SensoLyte® Green Glutaminyl Cyclase Activity Assay Kit**Fluorimetric***

Revision number: 2.2	Last updated: 27MAR23
Catalog #	**AS-72230**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect Glutaminyl Cyclase activity.
- ***Enhanced Value:*** It provides enough reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed in two hours.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Glutaminyl Cyclase Green Substrate	0.5 mM in DMSO, 50 µL
Component B	Pyroglutamated Fluorescent Substrate Standard	0.5 mM in DMSO, 15 µL
Component C	Glutaminyl Cyclase, Human Recombinant	50 µg/ml, 10 µL
Component D	Glutaminyl Cyclase Developer, 250X	25 µL
Component E	Assay Buffer	35 mL
Component F	Inhibitor of Glutaminyl Cyclase (1-Benzyl-Imidazole)	1 M in DMSO, 75 µL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.
- Plate cover: To prevent liquid evaporation during incubation step.

Storage and Handling

- Store kit Components A, B, E, F at -20 °C. Store Components C and D at -80 °C.
- Protect Components A and B from light and from moisture.

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Introduction

Many biologically active peptides and proteins have pyroglutamate (pGlu) as their first amino acid. This modification protects them from degradation by amino peptidases and in many cases determines functionality of protein or peptide.[1-6] However, pyroglutamate modification sometimes may lead to negative side effects. For example, pGlu modified beta-amyloid peptides (pGlu-Aβ) aggregate much faster compared to non-pyroglutamic ones and form toxic oligomers that may damage neurons.[7-9] Recent studies linked pGlu-Aβ to the Alzheimer's Disease because it was identified as a major component of amyloid plaques.[7-9]

pGlu can be formed when glutamine or glutamic acid spontaneously convert into pyroglutamate. However, cyclic reaction rate is dramatically increased in the presence of Glutaminyl Cyclase (QC), also known as Glutaminyl-peptide Cyclotransferase (QPCT). Human Glutaminyl Cyclase has been isolated from nerve tissue, and detected in cerebrospinal fluid and saliva.[6]

The SensoLyte® Green Glutaminyl Cyclase Activity Assay Kit provides a convenient, two-step homogeneous procedure for measuring enzyme activity from various sources using a green fluorescence substrate. During the first step, substrate is incubated with Glutaminyl Cyclase or enzyme-containing samples and converted into the pyroglutamate form. Secondly, glutaminyl cyclase developer is added to remove pGlu residue and generate the green fluorophore. It can be detected with excitation at 490 nm and emission at 520 nm. Produced fluorescence is proportional to the enzyme activity. This kit is ideal for high throughput screening (HTS) of glutaminyl cyclase activators and inhibitors. The long wavelength of the green fluorophore is less interfered by autofluorescence of cell components and test compounds. Assay sensitivity is 1.5 ng/mL of active enzyme.

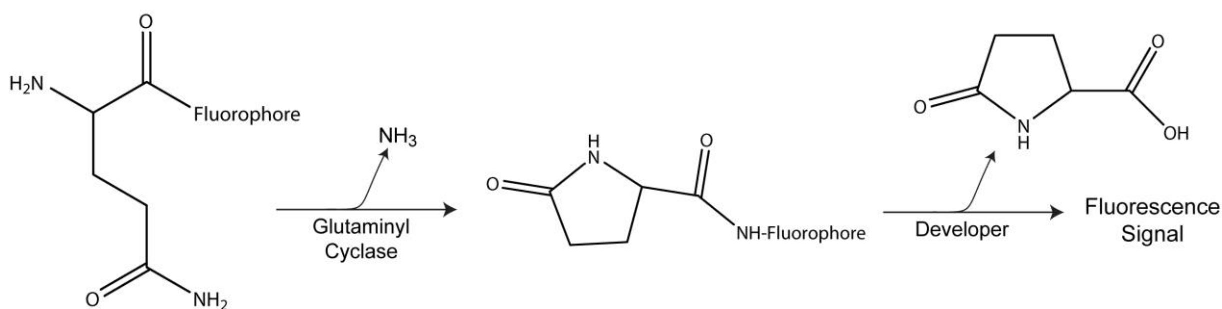

Figure 1. SensoLyte® Green Glutaminyl Cyclase Assay Principle.

