3.1 Introduction

All methodological aspects of the laboratory study are discussed in detail in this chapter. Section 3.2 focuses on the heavy metal cadmium, used as exposing substance. Its natural occurrence as well as the test concentrations used will be included in this discussion. Section 3.3 focuses on the test organism with the following section explaining the experimental design, in terms of the number of test organisms, the exposure period, and finally the exposure setup. Section 3.5 will explain aspects of the experiment setup system, as choice of exposure system in more detail. Following section 3.6 discuss water quality monitoring. While methods used for determining acute toxicity results and haematological and biochemical estimations are discussed in subsequent sections 3.8 and 3.9 respectively.

3.2 Cadmium as exposure substance

Heavy metals are found individually in a natural aquatic system very rarely. The multiple types of contaminations present, add to the difficulty of identifying the most active agents of toxicity. Unanimity does not exist about how multiple contaminant exposures differ from single contaminant exposures (Newman et al., 1991). Cadmium is a comparatively rare element that is usually closely associated with zinc ores. Geo-morphologically cadmium and
zinc occurs together and when processed, cadmium is yield as a byproduct of zinc with a ratio of 3 kg cadmium per 1 ton zinc (Mance. 1987). 10% of total Cd in the environment is derived from natural sources, whereas remaining 90% is derived from anthropogenic activity (Okada et al., 1997). The main sources for emissions to air, water and land are steel production, metal production; refining, cement manufacturing, pigment manufacturing, cadmium plating and battery manufacturing (Thornton, 1992).

Its large scale use dates back to the 1940’s, but it is only during the last few decades that serious consideration has been given to cadmium as an environment contaminant (Aylett, 1979). Therefore, it is possible that aquatic organisms will accumulate cadmium in an aquatic environment. Although small amounts of these metals are released by leaching of rocks and other natural processes, the levels of these metals in inland waters are often greatly increased by anthropogenic activities (Birch et al., 1996; Sanders el al., 1997).

The Cadmium Sulphate and Cadmium Chloride have an additive toxic effect on fish. The standards for assessing the effects of cadmium have on aquatic organisms and aquatic ecosystems are chronic and acute toxic effects. Cadmium was therefore, chosen as the exposure substance in this study. Two sets of experimental exposures were conducted. One set for acute toxicity estimation and another for chronic toxicity estimation for various haematological and biochemical parameters; and in each exposure, fish were exposed to cadmium (in form of cadmium chloride).

First we executed a limit test for estimating an approximate value of LC\textsubscript{50} concentration for 96 Hour acute toxicity test. With help of the derived
information we repeated the experiment twice for acute toxicity for precise result. The metal concentrations for chronic exposure were 0-10% concentrations of known LC$_{50}$ value for cadmium.

Cadmium was directly added to the water in the form of Cadmium Chloride (CdCl$_2$·2H$_2$O). In the Chloride form, Cadmium is less soluble in hard water or water with little amount of salts present. Therefore, we selected the water to be free from any hardness (reverse osmosis system treated). Although the exposure concentration of metal seems relatively small, the metal concentration was specifically chosen for the purpose of this study, as it may indeed prove to be harmful to an organism.

3.3 *Oreochromis niloticus* as test organism

In order to conduct a proper toxicity test, a test organism must be selected that is relatively sensitive to environmental pollution and other toxicants. All test organisms should preferably be of similar age (usually equated with similar size), free from disease and derived from the same source (EIFAC, 1976). Fishes are relatively sensitive to changes in their environment and have a relatively long life span. These animals can therefore, give an indication of the effect of both short and long-term exposure periods to environmental pollution and toxicants.

*Oreochromis niloticus* (Nile Tilapia) (Figure 3.1) was selected as the test organism for this study. Nile tilapia is the generic name for one group of fish belongs to the class of Actinopterygii under the order Perciformes of Cichlidae family. These fish are native to Africa but have become one of the most widely
farmed fish (Popma and Masser, 1999) with an increasing production output worldwide (Lem and Shehadeh, 1997). Nile tilapia inhabits fresh and brackish water of Africa, Middle East, Coastal India, Central and South America. It has become one of the most important food fish with an estimated 2 millions metric tons production worldwide in 2002 (FAO, 1999).

![Figure 3.1 Oreochromis niloticus (Nile Tilapia)](image)

This species have a relatively high tolerance for temperature and pH variance and is able to easily adapt to laboratory conditions. The fish were obtained from a local breeder in a village Napad, Dist. Anand, approximately four weeks prior to each experiment.

### 3.4 Experimental Design

#### 3.4.1 Acute Toxicity Tests

Before we can execute the acute toxicity test, it was necessary to execute limit test, in order to determine the concentrations to be used for acute tests. We exposed ten acclimated specimens to concentration 1 mg/L, 5 mg/L, 10
mg/L and 20 mg/L cadmium for 96 hours and observed the response. We found no morality in 1 mg/L cadmium exposure. This gave 100% confidence limit. On other hand, we found that no fish could survive for 96 hours in concentrations 10 mg/L and 20 mg/L. With help of these extreme data we decided to execute acute toxicity tests at concentrations from 1 mg/L to 5 mg/L. i.e. from 100% confidence level to half of 100% mortality level.

Seventy healthy, adult *Oreochromis niloticus* specimens were selected as test organisms. After an acclimatisation period of two weeks in a set of aquariums, the fish were transferred from the initial acclimation tank to six acute toxicity testing tanks (ten in each one), and allowed to further acclimatise for one week before the exposures commenced. The fish were not fed in the 24 hour period prior to the exposure. This minimizes the production of wastes in the exposure tanks which may also complex the test substance and affect its toxicity. It also avoids the unforeseen addition of possible chemical interference by components in the food (Mance. 1987). Then the experimental exposures were executed. The toxicant was added by means of a prepared stock solution. The concentration of exposure to each group is as per table 3.1.

