Laboratory One: General Introduction to the Nucleic Acid

Nucleic Acid is a macromolecule composed of chains of monomeric nucleotides. These molecules carry genetic information or form structures within cells. The most common nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Nucleic acids are universal in living things, as they are found in all cells and viruses.

1.1 Chemical Structure of Nucleic Acid

The monomers from which nucleic acids are constructed are called nucleotides. Nucleic acids are linear, unbranched polymers of nucleotides. Each nucleotide consists of three components (Figure 1.1).

1. Nitrogenous heterocyclic base, which is either a purine or a pyrimidine.
2. Pentose sugar.
3. Phosphate group.

Figure 1.1: The basic structure of RNA.
Nucleic acid types differ in the structure of the sugar in their nucleotides. DNA contains 2-deoxyribose while RNA contains ribose (where the only difference is the presence of a hydroxyl group). Also, the nitrogenous bases found in the two nucleic acid types are different: adenine, cytosine, and guanine are found in both RNA and DNA, while thymine only occurs in DNA and uracil only occurs in RNA.

Nucleic acids are usually either single-stranded or double-stranded, though structures with three or more strands can form. A double-stranded nucleic acid consists of two single-stranded nucleic acids held together by hydrogen bonds, such as in the DNA double helix. In contrast, RNA is usually single-stranded.

The sugars and phosphates in nucleic acids are connected to each other in an alternating chain, linked by shared oxygens, forming a phosphodiester bond. In conventional nomenclature, the carbons to which the phosphate groups attach are the 3' end and the 5' end carbons of the sugar. This gives nucleic acids polarity.

### 1.2 Types of Nucleic Acids

1. Ribonucleic acid

Ribonucleic acid, or RNA, is a nucleic acid polymer consisting of nucleotide monomers, which plays several important roles in the processes of transcribing genetic information from deoxyribonucleic acid (DNA) into proteins. RNA acts as a messenger between DNA and the protein synthesis complexes known as ribosomes, forms vital portions of ribosomes, and serves as an essential carrier molecule for amino acids to be used in protein synthesis. The three types of RNA include tRNA (transfer), mRNA (messenger) and rRNA (ribosomal).

2. Deoxyribonucleic acid

Deoxyribonucleic acid is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms. The main role of DNA molecules is the long-term storage of information and DNA is often compared to a set of blueprints, since it contains the instructions needed to construct other components of cells, such as proteins and RNA molecules. The DNA segments that carry this genetic information are called genes, but other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information.
DNA is made of four types of nucleotides, containing different nucleobases: the pyrimidines cytosine and thymine, and the purines guanine and adenine. The nucleotides are attached to each other in a chain by bonds between their sugar and phosphate groups, forming a sugar-phosphate backbone. Two of these chains are held together by hydrogen bonding between complementary bases; the chains coil around each other, forming the DNA double helix.

1.3 Nucleic Acid Components

1. Nucleobases

Nucleobases are heterocyclic aromatic organic compounds containing nitrogen atoms. Nucleobases are the parts of RNA and DNA involved in base pairing. Cytosine, guanine, adenine, thymine are found predominantly in DNA, while in RNA uracil replaces thymine. These are abbreviated as C, G, A, T, U, respectively.

2. Nucleosides

Nucleosides are glycosylamines made by attaching a nucleobase (often referred to simply as bases) to a ribose or deoxyribose (sugar) ring. In short, a nucleoside is a base linked to sugar. The names derive from the nucleobase names. The nucleosides commonly occurring in DNA and RNA include cytidine, uridine, adenosine, guanosine and thymidine.

3. Nucleotides and deoxynucleotides

A nucleotide consists of a nucleoside and one phosphate group. Nucleotides are the monomers of RNA and DNA, as well as forming the structural units of several important cofactors - CoA, flavin adenine dinucleotide, flavin mononucleotide, adenosine triphosphate and nicotinamide adenine dinucleotide phosphate. In the cell nucleotides play important roles in metabolism, and signaling.

1.4 Molecular Biology

Molecular biology is the study of biology at a molecular level. The field overlaps with other areas of biology and chemistry, particularly genetics and biochemistry. Molecular
biology chiefly concerns itself with understanding the interactions between the various systems of a cell, including the interactions between DNA, RNA and protein biosynthesis as well as learning how these interactions are regulated.

- **Techniques of molecular biology**
  1. Expression cloning
  2. Polymerase chain reaction (PCR)
  3. Gel electrophoresis
  4. Macromolecule blotting and probing
  5. Southern blotting
  6. Northern blotting
  7. Western blotting
  8. Arrays
  9. Allele Specific Oligonucleotide

### 1.5 Genetics

Genetics is the study of the effect of genetic differences on organisms. Often this can be inferred by the absence of a normal component (e.g. one gene). The study of "mutants" organisms which lack one or more functional components with respect to the so-called "wild type" or normal phenotype.

Genes correspond to regions within DNA, a molecule composed of a chain of four different types of nucleotides the sequence of these nucleotides is the genetic information organisms inherit. Each strand of DNA can act as a template for creating a new partner strand this is the physical method for making copies of genes that can be inherited.

The sequence of nucleotides in a gene is translated by cells to produce a chain of amino acids, creating proteins—the order of amino acids in a protein corresponds to the order of nucleotides in the gene. This relationship between nucleotide sequence and amino acid sequence is known as the **genetic code**. The amino acids in a protein determine how it folds into a three-dimensional shape; this structure is, in turn, responsible for the protein's function. Proteins carry out almost all the functions needed for cells to live. A change to the DNA in a gene can change a protein's amino acids, changing its shape and function: this can have a dramatic effect in the cell and on the organism as a whole.
1.6 Mutations

During the process of DNA replication, errors occasionally occur in the polymerization of the second strand. These errors, called mutations, can have an impact on the phenotype of an organism, especially if they occur within the protein coding sequence of a gene. Error rates are usually very low about 1 error in every 10 to 100 million bases due to the "proofreading" ability of DNA polymerases. Processes that increase the rate of changes in DNA are called mutagenic: mutagenic chemicals promote errors in DNA replication, often by interfering with the structure of base-pairing, while UV radiation induces mutations by causing damage to the DNA structure. Chemical damage to DNA occurs naturally as well, and cells use DNA repair mechanisms to repair mismatches and breaks in DNA nevertheless, the repair sometimes fails to return the DNA to its original sequence.
1.7 References


- Freisinger, E; Grollman; Miller; Kisker (2004). "Lesion (in) tolerance reveals insights into DNA replication fidelity". The EMBO journal 23 (7): 1494–505. doi:10.1038/sj.emboj.7600158. PMID 15057282.

Laboratory Two: Deoxyribonucleic acid (DNA) Extraction

2.1 Deoxyribonucleic Acid

DNA is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses. The main role of DNA molecules is the long-term storage of information. DNA is often compared to a set of recipe, or a code, since it contains the instructions needed to construct other components of cells, such as proteins and RNA molecules. The DNA segments that carry this genetic information are called genes, but other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information.

Within cells, DNA is organized into long structures called chromosomes (Figure 2.1). These chromosomes are duplicated before cells divide, in a process called DNA replication. Eukaryotic organisms (animals, plants, fungi, and protists) store most of their DNA inside the cell nucleus and some of their DNA in organelles, such as mitochondria or chloroplasts. In contrast, prokaryotes (bacteria and archaea) store their DNA only in the cytoplasm. Within the chromosomes, chromatin proteins such as histones compact and organize DNA. These compact structures guide the interactions between DNA and other proteins, helping control which parts of the DNA are transcribed.

2.2 Properties of DNA

1. DNA is a long polymer made from repeating units called nucleotides.
2. The DNA chain is 22 to 26 Ångströms wide (2.2 to 2.6 nanometres).
3. Each nucleotide unit is 3.3 Å (0.33 nm) long.
4. In living organisms, DNA does not usually exist as a single molecule, but instead as a pair of molecules that are held tightly together double helix structure.
5. DNA usually occurs as linear chromosomes in eukaryotes, and circular chromosomes in prokaryotes.

6. Human genome has approximately 3 billion base pairs of DNA arranged into 46 chromosomes.

![Figure 2.1: Packaging of DNA in the Nucleus.](image)

**2.3 DNA Extraction**

DNA Extraction is the removal of DNA from the cells.

**2.3.1 Clinical applications:**
Extraction of DNA is a key step in diagnosing disease and genetic disorders in addition to forensic analysis. As well as used in many diagnostic processes used to detect bacteria and viruses in the environment.
2.3.2 Kind of samples used for DNA extraction:
We can use all living cells to extract DNA. But in epidemiologic studies they prefer whole blood and blood spots as a first choice, amniotic fluid and chorionic villus sampling (CVS) for prenatal diagnosis (PND), buccal swab and hair follicles in forensics.

2.3.3 Choice of sample depends on:
1. Amount of DNA needed for analysis, which depends on what kind of mutation we are looking for, and the method used for DNA analysis.
2. The conditions and resources for collecting sample.

2.3.4 DNA extraction Methods:
The method should be:
1. Safe
2. Simple
3. Inexpensive
4. Yield good DNA quality based on:
   a) Concentration of DNA is sufficient for analysis.
   b) Purity; no contamination, lipids, proteins and RNA.
   c) Integrity; no DNA degradation

2.3.5 DNA extraction steps:
There are three basic and one optional step in a DNA extraction:
1. Breaking the cells open commonly referred to as cell disruption or cell lysis, to expose the DNA within.
2. Removing membrane lipids by adding a detergent.
3. Removing proteins by adding a protease (optional but almost always done).
4. Precipitating the DNA with an alcohol usually ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a **pellet** upon centrifugation. This step also removes alcohol-soluble salt.

