Pioneering GTPase and Oncogene Product Development since 2010

Configuration-specific Monoclonal Antibody Based

$G\alpha_i$ Activation Assay Kit

(30 Assays)

Cat. # 83001

FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC APPLICATIONS

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Gαi Activation Assay Kit Protocol
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Introduction

A. Background

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.

Gαi 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 Gαi 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 Gαi proteins involved in a signaling pathway.

B. Assay Principle

NewEast Biosciences Gαi Activation Assay Kit uses configuration-specific anti-Gαi-GTP Mouse monoclonal antibody to measure Gαi-GTP levels in cell extracts or in vitro GTPγS loading Gαi activation assays. Anti-Gαi-GTP mouse monoclonal antibody is first incubated with cell lysates containing Gαi-GTP. Next, the GTP-bound Gαi is pulled down by protein A/G agarose. Finally, the precipitated Gαi-GTP is detected through immunoblot analysis using anti-Gαi mouse monoclonal antibody.

C. Kit Contents

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

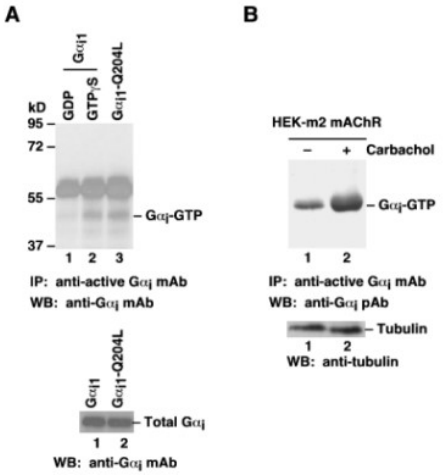
Reagent	Cat. #	Quantity	Storage
Anti-Gαi-GTP Mouse Monoclonal Antibody	26901	30 µL	-20°C
Protein A/G Agarose	30301	600 µL	4°C
5X Assay/Lysis Buffer	30303	30 mL	4°C
Anti-Gαi Mouse Monoclonal Antibody	26003	50 µL	-20°C
100X GTPγS	30302	50 µL	-20°C
100X GDP	30304	50 µL	-20°C
HRP-Goat Anti-Rabbit IgG	29002	50 µL	-20°C

D. Materials Needed but Not Supplied

1. Stimulated and non-stimulated cell lysates
2. Protease inhibitors
3. 4°C tube rocker or shaker
4. 0.5 M EDTA at pH 8.0
5. 1.0 M $MgCl_2$
6. 2X reducing SDS-PAGE sample buffer
7. Electrophoresis and immunoblotting systems
8. Immunoblotting wash buffer such as TBST (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20)
9. Immunoblotting blocking buffer (TBST containing 5% Non-fat Dry Milk or 3% BSA)
10. ECL Detection Reagents

E. Example Results

The following figure demonstrates example results seen with the Gαi Activation Assay Kit. For reference only.



Ar13 Activation Assay. A. CHO cells were transfected with wild-type Gαi1 (lanes 1 and 2) or constitutively active Gαi1-Q204L (lane 3). Cell lysates were treated with GDP (lane 1) or GTPγS (lane 3). Lysates were then incubated with an anti-active Gαi monoclonal antibody (Cat. # 26901) (top panel). The precipitated active Gαi was immunoblotted with an anti- Gαi monoclonal antibody (Cat. # 26003). The bottom panel shows the Western blot with anti-Gαi monoclonal antibody (Cat. # 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 Gαi monoclonal antibody (Cat. No. 26901) (top panel). The precipitated active Gαi was immunoblotted with an anti- Gαi rabbit polyclonal antibody (Cat. # 21006). The bottom panel shows the Western blot with anti-tubulin of the cell lysates.

Assay Procedure

A. Reagent Preparation

1X Assay/Lysis Buffer: Shake the 5X Stock Buffer (Cat. # 30301) briefly and dilute with 4 times deionized water to make 1X buffer. Just prior to usage, add protease inhibitors such as 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin.

