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Bilirubin ELISA Kit

12/19

(Catalog # E4794-100; 96 assays, Storage at 4°C)

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 ELISA Kit is based on Competitive ELISA principle. The micro-plate provided in this kit has been pre-coated with Bilirubin. During the reaction, Bilirubin in the samples or standard competes with Bilirubin coated on the plate for binding to the anti- Bilirubin antibody. Then Horseradish Peroxidase (HRP) conjugate is added to each micro plate well, and TMB substrate is for color development. There is a negative correlation between the OD value of samples and the concentration of Bilirubin. The concentration of Bilirubin in the samples can be calculated by comparing the OD of the samples to the standard curve.

II. Applications:

in vitro quantitative determination of Bilirubin concentrations in serum, plasma and other biological fluids.
Sensitivity: 0.19 µg/mL
Detection Range: 0.31-20 µg/mL

Specificity: No Significant cross-reactivity or interference between Bilirubin and analogues was observed.

Precision: Coefficient of variation is < 10%.

III. Sample Type:

Serum, plasma and other biological fluids

IV. Kit Contents:

Components	E4794-100	Part Number	Storage
Micro ELISA Plate	8 wells x12 strips	E4794-100-1	-20°C
Reference Standard	2 vials	E4794-100-2	-20°C
Biotinylated Detection Ab (100x)	120 µl	E4794-100-3	-20°C
HRP Conjugate (100x)	120 µl	E4794-100-4	-20°C (protect from light)
Reference Standard & Sample Diluent	20 ml	E4794-100-5	4°C
Biotinylated Detection Antibody Diluent	14 ml	E4794-100-6	4°C
HRP Conjugate Diluent	14 ml	E4794-100-7	4°C
Wash Buffer (25X)	30 ml	E4794-100-8	4°C
Substrate Reagent	10 ml	E4794-100-9	4°C (protect from light)
Stop Solution	10 ml	E4794-100-10	4°C
Plate Sealer	4	E4794-100-11	4°C

V. User Supplied Reagents and Equipment:

- Microplate reader capable of measuring absorbance at 450 nm
- Clean Eppendorf tubes for preparing standards or sample dilutions

VI. Storage and Handling:

Store at 4ºC**.**

VII. Reagent and Sample Preparation:

Bring all reagents to room temperature before use. Before using the kit, spin tubes and bring down all components to the bottom of tubes.

- **Wash Buffer (25X):** Dilute 30 ml of Concentrated Wash Buffer with 720 ml of deionized or distilled water to prepare 750 ml of Wash Buffer. (Note: if crystals have formed in the concentrate, warm it in a 40°C water bath and mix it gently until the crystals have completely dissolved)

- **Biotinylated Detection Antibody working solution:** Calculate the required amount (50 µL/well). Centrifuge the stock tube before use; dilute the 100x Concentrated Biotinylated Detection Antibody to 1x working solution with Biotinylated Detection Antibody Diluent.

- **HRP Conjugate working solution:** Calculate the required amount before the experiment (100 µL/well). In preparation, slightly more than calculated should be prepared. Dilute the 100x Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

- **Standard:** Centrifuge the standard at 10,000xg for 1 min. Add 1.0 ml of Standard and Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 20 μg/ml. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0 μg/ml. Prepare 7 tubes, add 500 μl of Standard and Sample Diluent to each tube. Pipette 500 μl of the 20 μg/ml stock solution to the first tube and mix up to produce a 10 μg/ml working solution. Transfer 500 μl of the solution into the other tube to form 2-fold serial dilutions of the highest standards to make the standard curve within the range of this assay.

VIII. Sample Preparation:

- **Serum:** Allow samples to clot for 2 hours at room temperature or overnight at 2-8°C before centrifugation for 15 min at 1000xg at 2-8°C. Collect the supernatant. Blood collection tubes should be disposable and be endotoxin free.

