolecules, etc.

Analytical procedures are used to measure the physical characteristics of biological samples. The purity, size, shape and density of macromolecules may be defined by centrifugation.

3.1.1 Learning unit outcomes

On completion of this learning unit, you should be able to:

- Discuss the principles and theory underlying centrifugation techniques
- Discuss the advantages of centrifugation techniques
- Discuss the application of centrifugation to the isolation and characterisation of biological molecules and cellular components

- Describe the methods used in preparative and analytical centrifugation
- Be able to select an appropriate technique for a specific application

3.2 Basic principles of centrifugation

Particles suspended in a solution are pulled downward by the earth's gravitational force. In a solution, particles whose mass or density is higher than that of the solvent, sink/sediment, while particles that are lighter float to the top. The greater the difference in mass or density, the faster the particles sink. This sedimentation movement is partially offset by the buoyancy of the particle. Because the earth's gravitational field is weak, a solution containing particles of very small masses usually remain suspended due to random thermal motion. However, the sedimentation of these particles can be enhanced by applying centrifugal forces. A centrifuge does the same thing: it increases sedimentation by generating centrifugal forces as great as one million times the force of gravity.

Let us consider a solution being spun in a centrifuge tube. The centrifugal force acting on a solute particle of mass (m), is

Centrifugal force, (F) $= m\omega^2 r$

F = intensity of the centrifugal force

Ω = angular velocity or rotation in rad/sec

M = effective mass of the sedimenting particle

R = distance of the migrating particles from the central axis of rotation

Important conclusions drawn from the equation:

- The sedimentation velocity of a particle is proportional to its mass.
- A dense particle moves more rapidly than a less dense one, because the opposing buoyant force is smaller for a dense particle.
- Shape, too, is important, because it affects the viscous drag. The frictional coefficient of a compact particle is smaller than that of an extended particle of the same mass.
- The sedimentation velocity also depends on the density of the solution (ρ).

3.3 Relative centrifugal field

Particles suspended in a fluid move, under the influence of gravity, towards the bottom of a vessel at a rate that depends, in general, on their size and density. Centrifugation utilises centrifugal forces which are greater than the force of gravity, to increase the sedimentation rate of particles. This is achieved by spinning the vessel containing the fluid and particles about an axis of rotation, so that the particles experience a centrifugal force acting away from the axis.

The force, measured in multiples of the earth's gravitational force, is known as the relative centrifugal field (RCF) or (more commonly) the g force. For example, an RCF of 500×g indicates that the centrifugal force applied is 500 times greater than the earth's gravitational force. The RCF generated by a rotor depends on the speed of the rotor in revolutions per minute (rpm) and the radius of rotation (i.e., the distance from the axis of rotation). The equation that permits us to calculate the RCF from a known rpm and radius of rotation is:

$$RCF = (1.119X10^{-5})\ (rpm)^2\ (r)$$

The equation relates RCF to revolutions per minute of the sample. It dictates that the RCF on a sample will vary with r, the distance of the sedimenting particles from the axis of rotation.

The force applied to the samples varies according to the size of the centrifuge: a larger centrifuge will have a longer radius and a smaller centrifuge will have a shorter radius. For example, when revolving at 2000 rpm, a larger centrifuge with a longer radius length will spin samples at a higher g force than a smaller centrifuge with a shorter radius length.

A centrifuge is used to separate particles or macromolecules

1. **Cells** – biological components in tissues and cells are separated by centrifugation, a principle widely used in biological laboratories. In fact, it is one of the most essential instruments to include when designing a laboratory.

2. **Sub-cellular components** – substances like the cytoplasmic fluid, nucleus, mitochondria and golgi bodies are separated by this principle.

    (a) *Proteins* – based on density, the protein in cells and tissues is separated using high-speed centrifugation

    (b) *Nucleic acids* – DNA, RNA, snRNA, etc. are separated using this method

3.3.2 Basis of separation

Size – the of the particle matters in the application of this principle. It works on the basis that the smaller the size, the more particles there will be towards the base.

Shape – the shape of a particle is important, in that ex-circular particles will settle down more easily than polygon-shaped particles.

Density – this refers to the main issue at play in the centrifugation principle: the denser the object, the lower it will settle.

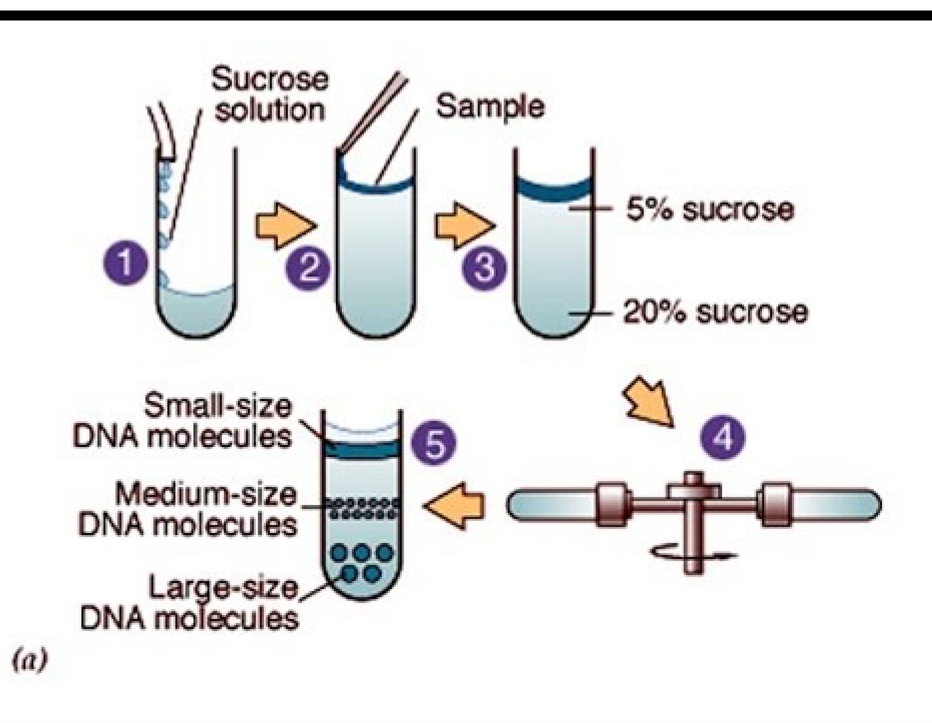

Figure 3.1: Centrifugation technique principle

3.4 Density gradient centrifugation

Density gradient centrifugation is used to separate macromolecules that differ only slightly in size or density. Two techniques are commonly used: in **zonal centrifugation**, the sample being separated (e.g., a cell extract or cells) is placed on top of the centrifugation solution as a thin layer. During centrifugation, the particles move through the solution due to their greater density. The rate of movement depends on their molecular mass. Centrifugation stops before the particles reach the bottom of the tube. Drilling a hole into the centrifugation tube and allowing the contents to drip out makes it possible to collect the different particles in separate fractions.

