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United Diagnostics Industry
P. O. Box 9466 - Dammam 31413 - K.S.A.
Tel. : (03) 812 1233 - 812 2004 - Fax : (03) 812 1704
Lic No. 1980 /S www.udignost.com

# BILIRUBIN TOTAL/DIRECT REAGENT SET

MOD. MALLOY-EVELYN COLORIMETRIC/ENDPOINT METHOD

**REF 019**

**FOR IN VITRO DIAGNOSTIC USE**

## INTENDED USE

Quantitative determination of bilirubin in serum using a modified Malloy-Evelyn colorimetric/Endpoint procedure.

## DIAGNOSTIC SIGNIFICANCE

Elevation of Total Serum bilirubin may occur due to (A) an excessive hemolysis or destruction of the red cells, e.g. hemolytic disease of the new born, (B) Liver diseases, e.g. hepatitis and cirrohosis (C) Obstruction of biliary tract, e. g. gall stones[1].

Both conjugated (direct) and unconjugated (indirect) bilirubin are increased, in the serum, in hepatitis. The relative proportion of the conjugated fraction increases with progression of the disease until eventually the liver loses its ability to carry out the conjugation reaction[1].

## EXPECTED VALUES IN SERUM[2].

| | |
|---|---|
| Direct | upto 0.5 mg/dL (8.6μ mol/L) |
| Total | upto 1.0 mg/dL (17.1 μ mol/L) |

## METHOD PRINCIPLE

Bilirubin in the serum is coupled with diazotized sulfanilic acid to form azobilirubin. The intensity of the purple color that is formed is proportional to the bilirubin concentration in the serum. Bilirubin glucuronide, the conjugated or direct bilirubin reacts with the diazo reagent in aqueous solution to form a colored diazo compound within 1 minute. The subsequent addition of methanol accelerates the reaction of unconjugated bilirubin in the serum, and a value of total bilirubin is obtained after letting the sample stand for 5 minutes. The total bilirubin value represents the sum of the bilirubin glucoronide (direct) and the unconjugated (indirect) bilirubin. The color formed in the reaction is measured photometrically at 540/546 nm.

The UDI procedure is a modified Malloy and Evelyn[3] method using absolute methanol as solvent.

## REAGENTS

1. ***SULFANILIC ACID REAGENT:*** 0.5% (w/v) Sulfanilic acid in 0.18 N Hydrochloric acid.Must be kept tightly capped and protected from excessive light.
2. ***SODIUM NITRITE REAGENT :*** 0.5% Sodium nitrite with Stabilizer. Keep tightly capped and protected from contamination.
3. ***METHANOL REAGENT :*** Absolute Methanol, Reagent grade. Must be kept tightly capped and protected against evaporation.
4. ***BILIRUBIN EQUIVALENT STANDARD (5mg/dL Total; 2.5 mg/dL Direct) : A*** solution containing 1.00% (w/v) Cobaltous acetate in 2N Acetic acid . Must be kept tightly capped and protected from excess light.

## REAGENT STORAGE AND STABILITY

Store all reagents in this reagent set at room temperature. All the reagents are stable upto expiration date indicated on the individual bottle label.

## CHEMICAL PRECAUTIONS

SULFANILIC ACID REAGENT contains 0.18 N *Hydrochloric acid* which is **Corrosive**. In case of contact, flush affected area with large amounts of water.METHANOL REAGENT is **poisonous and flammable**. Do not pipette by mouth and do not expose to open flame.

## INDICATIONS OF REAGENT DETERIORATION

**1.** Physical appearance

Darkening of the sodium nitrite reagent or appearance of turbidity that will not readily dissolve are signs of reagent deterioration.

2. Control assays

Failure to obtain accurate results in the assay of control materials may indicate reagent deterioration.

UDI cannot guarantee the stability of reagents which have been:

1. transferred from their original containers.
2. improperly stored.
3. contaminated during use.

## MATERIALS PROVIDED

Sulfanilic Acid Reagent, Sodium Nitrite Reagent, Methanol Reagent Bilirubin Equivalent Standard (5mg/dL Total; 2.5 mg/dL Direct)

## ADDITIONAL MATERIALS REQUIRED, BUT NOT PROVIDED

Reagent and sample pipettes, test vials or cuvettes, timer, test tube rack, control sera, spectrophotometer.

