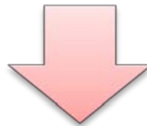
Alper Biotech GMF-β MoAb R&D Kit

Cat. No. AB010

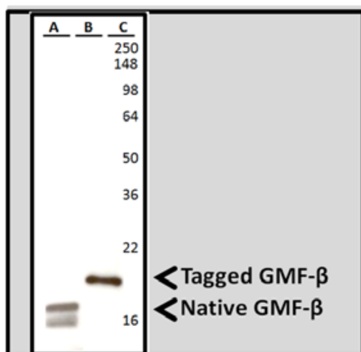
Alper Biotech GMF-β (Glia Maturation Factor-beta) R&D Kit is a sensitive research and development kit that is designed for researchers, and specific for the detection of GMF-beta protein in applications for western blot, indirect immunofluorescence staining assays, FACS application. **Anti-GMF-beta monoclonal antibody recognizes the native form of human GMF-beta protein**.

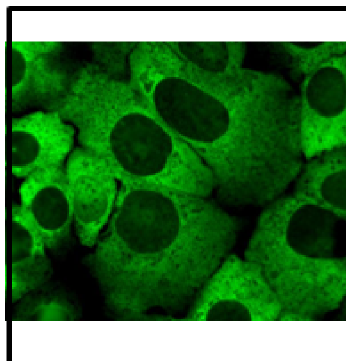
Intended Use:

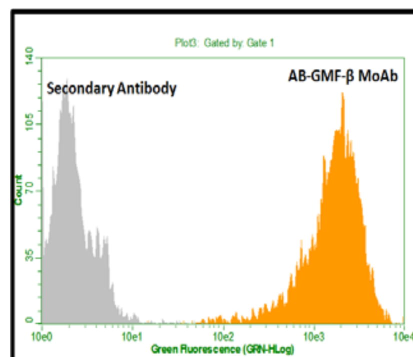
1 KIT 3 ASSAYS



1	2	3
Western Blot **WB**	**Indirect Immunofluorescence** **IF**	**Flow Cytometry** **FACS**







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This product is for research use only. Not for use in diagnostic procedures.

COMPONENTS IN THE KIT

- Cell Lysis Buffer (RIPA Buffer, 10 mL); **WB**
- Wash Buffer (10X, Phosphate Buffered Saline solution with 0.1% Tween 20, pH7.6, 30 mL); **WB**
- Non-fat Dry Milk (2X5 g); **WB**
- AB-GMF-beta MoAb (Human GMF-BETA Monoclonal Mouse IgG1 , 500 µg/mL, 300 µl, total 150 µg); **WB, IF, FACS**
- Horseradish Peroxidase-Congugated Anti Mouse IgG (5 µL); **WB**
- Primary Antibody Diluent (5 mL); **IF**
- Secondary Antibody Diluent (5 mL); **IF**
- Alexa Fluor 488-donkey anti-mouse antibody (2 mg/mL; 40 µl total 80 µg); **IF, FACS**
- FACS Buffer (total 40 mL in two bottles); **FACS**

STORAGE AND STABILITY

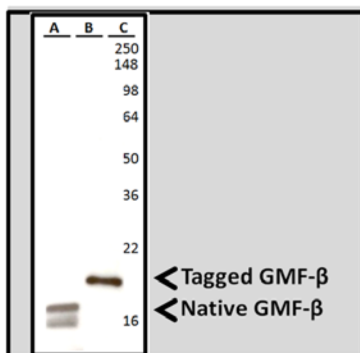
Store GMF-beta R&D Kit at 2-8 ^{0}C. The kit is stable for one year at 4 ^{0}C. For long term storage, aliquot the antibody into small volumes and store at -20^{0}C. Do not use after expiration date.

PRECAUTIONS

Take reasonable precautions when handling reagents. Use disposable gloves when handling suspected carcinogens or toxic materials (examples: Sodium Azide). Unused solution should be disposed of according to applicable local, state and federal regulations.

