

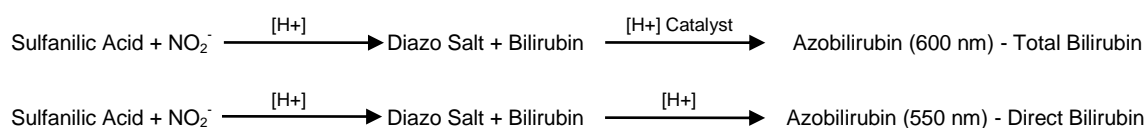
Bilirubin (Total and Direct) Colorimetric Assay Kit

(Catalog # K553-100; 100 assays; Store at -20 °C)

rev 01/21

I. Introduction:

Bilirubin, a degradation product of heme catabolism, is a non-polar molecule. There are two forms of bilirubin: water-soluble (conjugated or direct) and water-insoluble (unconjugated or indirect) bilirubin. Bilirubin is produced in the endoplasmic reticulum as unconjugated bilirubin, which binds to albumin in plasma and forms albumin-bilirubin complex. This complex is transported to the liver, where it is conjugated with glucuronic acid and forms conjugated bilirubin. Bilirubin has potent antioxidant, anti-inflammatory and autoimmune properties. Bilirubin concentration in human body depends on gender, drug intake, age, etc. Low serum bilirubin is directly correlated with pathological conditions including diabetes mellitus, metabolic syndrome, and cardiovascular diseases. However, high bilirubin indicates hemolysis, jaundice, Gilbert's syndrome, hepatitis, drug toxicity, and possible blockage of bile ducts. **BioVision's Bilirubin Assay Kit** utilizes the Jendrassik-Grof principle to detect bilirubin. Total bilirubin (unconjugated + conjugated) concentration is determined in the presence of a catalyst, where bilirubin reacts with a diazo- salt to form azobilirubin, which absorbs at 600 nm. Direct bilirubin (conjugated) is determined in the absence of catalyst (550 nm).



II. Application:

- Measurement of bilirubin concentration in serum

III. Sample Type:

- Biological fluids: Serum

IV. Kit Contents:

Components	K553-100	Cap Code	Part Number
Bilirubin Reagent 1	2.5 ml	NM	K553-100-1
Bilirubin Reagent 2	1 ml	Red	K553-100-2
Catalyst	15 ml	NM	K553-100-3
Total Bilirubin Probe	10 ml	NM	K553-100-4
Direct Bilirubin Probe	20 ml	WM	K553-100-5
Bilirubin Standard (0.2 µg/µl)	2 x 200 µl	Yellow	K553-100-6
DMSO (Anhydrous)	3.5 ml	Amber/NM	K553-100-7

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer or plate reader

VI. Storage Conditions and Reagent Preparation:

Store kit at -20 °C, protected from light. Briefly spin small vials prior to opening. Read the entire protocol before performing the assay. Once opened, all kit components can be stored and used for up to 6 months.

- **Bilirubin Reagent 1:** Bring to room temperature (RT) before use. Store at -2 0°C.
- **Bilirubin Reagent 2:** Ready to use as supplied. Light sensitive. Warm to RT before use. Store at -20 °C.
- **Catalyst:** Ready to use as supplied. Bring to RT before use. Store at -20 °C.
- **Total Bilirubin Probe:** Bring to RT before use. Store at -20 °C.
- **Direct Bilirubin Probe:** Ready to use as supplied. Warm to RT before use. Store at -20 °C.
- **Bilirubin Standard:** Ready to use as supplied. Bring to RT before use. **Light and Oxygen sensitive. Aliquot into amber vials and store at -20 °C.**
- **DMSO:** Warm to RT to dissolve the DMSO completely before use.

****All incubations are performed at RT (~25 °C), protected from light.**

VII. Bilirubin Assay Protocol:

1. **Sample Preparation:** Add 2-50 µl of undiluted serum to desired well(s) in a 96-well plate. Adjust the volume to 50 µl/well with 50% DMSO (mix 500 µl 100% DMSO (provided) and 500 µl ddH₂O for about 20 wells).

Notes:

- a). Bilirubin concentration varies over a wide range depending on the patient's age, gender, and pathological conditions. In healthy patients, bilirubin concentrations (in mg/dl) are: Total: (0.1- 1.2); Indirect: (0.1- 0.7); Direct: (0.1- 0.4).
- b). For Unknown Samples, we recommend doing a pilot experiment & testing several doses to ensure the readings are within the Standard Curve range.

2. Standard Curve Preparation: Dilute 50 μl of 0.2 $\mu\text{g}/\mu\text{l}$ Bilirubin Standard with 50 μl 100% DMSO (provided). Add 0, 2.5, 5, 10, 20, 40 μl of the diluted Bilirubin Standard (0.10 $\mu\text{g}/\mu\text{l}$) into a series of wells in a 96-well plate to generate 0, 0.25, 0.5, 1, 2, and 4 μg of Bilirubin/well. Adjust the final volume to 50 μl /well with 50% DMSO.

