β-Hydroxybutyrate (Ketone Body) Fluorometric Assay Kit

Item No. 700740

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
700191	β-HB Assay Buffer	1 vial
700192	β-Hydroxybutyrate Standard	2 vials
700741	β-HB Fluorometric Cofactors	2 vials
700742	β-Hydroxybutyrate Dehydrogenase	2 vials
700743	β-HB Developing Enzyme	2 vials
700004	Fluorometric Developer Reagent	2 vials/60 µg
700517	Potassium Carbonate Assay Reagent	1 vial/5 ml
700518	MPA Assay Reagent	1 vial/2 g
400017	96-Well Solid Plate (black)	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.

It is recommended to take appropriate precautions when using the kit reagents (*i.e.*, lab coat, gloves, eye goggles, etc.) as some of them may be harmful. MPA (metaphosphoric acid) and potassium carbonate are corrosive and harmful if swallowed. Contact with skin may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water for 15 minutes.

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 with the capacity to measure fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Optional: 10 kDa spin filters for sample filtration

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) Fluorometric Assay Kit provides a simple, reproducible, and sensitive tool for assaying β-HB from plasma, serum, urine, tissue homogenates, and cell lysates. 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. NADH reacts with the fluorometric developer to yield a highly fluorescent product which can be analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. The fluorescence is directly proportional to the β-HB concentration.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **β-HB 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 -20°C, the assay buffer is stable for at least six months.

2. **β-Hydoxybutyrate 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-HB Fluorometric Cofactors - (Item No. 700741)**

   Each vial contains a lyophilized powder of cofactors. Reconstitute the contents of the vial with 2 ml of β-HB Assay Buffer. Keep the reconstituted cofactor solution on ice. This is sufficient reagent to evaluate 80 wells. Prepare the additional vial as needed. The reconstituted cofactors are stable for one hour on ice.

4. **β-Hydroxybutyrate Dehydrogenase - (Item No. 700742)**

   Each vial contains a lyophilized powder of β-Hydroxybutyrate Dehydrogenase. Reconstitute the contents of the vial with 2 ml of β-HB Assay Buffer and put the vial on ice. One vial of the enzyme solution is sufficient to evaluate 80 wells. Prepare the additional vial as needed. The reconstituted enzyme is stable for one hour on ice.

5. **β-HB Developing Enzyme - (Item No. 700743)**

   Each vial contains lyophilized developing enzyme. Reconstitute the contents of the vial with 600 µl of β-HB Assay Buffer and put the vial on ice. One vial is sufficient to evaluate 60 wells. Prepare the additional vial as needed. The reconstituted enzyme is stable for one hour on ice.

6. **Fluorometric Developer Reagent - (Item No. 700004)**

   Each vial contains a lyophilized powder of fluorometric developer. Reconstitute the contents of the vial with 600 µl of HPLC-grade water *(do not use assay buffer)*. One vial is sufficient to evaluate 60 wells. Prepare the additional vial as needed. The developer solution is stable for four hours at room temperature.

7. **Potassium Carbonate Assay Reagent - (Item No. 700517)**

   The vial contains 5 ml of 5 M potassium carbonate. The reagent is ready to use as supplied.

8. **MPA Assay Reagent - (Item No. 700518)**

   The vial contains 2 g of metaphosphoric acid (MPA). To prepare 1 M MPA for deproteinating the samples, dissolve the 2 g of MPA in 25 ml of HPLC-grade water. Store the diluted acid solution at room temperature. The diluted acid is stable for three months at room temperature.

Sample Preparation

We recommend deproteinating samples upon collection and then storing at -80°C.

Plasma

Typically, normal human plasma has a β-HB range of 0.02-0.27 mM (0.2-2.81 mg/dl).[6,7,17]

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.
3. Deproteinate, using one of the following methods:

   MPA deproteination:

   a. Add 500 µl of plasma to a tube. To deproteinate, add 500 µl of 1 M MPA to the plasma, vortex, and place on ice for five minutes.

   b. Centrifuge at 10,000 x g for five minutes at 4°C to pellet the proteins. Remove the supernatant and add 75 µl of potassium carbonate to the supernatant to bring the pH to approximately 8.5.

   c. Centrifuge at 10,000 x g for five minutes at 4°C to remove any additional debris. Remove the supernatant for assaying.

   Ultrafiltration:

   a. Plasma should be filtered using 10 kDa spin filters, following the manufactor's protocol.
4. If not assaying the same day, freeze at -80°C. The deproteinated plasma sample will be stable for one month stored at -80°C.
5. To fall within range of the standard curve, it may be necessary to dilute samples 1:2 with assay buffer before assaying. Generally, it is recommended to assay each sample in several dilutions.

