Glutamate Fluorometric/Colorimetric Assay Kit

Item No. 702330

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
400548	Glutamate Assay Buffer (5X)	1 vial/5 ml	-20°C
400549	L-Glutamate Oxidase (LGOX)	1 vial	-20°C
400550	Glutamate Assay HRP	1 vial	-20°C
400610	MaxiProbe	1 vial/250 µl	-20°C
400552	Glutamate Standard	1 vial/50 µl	-20°C
400115	Half-Area 96-Well Solid Plate (black, clear bottom)	1 plate	RT

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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Glutamate Fluorometric/Colorimetric Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

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 as directed in the **Materials Supplied** section (see page 3) 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 fluorescence with excitation and emission wavelengths of 530 and 590 nm, respectively, or a plate reader capable of measuring absorbance at 570 nm
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of pure water; glass-distilled water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Anhydrous DMSO (cell culture grade is preferred)
5. Aluminum foil
6. Materials used for **Sample Preparation** (see page 10)
7. A 37°C incubator

INTRODUCTION

Background

Glutamate is a non-essential proteinogenic amino acid and metabolite involved in diverse biological processes such as the citric acid cycle, neurotransmission, glutathione and amino acid biosynthesis, gluconeogenesis, tubulin and folic acid polyglutamylation, and umami taste sensation.[1,2] It can be obtained through the diet in foods such as cheese, seaweed, tomatoes, soy sauce, fish sauce, and fermented beans, which all contain high levels of free glutamate.[3] The sodium salt of glutamate, monosodium glutamate (MSG), is a common food additive used as a flavor enhancer. Glutamate can also be biosynthesized *in vivo via* deamination of glutamine by glutaminase.[1,2] It is the most abundant intracellular amino acid and has tissue-dependent functions.[1] In the kidney, it is metabolized by glutamate dehydrogenase to produce ammonia and provide the carbon skeleton for glucose production. In the intestine, it is metabolized by alanine transaminase to generate L-alanine and α-ketoglutarate. In macrophages and monocytes, glutamate is a precursor for the biosynthesis of ornithine and, subsequently, arginine. Inhibition of the system x_c^- cystine/glutamate transporter blocks glutamate export and cystine import, leading to the depletion of intracellular glutathione and induction of endoplasmic reticulum stress and ferroptosis.[4] Glutamate is also an excitatory neurotransmitter that acts on ionotropic and metabotropic receptors to induce excitatory synaptic transmission and a biosynthetic precursor to the inhibitory neurotransmitter GABA.[5] Excessive glutamate release by presynaptic neurons induces excitotoxicity and is associated with several neurological disorders, including amyotrophic lateral sclerosis (ALS), stroke, Parkinson's disease, Alzheimer's disease, and Huntington's disease.[6] Excessive glutamate release also occurs during seizure activity and contributes to epileptogenesis and seizure-induced brain damage.[7]

About This Assay

Cayman's Glutamate Fluorometric/Colorimetric Assay Kit provides both fluorometric and colorimetric methods for measuring glutamate in biological samples such as cell culture media, cell lysates, and tissue homogenates. In the assay, L-glutamate oxidase (LGOX) catalyzes the conversion of glutamate and oxygen (O_2) to α-ketoglutarate (α-KG), ammonia (NH_3), and hydrogen peroxide (H_2O_2). In the presence of horseradish peroxidase (HRP), H_2O_2 reacts stoichiometrically with MaxiProbe to produce a fluorescent compound (Figure 1). The product's fluorescence can be easily quantified at excitation and emission wavelengths of 530 and 590 nm, respectively. Alternatively, the absorbance of the product can be measured at 570 nm. Background signals generated from H_2O_2 and other interferences found inherently in samples that are not produced by LGOX in the assay can be corrected by omitting the enzyme in sample background reactions.