## SPECIMEN

SERUM

While processing samples, protect from direct light as loss of bilirubin may occur.Bilirubin in serum is reportedly stable for 4-7 days, when stored in the dark at 2-8°C.

## PROCEDURE

NOTE: Due to the critical timing of the DIRECT BILIRUBIN REACTION, process each test separately. Serum blank must be prepared for each test and control.

**I MANUAL PROCEDURE (Endpoint with standard)**

λ = 540 nm.

Pipette into clean dry test tubes:

| | TEST BLANK | TEST |
|---|---|---|
| Sulfanilic Acid Reagent | 1.4 ml | 1.4 ml |
| Sodium Nitrite Reagent | -- | 0.025 ml |
| Distilled Water | 0.025 ml | -- |
| Mix and let stand for 1 min. then add (using timed intervals) | | |
| Sample | 0.1 ml | 0.1 ml |
| After exactly I, in. read the absorbance of test blank at 540 nm against dist.water. Use this to calculate Direct Bilirubin, then add: | | |
| Methanol Reagent | 1.5 ml | 1.5 ml |
| Mix by inversion and let stand for at least 5 min. | | |
| Read the absorbance of test and test blank at the same wave-length against dist.water, use this to calculate TOTAL BILIRUBIN. STANDARD: Carefully pour Bilirubin Equivalent Standard into a clean vial, read and record its absorbance aginst distilled water at 540nm. Pour back to its original container. | | |

## CALCULATION OF RESULTS USING STANDARD

**1.**The absorbance of Bilirubin Equivalent Standard represents:
Direct Bilirubin = 2.5 mg/dL
Total Bilirubin = 5.0 mg/dL

**2.** Direct Bilirubin (1minute reading):

$$\frac{\text{Abs (Test) - Abs (Test Blank)}}{\text{Abs (Bilirubin Equivalent Standard)}} \times 2.5 \text{ mg/dL} = \text{Direct Bilirubin (mg/dL)}$$

3. Total Bilirubin (5 minutes reading)

$\frac{\text{Abs (Test) - Abs (Test Blank)}}{\text{Abs (Bilirubin Equivalent Standard)}}$ x 5.0 mg/dL = Total Bilirubin (mg/dL)

4. To convert mg/dL to µmol/L, multiply the final results by 17.1

**EXAMPLE OF CALCULATION WITH STANDARD**

Assume that the 1 minute reading for the test = 0.079 and the test blank gave an absorbance of 0.04, while the absorbance of standard = 0.140. The Direct Bilirubin would then be calculated as follow:

$\frac{0.079 - 0.04}{0.140}$ x 2.5 mg/dL = 0.696 mg/dL or 11.9 µmol/L.

Assume that the 5 minutes reading for the test = 0.13 and the test blank gave an absorbance of 0.03, while the absorbance of standard = 0.140. TheTotal bilirubin would then be calculated as follows:

$\frac{0.13 - 0.03}{0.140}$ x 5 mg/dL = 3.57 mg/dL or 61.1 µmol/L.

**II MANUAL/SEMIAUTOMATED PROCEDURE**

**(Endpoint with Factor)** λ=546 nm

Perform Direct Bilirubin and Total Bilirubin separately.

Pipette into clean dry test tubes:

| | DIRECT BILIRUBIN | | TOTAL BILIRUBIN | |
|---|---|---|---|---|
| | Test Blank | Test | Test Blank | Test |
| Sulfanilic Acid Rgnt | 1.0 ml | 1.0 ml | 0.5 ml | 0.5 ml |
| Sodium Nitrite Rgnt | -- | 0.02 ml | -- | 0.02 ml |
| Mix and let stand for at least 1 min but no longer than 3 min., then add: (Use times intervals for Direct Bilirubin) | | | | |
| Sample | 0.05 ml | 0.05 ml | 0.05 ml | 0.05 ml |
| After exactly 1 min. read the absorbance of Test and Test Blank (of Direct bilirubin only) at 546 nm against distilled water. For Total Bilirubin add: | | | | |
| Methanol | -- | -- | 0.5 ml | 0.5 ml |
| Mix and let stand for 5 min. at room temperature and read absorbance of test and test Blank (of Total Bilirubin) at 546 nm against distilled water. | | | | |

**CALCULATION OF RESULTS**

USING FACTOR

**1.Direct Bilirubin**

(Abs. Test - Abs. Test Blank) x 25 = Direct Bilirubin (mg/dL).