Out of the six groups one group was exposed to no cadmium. i.e. was kept as control with reference to other groups. While rest exposure groups each of ten specimens, were exposed to the respective cadmium concentration of 1mg/L, 2mg/L, 3mg/L, 4mg/L and 5mg/L respectively. Being healthy specimens no death was noted in control group. Lethality was recorded for each group at an interval of 1, 2, 4, 6, 12, 24, 36, 48, 72 and 96 hours. From the collected data **LC$_{50}$** value was calculated.
### Materials and Methods

<table>
<thead>
<tr>
<th>[Cd]per liter</th>
<th>[Cd] for 50L tank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 mg/L)</td>
<td>0 mg</td>
</tr>
<tr>
<td>1 mg/L</td>
<td>50 mg</td>
</tr>
<tr>
<td>2 mg/L</td>
<td>100 mg</td>
</tr>
<tr>
<td>3 mg/L</td>
<td>150 mg</td>
</tr>
<tr>
<td>4 mg/L</td>
<td>200 mg</td>
</tr>
<tr>
<td>5 mg/L</td>
<td>250 mg</td>
</tr>
</tbody>
</table>

Table 3.1: Metal concentrations used for acute toxicity testing

Same experiment was repeated again to confirm the result achieved from first experiment.

#### 3.4.2 Chronic Toxicity Tests

One hundred fifty healthy, adult *Oreochromis niloticus* specimens were selected as test organisms. After an acclimatisation period of two weeks in a set of aquariums the fish were transferred from the initial acclimation tank to four chronic toxicity testing tanks (thirty in each), and allowed to acclimatise for one week before the exposures commenced. The fish were fed on a daily basis for the entire period of exposure. Then the experimental exposures were executed. The concentration of exposure to each group is as per table 3.2.

<table>
<thead>
<tr>
<th>[Cd]per liter</th>
<th>[Cd] for 50L tank</th>
<th>[Cd] for 300L tank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 mg/L)</td>
<td>0 mg</td>
<td>0 mg</td>
</tr>
<tr>
<td>0.1 mg/L</td>
<td>5 mg</td>
<td>30 mg</td>
</tr>
<tr>
<td>0.2 mg/L</td>
<td>10 mg</td>
<td>60 mg</td>
</tr>
<tr>
<td>0.3 mg/L</td>
<td>15 mg</td>
<td>90 mg</td>
</tr>
</tbody>
</table>

Table 3.2: Metal concentrations used for chronic toxicity testing
Out of the four groups one group was exposed to no cadmium. i.e. was kept as control with reference to other groups. While rest exposure groups each of thirty specimens, were exposed to the respective cadmium concentration of 0.1mg/L, 0.2mg/L and 0.3mg/L respectively. Being lower concentration of cadmium than required to cause lethal effects no death was observed in any of the groups. After exposure of 24 hours, 7 days and 15 days fish were sacrificed to collect blood samples from them. The collected blood samples were analyzed for measurement of different haematological parameters like Haemoglobin (Hb), Red Blood Cells (RBCs), Haematocrit (Hct), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) and biochemical parameters like Creatinine (CRT), Serum Glutamate Pyruvate Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT), Alkaline Phosphatase (ALP), Triglyceride, Glucose, Cholesterol and Total Protein.

3.5 Acute and Chronic toxicity exposure system

A flow-through system provides an ideal environment when exposing fish under controlled condition to a specific substance, because the desired test concentration can be sustained throughout the exposing period, while water is constantly flowing through the system.

For acute toxicity we have selected static type bio assay method. Therefore, there was no need to implement the flow-through system. The tank system used in this study (Figure 3.2), consisted of a reservoir tank of 500L which was used to hold reverse osmosis treated water required for the experiment where the specimens were acclimated.
For the exposure purpose we used six tanks each with capacity of 80L and used them to the level of 50L. Continuous aeration was provided throughout the exposure period. Biological filters were provided in each tank, in order to maintain the test vessel clean and test solution transparent.

For chronic toxicity estimation a longer period of exposure is required. This made it mandatory to implement the flow-through system. The flow-through system used in this study, consisted of a reservoir tank of 300L which was used to renew the test solution in the smaller exposure or holding tanks of 50L.
respectively, through water supply pipes (Figure 3.3). Each exposure tank accommodated 10 specimens. This number of specimens per tank was specifically chosen to avoid oxygen depletion, high levels of waste production per tank and stress induced by crowding. Four flow-through systems were prepared. As previously mentioned, all four systems were situated in the same environment. The systems were thoroughly cleaned and aged in an acid solution prior to the exposure period, to prevent any possible contamination from previous studies. Each individual system was fitted with a biological filter. The water in each tank was sufficiently aerated. The flow-tempo of the water for each system was maintained at approximately 16ml/min as the water moved through the system via outlet pipes. Test concentrations for the specific metals were determined taking the volume of the specific tanks into account. Detailed schematic diagram representing flow-through system is shown in Figure 3.3.

**3.6 Water Quality Monitoring**

Water quality is a term used to describe the chemical, physical and biological characteristics of water. When exposing fish under controlled conditions, as explained in the previous section, the condition and quality of the water must be maintained and monitored in such a way that the exposing substance will ultimately be the only variable in the system that will exert a possible stressful effect on the test organisms. Before the addition of the test substance, it is therefore, important for the prevailing conditions in the exposure system to be recorded.
Figure 3.3: The tank system for chronic toxicity
This record of water quality should demonstrate that conditions in the test system are stable and unlikely to stress the test organisms during the exposure (Mance, 1987). All other variables regarding water quality, for example temperature and pH, must be maintained at a constant but optimal level.

Water quality for each flow-through system in this study was monitored on a daily basis at random time intervals before and during the exposure period. Readings were recorded for five physical parameters: temperature, conductivity, Total Dissolved Solids (TDS), Dissolved Oxygen (DO) and pH. These observations were recorded for each individual holding tank as well as for each reservoir tank. The observations were taken using a glass thermometer, TDS meter, a Scientech conductivity meter, Scientech pH meter and DO meter.

