ab173234

G alpha i Activation

Assay Kit

Instructions for Use

For the simple and fast measurement of G alpha i activation.

This product is for research use only and is not intended for diagnostic use.
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1. 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 G alpha subunit with GTP in the presence of Gβγ, causing the dissociation of the G alpha subunit from the Gβγ dimer to form two functional units (G alpha and Gβγ). Both G alpha 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 regulate 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 involved in a signaling pathway.
2. Principle of the Assay

Abcam’s G alpha i Activation Assay Kit (ab173234) 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.

Abcam’s 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.

ab173234 uses 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.
3. Protocol Summary

Prepare buffers

Prepare samples and positive/negative controls

**Active G alpha i Pull-Down Assay**

Add sample to microcentrifuge tube

Add anti-active G alpha i monoclonal antibody

Add re-suspended protein A/G Agarose bead slurry

Incubate tubes at 4°C for 1 hour (with gentle agitation)

Pellet beads by centrifugation

Wash pellet 3 times with 1X Assay/Lysis Buffer

Pellet beads by centrifugation

Resuspend bead pellet in SDS-PAGE sample buffer.

Boil and centrifuge each sample
Electrophoresis and Transfer

Load pull-down supernatant to polyacrylamide gel.

Perform SDS-PAGE following your preferred protocol.

Immerse PVDF membrane in 100% Methanol before immersing it in transfer buffer.

Transfer gel proteins to a PVDF or nitrocellulose membrane following your preferred protocol.

Immunoblotting and Detection

Block the membrane with 5% non-fat dry milk or 3% BSA in TBST

Incubate membrane with anti-G alpha i monoclonal antibody

Wash membrane three times with TBST

Incubate membrane with the secondary antibody

Wash membrane three times with TBST

Use detection method of your choice (e.g. ECL)
4. Materials Supplied

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-active G alpha i, Mouse Monoclonal Antibody</td>
<td>1 x 22 µl</td>
</tr>
<tr>
<td>Note: This antibody specifically recognizes</td>
<td></td>
</tr>
<tr>
<td>G alpha i-GTP from all vertebrates</td>
<td></td>
</tr>
<tr>
<td>Protein A/G Agarose</td>
<td>1 x 400 µl</td>
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<tr>
<td>5X Assay/Lysis Buffer</td>
<td>2 x 15 ml</td>
</tr>
<tr>
<td>Anti-G alpha i, Mouse Monoclonal Antibody</td>
<td>1 x 22 µl</td>
</tr>
<tr>
<td>100X GTP gamma S (10 mM)</td>
<td>1 x 100 µl</td>
</tr>
<tr>
<td>100X GDP (100 mM)</td>
<td>1 x 100 µl</td>
</tr>
</tbody>
</table>

5. Storage

Store all kit components at 4°C.
6. Materials Required, Not Supplied

- Stimulated and non-stimulated cell lysates
- Protease inhibitors – we recommend Protease Inhibitor Cocktail (ab65621)
- 4°C tube rocker or shaker
- PBS, ice-cold
- 0.5 M EDTA, pH8.0
- 1 M MgCl$_2$
- 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)
- PVDF or nitrocellulose membrane
- Secondary Antibody against G alpha i monoclonal antibody – we recommend Goat polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) (ab97023)
- ECL Detection Reagents – we recommend Optiblot ECL Detect kit (ab133406)
7. Reagent Preparation

1X Assay/Lysis Buffer:

Mix the 5X Stock briefly and dilute to 1X in deionized water and keep cold. Just prior to usage, add protease inhibitors such as 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin.

Protein A/G Agarose:

Cut the end of a 200 μl tip and resuspend the bead slurry prior use.

8. Sample Preparation

A. Adherent Cells

1. Culture cells (10^7 cells, ~ 1x10-cm plate) 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/ 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 -80°C for future use.

B. 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 10^7 cells).

5. Lyse the cells by repeated pipetting up and down.

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 -80°C for future use.

C. *In vitro* GTP gamma S/GDP Protein Loading for positive and negative controls

Note: *In vivo* stimulation of cells will activate approximately 10% of the available G alpha i, whereas *in vitro* GTP gamma S protein loading will activate nearly 90% of G alpha i.

1. Aliquot 0.5 ml of each cell extract to two microfuge tubes (or use 1 µg of purified G alpha i protein).
2. To each tube, add 20 µL of 0.5 M EDTA (20 mM final concentration).
3. Positive control: add 5 µL of 100X GTP gamma S (100 µM, final concentration) to one tube.
4. Negative control: add 5 µL of 100X GDP (to 1 mM, final concentration) to the second tube.
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 (60 mM, final concentration).
9. Assay Procedure

A. Active G alpha i Pull-Down Assay

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 G alpha i monoclonal antibody to the tube.
4. Thoroughly resuspend the protein A/G Agarose bead slurry by vortexing or pipetting up and down.
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.
B. Electrophoresis and Transfer

1. Load 15 μL/well of pull-down supernatant to a 17% polyacrylamide gel.

Note: 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 your preferred protocol.

3. Following the transfer step, immerse the PVDF membrane in 100% Methanol for 15 seconds before immersing it in transfer buffer.

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

4. Transfer the gel proteins to a PVDF or nitrocellulose membrane following your preferred protocol.

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

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

2. Incubate the membrane with anti-G alpha i monoclonal antibody, freshly diluted 1/50 – 1/1000 (depending on the amount of G alpha i proteins in your samples) in 5% non-fat dry milk or 3% BSA/TBST, for 1-2 hours at room temperature with constant agitation or at 4°C overnight.

3. Wash the membrane three times with TBST, 5 minutes each time.
4. Incubate the membrane with the secondary antibody freshly diluted in 5% non-fat dry milk or 3% BSA/TBST for 30 min – 1 hour at room temperature with constant agitation. Use the dilution appropriate for your secondary antibody (for example ab97023 – Goat polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) can be use at 1/2000 – 1/20000).

5. Wash the membrane three times with TBST, at least 5 minutes each time.

6. Use the detection method of your choice, such as ECL.
10. Data Analysis

Example results for Western blotting

Note: The following figure demonstrates typical results seen with Abcam’s G alpha i Activation Assay Kit. Data below should be used 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 GTP gamma S (lane 3).

Top panel: Lysates were incubated with the anti-active G alpha i monoclonal antibody provided in the kit. The precipitated active G alpha i was immunoblotted with the anti-G alpha i monoclonal antibody provided in the kit.
Bottom panel: Western blot with anti-G alpha i monoclonal antibody of the cell lysates.

B: HEK293 cells stably expressing Human m2 mAChR were treated with (lane 2) or without (lane 1) carbachol.

Top panel: Lysates were incubated with the anti-active G alpha i monoclonal antibody provided in the kit. The precipitated active G alpha i was immunoblotted with an anti-G alpha i rabbit polyclonal antibody.

Bottom panel: Western blot with anti-tubulin of the cell lysates.
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