—A helping hand for your research

Configuration-specific Monoclonal Antibody Based

Gαo Activation Assay Kit

(30 Assays)

Cat. # 80901

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Gαo Activation Assay Kit Protcol

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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-ound 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.

Gαi family (including Gαo) 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 Gαo 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.

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

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

Assay Principle

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

Kit Contents

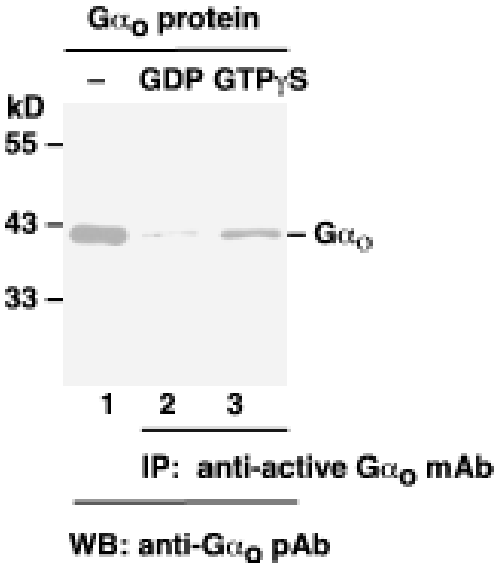
This kit contains enough reagents for approximately 30–35 pull–down assays.

Reagent	Cat. #	Quantity	Storage
Anti – active Gao Mouse Monoclonal Antibody	Cat. # 26907	1 X 35µl	–20℃
Protein A/G Agarose	Cat. # 30301	1X600µl	4℃
5X Assay/Lysis Buffer	Cat. # 30303	1X30mL	4℃
Anti– Gao Rabbit polyclonal Antibody	Cat. # 21015	1X50µl	–20℃
100x GTPγS	Cat. # 30302	1X50µl	4℃
100x GDP	Cat. # 30304	1X50µl	–80℃
HRP– Goat Anti–Rabbit IgG	Cat. # 29002	1X50µl	–20℃

Note: For GDP and GTPrS, aliquot into 10x5ul volumes, then store at–80 degrees.

Example of Results

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



Gαo activation assay. Purified Gαo proteins were loaded as a control (lanes 1) or immunoprecipitated after treated with GDP (lane 2) or GTPγS (lane 3). Immunoprecipitation was done with the anti-active Gαo monoclonal antibody (Cat. No. 26907). Immunoblot was with an anti-Gαo polyclonal antibody (Cat. No . 21015).

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Materials Needed but Not Supplied

Stimulated and non–stimulated cell lysates
Protease inhibitors
4℃ 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℃).
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℃ 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 107cells).
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 xg at 4℃).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at – 70℃ for future use.

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

Note: In vivo stimulation of cells will activate approximately 10% of the available Gao, whereas in vitro GTPγS protein loading will activate nearly 90% of Gao.

1. Aliquot 0.5 ml of each cell extract to two microfuge tubes (or use 1μg of purified Gao 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 GTPγS (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℃ 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 1μl anti–active Gao monoclonal antibody to the tube.
4. Thoroughly resuspend the protein A/G Agarose bead slurry by vortexing or titurating.
5. Quickly add 20 μL of resuspended bead slurry to each tube.
6. Incubate the tubes at 4℃ 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– Gao polyclonal antibody, freshly diluted 1:50~500 (depending on the amount of Gao proteins in your samples) in 5% non–fat dry milk or 3% BSA/TBST, for1–2 hr at room temperature with constant agitation or at 4℃ overnight.
6. Wash the blotted membrane three times with TBST, 5 minutes each time.
7. In cubate 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