This assay offers the option to measure fluorescence or absorbance. It is at the user's discretion to choose the mode of measurement (and corresponding standard curve preparation) that best fits their needs. When read fluorometrically, this assay has a range of 0.3-5 µM and a lower limit of detection (LLOD) of 0.3 µM. When read colorimetrically, the range is 2.6-80 µM, with an LLOD of 2.6 µM.

$$\text{Glutamate} + O_2 \xrightarrow{\text{LGOX}} \alpha\text{-KG} + NH_3 + H_2O_2$$

$$\text{MaxiProbe} + H_2O_2 \xrightarrow{\text{HRP}} \text{Compound detected at } Ex_{530}/Em_{590} \text{ or } A_{570}$$

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Glutamate Assay Buffer (5X) - (Item No. 400548)**

   This vial contains 5 ml of Glutamate Assay Buffer (5X). Thaw at room temperature and dilute the contents with 20 ml of pure water. Be certain to rinse the vial to remove any salts that may have precipitated. The Glutamate Assay Buffer (1X) will be stable for at least two months when stored at 4°C.

   NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with pure water.

2. **Glutamate Standard - (Item No. 400552)**

   This vial contains 50 µl of 10 mM glutamate, which is ready to use as supplied. It will be stable for at least six months when stored at -20°C. If not using the standard all at once, prepare aliquots and store at -20°C. Avoid multiple freeze-thaw cycles.

3. **L-Glutamate Oxidase (LGOX) - (Item No. 400549)**

   This vial contains a lyophilized powder of LGOX. Reconstitute the contents of the vial with 150 µl of pure water and place on ice. Do not vortex. One vial provides a sufficient volume to assay 100 wells. The reconstituted LGOX will be stable for at least two months when stored at -80°C. If not using the reconstituted enzyme all at once, prepare aliquots and store at -80°C. Avoid multiple freeze-thaw cycles.

4. **Glutamate Assay HRP - (Item No. 400550)**

   This vial contains a lyophilized powder of Glutamate Assay HRP. Reconstitute the contents of the vial with 150 µl of pure water and place on ice. Do not vortex. One vial provides a sufficient volume to assay 100 wells. The reconstituted Glutamate Assay HRP will be stable for two months when stored at 4°C.

5. **MaxiProbe - (Item No. 400610)**

   The vial contains 250 µl of MaxiProbe. Add 90 µl of DMSO to the vial and mix well. Once diluted, the vial will contain a sufficient amount to evaluate 100 wells. The MaxiProbe developer solution will be stable for one hour at room temperature if protected from light with the cap closed. If not using the diluted developer reagent all at once, prepare aliquots and store at -20°C. Avoid multiple freeze-thaw cycles.

Sample Preparation

Plasma

Collect blood in vacutainers containing heparin, EDTA, or sodium citrate for plasma samples. To obtain plasma, centrifuge blood at 1,000 x g for 15 minutes at 4°C. Pipette off the top plasma layer without disturbing the white buffy layer. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C. It is recommended to deproteinate plasma prior to the assay using ultrafiltration with 10 kDa spin filters, following the manufacturer's protocol. To fall within the range of the standard curve, it may be necessary to dilute samples with Glutamate Assay Buffer (1X) prior to the assay.

Serum

Collect blood in vacutainers without a coagulant for serum samples. Allow samples to clot undisturbed for 30-60 minutes at room temperature. To obtain serum, centrifuge at 1,000-2,000 x g for 15-30 minutes at 4°C. Pipette off the top serum layer. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C. It is recommended to deproteinate serum prior to the assay using ultrafiltration with 10 kDa spin filters, following the manufacturer's protocol. To fall within the range of the standard curve, it may be necessary to dilute samples with Glutamate Assay Buffer (1X) prior to the assay.

Tissue Homogenate

Collect tissue and process immediately or store at -80°C. Thoroughly rinse tissue with PBS to remove excess blood and mince into small pieces. Homogenize 50 mg of minced tissue in 1 ml of Glutamate Assay Buffer (1X) or an alternative buffer such as T-PER™ or RIPA (Cayman Chemical Item No. 10010263), then centrifuge at 15,000 x g for 10 minutes at 4°C. Transfer the supernatant to another tube and store on ice temporarily or at -80°C for long-term storage. Dilute the samples using Glutamate Assay Buffer (1X) prior to the assay. It is recommended to deproteinate tissue homogenate prior to the assay using ultrafiltration with 10 kDa spin filters, following the manufacturer's protocol. Unfiltered samples may be measured using the fluorometric method after sufficient dilution with Glutamate Assay Buffer (1X). *NOTE: The Glutamate Standard should be prepared using the same homogenate buffer or diluted homogenate buffer as in the samples.*

