BILIRUBIN

Total
Caffeine Method

INTENDED USE

Vitro bilirubin reagent is intended for the in vitro quantitative determination of total bilirubin in serum or plasma on manual system.

METHOD

Diazo method according to Jendrassik-Graf.[1]

BACKGROUND

Bilirubin is formed in the reticuloendothelial system during the degradation of aged erythrocytes. The heme portion from hemoglobin and from other-containing proteins is removed, metabolized to bilirubin, and transported as a complex with serum albumin to the liver. This process accounts for about 80% of bilirubin formed daily. Other sources of bilirubin include the breakdown of myoglobin and cytochromes and the catabolism of immature red cells in the bone marrow. In the liver, bilirubin is conjugated with glucuronic acid for solubilization to form conjugated or direct bilirubin for subsequent transport through the bile duct into the digestive tract where it is metabolized by bacteria to a group of products collectively known as stercobilinogen. Total bilirubin is the sum of the conjugated and unconjugated fractions. Pre-hepatic diseases or conditions such as hemolytic disease or liver diseases resulting in impaired entry, transport or conjugation within the liver cause elevation of unconjugated (indirect) bilirubin. Monitoring of bilirubin in newborns, particularly if premature, has special importance since the hepatic handling of bilirubin is immature leading to elevated unconjugated bilirubin. If not bound to albumin, unconjugated bilirubin is able to cross the blood brain barrier more easily, increasing the risk of cerebral damage. Total bilirubin is elevated in conditions causing obstruction of the bile duct, hepatitis, cirrhosis, in hemolytic disorders and several inherited enzyme deficiencies. There is information indicating elevated levels of direct bilirubin in patients with liver or biliary tract disease, even though, total bilirubin levels are normal. Therefore, the greatest diagnostic value of direct bilirubin assays stem from their ability to indicate occult liver disease[4].

ASSAY PRINCIPLE

Most methods currently used for the quantitative determination of bilirubin are based on the reaction between bilirubin and diazotised sulfanilic acid. In aqueous solution only the direct bilirubin (conjugated) bilirubin will react in this manner. On the other hand, in order to estimate total bilirubin the unconjugated bilirubin must be freed from attachment to albumin and rendered water soluble. In Malloy-Evelyn method methanol is used while caffeine/sodium benzoate is used in the Jendrassik-Grof method[1].
The series of reactions involved in the assay system is as follows:

1. Conjugated (direct) and unconjugated (indirect) bilirubin in the sample react with diazotized sulfanilic acid to form the red colored azobilirubin in presence of caffeine.

Sulfanilic acid + $NaNO_2$ $\xrightarrow{HCl}$ **diazotized sulfanilic acid**

Bilirubin + diazotized sulfanilic acid $\xrightarrow{pH\ 1.4}$ **azobilirubin**

The intensity of the color produced is directly proportional to bilirubin concentration. It is determined by measuring the increase in absorbance at 546 nm

EXPECTED VALUES

Total Bilirubin[2]	
Adults and	0.2 – 1.0 mg/dl
infants > 1 month	3.4 - 17 µmol/l
Newborns premature	10 - 14 mg/dl
(3-5 days)	171 - 239 µmol/l
Newborns (3-5 days)	4.0 – 8.0 mg/dl
	68 - 137 µmol/l
Newborns (<48 hrs)	6.0 – 10.0 mg/dl
	103 - 171 µmol/l
Newborns (<24 hrs)	2.0 – 6.0 mg/dl
	34 - 103 µmol/l

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 bilirubin results should always be assessed in conjunction with the patient's medical history, clinical examination, and other findings.

REAGENTS

R_1 Total Reagent
R_2 Total Sodium nitrite

- **Reagent Preparation & Stability**

All reagents are stable up to the expiry date given on label when stored at room temperature.

SPECIMEN

Serum.

For direct bilirubin plasma specimen may be used, but the only accepted anticoagulants are heparin and oxalate.

- **Specimen Preparation & Stability**

The specimen of choice is serum. Specimens should be assayed promptly after collection, since direct bilirubin is reportedly unstable[5]. If testing is delayed, specimens should be protected from exposure to light. Bilirubin remains stable in serum samples for 2 days at room temperature, 4 days at 4^0C, or 3 months at -20^0C, if care is taken to prevent exposure to light[6].

