—A helping hand for your research

Product Manual

Configuration-specific Monoclonal Antibody Based

$G\alpha_i$ Activation Assay Kit

Catalog Number：80301

20 assays

NewEast Biosciences
24 Whitewoods Lane
Malvern, PA 19355

Fax: 610-945-2008
Phone: 610-945-2007
Web: www.neweastbio.com
E-mail: sale@neweastbio.com

Table of Content

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

Product Description

A structurally diverse repertoire of ligands, from photons to large peptides, activates G protein-coupled receptors (GPCRs) to elicit their physiological functions. Ligand-bound GPCRs, in turn, function as guanine nucleotide exchange factors catalyzing the exchange of GDP bound on the Gα subunit with GTP in the presence of Gβγ, causing the dissociation of the Gα subunit from the Gβγ dimer to form two functional units (Gα and Gβγ). Both Gα and Gβγ subunits signal to various cellular signaling pathways. Based on the sequence and functional homologies, G proteins are grouped into four families: Gs, Gi, Gq, and G12.

$G\alpha_i$ family is the largest family of G proteins. They relay signals from many GPCRs to regualte various biological functions. There were no direct methods to measure the activation of $G\alpha_i$ proteins by receptors (until this assay kit). Most reports used one of the downstream pathway, i.e. the inhibition of adenylyl cyclases, as a readout. Alternatively, sensitivity to pertussis toxin (PTX) was used as an indicator of possible $G\alpha_i$ proteins invovled in a signaling pathway.

NewEast Biosciences $G\alpha_i$ Activation Assay Kit provides a direct measurement of the activation of $G\alpha_i$ proteins. This is a simple and fast tool to monitor the activation of $G\alpha_i$. Each kit provides sufficient quantities to perform 20 assays.

NewEast Biosciences $G\alpha_i$ Activation Assay Kit is based on the monoclonal antibody specifically recognizing the active GTP-bound $G\alpha_i$ proteins. This monoclonal antibody has much lower affinity towards the inactive $G\alpha_i$ proteins. Therefore, after activation by receptor signals, active GTP-bound $G\alpha_i$ proteins could be immunoprecipitated by this monoclonal antibody and further quantified by western blot with another anti- $G\alpha_i$ antibody.

Assay Principle

NewEast Biosciences $G\alpha_i$ Activation Assay Kit is an immunoprecipitation/western blot assay to measure the levels of active GTP-bound $G\alpha_i$ proteins, either from cell extracts or from in vitro GTPγS loaded $G\alpha_i$ proteins. Briefly, the anti-active $G\alpha_i$ monoclonal antibody will specifically bind to active $G\alpha_i$ protein. This antibody/ $G\alpha_i$ complex will then be pulled down by protein A/G agarose. The precipitated active $G\alpha_i$ proteins will be detected by immunoblots with another anti-$G\alpha_i$ antibody.

Kit Components

1. Anti-active $G\alpha_i$, Mouse Monoclonal Antibody (Catalog No. 26901): One vial – 22 µL (1 mg/mL) in PBS, pH 7.4, contained 50% glycerol. This antibody specifically recognizes GTP- $G\alpha_i$ from all vertebrates.

2. Protein A/G Agarose (Catalog No. 30301): One vial – 400 µL of 50% slurry.

3. 5X Assay/Lysis Buffer (Catalog No. 30303): One bottle – 30 mL of 250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 5 mM EDTA, 5% Triton X-100.

4. Anti-$G\alpha_i$, Mouse Monoclonal Antibody (Catalog No. 26003): One vial – 22 µL(1 mg/mL) in PBS, pH 7.4, contained 50% glycerol.

5. 100 X GTPγS (Catalog No. 30302): One vial –100 µL at 10 mM, use 5 µL of GTPγS for GTP-labeling of 0.5 mL of cell lysate.

6. 100 X GDP (Catalog No. 30304): One vial –100 µL at 100 mM, use 5 µL of GDP for GDP-labeling of 0.5 mL of cell lysate.

Storage

Store all kit components at 4°C until their expiration dates.