Serum

Typically, normal human serum has a β-HB range of 0.02-0.27 mM (0.2-2.81 mg/dl).[6,7,17]

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.
4. Deproteinate, using one of the following methods:

   MPA deproteination:

   a. Add 500 µl of serum to a tube. To deproteinate, add 500 µl of 1 M MPA to the serum, vortex, and place on ice for five minutes.

   b. Centrifuge at 10,000 x g for five minutes at 4°C to pellet the proteins. Remove the supernatant and add 75 µl of potassium carbonate to the supernatant to bring the pH to approximately 8.5.

   c. Centrifuge at 10,000 x g for five minutes at 4°C to remove any additional debris. Remove the supernatant for assaying.

   Ultrafiltration:

   a. Serum should be filtered using 10 kDa spin filters, following the manufacture's protocol.
5. If not assaying the same day, freeze at -80°C. The deproteinated serum sample will be stable for one month stored at -80°C.
6. To fall within range of the standard curve, it may be necessary to dilute samples 1:2 with assay buffer before assaying. Generally, it is recommended to assay each sample in several dilutions.

Urine

Typically, normal human urine has a β-HB range of 0-15 mmol/mol creatinine (0-0.133 µmol/mg creatinine).[18]

1. Collection of urine does not require any special treatments.
2. If high protein content of urine is suspected, proceed with one of the following methods of deproteination. If high levels of protein are not expected proceed to step 3.

   MPA deproteination:

   a. Add 500 µl of urine to a tube. To deproteinate, add 500 µl of 1 M MPA to the urine, vortex, and place on ice for five minutes.

   b. Centrifuge at 10,000 x g for five minutes at 4°C to pellet the proteins. Remove the supernatant and add 75 µl of potassium carbonate to the supernatant to bring the pH to approximately 8.5.

   c. Centrifuge at 10,000 x g for five minutes at 4°C to remove any additional debris. Remove the supernatant for assaying.

   Ultrafiltration:

   a. Urine should be filtered using 10 kDa spin filters, following the manufacture's protocol
3. If not assaying the same day, freeze at -80°C. The deproteinated urine sample will be stable for one month stored at -80°C.
4. To fall within range of the standard curve, it may be necessary to dilute samples 1:2 with assay buffer before assaying. Generally, it is recommended to assay each sample in several dilutions.

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 Lysate

1. Collect cells (~10 x 10^6 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 cell scraper.
2. Add 500 µl of β-HB Assay Buffer to the cell pellet and vortex. If additional buffers are to be used, refer to Interferences on page 26.
3. If high protein content of lyasate is suspected, proceed with one of the following methods of deproteination. If high levels of protein are not expected proceed to step 4.

   MPA deproteination:

   a. To deproteinate, add 500 µl of 1 M MPA to the cells, vortex, and place on ice for five minutes.

   b. Centrifuge at 10,000 x g for five minutes at 4°C to pellet the proteins. Remove the supernatant and add 75 µl of potassium carbonate to the supernatant to neutralize the acid and bring the pH to approximately 8.5.

   c. Centrifuge at 10,000 x g for five minutes at 4°C to remove any additional debris. Remove the supernatant for assaying.

   Ultrafiltration:

   a. Lysate should be filtered using 10 kDa spin filters, following the manufacture's protocol.
4. If not assaying the same day, freeze at -80°C. The deproteinated sample will be stable for one month stored at -80°C.
5. To fall within range of the standard curve, it may be necessary to dilute samples 1:2 with assay buffer before assaying. Generally, it is recommended to assay each sample in several dilutions.

Tissue Homogenate

1. Prior to dissection, rinse tissue with a phosphate buffered saline (PBS) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenate the tissue in 5-10 ml of cold β-HB Assay Buffer per gram weight of tissue. If additional buffers buffers are used, refer to Interferences on page 26. Assay buffer should contain protease inhibitors.
3. Deproteinate using one of the following methods:

   MPA deproteination:

   a. To deproteinate, add 500 µl of 1 M MPA to 500 µl of tissue homogenate, vortex, and place on ice for five minutes.

   b. Centrifuge at 10,000 x g for five minutes at 4°C to pellet the proteins. Remove the supernatant and add 75 µl of potassium carbonate to the supernatant to neutralize the acid and bring the pH to approximately 8.5.

   c. Centrifuge at 10,000 x g for five minutes at 4°C to remove any additional debris. Remove the supernatant for assaying.

