TOTAL BILIRUBIN

Diagnostic reagent for determination of Total Bilirubin concentration.
Liquid. Dual reagents *(Ratio: R1/R2: 4/1)*. Store at +2/+8°C. For in Vitro Diagnostic Use (IVD). Do not freeze.

Ref No	Pack
MH-042	75 mL
VH-042	75 mL

Changes made in the instructions for use are marked as grey.

INTENDED USE

The test is applied for the quantitative determination of total bilirubin in human serum and plasma.

GENERAL INFORMATION

Bilirubin was discovered in 1849 by Virchow, who called this yellow pigment "hematidine". The term bilirubin was introduced by Stadeler in 1864, and in 1874 Tarchanoff showed the direct relationship of bile pigments to Hb. Bilirubin is an orange-yellow pigment derived mainly from heme, a product of the red blood cell (RBC) cycle.[1] Important chemical properties of the bilirubin molecule are its insolubility in water and solubility in various non-polar solvents. Bilirubin from natural sources is almost entirely (99%) composed of the IXα isomer. The bilirubins IXβ and IXδ, resulting from cleavage of β- and δ-methene bridges, account for less than 0.5% of bilirubin isolated from bile. Ap- proximately 85% of the total bilirubin produced is derived from the heme moiety of Hb released from senescent erythrocytes that are destroyed in the reticuloendothelial cells of the liver, spleen, and bone marrow. The remaining 15% is produced from RBC precursors destroyed in the bone marrow (so-called ineffective erythropoiesis) and from the catabolism of other heme-containing proteins such as myoglobin, cytochromes and peroxidases.[1]

The first step in heme degradation is the catalysis of the reticuloendothelial cells by the microsomal heme oxygenase system. In the presence of nicotinamide adenine dinucleotide phosphate ($NADPH+H^+$) and O_2, the enzyme catalyzes three sequential oxygenations resulting in porphyrin ring opening (conversion of cyclic heme to linear biliverdin), carbon monoxide (CO) production and Fe^{+2} release. Next, biliverdin, a green pigment, is reduced by a reaction catalyzed by biliverdin reductase, which requires NADPH, to form red-orange bilirubin.[2,3]

The bilirubin formed (unconjugated=indirect) is poorly soluble in plasma and is therefore non-covalently bound to albumin and transported to the liver. In the liver, bilirubin dissociates from the carrier albumin molecule, enters the hepatocyte by facilitated diffusion and binds to intracellular proteins, particularly the ligandin protein. The solubility of bilirubin is here increased by the addition of two molecules of glucuronic acid to bilirubin.[3] Bilirubin uridine diphosphate (UDP)-glucuronyltransferase is the enzyme that catalyzes the reaction. As a result of the reaction, bilirubin diglucoronide (conjugated=direct bilirubin) is formed. Bilirubin diglucuronide returns to the cytosol, presumably via a transporter, and binds to its ligand, where it diffuses to the canalicular pole for secretion into bile or to the sinusoidal pole for secretion back into plasma.[1] Bilirubin diglucuronide is hydrolyzed and reduced by bacteria in the intestine to yield urobilinogen, a colorless compound. Most urobilinogen is oxidized by intestinal bacteria to sterkobilin, which gives stool its characteristic brown color. However, some of the urobilinogen is reabsorbed from the intestine and enters the portal blood. Part of this urobilinogen participates in the enterohepatic urobilinogen cycle, where it is taken up by the liver and then re-secreted into bile. The rest of the urobilinogen is transported through the blood to the kidney, where it is converted to yellow urobilin and excreted, giving urine its characteristic color.[3]

Since bilirubin is a powerful antioxidant, mild or moderately elevated serum bilirubin has beneficial effects.[4] Protective effects of bilirubin on atherogenesis and carcinogenesis have been demonstrated in both in vitro and in vivo studies.[5,6] However, high concentrations of unconjugated hyperbilirubinemia are toxic and lead to bilirubin encephalopathy (kernicterus), which causes massive destruction of neurons through apoptosis and necrosis due to inhibition of DNA synthesis and direct neurotoxicity.[7]