Materials Needed but Not Supplied

1. Stimulated and non-stimulated cell lysates
2. Protease inhibitors
3. 4 °C tube rocker or shaker
4. 1 M $MgCl_2$
5. 2X reducing SDS-PAGE sample buffer
6. Electrophoresis and immunoblotting systems
7. Immunoblotting wash buffer such as TBST (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05 % Tween-20)
8. Immunoblotting blocking buffer (TBST containing 5 % Non-fat Dry Milk or 3 % BSA)
9. PVDF or nitrocellulose membrane
10. Secondary Antibody
11. ECL Detection Reagents

Reagent Preparation

- 1X Assay/Lysis Buffer: Mix the 5X Stock briefly and dilute to 1X in deionized water. Just prior to usage, add protease inhibitors such as 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin.

Sample Preparation

Adherent Cells

1. Culture cells (one 10-cm plate, ~ 10^7 cells) to approximately 80-90 % confluence. Stimulate cells with activator or inhibitor as desired.
2. Aspirate the culture media and wash twice with ice-cold PBS.
3. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer to the cells (0.5 - 1 mL per 10 cm tissue culture plate).
4. Place the culture plates on ice for 10-20 minutes.
5. Detach the cells from the plates by scraping with a cell scraper.
6. Transfer the lysates to appropriate size tubes and place on ice.
7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.

Clear the lysates by centrifugation for 10 minutes (12,000 x g at 4 °C).

8. Collect the supernatant and store samples (~1-2 mg of total proteins) on ice for immediate use, or snap freeze and store at - 70 °C for future use.

Suspension Cells

1. Culture cells and stimulate with activator or inhibitor as desired.
2. Perform a cell count, and then pellet the cells by centrifugation.
3. Aspirate the culture media and wash twice with ice-cold PBS.
4. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer to the cell pellet (0.5 – 1 mL per 1 x 10^7cells).
5. Lyse the cells by repeated pipetting.
6. Transfer the lysates to appropriate size tubes and place on ice.
7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.

8. Clear the lysates by centrifugation for 10 minutes (12,000 x g at 4 °C).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at -70 °C for future use.

In vitro GTPγS/GDP Protein Loading for positive and negative controls

Note: In vivo stimulation of cells with receptor ligands might activate ~10 % of the available $G\alpha_i$ proteins, whereas in vitro GTPγS loading could activate ~50 % of the $G\alpha_i$ proteins that can be activated.

1. Aliquot 0.5 mL of each cell extract to two microfuge tubes.
2. To each tube, add 5 µL of 1M $MgCl_2$ (to 10 mM final concentration).
3. Add 5 µL of 100X GTPγS (to 100 µM, final concentration) to one tube (positive control).
4. Add 5 µL of 100X GDP (to 1 mM, final concentration) to the second tube (negative control).
5. Incubate the tubes at 30°C for 90 minutes with agitation.

Assay Procedure

I. Active $G\alpha_i$ Pull-Down Assay

1. Aliquot 0.5 – 1 mL of cell lysate to a microcentrifuge tube.
2. Adjust the volume of each sample to 1 mL with 1X Assay/Lysis Buffer.
3. Add 1 µL anti-active $G\alpha_i$ monoclonal antibody (Cat. No. 26901) to the tube.
4. Thoroughly resuspend the protein A/G agarose bead slurry by vortexing or titurating.
5. Add 20 µL of resuspended bead slurry to each tube.
6. Incubate the tubes at 4 °C for 1 hour with gentle agitation.
7. Pellet the beads by centrifugation for 10 seconds at 12,000 x g.
8. Aspirate and discard the supernatant, making sure not to disturb/remove the bead pellet.
9. Wash the bead 3 times with 0.5 mL of 1X Assay/Lysis Buffer, centrifuging and aspirating each time.
10. After the last wash, pellet the beads and carefully remove all the supernatant.
11. Resuspend the bead pellet in 20 µL of 2X reducing SDS-PAGE sample buffer.
12. Boil each sample for 5 minutes.
13. Centrifuge each sample for 10 seconds at 12,000 x g.

II. Electrophoresis and Transfer

1. Load 20 µL/well of pull-down supernatant to a polyacrylamide gel. Also, it's recommended to include a pre-stained MW standard (as an indicator of a successful transfer in step 3).
2. Perform SDS-PAGE as per the manufacturer's instructions.
3. Transfer the gel proteins to a PVDF or nitrocellulose membrane as per the manufacturer's instructions.

III. Immunoblotting and Detection (all steps are at room temperature, with agitation)

1. Following the electroblotting step, immerse the PVDF membrane in 100% Methanol for 15 seconds, and then allow it to dry at room temperature for 5 minutes.