During centrifugation, the solution tube is stabilised in the tube by a density gradient. This consists of solutions of carbohydrates or colloidal silica gel, the concentration of which increases from the surface of the tube to the bottom. Density gradients prevent the formation of convection currents which would impair the separation of the particles. **Isopycnic centrifugation**, which takes much longer than zonal centrifugation, starts with a CsCl solution in which the sample material (e.g., DNA, RNA or viruses) is homogeneously distributed. A density gradient only forms during centrifugation, because of the sedimentation and diffusion processes. Each particle moves to the region corresponding to its own buoyant density. Centrifugation stops once equilibrium is reached. The samples are obtained by fractionation, and their concentration is measured using the appropriate methods.

https://www.youtube.com/watch?v=KEXWd3_fM94

3.5 Moving boundary/zone centrifugation

In moving boundary (or differential) centrifugation, the entire tube is filled with the sample and centrifuged. Through centrifugation a separation of two particles occurs, but any particle in the mixture may end up in the supernatant or in the pellet, or it may be distributed in both fractions, depending on its size, shape, density and the conditions of centrifugation. The pellet is a mixture of all the sedimented components, and it is contaminated with whatever unsedimented particles were initially at the bottom of the tube. The only component which is purified is the slowest sedimenting one, but its yield is often very low. The two fractions are recovered by decanting the supernatant solution from the pellet. The supernatant can be re-

centrifuged at higher speed to obtain further purification, with the formation of a new pellet and supernatant.

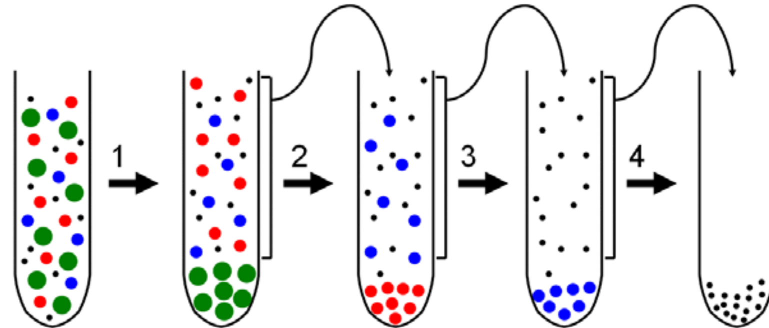

Figure 3.2: Differential centrifugation

Source:
https://en.wikipedia.org/wiki/Differential_centrifugation#/media/File:Differentielle_zentrifugation.png

Now click on the link below, which explains the principles of the centrifugation technique in greater detail.

https://www.youtube.com/watch?v=nJUuab-d3NQ

3.6 Rate zonal centrifugation

Particles of the same size (M) but different shapes (e.g., linear versus globular) will separate – the particle with the greater frictional coefficient (f) will move more slowly (rod-shaped moves slower than globular). This technique is called **velocity gradient centrifugation** (a gradient of sucrose is used to linearise the motion of the particles).

In rate zonal centrifugation, the sample is applied in a thin zone at the top of the centrifuge tube on a density gradient. Under centrifugal force, the particles begin sedimenting through the gradient in separate zones, according to their size, shape and density. The run must be terminated before any of the separated particles reach the bottom of the tube.

The link below will help you understand and explain ultracentrifugation processes, mechanisms and applications.

https://www.youtube.com/watch?v=K4_Xo0nKdSQ

Particles can be separated by density. When the density in the solvent equals the density of the particle, the denominator of the equation equals zero, therefore velocity equals zero – the particle reaches its equilibrium density in the solvent (known as equilibrium density gradient centrifugation or isopycnic banding).

3.7 Isopycnic centrifugation

When using the isopycnic technique, the density gradient column encompasses the whole range of densities of the sample particles. The sample is uniformly mixed with the gradient material. Each particle sediments only to the position in the centrifuge tube at which the gradient density is equal to its own density, and there it remains. The isopycnic technique therefore separate particles into separate zones solely on the basis of their density differences, independent of time. In many density gradient experiments, particles of both the rate zonal and the isopycnic principles may enter the final separations. For example, the gradient may be of such a density range that one component sediments to its density in the tube and remains there, while another sediments to the bottom of the tube. The self-generating gradient technique often requires long hours of centrifugation.

3.8 Safety measures

3.8.1 Safety while using the centrifuge

- The work surface must be level and firm. Do not use the centrifuge on an uneven or slanted work surface.
- Balance the tubes in the rotor! If you want to run a tube with 10 ml of liquid, insert another tube with 10 ml of water in the opposing hole on the rotor. If the liquid has a higher or lower density than water, you must balance the tubes by mass, not volume.
- Do not open the lid while the rotor is moving. Even though many centrifuges have a “safety shutoff” if the lid is opened, the only thing this does is to stop powering the

rotor. The rotor will keep spinning due to its own inertia, until friction slows and eventually stops it.

- If you see the centrifuge wobbling or shaking, turn it off or pull the plug. A little vibration is normal, but excessive amounts can mean danger. FIRST, double check that you have correctly balanced the tubes. If you have and the wobbling continues, contact the manufacturer or dealer and have the unit serviced. DO NOT continue to run a centrifuge that wobbles visibly when the rotor is spinning.
- Wear a face shield and/or safety goggles if you have to work anywhere near a centrifuge that is in use.
- Do not bump, jar or move the centrifuge while the rotor is spinning. Make sure you do not have the cord dangling from a table edge where someone can catch their foot in it and pull down the centrifuge.