   Ultrafiltration:

   a. Tissue should be filtered using 10 kDa spin filters, following the manufacture's protocol.
4. If not assaying the same day, freeze at -80°C. The deproteinated tissue sample will be stable for one month stored at -80°C.
5. To fall within the range of the standard curve, it may be necessary to dilute samples 1:2 with the assay buffer before assaying. Generally, it is recommended to assay each sample in several dilutions.

Sample Matrix Properties

Spike and Recovery

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

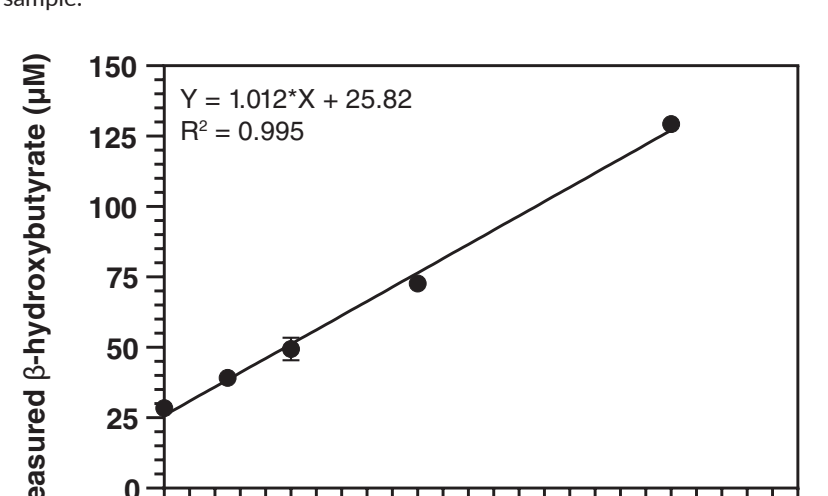

Figure 1. Spike and recovery of β-hydroxybutyrate in human plasma

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, a β-HB standard curve in duplicate has to be assayed with the samples. We suggest that each sample be assayed at least in duplicate in the presence and absence of β-Hydroxybutyrate Dehydrogenase (Item No. 700742). A typical layout of standards, samples, and sample backgrounds to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 29).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S5	S5	S9	S9	S13	S13	S17	S17
B	B	B	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17
C	C	C	S2	S2	S6	S6	S10	S10	S14	S14	S18	S18
D	D	D	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
E	E	E	S3	S3	S7	S7	S11	S11	S15	S15	S19	S19
F	F	F	B3	B3	B7	B7	B11	B11	B15	B15	B19	B19
G	G	G	S4	S4	S8	S8	S12	S12	S16	S16	S20	S20
H	H	H	B4	B4	B8	B8	B12	B12	B16	B16	B20	B20

A-H = Standards
S1-S20 = Sample Wells
B1-B20 = Sample Background Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multi-channel pipette 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 120 µl in all wells.
- All reagents except the enzymes and cofactors must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples at least in duplicate (triplicate preferred).
- The assay is performed at 37°C.
- Monitor the fluorescence with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Standard Preparation

Dilute 100 µl of the reconstituted β-Hydroxybutyrate Standard with 900 µl of β-HB Assay Buffer to yield a stock concentration of 100 µM. Take eight clean glass or polystyrene tubes and mark them A-H. Add the amount of the 100 µM Stock Solution and Assay Buffer to each tube as described in Table 1. Use the diluted standards within one hour.

Tube	β-HB Stock Solution (µl)	β-HB Assay Buffer (µl)	β-HB Concentration (µM)	β-HB Concentration (nmol/well)
A	0	600	0	0
B	15	585	2.5	0.13
C	30	570	5	0.25
D	60	540	10	0.50
E	120	480	20	1.0
F	180	420	30	1.5
G	240	360	40	2.0
H	300	300	50	2.5

Table 1. Preparation of β-hydroxybutyrate standards

Performing the Assay

1. **Standard Wells:** Add 50 µl of standard to standard wells.
2. **Sample Wells:** Add 50 µl of sample to sample wells and sample background wells.
3. **β-HB Fluorometric Cofactor:** Add 25 µl of reconstituted β-HB Fluorometric Cofactor to all wells.
4. **β-HB Developing Enzyme:** Add 10 µl of reconstituted β-HB Developing Enzyme to all wells.
5. **Fluorometric Developer Reagent:** Add 10 µl of reconstituted Fluorometric Developer Reagent to all wells.

NOTE: Alternatively, you may create a mastermix by gently mixing 1.5 ml flurometric cofactor, 600 µl developing enzyme, and 600 µl Fluorometric Developer Reagent. This is enough for 50 wells, including a 15% surplus. Add 45 µl of Mastermix to each well. Mastermix is stable for 1 hour on ice.