Cell Lysate

Thoroughly wash cells 2-3 times with warm PBS (37°C) to remove excess medium, which may contain glutamate. Collect cells (~10 x 10^7 cells) by centrifugation (*i.e.* 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, harvest using a cell scraper instead of proteolytic enzymes. Snap freeze the pellets if not lysing immediately and store at -80°C. To lyse cells, add 800 µl of RIPA buffer (Cayman Chemical Item No. 10010263) to the cell pellet and vortex. Centrifuge at 15,000 x g for 10 min at 4°C and use the supernatant for analysis. If not assaying the same day, store the cell lysates at -80°C. To fall within the range of the standard curve, it may be necessary to dilute samples with lysis buffer or Glutamate Assay Buffer (1X) prior to the assay. *NOTE: The Glutamate Standard should be prepared using the same lysis buffer or diluted lysis buffer as in the samples.*

Cell Culture Medium

Remove cells from the medium by centrifugation. To minimize interferences, a minimum of 100X and 10X dilutions of the culture medium are required for the fluorometric and colorimetric methods, respectively. Phenol red does not significantly interfere with the assay if diluted as recommended. Further dilutions might be required for some samples to fall within the range of the standard curve.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. It is suggested that each sample and standard be assayed at least in duplicate (triplicate is preferred). A typical layout of standards and samples to be measured in duplicate is shown in Figure 2 below. It is suggested that the contents of each well are recorded on the template sheet provided (see page 29).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S2	S2	S6	S6	S10	S10	S14	S14	S18	S18
B	B	B	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
C	C	C	S3	S3	S7	S7	S11	S11	S15	S15	S19	S19
D	D	D	B3	B3	B7	B7	B11	B11	B15	B15	B19	B19
E	E	E	S4	S4	S8	S8	S12	S12	S16	S16	S20	S20
F	F	F	B4	B4	B8	B8	B12	B12	B16	B16	B20	B20
G	S1	S1	S5	S5	S9	S9	S13	S13	S17	S17	S21	S21
H	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17	B21	B21

A-F = Standards
S1-S21 = Sample Wells
B1-B21 = Sample Background Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps 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 in the assay is 100 µl in all of the wells.
- All reagents should be prepared as described above. The LGOX and Glutamate Assay HRP should be kept on ice and all other reagents should be kept at room temperature before beginning the assay.
- 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 duplicate (triplicate is preferred), but it is at the user's discretion to do so.
- Monitor the fluorescence with excitation and emission wavelengths of 530 and 590 nm, respectively, or monitor the absorbance at 570 nm.

Standard Curve Preparation

NOTE: This assay can be read using fluorescence or absorbance. Choose the standard curve preparation that matches the format needed. Both standard curves do not need to be prepared.

Fluorometric Standard Curve Preparation

Dilute 5 µl of the 10 mM Glutamate Standard with 495 µl of Glutamate Assay Buffer (1X) to yield a 100 µM bulk standard. Label six clean glass or polystyrene test tubes A-F. Pipette 380 µl of Glutamate Assay Buffer (1X) to tube A. Pipette 200 µl of Glutamate Assay Buffer (1X) to tubes B-F. Transfer 20 µl of the bulk standard (100 µM) to tube A. Mix gently. Serially dilute the standard by transferring 200 µl from tube A to tube B. Mix gently. Next, transfer 200 µl from tube B to tube C. Mix gently. Repeat the process for tubes D-E. Do not add any standard to tube F. This tube is the zero point, which is the lowest point of the standard curve. The diluted standards will be stable for at least one hour at room temperature.

