



β -Hydroxybutyrate (Ketone Body) Colorimetric Assay Kit

Item No. 700190

www.caymanchem.com

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
700191	β -HB Assay Buffer	1 vial
700192	β -Hydroxybutyrate Standard	2 vials
700193	β -Hydroxybutyrate Enzyme Solution	3 vials
700194	β -Hydroxybutyrate Colorimetric Detector	3 vials
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.



WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbances between 445-455 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

β -Hydroxybutyrate (β -HB; 3-hydroxybutyric acid) is a “ketone body” which is produced in the liver, mainly from the oxidation of fatty acids, and is exported to peripheral tissues for use as an energy source. The term ‘ketone body’ refers to three molecules, acetoacetate, β -HB, and acetone. β -HB and acetoacetate transport energy from the liver to the other tissues and acetone is generated by spontaneous decarboxylation of acetoacetate.¹ The presence of ketosis may be normal or pathologic. Normally ketosis can indicate that lipid metabolism has been activated and the pathway of lipid degradation is intact. Normal ketosis is prevalent in many circumstances such as during fasting, after prolonged exercise or after a high fat diet. Pathological causes of ketosis include multiple organ failure, diabetes, childhood hypoglycemia, corticosteroid or growth hormone deficiency, intoxication with alcohol or salicylates and several inborn errors of metabolism.² In acutely ill patients, these ketone bodies can accumulate in the body to cause ketoacidosis, which leads to the potentially life threatening condition known as metabolic acidosis.³ The presence and degree of ketosis can be determined by measuring blood levels of β -HB.

Ordinarily, β -HB accounts for approximately 75% of the ketone bodies in serum.⁴⁻⁶ Measurement of β -HB provides a reliable index of the level of ketoacidosis, including the detection of subclinical ketosis.⁷⁻⁹ In diabetics, β -HB measurements (and blood glucose) can be used for the assessment of the severity of diabetic coma and is essential for the exclusion of hyperosmolar non-ketotic diabetic coma. The measurement of β -HB is also used to monitor insulin requirements, based on existing hyperketonemia.¹⁰ β -HB has more recently been evaluated for use in neurodegenerative diseases and inhibition of adipocyte lipolysis.¹¹⁻¹⁵

About This Assay

Cayman's β -HB (Ketone Body) Colorimetric Assay Kit provides a simple, reproducible, and sensitive tool for measuring β -HB levels in plasma, serum, urine, cell lysates, or tissue homogenates. The method for β -HB determination is based upon the oxidation of D-3-hydroxybutyrate to acetoacetate by the enzyme 3-hydroxybutyrate dehydrogenase.¹⁶ Concomitant with this oxidation, the cofactor NAD^+ is reduced to NADH. In the presence of diaphorase, NADH reacts with the colorimetric detector WST-1 to produce a formazan dye with an absorbance maximum at 445-455 nm (see Figure 1 on page 9). The absorbance of the dye is directly proportional to the β -HB concentration.

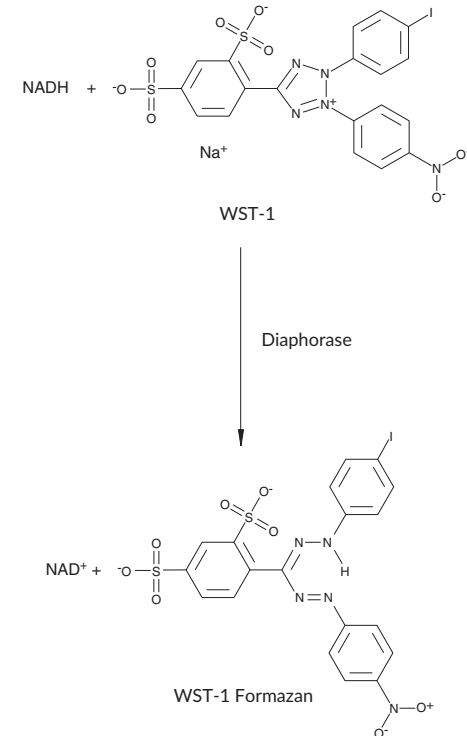
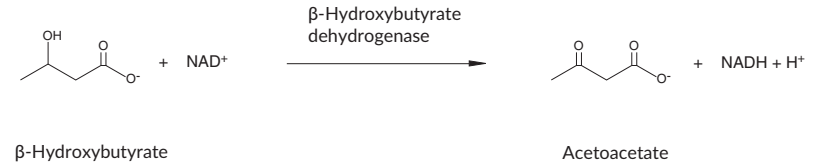