3.9 Precautions – working with bio-hazardous materials

Employ the following procedures for centrifugation when working with bio-hazardous materials:

- Examine whether the tubes and bottles are suitable for centrifugation; discard tubes with cracks or stress marks.
- When working with bio-hazardous materials, wipe the outside of the tubes with disinfectant prior to removing them from the biological safety cabinet and before placing them in the safety cups or rotors.
- Place all tubes in safety buckets or sealed rotors when centrifuging infectious materials.
- Inspect the "O" ring seal of the safety bucket and the inside of safety buckets or rotors. Open safety buckets or rotors in a biological safety cabinet.
- If any spills or leakages are apparent, the centrifuge rotor should be cleaned with a mild detergent, rinsed thoroughly with distilled water, and allowed to air dry completely (while in the bio-safety cabinet).
- Clean the rotor and centrifuge well after each use.

Learning Unit 4: Chromatography

4.1 Introduction

This learning section mainly focuses on different types of chromatography and their basic applications. Chromatography is a laboratory technique used for separating a mixture. All types of chromatography are based on a very simple concept: the sample to be examined is allowed to interact with two physically distinct entities – a mobile phase and a stationary phase. The molecules targeted for analysis are called analytes.

The mobile phase, which can be either liquid or gas, moves the sample components through the region containing the solid or liquid stationary phase called the sorbent. The stationary phase has the ability to bind some types of analytes, and this varies from one chromatographic method to another. Analytes that are preferentially bound by sorbent are observed to be retarded in their movement through their chromatographic system. Molecules that show weak affinity for the sorbent spend more time in the mobile phase and are more easily removed or eluted from the system.

The general process of moving a sample mixture through a chromatographic system is called development.

Modern separation methods are based on different types of chromatographic methods. The basic principle of any chromatography is due to the presence of two phases:

- **Mobile phase** – substances to be separated are mixed with this fluid; it may be a gas or a liquid; it continues to move through the chromatographic instrument
- **Stationary phase** – it does not move; it is packed inside a column; it is a porous matrix that helps separate the substances present in the sample. It works in accordance with a simple physical process called adsorption.

4.2 Learning unit outcomes

On completion of this learning unit, you should be able to:

- Define chromatography
- Describe the principles and important types of chromatographic techniques

4.3 Types of chromatograph

There are many types of chromatographic methods, some of which include:

- Paper and thin-layer chromatography
- Gas chromatography
- Liquid chromatography
- Gel filtration chromatography
- Ion exchange chromatography
- Affinity chromatography

4.3.1 Paper and thin-layer chromatography

This is an example of partition chromatography. Due to similarities in the theory and practice of these two procedures, they are considered together. This type of chromatography may be applied to two major types of problem: (i) the identification of unknown samples and (ii) the isolation of the components of a mixture.

In **paper chromatography**, the cellulose support is extensively hydrated, so that the distribution of the analyte occurs between the immobilised sorbent and the mobile developing solvent.

In **thin-layer chromatography**, the initial stationary liquid phase is the solvent used to prepare the thin layer of adsorbent. However, as the developing solvent molecules move through the sorbent, polar solvent molecules may bind to the immobilised support and become the sorbent.

4.3.1.1 Applications of planar chromatography

Paper and thin-layer chromatography require only a minute sample size, the analysis is fast and inexpensive, and detection is straightforward. Unknown samples are applied to a plate along with appropriate standards, and the chromatogram is developed as a single experiment. Purified substances can be isolated from developed chromatograms, however only tiny amounts are present.

Click on the link below to learn more about the procedures involved in paper and thin-layer chromatography.

https://www.youtube.com/watch?v=J8r8hN05xXk

4.3.2 Gas chromatography (GC)

The gas chromatography principle involves the separation of the components of the sample due to a separation occurring between the gaseous mobile phase and the stationary phase (usually silica). The components separated into gas emerge first, and the others later. The process detects compounds like fatty acids, essential organic solvents, flavoured oils, etc.

In GC, a liquid sample is injected into the column. The GC column is usually coated with stationary phase and placed inside an oven chamber. The sample is vapourised as it passes the column, which exceeds its boiling point. The sample compounds are carried to the column by gas (usually helium or nitrogen) and then to a detector.

Click on the link below to view the operations involved in gas chromatography.

https://www.youtube.com/watch?v=iX25exzwKhI

4.3.3 Liquid chromatography

This is a separation technique where the mobile phase is a liquid. It can be performed in a column or on a plane surface. Various types exist:

- HPLC – high-performance liquid chromatography
- FPLC – fast protein liquid chromatography

Click on the following links for more information on liquid chromatography.

https://www.youtube.com/watch?v=IUwRWn9pEdg

https://www.youtube.com/watch?v=I-CdTU5X4HA

4.3.4 Gel filtration chromatography

This chromatographic technique, also known as size exclusion or molecular sieve chromatography, involves the separation of molecules based on their molecular weight and size. It involves two phases:

- **Mobile phase** – liquid is mixed with the sample mixture
- **Stationary phase** – a gel matrix with a particular pore size allows smaller molecules to pass easily. It well suited to the separation of biomolecules that are sensitive to environmental conditions like temperature, pH, etc.

Click on the link below to learn more about the principle of ion exchange chromatography:
https://www.youtube.com/watch?v=q3fMqgT1do8

4.3.5 Affinity chromatography

Affinity chromatography separates proteins on the basis of a specific interaction between the molecules in the sample, and a compound called a ligand in the column.

Some of the ligands used in the matrix are:

- Enzymes
- Antibodies
- Metal ions (e.g., Nickel).

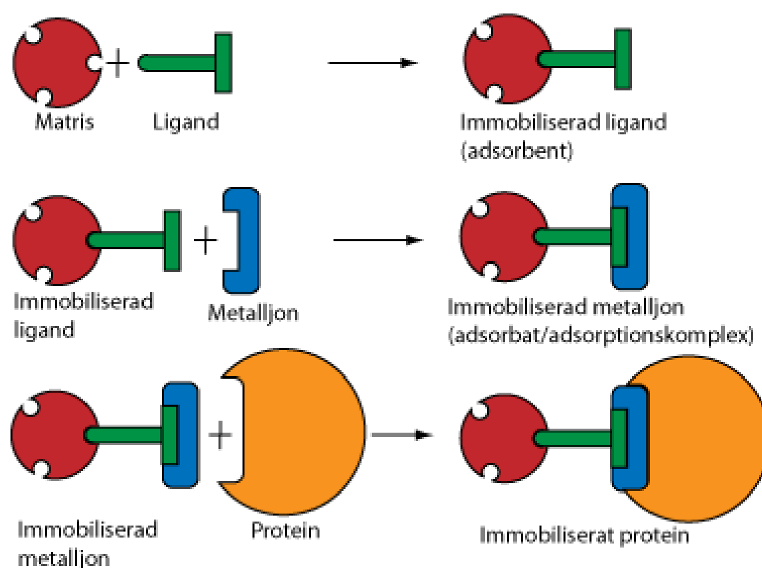

Figure 4.1 Affinity chromatography

Click on the link below for more information on affinity chromatography. The technique is highly specific and is mainly used for the purification of proteins and peptides.

https://sv.wikipedia.org/wiki/Immobilized_Metal_Ion_Affinity_Chromatography

Activity 4.1

1. Define chromatography and the principle on which it works.
2. Discuss the major components of a chromatographic system.