Accumulation of bilirubin in plasma and tissues causes the characteristic yellow discoloration of tissues known as icterus or jaundice.[1] The causes of jaundice are of three main types: hemolytic (=pre-hepatic), hepatocellular (=hepatic) and obstructive (=post-hepatic).[3,8] The liver has the capacity to conjugate and excrete more than 3000 mg of bilirubin per day, whereas normal bilirubin production is only 300 mg per day. In sickle cell anemia, pyruvate kinase and glucose 6-phosphate dehydrogenase deficiency diseases with intense hemolysis, the indirect bilirubin concentration is elevated if the conjugation capacity of the liver is exceeded.[3] There are two main causes of hemolytic anemia, congenital and acquired.[9] The main cause of increased hemolysis is a defective plasma membrane of red blood cells. This delicate cell membrane cannot resist the stress of circulating flow, resulting in ruptures that result in hemolysis, which in turn leads to increased serum

bilirubin levels.[10,11] Patients with hemolytic jaundice are seen anemia, yellowing of the sclera, dark yellow-brown urine, yellowish skin and high indirect bilirubin levels.[12]

In hepatic jaundice, there may be defects in the uptake, conjugation and excretion of bilirubin by the liver.[13-16] Similar to hemolytic jaundice, hepatic jaundice has two main causes, congenital and acquired.[16,17] Clinical symptoms of hepatic jaundice include abdominal pain, fever, vomiting and nausea, as well as complications such as anorexia, gastrointestinal bleeding, diarrhea, anemia, edema, weight loss and associated weakness; if left uncontrolled, kernicterus, coma and even death may occur.[18] Physiologic jaundice observed in newborns develops due to inadequate conjugation process due to delayed maturation and low activity of uridine 5-phosphate (UDP)-glucuronyl transferase enzyme and indirect bilirubin increases.[1] Hepatic jaundice may develop due to a genetic defect in the UDP-Glucuronyl transferase enzyme. For example, Gilbert-Meulengracht disease, one of the causes of hereditary non-hemolytic hyperbilirubinemia, can be given. Mutations in UGT1A1 result in[19] indirect hyperbilirubinemia due to impaired glucuronidation activity and cause mild jaundice.[19,20] Crigler-Najjar syndrome type 1 is an autosomal recessive disorder caused by the complete absence of UDP-Glucuronyl transferase activity. Affected patients have severe hyperbilirubinemia in the first few days of life, often leading to bilirubin encephalopathy. Patients with Crigler-Najjar syndrome type 2 retain some activity of UDP-Glucuronyl transferase enzymes. Therefore, total bilirubin levels are not very high and patients rarely develop bilirubin encephalopathy.[19,21] In severe liver damage with fulminant liver failure and end-stage cirrhosis, liver disease may primarily cause indirect hyperbilirubinemia.[1]

Direct bilirubin levels are elevated in posthepatic jaundice. In most cases of acute hepatitis and cholestasis (stasis or suppression of bile flow), direct bilirubin is elevated. Urinary bilirubin reflects increased plasma concentrations of direct bilirubin.[1] Since the most important cause of post-hepatic jaundice is extrahepatic biliary obstruction, it is also known as obstructive jaundice. Extrahepatic obstruction has two main causes, congenital and acquired.[22-30] Clinical manifestations of obstructive jaundice include dark urine, pale stools and generalized pruritus. A history of febrile biliary colic, weight loss, abdominal pain and abdominal mass are also signs of obstructive jaundice.[23] Obstructive jaundice may lead to various complications including cholangitis, pancreatitis, renal and hepatic failure.[3] Increased direct bilirubin is rarely seen in congenital defects in bilirubin excretion and in impaired bilirubin excretion occurring in sepsis or other acute diseases.[1] Dubin-Johnson (DJS) and Rotor syndrome are examples of posthepatic jaundice associated with congenital defects causing direct bilirubin increase. DJS is characterized by chronic, predominantly conjugated non-hemolytic hyperbilirubinemia and its phenotype is similar to Rotor syndrome. Unlike Rotor, biliary excretion of organic anions other than bile acids is also impaired in DJS.[21]

Common NSAIDs or hypolipidemics cause mild bilirubin elevation in susceptible individuals (for review see Ref. 31). On the other hand, oral contraceptives are known to reduce serum bilirubin concentrations by up to 30%.[32] Systemic concentrations of bilirubin can also be increased by many nutraceuticals and dietary supplements.[31] For example, patients with prostate cancer treated with the dietary supplement silybin[33] and hepatitis C patients receiving the same treatment[34] may experience mild indirect hyperbilirubinemia[35] due to the inhibitory effects of silymarin flavonoids on bilirubin conjugation in liver tissue.[36]