Protocol

Note: Please use Protocol A or B based on your needs.

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SensoLyte® Green Glutaminyl Cyclase Activity Assay Kit* *Fluorimetric**

<u>Protocol A.</u> Screening of Glutaminyl Cyclase inhibitors using purified enzyme.

1. Prepare working solutions.

<u>Note 1</u>: Allow all kit components to thaw before starting the experiment. Components C and D should be kept on ice.
<u>Note 2</u>: Briefly centrifuge Components C and D to completely recover enzymes.

<u>1.1 Glutaminyl Cyclase substrate solution</u>: Dilute Glutaminyl Cyclase substrate (Component A) 1:100 in the assay buffer (Component E). This will provide working substrate solution of 5 µM. 50 µL of this diluted substrate is enough for one-well reaction (96-well plate). For each experiment prepare fresh substrate solution.

Table 1. Glutaminyl Cyclase Substrate solution for one 96-well plate (100 assays).

Components	Volume
Glutaminyl cyclase substrate, 0.5 mM (Component A)	50 µL
Assay buffer (Component E)	4.95 mL
Total volume	5 mL

<u>1.2 Recombinant Glutaminyl Cyclase diluent</u>: Dilute human enzyme (Component C) with 3.99 ml of the assay buffer (Component E) to a final concentration of 125 ng/ml. This amount of enzyme is enough for a full 96-well plate at 5 ng/well of enzyme. If not using the entire plate, adjust the amount of enzyme to be diluted accordingly. Suggested volume of the diluted enzyme solution for one well in a 96-well plate is 40 µL.

<u>Note</u>: Do not vortex enzyme solutions. Dilute enzyme immediately before use. Prolonged storage or vigorous agitation of the diluted enzyme will cause enzyme denaturation. Store diluted enzyme on ice.

<u>1.3 Glutaminyl Cyclase Inhibitor</u>: Dilute the 1M inhibitor solution (Component F) 1000-fold in assay buffer to make the 1mM diluted inhibitor solution. Add 10 µl of the diluted inhibitor solution into each of the inhibitor control well.

<u>1.4 Reaction Developer</u>: Dilute Glutaminyl Cyclase Developer (Component D) 1:250 with the assay buffer (Component E). Each assay well requires 50 µL of the prepared enzyme developer.

Table 2. Glutaminyl Cyclase Developer solution for one 96-well plate (100 assays).

Components	Volume
Glutaminyl Cyclase Developer, (Component D)	20 µL
Assay buffer, (Component E)	4.98 mL
Total volume	5 mL

<u>Note1:</u> Prepare developer before use. Otherwise keep prepared solution on ice until use.
<u>Note2</u>: To stop Glutaminyl Cyclase reaction the inhibitor (Component F) can be added to the developer solution at 10 µL per mL of the developer solution. In that case developer will work as a bi-functional buffer that quenches Glutaminyl Cyclase and releases fluorophore to generate fluorescent signal.

2. **Set up the enzymatic reaction.**

2.1 Add test compounds and enzyme solution to the microplate wells. For one well of a 96-well plate, suggested volume of Glutaminyl Cyclase enzyme solution is 40 µL and 10 µL of test compound.

2.2 Establish the following control wells at the same time, as deemed necessary:

- Positive control contains Glutaminyl Cyclase enzyme without test compound.
- Inhibitor control contains enzyme and 1-BenzylImidazole.
- Vehicle control contains enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer (Component E) and test compound. Some test compounds may themselves be fluorescent or interfere with the Ex/Em wavelengths and thereby give false results.
- Substrate control contains assay buffer (Component E).

2.3 Using the assay buffer (Component E), bring the total volume of all controls to 50 µL.

3. **Run the enzymatic reaction.**

3.1 Add 50 µL of Glutaminyl Cyclase substrate solution into each well, except the test compound control wells. For best accuracy, it is advisable to have substrate solution equilibrated to the assay temperature. Mix reagents completely by shaking the plate gently for no more than 30 sec.