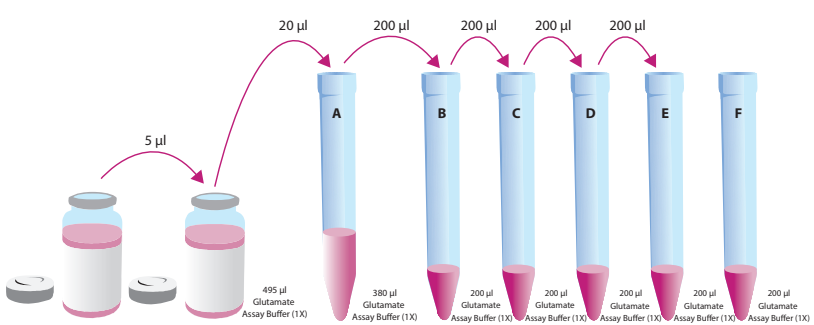


Figure 3. Preparation of the glutamate standards for the fluorometric assay format

Colorimetric Standard Curve Preparation

Dilute 20 µl of the 10 mM Glutamate Standard with 230 µl of Glutamate Assay Buffer (1X) to yield an 800 µM bulk standard. Label six clean glass or polystyrene test tubes A-F. Pipette 360 µl of Glutamate Assay Buffer (1X) to tube A. Pipette 200 µl of Glutamate Assay Buffer (1X) to tubes B-F. Transfer 40 µl of the bulk standard (800 µM) to tube A. Mix gently. Serially dilute the standard by transferring 200 µl from tube A to tube B. Mix gently. Next, transfer 200 µl from tube B to tube C. Mix gently. Repeat the process for tubes D-E. Do not add any standard to tube F. This tube is the zero point, which is the lowest point of the standard curve. The diluted standards will be stable for at least one hour at room temperature.

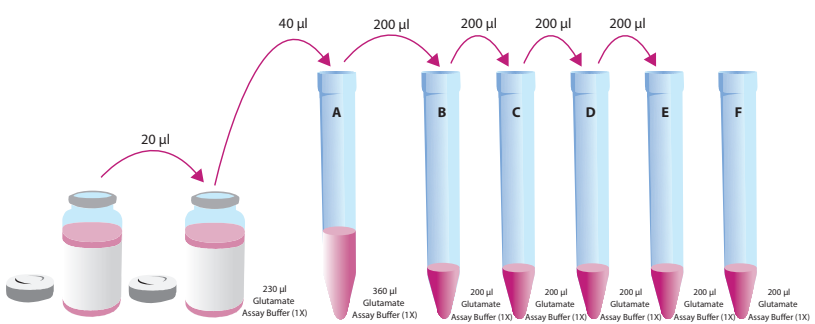


Figure 4. Preparation of the glutamate standards for the colorimetric assay format

Performing the Assay

1. **LGOX$^+$ Reaction Mix:** combine Glutamate Assay Buffer (1X), reconstituted LGOX, reconstituted Glutamate Assay HRP, and diluted MaxiProbe at a 47:1:1:1 ratio. Each sample and standard well will require 50 µl of the LGOX$^+$ Reaction Mix. Prepare a sufficient volume (10-25% surplus) according to the number of wells to be assayed. See Table 1 below for an example.
2. **LGOX$^-$ Background Mix:** combine Glutamate Assay Buffer (1X), reconstituted Glutamate Assay HRP, and diluted MaxiProbe at a 48:1:1 ratio. Each sample background well will require 50 µl of the LGOX$^-$ Background Mix. Prepare a sufficient volume (10-25% surplus) according to the number of wells to be assayed. See Table 1 below for an example.
3. **Standard Wells**: add 50 µl of standard per well in the designated wells on the plate (see **Sample plate format**, Figure 2, on page 12).

	LGOX$^+$ Reaction Mix	LGOX$^-$ Background Mix
# of Wells	48 wells	48 wells
Glutamate Assay Buffer (1X)	2,820 µl	2,880 µl
Reconstituted LGOX	60 µl	--
Reconstituted Glutamate Assay HRP	60 µl	60 µl
Diluted MaxiProbe	60 µl	60 µl