TEST PRINCIPLE

Colorimetric diazo method

Bilirubin reacts with diazotized sulfonylic acid in an acidic environment to form the red azobilirubin. The intensity of the resulting color is measured by absorbance reading at a wavelength of 500 nm and is proportional to the total bilirubin concentration in the sample. As with the Jendrassik-Gróf method,[37] caffeine is present in the reagent as a reaction accelerator. Benzoate also has a similar effect. Surfactant as a solubilizing agent is also present in the reagent.[38]

Annotation:

- Bilirubin is commonly measured by the Van Den Bergh reaction in which diazotized sulfanilic acid reacts with bilirubin to form red azodipyrroles, which are measured colorimetrically. In aqueous solution, water-soluble conjugated bilirubin (direct bilirubin) reacts rapidly (within one minute) with the reagent and is said to "react directly". Unconjugated (=indirect) bilirubin, which is much less soluble in aqueous solution, reacts more slowly.[3]

- The diazo method, described by Jendrassik and Grof in 1938[39] and later modified by Doumas et al., gives reproducible and reliable results for serum total bilirubin.[40-43] In this procedure, an aqueous solution of caffeine and sodium benzoate acts as an accelerator. Studies of the mechanism by which the caffeine-benzoate solution facilitates the reaction of unconjugated bilirubin with the diazo reagent have provided strong, albeit indirect, evidence that caffeine and perhaps benzoate remove unconjugated bilirubin from the incorporation sites on albumin.[1, 44-46]

REAGENT COMPONENTS

Reagent 1:

Sodium benzoate	: ≤ 0.30 M
Sodium acetate	: ≤ 0.50 M
Caffeine	: ≤ 0.15 M
Surfactant	

Reagent 2:

Sulphanilic acid	: ≤ 33 mM
Hydrochloric acid	: ≤ 0.20 M

REAGENT PREPARATION

Reagent is ready for use.

REAGENT STABILITY AND STORAGE

Reagents are stable at +2/+8°C till the expiration date stated on the label which is only for closed vials.

Once opened vials are stable for 30 days at +2/+8°C in optimum conditions. On board stability is strongly related to auto analyzers' cooling specification and carry-over values.

Reagent stability and storage data have been verified by using Clinical and Laboratory Standards Institute (CLSI) EP25-A protocol.[47]

SAMPLE REQUIREMENTS

Serum and plasma can be used and are collected according to the standard procedures. For plasma, sample collection tubes with Li heparin, Na heparin, K2-EDTA or K3-EDTA must be preferred. Contact with light must be avoided. Non-lipemic samples must be used. Multiple sample freezing and thawing should be avoided.

Bilirubin Total activity stability in serum and plasma[65]:

1 day at +20/+25ºC
7 days at +2/+8ºC
6 months at -20°C

Annotation:

- Significant and substantial decreases in serum bilirubin concentrations have been reported in serum samples not protected from light, with more prominent changes in samples with normal or low bilirubin concentrations.[35,48]

CALIBRATION AND QUALITY CONTROL

Calibration: The assay requires the use of an Arcal Auto Calibrator.

Arcal Auto Calibrator-Lyophilized
Ref.No: VT-003

Calibration stability is 30 days.Calibration stability depends on the application characteristics and cooling capacity of the autoanalyzer used.

It has been standardized according to the Doumas method/NIST SRM 916.

Control: Commercially available control material with established values determined by this method can be used. We recommend:

Arcon N Level 1 Control- Lyophilized
Ref.No: VT-001

Arcon P Level 2 Control- Lyophilized
Ref.No: VT-002

At least two level controls must be run once in every 24 hours. Each laboratory should determine its own quality control scheme and procedures. If quality control results are not within acceptable limits, calibration is required.

REFERENCE INTERVALS / MEDICAL DECISION LEVELS

Adults[66]	: 0.2 - 1.2 mg/dL
Newborn[67]	
Up to 24 hours	: < 6.0 mg/dL
Up to 48 hours	: < 10.0 mg/dL
3-5 days old	: < 8.0 mg/dL
7 days old	: <10 mg/dL

Annotation:

- Reference ranges for serum bilirubin concentrations vary among a variety of different populations, being lowest in African Americans[49] and highest in Asian populations.[35]
- Age, gender, ethnicity, diet, smoking status, alcohol intake, underlying liver disease, hemolytic disease, circadian rhythms, medications, physical activity, anthropometric parameters, medical history and/or fasting status should be considered as the most important variables affecting bilirubin concentrations.[35]
- One study has shown that increasing age per decade is associated with a 0.3 µmol/L decrease in serum bilirubin concentrations, but only in men.[49] In other studies, this association is unclear due to the misidentification of an elderly healthy control population.[35]