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. β -Hydroxybutyrate Assay Buffer - (Item No. 700191)

The vial contains 25 ml of 100 mM Tris-HCl, pH 8.5. Thaw the Assay Buffer at room temperature. Once thawed, the Assay Buffer is ready to use in the assay and for diluting reagents and samples. When stored at 4°C, the thawed Assay Buffer is stable for at least six months.

2. β -Hydroxybutyrate Standard - (Item No. 700192)

Each vial contains a lyophilized powder of DL-hydroxybutyrate. Reconstitute the contents of the vial with 1 ml of β -HB Assay Buffer (Item No. 700191). This reconstituted standard solution is used to prepare the β -HB standard curve. The reconstituted Standard is stable for six hours on ice. *NOTE: When reconstituted, the solution will become a 1.0 mM D-hydroxybutyrate solution.*

3. β -Hydroxybutyrate Enzyme Solution - (Item No. 700193)

Each vial contains a lyophilized enzyme mixture. Reconstitute the contents of the vial with 2.4 ml of β -HB Assay Buffer (Item No. 700191). The reconstituted Enzyme Solution is stable for two hours on ice. This Enzyme Solution is used to prepare the Developer Solution in step 5. One vial of the Enzyme Solution is sufficient to evaluate 48 wells.

4. β -HB Colorimetric Detector - (Item No. 700194)

Each vial contains 125 μ l of WST-1 solution. It is ready to use as supplied. Thaw the Detector Solution and store on ice. The Detector is also used to prepare the Developer Solution in step 5. One vial of the Colorimetric Detector is sufficient to evaluate 48 wells.

5. Developer Solution

Prepare the Developer Solution by adding 100 μ l of the β -HB Colorimetric Detector (Item No. 700194) to the vial of the reconstituted Enzyme Solution (Item No. 700193). Store the Developer Solution on ice. The Developer Solution is stable for one hour.

Sample Preparation

β -HB concentrations in plasma and serum can vary over a rather wide range, with normal levels measuring 0.02-1.5 mM and increasing to as high as 3-5 mM in diabetics. β -HB concentrations in urine can be as high as 30-50 mM during diabetic ketoacidosis.^{6,7,17,18}

Plasma

1. Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. If not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month while stored at -80°C.
3. Plasma should be filtered through 10 kDa MWCO spin filters (following the manufacturer's protocol) before assaying.

Serum

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.
4. Serum should be filtered through 10 kDa MWCO spin filters (following the manufacturer's protocol) before assaying.

Urine

Collection of urine does not require any special treatment. If not assaying the same day, freeze at -80°C.

It is recommended that the values obtained from urine samples be standardized to creatinine levels using Cayman's Creatinine ELISA Kit (Item No. 502330), Creatinine (urinary) Colorimetric Assay Kit (Item No. 500701), or a similar assay.

Cell Samples

1. Collect cells (~18 x 10⁶ cells) by centrifugation (i.e., 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Resuspend cell pellet in 1-2 ml of cold Assay Buffer.
3. Sonicate the cell suspension 20X at 1 second bursts.
4. Centrifuge cell suspension at 10,000 x g for 10 minutes at 4°C.
5. Remove the supernatant and store on ice. If not assaying on the same day, freeze at -80°C until use. The sample will be stable for at least one month. No further dilution is needed before assaying.
6. Resuspend the pellet in 1 ml of cold Assay Buffer and store on ice. If not assaying on the same day, freeze at -80°C until use. The sample will be stable for at least one month. No further dilution is needed before assaying.

