



## TECO DIAGNOSTICS

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## DIRECT AND TOTAL BILIRUBIN

### INTENDED USE

For the quantitative determination of direct and total bilirubin in serum.

### INTRODUCTION

Bilirubin is a metabolite of the heme portion of heme proteins, mainly hemoglobin. Normally it is excreted into the intestine and bile from the liver. The site of the catabolism of hemoglobin is the reticuloendothelial system (RES). Bilirubin is then released into the bloodstream where it binds tightly to albumin and is transported to the liver. Upon uptake by the liver, bilirubin is conjugated with glucuronic acid to form bilirubin mono and diglucuronide which are water-soluble metabolites. The metabolites will react with aqueous diazo reagent and are commonly referred to as "direct bilirubin".<sup>1</sup>

Elevation of total serum bilirubin may occur due to (1) excessive hemolysis or destruction of the red blood cells e.g. hemolytic disease of the newborn, (2) liver diseases e.g. hepatitis and cirrhosis (3) obstruction of the biliary tract e.g., gallstones.<sup>1</sup> There is information in the literature indicating elevated levels of direct bilirubin in patients with liver or biliary tract disease, even though, total bilirubin levels are normal.<sup>2</sup> Therefore, the greatest diagnostic value of direct bilirubin assays stem from their ability to indicate occult liver disease.

Most chemical methods for the determination of total bilirubin are based on the reaction between diazotized sulfanilic acid and bilirubin to produce azobilirubin, which absorbs maximally at 560 nm. Such tests are often run in the presence and absence of an organic solvent e.g., methanol to distinguish free bilirubin from conjugated bilirubin on a differential solubility basis.<sup>3</sup>

### PRINCIPLE

Bilirubin reacts with diazotized sulfanilic acid to produce azobilirubin, which has an absorbance maximum at 560 nm in the aqueous solution. The intensity of the color produced is directly proportional to the amount of direct bilirubin concentration present in the sample. The subsequent addition of methanol accelerates the reaction of unconjugated bilirubin in the serum, and a value for total bilirubin is obtained after five (5) minutes. The total bilirubin value represents the sum of the bilirubin glucuronide (direct) and the unconjugated (indirect) bilirubin. The color produced measured at 560 nm is proportional to the amount of the total bilirubin concentration present in the sample.

### REAGENTS

1. Bilirubin Reagent: Sulfanilic Acid 32 mM, Hydrochloric Acid 165 mM.
2. Bilirubin Nitrite Reagent: Sodium Nitrite 60mM.
3. Bilirubin Calibrator: N-1-naphthylethylenediamine dihydrochloride salt (5 mg/dl).
4. Methanol Reagent: Absolute methanol, reagent grade.

### WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use.
2. Specimens should be considered infectious and handled appropriately.
3. Do not pipette reagents by mouth. Avoid contact reagent with eyes, skin and clothing. Do not ingest. Wash hands after use.

### REAGENT STORAGE

1. All reagents are stored at room temperature (15 - 30°C).
2. Do not freeze reagents.
3. Avoid exposure to direct sunlight.

### REAGENT DETERIORATION

The reagent should be discarded if:

1. Sodium Nitrite reagent has a yellow discoloration.
2. Reagent fails to achieve assigned assay values of fresh control sera.

### SPECIMEN COLLECTION AND STORAGE

1. Hemolysis interferes with the test, i.e., hemolyzed samples should be avoided since they may give falsely low values.<sup>4</sup>
2. All specimens for this assay must be carefully protected from light.<sup>1</sup>
3. Bilirubin in serum is stable for 4 - 7 days when stored in the dark at 2 - 8°C.<sup>1</sup>

### INTERFERING SUBSTANCES

1. Young, et al., give an exhaustive list of drugs and other substances known to affect the circulating level of bilirubin.<sup>5</sup>
2. In this assay, as in all laboratory procedures, materials, which come in contact with specimens, should be clean and free of contamination by heavy metals, detergents, and other chemicals.

### MATERIALS PROVIDED

1. Bilirubin reagent
2. Bilirubin Nitrite reagent
3. Bilirubin Calibrator
4. Methanol reagent

### MATERIALS REQUIRED BUT NOT PROVIDED

1. Cuvettes
2. Pipettes
3. Timer
4. Appropriate automated chemistry analyzer or spectrophotometer capable of measuring at 560 nm

### MANUAL ENDPOINT PROCEDURE

*NOTE:* Due to the critical time of the Direct Bilirubin Reaction, process each patient separately. Serum blank must be prepared for each patient and control.

