EXPERIMENTAL PROCEDURES: ELISA TESTING

ELISA (Enyzme Linked Immunosorbent Assay) has become the method of choice for detecting the interaction between an antibody and its specific epitope. The following adaptions of the ELISA protocol have been used successfully with the Multipin system to scan sets of peptides and define specific epitopes. These protocols are intended for horse radish peroxidase labelled anti-species immunoglobulins using ABTS as the chromogenic substrate.

Topics in this article:

REAGENT SOLUTIONS

PBS 10x

0.1 M phosphate buffered saline (PBS) stock solution is used to prepare working strength PBS for use in ELISA tests.

53.7 g $Na_2HPO_4.2H_2O$

(or 42.8 g Na_2HPO_4 anhydrous

or 107.44 g $Na_2HPO_4.12H_2O$)

15.6 g $NaH_2PO_4.2H_2O$

340 g NaCl

Dissolve salts in hot purified water to give a final volume of 4 litres. Mix thoroughly and allow the solution to cool to room temperature. Adjust the pH to 7.2 with either 50% w/v NaOH or concentrated (37% w/w) HCl (take appropriate precautions when using these reagents). This solution is ten times the working strength and can by stored at room temperature for short periods, or in a refrigerator over extended periods. Discard the stock solution immediately if there are any signs of contamination.

PBS

0.01 M phosphate buffered saline pH 7.2 (PBS) is the working strength used in ELISA testing.

Dilute the PBS 10x stock solution 1/10 with purified water when required for use. Discard any unused portion of the working PBS solution.

Pre-coat Buffer

This buffer is used to pre-coat the gears. This reduces non-specific binding and gives a better signal-to-background ratio in ELISA assays. This buffer is also used as the diluent for the test antibodies.

0.01 M phosphate buffered saline pH 7.2

0.1% v/v Tween 20

0.1% w/v sodium azide

Conjugate Diluent

This is the diluent for the anti-species conjugate in the ELISA.

0.01 M PBS pH 7.20.1% v/v Tween 20

0.1% w/v sodium caseinate (USB)

Store the solution in a refrigerator for up to 24 hours. For longer term storage, freeze the solution at -20°C. Thaw as required. Do not add sodium azide as a preservative as the activity of the horse radish peroxidase will be destroyed.

Substrate Buffer Solution

This buffer is used as the solvent for the chromogenic substrate in ELISA.

35.6 g $Na_2HPO_4.2H_2O$

(or 28.4 g Na_2HPO_4 anhydrous)

33.6 g citric acid monohydrate

Dissolve the salts in a small amount of hot purified water. Make up to 2 litre with purified water at room temperature. Adjust the pH to 4.0 if necessary with 1 M Na_2HPO_4 solution (142 g Na_2HPO_4 anhydrous made up to 1 litre with purified water) or 0.8 M citric acid (168 g citric acid monohydrate made up to 1 litre with purified water).

Note

Substrate buffer should be stored in the refrigerator, and the amount required should be brought to room temperature before use each day. Substrate buffer should be used within two weeks of preparation and should be checked for signs of contamination immediately before use. If contamination is detected, discard the solution,

ABTS Substrate Solution

This is the chromogenic substrate for the horseradish peroxidase conjugate used to detect antibodies. Prepare the substrate solution immediately before use. Prepare 20 mL of substrate solution per block of gears to be tested. For accuracy, it is recommended that a minimum of 100 mL of substrate solution be prepared.

Equilibrate the substrate buffer solution to room temperature. Dissolve 0.5 mg/mL of diammonium 2,2′-azino-bis[3-ethylbenz-thiazoline-6-sulfonate] (ABTS) in substrate buffer. ABTS may be obtained from Boehringer Mannheim GmbH. When the ABTS has completely dissolved, add sufficient hydrogen peroxide solution (35% w/w - "120 vol") to give a final concentration of 0.01% w/v. It is important that hydrogen peroxide must not be added until the ABTS has completely dissolved.

CONJUGATE TEST

The conjugate used in an ELISA test is usually an antibody. Therefore it is possible that it will react directly and specifically with gear-bound peptides. This reaction could mask or be mistaken for the peptide binding of the primary antibody being tested. Therefore, we recommend that the reactivity of the conjugate with the peptides be checked before any testing with a primary antibody is carried out. If it is intended that antibodies from more than one species be tested, each of the conjugates should be checked. We have found that conjugates prepared by Kirkegaard & Perry Laboratories Inc. (Gaithersburg MD 20879, USA) are satisfactory.

