Reagent kit for the quantitative determination of total and direct bilirubin in serum. Diazosulfanilic acid method.

**Principle**

Sulfanilic acid reacts with sodium nitrite to form diazotized sulfanilic acid. In the presence of dimethylsulfoxide, total bilirubin reacts with diazotized sulfanilic acid to form azobilirubin. In the absence of dimethylsulfoxide, only the direct bilirubin reagents react to give azobilirubin.

**Reference value**

Serum:
- direct bilirubin <5.1 µmol/l (<0.3 mg/dl)
- total bilirubin <17.0 µmol/l (<1.0 mg/dl)

It is recommended that each laboratory should assign its own normal range.

**Reagents**

**A) Total Bilirubin**

1. Reagent (RT1)
   - Sulfanilic acid
   - Sodium nitrite
   - Dimethylsulfoxide

2. Reagent (R2)
   - Hydrochloric acid
   - Sulfanilic acid

**B) Direct Bilirubin**

1. Reagent (RD1)
   - Sulfanilic acid
   - Sodium nitrite

2. Reagent (R2)
   - Hydrochloric acid

**Sample**

Serum free of haemolysis.

Bilirubin is light sensitive and it is recommended that serum be stored in the dark. The reagent is not suitable for bilirubin determination of infants.

**PROCEDURE**

**Preparation of working reagent**

**A) Total bilirubin**

Mixing ratio of RT1 and R2: 125 ml/25 ml

**B) Direct bilirubin**

Mixing ratio of RD1 and R2: 125 ml/25 ml

Reagents can only be applied with previous mixing!

**Stability of the working reagent**

- 20-25°C: 1 day
- 2-8°C: 5 days

If the absorbance of working reagent is higher than 0.02 at 546 nm the reagent cannot be used.

**Assay Conditions**

- Wavelength: 555 nm (540-560 nm)
- Secondary wavelength: 600 nm
- Temperature: 37°C
- Cuvette: 1 cm light path
- Method: end point
- Read against: reagent blank

**Pipette into cuvette**

A) **Total bilirubin**:

<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>Calibrator</th>
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</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td>75 µl</td>
</tr>
<tr>
<td>Calibrator</td>
<td></td>
<td>75 µl</td>
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</tbody>
</table>

A) **Direct bilirubin**:

<table>
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</tr>
</thead>
<tbody>
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</tbody>
</table>

Mix and read the optical density (A) after exactly 3 minutes incubation.

**Calculation using calibration**

\[
\frac{A_{\text{Sample}}}{A_{\text{Calibrator}}} \times C_{\text{Calibrator}} = C_{\text{Sample}}
\]

A = optical density;  
C = concentration

**Quality control**

A quality control program is recommended for all clinical laboratories. The analysis of control material in both the normal and abnormal ranges with each assay is recommended for monitoring the performance of the procedure. Each laboratory should establish corrective measures to be taken if values fall outside the limits.

**PERFORMANCES DATA**

The following data were obtained using the Olympus 600 analyzer (37°C).

**Linearity**

The test is linear up to 340 µmol/l (20.0 mg/dl) bilirubin concentration.

**Sensitivity**

It is recommended that each laboratory establishes its own range of sensitivity as this is limited by the sensitivity of the spectrophotometer used. Under manual conditions however, a change of 0.001 Abs is equivalent to 0.575 µmol/l (0.034 mg/dl) direct Bilirubin and 1.42 µmol/l (0.08 mg/dl) total Bilirubin concentration at 540 nm.

**Correlation**

Comparative studies were done to compare our reagent with another commercial direct Bilirubin assay.

The results from these studies are detailed below.

Correlation coefficient: r=0.9957

Linear regression: \( y \) (µmol/l)= 1.05x-0.52

(x= other commercial reagent, y= own reagent)

**Bibliography**

Hijmans Van den Bergh A. A., Muller P.: Biochem, 77 ; 90, (1916).