



—A helping hand for your research

Configuration-specific Monoclonal Antibody Based

G α i Activation Assay Kit

(30 Assays)

Cat. # 80301

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Gai Activation Assay Kit Protocol

Cat# 80301

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 $G\alpha$ subunit with GTP in the presence of $G\beta\gamma$, causing the dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer to form two functional units ($G\alpha$ and $G\beta\gamma$). Both $G\alpha$ and $G\beta\gamma$ 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.

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

NewEast Biosciences Gai Activation Assay Kit provides a direct measurement of the activation of Gai proteins. This is a simple and fast tool to monitor the activation of Gai. Each kit provides sufficient quantities to perform 30 assays.

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

Assay Principle

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

Kit Contents

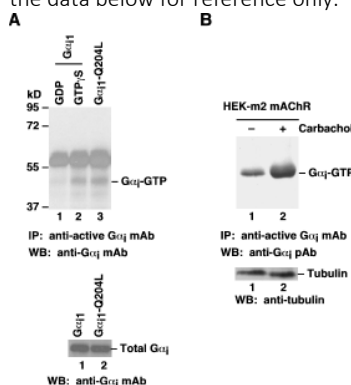
This kit contains enough reagents for approximately 30-35 pull-down assays.

Reagent	Cat. #	Quantity	Storage
Anti – active Gai Mouse Monoclonal Antibody	Cat. # 26901	1 X 35 μ l	-20 $^{\circ}$ C
Protein A/G Agarose	Cat. # 30301	1X600 μ l	4 $^{\circ}$ C
5X Assay/Lysis Buffer	Cat. # 30303	1X30mL	4 $^{\circ}$ C
Anti– Gai Mouse Monoclonal Antibody	Cat. # 26003	1X50 μ l	-20 $^{\circ}$ C
100x GTP γ S	Cat. # 30302	1X50 μ l	4 $^{\circ}$ C
100x GDP	Cat. # 30304	1X50 μ l	-80 $^{\circ}$ C
HRP– Goat Anti–Rabbit IgG	Cat. # 29002	1X50 μ l	-20 $^{\circ}$ C

Note: For GDP and GTP γ S, aliquot into 10x5ul volumes, then store at -80 degrees.

Example of Results

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



Gai activation assay. A. CHO cells were transfected with wild-type Gai1 (lanes 1 and 2) or constitutively active Gai1-Q204L (lane 3). Cell lysates were treated with GDP (lane 1) or GTP γ S (lane 3). Lysates were then incubated with an anti-active Gai monoclonal antibody (Cat. No. 26901) (top panel). The precipitated active Gai was immunoblotted with an anti-Gai monoclonal antibody (Cat. No. 26003). The bottom panel shows the Western blot with anti-Gai 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 Gai monoclonal antibody (Cat. No. 26901) (top panel). The precipitated active Gai was immunoblotted with an anti-Gai rabbit polyclonal antibody (Cat. No. 21006). The bottom panel shows the Western blot with anti-tubulin of the cell lysates.

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Materials Needed but Not Supplied

Stimulated and non-stimulated cell lysates
Protease inhibitors
4°C tube rocker or shaker
0.5 M EDTA, pH8.0
1 M MgCl₂
2X reducing SDS-PAGE sample buffer
Electrophoresis and immunoblotting systems
Immunoblotting wash buffer such as TBST
(10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20)
Immunoblotting blocking buffer
(TBST containing 5% Non-fat Dry Milk or 3% BSA)
ECL Detection Reagents

A 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

B Sample Preparation

Adherent Cells

1. Culture cells (one 10-cm plate, ~ 10⁷ 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.
8. Clear the lysates by centrifugation for 10 minutes (12,000 x g at 4°C).
9. 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 10⁷ cells).
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
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.

C In vitro GTP γ S/GDP Protein Loading for positive and negative controls

Note: In vivo stimulation of cells will activate approximately

10% of the available Gai, whereas in vitro GTP γ S protein loading will activate nearly 90% of Gai.

1. Aliquot 0.5 ml of each cell extract to two microfuge tubes (or use 1 µg of purified Gai protein).
2. To each tube, add 20 µl of 0.5 M EDTA (to 20 mM final concentration).
3. Add 5 µl of 100 X GTP γ S (to 100 µM, final concentration) to one tube (positive control).
4. Add 5 µl of 100 X GDP (to 1 mM, final concentration) to the second tube (negative control).
5. Incubate the tubes at 30°C for 30 minutes with agitation.
6. Stop loading by placing the tubes on ice and adding 32.5 µl of 1 M MgCl₂ (to 60 mM, final concentration).

D Affinity Precipitation of Activated G protein

1. Aliquot 0.5 – 1 mL of cell lysate (~1 mg of total cellular protein) 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 Gai monoclonal antibody to the tube.
4. Thoroughly resuspend the protein A/G Agarose bead slurry by vortexing or titrating.
5. Quickly 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 1 min at 5,000 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 5,000 x g

E Western blot analysis

1. Load 15 µL/well of pull-down supernatant to a polyacrylamide gel (17%). 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 following the manufacturer's instructions.
3. Transfer the gel proteins to a PVDF or nitrocellulose membrane following the manufacturer's instructions
4. 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.
5. 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- Gai Mouse Monoclonal antibody, freshly diluted 1:50~500 (depending on the amount of Gai proteins in your samples) in 5% non-fat dry milk or 3% BSA/TBST, for 1–2 hr at room temperature with constant agitation or at 4°C overnight.
6. Wash the blotted membrane three times with TBST, 5 minutes each time.
7. Incubate the membrane with a secondary antibody (e.g. Goat Anti-Rabbit IgG, HRP-conjugate), freshly diluted 1:1000 in 5% non-fat dry milk or 3% BSA/TBST, for 1 hr at room temperature with constant agitation.
8. Wash the blotted membrane three times with TBST, 5 minutes each time.
9. Use the detection method of your choice such as ECL