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Technical Datasheet

EZAssay™ Glutamine Glutamate Determination Kit

Product Code: CCK037

1. Introduction

In cell culture systems, L-Glutamine is required for a number of biochemical reactions such as peptide and protein synthesis, amino sugar synthesis and nucleic acid synthesis. It also serves as a source of carbon for oxidation reactions in some cells. L-Glutamate is an immediate deamination product of L-Glutamine. L-Glutamate is most abundant intracellular amino acid whereas L-Glutamine is the most abundant extracellular amino acid.

Estimation of L-glutamate and L-Glutamine levels in the culture medium is thus indicative of the cellular metabolism and health of the cells.

CCK037 is EZAssay™ Glutamine Glutamate Determination Kit designed for estimation of concentration of these amino acids in cell culture samples such as cell lysates and spent media.

2. About the assay

Determination of L-Glutamine and L-Glutamate is a two-step enzymatic reaction.

Step 1: Deamination of L-Glutamine

$$\text{Glutamine} + H_2O \xrightarrow[H^+]{\textit{Glutaminase}} \text{L-Glutamate} + NH_4^+$$

Step 2: Dehydrogenation of glutamate and reduction of NAD^+

$$\text{L-Glutamate} + NAD^+ + H_2O \xrightarrow{\textit{Glutamate dehydrogenase}} \alpha\text{-Ketoglutarate} + NH_4^+ + NADH$$

3. Kit contents

The reagents supplied in this kit are sufficient for 100 tests including standards and tests.

Code	Description	CCK037-100	Shipping Temperature
CCK037(A)	Acetate Buffer	1 x 25ml	2 – 8°C
CCK037(B)	Glutaminase	1 x 80µl	2 - 8°C
CCK037(C)	Tris EDTA Hydrazine Buffer	2x60 ml	–20°C
CCK037(D)	Nicotinamide adenine dinucleotide (NAD)	1 x 199mg	-20°C
CCK037(E)	Adenosine – 5 – diphosphate	1 x 42.7mg	-20°C
CCK037(F)	Glutamate dehydrogenase	2 x 1ml	-20°C
CCK037(G)	Glutamine standard	1 x 7.3 mg	-20°C
CCK037(H)	Glutamate standard	1 x3.7mg	-20°C

4. Materials required but not provided in the kit

- Adjustable pipettes and a repeat pipettor
- Clean glass test tubes
- Spectrophotometer
- Water bath

5. General guidelines

Accuracy

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.

- Use of a repeating pipettor is recommended to pipette reagents. This saves time and helps maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents several times.
- Care should be taken so that no bubbles are introduced into the tubes during pipetting or mixing of the reagents.

Procedural precautions

- Thaw all the reagents on ice after reconstitution and keep them on ice. Store reconstituted components at -20ºC in working aliquots.
- Do not leave the reagent bottles and sample bottles open for prolonged duration. Replace the caps of reagent bottles immediately after use and store them under storage conditions mentioned in section 3.

6. Directions for use

Users are advised to review entire procedure before starting the assay

6.1. Preparation of reagents

6.1.1. Glutaminase solution

1. Prepare 1:10 dilution of acetate buffer CCK037(A) in ultrapure water.
2. Take 8µl of Glutaminase CCK037(B) and add 1.0 ml of diluted acetate buffer. The dilution of Glutaminase enzyme with acetate buffer can be adjusted according to usage.
3. Store the solution at 2 – 8ºC until needed.

Note: It is recommended to prepare Glutaminase solution just prior to use.

If not consumed in single experiment, store the remaining solution in working aliquots at -20ºC. The appearance of Glutaminase enzyme is turbid or suspension. Please ensure to mix or swirl properly prior to use.

6.1.2. Nicotinamide adenine dinucleotide (NAD) solution

1. Add 10ml ultrapure water to the bottle of NAD CCK037(D).
2. Mix well until dissolved.
3. Store the solution at 2 – 8ºC until needed.

Note: If not consumed in single experiment, store the remaining solution in working aliquots at -20ºC.