Table 1. Master mix preparation

4. **Sample Wells**: add 50 µl of sample to at least two wells. To obtain reproducible results, the amount of glutamate in a sample should fall within the range of the assay. When necessary, samples should be diluted with Glutamate Assay Buffer (1X).
5. **Sample Background Wells:** Add 50 µl of sample to at least two wells.
6. Initiate the reactions by adding 50 µl of the LGOX$^+$ Reaction Mix to all standard and sample wells. Add 50 µl of the LGOX$^-$ Background Mix to the sample background wells. Mix well by gently pipetting up and down four times without generating bubbles.
7. Cover the plate with non-adhesive foil and incubate for 30 minutes at 37°C.
8. Remove the foil and read fluorescence with excitation and emission wavelengths of 530 and 590 nm, respectively. The background fluorescence intensity will increase over time. If the colorimetric method is used, read absorbance at 570 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence or absorbance of each standard, sample, and sample background well.
2. Subtract the average fluorescence or absorbance value of standard F from itself and all other standards. This is the corrected signal (CS).
3. Plot the CS values (from step 2 above) of each standard as a function of the concentration of glutamate. See Figures 5 and 6, on pages 21 and 22, for typical standard curves.
4. Subtract the average fluorescence or absorbance values of the sample background wells from the average fluorescence or absorbance values of the sample wells to yield the corrected sample measurement (CSM).
5. Calculate the glutamate concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the CSM for each sample, then multiply by the sample dilution factor.

$$[\text{glutamate}]\ (\mu M) = \left[\frac{\text{CSM - (y-intercept)}}{\text{slope}} \right] \text{x sample dilution factor}$$

Performance Characteristics

The assay range describes the lowest and highest concentrations in which glutamate can be reliably detected. The assay range is 0.3-5 µM for the fluorometric assay and 2.6-80 µM for colorimetric assay.

The lower limit of detection (LLOD) is the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value. The LLOD for the fluorometric assay is 0.3 µM, and the LLOD for the colorimetric assay is 2.6 µM.

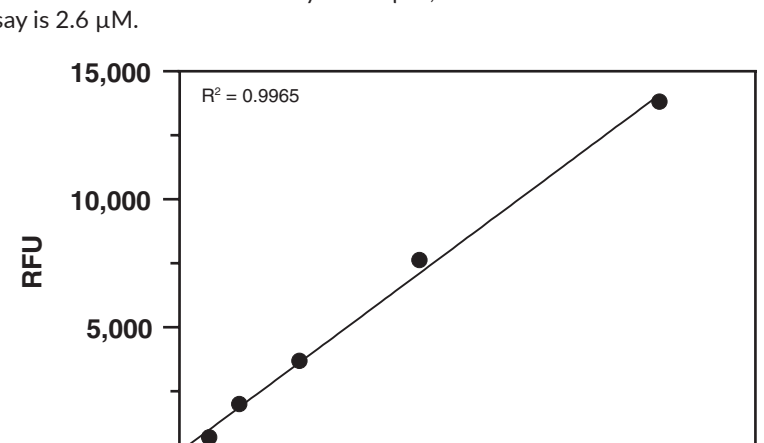


Figure 5. Glutamate standard curve for the fluorometric format

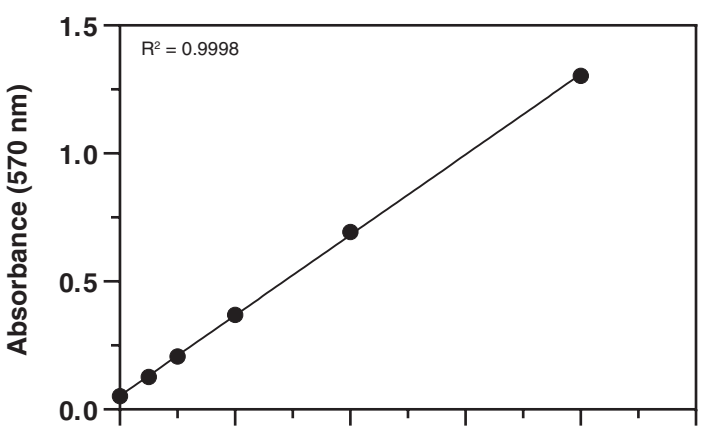


Figure 6. Glutamate standard curve for the colorimetric format

Parallelism

To assess parallelism, human plasma and serum, rat liver homogenate, cell lysates, and cell culture medium were processed as described in the **Sample Preparation** section, serially diluted with Glutamate Assay Buffer (1X), and evaluated using the Glutamate Fluorometric/Colorimetric Assay Kit. Measured glutamate concentrations were plotted against the dilution factor. The results are shown below.

