

# CONCEPT DIAGNOSTICS

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## DIRECT BILIRUBIN REAGENT

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For the quantitative determination of direct 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 that elevation of direct bilirubin levels in patients with liver or biliary tract diseases 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 is 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.

### REAGENTS

1. Bilirubin Reagent: Sulfanilic Acid 32mM, Hydrochloric Acid 165mM.
2. Bilirubin Nitrite Reagent: Sodium Nitrite 60mM.
3. Bilirubin Calibrator: N-1-naphthyl ethylenediamine dihydrochloride salt. (5 mg/dl).

### 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 PREPARATION

Direct bilirubin working reagent. Add 0.005 ml (5 µl) of sodium nitrite reagent per 1.0 ml of direct bilirubin reagent and mix reagent. Example: 0.05ml sodium nitrite/10ml direct bilirubin reagent, 0.1ml sodium nitrite/20ml direct bilirubin reagent, etc.

### REAGENT STORAGE

1. All reagents are stored at room temperature (15 - 30°C)
2. Combined working reagent can be stored for up to eight (8) hours when kept in an amber bottle at room temperature.
3. Do not freeze reagents.
4. Avoid exposure to direct sunlight.

### REAGENT DETERIORATION

The reagent should be discarded if:

1. Sodium Nitrite reagent has a yellow discoloration.
2. Working 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>

### INTERFERENCES

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.

### MATERIALS REQUIRED BUT NOT PROVIDED

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

### DIRECT BILIRUBIN PROCEDURE (AUTOMATED)

Refer to appropriate instrument application instructions

### DIRECT BILIRUBIN PROCEDURE (MANUAL)

1. Label test tubes, "Blank, Standard, Control, Patient". Each tube requires a **blank** tube.
2. Dispense 1.0ml of direct bilirubin reagent to all **blank** tubes.
3. Prepare a working reagent. See "REAGENT PREPARATION."
4. Dispense 1.0ml of the working reagent into the labeled test tubes.
5. Add 0.1ml (100µl) of each standard, control, and sample to its respective tube. Mix well.
6. Allow all tubes to stand for five (5) minutes at room temperature.
7. Set the wavelength of the instrument at 560nm. Zero with reagent blank. (Wavelength range: 500-550).
8. Read and record absorbance of all tubes.

\* USE MULTI PURPOSE CALIBRATOR TO REPLACE STANDARD.

### PROCEDURE NOTE

The final color produced is stable for 60 minutes.

1. For pediatric samples with bilirubins over 3.0 mg/dl, use 0.05ml (50 µl) of sample and then multiply the result by two (2).
2. If the spectrophotometer being used requires a final volume greater than 1.0ml for accurate readings, 3ml of reagent and 0.2ml (200 µl) of sample may be used.

### CALCULATIONS

Abs. = absorbance

Abs. of Unknown - Abs. of blank

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

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

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

### PROCEDURE 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 bilirubin should be analyzed routinely with each group of unknown specimens.

### EXPECTED VALUES<sup>1,6</sup>

Infant (after one month) and adults 0.0-0.5 mg/dl. It is strongly recommended that each laboratory establish its own normal range.

### PERFORMANCE

1. Linearity: 20 mg/dl
2. Sensitivity: Based on an instrument resolution of 0.001 absorbance, the present procedure has a sensitivity of 0.01 mg/dl.
3. Comparison: A comparison study between the present method with an available commercial product using the same identical method on forty (40) fresh serum samples and two commercial serum controls, ranging from 0.10 mg/dl to 0.67 mg/dl yielded a coefficient of 0.98 and a regression equation of  $Y = 0.99X + 0.01$ .
4. Precision studies:

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

	Level I	Level II
Mean (mg/dl) N = 16	0.13	0.33
S.D.	0.01	0.04
C.V.	7.0%	12.0%

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

	Level I	Level II
Mean (mg/dl) N = 20	0.10	0.23
S.V.	0.01	0.03
C.V.	10.0%	13.0%

### REFERENCES

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