6.1.3. Adenine dinucleotide phosphate (ADP) solution

1. Add 1ml ultrapure water to the bottle of ADP CCK037(E). Mix well until dissolved.
2. Store the solution at 2 – 8ºC until needed.

Note: If not consumed in single experiment, store the remaining solution in working aliquots at -20ºC.

6.1.4. Glutamine standard solution

1. Add 25ml ultrapure water to the bottle of Glutamine standard CCK037(G) to generate 2mM L-Glutamine solution.
2. Mix well until dissolved.
3. Store the solution at 2 – 8ºC until needed.

Note: If not consumed in single experiment, store the remaining solution in working aliquots at -20ºC.

6.1.5. Glutamate standard solution

1. Add 25ml ultrapure water to the bottle of Glutamate standard CCK037(H) to generate 1mM L-Glutamate solution.
2. Mix well until dissolved.
3. Store the solution at 2 – 8ºC until needed.

Note: If not consumed in single experiment, store the remaining solution in working aliquots at -20ºC.

6.1.6. Tris-EDTA-Hydrazine buffer

Cap the bottle tightly and mix well by swirling before use and aliquot into small portion and store at -20°C.

Note: Avoid prolonged exposure of hydrazine to air. Cap the bottle of hydrazine immediately after use.

6.2. Sample preparation

It is recommended to determine sample preparation method depending on type of sample being used.

6.3. Preparation of standard curve

The concentration of L-Glutamine and / or L-Glutamate in samples are determined using standard curves of L-Glutamine, L-Glutamate or both.

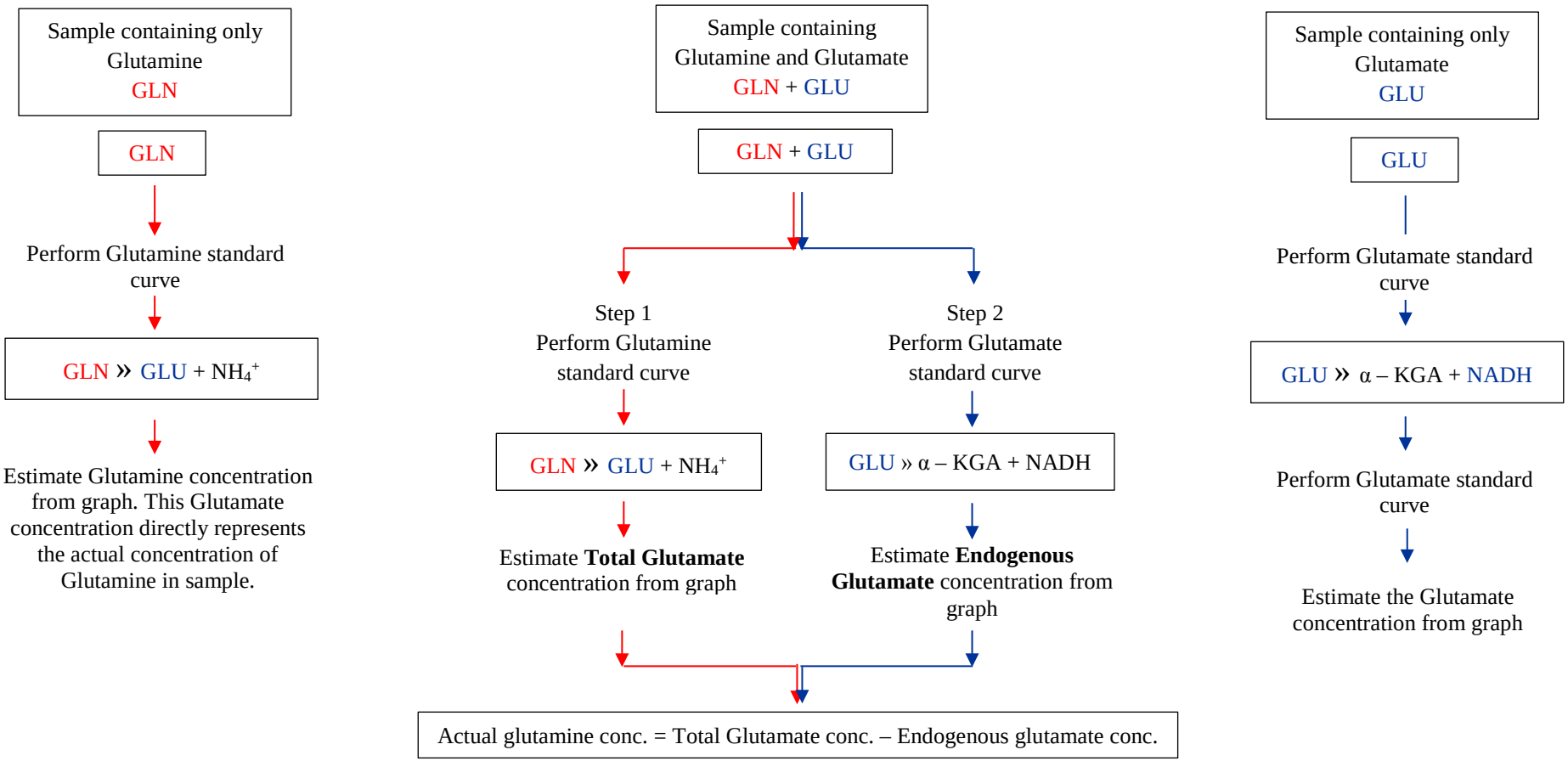
i. If sample contains only L-Glutamine, the standard curve of L-Glutamine is used to determine its concentration.

