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-ene-3-one and H₂O₂.
3. In presence of peroxidase (POD), the formed hydrogen peroxide formed effects the oxidative coupling of phenol and 4-aminoantipyrine (4-AAP) to form a red-colored quinoneimine dye.

Cholesterol esters + H₂O → Cholesterol + fatty acids
Cholesterol + O₂ → Cholest-4-ene-3-one + H₂O₂
2 H₂O₂ + 4-AAP + Phenol → Quinoneimine dye + 4 H₂O

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.

**EXPECTED VALUES**

<table>
<thead>
<tr>
<th>Risk classification</th>
<th>Total Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desirable</td>
<td>&lt; 200 mg/dl (5.2 mmol/l)</td>
</tr>
<tr>
<td>Borderline high</td>
<td>200 – 239 mg/dl [5.2 – 6.2 mmol/l]</td>
</tr>
<tr>
<td>High</td>
<td>&gt; 240 mg/dl (6.2 mmol/l)</td>
</tr>
</tbody>
</table>

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference range. For diagnostic purposes, the cholesterol results should always be assessed in conjunction with the patient’s medical history, clinical examination, and other findings.

**CHESTEROL (CHOD-PAP)**

Enzymatic Colorimetric Determination of Serum Cholesterol

**REAGENTS**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁</td>
<td>Cholesterol standard</td>
</tr>
<tr>
<td>R₂</td>
<td>Pipes buffer, pH 6.9</td>
</tr>
<tr>
<td></td>
<td>Phenol</td>
</tr>
<tr>
<td></td>
<td>Cholesterol oxidase</td>
</tr>
<tr>
<td></td>
<td>Cholesterol esterase</td>
</tr>
<tr>
<td></td>
<td>Peroxidase</td>
</tr>
<tr>
<td></td>
<td>4-Aminoantipyrine</td>
</tr>
</tbody>
</table>

**Reagent Preparation & Stability**

All reagents are ready for use and stable up to the expiry date given on label when stored at 2–8°C.

**SPECIMEN**

- Serum, or plasma*.
- The only acceptable anticoagulants are heparin and EDTA.

**Specimen Preparation & Stability**

No special preparation of the patient is necessary; however, it is recommended that prior to collection, patients should be following their usual diet and be in their usual state of health. Patients who are acutely ill, losing weight, pregnant or have had a myocardial infarction in the previous 3 months should be rescheduled.

Blood should be collected by venipuncture, after the patient has been in a seated position for at least 5 minutes. Tourniquet usage should be kept to a minimum and the specimen should be allowed to clot for 30 minutes at room temperature.

The best specimen is unhemolysed serum, and should be analyzed on the day of collection. When stored at 4°C, specimens are stable for 3-4 days; specimens are stable at −20°C for several months.

**PROCEDURE**

- **Manual Procedure**
  - Wavelength 500 - 550 nm
  - Cuvette 1 cm light path
  - Temperature 20-25 or 37°C
  - Zero adjustment against reagent blank
  - 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>10 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>Standard</td>
<td>......</td>
<td>10 μl</td>
<td>......</td>
</tr>
<tr>
<td>Specimen</td>
<td>......</td>
<td>......</td>
<td>......</td>
</tr>
</tbody>
</table>

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

- **Automated Procedure**

User defined parameters for different auto analyzers are available upon request

**CALCULATION**

Calculate the cholesterol concentration by using the following formulae:

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

\[ \text{mg/dl} \times 0.0259 = \text{mmol/l} \]

**LINEARITY**

When run as recommended, the assay is linear up to 800 mg/dl (20.7 mmol/l).

If result exceeds 800 mg/dl (20.7 mmol/l), specimen should be diluted with 0.9% NaCl solution and reassayed. Multiply the result by the dilution factor.

**SENSIVITY**

The sensitivity is defined as the change of analytical response per unit change in analyte concentration at a pathlength of 1 cm.

When run as recommended the sensitivity of this assay is 3.0 mg/dl (0.08 mmol/l).

**QUALITY CONTROL**

It is recommended that controls (normal and abnormal) be included in:

- Each set of assays, or
- At least once a shift, or
- When a new bottle of reagent is used, or
- After preventive maintenance is performed or a clinical component is replaced.

Commercially available control material with established cholesterol values may be routinely used for quality control.

Failure to obtain the proper range of values in the assay of control material may indicate:

- Reagent deterioration,
- Instrument malfunction, or
- Procedure errors.

The following corrective actions are recommended in such situations:

- Repeat the same controls.
- If repeated control results are outside the limits, prepare fresh control serum and repeat the test.
- If results on fresh control material still remain outside the limits, then repeat the test with fresh reagent.
- If results are still out of control, contact NS Biotec Technical Services.

**INTERFERING SUBSTANCES**

- **Anticoagulants**

  The only acceptable anticoagulants are heparin and EDTA.

- **Bilirubin**

  Bilirubin levels higher than 7.5 mg/dl decrease the apparent total cholesterol concentration significantly.

- **Drugs**

  Methylxindopa causes artificially low total cholesterol values at the tested drug level. For a more comprehensive review of drugs affecting cholesterol assays refer to the publication by Young\(^\text{13}\).

- **Haemoglobin**

  No interference from haemoglobin up to a level of 500 mg/dl.

- **Lipemia**

  No significant interference.

- **Others**

  Ascorbic acid levels higher than 7.5 mg/dl decrease the apparent total cholesterol concentration significantly.

  Other 3-beta-hydroxysteroids cause positive interference but are not normally present in significant quantities in human serum\(^\text{14}\).

**WARNING & PRECAUTIONS**

- NS Biotec cholesterol reagent is for in vitro diagnostic use only. Normal precautions exercised in handling laboratory reagents should be followed.

- Warm up working solution to the corresponding temperature before use.

  The reagent and sample volumes may be altered proportionally to accommodate different spectrophotometer requirements.

- Valid results depend on an accurately calibrated instrument, timing, and temperature control.

- The reagent blank will not exceed an absorbance of 0.06 but don’t use the reagent if it is turbid or if the absorbance is greater than 0.2 at 500 nm.

**BIBLIOGRAPHY**