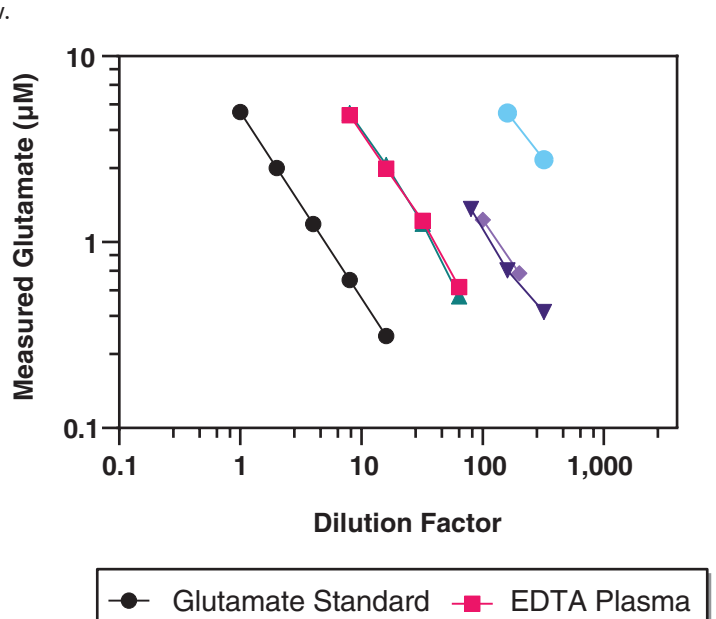


Figure 7. Parallelism of various matrices in the Glutamate Fluorometric/ Colorimetric Assay

Spike and Recovery

Human plasma was spiked with different amounts of glutamate, processed as described in the **Sample Preparation** section, serially diluted with Glutamate Assay Buffer (1X), and evaluated using the Glutamate Fluorometric/Colorimetric Assay Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

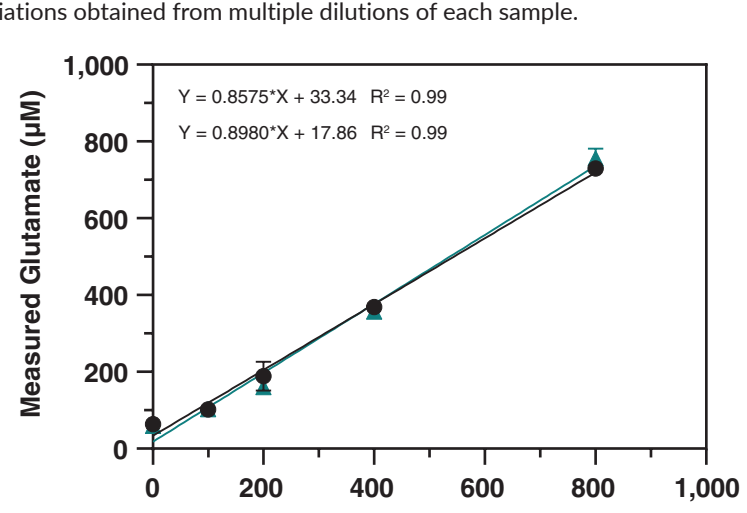


Figure 8. Spike and recovery of glutamate in human plasma

Precision

When a series of three plasma control measurements were performed on the same day, the intra-assay coefficients of variation were 9, 17, or 7%, respectively. When a series of three plasma control measurements were performed on different days under the same experimental conditions, the inter-assay coefficients of variation were 7, 5, or 13%, respectively.

Interferences

Antioxidants, reducing reagents, oxidizing reagents, and reagents that affect the pH of the final reaction interfere with this assay.

The following reagents were tested for interference in the assay.