Tissue Samples

1. Weigh tissue and then mince into small pieces.
2. Homogenize 350-400 mg of minced tissue in 2 ml of the Assay Buffer containing protease inhibitors of choice (see Interferences section).
3. Centrifuge at 1,000 x g for 10 minutes at 4°C.
4. Transfer the supernatant to another tube and centrifuge at 10,000 x g for 10 minutes at 4°C.
5. Remove the supernatant and store on ice. If not assaying on the same day, freeze at -80°C until use. The sample will be stable for at least one month. No further dilution is needed before assaying.
6. Resuspend the pellet in 1-2 ml of cold Assay Buffer and store on ice. If not assaying on the same day, freeze at -80°C until use. The sample will be stable for at least one month. Prior to assaying, dilute the sample 1:2-1:5 with cold Assay Buffer.

Sample Matrix Properties

Spike and Recovery

Human plasma and urine were spiked with different amounts of β -HB. Plasma samples were filtered as described in the Sample Preparation section and validated in the assay. Urine samples were serially diluted and validated in the assay. The error bars represent standard deviations from the multiple dilutions of each sample.

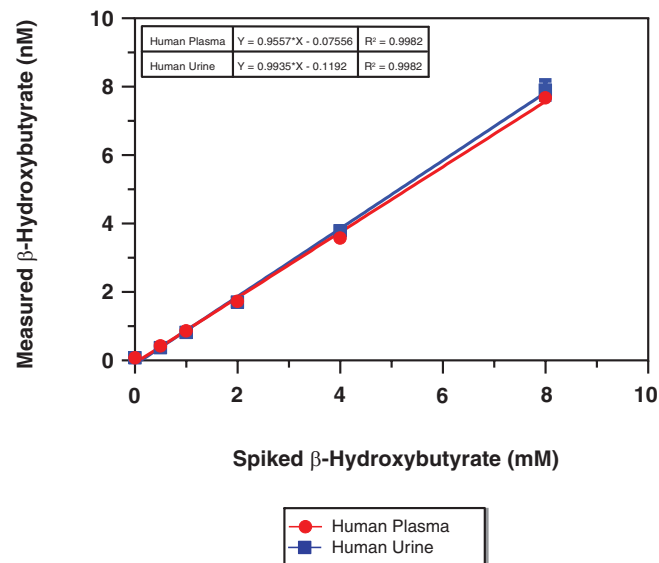


Figure 2. Spike and recovery of β -hydroxybutyrate in human plasma and urine

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of the β -HB standard curve and samples to be measured in triplicate is given in Figure 3. We suggest you record the contents of each well on the template sheet provided (see page 26).

	1	2	3	4	5	6	7	8	9	10	11	12
A	(A)	(A)	(A)	(S1)	(S1)	(S1)	(S9)	(S9)	(S9)	(S17)	(S17)	(S17)
B	(B)	(B)	(B)	(S2)	(S2)	(S2)	(S10)	(S10)	(S10)	(S18)	(S18)	(S18)
C	(C)	(C)	(C)	(S3)	(S3)	(S3)	(S11)	(S11)	(S11)	(S19)	(S19)	(S19)
D	(D)	(D)	(D)	(S4)	(S4)	(S4)	(S12)	(S12)	(S12)	(S20)	(S20)	(S20)
E	(E)	(E)	(E)	(S5)	(S5)	(S5)	(S13)	(S13)	(S13)	(S21)	(S21)	(S21)
F	(F)	(F)	(F)	(S6)	(S6)	(S6)	(S14)	(S14)	(S14)	(S22)	(S22)	(S22)
G	(G)	(G)	(G)	(S7)	(S7)	(S7)	(S15)	(S15)	(S15)	(S23)	(S23)	(S23)
H	(H)	(H)	(H)	(S8)	(S8)	(S8)	(S16)	(S16)	(S16)	(S24)	(S24)	(S24)

A-H = Standards

S1-S24 = Sample wells

Figure 3. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps to maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 μ l in all wells.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in triplicate, but it is the user's discretion to do so.
- The assay is performed at 25°C.
- Monitor the absorbance at 445-455 nm.

