ASSAYS OF BIOTINYLATED PEPTIDES

Peptides that have biotin coupled to their N- or C-terminus can be immobilised in solid-phase immunoassays by avidin or streptavidin. This method of immobilisation is independent of the sequence of the peptide being tested. The following is a description of a procedure that we have developed. Other ELISA enzyme/substrate systems may be used but the sensitivity of the test will vary accordingly. The conditions for each ELISA system should be optimised (eg. substrate concentration, pH, temperature and time etc.).

Topics in this article:

A. Reagent Solutions

The following reagents will be suitable for most ELISA work. However, there is one major exception. Do not use sodium caseinate in the ELISA if you have prepared an anti-peptide serum using peptide coupled to casein as the immunogen.

PBS 10x

10x phosphate buffered saline (PBS) stock solution (0.1 M) 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 $Na_2HPO_4.12H_2O$) (AR grade)

15.6 g $NaH_2PO_4.2H_2O$ (AR grade)

340 g NaCl (AR grade)

Dissolve the salts in hot distilled (or 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 the appropriate precautions when using these reagents. This solution is ten times the working strength. It can be stored at room temperature for a short time (ie. less than a week) or in a refrigerator over extended periods. Discard the stock solution if there are any signs of contamination.

PBS

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

Dilute the PBS 10x stock solution to 1:10 with distilled (or purified) water when required for use. Discard any unused portion of the working PBS solution.

PBS/Tween 20

This solution is used as the washing solution in the ELISA test.

0.1 % v/v Tween 20

0.01 M phosphate buffered saline, pH 7.2 (PBS)

This solution can be stored in a refrigerator for several days. Discard it if there is any sign of contamination.

PBS/Tween20/azide

This solution is used as the diluent of the primary antibody and peptides. It consists of: -

0.1 % v/v Tween 20

0.1 % w/v sodium azide

0.01 M phosphate buffered saline, pH 7.2 (PBS)

This solution can be stored in a refrigerator for several weeks. Discard it if there is any sign of contamination.

Conjugate diluent

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

1 % v/v sheep serum

0.1 % v/v Tween 20

0.1 % w/v sodium caseinate

0.01 M phosphate buffered saline, pH 7.2 (PBS)

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 because the activity of the horse radish peroxidase is destroyed by azide.

Note

This diluent is totally unsuitable for use in assays where the primary antibody to be tested is of sheep or goat origin. For sheep or goat antibodies modify the diluent by omitting the 1% v/v sheep serum and replacing with rabbit serum.

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 (AR grade)

Dissolve the salts in about 100 mL of hot distilled (or purified) water. Make up to 2 litres with distilled 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 distilled water) or 0.8M citric acid (168 g citric acid monohydrate made up to 1 litre with distilled water.)

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 checked for signs of contamination immediately before use. If contamination is detected, discard the solution, thoroughly wash the bottle and sterilize before reuse.

ABTS

This is the chromogenic substrate for the horseradish peroxidase conjugate used to detect antibodies. Prepare the substrate solution immediately before use. Prepare 12mL of substrate per plate to be tested. For accuracy, it is recommended that a minimum of 100 mL be prepared.

Equilibrate the substrate buffer solution to room temperature. Dissolve 0.5 mg/mL of diammonium 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonate) (ABTS) in substrate buffer. When the ABTS has completely dissolved, add sufficient hydrogen peroxide solution (30 to 35 % w/w - 100 to 120 volume AR grade) to give a final concentration of 0.01 % w/v. It is important that the hydrogen peroxide be added after the ABTS has dissolved completely. Because the concentration of H2O2 in the concentrated stock bottle can change over time, we recommend that it be checked regularly. This can be done volumetrically by titration against permanganate (1 mL of 0.1 N potassium permanganate is equivalent to 1.701 mg of acidified H_2O_2 - see European Pharmacopoeia for details).

Alternatively, the concentration can be assessed spectrophotometrically at 240 nm and taking the molar extinction coefficient as 43.6 $M^{-1}cm^{-1}$.

Blocking buffer

This solution is used to block non-specific absorption. The buffer consists of:

1.0 % w/v sodium caseinate

0.1 % v/v Tween 20

0.01 M phosphate buffered saline, pH 7.2 (PBS)

This solution can be stored in a refrigerator for several days. Discard it if there is any sign of contamination.

Note

If the antibody you wish to test is an anti-peptide serum in which the peptide was coupled to casein as a carrier, you should substitute another blocking agent for the sodium caseinate.

Streptavidin stock solution

Make a stock solution of streptavidin in purified water at a concentration of 2 mg/mL. We have found that Sigma catalog No. S-4762 (affinity purified, salt-free streptavidin) is suitable for these assays. This stock solution can be stored indefinitely if frozen at -20°C.

B. Coating the Plates

The plates for the assay must be coated with streptavidin. Select plates that are designed to absorb proteins to their surface. We have found Nunc-Immuno® MaxiSorb plates to be satisfactory (Nunc catalog No. 442404).