3.6.1 Temperature

Temperature expressed in terms of degrees Celsius (°C) is an important parameter regarding its effect on the solubility of oxygen in water, the rate of photosynthesis by algae and higher plants, the metabolic rates of aquatic organisms, and the sensitivity of organisms to toxic wastes, parasites and diseases. For the majority of heavy metals, it is not possible to infer any relationship between toxicity and water temperature (Mance, 1987). Optimal water temperature for *Oreochromis niloticus* is estimated between 31°C and 36°C. But this species can withstand temperatures ranging from 11°C to 42°C. For the purpose of this study, water temperature was kept constant at 30°C ± 2°C during both the short and long-term exposure periods.
3.6.2 Conductivity

Conductivity is a numerical expression of the ability of water to conduct an electrical current, resulting from the presence of charged species in solution (DWAF, 1993). Conductivity is influenced by an array of factors, for example the concentration and nature of the solutes, their degree of dissociation into ions, their electric charge, the mobility of the ions, and the temperature of the solution (DWAF, 1993). Conductivity was measured in micro Siemens per centimeter (μS/cm).

3.6.3 Total dissolved Salts

Total Dissolved Salts (TDS), is a measure of the amount of soluble material in a sample of water, which represents the total quantity of dissolved salts ionized in the solution. This parameter is usually expressed as mg/L.

3.6.4 Dissolved Oxygen (DO)

Analysis of dissolved oxygen measures the amount of gaseous oxygen (O₂) dissolved in an aqueous solution. The maintenance of adequate Dissolved Oxygen (DO) concentrations is critical for the survival and functioning of the aquatic biota. This parameter is commonly expressed as a concentration in terms of milligrams per liters (mg/L) or as a percentage (%). Dissolved oxygen concentrations for surface water ranges from 0 mg/L in extremely poor water conditions, to as high as 15 mg/L. Levels below 5 mg/L are, however stressful to most aquatic organisms.
3.6.5 pH

pH is a general measure of the acidity or alkalinity of a water sample and is indicated on a scale of 0 to 14 (Acidic < Neutral (7) <Alkaline). The pH of natural water is determined largely by geological and atmospheric influences. Human induced acidification from industrial effluents, mine drainage and acid precipitation can cause a lowering of the pH, leading to increasing concentration of several trace elements in rivers and lakes which may cause fish death (Reitz et al., 1996; Alloway et al., 1997).

3.7 External Investigation

After each specific exposure period (as mentioned in section 3.4.2), the nine specimens of each group were removed from the flow-through system and sacrificed for haematological and biochemical analysis. The body weight was recorded for each specimen. The condition of external features must also be recorded as it plays an important part in evaluating the general health of the specimen and also indicating whether the metal exposure may have caused any deviations in the external appearance of the exposed specimens.

3.8 Acute Toxicity estimation

The information derived from acute animal toxicology studies is essential for determining the potential toxicity of a chemical to humans and other life forms. The objective of acute studies is to identify the potential toxicology of chemicals, whether commercially available or in development. The underlying promise of using animals in acute testing is that the effects of administration of a synthetic agent on an animal mimic the possible outcomes that the agent
may produce in humans or other mammals. The system also implies that short term tests in animals are comparable to acute exposure in the human population.

Acute toxicology studies are designed to express the potency of a toxicant as a correlative dose–response relationship. The path by which a dose elicits a particular response is known as the dose–response (or concentration–effect) relationship. The observed response is a calculated observation, assuming that the response is a result of exposure to a chemical and is measured and quantified. The response also depends on the quantity of chemical exposure and administration within a given period. Two types of dose–response relationships exist, depending on number of subjects and doses tested. The graded dose–response (Figure 3.6), the relationship of test subjects to logarithmic increases in the dose or concentration of a chemical or quantal
dose–response (Figure 3.7), the relationship of test subjects to increases in the dose.

Because the LC$_{50}$ is a statistically calculated dose of a chemical that causes death in 50% of the animals tested. It is an example of a typical quantal dose–response curve. The doses administered are continuous or at different levels and the response is generally mortality (although gross injury, tumor formation, or other measurable criterion is used to determine a standard deviation or cut-off value). The time at which the response is measured is chosen empirically; in this case it is selected as 96 hours. The frequency of administration is assumed to be a single dose administered (static type bio assay) at the start of the period when the test subjects are acclimated to the environment.
For the derived quantal dose-response curves 96 Hour LC50 values for the toxicant were calculated using standard statistical methods. Here we have used Probit Analysis - Finney Method [Lognormal Distribution] and Least squares [Normal Distribution].

3.9 Chronic Toxicity estimation for haematological and biochemical parameters

The shorter duration of exposure in acute studies allows monitoring of the effects of a chemical at high doses in an abbreviated time period. Such acute exposures occur in a variety of circumstances, particularly in emergency, occupational, environmental and domestic settings. More frequently, clinical toxicology has shown that acute exposures to drugs of abuse and therapeutic drugs are of major international concern and treatment of accidental or intentional overdoses poses a significant challenge. For purposes of comparison, however, as much toxicity occurs with extended interactions with lower non-fatal concentrations of chemicals as occurs with short intense exposures.