Standard Preparation

Take eight clean test tubes and label them A-H. Add the amount of 1 mM β -HB Standard Solution and Assay Buffer to each tube as described in Table 1. We recommend that you store these diluted Standards for no more than one to two hours.

Tube	β -HB Stock Solution (μ l)	Assay Buffer (μ l)	β -HB Concentration (mM)
A	0	200	0
B	5	195	0.025
C	10	190	0.05
D	20	180	0.1
E	40	160	0.2
F	60	140	0.3
G	80	120	0.4
H	100	100	0.5

Table 1. Preparation of β -Hydroxybutyrate standard curve

Performing the Assay

1. **β -Hydroxybutyrate Standard Wells** - Add 50 μ l of each Standard (tubes A-H) to two or three wells (see suggested plate configuration, Figure 3, page 14).
2. **Sample Wells** - Add 50 μ l of the sample to two or three wells.
3. Initiate the reaction by adding 50 μ l of the Developer Solution to all wells being used.
4. Incubate the plate at 25°C in the dark for 30 minutes.
5. Read the absorbance at 445-455 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample.
2. Subtract the absorbance value of standard A (0 mM) from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of the final β -HB concentration (mM) (see Table 1, page 16). A typical β -HB standard curve is shown in Figure 4, on page 19.
4. Calculate the values of the β -HB samples using the equation obtained from the linear regression of the standard curve by substituting the corrected absorbance values for each sample into the equation.

β -Hydroxybutyrate (mM) =

$$\left[\frac{\text{Corrected absorbance} - (\text{y-intercept})}{\text{Slope}} \right] \times \text{Dilution}$$

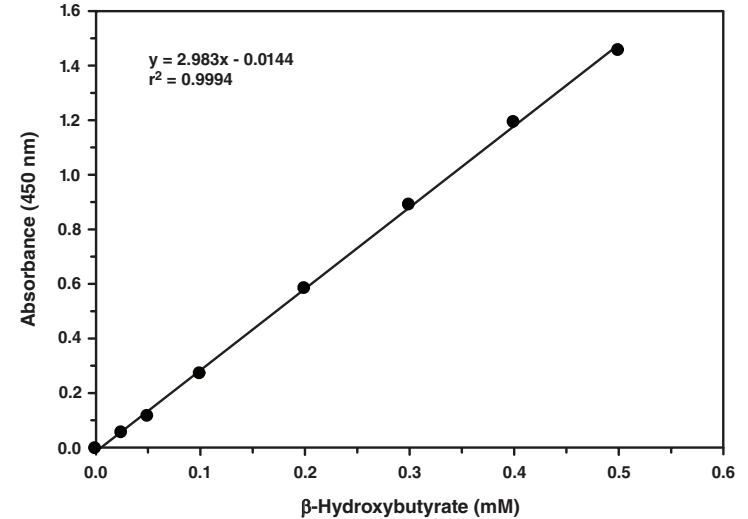


Figure 4. β -Hydroxybutyrate standard curve

Performance Characteristics

Precision:

When a series of 48 human plasma and urine samples were assayed on the same day, the intra-assay coefficient of variation was 4.05% and 3.68%, respectively. When a series of 48 human plasma and urine samples were assayed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 3.18% and 3.03%, respectively.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0-0.5 mM β -HB.