- Refinements of the technique include adding a **chelating agent** to sequester divalent cations such as Mg$^{2+}$ and Ca$^{2+}$. This stops nuclease enzymes from degrading the DNA.

### 2.3.6 Isolation of genomic DNA by phenol chloroform or enzymatic method

The most basic of all procedures in molecular biology is the isolation and purification of nucleic acids. The key step, the removal of proteins, can often be carried out simply by extracting aqueous solutions of nucleic acids with phenol, chloroform and isoamyl alcohol. Additional measures are required when nucleic acids are purified from complex mixtures of molecules such as cell lysates. In these cases, it is usual to remove most of the proteins by digestion with proteolytic enzymes such as proteinase K, which are active against a broad spectrum of native proteins before extracting with organic solvents.

Digestion of blood sample with Proteinase K will prepare a crude lysate by digesting cellular protein and SDS is used to break the disulphide bonds. Phenol is used to remove proteins. Chloroform facilitates the separation of the aqueous phase and organic phase and iso-amyl alcohol reduces foaming during extraction. Ethanol helps to precipitate DNA and remove the remaining salts.
Isolation of Genomic DNA by Phenol Chloroform

2.4 Practical Work

A. Materials & equipments:

1. Erythrocyte lysis buffer
2. 20% SDS
3. Proteinase K
4. Phenol
5. 1 M Tris
6. Chloroform-Isoamylalcohol
7. 3M Sodium Acetate
8. Absolute Ethanol
9. 70% Ethanol
10. TE Buffer (pH 8.0)
11. Micropipettes (100 – 1000 µl)
12. Micropipettes (10 – 100 µl)
13. Sterile blue tips (100 – 1000 µl)
14. Sterile yellow tips (10 – 100 µl)
15. 1.5 ml tubes
16. Water bath
17. Centrifuge (1.5 ml rotor)
18. Gloves
19. Fume hood
20. Racks (1.5 ml)
21. Filter paper

B. PROCEDURE

1. To 5ml of heparinized whole blood add three times equal volume of erythrocyte lysis buffer. Shake gently and keep it in ice for 15 minutes.

2. Remove tubes from ice and centrifuge in a refrigerated centrifuge at 3,500 rpm for 10 minutes at 4°C.

3. Discard supernatant and disturb the pellet with 1 ml of erythrocyte lysis buffer and make up to 5ml with buffer (repeat the step until white pellet is obtained).

4. To the sample add 300 µl of 20% SDS and mix gently for 3 to 4 times. To this mixture, add 40µl of Proteinase K and incubate at 37°C in a water bath overnight.

5. After overnight incubation, add 5ml of phenol mix slowly and centrifuge at 10,000 rpm for 10 minutes.
6. Transfer the supernatant to a fresh autoclaved tube. To this, add 5ml of Phenol: Chloroform-Isoamylalcohol (25:24:1). Mix gently and then centrifuge at 10,000 rpm for 10 minutes at 4°C.

7. Transfer the supernatant to a fresh autoclaved tube and add 5 ml chloroform and Isoamylalcohol (24:1). Mix and centrifuge at 10,000rpm for 10 minutes.

8. Transfer the supernatant to a fresh autoclaved tube and add 3 volumes of chilled ethanol and keep overnight at -20°C. Later centrifuge at 10000rpm for 10 minutes at 4°C.

9. Discard the supernatant and wash the pellet with 70% ethanol and allow it to dry.

10. After the pellet gets dried up, it is dissolved in 200μl of TE Buffer and transferred to 1.5ml eppendorf tube for storage.
2.5 References

Laboratory three: Ribonucleic acid (RNA) Extraction

3.1 Ribonucleic Acid

RNA is a biologically important type of molecule that consists of a long chain of nucleotide units. Each nucleotide consists of a nitrogenous base, a ribose sugar, and a phosphate. RNA is very similar to DNA, but differs in a few important structural details:

- In the cell, RNA is usually single-stranded, while DNA is usually double-stranded.
- RNA nucleotides contain ribose while DNA contains deoxyribose (a type of ribose that lacks one oxygen atom).
- RNA has the base uracil rather than thymine that is present in DNA.

RNA is transcribed from DNA by enzymes called RNA polymerases and is generally further processed by other enzymes. RNA is central to protein synthesis. Here, a type of RNA called messenger RNA carries information from DNA to structures called ribosomes. These ribosomes are made from proteins and ribosomal RNAs, which come together to form a molecular machine that can read messenger RNAs and translate the information they carry into proteins.

3.2 Types of RNA

Messenger RNA (mRNA) is the RNA that carries information from DNA to the ribosome, the sites of protein synthesis (translation) in the cell. The coding sequence of the mRNA determines the amino acid sequence in the protein that is produced. Many RNAs do not code for protein however (about 97% of the transcriptional output is non-protein-coding in eukaryotes). These so called non-coding RNAs ("ncRNA") can be encoded by their own genes (RNA genes).
A. In translation

1. **Messenger RNA (mRNA)** carries information about a protein sequence to the ribosomes.

2. **Transfer RNA (tRNA)** is a small RNA chain of about 80 nucleotides that transfers a specific amino acid to a growing polypeptide chain at the ribosomal site of protein synthesis during translation.

3. **Ribosomal RNA (rRNA)** is the catalytic component of the ribosomes. Eukaryotic ribosomes contain four different rRNA molecules: 18S, 5.8S, 28S and 5S rRNA.

4. **Transfer-messenger RNA (tmRNA)** is found in many bacteria and plastids. It tags proteins encoded by mRNAs that lack stop codons for degradation and prevents the ribosome from stalling.

B. In reverse transcription

5. Retrotransposons spread by copying DNA and RNA from one another.

6. Telomerase contains an RNA that is used as template for building the ends of eukaryotic chromosomes.

3.3 RNA Extraction

RNA Extraction is the removal of all or some types of RNA from the cells; depending on extraction methods are used.

3.3.1 Clinical applications:

Extraction of RNA has a major role in diagnosing disease and genetic disorders in addition to expression analysis. As well as used in many diagnostic processes used to detect RNA-bacteria and RNA-viruses in different samples.

3.3.2 Kind of samples used for RNA extraction:

1. Whole blood
2. Amniotic fluid
3. Chorionic villus sampling (CVS)
4. Living tissue
5. Paraffin embedded tissue and other.

3.3.3 RNA extraction methods:
The method should be:
5. Safe
6. Simple
7. Inexpensive
8. Yield good RNA quality based on:
   a) Concentration of RNA is sufficient for analysis.
   b) Purity; no contamination, lipids, proteins and DNA.
   c) Integrity; no RNA degradation

3.3.4 Column-based nucleic acid purification
Column-based nucleic acid purification is a solid phase extraction method to quickly purify nucleic acids. This method relies on the fact that the nucleic acid may bind (adsorption) to the solid phase (silica or other) depending on the pH and the salt content of the buffer (Figure 3.1), which may be a Tris-EDTA (TE) buffer or Phosphate buffer. Therefore, three stages are:
1. The sample is added to the column and the nucleic acid binds to the high pH and salt concentration of the binding solution.
2. The column is then washed (5 mM KPO4 pH 8.0 or similar, 80% EtOH).
3. The column can be eluted with buffer or simply water.
Figure 3.1: Silica gel column for RNA extraction.
RNA isolation from human blood samples

3.4 Practical Work

C. Materials & equipments:

9. Blood samples
2. kit (Qiagen)
3. Micropipettes (100 – 1000 µl)
4. Micropipettes (10 – 100 µl)
5. Sterile blue tips (100 – 1000 µl)
6. Sterile yellow tips (10 – 100 µl)
7. 1.5 ml tubes
8. Centrifuge (1.5 ml rotor)
9. Gloves
10. Racks (1.5 ml)
11. Filter paper

D. PROCEDURE

9. Centrifuge the RNAprotect Animal Blood Tube for 3 min at 5000 x g.

   Note: Be sure to incubate the tube for at least 2 h at room temperature (15–25°C) after blood collection to achieve complete lysis of blood cells.

   Note: If using an RNAprotect Animal Blood Tube (100 µl), transfer the blood sample to a new 1.5 ml collection tube (supplied; the same tube can be used in step 5). Alternatively, use only 450 µl RNase-free water in step 2 below.

10. Remove the supernatant by decanting or pipetting. Add 1 ml RNase-free water to the pellet, and close the tube. If decanting the supernatant, take care not to disturb the pellet, and dry the rim of the tube with a clean paper towel.

11. Vortex until the pellet is visibly dissolved, and centrifuge for 3 min at 5000 x g. Remove the entire supernatant by decanting or pipetting, and discard. Small debris remaining in the sample after vortexing does not affect subsequent RNA purification.

   Note: Incomplete removal of the supernatant will inhibit proteinase K digestion and dilute the lysate, affecting the conditions for binding RNA to the RNeasy MinElute membrane.