In addition, much regulatory emphasis is placed on long-term human and animal exposures to environmental toxins, occupational exposure to hazardous substances and chronic effects of food and dietary supplements. Consequently, there exists an important incentive for the scientific toxicology sector to develop test methods for predicting relative chronic hazards and risks of exposure to chemicals in daily life. As a result, simulation of these interactions requires the development of several different types of studies in order to mimic the human situation.
Chronic exposure is any relative time period for which continuous or repeated exposure beyond the acute phase is required for the same chemical to induce a toxic response. In contrast to acute studies, the objectives of chronic studies are to:

1. Determine toxicological effects of repeated administration of test chemicals on potential target organs at chronic dosages.
2. Establish dose–response (or concentration effect) relationships using various indicators over the selected dosage ranges and durations of exposure.
3. Experimentally verify a maximum dosage level that does not promote ostensible toxicity with repeated exposure/s.
4. Propose a mechanism of toxicity that complements or contrasts with acute studies of the chemical or class of agents.
5. Measure or assess the toxic effects of lower, more frequently administered doses of a chemical, thus analyzing for repeated cumulative exposure.
6. Determine cumulative effects of repeated exposure.
7. Examine the toxicological effects of increasing doses over extended periods.
8. Identify recovery of subjects after removal of the source of exposure.
9. Predict long-term adverse health effects in the species arising from intermittent, repeated or continuous exposure.

Finally, chronic studies generally complement acute studies, assuming that the conditions are structured to enhance the results obtained in acute experiments.
We exposed the specimens in group of 30 (10 X 3 to avoid oxygen depletion, high levels of waste production per tank and stress induced by crowding) to the decided concentration of Cadmium after primary and secondary acclimation. After exposure of 24 hours, 7 days and 15 days nine fishes were sacrificed to collect blood samples from them. The collected blood samples were analyzed for measurement of different haematological parameters like Hb, RBCs, Hct, MCV, MCH, MCHC and biochemical parameters like Creatinine, SGPT, Aspartate amino transferase, Alkaline Phosphatase, triglyceride, Glucose, Cholesterol and Total Protein.

Red blood cells count was done by using haemocytometry. PCV (Haematocrit) was determined by using microhaematocrit method. Haemoglobin was determined by using Sahli’s method (Acid haematin method)

The Mean Corpuscular Volume (MCV) estimation is the volume of the average red cell calculated from the number of red blood cells and haematocrit as described below:

$$ MCV = \frac{\text{Haematocrit (\%) } \times 10}{\text{RBC count } (10^6/\mu l)} $$

The Mean Corpuscular Haemoglobin (MCH) measurement is the concentration of haemoglobin by weight in the average red blood cell (expressed in units of picograms or micro-micrograms) and is calculated as described here:

$$ MCH = \frac{\text{Haemoglobin concentration (g/dl) } \times 10}{\text{RBC count } (10^6/\mu l)} $$
The Mean Corpuscular Haemoglobin Concentration (MCHC) is the ratio of the haemoglobin concentration to haematocrit (expressed as a percentage) and is calculated as described below:

\[
MCHC = \frac{\text{Haemoglobin concentration (g/dl) X 10}}{\text{Haematocrit (%)}}
\]

Methods used for estimation of these parameters are listed in Table 3.4.

<table>
<thead>
<tr>
<th>Parameter to be estimated</th>
<th>Method used for estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Trinders method</td>
</tr>
<tr>
<td>Total protein</td>
<td>Modified Biuret-End point assay</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>GPO–POD Enzymetic colorimetry</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Jaffe colorimetric-kinetic</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>CHOD–PAP End point</td>
</tr>
<tr>
<td>S.G.O.T. (AST)</td>
<td>IFCC method, NADH. Kinetic UV.</td>
</tr>
<tr>
<td>S.G.P.T. (ALT)</td>
<td>IFCC method, NADH. Kinetic UV.</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>p-NPP Kinetic</td>
</tr>
</tbody>
</table>

Table 3.3: Methods used for Biochemical estimation of different parameters

3.9.1 Glucose (Trinder) assay

The measurement of glucose concentrations in biological fluids has been well documented. Glucose testing can be diagnostically significant in diabetes and hypoglycemia. This procedure for the measurement of glucose employs a modification of the glucose oxidase / peroxidase (GOD-POD) method for
glucose described by Trinder (1969). Lott et al., (1975) in an evaluation of the Trinder procedure described it as highly specific, largely free of interferences, and that good agreement was obtained between the glucose oxidase and the hexokinase procedure. Methods for glucose including the glucose oxidase method have been extensively reviewed by Cooper (1973).

**Test principle**

\[
\beta - D - glucose + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} D - gluconic Acid + H_2O_2 \\
H_2O_2 + hydroxybenzoate + 4 - aminoantipyrine \xrightarrow{\text{Peroxidase}} Quinoneimine Dye + H_2O
\]

**Reagents**

Glucose Color Reagent: A solution containing (after reconstitution) a buffer (pH 7.25 at 25°C), 0.25 mmol/L 4-aminoantipyrine, 20 mmol/L p-hydroxybenzoate, > 40,000 U/L glucose oxidase (microbial), > 2000 U/L peroxidase (botanical), and preservatives.

Glucose Calibrator: Solution containing 90mg/dL (5mmol/L) glucose and preservatives.

**Reagent preparation, storage, and stability**

Add the required volume of deionized water. See vial label. Mix gently; allow 5 minutes for reconstitution, then re-mix gently.

**Specimen**

Plasma prepared from blood collected with an anticoagulant containing fluoride is the specimen of choice. Other plasmas and serum may be used if they are separated from the cells and assayed promptly.
Test condition

For the data presented in this insert, studies using this reagent were performed on an automated analyzer using an endpoint test mode, with a sample to reagent ratio of 1:100, and a wavelength reading of 505 nm.

Calibration

Calibration material should be used to calibrate the procedure. The frequency of calibration, if necessary, using an automated system is dependent on the system and the parameters used.

3.9.2 Quantitative determination of Total Protein

Assay principle

In the presence of alkaline Cupric Sulphate, the proteins produce a VIOLET colour. Intensity of the colour is proportional to protein concentration.

