竞争性分析 Epitope Mapping 实验方法

ABSTRACT

The simplest way to determine whether two monoclonal antibodies bind to distinct sites on a protein antigen is to carry out a competition assay. The assay can be used with antibodies that bind both conformational and linear epitopes, and it is most useful in the analysis of monoclonal antibody specificity because polyclonal sera typically recognize multiple different epitopes. This simple, ELISA-based protocol examines the ability of two hypothetical, newly derived monoclonal antibodies, XH1 and XH2, to compete with the well-characterized ZO-1 anti-YFP1 antibody for binding to the target antigen YFP1; hypothetical antibody PC10 is used as a control. First, 96-well plates are coated with the YFP1 antigen and direct binding assays are run to determine that all of the antibody preparations are active and able to react with the immobilized antigen. Second, the competition analysis itself is carried out. Note that the names of antibodies and antigens should be considered as generic.

MATERIALS

Reagents

Biotin-labeled ZO-1 antibody

H_2SO_4 (100 mM)

$NaHCO_3$ (0.1 M, pH 9.6) (Optional, see Step 1)

PBS

137 mM NaCl

2.7 mM KCl

10 mM Na2HPO4

2 mM KH2HPO4

To prepare 1 liter of PBS (Phosphate-buffered Saline), dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, and 0.24 g of KH2HPO4 in 800 ml of distilled H2O. Adjust the pH to 7.4 (or 7.2 if required) with HCl. Add H2O to 1 liter. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15 psi (1.05 kg/cm2) on liquid cycle or by filter sterilization. Store the buffer at room temperature. If necessary, PBS may be supplemented with 1 mM CaCl2 and 0.5 mM MgCl2. Can be made as a 10x stock.

PBS with BSA (1% [w/v]; should not contain azide)

PBS with Tween 20 (0.05%)

Rabbit anti-mouse IgG peroxidase conjugate

Streptavidin-peroxidase conjugate

TMB substrate

3, 3', 5, 5'-tetramethylbenzidine (TMB) (50 g/l stock), 50 µl

Sodium acetate (0.1 M, pH 6.6), 5 ml

Hydrogen peroxide (30%), 1 µl

Prepare fresh before each use.

Unlabeled control monoclonal antibody PC10

Unlabeled XH1 and XH2 antibodies

Unlabeled ZO-1 antibody

YFP1 (the target antigen)

Equipment

96-well microtiter plates

Incubator, preset to 70°C

Microplate reader

METHOD

Determination of Activity and Titer of Test and Control Antibodies

Prepare a stock solution of YFP1 at 20 µg/ml in NaHCO3 (0.1 M, pH 9.6) or in PBS. Add 50 µl to the wells of a 96-well microtiter plate and incubate for 2 hours at room temperature or overnight at 4°C.

Wash the wells three times in PBS/Tween (0.05%).

Fill the wells (200 µl) with PBS/BSA (1%) and incubate at room temperature for 1 hour.

Wash twice in PBS/Tween (0.05%).

In a separate 96-well plate, prepare a titration series of the biotin-labeled ZO-1 and the unlabeled ZO-1, XH1, XH2, and control PC10 antibodies over five 10-fold

dilutions using PBS/BSA (1%) as a diluent. The starting concentration of the antibody solutions should be about 10 µg/ml. Hybridoma supernatants can be used neat.

Transfer 50 µl of each dilution onto the antigen-coated plate and incubate for 2 hours at room temperature.

Wash the plate four times in PBS/Tween (0.05%).

Add 50 µl of rabbit anti-mouse peroxidase conjugate (diluted 1 in 5000 in PBS/BSA [1%]) to all of the wells containing unlabeled antibody. Add the streptavidin-peroxidase conjugate (diluted 1 in 5000 in PBS/BSA [1%]) to the wells that contain the biotinylated ZO-1 antibody.

Incubate for 2 hours at room temperature or overnight at 4°C, and then wash the plate four times in PBS/Tween (0.05%).

Add 50 µl of freshly prepared TMB substrate to each test well and incubate at room temperature for 5-15 minutes as the blue color develops in the wells containing the highest concentration of specific antibody.

After development of the blue color, stop the reaction by adding 50 µl of 100 mM H2SO4. Determine the absorbance of the now-yellow product read at 450-nm wavelength in a microplate reader. The positive antibodies should give a strong, clear signal that diminishes through the latter part of the dilution series. This establishes

that the specific unlabeled antibodies are able to bind to the target antigen, identifies those having very weak activity, and establishes a suitable concentration of labeled antibody to use in the competition assay.

The Competition Assay

Prepare antigen-coated plates as in Steps 1-4 above. The assay can be configured in a large number of different formats using either labeled antigen or labeled antibody. The key requirements remain the same, however-a demonstration that all of the antibodies are able to bind the test antigen in the assay and the labeling of one of the antibodies that allows it to be distinguished from the competitor antibody. Commonly, the antigen is immobilized on a 96-well plate. Some antigens are partially denatured on absorption to plastic, and in these cases, alternate means of immobilizing the antigen may be explored.

Incubate the wells with the dilution series of unlabeled antibodies as above for 2 hours at room temperature.

Without washing the wells, add 50 µl of the dilution of the biotin-labeled ZO-1 antibody that gave the strongest signal before the next point in the titration curve showing a strongly reduced signal, as determined in the previous procedure.

Wash the plate four times in PBS/Tween (0.05%).

Add streptavidin-peroxidase conjugate (diluted 1 in 5000 in PBS/BSA (1%)) to all of

the wells.

Incubate for 2 hours at room temperature or overnight at 4°C, and then wash the plate four times in PBS/Tween (0.05%).

Add 50 µl of freshly prepared TMB substrate to each test well and incubate at room temperature for 5-15 minutes. The blue color develops in the wells containing the highest concentration of specific antibody.

After development of the blue color, stop the reaction by adding 50 µl of H2SO4 (100 mM). Determine the absorbance of the now-yellow product read at 450-nm wavelength in a microplate reader.

A strong reduction in the signal of the binding of the labeled ZO-1 should be seen by the highest concentrations of the unlabeled ZO-1 (homotypic competition). The control PC10 antibody should not inhibit the signal at any concentration. Results for the newly derived XH1 and XH2 antibodies should be interpreted carefully. If, for example, the novel competing antibody XH2 reduces the signal, whereas the other new antibody XH1 does not, then it may be concluded that XH2 and ZO-1 recognize sterically competitive epitopes, whereas the XH1 epitope is clearly unique.