Reagent		Will Interfere (Yes or No)
Buffers	M-PER™	Yes, but could be countered by preparing standard in this buffer
	T-PER™	Yes, but could be countered by preparing standard in this buffer
	RIPA	Yes, but could be countered by preparing standard in this buffer

Table 2. Interferences

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s) C. The reaction mix was not sufficiently mixed	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 C. Make sure the reaction mix is mixed well
No signal was obtained above the background in the sample wells	A. The glutamate concentration in a sample is below the detection limit of this assay B. The sample type contains high levels of interfering substances such as antioxidants, reducing reagents, and/or oxidizing reagents	A. Re-assay using a lower dilution or extract more sample using less buffer B. Perform serial dilutions of the sample using Glutamate Assay Buffer (1X) to determine if glutamate is detectable at a higher dilution
Glutamate concentration in the sample wells was above the highest point in the standard curve	The glutamate concentration was too high in the sample	Dilute samples with Glutamate Assay Buffer (1X) and re-assay
The fluorometer exhibited 'MAX' value for the wells	The *gain* setting is too high	Reduce the *gain* and re-read

LGOX⁺ Reaction Mix:

Reconstituted or diluted Buffer:LGOX:HRP:Probe = 47:1:1:1

LGOX⁻ Background Mix:

Reconstituted or diluted Buffer:HRP:Probe = 48:1:1

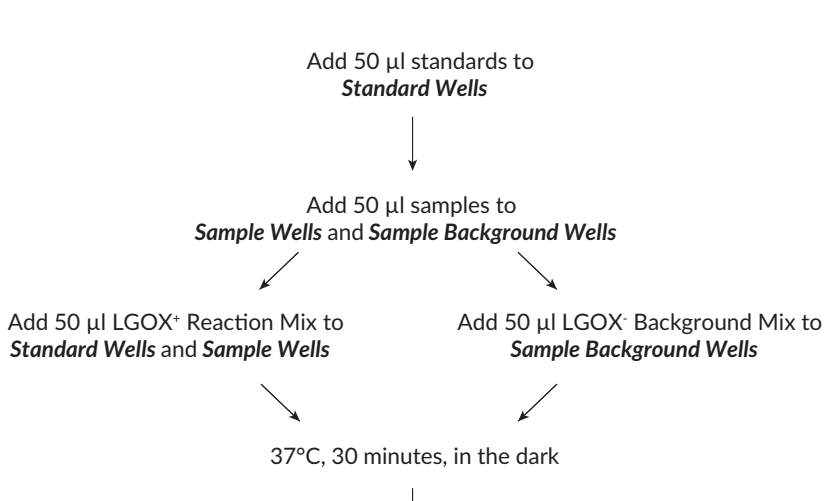
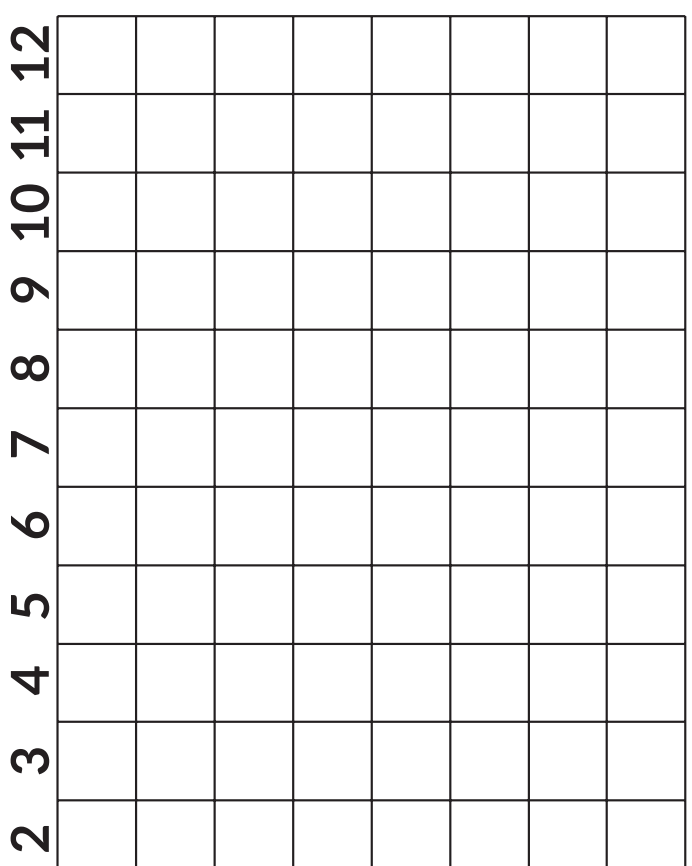


Figure 9. Assay summary



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NOTES

Warranty and Limitation of Remedy

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