PROTOCOLS FOR INDIVIDUAL ASSAYS

1 Western Blot (WB)



Western blot analysis of GMFbeta A, SKBR3 cell lysate; B, recombinant protein; C, Molecular Weight Markers using AB-GMF-beta MoAb R&D Kit. GMF-beta recombinant protein with Tag at C-terminal** (4 µg; Origene, Cat No.: TP302782)**; SKBR3 cell lysate** (75 µg of protein, prepared using RIPA buffer following the instruction for the kit)**.

Reagents provided in the kit

The materials listed are sufficient to perform 5 western blots (7.5 x 8 cm^2) probed with 10 ml of the working solution of primary and secondary antibodies.

- **Cell Lysis Buffer (RIPA Buffer); WB**
  10 mL; Add 1 tablet of Protease Inhibitor Cocktail and 100 µL of 200 mM sodium orthovanadate stock solution into 10 mL RIPA buffer prior to use.
- **Wash Buffer (10X); WB**
  30 mL, Phosphate Buffered Saline solution with 0.1% Tween 20 (PBST, pH7.6)
  Dilute at 1:10 using distilled or deionized water prior to use.
- **Non-fat Dry Milk; WB**
  10 g; Prepare 5% solution with PBST prior to use for blocking and antibody diluents.
- **AB-GMF-beta MoAb**
  500 µg/mL, 100 µl total (50 µg)
  Dilute in 5% milk-PBST solution at 1:500 prior to use.

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- **Horseradish Peroxidase-Congugated Anti Mouse IgG**
  5 µL; Dilute at 1:25000 in 5% milk-PBST solution prior to use.

Reagents and equipments required but not provided in the kit

Reagents:

Note: The reagents can be prepared by the individual laboratory or purchased from commercial sources. We provide the commercial sources where we purchase the reagents for our western blot experiments.

- **Protease Inhibitor Cocktail**: Commercially available from Roche (Cat No.: 11 836 170 001).
- **Sodium Orthovanadate**: Commercially available from Sigma (Cat No.: S6508); See the following protocol to prepare and GMF-betaivate sodium orthovanadate stock solution.
- **Running Buffer**: Commercially available from Invitrogen (Cat No.: LC2675)
- **Transfer Buffer**: Commercially available from Invitrogen (Cat No.: LC3675)
- **2X Laemmli Buffer**: Commercially available from BioRAD (Cat No.: 161-0737).
- **2-Mercaptoethanol**: Commercially available from Sigma (Cat No.: M-7522).
- **Polyvinylidene Difluoride (PVDF) Membrane**,
- **Detection Reagents**: Our laboratory uses ECL Plus Western Blotting Detection Reagents from GE Healthcare (Cat No.: RPN2132).
- **Chemiluminescence Film**: Commercially available.
- **Polyacrylamide Gel**: Our laboratory uses 14% NuPAGE Novex Bis-Tris Mini Gels from Invitrogen (Cat No.: EC6488 BOX).
- **Distilled or Deionized Water**

Lab Equipment:

- 1 mL Syringe, 20 Gauge needle
- Rotary platform shaker
- Apparatus for gel electrophoresis, transfer
- Autoradiography film, film cassette and imaging instrument.
- Other general lab equipments such as microcentrifuge, microcentrifuge tubes, forceps, washing trays, etc.

Procedure

A. Sample preparation

Preparation of 200 mM sodium orthovanadate stock solution

1. Dissolve 1.84 g of sodium orthovanadate (Sigma, S6508) in 45 mL purified water in a small beaker with a stir bar.
2. Adjust the pH to 10 using either 1 N NaOH or 1 N HCl, with stirring. The starting pH of the sodium orthovanadate may vary. At pH 10, the solution will be yellow.
3. Boil solution until colorless (approximately 10 min). All crystals should dissolve.
4. Cool to room temperature.
5. Readjust pH to 10 and repeat steps 3 and 4 until solution remains colorless and pH stabilizes at 10. Adjust the final volume to 50 mL with purified water.
6. Store the acivated sodium orthovanadate at -20 °C in aliquots.