12. Add 240 µl Buffer RSB, and vortex until the pellet is visibly dissolved.
13. Pipet the sample into a 1.5 ml collection tube (supplied). Add 200 μl Buffer RBT and 20 μl proteinase K. Mix by vortexing for 5 s, and incubate for 10 min at 55°C in a shaker–incubator at 400–1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C for step 18.

*Note: Do not mix Buffer RBT and proteinase K together before adding them to the sample.*

14. 6. Pipet the sample into a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 3 min at full speed (do not exceed 20,000 x g).

15. Carefully transfer the entire supernatant of the flow-through from the QIAshredder spin column to a new 1.5 ml collection tube (supplied) without disturbing the pellet.

16. Add 240 μl ethanol (96–100%), and mix by vortexing.

17. Pipet the sample into an RNeasy MinElute spin column (pink) placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm). Discard the flow-through.* Reuse the collection tube in step 10.

18. Add 350 μl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.* Reuse the collection tube in step 13.

*Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.*

19. Add 10 μl DNase I stock solution to 70 μl Buffer RDD in a 1.5 ml microcentrifuge tube (not supplied). Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

*Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.*

20. Pipet the DNase I incubation mix (80 μl) directly onto the RNeasy MinElute spin column membrane, and incubate on the benchtop (20–30°C) for 15 min.

*Note: Be sure to add the DNase I incubation mix directly to the spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.*

21. Add 350 μl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.* Reuse the collection tube in step 14.

*Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.*
22. Add 500 μl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Reuse the collection tube in step 15.

*Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).*

23. Add 500 μl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm).

*Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.*

24. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.

*Note: To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise). It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.*

25. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube, and pipet 14–30 μl Buffer REB directly onto the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA. Be sure to add Buffer REB directly to the spin column membrane. This wets the entire membrane, ensuring maximum elution efficiency.

26. Incubate the RNA eluate for 5 min at 65°C in the shaker–incubator without shaking. After incubation, chill immediately on ice. This incubation at 65°C denatures the RNA for downstream applications. Do not exceed the incubation time or temperature.

27. If the RNA eluate will not be used immediately, store at −20°C or −70°C. Since the RNA remains denatured after freezing and thawing, it is not necessary to repeat the incubation at 65°C.
3.5 References


Laboratory Four: Estimation of Nucleic Acid

4.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The purpose of the gel might be:

1. To look at the DNA
2. To quantify it
3. To isolate a particular band.

Separation of the molecules is achieved by moving negatively charged nucleic acid molecules through an agarose matrix in an electric field. Shorter molecules move faster and migrate further than longer ones. The DNA is visualized in the gel by addition of ethidium bromide (Figure 4.1).

4.1.1 Applications:

1. Allows the rough estimation of DNA quantity and quality.
2. Estimation of the size of DNA molecules using a DNA ladder which contains DNA fragments of various known sizes.
3. Analysis of PCR products after polymerase chain reaction to assess for target DNA amplification.
4. Separation of restriction enzyme digested DNA including genomic DNA, prior to Southern Blot transfer.
5. Quantity is assessed using lambda DNA ladder which contains specific amounts of DNA in different bands.
6. Quality of DNA is assessed by observing the absence of streaking or fragments (or contaminating DNA bands).

Figure 4.1: Gel electrophoresis steps for Nucleic acid separation.

4.1.2 Factors affecting the rate of DNA migration in agarose gels:
   
   1. Molecular size of the DNA.
   2. Agarose concentration.
   3. Conformation of DNA.
   5. Direction of electrical field.
   6. Composition of the electrophoresis buffer.
   7. Presence of intercalating dyes.
4.1.3 Equipment for conducting agarose gel electrophoresis:

1. Electrophoresis chamber (Figure 4.2).
2. Power supply (Figure 4.2).
3. Gel casting trays (Figure 4.2).
4. Sample combs to form sample wells.
5. Running buffer.
7. Ethedium bromide.
   
   *Note it is a mutagen and should be handled as a hazardous chemical- wear gloves while handling.*
8. Transilluminator.

4.1.4 Agarose gel electrophoresis buffers:

**Running buffers**: different buffers can be used for agarose electrophoresis depending on the size of the DNA. TAE buffer (Tris Acetate EDTA) is the most common used buffer. TBE buffer (Tris Borate/EDTA) is often used for smaller DNA fragments (i.e less than 500bp).

**Loading buffer**: this buffer mixed with the sample, it gives color and density to the samples makes it easy loading in the wells. It consists of bromophenol blue the tracing dye, sucrose and water.

4.1.5 Markers:

- Different kinds of markers most of them are bacteriophage DNA cut with restriction enzymes (Figure 4.3).
- Storing markers ready mixed with loading buffer at 4C,
- Choosing marker with a good resolution for fragment size you expect to see in your sample lanes.
4.1.6 Visualization:

After electrophoresis is complete a transiluminator (an ultraviolet light box) is used to visualize ethidium bromide-stained DNA in gels.

*Note: you have to wear a protective eyewear when observing DNA bands to prevent damage to the eyes from UV light.*

4.2 Spectrophotometric Method

Because DNA and RNA absorb ultraviolet light, with an absorption peak at 260nm wavelength, spectrophotometers are commonly used to determine the concentration of DNA in a solution. Inside a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample.

Using the Beer-Lambert Law it is possible to relate the amount of light absorbed to the concentration of the absorbing molecule. At a wavelength of 260 nm, the average extinction coefficient for double-stranded DNA is 0.020 (μg/ml)-1 cm-1, for single-stranded DNA and RNA it is 0.027 (μg/ml)-1 cm-1 and for short single-stranded oligonucleotides it is dependent on the length and base composition. Thus, an optical density (or "OD") of 1 corresponds to a concentration of 50 μg/ml for double-stranded DNA. This method of calculation is valid for up to an OD of at least 2. A more accurate extinction coefficient may be needed for oligonucleotides; these can be predicted using the nearest-neighbor model.

4.2.1 Sample purity:

It is common for nucleic acid samples to be contaminated with other molecules (eg, protein, phenol, and other organic compounds). Because these molecules have their own characteristic absorption spectra, the absorption at other wavelengths is often compared to 260nm absorption in order to assess sample purity.
4.2.2 Protein contamination and the 260:280 ratio:

The ratio of absorptions at 260nm vs 280nm is commonly used to assess the purity of protein with respect to DNA contamination, since protein (in particular, the aromatic amino acids) tends to absorb at 280nm.

4.2.3 Spectrophotometric method:

1. It is an analytical method used for determining the purity (quality) & quantity of the isolated DNA.
2. The absorbance is measured at 260nm, at this wavelength an absorbance of 1.0 corresponds to 50 µg per ml of dsDNA, 40µg/ml of ssDNA or RNA & 20µg/ml of oligo nucleotides.
4. For pure DNA and RNA the ratio is approximately 1.8 and 2.0 respectively.
5. If DNA is contaminated with proteins then the ratio will be < 1.8
6. If DNA is contaminated with RNA then the ratio will be > 2.0
I. Agarose Gel Electrophoresis

4.3.1 Practical Work

A. Materials & equipments:
1. DNA samples
2. 1X TBE buffer
3. Ethidium bromide
4. 6X Loading dye
5. DNA size marker
6. 0.25 M HCl
7. 0.25 M NaOH
8. Agarose
9. Micropipettes (1 – 10 µl)
10. Sterile white tips (1 – 10 µl)
11. Horizontal gel electrophoresis
12. Power supply
13. Autoclave
14. Water bath
15. UV transilluminator
16. Water bath
17. Gloves
18. Parafilm

B. Procedure:
1. Prepare a 1% agarose solution, measure 3 g agarose into a flask and add 150 ml 1X buffer. Microwave until agarose is dissolved and solution is clear.

2. Allow solution to cool to about 55°C before pouring. Add 10 µl of Ethidium bromide.

3. Prepare gel tray and place comb in gel tray.

4. Pour 50°C gel solution into tray and allow gel to solidify about 20 minutes at room temperature.

5. To run, gently remove the comb, place tray in electrophoresis chamber, and cover (just until wells are submerged) with electrophoresis buffer (the same buffer used to prepare the agarose).

6. To prepare samples for electrophoresis, add 1 µl of 6x gel loading dye for every 5 µl of DNA solution. Mix well & load. Don’t forget to load the DNA size marker.

7. Electrophorese at 50-150 volts until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.

8. Visualize DNA under the UV transilluminator.
II. DNA Spectrophotometry

4.3.11 Practical Work

A. Materials & equipments:
1. DNA samples
2. Tris-EDTA
3. Spectrophotometer
4. Quartz cuvettes
5. Micropipettes (1 – 10 µl)
6. Sterile white tips (1 – 10 µl)
7. Wipes
8. 1.5 ml tubes

B. Procedure:
1. Turn on the spectrophotometer about 10 minutes ahead of time to let the H2-deuterium lamp (for UV) warm up. You will take readings at 260 and 280 nm.
2. Dilute the DNA samples (1:200) in TE.
3. BLANK the spectrophotometer against TE alone at 260 nm.
4. Read the absorbance of your diluted DNA samples at 260 nm.
5. BLANK the spectrophotometer against TE alone at 280 nm.
6. Read the absorbance of your diluted DNA samples at 280 nm.
8. Calculate the DNA concentration in your diluted sample using the Beer-Lambert law.
4.4 References


Laboratory Five: Polymerase Chain Reaction (PCR)

5.1 PCR

PCR is a technique for amplifying DNA sequences in vitro. It can amplify a specific sequence of DNA between two regions of known sequence by as many as one billion times. It is important in biotechnology, forensics, medicine, and genetic research.

