POINTE SCIENTIFIC, INC.
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Direct Bilirubin Reagent Set

Intended Use
For the quantitative determination of direct bilirubin in serum. For *in vitro* diagnostic use only.

Method History
Since the introduction of the diazo method for bilirubin determination by Ehrlich in 1883,[1] several modifications have been proposed to enhance the reaction. The Malloy and Evelyn method[2] employs methanol to catalyze the azo-coupling reaction of the indirect Bilirubin, as well as to keep the azobilirubin in solution. A serious disadvantage of this method lies in the fact that protein may be precipitated by the methanol solution to yield falsely lowered results.

In 1938, Jendrassik and Grof.[3] presented an assay that gave reliable results. The method is, however, cumbersome and involves several pipetting steps.

The method presented here was developed by Wahlefeld et al.[4]The diazo reagent is 2,5-dichlorophenyldiazonium tetrafluoroborate (DPD) which reacts very rapidly in coupling with Bilirubin under acidic conditions. The resulting procedure is simple, yet exhibits good correlation when compared with the method of Jendrassik and Grof.

Principle
Direct Bilirubin is coupled with a diazonium salt (DPD) in a strongly acid medium (pH 1 – 2).

$$\text{Bilirubin} + \text{DPD} \xrightarrow{\text{acid}} \text{Azobilirubin}$$

The intensity of the color of the azobilirubin produced is proportional to the Direct Bilirubin concentration and can be measured photometrically.

Reagents
1. Direct Bilirubin R1 reagent: acid buffer 50 mmol/L
2. Direct Bilirubin R2 reagent: acid buffer >30 mmol/L, >2.0 mmol/L DPD and stabilizers

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.

Reagent Preparation
Reagents are supplied ready to use.

Reagent Storage
1. Packaged reagents may be stored at 2-8°C
2. Do not freeze reagents.
3. Avoid exposure to direct sunlight.

Reagent Deterioration
1. Do not use if reagents show evidence of microbial contamination (turbidity).
2. If the R2 develops very slight precipitation that re-dissolves when the R2 is warmed gently, the reagent may be used.
3. R2 reagent containing a precipitate that does not re-dissolve and results in product discoloration should not be used.
4. Do not use if reagent fails to achieve assigned assay values of fresh control sera.

Specimen Collection and Storage
1. Fresh, unhemolyzed serum is recommended.[5]
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.[6]
3. Bilirubin in serum is stable for three months when stored frozen (-20°C) and protected from light.[6]
4. Direct sunlight may cause up to a 50% decrease in bilirubin within one hour.[7]
5. Specimen collection should be carried out in accordance with NCCLS M29-T2. No method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood samples should be considered potentially infectious.

Interferences
1. All interference studies were performed according to the procedures recommended in NCCLS guideline No. EP7-P for interference testing in clinical chemistry.[8]
2. Hemoglobin has been found to interfere with this assay. Fresh, unhemolyzed serum is recommended.
3. Serum Triglycerides up to 500 mg/dl do not interfere with results.
4. A number of drugs and substances affect bilirubin results. See Young, et al.[9]

Materials Provided
Direct bilirubin reagents R1 and R2.

Materials Required but not Provided
1. Controls
2. Calibrator
3. Beckman Coulter AU™ analyzer
4. Application and instrument manuals

Procedure (Beckman Coulter AU™400 application)

SPECIFIC TEST PARAMETERS

TEST NUMBER: **#** TEST NAME: **D. Bilirubin** ∇ TYPE: **Serum** ∇ OPERATIONAL: **Yes** ∇
SAMPLE VOL.: **6** DIL. VOL.: **0** PRE-DILUTION RATE: **1**
REAGENTS: R1 VOLUME: **140** DIL. VOL.: **0** MIN. OD MAX. OD
R2 VOLUME: **35** DIL. VOL.: **0** L H
REAGENT OD LIMIT:
WAVELENGTH: PRI. **540** ∇ SEC. **660** ∇ FIRST L: **-0.100** FIRST H: **0.400**
METHOD: **END** ∇ LAST L: **-0.100** LAST H: **0.400**
REACTION SLOPE: **+** ∇ DYNAMIC RANGE: **#**
MEASURING POINT 1: FIRST: **0** LAST: **27** L: **#** H: **#**
MEASURING POINT 2: FIRST: **0** LAST: **9** CORRELATION FACTOR:
LINEARITY: % A: **1.000** B: **0.000**
NO LAG TIME: ∇ ON BOARD STABILITY PERIOD: **#**

