**PRINCIPLE**

Bilirubin is converted to coloured azobilirubin by diazotized sulfanilic acid and is measured photometrically. Of the two bilirubin fractions in serum – bilirubin-glucuronide and free bilirubin which is bound to albumin – only the former reacts directly, while free albumin reacts after being displaced from protein by an accelerator. The difference of two measurements total bilirubin (with accelerator) and direct bilirubin (without accelerator) enables the calculation of indirect bilirubin. The terms «direct» and «indirect» bilirubin refers exclusively to the reaction characteristics in the presence or absence of an accelerator or solubilizer and are only approximate equivalents of the two bilirubin fractions.1,2

**REAGENT COMPOSITION**

- **RT** Sulfanilic acid 29 mmol/L, hydrochloric acid 0.24 mol/L, Duposol® 3% (w/v).
- **RD** Sulfanilic acid 29 mmol/L, hydrochloric acid 0.24 mol/L.
- **RN** Sodium nitrite 11.6 mmol/L.

**Ancillary kit:**

- **CAL** Bilirubin Calibrator. REF. 1912005

  Bilirubin freeze-dried into a protein matrix. Concentration value is traceable to Standard Reference Material 916a. The concentration of total T and direct D Bilirubin is stated on the label and is lot-specific. The target values are derived using LINEAR reagents on the Cobas Mira.®

**STORAGE AND STABILITY**

- Store temperature stated on the label.
- All the kit compounds are stable until the expiry date stated on the label. Do not use reagents over the expiration date.
- Store the vials tightly closed, protected from light and prevented from contamination during use.
- **Discard If appear signs of deterioration:**
  - Presence of particles and turbidity.
  - Blank absorbance (A) at 540 nm > 0.050 in 1cm cuvette.
  - Reagent RN if it develops a yellow coloration.

**REAGENT PREPARATION**

- **Working reagents.** Mix 1 mL RN + 4 mL RT (Total) or 1 mL RN + 4 mL RD (Direct). Stable for 8 days at 2-8°C.

- **Calibrator.** Reconstitute the vial by adding exactly 1.0 mL of distilled water. Mix carefully and let stand for 5-10 minutes before use. The stability of bilirubin in the dark is: 8 hours 16-25°C, 2 days 2-8°C and 28 days –20°C frozen once.

**SAMPLES**

- Fresh hemolysis-free serum. Store in the dark until use.
- Samples can be frozen at –15°C or below in which case bilirubin is stable for 2 months.

**INTERFERENCES**

- Lipemia (intralipid < 5 g/L) does not interfere.
- Direct Bilirubin. (Hemoglobin 2 g/L) may affect the results.
- Total Bilirubin. (Hemoglobin 16 g/L) does not interfere.
- Other drugs and substances may interfere.3
- Lipemic samples interfere with the assay. The interference can be corrected by preparing a sample blank before applying the general formula of calculation.

**MATERIALS REQUIRED**

- Photometer or colorimeter capable of measuring absorbance at 540 ± 20 nm.
- Constant temperature incubator set at 37°C. Use the same temperature for assay of calibrator, controls and samples.
- Pipettes to measure reagent and samples.

**PROCEDURE**

1. Pipette into labelled tubes:

<table>
<thead>
<tr>
<th>TUBES</th>
<th>Reagent Blank</th>
<th>Sample Blank</th>
<th>Sample</th>
<th>CAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>100 µL</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sample</td>
<td>–</td>
<td>100 µL</td>
<td>100 µL</td>
<td>–</td>
</tr>
<tr>
<td><strong>CAL</strong></td>
<td>–</td>
<td>–</td>
<td>100 µL</td>
<td>–</td>
</tr>
<tr>
<td><strong>RT</strong></td>
<td>–</td>
<td>1.0 mL</td>
<td>–</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1.0 mL</td>
<td>–</td>
<td>1.0 mL</td>
<td>1.0 mL</td>
</tr>
</tbody>
</table>

2. Mix thoroughly and let the tubes stand for 2 minutes at room temperature.
3. Read the absorbance (A) of the sample blanks at 540 nm against distilled water.
4. Read the absorbance (A) of the samples at 540 nm against the reagent blank.

The color is stable for at least 60 minutes at room temperature.

