MANUAL

β-Hydroxybutyrate (Ketone Body) Assay Kit (Colorimetric)

Catalog #: MA-BHB

ISO 13485:2016

Introduction

β-Hydroxybutyrate is a “ketone body” produced in the liver and exported to peripheral tissues for use as an energy source. “Ketone body” includes three molecules: acetoacetate, β-hydroxybutyrate, and acetone. β-hydroxybutyrate and acetoacetate transport energy from the liver to other tissues, while acetone is generated by spontaneous decarboxylation of acetoacetate. High levels of ketone bodies can lead to ketosis, or as in extreme type 1 diabetes, ketoacidosis. Prolonged ketosis may lead to a life-threatening metabolic acidosis. Pathological ketosis may indicate various conditions, including organ failure, hypoglycemia in children, diabetes, alcohol intoxication, corticosteroid, or growth hormone insufficiency. The degree of ketosis can be determined by monitoring the blood levels of β-hydroxybutyrate.

β-Hydroxybutyrate (Ketone Body) Assay Kit provides a simple, reproducible, and sensitive tool for measuring β-hydroxybutyrate concentration in plasma, serum, cell lysates, urine, and other biological liquid samples. This assay employs an enzymatic cycling reaction. Initially, β-hydroxybutyrate is oxidized to acetoacetate by the β-Hydroxybutyrate Enzyme. Concomitant with this oxidation, the cofactor NAD^+ is reduced to NADH. Then NADH reacts with the colorimetric probe producing a colored product that can be measured at 450 nm. The optical density measured is directly proportional to the concentration of β-hydroxybutyrate present in the sample.

Storage

The entire kit should be stored below -20 °C for up to 6 months from the date of shipment. For prepared reagent storage, see table below.

Component	Size / Description	Storage After Preparation
Microplate	A 96-well (12 strips x 8 wells) plate	RT
Assay Buffer	12 ml	2–8 °C, 3 months
β-Hydroxybutyrate Standard	2 vials (Lyophilized)	Do not store and reuse.
β-Hydroxybutyrate Enzyme	1 vial	2–8 °C, 1 week
Co-Enzyme	1 vial (Lyophilized)	Aliquot and store at -20 °C
Cofactor	2 vials (Lyophilized)	Do not store and reuse.
Colorimetric Probe	1 vial (Lyophilized)	Aliquot and store at -20 °C

RT = room temperature

Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Tubes to prepare sample dilutions
4. 50 ml conical tubes

Sample Tips and General Considerations

NOTE: Optimal methods of sample preparation will need to be determined by each researcher empirically based on researched literature and knowledge of the samples.

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins.
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.

1. Cell lysates can be prepared as follows:

For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, and pellet the cells by spinning down the cells at 1,000 x g for 10 min) making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at $2x10^7$ cells/ml in lysis buffer containing protease inhibitors. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 minutes.
It is recommended that sample protein concentrations should be determined using a total protein assay. Lysates should be used immediately or aliquot and stored at -70 °C. Thawed lysates should be kept on ice prior to use.
General tips for preparing lysate samples can be viewed on the online Resources page of the website: https://www.raybiotech.com/tips-on-sample-preparation/

2. Plasma samples:

Collect blood with an anticoagulant such as citrate, EDTA, or oxalate and mix by inversion. Centrifuge the blood at 1000 x g at 4°C for 10 minutes. Collect plasma supernatant without disturbing the white buffy layer. Sample should be tested immediately or frozen at -80°C for storage. Typically, normal human plasma has β-hydroxybutyrate concentrations in the range of 20-1500 µM. The sample may be assayed directly or diluted as necessary in Assay Buffer.

3. Serum samples:

Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Samples should be tested immediately or frozen at -80°C for storage. Typically, normal human serum has β-hydroxybutyrate concentrations in the range of 20-1500 µM. The sample may be assayed directly or diluted as necessary in Assay Buffer.

4. Urine samples:

To remove insoluble particles, spin at 10,000 x g for 5 min. The supernatant may be assayed directly or diluted as necessary in Assay Buffer.

NOTE:

1. *Enzymes present in samples may deplete NADH rapidly and affect results. Samples should be deproteinized before using within the assay. A spin filter with a 10 kDa cutoff is recommended for efficient and clean separation.*
2. *Avoid samples containing SH groups like DTT, β-mercaptoethanol, or reduced gluathione.*
3. *Borate, methanol (5%), DMSO (5%), glycerol (5%), and BSA (~1%) may interfere with the assay.*
4. *Optimal experimental conditions for samples must be determined by the investigator. A set of serial dilutions is recommended for samples to achieve optimal assay results.*

Reagent Preparation

REAGENT	PREPARATION
Microplate	No preparation
Assay Buffer	
β-Hydroxybutyrate Standard	See "Standard Preparation" section.
β-Hydroxybutyrate Enzyme	Thaw on ice. Ready to use.
Co-Enzyme	Reconstitute with 36 µL Ultrapure water.
Cofactor	For each vial, reconstitute with 305 µL Assay Buffer.
Colorimetric Probe	Reconstitute with 40 µL Ultrapure water.
Working Solution	Add 42 µL β-Hydroxybutyrate Enzyme, 16 µL reconstituted Co-Enzyme, 300 µL reconstituted Cofactor, and 18 µL reconstituted Colorimetric Probe into 2374 µL Assay Buffer to make the **Working Solution**. This is enough to assay 50 wells. Prepare the additional vial as needed. The Working Solution is stable for 1 day at 2-8°C in the dark.