SPECIFIC TEST PARAMETERS

VALUE FLAG: **#** ∇ LEVEL L: **#** LEVEL H: **#**

NORMAL RANGES:	SEX	AGE L YEAR	AGE L MONTH	AGE H YEAR	AGE H MONTH	L	H
○ 1.	# ∇	#	#	#	#	#	#
○ 2.	# ∇	#	#	#	#	#	#
○ 3.	# ∇	#	#	#	#	#	#
○ 4.	# ∇	#	#	#	#	#	#
○ 5.	# ∇	#	#	#	#	#	#
○ 6.	# ∇	#	#	#	#	#	#
7. NONE SELECTED						#	#
8. OUT OF RANGE		L	H			#	#

PANIC VALUE: **#** **#** **#** UNIT: **mg/dl** DECIMAL PLACES: **1**

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CALIBRATION SPECIFIC PARAMETERS

CAL TYPE: **AB** ∇ FORMULA **Y=AX+B** ∇ COUNTS: **2** PROCESS: **CONC.** ∇

	CAL. NO.	OD	CONC.	FAC/OD-L	FAC/OD-H
POINT 1.	**#**		**#**	**-9999999**	**9999999**
POINT 2.					
POINT 3.					
POINT 4.					
POINT 5.					
POINT 6.					
POINT 7.					

1-POINT CAL. POINT: ○ WITH CONC-0

MB TYPE FACTOR: CALIBRATION STABILITY PERIOD: **#**

#: User-Defined

The above reagent parameters are intended to serve as a guide for use with Pointe Scientific, Inc. reagent. The parameters are based on data generated by Pointe Scientific, Inc. Please note: These parameters should be used in conjunction with your laboratory Quality Control Program for validation.

NOTE: For other instrument specific applications please contact Pointe Scientific, Inc. Technical Service Department at 1-800-445-9853

Pediatric Volumes

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

Calibration

Follow instrument application instructions for calibration. Refer to instrument manual instructions for calibration procedures and frequency. It is recommended that each laboratory determine its own frequency of calibration.

Quality Control

The validity of the reaction should be monitored by use of the control sera with known normal and abnormal direct bilirubin values. These controls should be run at least with every working shift in which direct bilirubin assays are performed. It is recommended that each laboratory establish its own frequency of control determination.

Calculations

Abs. = Absorbance
Unk. = Unknown
Cal. = Calibrator

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

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

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

Expected Values (Direct)[7,10]

Adults and infants (over one month): 0 – 0.5 mg/dl
It is strongly recommended that each laboratory establish its own normal range.

Limitations

1. Samples with values above 10 mg/dl must be diluted 1:1 with isotonic saline, re-assayed and the final answer multiplied by two.
2. Hemoglobin has been found to interfere with this assay.
3. Triglycerides up to 500 mg/dl do not interfere with results.

Performance

1. Assay Range: 0.0 - 10.0 mg/dl
2. Comparison: A comparison study performed between the Beckman Coulter AU400 and Roche Hitachi 717 using this method resulted in a correlation coefficient of r = 0.950 with a regression equation of y=1.092x+0.10. (n = 38, range 0.0 – 1.3 mg/dl)
3. Precision:
   Within - day precision study was performed using three levels of material.
   Between - day precision study was performed using two levels of control material with 2 runs per day and 2 replicates per run.

Within Day (N=20)			Day to Day		
Mean	**S.D.**	**C.V.%**	**Mean**	**S.D**	**C.V.%**
0.7	0.00	0.0	0.7	0.05	7.1
4.2	0.15	3.6	4.6	0.30	6.5
7.2	0.11	1.5			

Precision and Linearity studies were performed following modifications of CLSI Protocols EP5 and EP6[11] using a Beckman Coulter AU™400 analyzer

References

1. Ehrlich, P., Charite Ann. 8:140(1883).
2. Malloy, H.T., Evelyn, K.A., J. Biol. Chem. 119:481 (1937).
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4. Wahlefeld AW, et al. Scand J Clin Lab Invest. 29 Supplement 126(1972).
5. Michaelsson, M. Scand. J. Clin. Lab. Invest (Suppl. 49) 13:1 (1961)
6. Martinek, R.G., Clin. Chem. Acta 13:161 (1966).
7. Tietz, N.W. Fundamentals of Clinical Chemistry, Philadelphia, W.B. Saunders, P. 1028 (1976).
8. NCCLS document, "National Evaluation Protocols for Interference Testing", Evaluation Protocol Number 7, Vol. 4, No. 8, (June 1984).
9. Young, D.S., Effects of Preanalytical Variables on Clinical Laboratory Tests, Washington DC, AACC Press, (1997)
10. Gambino, S.R., et al, Bilirubin Assay (Revised), Commission on Continuing Education, Am. Soc. of Clin. Path., Chicago, (1968).
11. CLSI protocols EP5-A2, Vol. 24 No. 25, 2nd Ed, (2004) and CLSI EP6-A, Vol. 23 No. 16 (2003)

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Rev. 02/13 P803-OB936-01