Answers

1. When discussing the major components of a chromatographic system, did you mention high performance and fast protein?
2. In this case, you need to mention molecular weight and size.

Learning Unit 5: Electrophoresis

5.1 Introduction

The movement of particles under a spatially uniform electric field in a fluid is called electrophoresis. The rate of migration of particles depends on the strength of the field, on the net charge size and shape of the molecules, and on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used to study the properties of a single charged species, and as a separation technique.

Electrophoresis provides the basis for a number of analytical techniques used for separating molecules by size, charge, or binding affinity. An example is the separation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or protein molecules using an electric field applied to a gel matrix. Such a gel matrix is usually either polyacrylamide or agarose.

5.2 Learning unit outcomes

On completion of this learning unit, you should be able to:

- Define electrophoresis
- Describe the principle and important types of electrophoretic methods
- Explain the principle and components of electrophoresis
- Explain the various uses of electrophoresis

5.3 Methods of electrophoresis

The major difference between the various methods is the type of support medium. Cellulose or cellulose acetate is used as a support medium for low molecular-weight biochemicals like amino acids and carbohydrates, whereas polyacrylamide or agarose is widely used as a support medium for larger molecules.

5.3.1 Polyacrylamide gel electrophoresis (PAGE)

Gels formed by the polymerisation of an acrylamide have several positive features in electrophoresis, including

- high resolving power for small and moderately sized proteins and nucleic acids
- the acceptance of relatively large samples
- minimal interactions between migrating molecules and the matrix
- the physical stability of the matrix.

5.3.2 Discontinuous gel electrophoresis

Three significant characteristics of this method include

- the presence of two gel layers – a lower or resolving gel and an upper or stacking gel
- the buffers used to prepare the two gel layers are of different ionic strengths and each has a different pH
- the stacking gel has a lower acrylamide concentration, so its pores are larger.

These three changes in experimental conditions cause the formation of highly concentrated bands of sample in the stacking gel, and the greater resolution of the sample components in the lower gel.

5.3.3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

One of the most common means of analysing proteins by electrophoresis is by using Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis. SDS is a detergent which denatures proteins by binding to the hydrophobic regions and essentially coating the linear protein sequence with a set of SDS molecules. The SDS is negatively charged, and thus becomes the dominant charge of the complex. The number of SDS molecules that bind is simply proportional to the size of the protein. Therefore, the charge-to-mass ratio should not change with size. In solution (water), in principle all different-sized proteins covered with SDS would run at about the same mobility. However, the proteins are not run through water. Instead they are run through an inert polymer, polyacrylamide. The density and pore size of this polymer can vary according to how you make it (concentration of monomer and cross-linking agent).

Thus, the size of the molecules that pass through the matrix can vary. This determines in what molecular weight range the gel will have the highest resolving power.

5.3.4 Agarose gel electrophoresis

A simple way of separating large fragments of DNA/ RNA from one another by size is to use an agarose gel. Agarose is another type of matrix used for many purposes (such as supporting the growth of bacteria on plates). DNA/RNA does not need a detergent since it already has a large number of negative phosphate groups which are evenly spaced. Thus, as with SDS-PAGE, the charge-to-mass ratio is constant. Also, as is the case with SDS-PAGE, the separation results from the matrix itself. The range of size sensitivity can be varied by changing the density of the agarose.

DNA denaturing polyacrylamide gels (often called sequencing gels) can also be used. To look at smaller DNA molecules with much higher resolutions, the DNA is generally denatured via heat and run through a thin polyacrylamide gel that is kept near the denaturing temperature. These gels usually contain additional denaturing compounds such as urea. Two pieces of DNA that differ in size by one base can be distinguished from each other in this way.

5.3.5 Pulsed field gel electrophoresis

This is a method developed for separating large DNA molecules. Whereas standard DNA gel electrophoresis commonly resolves fragments up to ~50 kb in size, PFGE fractionates DNA molecules up to 10 Mb. The mechanism driving these separations exploits the fact that very large DNA molecules unravel and "snake" through a gel matrix, and such electrophoretic trajectories are perturbed in a size-dependent manner by carefully oriented electrical pulses.

PFGE has enabled the rapid genomic analysis of microbes and mammalian cells, and motivated the development of large-insert cloning systems such as bacterial and yeast artificial chromosomes.

The following article will assist you in understanding the application of pulsed field gel electrophoresis in clinical studies:

Parizad, EG, Parizad, EG & Valizadeh, A. 2015. The application of pulsed field gel electrophoresis in clinical studies. *Journal of Clinical and Diagnostic Research* 10(1): 1–3.

5.3.6 Two-dimensional electrophoresis (2-DE) of proteins

Two-dimensional gel electrophoresis is a highly effective means of separating proteins by utilising two important properties – their isoelectric point and their size. The protein mixture is first placed onto a gel and allowed to undergo isoelectric focusing. This separates the proteins based on the pH value at which they have a net charge of zero. This is the horizontal portion of the technique, because the proteins move either left or right along the horizontal gel slab. The slab is then placed into an SDS-PAGE apparatus, which begins to separate the proteins based on size. Since the movement is down along the y-axis, we call this the vertical component of the setup. Movement in the SDS-PAGE is perpendicular with respect to the movement of the proteins in the isoelectric focusing setup. That is precisely why we call this method two-dimensional gel electrophoresis. Click on the links below for more information.

https://www.youtube.com/watch?v=x62WZGd3VCM

https://www.youtube.com/watch?v=mXkIWIE0H-0

5.3.7 Capillary electrophoresis

Capillary electrophoresis (CE) can be used to separate ionic species by their charge and frictional forces and their hydrodynamic radius. In traditional electrophoresis, electrically charged analytes move in a conductive liquid medium under the influence of an electric field. The CE technique was designed to separate species based on their size-to-charge ratio in the interior of a small capillary filled with an electrolyte.