5.1.1 PCR properties:

- Rapid & easy.
- Sensitive.
- Robust.
- Widespread applications.

5.1.2 Basic requirements for PCR reaction:

1. DNA sequence of target region must be known (Figure 5.1).

2. Primers, typically 18-25 bases in size. These can be readily produced by commercial companies (Figure 5.1). Can also be prepared using a DNA synthesizer.

3. Thermo-stable DNA polymerase. E.g. Taq polymerase which is not inactivated by heating to 95°C (Figure 5.1).

Figure 5.1: PCR requirement.
4. dNTPs (Figure 5.1).
5. DNA thermal cycler, machine which can be programmed to carry out heating and cooling of samples over a number of cycles.

### 5.1.3 Standard PCR reaction:

1. DNA template that contains the DNA region (target) to be amplified. 1-5uL.
2. Two primers that are complementary to the 3’ (three prime) ends of each of the sense and anti-sense strand of the DNA target. 1-3uL.
3. Master mix consist of:
   1. **Taq polymerase** or another DNA polymerase with a temperature optimum at around 70 °C. 1-2U.
   2. **Deoxynucleoside triphosphates** (dNTPs; also very commonly and erroneously called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand. 1-25uL.
   3. **Buffer solution**, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase. 4-7uL
   4. **Divalent cations**, magnesium or manganese ions; generally Mg2+ is used, but Mn2+ can be utilized for PCR-mediated DNA mutagenesis, as higher Mn2+ concentration increases the error rate during DNA synthesis. 1-2uL.
   5. **Monovalent cation**: potassium ions. 1-2uL.

### 5.1.4 The Cycling Reactions

**A. Denaturation** (Figure 5.2).
- Temperature: 93-95°C.
- Double stranded DNA melts to form single stranded DNA.

**B. Annealing** (Figure 5.2).
- Temperature: 50-70°C (dependant on the melting temperature of the expected duplex).
- Primers bind to their complementary sequences.
C. Extension (Figure 5.2).

- Temperature: 72°C
- DNA polymerase binds to the annealed primers and extends DNA at the 3’ end of the chain.

![PCR: Polymerase Chain Reaction]

Figure 5.2: The PCR cycling steps.

5.1.5 Standard PCR method:

- Prepare mix containing primers, dNTP’s, buffer, Taq polymerase and water sufficient for all reaction tubes.
- Aliquot appropriate volume to each tube.
- Add DNA to each tube using a new tip for each sample.
- Load tubes on PCR machine.
- If PCR machine does not have heated lid, 1 drop of mineral oil should be added to each tube before loading then start the program.
5.2 Properties of Polymerase

1. Taq polymerase originally isolated from Thermus aquaticus.
2. Heat stable (half life of about 30 min at 95°C).
3. Taq DNA polymerase has no proof-reading function in 3’ to 5’ direction.
4. Primer extension occurs at up to 100 bases/sec.
5. Plateau is eventually reached.

5.3 Trouble Shooting

Table 5.1: Common PCR problems and possible reasons.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible reasons</th>
</tr>
</thead>
<tbody>
<tr>
<td>No product</td>
<td>Primers annealing?</td>
</tr>
<tr>
<td>Product of incorrect size</td>
<td>Primers annealing elsewhere in genome?</td>
</tr>
<tr>
<td>Several Products formed</td>
<td>Contamination?</td>
</tr>
<tr>
<td></td>
<td>Several annealing sites?</td>
</tr>
</tbody>
</table>

5.3.1 PCR rxn inhibitors:

- protease K: digest polymerase
- phenol: denature the polymerase
• EDTA: chelates Mg+2

5.4 Advantages of PCR

1. Uses less patient DNA.
2. Result obtained more quickly about 3hr for PCR.
3. Usually not necessary to use radioactive material for PCR.
4. Precise in determining sizes of alleles.
5. Can be used to detect point mutations.

5.5 Disadvantages of PCR

1. Target DNA sequence must be known.
2. Errors made by Taq polymerase.
3. Size limitations particularly in GC rich triplet repeats.

5.6 PCR Based Technologies

• Multiplex PCR: Involves using more than one set of primers in the same reaction tube.
• QF-PCR: Precise measure of input DNA required for quantitative PCR reaction. The fluorescent assay determines peak areas equivalent to PCR product yield for each exon.
• RT-PCR: Uses mRNA as a template to produce a cDNA copy. The cDNA amplified sequences can be used to screen for possible mutations directly. And others.
Polymerase Chain Reaction for *TPMT* Gene

5.7 *Practical Work*

A. Materials & equipments

1. DNA samples
2. Nuclease-free H2O
3. Ready Mix (2X)
4. *TPMT*-1 Primer
5. *TPMT*-2 Primer
6. Micropipettes (1 – 10 µl)
7. Micropipettes (10 – 100 µl)
8. Sterile yellow tips (10 – 100 µl)
9. Sterile white tips (1 – 10 µl)
10. 1.5 ml tubes
11. 0.2 ml PCR tubes
12. Ice
13. Gloves
14. Thermal cycler

B. Primers

Primers were designed from the *TPMT* gene (EMBL database, accession no. ENSG00000137364).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ …… 3’)</th>
<th>Position on the gene</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>TPMT</em>-1</td>
<td>GCAATCGCTAAAGAACTAAG</td>
<td>693-712</td>
<td>321</td>
</tr>
<tr>
<td><em>TPMT</em>-2</td>
<td>GGGACCAACATAACCTAATA</td>
<td>859-914</td>
<td></td>
</tr>
</tbody>
</table>

C. PCR Mixture components

1. Melt all the PCR reagents on ice.
2. Prepare a master mixes (MM) as follows
<table>
<thead>
<tr>
<th>Component (Concentration)</th>
<th>Concentration in the reaction</th>
<th>Volume per Sample (µL)</th>
<th>No. of samples</th>
<th>Volume added to MM (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Ready mix (2X)</td>
<td>1X</td>
<td>12.5</td>
<td>X 5</td>
<td>62.5</td>
</tr>
<tr>
<td><em>TPMT</em>-1 (10 pmol)</td>
<td>0.5 pmol</td>
<td>1</td>
<td>X 5</td>
<td>5</td>
</tr>
<tr>
<td><em>TPMT</em>-2 (10 pmol)</td>
<td>0.5 pmol</td>
<td>1</td>
<td>X 5</td>
<td>5</td>
</tr>
<tr>
<td>Nuclease-free H2O</td>
<td>-</td>
<td>9.5</td>
<td>X 5</td>
<td>47.5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>24</td>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>

3. Distribute the mix into three 0.2 ml tubes (24 µl per tube). Don’t forget to label the tubes appropriately.

4. Add 1 µL of DNA from the sample one tube. Add 1 µL of Nuclease-free H2O into the negative control tube.

**D. Amplification Conditions**

Put the tubes in the thermal cycler. Program the thermal cycler as follows

<table>
<thead>
<tr>
<th>PCR Step</th>
<th>Temperature</th>
<th>Time Duration</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>45 sec</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Store</td>
<td>4°C</td>
<td>∞</td>
<td>∞</td>
</tr>
</tbody>
</table>
E. Results Analysis

PCR products are resolved by electrophoresis on 2% agarose gel stained with ethidium bromide and visualized under U.V. transilluminator.
5.8 References

Laboratory six: Restriction Fragment Length Polymorphism (RFLP)

6.1 RFLP
RFLP is widely used technique to detect known mutations and variations using specific restriction endonucleases. Genetic defects can be diagnosed by RFLP, as normal and defective genes give rise to different restriction patterns, if change is in the recognition site of restriction enzyme.

6.2 Principle
Restriction endonucleases recognize specific sequences and cut double strand DNA within their recognition sequence to produce fragments. Changes in DNA sequences may generate or abolish or alter the position of recognition site for restriction endonucleases. Fragments thus produced get separated on agarose gel electrophoresis and are visualized after staining with ethidium bromide which enables analysis of sequence variations of discrete region.

6.2.1 Major characteristics of restriction enzymes (Res):
- Enzymes originally isolated from bacteria.
- Cut double-stranded DNA at or near to specific sites.
- Most recognition sites are palindromes e.g. GTATAc.
  - The sequences read 5’ to 3’ the same on both strands.
  - These sequences normally between 4-12 base pairs in length.
- Cutting can produce ‘blunt’ ends or ‘sticky’ ends.
  - Sticky ends are useful in constructing recombinant DNA molecules.
6.2.2 Component of the reaction:

The digestion conditions differ from one enzyme to another (pH, [salt], incubation temp) and depends on the type of DNA to be digested (PCR product, Genomic DNA..Etc)

But usually all digestion rxn must contain:

- Nuclease free water
- Buffer (to provide the optimum conditions for enzyme activity: PH, [SALT] and cofactor if needed)
- Enzyme.