3. Preparation of Reagent Mix: Mix enough reagents for the total number of well(s) to be assayed including Standards, Samples, and Background Controls just before starting the assay.

	<u>Standard/Sample</u>	<u>Background Control</u>
Bilirubin Reagent 1	20 μl	20 μl
Bilirubin Reagent 2	5 μl	—
ddH ₂ O	—	5 μl

4. Total Bilirubin Assay: Add 100 μl of Catalyst to each well, mix well and incubate for at least 15 min at RT, protected from light. Then add 25 μl of Reagent Mix to each well as shown above, mix and incubate for 15 min at RT, protected from light. Add 75 μl of Total Bilirubin Probe to all Standards, Samples, and Background Control wells. Mix well and incubate the plate for 15 min at RT, protected from light. Record the endpoint absorbance at 600 nm on a plate reader.

5. Direct Bilirubin Assay: Add 100 μl of 50% DMSO to each well and mix well. Add 25 μl of Reagent Mix to each well, mix and incubate for at least 30 min at RT, protected from light. Add 75 μl of Direct Bilirubin Probe to all Standards, Samples, and Background Control wells. Incubate the plate for 15 min at RT, protected from light and record the endpoint absorbance at 550 nm in a plate reader.

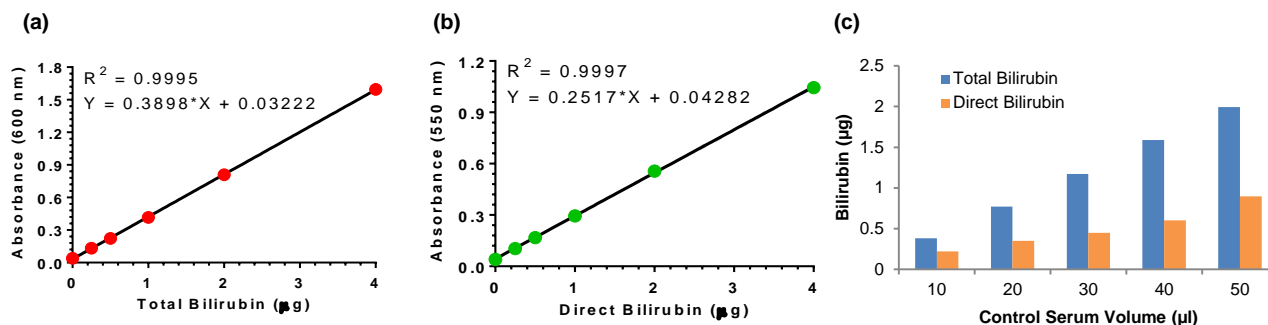
6. Calculation: Subtract 0 Total Bilirubin Standard reading from all Standard readings. Plot the Linear Total Bilirubin Standard Curve. If the Sample Background Control is significant (i.e. if Sample Background Control for Total Bilirubin/Direct Bilirubin has intrinsic high absorbance at 600/550 nm), then subtract Sample Background Control reading from Sample readings. Apply the corrected OD to the Total Bilirubin Standard Curve to get B μg of Total Bilirubin in the Sample well. Likewise, apply the corrected Sample OD to the Direct Bilirubin Standard Curve to get B μg of Direct Bilirubin in the Sample well (Fig. b).

$$\text{Sample Total or Direct Bilirubin Concentration (C)} = \text{B/V} \times \text{D } \mu\text{g}/\mu\text{l}$$

Where: **B** is the amount of Total/Direct Bilirubin in the sample well (μg)
V is the sample volume added into the reaction well (μl)
D is the sample dilution factor

Total Bilirubin = Unconjugated Bilirubin + Conjugated Bilirubin

Bilirubin Molecular Weight: 584.7 kDa
 10 mg/ml \equiv 10 $\mu\text{g}/\mu\text{l}$ \equiv 10000 $\mu\text{g}/\text{ml}$ \equiv 1 g/dl



Figures: (a). Total Bilirubin Standard Curve (0-4 μg). (b). Direct Bilirubin Standard Curve (0-4 μg). (c). Total and Direct Bilirubin concentration in normal human serum. Different volumes of human serum (10- 50 μl) were assayed following kit protocols. Reported concentrations (in mg/dl): Total Bilirubin: 4; Direct Bilirubin: 1.5. Experimental concentrations (calculated as the average of estimated bilirubin in five different human serum volumes ranging from 10 to 50 μl): Total: 3.8 mg/dl; Direct: 1.75 mg/dl.

VII. Related Products:

Albumin (BCG) Colorimetric Assay Kit (K554)
 Human Serum Albumin (4016)
 Albumin, Human plasma (7546)

FOR RESEARCH USE ONLY! Not to be used on humans.