**2. Total Bilirubin**

(Abs. Test - Abs. Test Blank) x 25 = Total Bilirubin (mg/dL).

3. To convert mg/dL to µmol/L multiply the final results by 17.1.

**EXAMPLE OF CALCULATION WITH FACTOR**

**Direct Bilirubin** Assume that the 1 minute absorbance reading for Test Blank is 0.020 and for Test is 0.048. The Direct Bilirubin would then be calculated as

(0.048 - 0.020) x 25 =0.7 mg/dL or 11.97 µmol/L.

**Total Bilirubin**: Assume that the 5 minute absorbance reading for Test Blank is 0.025 and for Test is 0.168. The Total Bilirubin would then be calculated as (0.168 - 0.025) x 25 =3.57 mg/dL or 61.1 µmol/L.

**STABILITY OF END POINT REACTION**

Direct Bilirubin color formation is stable only for about 60 seconds whereas the total Bilirubin color formation is stable for atleast 60 minutes.

**PERFORMANCE CHARACTERISTICS**

***1. LINEARITY***

This method is linear for total bilirubin upto 20 mg/dL.

**2. TOTAL BILIRUBIN**

COMPARISON : UDI reagent tested on manual instrument(y) was compared with similar UDI reagent tested on other systems(x). The systematic difference between the results were within CLIA specified limits,

N = 25

Correlation Coefficient 0.970

Regression Equation y=0.91x+0.39

**PRECISION:**

| | Mean mg/dL | S.D. | C.V.% |
|---|---|---|---|
| Within run | 5.2 | 0.23 | 4.43 |
| Run to run | 4.6 | 0.22 | 4.91 |

**3. DIRECT BILIRUBIN**

COMPARISON : UDI reagent tested on manual instrument(y) was compared with similar UDI reagent tested on other systems(x). The systematic difference between the results were within CLIA specified limits, N = 25

Correlation Coefficient 0.994

Regression Equation y=0.943x+0.14

**PRECISION:**

| | Mean mg/dL | S.D. | C.V.% |
|---|---|---|---|
| Within run | 1.9 | 0.08 | 4.54 |
| Run to run | 2.3 | 0.07 | 3.01 |

**PROCEDURE NOTES**

1. If the Bilirubin values exceed 20 mg/dL in the sample or if the linear capability of the photometer is exceeded, the sample should be retested using a 1:1 dilution with distilled water. Multiply the results by 2.
2. Hemolysis interferes with the reaction to give falsely decreased values,severe lipemia may also cause loss of precision[4].
3. The following substances have been reported to contribute to falsely elevated results: Urea, histidine, indican, tyrosine,[5] aminophenol, epinephrine, isoproterenol[6] L-DOPA, methyldopa[7] and dextran, which causes turbidity with methanol. Metabolites of novobiocin and theophylline reportedly depress color formation[8].

**QUALITY CONTROL**

For accuracy and precision check, we recommend the use of normal and abnormal UDI controls based on human serum.

ORDERING INFORMATION:

**UDITROL 'N' (Normal Serum Control) REF # 070N-010 2x5 ml**

**UDITROL 'A' (Abnormal Serum Control) REF # 070A-010 2x5 ml**

**REFERENCES**


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## PRODUCT AVAILABILITY

### BILIRUBIN TOTAL /DIRECT (Colorimetric)

| | |
|---|---|
| **REF # 019-160** | **2 x 225 ml** |
| **REF # 019-085** | **2 x 120 ml** |
| **REF # 019-400** | **5 x 250 ml** |

EC REP
(Authorized representative in the European Community)

mdi Europa GmbH
Wittekamp 30
D-30163 Hannover
Germany

CE