6.2.3 Nomenclature:

Since their discovery in the 1970s, more than 100 different restriction enzymes have been identified in different bacteria. Each enzyme is named after the bacterium from which it was isolated using a naming system based on bacterial genus, species and strain. For example, the name of the EcoRI restriction enzyme was derived as shown in the Table 1.
Table 6.1: An example of RE nomenclature.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>Escherichia</td>
<td>Genus</td>
</tr>
<tr>
<td>co</td>
<td>Coli</td>
<td>Species</td>
</tr>
<tr>
<td>R</td>
<td>RY13</td>
<td>Strain</td>
</tr>
<tr>
<td>I</td>
<td>First identified</td>
<td>order of identification in the bacterium</td>
</tr>
</tbody>
</table>

6.3 Example of Restriction Reaction

- E.g. MspI enzyme

  A. Digestion of PCR product:

     - NFW 18 µl
     - 10X buffer 2 µl
     - Enzyme 0.5-1µl
     - PCR product 10 µl
     - Incubate at 37°C from 1-16 hr.’s
     - MspI is inactivated by incubation at 80°C for 20 min.

  B. Data analysis:

     Figure 6.2 discusses the all possible results of DNA fragments.
6.4 Applications

1. Assist insertion of genes into plasmid vectors during gene cloning and protein expression experiments.

2. Forensic scientists can use restriction enzymes for “DNA fingerprinting” – to identify a suspect based on the patterns their DNA makes when cut with specific enzymes.

3. Used to distinguish gene alleles (genotyping). By specifically recognizing single base changes in DNA, known as single nucleotide polymorphisms (SNPs).

4. Restriction enzymes are used to digest genomic DNA for gene analysis by southern blot.

Figure 6.2: An example of MspI enzyme cutting sites.
Restriction Fragment Length Polymorphism for Sickle Cell Anaemia

6.5 Practical work

A. Materials & equipments

10. DNA samples
11. Nuclease-free H2O
12. Ready Mix (2X)
13. sicF Primer
14. sicR Primer
6. Micropipettes (1 – 10 µl)
7. Micropipettes (10 – 100 µl)
8. Restriction enzyme kit (MstII)
9. Sterile yellow tips (10 – 100 µl)
10. Sterile white tips (1 – 10 µl)
11. 1.5 ml tubes
12. 0.2 ml PCR tubes
13. Ice
14. Gloves
15. Thermal cycler
16. Water bath

B. Primers

Primers were designed to encompass the entire 3’ repeat elements and avoid the variable regions within the β-globin gene gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ …… 3’)</th>
<th>Position on the gene</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>sic-F</td>
<td>5’-TAGAG ATGCTTACAGG-3’</td>
<td>1513-1531</td>
<td>660 bp</td>
</tr>
<tr>
<td>sic-R</td>
<td>5’-GCTTCCGATTGTTGCAGGC-3’</td>
<td>2188-2168</td>
<td></td>
</tr>
</tbody>
</table>

C. PCR Mixture components

1. Melt all the PCR reagents on ice.
2. Prepare a master mixes (MM) as follows

<table>
<thead>
<tr>
<th>Component (Concentration)</th>
<th>Concentration in the reaction</th>
<th>Volume (µL) per Sample</th>
<th>No. of samples</th>
<th>Volume (µL) to added to MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Ready mix (2X)</td>
<td>1X</td>
<td>12.5</td>
<td>X 5</td>
<td>62.5</td>
</tr>
<tr>
<td>Coa-F</td>
<td>0.4 pmol</td>
<td>1</td>
<td>X 5</td>
<td>5</td>
</tr>
<tr>
<td>Coa-R</td>
<td>0.4 pmol</td>
<td>1</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Nuclease-free H2O</td>
<td>-</td>
<td>9.5</td>
<td>X 5</td>
<td>47.5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>24</td>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>
3. Distribute the mix into three 0.2 ml tubes (24 µl/tube). Don’t forget to label the tubes appropriately.
4. Add 1 µL of DNA from the sample one tube. Add 1 µL of Nuclease-free H2O into the negative control tube.

**D. Amplification Conditions**

Put the tubes in the thermal cycler. Program the thermal cycler as follows:

<table>
<thead>
<tr>
<th>PCR Step</th>
<th>Temperature</th>
<th>Time Duration</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 sec</td>
<td>35</td>
</tr>
<tr>
<td>Anealing</td>
<td>62°C</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10min</td>
<td>1</td>
</tr>
<tr>
<td>Store</td>
<td>4°C</td>
<td>∞</td>
<td>∞</td>
</tr>
</tbody>
</table>

**E. Analysis**

PCR products are resolved by electrophoresis on 2% agarose gel stained with ethidium bromide and visualized under U.V. transilluminator.

**F. Restriction Digestion of Coagulase PCR product**

1. Combine the following reaction components at room temperature in the order indicated:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL) per Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>17</td>
</tr>
<tr>
<td>10X FastDigest Buffer</td>
<td>2</td>
</tr>
<tr>
<td>PCR product</td>
<td>10</td>
</tr>
<tr>
<td>Fast Digest MstII Enzyme</td>
<td>1</td>
</tr>
<tr>
<td>Total volume</td>
<td>30</td>
</tr>
</tbody>
</table>

2. Mix gently and spin down.
3. Incubate at 37°C in a heat block or water thermostat for 1 hr.
4. Inactivate the enzyme by heating for 5 min at 65°C (optional).
5. Analyze the resulting RFLP patterns by gel electrophoresis on 3% agarose gel.
6.6 References

Laboratory Seven: DNA Sequencing

7.1 DNA Sequencing

The determination of the precise sequence of nucleotides in a sample of DNA is called DNA sequencing.

The most popular method for doing this is called the dideoxy method or Sanger method (named after its inventor, Frederick Sanger, who was awarded the 1980 Nobel prize in chemistry for this achievement).

The dideoxy method gets its name from the critical role played by synthetic nucleotides that lack the -OH at the 3’ carbon atom (red arrow) (Figure 7.1). A dideoxynucleotide (dideoxythymidine triphosphate ddTTP is the one shown here) can be added to the growing DNA strand but when it is, chain elongation stops because there is no 3’ -OH for the next nucleotide to be attached to. For this reason, the dideoxy method is also called the chain termination method.

![Figure 7.1: The difference in structure between dTTP, and ddTTP.](image-url)
7.2 The Procedure

- The DNA to be sequenced is prepared as a single strand.
- This template DNA is supplied with a mixture of all four normal (deoxy) nucleotides in sample quantities
  - dATP
  - dGTP
  - dCTP
  - dTTP
- A mixture of all four dideoxynucleotides, each present in limiting quantities and each labeled with a "tag" that fluoresces a different color:
  - ddATP
  - ddGTP
  - ddCTP
  - ddTTP
- DNA polymerase I is used.

Because all four normal nucleotides are present, chain elongation proceeds normally until, by chance, DNA polymerase inserts a dideoxy nucleotide instead of the normal deoxynucleotide. If the ratio of normal nucleotide to the dideoxy versions is high enough, some DNA strands will succeed in adding several hundred nucleotides before insertion of the dideoxy version halts the process.

At the end of the incubation period, the fragments are separated by length from longest to shortest. The resolution is so good that a difference of one nucleotide is enough to separate that strand from the next shorter and next longer strand. Each of the four dideoxynucleotides fluoresces a different color when illuminated by a laser beam and an automatic scanner provides a printout of the sequence.

7.2.1 Putting all four deoxynucleotides into the picture:

The spacing between the bands isn't all that easy to figure out. Imagine, though, that we ran the reaction with all four of the dideoxy nucleotides (A, G, C and T) present, and with
different fluorescent colors on each, look at the figure 7.2 below. The sequence of the DNA is rather obvious if you know the color codes, so just read the colors from bottom to top: TGCGTCCA-(etc).

*Note: Black here it shows up better than yellow*

Figure 7.2: Automated dideoxy DNA sequencing with fluorescent ddNTPs.

7.2.2 A scan of one gel lane:

We don't even have to read the sequence from the gel the computer does that for us. Below is an example of what the sequencer's computer shows us for one sample (figure 7.3). This is a plot of the colors detected in one lane of a gel (one sample), scanned from smallest fragments to largest. The computer even interprets the colors by printing the nucleotide sequence across the top of the plot. This is just a fragment of the entire file, which would span around 900 or so nucleotides of accurate sequence.

The sequencer also gives the operator a text file containing just the nucleotide sequence, without the color traces.
Figure 7.3: A Electropherogram printout from automated sequencer for determining part of a DNA sequence.
DNA Sequencing

7.3 Practical work

1. Purification of PCR product
2. DNA sequencing reaction
3. Clean-up of Sequencing Reactions
I. Purification of PCR product

A. Materials & equipments:

1. QIAquick PCR purification Kit
2. 96-100% ethanol
3. Racks (1.5 ml)
4. Gloves
5. Centrifuge (1.5 ml rotor)
6. Micropipettes (100 – 1000 µl)
7. Micropipettes (10 – 100 µl)
8. Sterile blue tips (100 – 1000 µl)
9. Sterile yellow tips (10 – 100 µl)
10. 1.5 ml tubes

B. Procedure:

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene. For example, add 500 µl of Buffer PB to 100 µl PCR samples (not including oil).
2. Place a QIAquick spin column in a provided 2 ml collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
4. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
5. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed. IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
7. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for
increased DNA concentration, add 30 μl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

**IMPORTANT:** Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 μl from 50 μl elution buffer volumes, and 28 μl from 30 μl elution buffer.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at −20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
II. DNA sequencing

A. Materials & equipments:

1. Purified DNA samples
2. Nuclease-free H2O
3. RR buffer
4. Sequencing buffer
5. Forward primer
6. Sterile white tips (1–10 µl)
7. Micropipettes (1–10 µl)
8. 0.2 ml PCR tubes
9. 1.5 ml PCR tubes
10. Gloves
11. Ice

B. Procedure:

1. Melt all the RT-PCR reagents on ice.
2. Prepare a master mixs (MM) as follows.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in the reaction</th>
<th>Volume (µL) per Sample</th>
<th>No. of samples</th>
<th>Volume (µL) to added to MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing buffer</td>
<td></td>
<td>4</td>
<td>X 2</td>
<td>8</td>
</tr>
<tr>
<td>RR buffer</td>
<td></td>
<td>2</td>
<td>X 2</td>
<td>4</td>
</tr>
<tr>
<td>Forward primer (10 pmol)</td>
<td></td>
<td>1</td>
<td>X 2</td>
<td>2</td>
</tr>
<tr>
<td>Nuclease-free H2O</td>
<td></td>
<td>4</td>
<td>X 2</td>
<td>8</td>
</tr>
<tr>
<td>Purified DNA</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. Distribute the mix into two 0.2 ml tubes (16 µl/tube). Don’t forget to label the tubes appropriately.

