

Intended Use

For the quantitative determination of direct bilirubin in serum.

Method History

A reaction in which bilirubin is coupled with diazotized sulfanilic acid (*p*-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 reacts quickly 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.

Principle

Sulfanilic acid reacts with sodium nitrite to produce diazotized sulfanilic acid (diaz). Direct bilirubin couples with diazo to produce azobilirubin. The intensity of the color produced is directly proportional to the amount of direct bilirubin present in the sample.

Reagents

1. Direct bilirubin reagent: Sulfanilic acid 32 mM in dilute hydrochloric acid.
2. Sodium nitrite reagent: Sodium nitrite 60mM.

Reagent Preparation

Direct Bilirubin working reagent: Add 0.005ml (5ul) of nitrite reagent per 1.0ml of direct bilirubin reagent. Mix. Example: 0.05ml (50ul) nitrite / 10ml direct bilirubin reagent, 0.10 (100ul) nitrite / 20ml direct bilirubin reagent.

Reagent Storage

1. Packaged reagents may be stored at room temperature.
2. Combined working reagent can be stored for up to 24 hours when kept in an amber bottle at room temperature or 10 days at 2-8°C.
3. Do not freeze reagents.
4. Avoid exposure to direct sunlight.

Reagent Deterioration

Do not use if:

1. Sodium nitrite reagent has a yellow discoloration.
2. Working reagent fails to achieve assigned assay values of fresh control sera.

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
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.³

3. Bilirubin in serum is stable for three months when stored frozen (-20°C) and protected from light.³
4. Direct sunlight may cause up to a 50% decrease in bilirubin within one hour.⁴

Interferences

A number of drugs and substances affect bilirubin results. See Young, et al.⁵

Materials Provided

1. Direct 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)
5. Bilirubin calibrator

Procedure (Automated)

Refer to specific instrument application instructions.

Procedure (Manual)

	<u>Test</u>	<u>Blank</u>
Working reagent (ml)	1.0	-----
Direct bilirubin reagent (ml)	-----	1.0
Sample (ml)	0.10	0.10

1. Label test tubes: "Blank", "Standard", "Control", "Patient", etc. Each tube also requires a blank tube.
2. Pipette 1.0ml of direct 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 0.10ml (100ul) 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.

NOTE: Color will continue to increase slowly due to the presence of the indirect fraction. Timing must be exact for precise measurement of the direct fraction.

Pediatric Volumes

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

Alternative Volumes

For instruments that require a total volume greater than 1.0ml for accurate reading use 3.0ml reagent and 0.200ml (200ul) sample. Follow above directions.

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.

Direct Bilirubin Reagent Set

Calibration

Use an appropriate serum based calibrator.

Calculations

Abs. = Absorbance

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

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 = 4.0 mg/dl

$$\text{Then: } \frac{0.35 - 0.01}{0.25 - 0.01} \times 4 = \frac{0.34}{0.24} \times 4 = 5.7 \text{ mg/dl}$$

Quality Control

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

Expected Values (Direct)^{4,6}

Adults and infants (over one month): 0 – 0.5 mg/dl

It is strongly recommended that each laboratory establish its own normal range.

Performance

1. Linearity: 20 mg/dl
2. Comparison: Testing performed between this and a similar method yielded a coefficient of correlation of 0.998 with a regression equation of $y = 1.04x + 0.07$.
3. Precision:

Within Run			Run to Run		
Mean	S.D.	C.V.%	Mean	S.D.	C.V.%
0.98	.025	2.6	0.96	.028	2.9

References

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4. Tietz, N.W., Fundamentals of Clinical Chemistry, Philadelphia, W.B. Saunders, p.1028 (1976).
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6. Gambino, S.R., et al, Bilirubin Assay (Revised), Commission on Continuing Education, Am. Soc. of Clin. Path., Chicago, (1968).

Manufactured by Pointe Scientific, Inc.
5449 Research Drive Canton, MI 48188

"European Authorized Representative"
(O.E.A.R.C.) Av. De Tervueren 34 bte
44 B-1040 Brussels, Belgium



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