Preparation of lysate from cell culture

1. Place the cell culture flask or dish on ice and wash the cells with ice-cold PBS.
2. Drain the PBS, then add ice-cold lysis buffer (1 mL per 10^7 cells).
3. Scrape adherent cells off the flask/dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microcentrifuge tube.
4. Carefully pass the cell suspension through a 1 mL syringe assembled with a 20 Gauge needle for 10 times.
5. Centrifuge for 10 minutes at 9.600 x g in a microcentrifuge at 4°C.
6. Gently remove the tubes from the centrifuge and place on ice, transfer the supernatant into a fresh tube kept on ice, and discard the pellet.

Determination of protein concentration

Perform a BCA assay to determine the concentration of each sample. Prepare samples for western blot or freeze at -80°C for later use.

B. Western Blot

Preparation of samples for loading into gels

Add 50 µL of 2-mercaptoethanol to 950 µL of 2X Laemmli buffer. Combine with sample at 1:1 ratio. Boil the resulting mixture at 95-100°C for 3 minutes.

Preparation of PAGE gels

A polyacrylamide gel can be prepared according to standard lab protocol or purchased from commercial ready-made gels.

Note: **Acrylamide is a potent cumulative neurotoxin: wear gloves at all times**.

Place gels in the electrophoresis tank as instructed by the manufacturer and add running buffer, which can be prepared according to standard lab protocol or be purchased from commercial resources.

Loading samples and running the gel
1) Load a suitable range of molecular weight marker (commercially available) into a well to determine protein size and monitor the progress of electrophoresis.
2) Load the samples individually into each well (50-100 µg total protein per mini-gel well).
3) Run the gel using the recommended voltage as instructed by the manufacturer (normally 80-100 v), and turn off power when the dye molecule (the "migration front") reaches the bottom of the gel. Take out the gel and proceed immediately to transfer process.

Transfer of protein
The transfer process varies dependent on apparatus and system used and should be completed following the manufacturer's protocol of the apparatus. The following procedure is for a standard wet transfer using PVDF membrane.
1) Prepare transfer buffer according to standard lab protocol or purchase from commercial.
2) Soak PVDF membrane in 100% methanol for 1 minute, wash in distilled water for 5 minutes, and then equilibrate in transfer buffer for at least 10 minutes.
3) Prepare "Sandwich" (sponge/paper/gel/membrane/paper/sponge) and place into the trunk of transfer apparatus and run.
Note: The membrane should face to the positive charge.

Blocking the membrane
1) After transfer, remove the membrane into western tray. Rinse the membrane with PBST three times for 5 minutes each.
2) Block membrane with 5% non-fat milk PBST solution for 1 hour under agitation.

Incubation with the primary antibody
1) Dilute the GMF-beta antibody at 1:500 in 5% milk-PBST, and mix.
2) Incubate membrane in GMF-beta antibody solution overnight at 4°C under agitation.
2) Briefly wash membrane with PBST to remove excessive primary antibody, followed by an additional five washes for 5 minutes each.

Incubation with the secondary antibody
1) Dilute the secondary antibody at 1:25000 in 5% milk-PBST, and mix.
2) Incubate membrane in the second antibody solution for 1 hour at room temperature under agitation.
2) Briefly wash membrane with PBST to remove excessive secondary antibody, followed by an additional five washes for 5 minutes each.

Signal development
The membrane can be developed with various methods. We recommend chemiluminescent detection with ECL Plus Western Blotting Detection System (GE Healthcare).
1) Prepare the chemiluminescence development substrate mixture following the manufacturer's instruction (equilibrate to room temperature before use). When using ECL Plus system, mix Solution A with Solution B at a ratio of 40:1. About 0.2 mL of the working solution is sufficient to cover 1 cm^2 of membrane.
2) Place the membrane on a plastic flat film and drain off excess wash buffer, add the mixed detection reagent onto the membrane and allow the solution to cover the membrane.
3) Incubate for 2 minutes at room temperature.
4) Remove excess chemiluminescence reagent and wrap the membrane in plastic folder. Place inside X-ray cassette.
5) Expose to film for 30 second and then develop. Vary exposure time as necessary for optimum result.