5.3.8 Immunoelectrophoresis

Immunoelectrophoresis is a general name for a number of biochemical methods for separating and characterising proteins based on electrophoresis and their reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins (also known as antibodies) reacting with the proteins, to be separated or characterised.

5.3.9 Gel electrophoresis applications

Gel electrophoresis is used in forensics, molecular biology, genetics, microbiology and biochemistry. The results can be analysed quantitatively by visualising the gel with UV light and a gel-imaging device. The image is recorded with a computer-operated camera, and the intensity of the band or spot of interest is measured and compared against standard markers loaded on the same gel. Depending on the type of analysis being performed, other techniques are often implemented in conjunction with the results of gel electrophoresis, providing a wide range of field-specific applications.

5.3.10 Protein and nucleic acid blotting

5.3.10.1 Southern blotting

This is a method for the transfer of DNA restriction fragments from agarose gels to cellulose nitrate (nitrocellulose) membranes, prior to the hybridisation of the resulting blot-immobilised DNA to radioactively labelled DNA probes.

5.3.10.2 Northern blotting

This technique is used in molecular biology research to study gene expression by detecting RNA (or isolated mRNA) in a sample.

Click on the links below to learn more about the southern and northern techniques.

https://www.youtube.com/watch?v=qjYJQpiKlSU

https://www.youtube.com/watch?v=EoTq-Ql_Zzc

5.3.10.3 Western blotting

This technique is sometimes called the protein immunoblot; it is an analytical technique widely used in molecular biology, immunogenetics and other related disciplines to detect specific proteins in a sample of tissue homogenate or extract.

This technique combines an electrophoresis step with a step that transfers (blots) the separated proteins onto a membrane. Western blot is often used as a follow-up test to confirm the

presence of an antibody and help diagnose a condition. Examples of its use include confirmatory HIV and Lyme disease testing.

Click on the link below to learn more about the western blotting technique.

https://www.youtube.com/watch?v=v-O103PLhm8

Activity 5.1

1. What physical characteristics of a biomolecule influence its rate of movement in an electrophoresis matrix?
2. Draw a slab gel to show the results of non-denaturing electrophoresis of the following mixture of proteins. The molecular weight is given for each.
   Lysozyme (13930)
   Serum albumin (65400)
   Chymotrypsin (21600)
3. Explain the purpose of each of the chemical reagents used for PAGE
   (i) Acrylamide
   (ii) TEMED
   (iii) coomassie blue
   (iv) sodium dodecyl sulfate

Learning Unit 6: Histopathology

6.1 Introduction

Histopathology is the microscopic examination of biological tissues, to observe the appearance of diseased cells and tissues in very fine detail. Surgical pathology includes gross and microscopic examinations of resected specimens and biopsies by histopathologists for tissue diagnosis. Several steps are followed to get the tissue in a form which will allow a diagnosis to be made under a light microscope.

6.2 Learning unit outcomes

On completion of this learning unit, you should be able to:

- Explain the purpose of fixation, the quality of fixation and the functions of fixatives
- Define dehydration, clearing, infiltration and embedding
- Give a description of how tissues are decalcified
- Classify microtomes and describe section cutting
- Explain how low temperature works in histopathology, the techniques used and how frozen sections are prepared
- Discuss the most common stains used in histopathology, as well as staining methods
- Describe how various tissues are identified

6.3 Grossing

Grossing is the process whereby pathology specimens are inspected with the naked eye to obtain diagnostic information. The following points should be noted before the tissue is processed for microscopic examination:

- ➢ Identification of the specimen – confirmation of the patient and the anatomical site from which the specimen was obtained
- ➢ Clinical details
- ➢ Gross description – written record of the physical appearance of the specimen

- Only a small portion from the large specimen can be subjected to microscopic examination, hence during the gross inspection, a skilled person should do the examination
- Only soft tissue can be cut into small blocks and processed directly
- Bony specimens need to be decalcified prior to processing
- Bones and teeth require special treatment

The following items should be in a gross room:

- ➢ A cutting board. The fluid from the board must run directly into a sink
- ➢ Shelves for specimen containers
- ➢ Ready access to hot and cold water
- ➢ Ready access to formalin
- ➢ Box of instruments containing forceps of various size, scissors of various types and sizes, a probe, a bone-cutting saw or electric bone cutter, a scalpel handle, disposable blades, a long knife, and a ruler to measure the size of lesions and specimens
- ➢ Box with cassettes and labels

6.4 Laboratory hazards and safety measures

6.4.1 Gross room

- Formalin vapours are irritants, affecting the eyes and throat. An exhaust may be used as outlet for vapours.
- Always use a mask, apron, eye glasses and gloves, as protection from
    - ➢ infected material
    - ➢ formalin vapours
    - ➢ spilt blood or any other fluid
- Keep the grossing table clean with an antiseptic solution.
- All specimens should be in container with 10% formalin and covered with a lid.
- After grossing, the specimen should be kept according to accession numbers.

6.5 Histopathology laboratory

The laboratory should be large enough to accommodate various types of equipment and must allow personnel to work with ease. The following equipment is kept in this laboratory:

- Tissue processor
- Tissue embedding table
- Microtome
- Tissue warming plate
- Tissue flotation bath
- Slide stainer or glassware for manual staining
- Table to label and dispatch the slides

The handling of the tissues, as well as the description and functioning of various types of equipment are detailed in the respective lessons.

