BILIRUBIN (TOTAL AND DIRECT)		BILIRUBIN (TOTAL)		BILIRUBIN (DIRECT)	
COD 11515 2x50+2x50mL	COD 11555 500 + 500 mL	COD 11510 4 x 50 mL	COD 11544 2 x 500 mL	COD 11511 4 x 50 mL	COD 11545 2 x 500 mL
STORE AT 2-30ºC					
Reagents for measurement of bilirubin concentration Only for *in vitro* use in the clinical laboratory					

BILIRUBIN (TOTAL AND DIRECT)
BILIRUBIN (TOTAL) BILIRUBIN (DIRECT)

BioSystems

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BILIRUBIN
DIAZOTIZED SULFANILIC

PRINCIPLE OF THE METHOD

Direct bilirubin in the sample reacts with diazotized sulfanilic acid forming a coloured complex that can be measured by spectrophotometry. Both direct and indirect bilirubin couple with diazo in the presence of cetrimide[1,2]. The terms "direct" and "total" refer to the reaction characteristics of serum bilirubin in the absence or presence of solubilizing (accelerating) reagents. The "direct" and "indirect" bilirubin are only approximately equivalent to the conjugated and unconjugated fractions.

CONTENTS

	Reagent AT	Reagent AD	Reagent BT	Reagent BD
COD 11515	2 x 40 mL	2 x 40 mL	2 x 10 mL	2 x 10 mL
COD 11555	400 mL	400 mL	1 x 100 mL	1 x 100 mL
COD 11510	4 x 40 mL	—	4 x 10 mL	—
COD 11544	2 x 400 mL	—	2 x 100 mL	—
COD 11511	—	4 x 40 mL	—	4 x 10 mL
COD 11545	—	2 x 400 mL	—	2 x 100 mL

COMPOSITION

BILIRUBIN (TOTAL)

AT. Reagent. Sulfanilic acid 29 mmol/L, hydrochloric acid 0.2 mol/L, cetrimide 50 mmol/L.
DANGER: H314: Causes severe skin burns and eye damage. P280: Wear protective gloves/protective clothing/eye protection/face protection. P303+P361+P353: IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

BT. Reagent. Sodium nitrite 11.6 mmol/L.

BILIRUBIN (DIRECT)

AD. Reagent. Sulfanilic acid 35 mmol/L, hydrochloric acid 0.24 mol/L.
DANGER: H314: Causes severe skin burns and eye damage. P280: Wear protective gloves/protective clothing/eye protection/face protection. P303+P361+P353: IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

BD. Reagent. Sodium nitrite 3.5 mmol/L.

For further warnings and precautions, see the product safety data sheet (SDS).

STORAGE

Store at 2-30ºC.

Reagents are stable until the expiry date shown on the label when stored tightly closed and if contaminations are prevented during their use.

Indications of deterioration:

– Reagents: Presence of particulate material, turbidity, absorbance over 0.09 at 540 nm (1 cm cuvette).

AUXILIARY REAGENTS

S. Bilirubin Standard (cod 11513). Reconstitute with 5.0 mL of distilled water (Note 1). Concentration is given on the label. Concentration value is traceable to the Standard Reference Material 916a (National Institute of Standards and Technology, USA). Protect the reconstituted Standard from light. Stable for 4 hours at 15-30ºC or for 2 months at –18ºC when frozen in aliquots.

REAGENT PREPARATION

Working Reagent: Transfer the contents of one Reagent BT vial into a Reagent AT bottle for total bilirubin determination, or one Reagent BD vial into a Reagent AD bottle for direct bilirubin determination (Note 2). Mix thoroughly. Other volumes can be prepared in the proportion: 1 mL Reagent BT + 4 mL Reagent AT or 1 mL Reagent BD + 4 mL Reagent AD. Stable for 20 days at 2-8ºC.

ADDITIONAL EQUIPMENT

– Analyzer, spectrophotometer or photometer with cell holder thermostatable at 37ºC and able to read at 540 ± 20 nm.
– Cuvettes with 1 cm light path (if factor is used in calculations).

SAMPLES

Serum collected by standard procedures.

Bilirubin in serum is stable for 7 days at 2-8ºC if serum is protected from light[3].

PROCEDURE FOR TOTAL BILIRUBIN

1. Pipette into labelled test tubes: (Notes 1, 3)

	Reagent Blank	Sample Blank	Sample	Standard
Distilled water	100 µL	—	—	—
Sample	—	100 µL	100 µL	—
Standard (S)	—	—	—	100 µL
Reagent (AT)	—	1.0 mL	—	—
Working Reagent	1.0 mL	—	1.0 mL	1.0 mL

2. Mix thoroughly and let stand the tubes for 2 minutes at room temperature.
3. Read the absorbance (A) of the Sample Blanks at 540 nm against distilled water.
4. Read the absorbance (A) of the Samples and of the Standard at 540 nm against the Reagent Blank.

PROCEDURE FOR DIRECT BILIRUBIN

1. Pipette into labelled test tubes: (Notes 1, 3).

	Reagent Blank	Sample Blank	Sample
Distilled water	100 µL	—	—
Sample	—	100 µL	100 µL
Reagent (AD)	—	1.0 mL	—
Working Reagent	1.0 mL	—	1.0 mL

2. Mix thoroughly and let the tubes stand for exactly 5 minutes at 37ºC.
3. Read the absorbance (A) of the Sample Blanks at 540 nm against distilled water.
4. Read the absorbance (A) of the Samples at 540 nm against the Reagent Blank.