- **Plasma:** Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000xg at 2-8℃ within 30 min of collection. Collect the supernatant. **Hemolysed samples are not suitable for ELISA assay!**

- **Cell lysates:** For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000xg. Discard the medium and wash the cells 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 μl of pre-cooled PBS to keep the cells suspended. Repeat the freeze-thaw process several times until the cells are fully lysed. Centrifuge for 10 min at 1500xg at 2-8℃. Remove the cell fragments; collect the supernatant to carry out the assay. Avoid repeated freeze-thaw cycles.

- **Tissue homogenates:** It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolysed blood may affect the results, so the tissues should be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 min at 5000xg to get the supernatant.

- **Cell culture supernatant or other biological fluids:** Centrifuge samples for 20 min at 1000×g at 2-8℃. Collect the supernatant to carry out the assay

IX. Assay Protocol:

Note: Bring all reagents and samples to room temperature 30 minutes prior to the assay. It is recommended that all standards and samples be run at least in duplicate. A standard curve must be run with each assay.

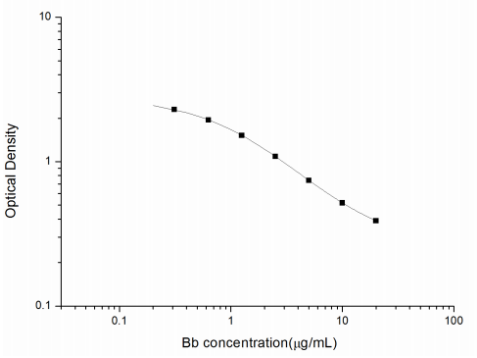
1. Add the **Standard working solution** to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (50 uL for each well). Add the samples to the other wells (50 uL for each well). Immediately add 50 μl of **Biotinylated Detection Antibody working** solution to each well. Cover with the Plate sealer. Incubate for 45 min at 37°C.

   Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Aspirate or decant the solution from each well, add 350 μl of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.
3. Add 100 μl of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.
4. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 2.
5. Add 90 μl of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C.

   Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min.
6. Add 50 μl of **Stop Solution** to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.
7. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

X. Calculation:

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.
If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.



Typical standard curve and data is provided below for reference only. A standard curve must be run with each assay

Concentration(μg/mL)	20	10	5	2.5	1.25	0.63	0.31	0
OD	0.39	0.518	0.742	1.086	1.52	1.948	2.298	2.78

XI. Precision:

Intra-assay Precision (Precision within an assay): 3 samples with low, mid-range and high level Human AGP1 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, mid-range and high level Human AGP1 were tested on 3 different plates, 20 replicates in each plate

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (µg/mL)	4.90	11.50	45.40	5.10	10.60	48.20
Standard deviation	0.30	0.70	2.10	0.30	0.40	2.50
C V (%)	6.12	6.09	4.63	5.88	3.77	5.19

XII. Recovery:

The recovery of Human AGP1 spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices

Sample Type	Range (%)	Average Recovery (%)
Serum (n=5)	87-103	94
EDTA plasma (n=5)	88-102	94
Cell culture media (n=5)	90-107	97

XIII. Linearity:

Samples were spiked with high concentrations of Human AGP1 and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma(n=5)	Cell culture media(n=5)
1:2	Range (%)	86-99	96-112	94-108
	Average (%)	93	102	102
1:4	Range (%)	99-112	91-103	92-104
	Average (%)	106	96	97
1:8	Range (%)	100-117	91-105	88-101
	Average (%)	107	96	94
1:16	Range (%)	95-109	91-107	84-100
	Average (%)	103	98	91

XIV. RELATED PRODUCTS:

- Heme Oxygenase 1 (HO1) (Human) ELISA Kit (E4507)
- Bilirubin (Total and Direct) Colorimetric Assay Kit (K553)
- Heme Oxygenase 1 (HO1) (Rat) ELISA Kit (E4525)
- Alpha-Fetoprotein (AFP) ELISA (K4242)
- Heme Oxygenase 1 (HO1) (Mouse) ELISA Kit (E4524)

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