6.6 Handling of specimen

The specimen should be transported in a glass, plastic/metal container or in a plastic bag, in 10% formalin. If formalin is not available, place the specimen in a refrigerator at 4 ℃ to slow down autolysis. The container should have an opening large enough so that the tissue can be removed easily after it has hardened by fixation. However, fresh material is needed for the following purpose:

- Frozen section
- Immunocytochemistry
- Cytological examination
- Microbiological sampling before histopathology
- Chromosome analysis
- Research purpose
- Museum display

6.6.1 General principles of gross examination

- Proper identification and orientation of the specimen

- Unlabelled specimens should never be processed
- A properly completed histopathology requisition form should contain the patient's name, age, sex, relevant clinical data, surgical findings, nature of operation and type of tissue submitted
- A careful search and examination of all the tissue in the order in which it was submitted
- The surgeon should be instructed to submit all the material s/he removed, rather than selecting a portion from it
- The specimen should be placed on the cutting board in the correct anatomical position, before recording the following information:
    - Type of specimen
    - Structure included
    - Dimensions
    - Weight
    - Shape
    - Colour
    - Consistency
    - Surgical margin, whether included or not showing tumour involvement
- Measurements are usually given in centimetre (cm), unless the specimen is very small in which case millimetre (mm) can be used
- Endometrial and prostatic tissue should be measured by aggregate pieces in volume
- Endoscopic biopsies should be numbered

6.6.2 Sampling for histopathological examination

- Tissue submitted for histopathology must not be more than 3 mm thick and not larger than the diameter of the slides used. Most specimens from solid tissue are cut in the form of pieces measuring 10 to 15 mm on the slides, and 2 to 3 mm in thickness. Adipose tissue must be cut even thinner
- Discrete areas of calcification or ossification should be removed and decalcified in nitric acid
- Small fragments of tissue must be wrapped in thin paper
- If the fragments are very small, they should be stained with haematoxylin to facilitate their identification by the histopathologist

- All tissue should be submitted in a diagnostic endometrial curettage. However, if the procedure was done for an incomplete abortion, and gross examination shows obvious product of conception, one representative section is more than adequate
- Determining surgical margins is helpful by painting them with India ink or a similar pigment prior to sectioning

6.6.3 Histological technique

The histological technique deals with the preparation of tissue for microscopic examination. The aim of good histological technique is to preserve the microscopic anatomy of tissue and harden it so that a very thin section (4–5 micron) can be made. After staining, the section should represent the actual anatomy of the tissue as closely as possible. This is achieved by passing the total selected part of the tissue through a series of processes.

These processes are:

- Fixation
- Dehydration
- Cleaning
- Embedding
- Cutting
- Staining

6.6.4 Fixation

This is the process by which the constituents of cells and tissue are fixed in a physical – and partly also chemical – state so that they will withstand subsequent treatment with various reagents, with minimum loss of architecture. This is achieved by exposing the tissue to chemical compounds called fixatives.

6.6.4.1 Mechanism of action of fixatives

Most fixatives act by denaturing or precipitating proteins, which then form a sponge or meshwork that tends to hold the other constituents.

A good fixative is the most important factor in the production of satisfactory results in histopathology. The following factors are important:

- Fresh tissue
- Proper penetration of tissue by fixatives
- Correct choice of fixatives

No fixative will penetrate a piece of tissue thicker than 1 cm. For dealing with specimens thicker than this, the following methods are recommended:

- ➢ Solid organ: Cut slices (as necessary) no thicker than 5 mm.
- ➢ Hollow organ: Either open or fill with fixative or pack lightly with wool soaked in fixative.
- ➢ Large specimen which requires dissection: Inject fixative along the vessels (or bronchi as in case of lung tissue) so that it reaches all parts of the organ.

6.6.4.2 Properties of an ideal fixative

- Prevents autolysis and bacterial decomposition
- Preserves tissue in its natural state and fixes all components
- Makes the cellular components insoluble to the reagent used in tissue processing
- Preserves tissue volume
- Avoids excessive hardness of tissue
- Allows enhanced staining of tissue
- Should be non-toxic and non-allergic for the user
- Should not be very expensive

6.6.4.3 Temperature

Fixation can be carried out at room temperature. Tissue should not be frozen once it has been placed in the fixative solution, for a peculiar ice crystal distortion will result.

6.6.4.4 Speed of fixation

With most fixatives, the speed of fixation is almost 1 mm/hour. Therefore, a fixation time of several hours is needed for most specimens.

6.6.4.5 Amount of fixative fluid

This should be approximately 10–20 times the volume of the specimen. The fixative should surround the specimen on all sides.

6.6.4.6 Factors affecting fixation

- Size and thickness of the piece of tissue
- Tissue covered by large quantities of mucous fix slowly, as does tissue covered by blood, or organs containing very large quantities of blood
- Fatty and lipomatous tissue fixes slowly
- Fixation is accelerated by agitation
- Fixation is accelerated by maintaining a temperature of around 60 ºC

6.6.4.7 Classification of fixatives

A. Tissue fixatives

- Buffered formalin
- Buffered gluteraldehyde
- Zenker's formal saline
- Bowen's fluid

B. Cytological fixatives

- Ethanol
- Methanol
- Ether

C. Histochemical fixatives

- Formal saline
- Cold acetone

- Absolute alcohol

6.6.4.8 Routine formalin

Formalin is sold as 40% w/w solution of formaldehyde gas in water. It is used as 10% solution in water or normal saline. It does not precipitate protein, but combined with the NH_2 group to form an insoluble gel, it preserves all elements including fats. It keeps phospholipids insoluble in fat solvents. Tissue can remain in it for prolonged periods without distortion. Compatible with most special stains, it is the cheapest and most popular fixative.

6.6.4.9 Ethyl alcohol

It is used at 90–100% strength. It precipitates albumin but not nucleoprotein. It causes shrinkages and hardening of tissues, and destroys mitochondria. It preserves glycogen and is useful for the histochemistry of glycogen, uric acid, iron, etc.

6.6.5 Tissue processing

To cut thin sections of a piece of tissue, it should have suitable hardness and consistency when presented to the knife edge. These properties can be imparted by infiltrating and surrounding the tissue with paraffin wax, colloidin or low-viscosity nitrocellulose, various types of resins or by freezing. This is called tissue processing, which is done in stages. It can be subdivided into dehydration, clearing, impregnating and embedding. All specimens must be properly labelled before commencing with processing.

For labelling, do not use a pen containing ordinary ink. Printed, graphite pencil, written, typewritten or India ink-written labels are satisfactory.

A system of transportation is required to carry the tissue through various steps of processing. The cut specimens are put in muslin cloth together with their labels, and then transported from reagent to reagent in metal containers with perforated walls, so that the reagent enters the tissue.

Tissue processing is a lengthy procedure requiring 24 hours, and it can be done manually or mechanically.

6.6.5.1 Manual tissue processing

In this process the tissue is moved from one container of reagent to another, by hand.