4. Add 4 µL of purified DNA from the sample one tube.

5. Put the tubes in the thermal cycler. Program the thermal cycler as follows:
   25 cycles compromising: 96°C for 10s, 50°C for 5 s and 60°C for 4 min.
III. Clean-up of Sequencing Reactions

A. Materials & equipments:

1. NucleoSEQ kit.
2. Micropipettes (100 – 1000 µl).
3. DNA sequencing reaction.
5. Nuclease-free H2O.
6. Sterile blue tips (100 – 1000 µl).

B. Procedure:

1. Spin down dried gel resin at 750 x g for 30 s
2. Hydrate gel resin with 600 µl water, vortex, and incubate at least 30 min for complete hydration
3. Remove bottom plug and spin down hydrated gel resin at 750 x g for 2 min
4. Load sample to the center of the column
5. Spin for 4 - 6 min at 750 x g to recover the purified sample
7.4 References

Laboratory Eight: Reverse Transcription Polymerase Chain Reaction (RT-PCR)

8.1 Introduction
RT-PCR is a variant of polymerase chain reaction (PCR), a laboratory technique commonly used in molecular biology to generate many copies of a DNA sequence, a process termed "amplification". In RT-PCR, however, an RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR. Reverse transcription PCR is not to be confused with real-time polymerase chain reaction which is also sometimes (incorrectly) abbreviated as RT-PCR.

8.2 RT-PCR Principles and Procedure
RT-PCR utilizes a pair of primers, which are complementary to a defined sequence on each of the two strands of the cDNA. These primers are then extended by a DNA polymerase and a copy of the strand is made after each cycle, leading to logarithmic amplification.

8.2.1 RT-PCR includes three major steps:
1. The first step is reverse transcription (RT).
   - In which RNA is reverse transcribed to cDNA using reverse transcriptase and primers.
   - This step is very important in order to perform PCR since DNA polymerase can act only on DNA templates.
   - The RT step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR).
The reaction temperature is between 40°C and 50°C, depending on the properties of the reverse transcriptase used.

2. The second step involves the denaturation and annealing.
   - Denaturation of the dsDNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction.
   - Then, the temperature is decreased until it reaches the annealing temperature which can vary depending on:
     i. The set of primers used.
     ii. Melting temperature (Tm) of the primers and probes (if used).
     iii. Length and composition of the primers. This is the result of the difference of hydrogen bonds between A-T (2 bonds) and G-C (3 bonds).
     iv. An annealing temperature about 5 degrees below the lowest Tm of the pair of primers is usually used.

3. The final step of PCR amplification is DNA extension from the primers.
   - This is done with thermostable Taq DNA polymerase.
   - Usually at 72°C, the temperature at which the enzyme works optimally.
   - The length of the incubation at each temperature, the temperature alterations, and the number of cycles are controlled by a programmable thermal cycler.

Note: The analysis of the PCR products depends on the type of PCR applied. If a conventional PCR is used, the PCR product is detected using agarose gel electrophoresis and ethidium bromide (or other nucleic acid staining).

8.2.2 Disadvantage of RT-PCR:

1. Yields results are not always reliable, due to ethidium bromide which has a low sensitivity.

2. The specificity of the assay is mainly determined by the primers, which can give false-positive results.
3. There is an increased cross-contamination risk of the samples since detection of the PCR product requires the post-amplification processing of the samples.

4. RT-PCR is a semi- or even a low-quantitative technique, whereas the amplicon can be visualized only after the amplification ends.

Real-time RT-PCR provides a method in which the amplicons can be visualized as the amplification progresses using a fluorescent reporter molecule. There are three major kinds of fluorescent reporters used in real time RT-PCR, which are:

   a. Non-specific DNA Binding Dyes such as SYBR Green I.
   b. TaqMan Probes.
   c. Molecular Beacons (including Scorpions).

8.2.3 Use of reverse transcription polymerase reaction:

The exponential amplification via reverse transcription polymerase chain reaction provides for a highly sensitive technique in which a very low copy number of RNA molecules can be detected.

1. RT-PCR is widely used in the diagnosis of genetic diseases.

2. RT-PCR is used to determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression, semiquantitatively.

3. RT-PCR can also be very useful in the insertion of eukaryotic genes into prokaryotes.

4. RT-PCR is commonly used in studying the genomes of viruses whose genomes are composed of RNA, such as Influenzavirus A and retroviruses like HIV.
RT-PCR

8.3 Practical work

A. Materials & equipments:

1. Human RNA samples
2. Nuclease-free H2O
3. Forward primer F
4. Reverse primer R
5. RT-PCR Buffer (2X)
6. RT-PCR Enzyme Mix (25X)
7. RNAse inhibitor (40,000 U/ml)
8. Micropipettes (10 – 100 µl)
9. Micropipettes (1 – 10 µl)
10. Sterile yellow tips (10 – 100 µl)
11. Sterile white tips (1 – 10 µl)
12. 1.5 ml tubes
13. 0.2 ml PCR tubes
14. Ice
15. Gloves

B. Procedure:

1. Melt all the RT-PCR reagents on ice.
2. Prepare a master mix (MM) as below.
3. Distribute the mix into two 0.2 ml tubes (20 µl/tube). Don’t forget to label the tubes appropriately.
4. Add 5 µL of RNA from the sample one tube. Add 5 µL of Nuclease-free H2O into the negative control tube.
5. Put the tubes in the thermal cycler. Program the thermal cycler as follows:
6. Reverse transcription at 50°C for 30 min followed by denaturation at 94°C for 10 min, then 45 cycles compromising: 94°C for 30s, 58°C for 1 min, 72°C for 30s. The final extension is done at 72°C for 10 min.
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in the reaction</th>
<th>Volume (µL) per Sample</th>
<th>No. of samples</th>
<th>Volume (µL) to added to MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (2X)</td>
<td>1X</td>
<td>12.5</td>
<td>X 2</td>
<td>25</td>
</tr>
<tr>
<td>Forward primer (10 pmol)</td>
<td>0.4 pmol</td>
<td>1</td>
<td>X 2</td>
<td>2</td>
</tr>
<tr>
<td>Reverse primer (10 pmol)</td>
<td>0.4 pmol</td>
<td>1</td>
<td>X 2</td>
<td>2</td>
</tr>
<tr>
<td>RT-PCR Enzyme Mix (25X)</td>
<td>1X</td>
<td>1</td>
<td>X 2</td>
<td>2</td>
</tr>
<tr>
<td>RNAse inhibitor (40 U / µl)</td>
<td>20 U</td>
<td>0.5</td>
<td>X 2</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease-free H2O</td>
<td>-</td>
<td>4</td>
<td>X 2</td>
<td>8</td>
</tr>
<tr>
<td>RNA</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>25</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
8.4 References

- Hunt M. (2006) Real time PCR tutorial - Copyright 2006, The Board of Trustees of the University of South Carolina
Laboratory Nine: Bioinformatics

9.1 Introduction

Bioinformatics is the application of computer science to the field of molecular biology. The term bioinformatics was coined by Paulien Hogeweg in 1979 for the study of informatic processes in biotic systems. Its primary use since at least the late 1980s has been in genomics and genetics, particularly in those areas of genomics involving large-scale DNA sequencing. Bioinformatics now entails the creation and advancement of databases, algorithms, computational and statistical techniques, and theory to solve formal and practical problems arising from the management and analysis of biological data. Over the past few decades rapid developments in genomic and other molecular research technologies and developments in information technologies have combined to produce a tremendous amount of information related to molecular biology. It is the name given to these mathematical and computing approaches used to glean understanding of biological processes.

9.2 Common Activities in Bioinformatics Include

1. Mapping and analyzing DNA and protein sequences.
2. Aligning and compare different DNA and protein sequences.
3. Creating and viewing 3-D models of protein structures.

9.3 Aims of Bioinformatics

- Increase our understanding of biological processes.
- Developing and applying computationally intensive techniques (e.g., pattern recognition, data mining, machine learning algorithms, and visualization).
Increase research efforts in the field include sequence alignment, gene finding, genome assembly, drug design, drug discovery, protein structure alignment, protein structure prediction, prediction of gene expression and protein-protein interactions, genome-wide association studies and the modeling of evolution.

9.4 Software and Tools

Software tools for bioinformatics range from simple command-line tools, to more complex graphical programs and standalone web-services available from various bioinformatics companies or public institutions.