The actual concentration of the conjugate should be optimised. The ideal concentration is found by assaying with different concentrations of the conjugate. The sensitivity of the assay will tend to "plateau" with increasing concentration. The optimum concentration is the lowest concentration (highest dilution) that gives the maximum sensitivity. This concentration will also minimize interference to the assay by specific reaction of peptides with the conjugate. We have found the Kirkegaard & Perry conjugates are satisfactory when used at a concentration of 0.25 µg/mL.

Pre-Coating Gears ("blocking" step)

Pre-coating gears reduces non-specific binding, giving a better signal-to-background ratio.

Dispense 200 µL of the pre-coat buffer into each well of a microtitre plate. Place the gears in the wells and incubate for 60 min at room temperature, preferably on a shaker table (at 100 rpm).

Note

This step may be performed by using a suitable size bath to contain sufficient volume (>30 mL) of the pre-coat buffer.

PBS Wash

Remove the gears from the pre-coat buffer and flick any excess buffer from them. Wash the gears in a bath containing ≥ 700 mL of 0.01 M PBS (pH 7.2) for 10 minutes at room temperature with agitation.

Conjugate Reaction

Dilute the appropriate conjugate preparation to its working concentration with conjugate diluent that has equilibrated to room temperature. Dispense 200 µL/well into microtitre plates. Flick excess PBS from the gears. Place the washed gears into the wells filled with conjugate and incubate at room temperature for 60 minutes with agitation.

PBS Washes

Remove gears from the conjugate solution and wash in a bath containing ≥ 700 mL of 0.01 M PBS (pH 7.2). Wash should be 10 min at room temperature with agitation. Note that froth may develop in the wash solution Rinse the block with purified water to remove any froth.

Substrate Reaction

Note

Prepare the substrate solution immediately before use. Ensure that it is equilibrated to room temperature.

Dispense 200 µL/well of ABTS substrate solution into ELISA reading plates (e.g., flat-bottom polystyrene plates). Flick any excess water from the gears and place them in the substrate solution. Ensure that the plates are correctly labelled and the blocks correctly oriented in the plates. Incubate the gears at room temperature on a shake table (100 rpm) for 45 minutes. Stop the enzyme action by removing the gears from the substrate solution.

Reading Results

The optical density of the substrate in each well should be read as soon as possible after incubation. The reacted substrate solutions should be evenly mixed in the wells before reading.

The optical density is read at 405 nm in a suitable Plate Reader. If the Plate Reader is capable of "dual wavelength" mode of operation, the optical density at 492 nm can be subtracted to correct for "background". Background in this case includes colour present in the substrate solution before use, and optical defects of individual wells of the ELISA plate.

PRIMARY ANTIBODY TEST

The assay with the primary antibody is a typical, indirect ELISA, with extra steps added to the conjugate reactivity test described above. After the pre-coat "blocking" step add the following two steps. Then continue with the conjugate reaction and subsequent steps.

First Antibody (primary antibody)

Dispense 200 µL/well of suitably diluted primary antibody into microtitre plates (antiserum is normally diluted in pre-coat buffer containing 0.1% w/v sodium azide). Flick excess pre-coat buffer from the gears and place the gears in the wells of the plate containing the dispensed primary antibody.

Note

This step may be performed by using a suitable size bath to contain sufficient volume (>30 mL) of the pre-coat buffer. Typically there is a 10 fold increase in sensitivity using the bath incubation for the primary antibody.

Incubate the gears in this plate or bath overnight in a refrigerator (or cold room) on a shaker table. We have found the following dilutions of antibodies to be suitable for the first test. The test should be repeated if these dilutions are not suitable. Ascites fluid and human antisera- 1:1000; hyperimmunized animal sera- 1:5000; supernatant of hybridoma cell cultures- 1:20; naturally infected (as opposed to hyperimmunized) animal sera- 1:1000.

PBS Wash

Remove gears from the primary antibody preparation and wash in a bath containing ≥ 700 mL of 0.01 M PBS (pH 7.2). for 10 min at room temperature with agitation. These steps replace the single PBS wash in the conjugate test.