6.6.5.2 Mechanical tissue processing

Automatic tissue processors are available. Such processors contain different jars with reagents. These are arranged in a sequence. The tissue is moved from one jar to another by a mechanical device. Timings are controlled by a timer, which can be adjusted to measure hours and/or minutes. The temperature is maintained at around 60 ℃.

Processing, be it manual or mechanical, involves the same steps.

Sequence of manual tissue processing:

a) Dehydration

Tissues are dehydrated using increasingly strong alcohol; e.g. 50%, 70%, 90% and 100%.

The duration for which tissues are kept in each strength of alcohol depends on the size of the tissue, the fixative used and the type of tissue (e.g., after fixation in an aqueous fixative, delicate tissue needs to be dehydrated slowly, starting with 50% ethyl alcohol directly, whereas most tissue specimens may be placed in 70% alcohol). Delicate tissue will undergo a high degree of shrinkage in too great a concentration of alcohol.

The volume of alcohol should be 50–100 times that of the tissue.

b) Clearing

During dehydration, water in the tissue is replaced by alcohol. The next step is for the alcohol to be replaced by paraffin wax. As paraffin wax is not alcohol soluble, the alcohol must be replaced with a substance in which wax is soluble. This step is called clearing.

The clearing of tissue is achieved by using any of the following reagents:

- Xylene
- Chloroform
- Benzene
- Carbon tetrachloride
- Toluene

Xylene is commonly used. Small pieces of tissue are cleaned in 0.5 – 1 hour, whereas larger pieces (5 cm or more thick) are cleaned in 2–4 hours.

c) Impregnation with wax

This is allowed to occur at the melting point of paraffin wax, which is at 54–60 ºC. The volume of the wax should be about 25–30 times the volume of the tissue. The duration of impregnation depends on the size and type of tissue, and the clearing agents employed. Longer periods are required for larger pieces and for harder tissue (like bone and skin, compared to liver, kidney, spleen, lung, etc.). Using xylene is the easiest way to remove wax. In total, four hours is sufficient for routine impregnation.

Types of wax employed for impregnation:

- Paraffin wax
- Water-soluble wax
- Other material like colloidin, gelatin, paraplast, etc.

Paraffin wax is used routinely. It has a hard consistency, so a section of 3–4 micron thickness can be cut.

d) Blocking

Impregnated tissues are placed in a mould with their labels, and then freshly melted wax is poured into the mould and allowed to settle and solidify. Once the block has cooled sufficiently to form a surface skin, it should be immersed in cold water to cool it rapidly.

After the block has completely cooled down, it should be cut into individual blocks, after which each must be trimmed. Labels must be made to adhere on the surface of the block by melting the wax with a metal strips which have been sufficiently warmed.

6.6.6 Staining

Staining is a process by which colour is given to a section. Hundreds of stains are available.

Classification of stains

Generally, stains are classified as:

- Acid stains
- Basic stains
- Neutral stains

All dyes are composed of acid and basic components. Dye is a compound which can colour fibres and tissue constituents.

Acid dyes – in an acid dye, the basic component is coloured while the acid component is colourless. Acid dyes stain basic components (e.g. eosin stains cytoplasm). The colour imparted is a shade of red.

Basic dyes – in a basic dye, the acid component is coloured while the basic component is colourless. Basic dyes stain acidic components (e.g., basic fuchsin stains nuclei). The colour imparted is a shade of blue.

Neutral dyes – when an acid dye is combined with a basic dye a neutral dye is formed. As it contains both coloured radicals, it gives different colours to each of the cytoplasm and nucleus. This is the basis of a Leishman stain.

6.6.6.1 Procedure of staining

Every stain should be used according to a specified method. Staining can be done either manually or in an automatic stainer.

Manual staining – in a small laboratory where a few slides are stained daily, this is the method of choice. Although it is time consuming, it is economical. Different reagent containers are placed in a special sequence and the slides are moved manually from one container to another.

Automatic staining – in a busy histopathology laboratory where hundreds of slides are stained daily, an automatic stainer is required. This method uses different containers of staining reagents, arranged according to the desired sequence. It has a timer which controls the period for which slides should remain in a given container. A mechanical device shifts the slides from one container to the next, after a specified period. The advantages of using an automated stainer are:

- It reduces manpower
- It accurately controls the timing of staining
- Large number of slides can be stained simultaneously
- Lower quantities of reagents are used

Slides, whether they are stained manually or by automatic stainer, follow the same sequence.

Haematoxylin and Eosin staining – these are the most commonly used routine stains in any histopathology laboratory.

6.6.6.2 Frozen sectioning

This is a technique in which tissue is frozen rapidly at -20 ℃, and sections are cut and stained. In this way, tissue can be examined microscopically within 5–10 minutes of its removal from the body. It reduces the time of processing from 18 hours to five minutes. It has the disadvantage that only 8–16 micron-thick sections can be cut, and finer details of tissue cannot be examined. Frozen sectioning is performed on a machine called a cryostat.

The following are the situations in which frozen sections are helpful:

- When rapid diagnosis regarding the benign or malignant nature of a lesion is required, to decide the extent of surgery while the patient is still on the operating table
- When a study of fat, proteins or antigenic markers is required, as the routine processing of tissue destroys them.

Activity 6.1

1. Describe the steps involved in the processing of surgical specimens for a histopathological examination.
2. What does grossing involve?
3. How should the gross room be built?

Answer

See https://library.med.utah.edu/WebPath/HISTHTML/HISTOTCH/HISTOTCH.html and http://grossing-technology.com/home/grossing-in-dermatopathology-manual/skin-excision/

Learning Unit 7: Polymerase chain reaction (PCR)

7.1 Introduction

A polymerase chain reaction is a technique for making copies or amplifying a specific sequence of DNA within a short period of time. It allows a specific sequence of DNA/RNA (usually fewer than 300 base pairs) to be amplified more than a million-fold, i.e., millions of copies of a segment of a DNA can be made within a few hours. There are many variations and different applications in PCR technology. For example, real-time time or quantitative PCR uses specialised thermal cyclers that enable researchers to quantify amplification reactions as they occur. In this learning unit, we focus mainly on conventional PCR.

PCR has a wide array of applications in research and medicine, such as making DNA probes, studying gene expression, amplifying minute amounts of DNA to detect viral pathogens and bacterial infections, amplifying DNA to diagnose genetic conditions, or detecting trace amounts of DNA from tissue at a crime scene.