9.4.1 Web services in bioinformatics:

Simple Object Access Protocol (SOAP) has been developed for a wide variety of bioinformatics applications allowing an application running on one computer in one part of the world to use algorithms, data and computing resources on servers in other parts of the world. The main advantages derive from the fact that end users do not have to deal with software and database maintenance overheads.

Basic bioinformatics services are classified by the European Bioinformatics Institute (EBI) into three categories:

1. SSS (Sequence Search Services).
2. MSA (Multiple Sequence Alignment).
3. BSA (Biological Sequence Analysis).

The availability of these service-oriented bioinformatics resources demonstrate the applicability of web based bioinformatics solutions, and range from a collection of standalone tools with a common data format under a single, standalone or web-based interface, to integrative, distributed and extensible bioinformatics workflow management systems.

9.5 Primer

Primer is a strand of nucleic acid that serves as a starting point for DNA or RNA synthesis. They are required because the enzymes that catalyze replication, DNA
polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3’-end of the primer, and copies the opposite strand.

In most cases of natural DNA replication, the primer for DNA synthesis and replication is a short strand of RNA (which can be made de novo).

Many of the laboratory techniques of biochemistry and molecular biology that involve DNA polymerase, such as DNA sequencing and the polymerase chain reaction, require DNA primers.

9.5.1 Properties of synthesis primer:

1. Primers are usually short, with a length of about twenty bases.
2. Chemically synthesized oligonucleotides.
3. They are hybridized to a target DNA, which is then copied by the polymerase.

9.5.2 PCR primer design:

Pairs of primers should have similar melting temperatures since annealing in a PCR occurs for both simultaneously. A primer with a Tm significantly higher than the reaction's annealing temperature may mis-hybridize and extend at an incorrect location along the DNA sequence, while Tm significantly lower than the annealing temperature may fail to anneal and extend at all. Here are some helpful websites for primer design and SNP genotype:

1. **NCBI (National Center for Biotechnology Information)**


2. **Primer3**

   [http://frodo.wi.mit.edu/primer3](http://frodo.wi.mit.edu/primer3)

3. **Database of single nucleotide polymorphism (dbSNP)**


4. **UCSC Genome Brower**
http://genome.ucsc.edu/cgi-bin/hgPcr?command=start

6. **Ensemble Genome Browser**

http://www.ensembl.org

Primer sequences need to be chosen to uniquely select for a region of DNA, avoiding the possibility of mis-hybridization to a similar sequence nearby. A commonly used method is **BLAST** search whereby all the possible regions to which a primer may bind can be seen. Both the nucleotide sequence as well as the primer itself can be BLAST searched. The free NCBI tool Primer-BLAST integrates primer design tool and BLAST search into one application. Alternatively use of software such as **Beacon designer**, may yield to specific primers.

9.5.3 **Points should be taken in consideration:**

- Mononucleotide repeats should be avoided, as loop formation can occur and contribute to mishybridization.

- Primers should not easily anneal with other primers in the mixture (either other copies of same or the reverse direction primer).

  *This phenomenon can lead to the production of 'primer dimer' products contaminating the mixture.*

- Primers should also not anneal strongly to themselves, as internal hairpins and loops could hinder the annealing with the template DNA.

- When designing a primer for use in TA cloning, efficiency can be increased by adding AG tails to the 5' and the 3' end.

- Remember that the reverse primer has to be the reverse complement of the given cDNA sequence.
9.6 References

- Hunt M. (2006) Real time PCR tutorial - Copyright 2006, The Board of Trustees of the University of South Carolina
Laboratory Ten: Cytogenetics

10.1 Introduction

Cytogenetics determines the chromosomal make up of an individual by using Karyotype (Figure 10.1) from different samples such:

1. Postnatal including blood samples.
2. Prenatal including amniotic fluid, Chorionic Villu Tissues (CVS) and bone marrow biopsy.
3. Cancer including solid tumours.

10.2 Referral Reasons

- Dysmorphic features.
- Developmental delay.
- Short stature.
- Failure to develop secondary sex characteristics.
- Infertility.
- Recurrent miscarriages.
- Family history of Down syndrome.
- Indeterminate gender at birth.
- New born babies with suspected chromosome abnormality.
- Parents of abnormality found in PND.
10.3 Cell culture Techniques

- Living cells that are maintained in vitro in artificial media of serum and nutrients for the study and growth of certain strains, experiments in controlling diseases, or study of the reaction to certain drugs.
- Many laboratories refer to tissue culture as “black art” due to the numerous variables that make it difficult to determine.

![Figure 10.1: Peripheral blood Karyotyping steps.](image)

- Almost every problem encountered in cell culture can be identified as one of the following:
  1. The cells are growing poorly or not at all.
  2. The cells have an abnormal morphology.
- Each of these problems can usually be traced to one of the four following items:
  1. Materials: They were poor quality, inappropriate, or contaminated.
  2. Equipment: It may need to be calibrated.
3. Environment: The cultures were exposed to the wrong type of environment.

4. Cells: They were exposed to toxicity, contamination, or nutritional deficiency.

5. Technique: The procedures or protocols were not correct for the cell type.

10.4 Lymphocytes Culture

- Chromosomal analysis can be done on any type of cells but as a clinical laboratory it is important to choose the type of cells according to certain criteria:
  1. Sample tissue should be representative to all cell lines. This is especially important when studying cases in which mosaicism could be present.
  2. Easy to obtain such that the sampling is done in a noninvasive manner.
  3. Inexpensive to culture.
  4. Require short culture duration.
  5. To yield high-quality metaphase chromosomes in abundance.

- Lymphocytes accommodate the five criteria listed above.

- Lymphocytes are the cells of choice for chromosomal analysis because they are nucleated, unlike the RBC's, which lose their nucleus, as they mature.

- T cells making up about 70% of all lymphocytes in healthy persons.

- Invitro mitogens mimic the effect of foreign Antigen that gives mitotically active cells.

- Phytohemagglutinin (PHA) is principally a T lymphocyte mitogen.

- They can be obtained from peripheral blood or cord blood in case of new born babies.

10.5 Cell Culture Conditions

- Lymphocytes remain suspended in the culture media, so they don't need culture flasks they are cultured in plastic screw capped centrifugation tubes.
• Major Media component:
  ✓ 15% serum (bovin, human, horse), amino acids (L-glutamine), Vitamins, Salts, Glucose, growth factors, antibiotics (penicillin, streptomycin), Buffers, PHA.

• Media can be in several forms, powdered or liquid.

• Temperature, PH, and Humidity control:
  ✓ Temperature always between 37-37.5 C,
  ✓ PH of 7.2-7.4; controlled via
    o Bicarbonate buffer in the media.
    o Providing the incubator with a constant, controlled flow, 5% CO2.

• Humidified, gas flow incubator is recommended (Figure 10.2 A).

• The following culture techniques are done in strictly aseptic sterile conditions under vertical laminar flow hood Figure (Figure 10.2 B).

![Figure 10.2: A: Gas flow incubator. B: Vertical laminar flow hood.](image)

• Collect samples aseptically in green-top lithium or sodium tubes. The sample must be accompanied with a proper request form indicating the name of the patient written clearly, the status of the patient, history, the date and the signature of the referring doctor.
- The inoculum of blood to be added to a culture is varying depending on age and patient status.
- 0.8 ml of PB in case of normal adult, and 0.5ml PB in case of newborns.
- The specimen must never be frozen, because live cells required for culture.
- Peak of mitotic activity at approximately 72 hrs.

10.5.1 Procedure:

1. Mix the sample well and transfer 0.6 ml to 10 ml screw-capped plastic centrifugation tube containing PB-Max® culture media.

2. Close the tube, invert it gently and incubated at 37 °C for 72 hours in slant position.
10.6 References

Laboratory Eleven: Harvest and Staining Protocols

11.1 Harvest Protocols

- The criteria for harvesting are determined by the method of culture.
- The harvesting protocols for any type of cells starts by adding mitotic arrestant such as Colcemid.
- Colcemid is a synthetic analog of Colchicine, a derivative of autumn crocus (Cholchicum autumnal).
- Colcemid arrests cells at metaphase stage in which chromosomes are lined at the equatorial plate before sister chromatides are pulled to opposite poles by spindle fibers for incorporation into the two daughter cells.
- The mechanism by which the Colcemid arrests cells at metaphase stage is by preventing spindle fibers formation and thereby preventing pulling the two sister chromatides to the daughter cells.
- After Colcemid treatment the hypotonic treatment comes next. Treating cells with the hypotonic solution resulting osmotic potential induce inward movement of water across cell membrane causing cell to swell. It is also important for the lysis of RBCs.
- The next step in harvesting is cells fixation, which is initiated by adding a few drops of the fixative to the hypotonic, treated cells. This will stop the activity of the hypotonic solution and prepare cells to receive higher concentrations of the fixative. The brown supernatant is the color of the methemoglobin, which is formed after the addition of fixative to the culture.
- Multiple fixative steps should be carried out (until a clear supernatant is obtained) as this is responsible for getting rid of red cell ghosts and methemoglobin,
removing water from the cells, killing them and finally preserving them, it also hardens membranes and chromatin.