Medical Decision Levels:

- **7-17 mg/dL:** Serum total bilirubin levels above 17 mg/dL may be pathologic.[50]

- **5-6 mg/dL:** Physiologic jaundice usually develops 24 hours after birth. It peaks at approximately 48-96 hours and resolves in 2-3 weeks in term babies. Total bilirubin peaks at approximately 72 hours in term babies and after 72 hours in preterm babies.[19] A total serum bilirubin peak of 5-6 mg/dL may be seen in a developing newborn. The main increased fraction is indirect bilirubin.[50]
  Jaundice is considered pathologic if it occurs on the first day of life, total bilirubin is above the 95th percentile according to age-specific bilirubin monograms, levels increase more than 5 mg/dL/day or more than 0.2 mg/dL/hour, and jaundice lasts more than 2 to 3 weeks in term infants.[19]

- **>25 mg/dL:** This is the serum total bilirubin threshold level recommended by the American Academy of Pediatrics (AAP) in its 2004 guidelines for phototherapy and exchange transfusion. However, there is no reason not to start phototherapy earlier or at lower bilirubin concentrations than recommended by the AAP if multiple risk factors are present or if deemed necessary based on the clinical evaluation.[51]

Annotation:

- In kernicterus, especially when indirect bilirubin exceeds a certain concentration, it can cross the blood-brain barrier as it is fat-soluble, unlike direct bilirubin which is water-soluble. It accumulates in brain tissue, particularly in the basal ganglia. The neurotoxicity of indirect bilirubin leads to various neurologic sequelae.[52]

- **Neonatal pre-discharge serum total bilirubin predictive value:** This value is plotted on an hour-specific bilirubin nomogram that describes the range of serum total bilirubin levels observed in a racially diverse population of healthy, term and near-term newborns during the first postnatal week. Thus, if the percentile curves for serum total bilirubin values are ≥8 mg/dL at approximately 24 hours, ≥14 mg/dL at approximately 48 hours, and ≥17 mg/dL at approximately 84 hours, it means that these levels are above the 95th percentile for age in hours after birth, and this level of hyperbilirubinemia is considered relevant. This usually indicates the need for close observation, possible further evaluation and sometimes intervention to prevent brain damage without resorting to exchange transfusion.[53]

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary, determine its own reference range.

Reference interval has been verified by using CLSI EP28-A3c protocol.[54]

PERFORMANCE CHARACTERISTICS

Measuring Interval

According to CLSI EP34-ED1:2018, "Measuring Interval" refers to the interval where the analyte concentration is measured with intended accuracy in terms of medical and laboratory requirements without dilution, concentrating or any kind of pre-treatment that is between the analyte's lower limit of quantitation (LLoQ) and upper limit of quantitation (ULoQ).[55]

The determined analytic measuring interval for Total Bilirubin is 0.15 – 20 mg/dL.

Detection Capability

Limit of Detection (LoD): 0.1 mg/dL

Limit of Quantitation (LoQ): 0.15 mg/dL

LoQ values are based on Coefficient of Variation Percentage (CV) ≤ 20%.

LoD and LoQ values have been verified by using CLSI EP17-A2:2012 protocol.[56]

Linearity

This method shows measurement linearity in the activities up to 20 mg/dL. Autoanaylzer's auto-dilution system can be used if the concentrations have higher values. See device manual for further information.

For the manual dilution procedure, dilute the sample 1:5 using 0.90% isotonic. After this process, multiply the result of the reworked sample by the dilution factor. Do not report the sample result after dilution if it is marked as lower than the linear lower limit. Rerun with a suitable dilution.

Linearity Studies data have been verified by using CLSI EP06-A:2003 protocol.[57]

Precision

Running system has been developed according to 20x2x2 "The Single Site" protocol. Repeatability and Within-Laboratory Precision/Within-Device values have been obtained according to the running results.

According to the protocol in use, 2 separate runs per day have been made for 20 days (no obligation for being consecutive days). This protocol has been applied to each low and high samples separately and 80 results have been obtained for each one. Statistically, the results have been obtained using 2-factor Nested-ANOVA model.[58]

Repeatability (Within Run) and Repeatability (Day to Day) SD (standard deviation) and CV% values of Bilirubin Total have been given in the table 1 and 2 respectively.

