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Antibody Protocol Collection

This collection contains protocols for each antibody application. We recommend following dilutions as a starting point for use of our antibodies, but the optimal concentrations/dilutions should be determined by the end user. For specific antibody, please also check Materials and methods section in the reference indicated on the antibody webpage/datasheet.

- WB: 1:250-1:1000
- FACS/IF: 1:50-1:200
- IHC: 1:100-1:500
- IP: 2 ug-5ug

Flow cytometry- intracellular staining

Reagents

- FACS Buffer: 10g BSA, 1g NaN_3, 1l PBS
- PBS, 4%PFA, 0.05% Saponin
- Primary monoclonal antibody
- Fluorochrome-labeled anti mouse IgG antibody

Procedure:

1. Resuspend cells in PBS and place in a 1ml microfuge tube, pellet cells at 4000 rpm for 5'. Aspirate the PBS but do not allow the pellet to dry. Prior to addition of reagents, resuspend the cells in the residual PBS by tapping the microfuge tube. This procedure is used for all centrifugation/washing steps.
2. Fix cells: Add 500ul of PBS, resuspend cells and than add 500ul of 4%PFA. Leave on RT for 20'.
3. Pellet the cells as in step 1, remove paraformaldehyde and wash 2x in PBS.
4. Staining with primary antibody: Resuspend cells in 50ul of primary antibody diluted in 0,05% saponin in FACS buffer. Leave on RT for 20'.
5. Wash 1x in 0,05% saponin in FACS buffer and 1x in PBS.
6. Staining with secondary antibody (fluorochrome-labeled): resuspend cells in 50ul of anti mouse -conjugated secondary antibody. Leave on RT for 20'.
7. Wash 1x in 0,05% saponin in FACS buffer and 1x in FACS buffer.
8. Resuspend cells in 200ul of FACS medium and proceed with reading. Check for cell clumping before analyzing the cells. It may be necessary to filter the sample through nylon filters before analysis to remove any cell clumps.

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Flow cytometry- surface staining

Reagents

- FACS Buffer: 10g BSA, 1g NaN_3, 1l PBS
- Primary monoclonal antibody
- Fluorochrome-labeled anti mouse IgG antibody

Procedure:

1. Resuspend cells in PBS and distribute in 96-well plate (round bottom), pellet cells at 1300 rpm for 5'. Flick off the supernatant.
2. Staining with primary antibody: Resuspend cells in 50ul of primary antibody diluted in FACS buffer. Leave on ice for 25'.
3. Wash unbound antibody by adding 140ul FACS buffer and pellet at 1300rpm for 5'. Flick off the supernatant.
4. Wash additional 1x in FACS buffer.
5. Staining with secondary antibody (fluorochrome-labeled): Resuspend cells in 50ul of anti mouse-conjugated secondary antibody. Leave on ice for 20'.
6. Wash unbound antibody by adding 140ul FACS buffer and pellet at 1300rpm for 5'. Flick off the supernatant.
7. Wash 1x in FACS buffer.
8. Resuspend cells in 200ul of FACS medium and proceed with reading. Check for cell clumping before analyzing the cells. It may be necessary to filter the sample through nylon filters before analysis to remove any cell clumps.

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Western blot Protocol

Reagents:

- Lysis buffer: RIPA buffer: 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris-HCl pH 8.0, Protease Inhibitors
- Loading buffer: Laemmli 2X buffer: 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl. Adjust pH to 6.8.
- Running buffer: 25 mM Tris base, 190 mM glycine, 0.1% SDS. Adjust pH to 8.3.
- Transfer buffer: 25 mM Tris base, 190 mM glycine, 20% methanol. Adjust pH to 8.3.
- Blocking buffer: 5% milk in TBST buffer.
- Antibodies

Procedure:

Preparation of lysate from cell culture:

1. Place the cell culture dish in ice and wash the cells with ice-cold PBS. Aspirate the PBS, then add ice-cold lysis buffer. Scrape adherent cells off the dish and gently transfer the cell suspension into a pre-cooled microcentrifuge tube. Maintain constant agitation for 30 minutes at 4°C.
2. Spin at 16,000 x g for 20 minutes in a 4°C pre-cooled centrifuge. Transfer the supernatant to a fresh tube kept on ice, and discard the pellet.
3. Add equal volume of 2X Laemmli Sample Buffer to volume of cell lysate.
4. Boil cell lysate in sample buffer at 95°C for 5 minutes.
5. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight markers. Load 2µg of purified protein or 75ug of protein from cell lysate.
6. Run the gel for 1 to 2 hours at 100 V. Time and voltage may require some optimization, we recommend following the manufacturer's instructions.