11.1.1 Procedure:

1. Add 100 µl Colcemid to the culture for 20 minutes at 37°C.
2. Spin 8 minutes at 1000 RPM.
3. Discard the supernatant, add 10 ml of pre-warmed hypotonic solution (0.075 M KCl) dropwise and incubate 20 minutes at 37°C.
4. Add 5 drops conditioning fixative 3:1 methanol: acetic acid and spin 8 minutes at 1000 RPM.
5. Discard the supernatant and resuspend pellets.
6. Add 10 ml fixative to pellets and leave for 30 minutes at room temperature.
7. Repeat step 6, 2 times (7ml for 20 minutes and 5ml for 15 minutes).

Preserve at -4°C. pellets can be preserved at -4°C for a long period of time.

11.2 Dropping Protocol

The theory of air drying of cells fixed in methanol: acetic acid and supported by the layer of fixative on the slide in the first few seconds of application to the slide, is that as the fixative evaporates the layer of the fixative on the slide becomes thinner and the meniscus pushes down on the top of the cell, enlarging the area of the cell and pressing the metaphase chromosomes between the upper and lower membranes, and thereby spreading them out (Figure 11.1).

![Figure 11.1: Dropping technique.](image-url)
11.2.1 Procedure:

1. Replace the fixative with a new one. Using a fine glass pasture pipette make a gentle mix without making air bubbles, as this will cause cells to clump.
2. Clean the slides with methanol, that will help to et a uniformity in spreading.
3. Carry the slide in 45 degrees and drop the sample from about one meter apart.
4. As a test slide drop about 8 drops on the slide, leave to air dry completely and observe the mitotic index and metaphases compactness.

Aging: Put the slides in the oven at 60°C over night or at 90°C for 90 minutes, that will driving off water and get better banding pattern.

11.3 Slide Staining

11.3.1 Giemsa-trypsin (GTG banding):

1. Giemsa stain is a complex mixture of dyes. The main components are the basic aminophenothiazin dyes azure A, azure B, azure C, thionin, and mythelen blue, and the acidic dye eosin.

2. Wang and Federoff suggested that typsin hydrolyses the protein component of the chromatin, thereby allowing the Giemsa dye to react with the exposed DNA, other reports suggested that trypsin and other agents might act by chelation rather than by protein digestion.

3. Appropriately stained chromosomes are neither too dark nor too pale to analyze at the microscope.

4. Under-trypsinized chromosomes have indistinct bands and little contrast. They are usually fuzzy in appearance.

5. Over-trypsinized chromosomes have sharp bands and often appear frazzled at the ends. Eventually overtrypsinized chromosomes are very pale after staining and may appear ghost-like and very swollen.

6. Immersing slides in fetal calf serum is recommended to stop the activity of the trypsin since the serum contains α1-antitrypsin which inhibits the trypsin from further digestion action.
11.3.2 **GTG staining procedure:**

1. Let the dropped slides to age by drying completely at room temperature and then incubating them at 60°C over night or alternatively at 90°C for one hour.

2. Prepare the following jars:
   
   i. A jar containing 75ml Phosphate buffer (pH 6.8). Put the jar in 37°C water bath and wait until the Phosphate buffer reaches 37°C. Add 1.0ml of 0.025% trypsin solution to the pre-warmed Phosphate buffer and stir it thoroughly.
   ii. A jar containing Phosphate buffer at room temperature.
   iii. A jar containing Phosphate buffered saline (PBS) at room temperature. Add 3.5ml Giemsa for a final concentration of 4%.
   iv. A jar containing distilled water.

3. Make sure that the slides are cooled down to room temperature.

4. Immerse the slide in the trypsin solution for 15-20 seconds.

5. Directly wash the slide in the Phosphate buffer jar at room temperature.

6. Transfer the slide to Giemsa stain jar for 90-120 seconds.

7. Rinse the slide in the distilled water jar and leave to dry completely.

8. Examine the slide and determine the proper times for the trypsin and the stain (Figure 11.2).

![A metaphase spread from blood sample followed by trypsin banding using Giemsa staining procedure.](image)

**Figure 11.2:** A metaphase spread from blood sample followed by trypsin banding using Giemsa staining procedure.
11.4 Karyotype and Ideogram

Chromosome Structure: Various stages in condensation of DNA and Chromatin to form a metaphase chromosome (Figure 11.3).

![Diagram showing DNA helix, nucleosomes, chromatin fibre, loop, and chromosome](image)

Figure 11.3: Chromosome condensation to form metaphase chromosome.

Karyotyping: Organization of the chromosomes of an individual, lined up using computer image processing according to:

1. **Size**, the largest to smallest (Figure 11.4).
2. **Location of centromere** (Figure 11.5).
3. **Banding pattern**: A ‘band’ is defined as that part of a chromosome which is clearly distinguishable from its adjacent segments by appearing darker or lighter with one or more banding techniques.
4. Bands stains darkly with Giemsa have DNA rich in AT pairs, these bands also seems to be less active portions of the chromosomes in terms of genetic and metabolic impact. The sections that do not take up these dyes but remain pale appear to be the major areas for active genes.
5. **Schematic diagram of banding patterns** (Figure 11.6) is from ISCN to identify the whole chromosomes banded sections numerically.
6. **High resolution banding** (prometaphase and prophase chromosomes) (Figure 11.7) can be obtained by treatment of the cultures with chemical agent to produce cell synchrony such as (Methotrexate) then adding a release agent to prevent contraction such as (Thymidine).
Figure 11.4: Chromosomes description.

Figure 11.5: Chromosomes structure.

Figure 11.6: Chromosomes ideogram.
Figure 11.6: Resolution G-banding: G-banded chromosome 22 at various levels from 400-850 band level. Illustrate how a prophase and prometaphase bands coalesce to form metaphase bands.
11.5 References

Laboratory Twelve: Chromosomal Abnormalities

A chromosome anomaly, abnormality or aberration reflects an atypical number of chromosomes or a structural abnormality in one or more chromosomes. A Karyotype refers to a full set of chromosomes from an individual which can be compared to a "normal" Karyotype for the species via genetic testing. A chromosome anomaly may be detected or confirmed in this manner. Chromosome anomalies usually occur when there is an error in cell division following meiosis or mitosis. There are many types of chromosome anomalies. They can be organized into two basic groups, numerical and structural anomalies.

12.1 Numerical Abnormalities

This is called Aneuploidy (an abnormal number of chromosomes), and occurs when an individual is missing either a chromosome from a pair (monosomy) or has more than two chromosomes of a pair (Trisomy, Tetrasomy, etc).

In humans, an example of a condition caused by a numerical anomaly is Down Syndrome (Figure 12.2), also known as Trisomy 21 (an individual with Down Syndrome has three copies of chromosome 21, rather than two copy in case of normal Karyotype (Figure 12.1)). Turner Syndrome is an example of a monosomy where the individual is born with only one sex chromosome, an X (Figure 12.3). More over Klinefelter's syndrome, 47, XXY, or XXY syndrome is a condition in which males have an extra (an aneuploidy) X sex chromosome (Figure 12.4).

Euploidy it is of three types: monoploidy (1n), diploide (2n), polyploid. and this polyploidy is further divided into auto, endo and allopolyploid.

12.2 Structural Abnormalities

When the chromosome's structure is altered. This can take several forms:
- **Deletions**: A portion of the chromosome is missing or deleted. Known disorders in humans include Wolf-Hirschhorn syndrome, which is caused by partial deletion of the short arm of chromosome 4; and Jacobsen syndrome, also called the terminal 11q deletion disorder.

- **Duplications**: A portion of the chromosome is duplicated, resulting in extra genetic material. Known human disorders include Charcot-Marie-Tooth disease type 1A which may be caused by duplication of the gene encoding peripheral myelin protein 22 (PMP22) on chromosome 17.

- **Translocations**: When a portion of one chromosome is transferred to another chromosome. There are two main types of translocations. In a reciprocal translocation, segments from two different chromosomes have been exchanged. In a Robertsonian translocation (Figure 12.5), an entire chromosome has attached to another at the Centromere in humans these only occur with chromosomes 13, 14, 15, 21 and 22.

- **Inversions**: A portion of the chromosome has broken off, turned upside down and reattached, therefore the genetic material is inverted (Figure 12.6).

- **Rings**: A portion of a chromosome has broken off and formed a circle or ring (Figure 12.7). This can happen with or without loss of genetic material.

- **Isochromosome**: Formed by the mirror image copy of a chromosome segment including the centromere.

*Chromosome instability syndromes* are a group of disorders characterized by chromosomal instability and breakage. They often lead to an increased tendency to develop certain types of malignancies.

### 12.3 Chromosome Nomenclature

International System for Human Cytogenetic Nomenclature (ISCN), since 1995 they start a nomenclature system for the chromosomes and chromosomal abnormality, which include:

A. Number of chromosomes.

B. Structural abnormality of chromosomes.

C. Numerical abnormality of chromosomes. See the examples below.
Figure 12.1: Normal Male 46,XY karyotype.

Figure 12.2: 47,XY,+21 Down syndrome karyotype.
Figure 12.3: 45,X Turner syndrome karyotype. Note unpaired X at lower right.

Figure 12.4: 47,XXY Klinefelter syndrome karyotype.
Figure 12.5: Robertsonian translocation 45,XX,der(13;14)(q10;q10).

Figure 12.6: An inversion loop in the A arm of a chromosome from an Axarus species midge.
Figure 12.7: Formation of a ring chromosome.
12.4 References