Clinical significance

The proteins are macromolecular organic compounds, widely distributed in the organism. They act like structural and transport elements. The determination of total proteins is useful in the detection of:

- High protein levels caused by hemoconcentration like in the dehydrations, hyper-globulinaemia or increase in the concentration of specific proteins.
- Low protein level caused by hemodilution, by hemorrhage, malnutrition and hypoalbuminaemia. or excessive protein catabolism (Burtis A et al., 1999, Tietz N W et al., 1995).
Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biuret Reagent</td>
<td>100mL</td>
</tr>
<tr>
<td>Cupric Sulphate</td>
<td>6 mmol/L</td>
</tr>
<tr>
<td>Sodium and Potassium Tartrate</td>
<td>21mMols/L</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>750mMols/L</td>
</tr>
<tr>
<td>Potassium Iodide</td>
<td>6 mMols/L</td>
</tr>
<tr>
<td>Standard (Albumin)</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Value of Albumin Standard</td>
<td>8.0Gms/dL</td>
</tr>
</tbody>
</table>

Preparations

Reagents are ready to use no preparation is required.

Procedure

1. Assay Conditions:
   
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction:</td>
<td>End-Point</td>
</tr>
<tr>
<td>Temperature:</td>
<td>30° + 5°C</td>
</tr>
<tr>
<td>Wavelength:</td>
<td>550 + 20nM</td>
</tr>
<tr>
<td>Standard Concentration:</td>
<td>4.0 Gms/dL</td>
</tr>
<tr>
<td>Absorbance Range:</td>
<td>0-2°A</td>
</tr>
<tr>
<td>Cuvette Path Length:</td>
<td>1 cM</td>
</tr>
<tr>
<td>Reaction Time:</td>
<td>10 Mins at RT/5 Mins at 37 C</td>
</tr>
<tr>
<td>Protein Standard Value:</td>
<td>8.0Gms/dL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>---</td>
<td>100 µl</td>
<td>---</td>
</tr>
<tr>
<td>Specimen</td>
<td>---</td>
<td>---</td>
<td>100 µl</td>
</tr>
</tbody>
</table>
2. Mix well Incubate for 10 minutes at RT or 5 minutes at 37°C. Read at 550 nm (550+20 nm) or GREEN filter against Blank. The final colour is stable for approximately 30 minutes.

**Calculation**

Compute O.D. \( \text{TEST} \)

\[
\text{Total Proteins in Gms/dL} = \frac{O.D. \text{TEST}}{O.D. \text{STD}} \times C_{\text{STD}}
\]

### 3.9.3 Quantitative determination of Triglycerides IVD

**Principle of the method**

Sample triglycerides incubated with lipo-protein lipase (LPL), liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. Glycerol-3-phosphate (G3P) is then converted by glycerol phosphate dehydrogenase (GPO) to dihydroxy acetone phosphate (DAP) and hydrogen peroxide (H\(_2\)O\(_2\)). In the last reaction hydrogen peroxide (H\(_2\)O\(_2\)) reacts with 4-aminophenazone (4-AP) and p-chlorophenol in presence of peroxidase (POD) to give a red colored dye:

\[
\text{Triglycerides} + H_2O^{\text{LPL}} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol} + \text{free fatty acid} \\
\text{Glycerol} + ATP^{\text{kinase}} \xrightarrow{\text{Glycerol phosphate dehydrogenase}} \text{G3P} + ADP \\
\text{G3P} + O_2^{\text{POD}} \xrightarrow{\text{DAP} + H_2O_2} \text{H}_2\text{O}_2 + 4 - \text{AP} + p - \text{Chlorophenol} \xrightarrow{\text{POD}} \text{Quinone} + H
\]
The intensity of the color formed is proportional to the triglycerides concentration in the sample (Buccolo G et al., 1973, Kaplan A et al., 1984).

**Clinical significance**

Triglycerides are fats that provide energy for the cell. Like cholesterol, they are delivered to the body’s cells by lipo proteins in the blood. A diet with a lot of saturated fats or carbohydrates will raise the triglyceride levels. The increases in serum triglycerides are relatively nonspecific. For example liver dysfunction resulting from hepatitis, extra hepatic biliary obstruction or cirrhosis, diabetes mellitus is associated with the increase (Burtis A et al., 1999, Tietz N W et al., 1995.)

Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

**Reagents**

<table>
<thead>
<tr>
<th>R1 Buffer</th>
<th>GOOD pH7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-Chlorophenol</td>
</tr>
<tr>
<td>Triglycerides cal</td>
<td>Triglycerides aqueous primary standard</td>
</tr>
<tr>
<td>R2 Enzymes</td>
<td>Lipoproteinlipase (LPL)</td>
</tr>
<tr>
<td></td>
<td>Glycerolkinase(GK)</td>
</tr>
<tr>
<td></td>
<td>Glycerol-3-oxidase(GPO)</td>
</tr>
<tr>
<td></td>
<td>Peroxidase (POD)</td>
</tr>
<tr>
<td></td>
<td>4– Aminophenazone(4-AP)</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td></td>
<td>50 mmol/L</td>
</tr>
<tr>
<td></td>
<td>2 mmol/L</td>
</tr>
<tr>
<td></td>
<td>200mg/dL</td>
</tr>
<tr>
<td></td>
<td>150000 U/L</td>
</tr>
<tr>
<td></td>
<td>500U/L</td>
</tr>
<tr>
<td></td>
<td>2500 U/L</td>
</tr>
<tr>
<td></td>
<td>440U/L</td>
</tr>
<tr>
<td></td>
<td>0.1 mmol/L</td>
</tr>
<tr>
<td></td>
<td>0.1 mmol/L</td>
</tr>
</tbody>
</table>

**Preparation**

Working reagent (WR): Dissolve (→) the contents of one vial R2 Enzymes into one bottle of R 1 Buffer.
Ref: 1001310 Working reagent (WR): Dissolve (→) the contents of one vial R2 Enzymes in 10mL of R1 Buffer.

Cap and mix gently to dissolve contents.

WR stability: 6 weeks at 2-8°C or 1 week at room temperature (15-25°C).