PROCEDURE

- **Total Bilirubin**

Wavelength	546 nm
Cuvette	1 cm light path
Temperature	20-25 ^{0}C
Zero adjustment	against specimen blank
Specimen	Serum

	Specimen blank	Specimen
R_1	1 ml	1 ml
R_2	……	One drop
Specimen	50 µl	50 µl
Mix well, let stand 10 minutes at room temperature.		

Measure the absorbance of specimen ($A_{specimen}$) against specimen blank.

CALCULATION

Calculate the bilirubin concentration by using the following formulae:

Total Bilirubin Concentration=
Specimen absorbance X 26.9 =mg/dl

Unit conversion

mg/dl x 17.1= µmol/dl

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 bilirubin 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 Vitro Technical Services.

Medical Device Safety Services
MDSS GmbH
Burckhardtstr. 1
30163 Hannover, Germany
Tel.: +49 511 6262 8630
Fax: +49 511 6262 8633

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INTERFERING SUBSTANCES

- **Anticoagulants:**
  The only accepted anticoagulants are heparin and oxalate for direct bilirubin.
- **Drugs:**
  Young[7] in 1990 has published a comprehensive list of drugs and substances, which may interfere with this assay.
- **Hemolysis:**
  Avoid hemolyzed specimens. Even slight hemolysis interferes with the test. Haemoglobin interference is dependent on both analyte and hemoglobin concentration.
  The interference becomes decreasingly significant with increasing concentration of total bilirubin.
- **Lipemia:**
  Avoid lipemic specimens. Even slight lipemia interferes with the test.

WARNING & PRECAUTION

- Vitro bilirubin reagent is for in vitro diagnostic use only. Normal precautions exercised in handling laboratory reagents should be followed.
- 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.
- All specimens must be protected from light. Serum bilirubin will decrease 50% in one hour if kept at room temperature and at direct sunlight.
- Don't use the reagent if it is turbid.

PERFORMANCE CHARACTERISTICS

Imprecision
Reproducibility was determined using in an internal protocol. The following results were obtained.

Within Run		
Total Bilirubin		
Number of samples	40	40
Mean (mg/dl)	1.15	4.22
SD (mg/dl)	0.02	0.04
CV (%)	2.02	1.05

Between Day		
Total Bilirubin		
Control	Level I	Level II
Number of samples	40	40
Mean (mg/dl)	1.15	4.27
SD (mg/dl)	0.02	0.13
CV (%)	1.91	3.2

Method Comparison
Comparison studies were carried out using a similar commercially available Total Bilirubin reagent as a reference. Serum samples were assayed in parallel and the results compared by least squares regression. The following statistics were obtained.
Number of sample pairs 929
Range of sample results 0.05-26 mg/dl
Mean of reference results 1.2 mg/dl
Mean of Total Bilirubin results 1.2 mg/dl
Slope 1.10
Intercept 0.12 mg/dl
Correlation coefficient 1.00

Sensitivity
The sensitivity is defined as the change of analytical response (ΔA/min) per unit change in analyte concentration at a pathlength of 1 cm.

When run as recommended the sensitivity of this assay is 0.01 mg/dl (0.17 mol/l).

LINEARITY

When run as recommended, the assay is linear up to 25 mg/dl (0.428 mmol/l).

If result exceeds 25 mg/dl (0.428 mmol/l), specimen should be diluted with 0.9% NaCl solution and reassayed. Multiply the result by the dilution factor

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4. **Gambino, SR et al., (1967):** JAMA 201: 1047.
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6. **Winkelmann, J, Cannon, DC and Jacobs, SL (1974):** Liver function tests, including bile pigments. In: Henry RJ, Cannon DC, Winkelman, JW, eds. Clinical chemistry, principles and technics. 2nd ed. New York: Harper & Row; 1o42-1079.
7. **Young, DS (1990):** Effects of Drugs on Clinical Laboratory Tests. Third Edition. 1990: 3: 6-12.

SYMBOL DECLARATION

	Manufacturer
	Consult instructions for use
LOT	Batch code (Lot #)
REF	Catalog number
	Temperature limitation
IVD	In vitro diagnostic medical device
	Use by
	Caution. Consult instructions
	Keep away from light

ORDERING INFORMATION

REF	SIZE
1071	1 x 100 ml
1072	2 x100 ml

Manufactured in Egypt by:
Vitro Scient
www. vitroscient.com

Technical Support:
+202 26439699
info@vitroscient.com

orders:
+202 26439698
order@vitroscient.com

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