ii. If sample contains both L-Glutamine and L-Glutamate, endogenous L-Glutamate must be determined and subtracted from L-Glutamate derived from deamination of L-Glutamine. Hence for such samples, standard curves of both, L-Glutamine and L-Glutamate are used.

iii. If sample contains only L-Glutamate, the standard curve of L-Glutamate is used to determine its concentration.

Figure 1: Estimation of glutamine and glutamate in different samples

Key- GLN: Glutamine
GLU: Glutamate
GLN + GLU: Glutamine + Glutamate



6.4. L-Glutamine standard curve

1. In clean glass test tubes, prepare serial dilutions of standard L-Glutamine solution CCK037 (G).
2. Label the tubes properly with respective glutamine concentration.
3. For samples containing only L-glutamine, label the tube as 'GLN'.
   For sample containing L-glutamine and L-glutamate both, label the tube as 'GLN + GLU'.
   Note: If the test sample is known to contain glutamine concentration greater than 2mM, it must be diluted with acetate buffer.
4. Refer table 1 for assay scheme.
5. Calculate corrected absorbance by subtracting background absorbance of each tube from respective actual absorbance.
6. Plot a standard curve of glutamine with glutamine concentrations on X-axis and corrected absorbance on Y-axis.
   Determine slope of the curve. Extrapolate the concentration of glutamine in test samples from the slope.
7. Multiply the obtained concentration by dilution factor.
   Note: Refer figure 2 for L-Glutamine standard curve. ***DO NOT*** *use the same for interpretation of actual data. A new standard curve must be set up every time the assay is run.*
8. Perform glutamate standard curve when using GLU + GLN samples.
9. Calculation of glutamine concentration

a. For ONLY Glutamine containing sample

$$\text{Total Glutamine } (mM) = \frac{\text{Corrected OD of Sample} - \text{c}}{\text{m(Slope)}x\ 0.25\ or\ sample\ volume} \text{ x Dilution factor}$$

y=mx+c (equation of line)

(here the concentration of Glutamine is actually glutamine converted to glutamate)

b. For Glutamine and Glutamate containing sample

$$\text{Total Glutamine } (mM) = \frac{\text{Corrected OD of Sample} - \text{c}}{\text{m(Slope)}x\ 0.25\ or\ sample\ volume} \text{ x Dilution factor}$$

Calculate the concentration of endogenous L-Glutamate from glutamate standard curve. Subtract the endogenous glutamate from total glutamate to obtain actual glutamine concentration in the sample.