Table 1. Total Bilirubin Repeatability (Within Run) results obtained from samples in two different concentrations

Mean Concentration	SD	CV%	n
0.68 mg/dL	0.01	2.25	80
5.18 mg/dL	0.02	0.55	80

Annotation:

- This working system has been named "Within-Run Precision" in the previous CLSI - EP05-A2 manual.[59]

Table 2. Total Bilirubin Repeatability (Day to Day) results obtained from samples in two different concentrations

Mean Concentration	SD	CV%	n
0.68 mg/dL	0.03	3.74	80
5.18 mg/dL	0.15	2.90	80

Annotation:

- This working system has been named "Total Precision" in the previous CLSI - EP05-A2 manual.[59]

Method Comparison

As a result of the statistical evaluation of the method comparison data:

Passing-Bablock equation:[60]
y= 1.179x – 0.184 mg/dL
r=0.96

Interference

Endogenous interferant and analyte concentrations that have been used in the Total Bilirubin scanning tests has been determined according to "CLSI EP37-ED1:2018" and "CLSI EP07-ED3:2018" manuals.[61,62]

The total acceptable error rate, which is going to be used to detect whether the observed differential value obtained from Bilirubin Total interference scanning test is appropriate, is determined as ±10%.[63]

In Total Bilirubin test results, no significant interaction has been observed in the determined endogenous interferant and analyte concentrations or between interferants and analyte.

Interferant-Concentration	Total Bilirubin Target (mg/dL)	N*	Observed Recovery %
Indicane (Indoxyl Sulfate) 0.08 mmol/L	1.06	3	106

** Total acceptable error rate determined as interference limit and repeatability (within run) pre-detected for the related method were used for the calculations of how many times the control and test samples prepared as a serum pool are going to be run repetitively. In the calculations, the accepted error rate for type 1 (α error) was 5% and for type 2 (β error) was 10% (90% power).*[62]

Annotation:

- Non-hemolyzed and non-lipemic samples should be used.
- Some drugs, such as naproxen metabolites, may interfere with the diazo method and cause erroneous measurements.[64]

It should be noted that endogenous interferants, as well as various medicines and metabolites, anticoagulants (e.g. Heparin, EDTA, citrate, oxalate) and preservatives (e.g. sodium floride, iodoacetate, hydrochloride acide) such as additives, materials that may contact with samples during collection and processing (serum separator devices, sample collection containers and contents, catheters, catheter wash solutions, skin disinfectants, hand cleaners and lotions, glass washing detergents, powder gloves), dietary substances known to affect some specific tests (caffeine, beta-carotene, poppy seeds, etc.), or some substances present in a sample that cause foreign proteins (heterophilic antibodies, etc.), autoimmune response (autoantibodies, etc.), or due to malignancy (for example, interference by paraproteins with phosphate testing and indirect ion selective electrode methods) may show some negative effects that will cause various attempts and some misjudgements.[62]

These performance characteristics have been obtained using an autoanalyzer. Results may vary slightly when using different equipment or manual procedures.

WARNINGS AND PRECAUTIONS

IVD: For in Vitro Diagnostic use only.
Do not use expired reagents.
Reagents with two different lot numbers should not be interchanged.
For professional use.
Follow Good Laboratory Practice (GLP) guidelines.

CAUTION: Human source samples are processed with this product. All human source samples must be treated as potentially infectious materials and must be handled in accordance with OSHA (Occupational Safety and Health Administration) standards.

Danger

EUH032 :Releases a very toxic gas if contacts with acid.
H317 :May cause allergic skin reaction.

Precaution

P280 :Use protective gloves / clothes / glasses / mask.
P264 :Wash your hands properly after using.
P272 :Contaminated work clothes should not be allowed to be used outside of the workplace.

Intervention

P302+P352 :Wash with plenty of water and soap if it contacts with skin.
P333+P313 :Seek medical help if it irritates your skin or develops rash.
P362+P364 :Remove contaminated clothes and wash properly before using.

Disposal

P501 :Dispose the vials and contents according to the local regulations.

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SYMBOLS

IVD	In Vitro Diagnostic Medical Device
LOT	Lot Number
R1	Reagent 1
R2	Reagent 2
GTIN	Global Trade Item Number
REF	Reference Number
GLP	Good Laboratory Practices
FOR USE WITH	Identifies Products to Be Used Together
PRODUCT OF TURKEY	Product of Turkey
	Manufacturer
	Expiration Date
	Temperature Limits
	Consult Instructions for Use
	Caution
	Number of Tests