   Note: If Nitrocellulose is used instead of PVDF, this step should be skipped.

2. Block the membrane with 5 % non-fat dry milk or 3 % BSA in TBST for 1 hr at room temperature with constant agitation.

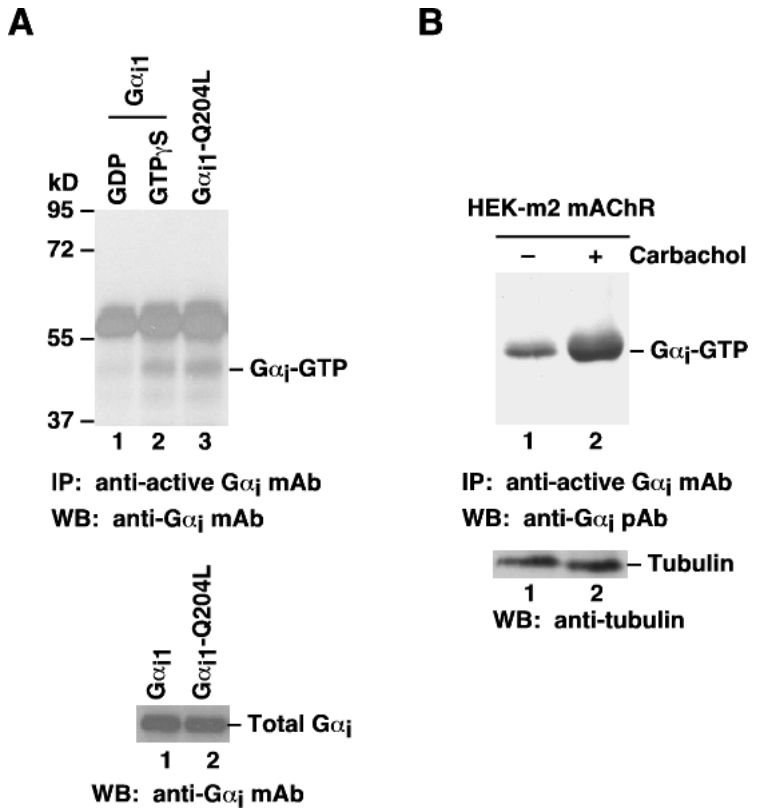
   Incubate the membrane with anti-$G\alpha_i$ monoclonal antibody (Cat. No. 26003), freshly diluted 1:100 ~ 1000 in 5 % non-fat dry milk or 3 % BSA/TBST, for 1-2 hr at room temperature with constant agitation.

   Note: To conserve antibody, incubations should be performed in a plastic bag.

3. Wash the blotted membrane three times with TBST, 5 minutes each time.
4. Incubate the membrane with a secondary antibody (e.g. goat anti-mouse IgG, HRP-conjugate), freshly diluted in 5 % non-fat dry milk or 3 % BSA/TBST, for 1 hr at room temperature with constant agitation.
5. Wash the blotted membrane three times with TBST, 5 minutes each time.
6. Use the detection method of your choice.

Example of Results

The following figure demonstrates typical results seen with NewEast Biosciences $G\alpha_i$ Activation Assay Kit. One should use the data below for reference only.



$G\alpha_i$ activation assay. **A.** CHO cells were transfected with wild-type $G\alpha_{i1}$ (lanes 1 and 2) or constitutively active $G\alpha_{i1}$-Q204L (lane 3). Cell lysates were treated with GDP (lane 1) or GTPγS (lane 3). Lysates were then incubated with an anti-active $G\alpha_i$ monoclonal antibody (Cat. No. 26901) (top panel). The precipitated active $G\alpha_i$ was immunoblotted with an anti- $G\alpha_i$ monoclonal antibody (Cat. No. 26003). The bottom panel shows the Western blot with anti- $G\alpha_i$ monoclonal antibody (Cat.No. 26003) of the cell lysates. B. HEK293 cells stably expressing human m2 mAChR were treated with (lane 2) or without (lane 1) carbachol. Cell lysates were then incubated with an anti-active $G\alpha_i$ monoclonal antibody (Cat. No. 26901) (top panel). The precipitated active $G\alpha_i$ was immunoblotted with an anti- $G\alpha_i$ rabbit polyclonal antibody (Cat. No. 21006). The bottom panel shows the Western blot with anti-tubulin of the cell lysates.