Trouble Shooting

No signal or the signal is weak
GMF-beta protein is not present in target cells
Check literature, run western blot with a positive control to verify GMF-beta protein present in the test target cells

The antibody does not recognize antigen in test species
The primary antibody provided in the kit recognizes human GMF-beta antigen. For other species, users should test the human GMF-beta cross-reactivity across species. Run a positive control when testing other species .

Insufficient antigen protein loaded into the gel
Load an appropriate amount of sample into each well of the gel (at least 50-100 ug protein per well depending on the abundance of GMF-beta antigen in the target cells).

Insufficient primary antibody bound to antigen protein
Incubate with primary antibody for greater periods of time (it is recommended to incubate with primary antibody overnight followed by 1 hour incubation at room temperature); do not wash the membrane excessively.

Insufficient Transfer
Check transfer efficiency by dying the gel with coomassie blue or transiently coloring the blot with Ponceau Red ; optimize the transfer time and the electrical current ; check that the transfer was performed in the correct orientation; ensure that the PVDF membrane has been pre-soaked in MeOH then in transfer buffer.

Inactive detection of substrate.
Check the expiration date of detection kit and use fresh substrate.

High background
Insufficient washing of unbound antibodies
Increase the number of washing steps or lengthen washing time to 10 minutes each.

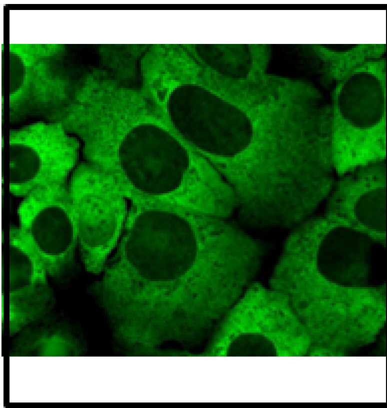
Excessive signal development time.
Reduce exposure time. Vary the exposure time for optimal result.

Multiple bands.
The antigen protein in sample has been digested (more probable if bands are of lower molecular weight)
Prepare fresh cell lysate following the instructions and ensure that the samples are kept either on ice or at 4ºC throughout the process; Aliquot unused lysate and store at -80 ºC. Add protease inhibitors and sodium orthovanadate in lysis buffer.

Uneven white "spots" on the blot
When preparing the gel for transfer, ensure that air bubbles have been removed.

Black dots on the blot
When prepare 5% milk solution, ensure that the dry milk has been fully dissolved. Filter the solution if necessary.

2 Indirect Immunofluorescence Staining (IF)



Expression of cytoplasmic GMFbeta in SKBR3 cells using AB-GMF-beta MoAb in Indirect-immunofluorescence staining assay.

Reagents provided in the kit

The materials listed are sufficient for 20 tests. The number of tests is based on the use of 200 µL each of ready-to-use reagent per slide.

- **Primary Antibody Diluent ; IF**
  5 mL; Ready-to-use
  Contains 0.2% Sodium Azide. Store unused working solution at 4 ^{0}C.
- **AB-GMF-beta MoAb**
  500 µg/mL; 100 µl total (50 µg)
  Dilute in primary antibdoy diluent immediately before use (recommend use at 1:100 dilution).
- **Secondary Antibody Diluent ; IF**
  5 mL; Ready-to-use
  Contains 0.2% Sodium Azide. Store unused working solution at 4 ^{0}C.

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- **Alexa Fluor 488-Goat Anti-Mouse Antibody**
  2 mg/mL; 20 µl total (40µg)
  Dilute in secondary antibody diluent immediately before use (recommend use at 1:200 dilution).