Actual L-Glutamine = Total L-Glutamate – Endogenous L-Glutamate

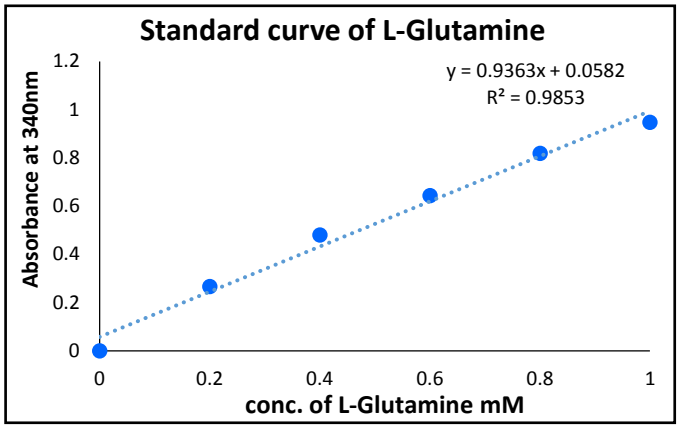


Figure 2: Glutamine standard curve

DO NOT *use this graph to interpret actual results. It is ONLY for reference.*

Table 1: L-Glutamine standard curve assay scheme

REACTION A								
	Glutamine concentration (mM)						**Test sample**	
Reagents (µl)	**0**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**GLN**	**GLU + GLN**
Acetate buffer	200	200	200	200	200	200	200	200
Glutaminase	100	100	100	100	100	100	100	100
Glutamine solution	0	100	200	300	400	500	0	0
Test sample	-	-	-	-	-	-	250	250
Water	700	600	500	400	300	200	450	450
Total volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000
Mix the contents thoroughly by shaking the tubes/ pipetting up and down. Incubate at 37°C for 1 hour (preferably water bath).								
Reaction B- Take separate labelled tubes as per reaction A								
Tris-EDTA-Hydrazine buffer	1000	1000	1000	1000	1000	1000	1000	1000
NAD solution	100	100	100	100	100	100	100	100
ADP solution	10	10	10	10	10	10	10	10
From Reaction A	500	500	500	500	500	500	500	500
Water	390	390	390	390	390	390	390	390
i. Mix well and transfer required amount solution from ‘Blank’ tube to spectrophotometer cuvette. ii. Adjust the baseline absorbance to zero at 340nm using ‘Blank’. iii. Transfer the contents of cuvette back to the tube. iv. Read absorbance of other tubes at 340nm to obtain background absorbance. v. After every reading transfer the contents to respective tubes for further reaction. vi. Add 20µl to each tube and mix well.								
Glutamate dehydrogenase (µl)	20	20	20	20	20	20	20	20
i. Incubate the tubes at room temperature for 40 minutes. ii. Mix well and transfer required amount solution from ‘Blank’ tube to spectrophotometer cuvette. iii. Adjust the baseline absorbance to zero at 340nm using ‘Blank’. iv. Transfer the contents of cuvette back to the tube. v. Read absorbance of other tubes at 340nm to obtain actual absorbance generated due to reduction of NAD to NADH.								

6.5. Glutamate standard curve

1. In clean glass test tubes, prepare serial dilutions of standard L-Glutamate solution CCK037 (H).
2. Label the tubes properly with respective glutamate concentrations and test samples.
   Note: If the test sample is known to contain glutamate concentration greater than 1mM, it must be diluted with acetate buffer.
3. Refer table 2 for assay scheme.
4. Calculate corrected absorbance by subtracting background absorbance of each tube from respective actual absorbance.
5. Plot a standard curve of glutamate with glutamate concentrations on X-axis and corrected absorbance on Y-axis.
6. Determine slope of the curve.
7. Extrapolate the concentration of Glutamate in test samples from the slope.
8. Multiply the obtained concentration by dilution factor.
9. Calculate the concentration of L-Glutamate in the sample.

$$\text{Glutamate (mM)} = \frac{\text{Corrected OD of Sample} - c}{\text{m(Slope)x0.25 or sample volume}} \text{ x Dilution factor}$$

Note: Refer figure 3 for L-Glutamate standard curve. A new standard curve must be set up every time the assay is run.