Figure 7.1: Thermal cycler

Source:
https://en.wikipedia.org/wiki/Polymerase_chain_reaction#/media/File:PCR_masina_kasutamine.jpg

7.2 Learning unit outcomes

On completion of this learning unit, you should be able to:

- Understand the purpose of PCR
- Explain briefly how PCR mechanisms work.

7.3 Components of PCR

The concept behind a PCR reaction is remarkably simple. Target DNA to be amplified is added to a thin-walled tube (Eppendorf tube) and mixed with the listed components below:

- Nuclease-free water to fill up reaction to 50 or 100 μl
- Two primers (forward and reverse) – these are short, single-stranded DNA oligonucleotides, usually around 20–30 nucleotides long. These primers are complementary to nucleotides flanking opposite ends of the target DNA to be amplified
- Nucleotides (the 4 dNTPs: A, T, C, G) – single units of the bases which are essentially "building blocks" for new DNA strands
- Heat-stable DNA polymerase (*Taq* DNA polymerase) – this is stable at high temperatures; it can withstand temperature changes necessary for PCR, without being denatured. The repeated heating and cooling required for PCR will denature and destroy most DNA polymerases after a few cycles
- Buffer and cofactor $MgCl_2$
- Thermal cycler

The PCR Eppendorf tube containing reaction mix is placed in a thermal cycler (figure 7.1). In the simplest sense, a thermal cycler is a sophisticated heating block that is capable of rapidly changing temperature over short time intervals. The thermal cycler takes the sample through a series of reactions called the PCR cycle.

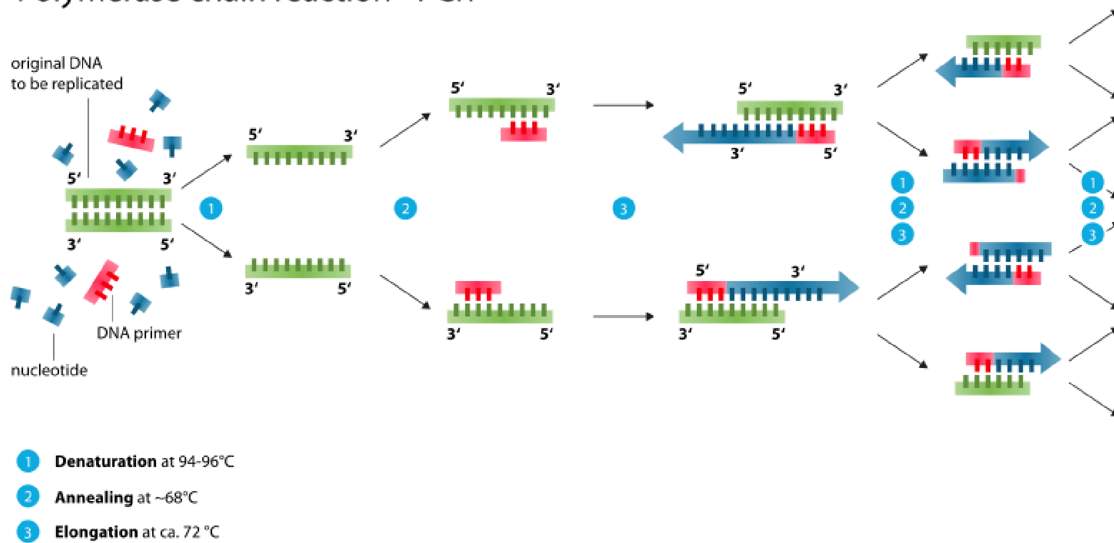

Figure 7.2: An overview of DNA amplification by PCR

Source:

https://en.wikipedia.org/wiki/Polymerase_chain_reaction#/media/File:Polymerase_chain_reaction.svg

7.4 The PCR cycle

Each cycle of the PCR follows three steps/stages:

- **Denaturation** – this is the first stage, where the reaction tube is heated to approximately 94–96 ℃, causing a separation of the target DNA into single strands
- **Primer annealing/hybridisation** – this is the second stage, where the tube is cooled slightly to between 55 ℃ and 65 ℃, which allows the primer to hydrogen bond to the complementary bases at opposite ends of the target sequence
- **Primer extension** – during this third and final stage of the PCR, the temperature is raised slightly to about 70–75 ℃ and DNA polymerase copies the target DNA by binding to the 3' ends of each primer and using primers as templates. DNA polymerase adds nucleotides to the 3' end of each primer to synthesise a complementary strand.

The cycle is usually repeated 25–40 times. A thermal cycler automatically changes the temperature at the correct time for each stage. At the end of one complete cycle, the amount of target DNA has been doubled.

The PCR amplified product can be detected using gel electrophoresis to view the band containing DNA fragments.

7.5 Primer features

Primers are short pieces of single-stranded DNA specific for each target. Primers can be specific (long 17–24 b) and random (short 10–15 b).

As the primer increases in size, the chances of matching the target size increase. The longer the primer, the higher the annealing temperature.

7.6 Taq DNA polymerase features

DNA polymerase-enzyme is responsible for the synthesis of new DNA strand in a 5'–3' direction.

Taq DNA polymerase is heat-stable and has optimal enzymatic activity at 72 ºC. Its enzymatic half-life (at 95 ºC) is 40 min and it extends the DNA chain by adding ~ 1.0 kb per min.

7.7 Advantages of PCR

- PCR is fast (2–5 hours)
- DNA or RNA can be amplified
- High-yield amplification can be achieved (106–109 amplifications)
- DNA from one cell equivalent can be amplified
- Works on damaged DNA
- PCR products can be directly sequenced
- Flexible

7.8 Disadvantages of PCR

- Need information about target DNA sequence
- Highly susceptible to contamination or false amplification
- Amplification may not be 100% specific
- Specificity of amplification is dependent on temperature and Mg++ concentration
- Analysis and product detection usually take longer than the PCR reaction itself
- There is an upper limit to the size of DNA synthesised by PCR

Click on the link below to learn more about PCR.

https://www.youtube.com/watch?v=vi7MeqD2_FY

Activity 7.1

1. PCR is performed in cycles of three steps. Discuss each step involved.
2. Why is PCR used to detect infectious agents that are often hard to diagnose?
3. If you performed a PCR experiment starting with only one copy of double-stranded DNA, approximately how many molecules would be produced after 15 cycles of amplification?