Reagents and equipments required but not provided in the kit

Reagents:

- 37% Formaldehyde: prepare 10% solution prior to use
- Phosphate buffered saline (PBS, pH7.6)
- Triton X-100
- Distilled or deionized water

Lab Equipment:

- General lab equipment for immunofluorescence such as glass bottomed chamber slides, forceps, cover slips, timer, pipettes, etc.
- Fluorescent/Confocal Microscope equipment and accessories

Staining Procedure

Note: **Do not allow cells to dry out throughout the staining process**

Step 1. After removing upon from the incubator, aspirate the media and fix cells for 10 minutes at room temperature by adding 10% formaldehyde solution to cover cells (final concentration at 3.7% paraformaldehyde in PBS).
Step 2. Remove/aspirate the formaldehyde solution and wash cells three timeswith PBS for 3 minutes each.
Step 3. Permeabilize cells with PBS with 0.1% Triton X-100 for 10 minutes.
Step 4. Remove/aspirate PBS with 0.1% Triton X-100 solution and wash cells three times with PBS for 3 times each.
Step 5. Dilute the primary GMF-beta antibody 1:20 to 1:100 depending on the cell density with primary antibody diluent and add onto the cells and incubate for 10-30 minutes at room temperature after completely removing PBS.
Step 6. Wash cells three times with PBS for 3 minutes each.
Step 7. Dilute Aexa fluor-488 goat-anti-mouse antibody 1:200 with secondary antibody diluent and add onto cells and incubate 10-20 minutes at room temperature after completely removing PBS.
Step 8. Wash cells three times with PBS. Repeat this washing step three times with deionzied or distilled water.
Step 7. Add mounting medium and visualize cells with a fluorescence microscopy.

Trouble Shooting

Weak or no staining

GMF-beta protein is not present in target cells
Check literature, prepare cell lysate and run western blot with a positive control to verify GMF-beta protein present in the test target cells

The antibody does not recognize antigen in test species
The primary antibody provided in the kit recognizes human GMF-beta antigen. For other species, users should test the cross-reactivity across species. Run a positive control when testing other species.

The concentration of primary antibody is too low or the incubation time with antibody is too short
Increase the concentration of GMF-beta antibody from 1:100 up to 1:20 or increase the incubation time with the primary antibody solution.

The fluorochrome fluorescent has faded
To avoid the fluorescent fading of fluorochrome, keep the fluorochrome-labeled secondary antibody away from light during storage and staining process. Change a fresh one If fading has happened.

Over staining or high background

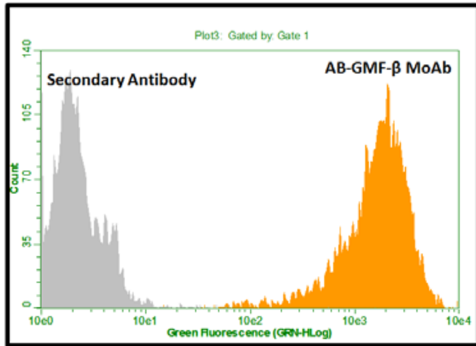
The concentration of GMF-beta antibody is too high or incubation time is too long
Reduced the concentration of GMF-beta antibody or reduce the incubation time with the primary antibody

The temperature is too high when doing staining
Check the room temperature range is at 20-25 ºC when doing staining.

The slide is not rinsed thoroughly
Use fresh washing buffer for each time rinse; Rinse at least three times between steps.

The slide dried during staining process
Do not allow sections to dry during staining process; use humid container during incubation of primary antibody.

3 Flow Cytometry (FACS)



FACS analysis of stained MDA-MB-231 breast cancer cells (5000 single cells) with AB-GMF-beta MoAb using Guava-EasyCyteMini flow cytometry (Millipore) equipped with GuavaExpressPro software.

Reagents provided in the kit

The materials listed are sufficient for nine labeling. The number of labeling is based on the use of 200 µL each of ready-to-use reagent per labeling.

- FACS Buffer (30 mL, 10 mL); **FACS**
  Ready-to-use
- **AB-GMF-beta MoAb**
  500 µg/mL; 100 µl total (50 µg)
  Dilute in FACS Buffer, immediately before use (recommend to start at 1:50 dilution).
- **Alexa Fluor 488-Goat Anti-Mouse Antibody**
  2 mg/mL; 20 µl total (40 µg)
  Dilute in FACS Buffer, immediately before use (recommend use at 1:700 dilution).