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B. Sample Preparation

Adherent Cells

1. Culture cells (one 10-cm plate, ~10^7 cells) to approximately 80-90% confluence. Stimulate the 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 (See Reagent Preparation) 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 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 centrifuging at 12,000 x g and 4°C for 10 minutes.
9. Collect the supernatant and store the sample (~1–2 mg of total protein) 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 through 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 (See Reagent Preparation) to the cell pellet (0.5-1 mL per 10^7 cells).
5. Lyse the cells by repeated pipetting.
6. Transfer the lysates to appropriate size tubes and place them on ice.
7. If nuclear lysis occurs, the cell lysates may become 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 centrifuging at 12,000 x g and 4°C for 10 minutes.
9. Collect the supernatant and store sample on ice for immediate use, or snap freeze and store at -70°C for future use.

C. In vitro GTPγS/GDP Protein for Positive and Negative controls

Note: In vivo stimulation of cells will activate approximately 10% of the available Ar13, whereas in vitro GTPγS protein loading will activate nearly 90% of Ar13.

1. Aliquot 0.5 mL of cell extract (or 1 µg of purified Ar13 protein) into two microcentrifuge tubes.
2. To each tube, add 20 µL of 0.5 M EDTA (final concentration of 20 mM).
3. Add 5 µL of 100 X GTPγS (Cat. # 30302) to the first tube as a positive control.
4. Add 5 µL of 100 X GDP (Cat. # 30304) to the second tube as a negative control.
5. Incubate both 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 $MgCl_2$ (final concentration of 60 mM).

D. Affinity Precipitation of Activated G Protein

1. Aliquot 0.5-1 mL of cell lysates (about 1 mg of total cellular protein) to a microcentrifuge tube.
2. Adjust the volume to 1 mL with 1X Assay/Lysis Buffer (See Reagent Preparation).
3. Add 1 µL anti-Ar13-GTP antibody (Cat. # 26901).
4. Prepare the protein A/G Agarose bead slurry (Cat. # 30301) by resuspending through vertexing or titrating.
5. Quickly add 20 µL of resuspended bead slurry to above tube.
6. Incubate the tube at 4°C for 1 hour with gentle agitation.
7. Pellet the beads through centrifugation at 5,000 x g for 1 min.
8. Aspirate and discard the supernatant (making sure not to disturb or remove the bead pellet).
9. Wash the beads 3 times with 0.5 mL of 1X Assay/Lysis Buffer, centrifuging and aspirating each time.
10. After the third wash, pellet the beads through centrifugation and carefully remove all the supernatant.
11. Resuspend the bead pellet in 20 µL of 2X reducing SDS-PAGE sample buffer.
12. Boil the sample for 5 minutes.
13. Centrifuge it at 5,000 x g for 10 seconds.

E. Western Blot Analysis

1. Load 15 µL/well of pull-down supernatant to a polyacrylamide gel (17%). It is recommended to include a pre-stained MW standard (as an indicator of a successful transfer in step 3 below).
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.

Note: Steps 4-11 are at room temperature with agitation

4. Following electroblotting, 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, step 4 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.
6. Wash the blotted membrane three times with TBST, 5 minutes each time.
7. Incubate the membrane with anti-Ar13 Mouse Monoclonal Antibody (Cat. # 26003), which is freshly diluted 1:50~500 (depending on the amount of Ar13 proteins in your sample) in 5% non-fat dry milk or 3% BSA in TBST, for 1-2 hr at room temperature with constant agitation or at 4°C overnight.
8. Wash the blotted membrane three times with TBST, 5 minutes each time.
9. Incubate the membrane with a secondary antibody (Cat. # 29002), which is freshly diluted 1:1000 in 5% non-fat dry milk or 3% BSA in TBST, for 1 hr at room temperature with constant agitation.
10. Wash the blotted membrane three times with TBST, 5 minutes each time.
11. Use the detection method of your choice such as ECL.