3.2 Cover and incubate plate for 30 minutes at 37 C°.

3.3 Add 50 µL of the prepared Glutaminyl Cyclase developer and mix thoroughly.

3.4 Incubate the plate for additional 60 min at 37 C°.

3.5 Measure fluorescence signal at Ex/Em=490 nm/520 nm.

3.6 An example of the inhibition of Glutaminyl Cyclase by 1-BenzylImidazole is shown in Figure 2.

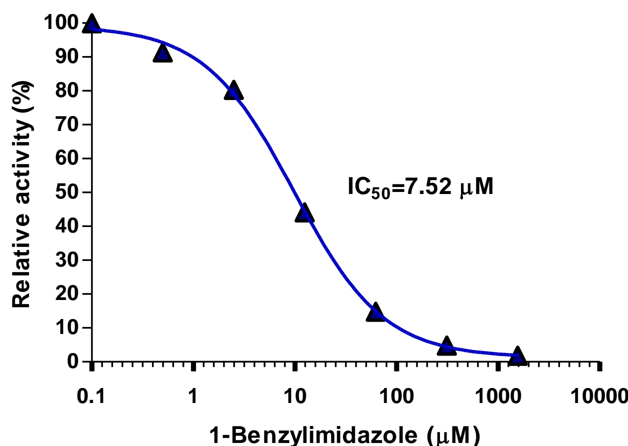

Figure 2. 1-Benzyl-Imidazole inhibition of Glutaminyl Cyclase activity measured with SensoLyte® Green Glutaminyl Cyclase Activity Assay Kit.

4. Data Analysis

4.1 The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).

4.2 For kinetic analysis of Glutaminyl Cyclase activity, add enzyme and substrate solutions into multiple wells and stop reaction at different time points with 10 µl/well of 50mM 1-Benzyl-Imidazole (for example at time=5, 10, 15, 20, and 30 minutes). After all reactions wells are quenched, follow Steps 3.3-3.5 of this protocol.
Refer to the Appendix I for instrument calibration procedure.

Protocol B. Measuring Glutaminyl Cyclase activity in biological samples.

1. Prepare Glutaminyl Cyclase containing biological samples.

1.1 Prepare saliva samples:

- Collect saliva and immediately centrifuge at 10,000 rpm for 10-15 minutes at 4 °C.
- Collect the supernatant and store at -70°C until use.
- Saliva should be diluted with the assay buffer (component E) prior to testing. Dilution ratio of 2-5 should provide adequate results.

1.2 Obtain Cerebrospinal Fluid (CSF) samples:

- Usually CSF is collected using Lumbar puncture without further processing.
- Store CSF samples at -70°C until use.
- CSF should be diluted with the assay buffer (component E) prior to testing. Dilution ratio of 2-5 should provide adequate results.

2. Prepare working solutions.

Note 1: Allow all kit components to thaw before starting the experiment. Components C and D should be kept on ice.
Note 2: Briefly centrifuge Components C and D to completely recover enzymes.

2.1 Glutaminyl Cyclase substrate solution: Dilute substrate (Component A) 1:100 in the assay buffer (Component E). This will provide working substrate solution of 5 µM. 50 µL of this diluted QC

substrate is enough for one-well reaction (96-well plate). For each experiment prepare fresh substrate solution.

Table 3. Glutaminyl Cyclase substrate solution for one 96-well plate (100 assays).

Components	Volume
Glutaminyl Cyclase substrate, 0.5 mM (Component A)	50 µL
Assay buffer (Component E)	4.95 mL
Total volume	5 mL

2.2 Glutaminyl Cyclase diluent: If using purified enzyme as a control, dilute human recombinant Glutaminyl Cyclase (Component C) with 4.99 ml of the assay buffer (Component E) to a final concentration of 100 ng/ml. Suggested volume of the diluted enzyme solution for one well of a 96-well plate is 50 µL.

Note: Do not vortex enzyme solutions. Dilute enzyme immediately before use. Prolonged storage or vigorous agitation of the diluted enzyme will cause enzyme denaturation. Store diluted enzyme on ice.

2.3 Glutaminyl Cyclase Inhibitor: Dilute the 1M inhibitor solution (Component F) 1000-fold in assay buffer to make the 1mM diluted inhibitor solution. Add 10 µl of the diluted inhibitor solution into each of the inhibitor control well.

2.4 Reaction Developer: Dilute Glutaminyl Cyclase Developer (Component D) 1:250 with the assay buffer (Component E) as described in Table 4. Each assay requires 50 µL of the prepared enzyme developer.