CALCULATIONS

The bilirubin concentration in the sample is calculated using the following general formula:

$$\frac{A_{Sample} - A_{Sample\ Blank}}{A_{Standard}} \times C_{Standard} = C_{Sample}$$

In calculations of direct bilirubin, use the absorbance value obtained for the standard in the total bilirubin procedure (Note 4).

Mass concentration (mg/dL) x 17.1 = substance concentration (µmol/L).

REFERENCE VALUES

Adults:

Total[4]:	Up to 2.0 mg/dL = 34 µmol/L
Direct[5]:	Up to 0.3 mg/dL = 5 µmol/L

Newborns[4] (total bilirubin) :

Age	premature	full-term
Up to 24 h	1.0-8.0 mg/dL = 17-137 µmol/L	2.0-6.0 mg/dL = 34-103 µmol/L
Up to 48 h	6.0-12.0 mg/dL = 103-205 µmol/L	6.0-10 mg/dL = 103-171 µmol/L
3-5 days	10-14 mg/dL = 171-239 µmol/L	4.0-8.0 mg/dL = 68-137 µmol/L

These ranges are given for orientation only; each laboratory should establish its own reference ranges.

QUALITY CONTROL

It is recommended to use the Biochemistry Control Serum level I (cod. 18005, 18009 and 18042) and II (cod. 18007, 18010 and 18043) to verify the performance of the measurement procedure.

Each laboratory should establish its own internal Quality Control scheme and procedures for corrective action if controls do not recover within the acceptable tolerances.

METROLOGICAL CHARACTERISTICS

– Detection limit (Total bilirubin): 0.003 mg/dL = 0.05 µmol/L
– Detection limit (Direct bilirubin): 0.02 mg/dL = 0.34 µmol/L
– Linearity limit: 20 mg/dL = 343 µmol/L. For higher values dilute sample 1/3 with distilled water and repeat measurement.
– Repeatibility (within run):

Total bilirubin	CV	n	Direct bilirubin	CV	n
0.59 mg/dL = 10.1 µmol/L	3.0 %	20	0.77 mg/dL = 13.2 µmol/L	1.2 %	20
6.74 mg/dL = 115.2 µmol/L	1.0 %	20	1.36 mg/dL = 23.2 µmol/L	0.5 %	20

– Reproducibility (run to run):

Total bilirubin	CV	n	Direct bilirubin	CV	n
0.59 mg/dL = 10.1 µmol/L	3.6 %	25	0.77 mg/dL = 13.2 µmol/L	2.3 %	25
6.74 mg/dL = 115.2 µmol/L	3.3 %	25	1.36 mg/dL = 23.2 µmol/L	0.9 %	25

– Sensitivity (total bilirubin): 88 mA·dL/mg = 5.15 mA·L/µmol
– Sensitivity (direct bilirubin): 100 mA·dL/mg = 5.85 mA·L/µmol
– Trueness: Results obtained with this reagent did not show systematic differences when compared with reference reagents (Note 4). Details of the comparison experiments are available on request.
– Interferences: Hemoglobin (10 g/L) does not interfere. Lipemia (triglycerides > 15 g/L) may intefere. Other drugs and substances may interfere[6].

These metrological characteristics have been obtained using an analyzer. Results may vary if a different instrument or a manual procedure are used.

DIAGNOSTIC CHARACTERISTICS

Bilirubin is a waste product derived from the heme moiety of the hemoglobin released from senescent or damaged erythrocytes, that are destroyed in the reticuloendothelial cells. After production, bilirubin is transported to the liver in association with albumin. Inside the hepatocytes bilirubin is conjugated with glucuronic acid and it is excreted into bile. A number of inherited and acquired diseases affect production, uptake, metabolism, and excretion of bilirubin, resulting in hyperbilirubinemia[4,7].

Unconjugated hyperbilirubinemia is seen in newborns (physiological jaundice), in increased red cell destruction (hemolytic anemia, extensive hematoma), in ineffective erythropoiesis and in some rare genetic diseases (Gilbert's syndrome, Crigler-Najjar syndrome).

Conjugated hyperbilirubinemia is associated to a decreased excretion of bile due to liver diseases (hepatitis or cirrhosis) or to intrahepatic or extrahepatic cholestasis.

Jaundice is a clinical manifestation of hyperbilirubinemia, consisting of deposition of bile pigments in the skin, resulting in a yellowish staining of the skin and mucous membranes.

Clinical diagnosis should not be made on the findings of a single test result, but should integrate both clinical and laboratory data.

NOTES

1. For bilirubin determination in newborns, reconstitute the Standard with 1.0 mL of distilled water. The Standard concentration will be that stated on the vial label multiplied by 5. Reduce sample volume (water, standard, serum) to 50 µL and use the concentrated Standard. Method linearity is then doubled (up to 40 mg/dL = 686 µmol/L).
2. It is advisable to wash the Reagent B vial with a small volume of the prepared mixture in order to completely rinse the vial and avoid any losses.
3. These reagents may be used in several automatic analysers. Instructions for many of them are available on request.
4. Calibration with the provided aqueous standard may cause a matrix related bias, specially in some analyzers. In these cases, it is recommended to calibrate using a serum based standard (Biochemistry Calibrator, cod. 18011 and 18044).

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