ADMEbio
for proteomic research

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Blocking Peptide Protocol

We describe below general recommendations for using blocking peptides in western blot and immunostaining techniques. The precise conditions should be optimized for a particular assay.

1. Protocol:

Peptides are used to block antibody binding to its target. In order to visualize the inhibitory effect of the peptide, they are typically used at a 2X to 100X excess compared to antibody molarity. We recommend using a 0.15X, 3.0X and 30.0X excess of peptide first, and then to change this range if a more accurate study is required.

Calculation

WuXi Bioscience antibodies are generally manufactured at 0.5 mg/ml. Using the antibody at 1:1000 dilution as recommended corresponds to 0.5ug/ml. Estimating the MW of an antibody at 150,000 Da, the final antibody concentration is ca. 3.3nM. A peptide of 15 residues long has an average MW of 1650 Da (110 Da multiplied by 15). For an excess of 30.0X of peptide over the antibody used at 3.3nM, a concentration of 3.3nM ×1.650×30 = 0.165ug/ml, is needed. 0.165ug/ml is 30X excess.

Important Note

It is very important to mix the antibody with the peptide prior to incubation with the cell lysate or onto the slide. Otherwise, you may not be able to disrupt antibody binding from native target.

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I. Materials and Reagents:

1. Lysis buffer:

- 0.15M NaCl
- 5mM EDTA, pH 8;
- 1% Triton X100;
- 10mM Tris-Cl, pH 7.4
- Just before use add:
- 5M DTT 1:1,000;
- 100mM PMSF in isopropanol 1:1,000;
- 5M ε–aminocaproic acid 1:1,000

2. 2X Sample Buffer:

- 130mM Tris-Cl, pH8.0;
- 20% (v/v) Glycerol;
- 4.6% (w/v) SDS;
- 0.02% Bromophenol blue;
- 2% DTT

3. 1.5M Tris-HCl Resolving Gel Buffer:

500ml 91g Trizma base and adjust pH to 8.8 with 12N HCl

4. 1.0M Tris-HCl Stacking Gel Buffer:

500ml 60.55g Trizma base and adjust pH to 6.8

5. Resolving Gel: 10ml of a 12% gel

- 4ml 30% acrylamide/bisacrylamide (29:1 mix)
- 2.5ml Resolving Gel buffer
- 100µl 10% SDS
- 3.3ml water
- 4µl TEMED
- 100µl 10% ammonium persulfate

6. Stacking Gel: 10ml

- 1.4 ml 30% acrylamide/bisacrylamide (29:1mix)
- 2.5ml Stacking Gel buffer
- 100µl 10% SDS
- 6ml water
- 10µl TEMED
- 100µl 10% ammonium persulfate

7. 10x Running Buffer: 1L

- 30.3g Trizma base (= 0.25M)
- 144g Glycine (= 1.92M) 10g;
- add SDS (= 1%) finally.
- Do not need to adjust the pH.

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8. 10x transfer Buffer: 1L
   - 30.3g Trizma base (= 0.25M)
   - 144g Glycine (= 1.92M)
   - pH should be 8.3; do not need to adjust pH.

   - To make 2L of 1x transfer buffer:
   - 400ml Methanol and 200ml 10X TBS buffer

9. Blocking Buffer: 0.5L
   - 3-5% nonfat milk in TBST (0.05%Tween 20) or 5% BSA in TBST (0.05%Tween 20)
   - Make up in TBS and sterile filter. Then add Tween 20 to final 0.05%.
     Keep at 4°C to prevent bacterial contamination.

10. Stripping Buffer:
    - 0.5L (sterile filter solution and keep at 4°C)
    - 0.2M Glycine, pH 2.5, 0.05% Tween 20

II. Experimental Procedure:

1. Preparation of cell lysates:
   - Collect cells (confluent 10cm2 cell culture dish) by trypsinization or scrape and then spin at 3000rpm for 5 minutes at 4°C.
   - Abandon the supernatant, wash the cells twice with PBS, spin down (12,000rpm for 1 minute at 4°C) and collect cells on ice.
   - Split the cells with appropriate amount of Cell Lysis Buffer on ice for 30 minutes, and sonicate on ice for 10-30 seconds three to six times or until there's no cell clusters.
   - Spin at 12,000 rpm for 20-30 minutes at 4°C.
   - Transfer the supernatant to a new tube on ice and discard the pellet.
   - Measure the protein concentration (Bradford assay, A280, or BCA).
   - Add x µl (x=volume of the cell lysates/4) 5X sample buffer.
   - Boil for 5-10 minutes and cool at RT/on ice for 5 minutes.
   - Spin at 12,000rpm for 10 seconds to bring down condensation prior to loading gel.

2. Preparation of gel:
   - Assemble the plastic plates and spacers (1.0mm thick).
   - Pour the running gel to approximately 1cm below the wells of the comb (~6ml).
   - Seal with 1ml water-saturated 1-butanol. (Either proceed or stop here and store gel overnight.)
   - After gel has set, pour of butanol and rinse with deionized water.
   - Pour the Stacking Gel (~2 ml) and insert the comb immediately.
   - When the Stacking Gel has set, place in gel rig and immerse in buffer.
   - Prior to running the gel, flush the wells thoroughly with running buffer.

3. Running the gel:
   - After flash spinning the samples, load samples into the wells.
   - Run with constant voltage (120V). Typical running time is about 1.5hr.

4. Preparation of membrane:
   - Cut a piece of PVDF membrane (Millipore Immobilon-P#IPVH 000 10).