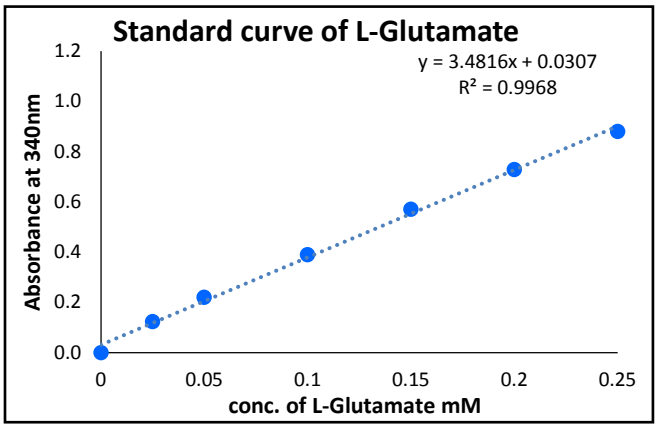


Figure 3: Glutamate standard curve
***DO NOT** use this graph to interpret actual results. It is ONLY for reference.*

Table 2: L-Glutamate standard curve assay scheme

	Glutamate concentration (mM)							Test sample
Reagents (µl)	**0**	**0.025**	**0.05**	**0.1**	**0.15**	**0.2**	**0.25**	**GLU**
Tris-EDTA-Hydrazine buffer	1000	1000	1000	1000	1000	1000	1000	1000
NAD solution	100	100	100	100	100	100	100	100
ADP solution	10	10	10	10	10	10	10	10
Glutamate standard solution	0	50	100	200	300	400	500	-
Water	890	840	790	690	590	490	390	640
Test sample	-	-	-	-	-	-	-	250
i.	ii. Mix the contents thoroughly by shaking the tubes. iii. Transfer required amount of solution from 'Blank' tube to spectrophotometer cuvette. iv. Adjust the baseline absorbance to zero at 340nm using 'Blank'. v. Transfer the contents of cuvette back to the tube. vi. Read absorbance of other tubes at 340nm to obtain background absorbance. vii. After every reading transfer the contents to respective tubes for further reaction.							
Glutamate dehydrogenase (µl)	20	20	20	20	20	20	20	20
i.	ii. Incubate the tubes at room temperature for 40 minutes. iii. Mix well and transfer required amount of solution from 'Blank' tube to spectrophotometer cuvette. iv. Adjust the baseline absorbance to zero at 340nm using 'Blank'. v. Transfer the contents of cuvette back to the tube. vi. Read absorbance of other tubes at 340nm to obtain actual absorbance.							

7. Storage and shelf life

On receipt store the components at temperature mentioned on individual labels.

After reconstitution, store the solutions in working aliquots at recommended temperatures (Refer section 6.1). Use before expiry date mentioned on the product label.

8. Advantages

- **Sensitivity and accuracy:** As the assay is based on an enzyme-substrate reaction, it is very sensitive.

9. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
Assay not working	Ice cold reagents used for the assay	Equilibrate the reagents to room temperature and repeat the assay.
Very low absorbance values	Improperly thawed reagents	Thaw the reagents completely, equilibrate at room temperature and repeat the assay
	Improper selection of filter for reading the absorbance	Choose appropriate filter i.e. 340nm
Very high absorbance values of samples	Concentration of L-Glutamine and / or L-Glutamate too high in the sample	Dilute the sample and repeat the assay
Random absorbance values/ poor consistency of replicates	Inaccurate pipetting technique or inaccurate equipment	Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents
	Use of old or inappropriately stored reagents	Store the reagents at recommended temperatures.
	Air bubbles formed in the cuvette / tube	Mix the contents gently to avoid formation of air bubbles. Perform reverse pipetting.
	Multiple freeze-thaw of the reagents	Aliquot the samples in working aliquots and freeze for future use.