Standard Preparation

To prepare a dilution series of the β-Hydroxybutyrate Standard in the concentration range of 0 µM – 1000 µM (see Table below),

1. Label 8 microtubes #1 through 8 with the following concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.625, 0 µM.
2. Pipette 294 µL Assay Buffer into labeled tube #1 and 150 µL Assay Buffer into labeled tube #2 through #8.
3. Briefly spin down β-Hydroxybutyrate Standard tube provided, add 20 µL Assay Buffer to make 50 mM stock, and mix well.
4. Pipette 6 µL of 50 mM stock into tube#1, mix thoroughly, and quick spin to make a 1000 µM β-Hydroxybutyrate Standard stock.
5. To make the 500 µM standard, pipette 150 µL of tube #1 into the tube labeled #2. Mix thoroughly and quick spin.
6. Repeat this step with each successive concentration, preparing a dilution series as shown in the Table below. Each time, use 150 µl of the prior concentration until the 15.625 µM is reached. Mix each tube thoroughly before the next transfer. Standards should be prepared fresh and used immediately.

Labeled Tubes	β-Hydroxybutyrate Standard (µL)	Assay Buffer (µL)	Standard Conc. (µM)
1	6 µL of 50 mM stock	294 µL	1000
2	150 µL of Tube #1	150 µL	500
3	150 µL of Tube #2	150 µL	250
4	150 µL of Tube #3	150 µL	125
5	150 µL of Tube #4	150 µL	62.5
6	150 µL of Tube #5	150 µL	31.25
7	150 µL of Tube #6	150 µL	15.625
8	0µL	150 µL	0

Assay Procedure

Each β-Hydroxybutyrate Standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add 50 µL of the diluted β-Hydroxybutyrate Standards or samples to the 96-well microtiter plate.
2. Initiate the reaction by adding 50 µL Working Solution (see “Reagent Preparation”) to each well. Carefully shake the plate for a few seconds to mix.
3. Incubate the plate for 30 minutes at room temperature with mixing in the dark.
4. Measure the absorbance at 450nm using a plate reader.

Calculation of Results

Subtract the blanks

Average the absorbance value of the blank wells (Standard 0 µM) and subtract this from the absorbance values of all the other wells. These are the corrected absorbances.

Plotting the standard curves

Make a plot of the corrected absorbance at 450nm as a function of β-Hydroxybutyrate concentration.

Determination of sample β-Hydroxybutyrate concentration

β-Hydroxybutyrate (μM) = $\frac{OD_{Sample}-OD_{Blank}}{Slope} \times DF$

OD_{Sample} = Optical density (OD) reading of the Sample

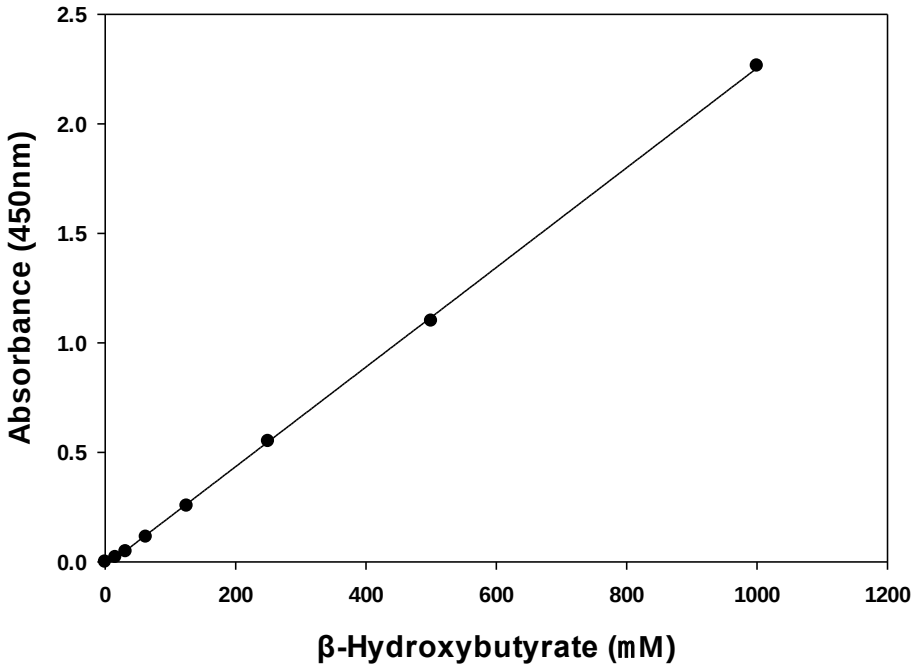
OD_{Blank} = Optical density (OD) reading of the Blank (Standard 0 μM)

Slope is from the plot of β-Hydroxybutyrate concentration vs. Absorbance (as shown in Typical data below).

DF = Sample dilution factor (DF = 1 for undiluted Samples)

Note: If the calculated β-Hydroxybutyrate concentration of the sample is higher than 1000 μM, dilute the sample in Assay Buffer and repeat the assay.

A. Typical Data



These standard curves are for demonstration only. A standard curve must be run with each assay.

B. Sensitivity

The minimum detectable concentration of β-Hydroxybutyrate is 10 µM.
Minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank.

C. Reproducibility

Intra-assay Precision (Precision within an assay):
To assess intra-assay precision, 16 wells per sample (total of 4 samples) were tested on a single plate. The intra-assay coefficient of variation was found to be 3.4%.

Inter-assay Precision (Precision between assays):
To assess inter-assay precision, 4 samples were tested in separate assays (n=4). The inter-assay coefficient of variation was found to be 6.5%.

This product is for research use only.