6. **β-HB Assay Buffer:** Add 25 µl of β-HB Assay Buffer to sample background wells.
7. **β-Hydroxybutyrase Dehydrogenase:** Initiate reactions by adding 25 µl of β-Hydroxybutyrase Dehydrogenase to standard and sample wells. DO NOT add to the sample background wells.
8. Carefully shake the plate for a few seconds to mix.
9. Cover the plate with the plate cover and incubate for 30 minutes at 37°C.
10. Remove the plate cover and read fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

See Tables 2 and 3 for pipetting summary on pages 20 and 21.

	Standard Wells (µl)	Sample Wells (µl)	Sample Background Wells (µl)
Standard	50	--	--
Sample	--	50	50
β-HB Fluorometric Cofactor	25	25	25
β-HB Developing Enzyme	10	10	10
Fluorometric Developer Reagent	10	10	10
β-HB Assay Buffer	--	--	25
Initiate reactions			
β-Hydroxybutyrate Dehydrogenase	25	25	--

Table 2. Pipetting summary

	Standard Wells (µl)	Sample Wells (µl)	Sample Background Wells (µl)
Standard	50	--	--
Sample	--	50	50
Mastermix	45	45	45
β-HB Assay Buffer	--	--	25
Initiate reactions			
β-Hydroxybutyrate Dehydrogenase	25	25	--

Table 3. Pipetting summary with mastermix

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard, sample, and sample background.
2. Subtract the fluorescence value of standard A (0 µM) from itself and all other standards. This is the corrected fluorescence.
3. Plot the corrected fluorescence values (from step 2 above) of each standard as a function of the final β-hydroxybutyrate concentration (µM) from Table 1. See Figure 3, on page 23, for a typical standard curve.
4. Subtract the average fluorescence value of the sample background from the average fluorescence value of the sample wells to yield the corrected sample fluorescence value (CSF).
5. Calculate the β-hydroxybutyrate concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the CSF values for each sample.

$$\text{β-Hydroxybutyrate (µM)} = \left[\frac{\text{CSF - (y-intercept)}}{\text{Slope}}\right] \text{ x Sample dilution*}$$

$$\text{nmol/well} = \text{µM x } 0.05$$

**If MPA was used for deproteination, include a factor of 2.15 in the sample dilution to account for the addition of MPA and potassium carbonate.*

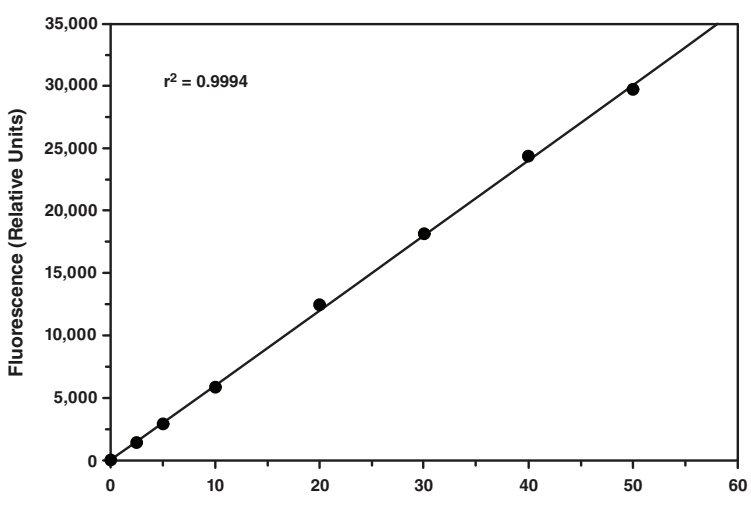

Figure 3. β-Hydroxybutyrate standard curve

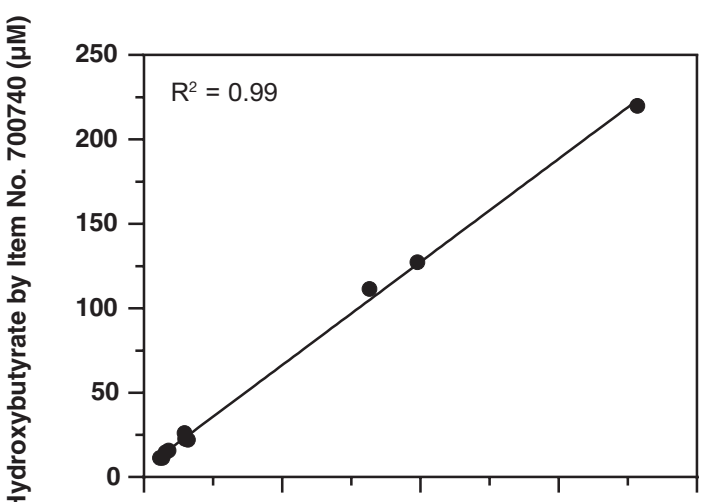

Figure 4. β-Hydroxybutyrate Fluorometric Assay Kit measurements with correlation to LC-MS/MS Endogenous and spiked β-HB was measured in ten plasma and serum samples by **β-HB Fluorometric Assay Kit (Item No. 700740) and LC-MS/MS for comparison**.