**Triglycerides cal**

Once open is stable up to 1 month when stored tightly closed at 2-8°C, protected from light and contaminations prevented during their use.

Signs of reagent deterioration:

- Presence of particles and turbidity.
- Blank absorbance (A) at 505 nm >0.14.

**Procedure**

1. Assay conditions:
   - Wavelength: 505 nm (490-550)
   - Cuvette: 1 cm light path
   - Temperature: 37°C / 15-25°C

2. Adjust the instrument to zero with distilled water.

3. Pipette into a cuvette:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR(mL)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Standard(µL)</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Sample(µL)</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>
4. Mix and incubate for 5 min. at 37°C or 10 min. at room temperature.

5. Read the absorbance (A) of the samples and Standard, against the Blank.

   The colour is stable for at least 30 minutes.

**Calculations**

\[
\frac{(A)_{Standard}}{(A)_{Sample}} \times 200 \text{ (Standard conc.)} = \text{mg/dL triglycerides in the sample}
\]

Conversion factor: mg/dL x 0.0113 = mmol/L.

**3.9.4 Quantitative determination of creatinine IVD**

**Principle of the method**

The assay is based on the reaction of creatinine with sodium picrate as described by Jaffé. Creatinine reacts with alkaline picrate forming a red complex. The time interval chosen for measurements avoids interferences from other serum constituents. The intensity of the color formed is proportional to the creatinine concentration in the sample (Murray R.L. 1984).

**Clinical significance**

Creatinine is the result of the degradation of the creatine, component of muscles, it can be transformed into ATP, that a source of high energy for the cells. The creatinine production depends on the modification of the muscular mass, and it varies little and the levels usually are very stable. Creatinine is excreted by the kidneys. With progressive renal insufficiency there is retention in blood of urea, creatinine and uric acid. Elevated creatinine level may be indicative of renal insufficiency (Murray R.L. 1984, Burtis A et al., 1999, Tietz N W et al., 1995).
Reagents

<table>
<thead>
<tr>
<th></th>
<th>Picric reagent</th>
<th>Picric Acid</th>
<th>17.5 mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Picric reagent</td>
<td>Picric Acid</td>
<td>17.5 mmol/L</td>
</tr>
<tr>
<td>R2</td>
<td>Alkaline reagent</td>
<td>Sodium Hydroxide</td>
<td>0.29 mol/L</td>
</tr>
<tr>
<td>CREATININE CAL</td>
<td>Creatinine aqueous primary standard 2 mg/dL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Preparation

Working reagent (WR): Mix equal volumes of R1 Picric Reagent and R2 Alkaline reagent

Samples

Serum or heparinized plasma

Procedure

1. Assay conditions:
   - Wavelength: 492 nm
   - Cuvette: 1 cm. light path
   - Temperature 37/15-25ºC

2. Adjust the instrument to zero with distilled water.

3. Pipette into a cuvette:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 1 (mL)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Standard(mL)</td>
<td>--</td>
<td>100</td>
<td>--</td>
</tr>
<tr>
<td>Sample (mL)</td>
<td>--</td>
<td>--</td>
<td>100</td>
</tr>
</tbody>
</table>

4. Mix and start stopwatch.

5. Read the absorbance (A1) after 30 seconds and after 90 seconds (A2) of the sample addition.

6. Calculate: \[ \Delta A = A2 - A1 \]

Calculations
Materials and Methods

3.9.5 Quantitative determination of Cholesterol

Assay principle

The series of reactions involved in the assay system are as follows:

1. Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase (CE) to cholesterol and free fatty acids.

2. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase (CHOD) to cholest-4-en-3-one and H$_2$O$_2$.

3. In presence of peroxidase (POD), the formed hydrogen peroxide effects the oxidative coupling of phenol and 4-aminoantipyrine (4-AAP) to form a red-colored quinoneimine dye.

\[
\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol Estarase}} \text{Cholesterol} + \text{fatty acids}
\]

\[
\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Estarase}} 4 - \text{Cholestene} - 3 - \text{one} + H_2O_2
\]

\[
2H_2O_2 + 4 - AAP + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H2O
\]

The intensity of the color produced is directly proportional to cholesterol concentration. It is determined by measuring the increase in absorbance at 500 – 550 nm.

Clinical significance

Cholesterol is a steroid with a secondary hydroxyl group in the C3 position, and found in blood, bile, and brain tissue. It serves as a precursor to bile acids, steroids and vitamin D. It is synthesized in many types of tissue, but...
particularly in the liver and intestinal wall. Approximately, 75% of cholesterol is newly synthesized and a 25% originates from dietary intake.

Measurements of serum cholesterol levels are important in the diagnosis and classification of hyper-lipo-proteinemias. Elevated cholesterol levels may occur with hypothyroidism, nephrotic syndrome, diabetes, and various liver diseases. There is a correlation between elevated serum cholesterol levels and the incidence of coronary artery diseases. Normal cholesterol levels are affected by stress, diet, age, gender, hormonal balance, and pregnancy. Depressed levels are associated with hyperthyroidism and severe liver diseases (Searcy, RL 1969, Ellefson et al., 1976)

**Reagents**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Cholesterol Standard</td>
<td>200mg/dL</td>
</tr>
<tr>
<td>R2</td>
<td>Pipes buffer (pH 6.90)</td>
<td>50 mmol/L</td>
</tr>
<tr>
<td></td>
<td>Phenol</td>
<td>24mmol/L</td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride</td>
<td>0.5 mmol/L</td>
</tr>
<tr>
<td></td>
<td>Cholesterol Esterase</td>
<td>≥ 200 U/L</td>
</tr>
<tr>
<td></td>
<td>Cholesterol Oxidase</td>
<td>≥ 250 U/L</td>
</tr>
<tr>
<td></td>
<td>Peroxidase</td>
<td>≥ 1000 U/L</td>
</tr>
<tr>
<td></td>
<td>4-Aminoantipyrine</td>
<td>0.5 mmol/L</td>
</tr>
</tbody>
</table>

**Preparations**

Dissolve contents of reagent 2 (R2) with the amount of reagent 1 (R1) indicated on the vial label. The working reagent is stable for 90 days at 2-8°C.