**DIRECT BILIRUBIN**

1. Pipette into labelled tubes:

<table>
<thead>
<tr>
<th>TUBES</th>
<th>Reagent Blank</th>
<th>Sample Blank</th>
<th>Sample</th>
<th>CAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>100 µL</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sample</td>
<td>–</td>
<td>100 µL</td>
<td>100 µL</td>
<td>–</td>
</tr>
<tr>
<td><strong>CAL</strong></td>
<td>–</td>
<td>–</td>
<td>100 µL</td>
<td>–</td>
</tr>
<tr>
<td><strong>RD</strong></td>
<td>–</td>
<td>1.0 mL</td>
<td>–</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1.0 mL</td>
<td>–</td>
<td>1.0 mL</td>
<td>1.0 mL</td>
</tr>
</tbody>
</table>
2. Mix thoroughly and let the tubes stand for exactly 5 minutes at 37°C.
3. Read the absorbance (A) of the sample blanks at 540 nm against distilled water.
4. Read the absorbance (A) of the samples at 540 nm against the reagent blank.

**CALCULATIONS**

\[
A_{\text{Sample}} - A_{\text{Sample blank}} \times \frac{C_{\text{Cal}}}{A_{\text{Cal}}} = \text{mg/dL total or direct bilirubin}
\]

Samples with concentrations higher than 20 mg/dL should be diluted 1:2 with saline and assayed again. Multiply results by 2.

If results are to be expressed as SI units apply:

\[
\text{mg/dL} \times 17.1 = \mu\text{mol/L}
\]

**REFERENCE VALUES**

**ADULTS**

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Direct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 1.0 mg/dL</td>
<td>Up to 0.2 mg/dL</td>
<td></td>
</tr>
</tbody>
</table>

**NEWBORNS (TOTAL BILIRUBIN)**

<table>
<thead>
<tr>
<th>Age</th>
<th>Premature</th>
<th>Full-term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 24 h</td>
<td>1.0 - 6.0 mg/dL</td>
<td>2.0 - 6.0 mg/dL</td>
</tr>
<tr>
<td>Up to 48 h</td>
<td>6.0 - 8.0 mg/dL</td>
<td>6.0 - 7.0 mg/dL</td>
</tr>
<tr>
<td>3-5 days</td>
<td>10.0 - 15.0 mg/dL</td>
<td>4.0 - 12.0 mg/dL</td>
</tr>
</tbody>
</table>

It is recommended that each laboratory establishes its own reference range.

**QUALITY CONTROL**

The use of a standard to calculate results allows to obtain an accuracy independent of the system or instrument used. To ensure adequate quality control (QC) each run should include a set of controls (normal and abnormal) with assayed values handled as unknowns.

**NOTES**

- For bilirubin determination in newborns pipette 50 µL of sample or standard, and follow the exposed procedure.
- This method may be used with different instruments. Any application to an instrument should be validated to demonstrate that results meet the performance characteristics of the method. It is recommended to validate periodically the instrument. Contact to the distributor for any question on the application method.
- Clinical diagnosis should not be made on findings of a single test result, but should integrate both clinical and laboratory data.

**ANALYTICAL PERFORMANCE**

- **Detection Limit (Direct Bilirubin)**: 0.09 mg/dL
- **Detection Limit (Total Bilirubin)**: 0.03 mg/dL
- **Linearity**: Up to 20 mg/dL
- **Precision**:

<table>
<thead>
<tr>
<th>Direct Bilirubin</th>
<th>Total Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td>Between-run</td>
</tr>
<tr>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>0.79</td>
<td>0.03</td>
</tr>
<tr>
<td>1.82</td>
<td>0.06</td>
</tr>
<tr>
<td>0.79</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Sensitivity**

- **Sensitivity (Direct Bilirubin)**: 0.171 A / mg/dL
- **Sensitivity (Total Bilirubin)**: 0.073 A / mg/dL

**Correlation (Total Bilirubin)**: This assay (y) was compared with a similar commercial method (x). The results were:

\[
N = 52, \quad r = 0.96, \quad y = 0.99x + 0.113
\]

The analytical performance has been generated using on automatic instrument. Results may vary depending on the instrument.

**REFERENCES**