Performance Characteristics

Sensitivity:

The limit of detection (LOD) for the assay is 1.2 µM.

The lower limit of quantification (LLOQ) for the assay is 1.5 µM.

Precision:

When a series of 48 human plasma samples were assayed on the same day, the intra-assay coefficient of variation was 4.1%. When a series of 48 human plasma samples were assayed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 4.6%.

RESOURCES

Interferences

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

Reagent		Will Interfere (Yes or No)
Buffers	Borate	No
	HEPES	Yes
	MES	No
	Phosphate	No
Detergents	Polysorbate 20 (≤1%)	No
	Triton X-100 (0.1%)	Yes
Protease Inhibitors/ Chelators/ Enzymes	Antipain (100 µg/ml)	No
	Chymostatin (10 µg/ml)	No
	EDTA (1 mM)	No
	EGTA (1 mM)	No
	Leupeptin (10 µg/ml)	No
	PMSF (200 µM)	No
	Trypsin (10 µg/ml)	No
Solvents	Dimethylsulfoxide (5%)	No
	Ethanol (5%)	No
	Methanol (5%)	No
Others	BSA (1%)	Yes
	Glycerol (≤10%)	No
	Sucrose (250 mM)	No

Troubleshooting

Problem	Recommended Solutions
Erratic values; dispersion of duplicates/ triplicates	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 fluorescence detected above background in the sample wells	A. Re-assay the sample using a lower dilution B. Check the **Interference** section for possible interferences (see page 23)
The β-Hydroxybutyrate concentration was above the highest point on the standard curve	Dilute samples with Assay Buffer and re-assay; *NOTE: Remember to account for the dilution factor when calculating β-hydroxybutyrate concentration*
The fluorometer exhibited 'MAX' values for the wells	Reduce the *gain* and re-read

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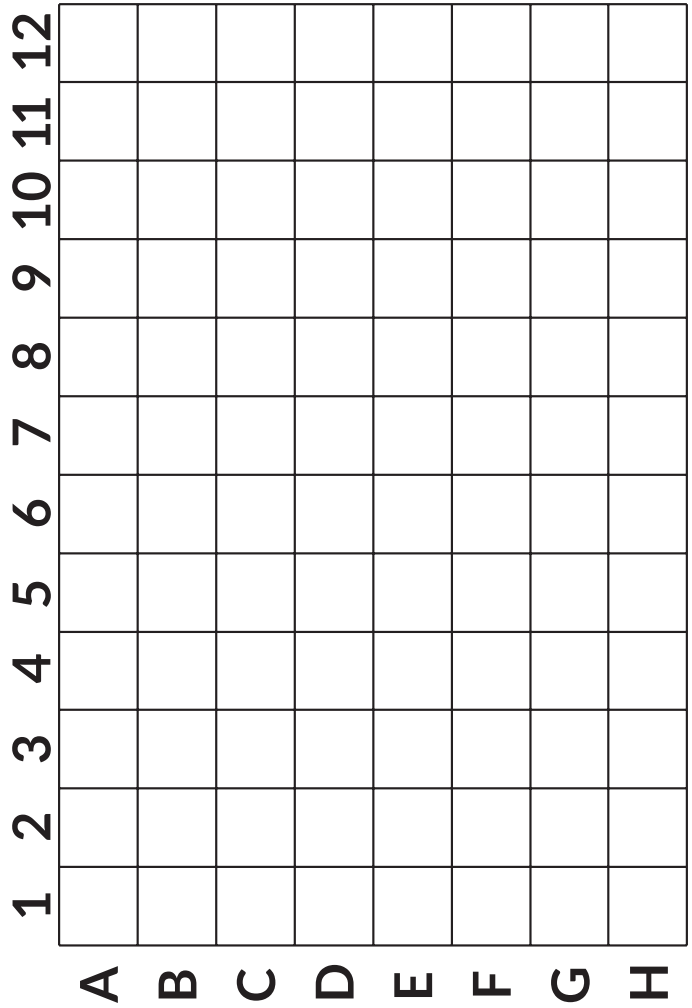

NOTES

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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