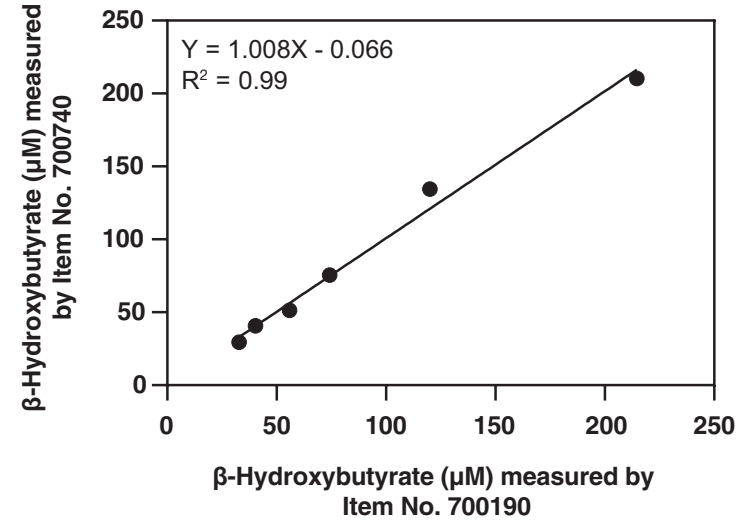


Figure 5. β -Hydroxybutyrate Fluorometric Assay Kit (Item No. 700740) measurements with correlation to β -hydroxybutyrate Colorimetric Kit (Item No. 700190) Endogenous and spiked β -HB was measured in 6 plasma samples by β -HB Fluorometric Assay Kit (Item No. 700740) and β -HB Colorimetric Kit (Item No. 700190) for comparison.

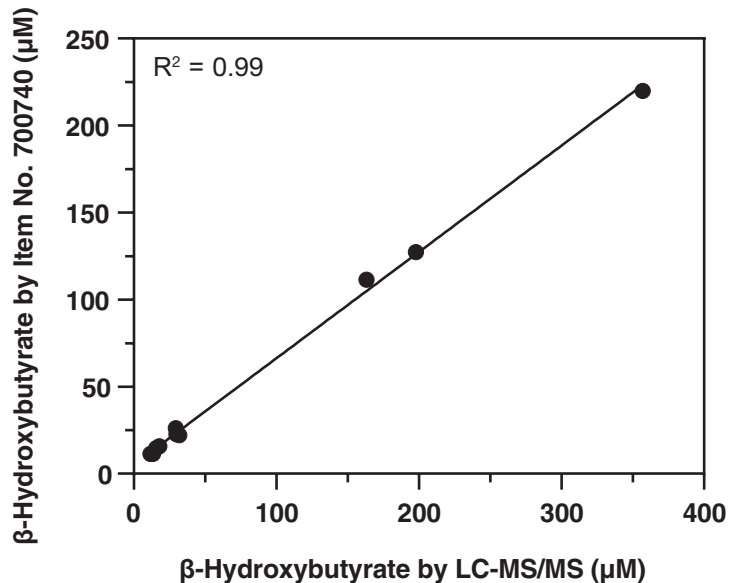


Figure 6. β -Hydroxybutyrate Fluorometric Assay Kit measurements with correlation to LC-MS/MS Endogenous and spiked β -HB was measured in ten plasma and serum samples by β -HB Fluorometric Assay Kit (Item No. 700740) and LC-MS/MS for comparison.

Interferences

The following reagents were tested in the assay for interference in the assay:

Reagent	Will Interfere (Yes or No)	
Buffers	Tris	No
	Borate	Yes
	HEPES	No
	Phosphate	No
	MES	No
Detergents	Polysorbate 20 ($\leq 1\%$)	No
	Triton X-100 ($\leq 1\%$)	No
Protease Inhibitors/ Chelators/ Enzymes	EDTA (1 mM)	No
	EGTA (1 mM)	No
	Trypsin (10 $\mu\text{g}/\text{ml}$)	No
	PMSF (1 mM)	No
	Leupeptin (10 $\mu\text{g}/\text{ml}$)	No
	Antipain (10 $\mu\text{g}/\text{ml}$)	No
	Chymostatin (10 $\mu\text{g}/\text{ml}$)	No
Solvents	Ethanol (5%)	No
	Methanol (5%)	Yes
	Dimethylsulfoxide (5%)	Yes
Others	BSA (1%)	Yes
	Sucrose (250 mM)	Yes
	Glycerol (5%)	Yes

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/ technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No β -HB was detected in the sample	A. β -HB concentration was too low B. The sample was too dilute	Do not dilute samples and re-assay
Sample absorbance values are above highest point in standard curve	A. β -HB concentration was too high in the sample B. The sample was too concentrated	Dilute samples with Assay Buffer and re-assay; <i>NOTE: Remember to account for the dilution factor when calculating β-HB concentration</i>

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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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