1. Label test tubes "Blank, Standard, Control, Patient", etc. Each tube requires a **Blank** tube.
2. Dispense 2.8 ml of Bilirubin Reagent to all tubes including blank tubes.
3. Add 50 µl (0.05 ml) of Bilirubin Nitrite Reagent to all labeled test tubes except blank tubes, mix and let stand for one (1) minute. (Use distilled water for all the blank tubes).
4. Transfer 200 µl (0.2 ml) of serum to its respective labeled and blank tube, gently mix and set timer for one (1) minute. (Use distilled water for standard blank tube).
5. Set the wavelength of the photometer at 560 nm and zero with blank tube. (Wavelength range: 500 - 560 nm).
6. After exactly one (1) minute, record absorbance and use this to calculate Direct Bilirubin.
7. Add 3.0 ml of Methanol Reagent to all tubes, mix by inversion and let stand for five (5) minutes.

8. After five (5) minutes, read and record absorbance of all tubes.
9. Use this to calculate Total Bilirubin.

\* *TC MULTI-PURPOSE CALIBRATOR MAY BE USED TO REPLACE STANDARD.*

#### STABILITY OF ENDPOINT REACTION

Direct bilirubin color formation is stable for thirty (30) minutes whereas the total bilirubin color formation is stable for sixty (60) minutes.

#### CALCULATIONS

Abs. = absorbance

$$\frac{\text{Abs. of unknown} - \text{Abs. of blank}}{\text{Abs. of calibrator} - \text{Abs. of calibrator blank}} \times \text{Concentration of calibrator (mg/dl)} = \text{Bilirubin (mg/dl)}$$

*Example:*

Absorbance of unknown	= 0.132
Absorbance of unknown blank	= 0.120
Absorbance of calibrator	= 0.450
Absorbance of calibrator blank	= 0.000
Concentration of calibrator	= 5.0 mg/dl

Then

$$\frac{0.132 - 0.120}{0.450 - 0.000} \times 5 = \frac{0.012}{0.450} \times 5 = 0.13 \text{ mg/dl}$$

#### LIMITATIONS

1. Sera with values above 20 mg/dl must be diluted 1:1 with isotonic saline, reassayed and the final answer multiplied by two (2).
2. Serum hemoglobin levels of up to 1.0 g/dl do not interfere with results.

#### QUALITY CONTROL

Normal and abnormal control sera of known concentrations of direct and total bilirubin should be analyzed routinely with each group of unknown specimens.

#### EXPECTED VALUES<sup>5</sup>

Direct up to 0.5 mg/dl

Total up to 1.0 mg/dl

It is recommended that each laboratory establish its own range of expected values, since differences exist between instruments, laboratories, and local populations.

#### PERFORMANCE CHARACTERISTICS

1. Linearity: 20 mg/dl.
2. Sensitivity: Based on an instrument resolution of 0.001 absorbance, the present procedure has a sensitivity of 0.010 mg/dl.
3. Comparison: A comparison study between the present method with an available commercial product using the same identical method on twenty (20) fresh serum samples and two commercial serum controls, ranging from 0.20 mg/dl to 7.2 mg/dl yielded a coefficient of 0.97 and a regression equation of:  
 $y = 0.98x + 0.001$ .

#### 4. Precision:

*Run-to-run*: Two commercial control sera were assayed for a period of 21 days and the following day-to-day precision was obtained.

	<u>Level I</u>	<u>Level II</u>
Mean (mg/dl) N = 16	0.20	7.2
S.D.	0.02	0.36
C.V.	10.0%	5.8%

*Within Run*: Two Commercial control sera were assayed 20 times and the following within run precision was obtained.

	<u>Level I</u>	<u>Level II</u>
Mean (mg/dl) N = 20	0.24	7.6
S.D.	0.02	0.36
C.V.	10.0%	4.7%

#### REFERENCES

1. Tietz, N.W.: *Fundamentals of Clinical Chemistry*. W. B. Saunders Co., Philadelphia, p. 1028 (1976).
2. Gambino, S.R., et al., *JAMA* 201:1047 (1967).
3. Walters, M. Gerand, H., *Microchem J.* 15; 231 (1970).
4. Michaelsson, M.: *Scand. J., J. Clin. Lab. Invest.* (Suppl. 49) 13, 1 (1961).
5. Young, D.S., et. al.: *Clin. Chem.* 21, 10 (1975).
6. Gambino, S.R., et. al.: Bilirubin Assay (Revised), Commission on Continuing Education. *Am. Soc. of Clin. Path.*, Chicago (1968).

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Manufactured by:



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