POINTE SCIENTIFIC, INC.
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Total Bilirubin Reagent Set

Intended Use
For the quantitative determination of total bilirubin in serum.

Method History
A reaction in which bilirubin is coupled with diazotized sulfanilic acid (ρ-diazobenzenesulfonic acid) to produce an azo dye was first described by Ehrlich in 1884.[1] The color of this derivative is pink in an acid medium and blue in an alkaline one. The measurement of the blue form has been more popular because of greater sensitivity, etc.

Two types of serum bilirubin can be distinguished and quantitated by the diazo reaction. The *direct* form, consists of conjugated, water-soluble derivatives and reacts in the absence of an accelerating or solubilizing agent. The *indirect* form, consists of free, unconjugated bilirubin bound to serum albumin. This form only reacts in the presence of an accelerating agent. The sum of these two forms is termed *total* bilirubin. The differentiation between direct and indirect is important in diagnosing causes of hyperbilirubinemia.

Many substances promote the coupling of diazo reagent with unconjugated bilirubin. Methanol was first suggested by Evelyn and Malloy[2] in the 1930's with caffeine/sodium benzoate of Jendrassik and Grof[3] a little later. Many modifications and improvements on the above two methods have been reported to date.[4,5,6]

Other solubilizing and accelerating agents have also been tried including 2-methoxyethanol[7] and urea.[8] Dimethyl sulfoxide (DMSO) was investigated as a possible solvent for total bilirubin in 1968.[9] The present procedure uses DMSO based on a modification of Walters and Gerarde.[10] The method is sensitive, accurate, and easy to perform. It compares very favorably with that of Evelyn and Malloy and Jendrassik and Grof.

Principle
Sulfanilic acid reacts with sodium nitrite to produce diazotized sulfanilic acid (diazo). Direct and indirect bilirubin couple with diazo to produce azobilirubin in the presence of dimethyl sulfoxide (DMSO). The intensity of the color produced is directly proportional to the amount of total bilirubin concentration present in the sample.

Reagents
1. Total bilirubin reagent: Sulfanilic acid 16 mM, hydrochloric acid 164mM, dimethyl sulfoxide 4.4M, surfactant.
2. Sodium nitrite reagent: Sodium nitrite 0.6M.

Reagent Preparation
Total bilirubin working reagent: Add 0.01 ml (10ul) of nitrite reagent per 1.0 ml of total bilirubin reagent. Mix.

Example: 0.1ml nitrite/10ml total bilirubin reagent, 1 ml nitrite/100ml total bilirubin reagent, etc.

Reagent Storage
1. Packaged reagents are stored at 2-8°C.
2. Combined working reagent can be stored in an amber bottle for up to 48 hours at room temperature and 30 days refrigerated.
3. Do not freeze reagents.
4. Avoid exposure to direct sunlight.

Reagent Deterioration
Do not use if:
1. Sodium nitrite reagent has a dark yellow discoloration.
2. Working reagent fails to achieve assigned assay values of fresh control sera.

NOTE: Working reagent will normally develop a light yellow/orange color upon standing.

Precautions
1. Reagents are toxic and corrosive. Do not pipette by mouth. Avoid contact with skin and clothing.
2. This reagent is for *in vitro* diagnostic use only.

Specimen Collection and Storage
1. Fresh, unhemolyzed serum is recommended.
2. Samples should be analyzed within two hours of collection if kept at room temperature in the dark and within twelve hours if kept refrigerated (2-8°C) and protected from light.[11]
3. Bilirubin in serum is stable for three months when stored frozen (-20°C) and protected from light.[11]
4. Direct sunlight may cause up to a 50% decrease in bilirubin within one hour.[12]

Interferences
A number of drugs and substances affect bilirubin results. See Young, et al.[13]

Materials Provided
1. Total bilirubin reagent.
2. Sodium nitrite reagent.

Materials Required but not Provided
1. Accurate pipetting devices.
2. Timer.
3. Test tubes/rack
4. Spectrophotometer with ability to read 555 nm (540-560 nm).

Procedure (Automated)
Refer to specific instrument application instructions.

Procedure (Manual)

	Test	Blank
Working reagent (ml)	1.0	------
Total bilirubin reagent (ml)	------	1.0
Sample (ml)	0.05	0.05

1. Label test tubes: "Blank", "Standard", "Control", "Patient", etc. Each tube also requires a blank tube.
2. Pipette 1.0ml of total bilirubin reagent to all blank tubes.
3. Prepare a working reagent. See "Reagent Preparation".
4. Pipette 1.0ml of the working reagent to all test tubes.
5. At timed intervals add 0.05ml (50ul) of sample to respective tubes. Mix.
6. Allow all tubes to stand for exactly for five minutes at room temperature (one minute at 37°C).
7. Zero spectrophotometer with reagent blank at 555nm (540-560nm).
8. Read and record the absorbance of all tubes.
9. See "Calculations" to obtain results.

Procedure Notes
1. Final color is stable for thirty minutes.
2. For instruments that require a total volume greater than 1.0 ml for accurate reading, use 3.0 ml reagent and 0.200 ml (200ul) of sample. Follow above directions.

Pediatric Volumes
For pediatric samples with bilirubin over 3.0 mg/dl, run a 1:1 dilution with saline. Multiply result by two.

Calibration
Use an appropriate standard or serum calibrator.

Calculations
Abs. = Absorbance

$$\frac{\text{Abs. of Unk.} - \text{Abs. of Unk. Blank}}{\text{Abs. of Calib.} - \text{Abs. of Cal. Blank}} \times \text{Conc. of Calib. (mg/dl)} = \text{Total Bilirubin (mg/dl)}$$

Sample: If Abs. of Unknown = 0.35, Abs. of Unknown Blank = 0.01, Abs. of Calibrator 0.25, Abs. of Calibrator Blank = 0.01, Concentration of Calibrator = 5.0 mg/dl

Then: $\frac{0.35 - 0.01}{0.25 - 0.01} \times 5 = \frac{0.34}{0.24} \times 5 = 7.1$ mg/dl

Quality Control
The integrity of the reaction should be monitored by use of control sera (normal and abnormal) with known bilirubin concentrations.

Expected Values[14]
Total: Adults 0.2 –1.2 mg/dl

Limitations
1. Serums with values above 20 mg/dl must be diluted 1:1 with isotonic saline, re-assayed and the final answer multiplied by two.
2. Serum hemoglobin levels of up to 1.0 g/dl do not interfere with results.

Performance
1. Linearity: 20 mg/dl
2. Comparison: Testing performed between this and a similar method yielded a coefficient of correlation of 0.987 with a regression equation of y=0.98x + 0.02.
3. Precision:

Within Run			Run to Run		
Mean	S.D.	C.V.%	Mean	S.D.	C.V.%
0.9	0.05	5.6	0.9	0.05	5.6
7.7	0.05	0.6	7.9	0.14	1.8

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