Prepare a solution of streptavidin by diluting the streptavidin stock solution 1:400 in purified water. Note that this dilution should be done in a container whose surface does not absorb proteins (e.g. polypropylene or polyethylene test tubes or bottles). The final concentration of streptavidin is 5 µg/mL. Dispense 100 µL of the solution into each well of the plates. Leave the plates exposed to the air at 37°C overnight to allow the solution to evaporate to dryness.

If desired, the plates can be stored in a refrigerator at 4°C. Pack the coated plates in a plastic bag with silica gel as a desiccant before storage. The coated plates will retain their activity for at least 4 weeks.

Each well is coated with enough streptavidin to bind approximately 30 pmol of biotinylated peptide.

C. Assay

1. Block non-specific absorption by dispensing 200 µL of blocking buffer (containing sodium caseinate as a block agent) into each well of the dry, streptavidin-coated plate. Allow to incubate for 1 hour at 20oC. After incubation, "flick" out the solution from the wells.

2. Wash the plates with PBS/Tween 20. This is done by flooding the plate with PBS/Tween 20 solution, ensuring that all wells are completely full of solution. Then vigorously flick the solution from the wells. Repeat this washing step 4 times. After the washings, remove excess buffer by vigorously "slapping" the plates, well side down, on a benchtop that has been covered with an absorbent material (e.g. paper towels).

3. If the peptides are a dry powder, dissolve them. We recommend that the peptides be reconstituted in 200 µL of either a pure solvent (e.g. dimethyl sulfoxide or dimethyl formamide) or solvent/water mixture (e.g. 40% acetonitrile in water). After reconstitution, the peptide solutions should be stored in a freezer (i.e. at -20°C or lower). Obviously, this reconstitution step is not needed if the peptide was cleaved directly into aqueous solution, for example, DKP peptides. In this case, the cleavage solution can be used "as is" for dilution to the working concentration.

4. Just before use, the peptide solutions are diluted to a working strength of 1:1000 with PBS/Tween 20/azide solution. The peptide stock solution can be diluted further (down to 1:5000), however there may be some loss in ELISA sensitivity if used too dilute. These solutions can be stored for one day at 4°C. For longer storage, the diluted peptide solutions should be frozen at -20°C.

Transfer 100 µL of each of the diluted peptide solutions into the corresponding well positions of the streptavidin-coated plate. Place the plate on a shaker table and allow the reaction to proceed for 1 hour at room temperature. For convenience, several sets of immobilised peptides may be prepared simultaneously and assayed at a later date.

After incubation, "flick" out the solution and repeat the washing procedure described in step 2. If the plates are not going to be used immediately, they should be dried at 37°C before storing in the dry state at 4°C.

5. Dilute the serum to be tested, using PBS/Tween 20/azide solution. The optimum dilution will depend on the source and amount of antibodies present. The recommended dilutions are 1:1000 for hyperimmune serum from experimental animals and ascites fluid from hybridoma-bearing mice, and 1:500 for human serum.

Add 100 µL of the diluted serum to each of the wells of the plates containing captured peptides. Place the plate on a shaker table and incubate with agitation for 1 hour at 20°C. Alternatively, the serum can be incubated overnight at 4°C. Better sensitivity has been found for some antibodies with the overnight reaction.

6. Remove the incubation mixture by flicking the plate and repeat the washes as described for step 2.

7. Detect bound antibody with a suitable dilution of conjugate solution consisting of a saturating level of horse radish peroxidase-labelled anti-species antibody. We have found the conjugate supplied by Kirkegaard and Perry to be suitable. We reconstitute a 0.5 mg pack size with 1 mL of 50 % glycerol (v/v) in purified water. This stock solution is stored at -20°C until required and diluted 1:2000 with conjugate diluent when needed (excess of the diluted conjugate is discarded).

Note

The diluent must not contain azide because this would destroy the activity of the peroxidase.

Dispense 100 µL of the dilute conjugate into each well and incubate at 20°C for 1 hour with agitation.

8. Remove the incubation mixture by flicking the plate and repeat the washes as described in step 2. Finally, wash the plate twice with PBS only (i.e. containing no Tween 20) to remove traces of Tween.

9. Detect the presence of peroxidase by adding 100 µL of freshly prepared ABTS solution to each well. This can be incubated for up to 45 minutes at 20°C. The plates are then read at a wavelength of 405 nm. A suitable reference wavelength is 495 nm if your plate reader has dual wavelength mode.

Note

A test should be done using pre-immune, negative or normal serum to verify that any binding observed was due to specific antibodies. We recommend that this test be done in parallel with the test antibody. Similarly, a negative control test can be performed by omitting the incubation with specific antibody. Any positives in this test would be due to conjugate binding directly to the captured peptide.

Mimotopes International
11 Duerdin St, Clayton
VIC, Australia 3168
mimotopes@mimotopes.com
www.mimotopes.com

Mimotopes Asia Pacific
Tel: +61 3 9565 1111
Fax: +61 3 9565 1199
australia@mimotopes.com

Mimotopes Europe
Tel: +44 870 460 1500
Fax: +44 870 460 1501
europe@mimotopes.com

Mimotopes US East
Tel: +1800 633 8161
Fax: +1800 424 3970
useast@mimotopes.com

Mimotopes US West
Tel: +1800 644 1866
Fax: +1800 655 1866
uswest@mimotopes.com