**Procedure**
1. Wavelength: 500 - 550 nm
2. Cuvette: 1 cm light path
3. Temperature: 20-25 or 37°C
4. Zero adjustment: against reagent blank
5. Specimen: Serum or plasma

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>---</td>
<td>10 µl</td>
<td>---</td>
</tr>
<tr>
<td>Specimen</td>
<td>---</td>
<td>---</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Mix, incubate for 5 minutes at 37°C or 10 minutes at 20-25°C. Measure the absorbance of specimen and standard against reagent blank. The color is stable for 60 minutes.

**Calculation**

Calculate the cholesterol concentration by using the following formulae:

\[
\text{Cholesterol Concentration} = \frac{\text{Absorbance of Specimen}}{\text{Absorbance of Standard}} \times \text{Standard value}
\]

Unit conversion: mg/dl x 0.0259 = mmol/l

**3.9.6 Quantitative determination of Aspartate Amino-Transferase GOT (AST) IVD**

**Principle of the method**

Aspartate Amino-Transferase (AST) formerly called glutamate oxaloacetate (GOT) catalyses the reversible transfer of an amino group from aspartate to α-ketoglutarate forming glutamate and oxalacetate. The oxalacetate produced is reduced to malate by malate dehydrogenase (MDH) and NADH:
The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of AST present in the sample (Murray R. 1984).

**Clinical significance**

The AST is a cellular enzyme, is found in highest concentration in heart muscle, the cells of the liver, the cells of the skeletal muscle and in smaller amounts in other weaves. Although an elevated level of AST in the serum is not specific of the hepatic disease, is used mainly to diagnostic and to verify the course of this disease with other enzymes like ALT and ALP.

Also it is used to control the patients after myocardial infarction, in skeletal muscle disease and other (Murray R. 1984, Burtis A et al., 1999, Tietz N W et al., 1995). Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

**Reagents**

<table>
<thead>
<tr>
<th>R1 Buffer</th>
<th>TRIS pH 7.8</th>
<th>80 mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Aspartate</td>
<td>200 mmol/L</td>
</tr>
<tr>
<td>R 2 Substrate</td>
<td>NADH</td>
<td>0.18 mmol/L</td>
</tr>
<tr>
<td></td>
<td>Lactate dehydrogenase (LDH)</td>
<td>800 U/L</td>
</tr>
<tr>
<td></td>
<td>Malate dehydrogenase (MDH)</td>
<td>600 U/L</td>
</tr>
<tr>
<td></td>
<td>α-Ketoglutarate</td>
<td>12 mmol/L</td>
</tr>
</tbody>
</table>

**Preparation**
Working reagent (WR): Dissolve one tablet of R2 Substrate with one vial of R1 Buffer.

Ref: 1001160 Dissolve (→) one tablet of R2 Substrate in one vial of R1.
Ref: 1001161 Dissolve (→) one tablet of R2 Substrate in 15 mL of R1.
Ref.: 1001162 Dissolve one tablet of R2 Substrate in 50 mL of R1.

Procedure

1. Assay conditions:
   - Wavelength: 340 nm
   - Cuvette: 1 cm. light path
   - Constant temperature: 25°C /30°C / 37°C

2. Adjust the instrument to zero with distilled water or air.

3. Pipette into a cuvette:

<table>
<thead>
<tr>
<th>WR(mL)</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample(µL)</td>
<td>100</td>
</tr>
</tbody>
</table>

4. Mix, incubate for 1 minute.

5. Read initial absorbance (A) of the sample, start the stopwatch and read absorbances at 1 minute intervals thereafter for 3 minutes.

6. Calculate the difference between absorbances and the average absorbance differences per minute (A/min).

Calculations

\[
A/\text{min} \times 1750 = \text{U/L of AST}
\]
Units: One international unit (IU) is the amount of enzyme that transforms 1 mol of substrate per minute, in standard conditions. The concentration is expressed in units per litre of sample (U/L).

Temperature conversion factors

To correct results to other temperatures multiply by:

<table>
<thead>
<tr>
<th>Assay temperature</th>
<th>Conversion factor to 25°C</th>
<th>Conversion factor to 30°C</th>
<th>Conversion factor to 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>1.00</td>
<td>1.37</td>
<td>2.08</td>
</tr>
<tr>
<td>30°C</td>
<td>0.73</td>
<td>1.00</td>
<td>1.54</td>
</tr>
<tr>
<td>37°C</td>
<td>0.48</td>
<td>0.65</td>
<td>1.00</td>
</tr>
</tbody>
</table>

3.9.7 Quantitative determination of Alanine Amino-Transferase GPT (ALT) IVD

Principle of the method

Alanine Amino-Transferase (ALT) or Glutamate Pyruvate Transaminase (GPT) catalyses the reversible transfer of an amino group from alanine to \( \alpha \)-ketoglutarate forming glutamate and piruvate.

The piruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH:

\[
\text{Alanine} + \alpha - \text{Ketoglutarate}^{ALT} \overset{\text{ALT}}{\rightarrow} \text{Glutamate} + \text{Piruvate}
\]

\[
\text{Piruvate} + NADH + H^+^{LDH} \rightarrow \text{Lactate} + NAD^+
\]
The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ALT present in the sample.

**Clinical significance**

The ALT is a cellular enzyme, found in highest concentration in liver and kidney. High levels are observed in hepatic disease like hepatitis, diseases of muscles and traumatisms; its better application is in the diagnosis of the diseases of the liver.

When they are used in conjunction with AST aid in the diagnosis of infarcts in the myocardium, since the value of the ALT stays within the normal limits in the presence of elevated levels of AST (Murray R. 1984, Burtis A et al., 1999, Tietz N W et al., 1995).

Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

**Reagents**

<table>
<thead>
<tr>
<th>R1 Buffer</th>
<th>TRIS pH 7.8</th>
<th>100 mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Alanine</td>
<td>500 mmol/L</td>
</tr>
<tr>
<td>R2 Substrate</td>
<td>NADH</td>
<td>0.18 mmol/L</td>
</tr>
<tr>
<td></td>
<td>Lactate dehydrogenase (LDH)</td>
<td>1200 U/L</td>
</tr>
<tr>
<td></td>
<td>-Ketoglutarate</td>
<td>15 mmol/L</td>
</tr>
</tbody>
</table>

**Preparation**

Working reagent (WR):

Ref: 1001170 Dissolve (→) one tablet of R2 Substrate in one vial of R1.

Ref: 1001171 Dissolve (→) one tablet of R2 Substrate in 15 mL of R1.
Ref: 1001172 Dissolve \( \rightarrow \) one tablet of R2 Substrate in 50 mL of R1.

**Samples**

Serum or plasma

**Procedure**

1. Assay conditions:
   - Wavelength: 340 nm
   - Cuvette: 1 cm light path
   - Constant temperature: 25ºC / 30ºC / 37ºC

2. Adjust the instrument to zero with distilled water or air.

3. Pipette into a cuvette:

<table>
<thead>
<tr>
<th>WR(mL)</th>
<th>Sample(L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>100</td>
</tr>
</tbody>
</table>

4. Mix, incubate for 1 minute.

5. Read initial absorbance (A) of the sample, start the stopwatch and read absorbances at 1-minute intervals thereafter for 3 minutes.

6. Calculate the difference between absorbances and the average absorbance differences per minute (A/min).

**Calculations**

\[ A/\text{min} \times 1750 = \text{U/L of ALT} \]

Units: One international unit (IU) is the amount of enzyme that transforms 1 mol of substrate per minute, in standard conditions. The concentration is expressed in units per liter of sample (U/L).

**Temperature conversion factors**
To correct results to other temperatures multiply by:

<table>
<thead>
<tr>
<th>Assay temperature</th>
<th>Conversion factor to 25°C</th>
<th>Conversion factor to 30°C</th>
<th>Conversion factor to 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>1.00</td>
<td>1.32</td>
<td>1.82</td>
</tr>
<tr>
<td>30°C</td>
<td>0.76</td>
<td>1.00</td>
<td>1.39</td>
</tr>
<tr>
<td>37°C</td>
<td>0.55</td>
<td>0.72</td>
<td>1.00</td>
</tr>
</tbody>
</table>

3.9.8 Quantitative determination of Alkaline Phosphatase

Assay principle

\[ pNP + H_2O \xrightarrow{ALP} \text{Nitrophenol} + Pi \]

The rate of Nitrophenol produced by the catalytic action of ALP is measured at 405nm which is directly proportional to the quantity of Alkaline Phosphatase (Wenger C. et al., 1984, Rosalki S et al., 1993).

Clinical significance

Alkaline phosphatase is an enzyme present in almost all weaves of the organism, being particularly high in bone, liver, intestine and kidney. Both increases and decreases of plasma ALP are of importance clinically.
Causes of increased plasma ALP: Paget's disease of bone, obstructive liver disease, hepatitis, hepatotoxicity caused by drugs or osteomalacia.

Causes of decreased plasma ALP may be vitamin deficiency (Wenger C. et al., 1984, Burtis A et al., 1999, Tietz N W et al., 1995).

**Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNP</td>
<td>10 mMol/L</td>
</tr>
<tr>
<td>Diethanolamine</td>
<td>1 Mol/L</td>
</tr>
<tr>
<td>MgCl</td>
<td>0.5 mMol/L</td>
</tr>
</tbody>
</table>

**Preparations**

Dissolve the substrate (Reagent No.1) with buffer (Reagent No.2) 10 mL and a uniform solution may take place after 30 minutes which is ready to use.

**Procedure**

1. Assay conditions:
   - Temperature: 37°C
   - Wavelength: 405nM (400-415 nM)
   - Factor: 5454
   - Absorbance Range: 0-2°A
   - Cuvette Path Length: 1cm
   - Delay Time: 60 seconds
   - Interval: 20 seconds
   - Dynamic Range: 10-1150 IU/L
Max. limit of Blank Reagent: 0.700

<table>
<thead>
<tr>
<th>Reagent Volume</th>
<th>500ul</th>
<th>1000 ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Volume</td>
<td>5ul</td>
<td>10 ul</td>
</tr>
</tbody>
</table>

Pipette sample to working reagent at the same time mix and note the time. Feed the cuvette of the analyser thermostatically controlled to 30 or 37°C. Read exactly after 60 seconds and continue recording the optical density (OD) at an interval of 20 seconds for 3 times. Calculate the average absorbance rate per minute and multiply with the factor or if the machine is capable of receiving a programme run the test under a programme given in the system parameters.

A specific programme datasheet for each analyser may be provided upon request.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>37°C</th>
<th>37°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Volume</td>
<td>10µL</td>
<td>10µL</td>
<td>10µL</td>
</tr>
<tr>
<td>Reagent Volume</td>
<td>1000µL</td>
<td>700µL</td>
<td>500µL</td>
</tr>
<tr>
<td>FACTOR</td>
<td>5454</td>
<td>3834</td>
<td>2754</td>
</tr>
</tbody>
</table>

Rerun the test if the average rate of absorbance per minute is over 0.211 A at 405 nM using a dilution of sample with 0.9% sodium chloride solution. Multiply the result by dilution factor i.e. by 2 for 1:1 dilution.

**Calculation**

\[
\text{Alkaline Phosphatase} = \frac{\Delta A}{\text{Minute}} \times \text{Factor}
\]
3.10 References