Reagents and equipments required but not provided in the kit

Reagents:

- 37% Formaldehyde: prepare 2% solution prior to use
- Phosphate buffered saline (PBS, pH7.6)
- Triton X-100
- Distilled or deionized water

Lab Equipment:

- General lab equipment for immuno-staining such as tubes, centrifuge, timer, pipettes, etc.
- Flow Cytometry equipment and accessories

Procedure

FACS Format

A. Unstained control cells (washed with PBS only)
B. Control cells stained with secondary antibody only (if preferred, isotype control antibody can be used as a primary control antibody)
C. Test cells stained both with primary and secondary antibodies
Total cell number required for above three groups is 10^6cells.

Cell Labeling

Step 1. Wash cells with cold PBS three times by centrifugation. Keep the cells either on ice or at 4°C until the labeling procedure is completed.
Step 2. Fix cells with 2% formaldehyde solution for 10 minutes. Centrifuge, remove formaldehyde, and then permeabilize cells by suspending cells in PBS with 0.1% Triton X-100 for 10 minutes.
Step 3. Wash cells with FACS buffer three times by centrifugation. (At this step, only control unstained cells (**A**) are ready for analysis and must be kept on ice until they are analyzed by Flow Cytometry)
Step 4. Dilute primary AB-GMF-beta MoAb in FACS Buffer (1:20-1:100) and add onto test cells (**C**). Gently suspend cells by up-down pipetting, and incubate on ice for one hour. Wash cells with FACS Buffer three times by centrifugation.
Step 5. Dilute Alexa Fluor 488-labeled secondary goat anti-mouse antibody 1:700 in FACS Buffer and, add onto control cells (**B**), and test cells (**C**). Gently suspend cells by up-down pipetting and incubate on ice or at 4°C for one hour. Wash cells three times with FACS Buffer by centrifugation.

Step 6. Analyze cells with a Flow Cytometry.

Trouble Shooting

No Signal

GMF-beta protein is not present in target cells

Check literature, prepare cell lysate and run western blot to verify GMF-beta protein present in the test target cells

The antibody does not recognize antigen in test species

The primary antibody provided in the kit recognizes human GMF-beta antigen. For other species, users should test the cross-reactivity across species. Run a positive control when testing other species.

***Incorrect laser is used or laser is not aligned to excite* fluorochrome**

The secondary antibody provided in this kit is conjugated to Alexa Fluor 488. The excitation and emission are Ex-Max 495 nm/Em-Max 519 nm. Check and make sure to use correct laser channel and lasers are aligned correctly.

Signal is not correctly compensated

Check positive single color control is set up and adjust compensation correctly on Flow Cytometer.

Weak fluorescent signal/weak cell labeling

The antibody concentration used is too low

Depending on abundance of GMF-beta antigen in target cells, the concentration of GMF-beta antibody can be increased from 1:100 up to 1:20.

Excess cells are used for labeling

Reduce cell numbers for labeling to recommended density.

The fluorochrome fluorescent has faded

To avoid the fluorescent fading of fluorochrome, keep the fluorochrome-labeled secondary antibody away from light during storage and staining process. Change a fresh one If fading has happened.

Incorrect offset/gain setting on Flow Cytometer

Use positive control to set up offset/gain correctly. Ensure fluorescent signal from stained cells is not being cut off.

Non-specific staining and high background

Autofluorescence

Always include a negative control into test panel to set up the machine voltages and check autofluorescence. The negative control is a no antigen control or the cells stained with pre-immune serum and then fluorochrome -labeled secondary antibody.

Insufficient washing of unbound antibodies

Increase the number of washing steps or lengthen the time periods of washes.

The antibody concentration used is too high or incubation time is too long

Depending on abundance of GMF-beta antigen in target cells, the concentration of GMF-beta antibody can be reduced, ranging from 1:20 to 1:100