Transferring the protein from the gel to the membrane

1. The membrane can be either nitrocellulose or PVDF. "Activate" PVDF with methanol for one minute and rinse with transfer buffer before preparing the stack. Transfer for 1 hour at 100 V. The time and voltage may require some optimization. We recommend following the manufacturer's instructions.

Antibody staining

1. Block the membrane for 1 hour at room temperature or overnight at 4°C using 5% blocking buffer.
2. Incubate membrane with appropriate dilutions of primary antibody in TBST overnight at 4°C.
3. Wash the membrane in four washes of TBST, 15 minutes each.
4. Incubate the membrane with the recommended dilution of labeled secondary antibody in TBST at RT for 1 hour. Wash the membrane in TBST, 1-2 hours.
5. For signal development, follow the kit manufacturer's recommendations.

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IF staining protocol

Reagents:

- Methanol, PBS
- Primary monoclonal antibody
- Fluorochrome-labeled anti mouse IgG antibody
- Mounting media

Procedure (for adherent cells)

1. Seed adherent cells on sterilized coverslips or appropriate plates (compatible with standard fluorescence microplate readers)
2. Aspirate media and fix adherent cells with ice-cold 100% methanol for 5'.
3. Wash 2x in PBS.
4. Incubate coverslips/plates with 40ul of primary antibody in PBS for 45' at RT.
5. Wash 2x in PBS.
6. Incubate coverslips with 40ul of fluorochrome-labeled anti mouse IgG antibody in PBS for 20' at RT in dark.
7. Wash 2x in PBS and 1x in ddH2O.
8. Carefully remove the coverslips from the wells and blot to remove any excess water. Dispense 1 drop of anti-fade mounting medium onto the microscope slide per coverslip. Mount the coverslip with the cells facing towards the microscope slide.
9. Visualize using a fluorescence microscope and filter sets appropriate for the label used.

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IHC staining protocol (for FFPE slides)

1. Deparaffinize sections in xylene, 3x8min.
2. Hydrate with 100% ethanol, 2x5min.
3. Hydrate with 90 % ethanol, 5 min.
4. Hydrate with 70 % ethanol, 5 min.
5. Wash in distilled water, 5 min.

Antigen Unmasking

For Citrate buffer pH 6.0 or Tris-EDTA buffer pH 9.0: Heat slides in a microwave submersed in unmasking solution until boiling is initiated; follow with 10 min at a sub-boiling temperature (95°-98°C).

Cool slides on bench top for 30 min.

Wash in distilled water, 2x3 min.

Wash in PBS, pH 7.2, 5 min

Serum Blocking:

Incubate sections with Normal Goat Serum, 5 % (in 1% BSA-TBS).....20 min at room temperature.

Primary Antibody: incubate sections with primary antibody in antibody dilution buffer (1% BSA-TBS) 300 µl to each section overnight at 4C in a humidified chamber.

Wash in PBS, pH 7.2, 2x5 min

Peroxidase –Blocking Solution (DAKO, S2023), 5 min

Wash in PBS, pH 7.2, 3x5 min

Secondary Antibody: incubate sections with secondary antibody – peroxidase labeled for 35 minutes in a humidified chamber at room temperature.

Wash in PBS, pH 7.2, 3x5 min

Chromogen/Substrate: Use according manufacturer's recommendations.

Counterstain : hematoxylin

Rinse in running water 15 min

Wash in distilled water, 5 min.

Dehydrate through 70% ethanol for 2 min, 95% ethanol for 2 min, 100% ethanol for 2x3min.

Clear in xylene for 3x5min. Coverslip with mounting medium.