Table 4. Glutaminyl Cyclase Developer solution for one 96-well plate (100 assays).

Components	Volume
Glutaminyl Cyclase Developer, (Component D)	20 µL
Assay buffer, (Component E)	4.98 mL
Total volume	5 mL

Note1: Prepare developer before use. Otherwise keep prepared solution on ice until use.
Note2: To stop Glutaminyl Cyclase reaction the inhibitor (Component F) can be added to the developer solution at 10 µL per mL of the developer solution. In that case developer will work as a bi-functional buffer that quenches Glutaminyl Cyclase and releases fluorophore to generate fluorescent signal.

3. Set up enzymatic reaction.

3.1 Add 50 µL of Glutaminyl Cyclase containing biological sample.

3.2 Set up the following control wells at the same time, as deemed necessary:

- Positive control contains purified Glutaminyl Cyclase enzyme.
- Inhibitor control contains test sample and 1-Benzyl-Imidazole.

- Substrate control contains assay buffer.

3.3 Using the assay buffer, bring total volume of all controls to 50 µL if necessary.

4. Run the enzymatic reaction.

4.1 Add 50 µL of the Glutaminyl Cyclase substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature (37 ºC usually gives higher signal-to-background ratio). Mix the reagents completely by shaking the plate gently for 30 sec.

4.2 Incubate covered plate at 37 °C for 30 minutes. For samples with low amount of the enzyme, suggested incubation time is 45 minutes.

4.3 Add 50 µL of the prepared Glutaminyl Cyclase developer and mix thoroughly.

4.4 Incubate the plate for an additional 60 min at 37 C°.

4.5 Measure fluorescence intensity at Ex/Em=490 nm/520 nm.

5. Data Analysis

5.1 The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).

5.2 For kinetic analysis of Glutaminyl Cyclase activity, add enzyme containing samples and substrate solutions into multiple wells and stop reaction at different time points with 10 µl/well of 50mM 1-Benzyl-Imidazole (for example at time=5, 10, 15, 20, and 30 minutes). After all reactions wells are stopped, follow Steps 4.3-4.5 of this protocol.
Refer to the Appendix I for instrument calibration procedure.

Appendix I. Instrument Calibration

- The standard for this assay is pyroglutamated substrate (Component B). It is not fluorescent until acted upon by the developer. Production of fluorescence is complete within 30 min incubation (if using in the protocol below), and is directly proportional to the concentration of the pyroglutamated standard.
- Pyroglutamated standard: Dilute 0.5 mM pyroglutamated standard (Component B) 1:200 in the assay buffer (Component E) to obtain 2.5 µM stock solution. Perform 2-fold serial dilutions with this diluted standard to obtain 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 µM pyroglutamated solutions, including an assay buffer blank. Add 100 µL/well of these serially diluted standard solutions into the plate wells.

- Add 50 µL of Glutaminyl Cyclase developer (prepare as in Step 3 from Protocol A) to each well. Mix reagents by shaking plate gently for 3 to 5 sec.
- Incubate the plate for 60 min at 37 °C.
- Measure fluorescence intensity of the reference standard wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot pyroglutamated standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 3.

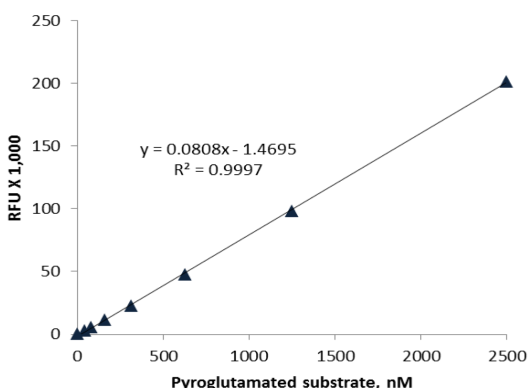

Figure 3. Example of pyroglutamated reference standard curve.
Pyroglutamated standard was serially diluted with the assay buffer, and after 30 min incubation with Glutaminyl Cyclase developer, fluorescence was recorded at Ex/Em=490 nm/520 nm (Flexstation 384II, Molecular Devices).

Note: The concentration of pyroglutamated reference standard solutions are 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0 µM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of enzymatic reaction in the final product.

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