Substrate Reaction

The reaction can be stopped by removing the gears from the plate. This can be done after 10 minutes if it appears that the reaction will give an optical density of 2 or greater (this is off the scale of many plate readers) if allowed to continue. However, this is not recommended and it is better to adjust the concentration of the primary antiserum so that on-scale readings are given with a 45 minutes incubation. However, if the substrate reaction is stopped after 10 minutes incubation, the plates should be given an extra 10 minutes shaking before reading. This ensures even mixing of the solution in the wells. Precipitation of the chromophore may occur if this precaution is not taken. Such precipitation may be observed when very strong colour has developed during the substrate reaction.

Quality Control

The reactivity by ELISA of positive (PLAQ) and negative (GLAQ) control peptides may be used as a measure of the quality of the peptide synthesis carried out. As control peptides are prepared on each block of peptides synthesized, they provide some measure of the variation in quality of synthesis from block to block and also from synthesis to synthesis.

Two sets of control peptide gears are supplied with the kit; one set has the positive control sequence PLAQGGGG, the other set has the negative control sequence GLAQGGGG. These control gears are distinguished from other gears by their stem colours. The positive control has a red stem and the negative control gear has a greeen.

These control peptides have been tested at our laboratories and gave satisfactory reactivity by ELISA with the Control Antibody provided. The reactivity of these peptides with the Control Antibody may be used as an indication of the sensitivity being achieved in your ELISA assay. Comparison with the block controls made by you gives an indication of the quality of the synthesis.

The monoclonal antibody provided, (in the bottle marked CONTROL ANTIBODY), reacts with the PLAQ but not the GLAQ peptide. It should be reconstituted with the indicated volume of purified water and requires no further dilution prior to use.

- Ensure that the antibody is completely dissolved before dispensing.
- The monoclonal antibody preparation contains bovine serum albumin, Tween 20, phosphate buffered saline and sodium azide (0.1%) and should be stored in a refrigerator between uses after reconstitution.

Control peptides may be tested independently of the rest of the peptide set, using the recommended ELISA procedure.

REMOVAL OF ANTIBODY

A major benefit of the Multipin System is that peptides can be reused after testing. The peptides are covalently coupled to gears and can be treated harshly to ensure that all antibody is removed.

Materials Required

0.1 M phosphate buffer with 1% w/v sodium dodecyl sulphate (SDS).

pH 7.2 at 60°C (adjust with 50% w/v NaOH or orthophosphoric acid).

2-mercaptoethanol

Hydrogen peroxide 35% w/w ("120 vol")

Methanol

Sonication bath (The sonication baths that we have found to be satisfactory have liquid surface dimensions of 25x38 cm. Power is applied at about 7 kW/m^2 to the transducers at 25 kHz.)

Caution

> *Mercaptoethanol is a toxic chemical that can be absorbed by the skin and lungs. The disruption procedure should be carried out in a fully functional and operating fume hood.*

"Disruption" Procedure

1. Thoroughly rinse the sonication bath with purified water and then add the 0.1 M phosphate buffer containing 1% SDS, preheated to 60°C. Add 2-mercaptoethanol to give a final concentration of 0.1% v/v. Allow the temperature to equilibrate to between 55°C and 65°C. Higher temperatures will damage the peptides, and lower temperatures may lead to ineffective removal of antibody.

2. Place the blocks in the bath with the gears downward. The blocks should just float in the disruption buffer. Sonicate for 10 minutes.

3. Rinse the blocks twice in purified water, pre-heated to 60°C, for 30 seconds.

4. Wash the blocks on a shake table in a bath of purified water at an initial temperature of 60°C for at least 30 minutes.

5. Shake off any excess water and totally immerse the blocks methanol for at least 15 seconds. Extreme care should be taken at this step to avoid exposure to methanol fumes and to avoid sources of ignition. Methanol is highly flammable.

6. Allow the blocks to air dry for at least 15 mins. They are ready for a further test. Store the blocks sealed in a plastic bag with desiccant in a refrigerator if they are not to be used immediately.

7. Before discarding the used disruption buffer, add 2 mL of hydrogen peroxide ("120 vol") per litre and allow to stand for 5 minutes. This will destroy any remaining 2-mercaptoethanol.

Confirmation of Antibody Removal

The effectiveness of antibody removal can be easily checked by repeating the test for conjugate reactivity as described above. If antibodies have not been completely removed, the scan may be similar before and after disruption. Any peaks in this test for carry-over of bound antibody should be lower than the first scan, indicating partial, but not complete, removal of antibody.

If antibody is shown to be present, repeat the sonication step. Do not increase the temperature above 65°C as this may adversely affect the peptides. It is better to repeat the sonication step rather than make the disruption conditions harsher.

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