Antigen Competition to Identify Sequential (Linear) Epitopes

Introduction

Antibody-defined epitopes of protein antigens can be broadly classified as sequential (=linear, continuous) or assembled (=discontinuous). The pin-peptide scanning technique can be used to locate all the sequential epitopes for protein antigens of known sequence [1,2,3,4]. However, not all peptide-binding antibodies present in a polyclonal anti-protein serum can be shown to bind to the native antigen [4]. Further, it has been claimed that many of the antibodies shown to bind to peptides were generated to denatured forms of the antigen [5,6], although the opposite has also been claimed [7]. Where sufficient amounts of antigen are available the most direct way of identifying peptides that represent true sequential epitopes is to show that antigen in solution is able to block the binding of the antibody to the pin-peptide [3,4].

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Procedure

If a duplicate set of pin-peptides homologous with the antigen of interest is available for testing, and the antigen is in plentiful supply, then the antigen can be used in a simultaneous competition test on all the antibody-binding peptides. However, where the antigen is in short supply it may only be possible to test a small selection of peptides.

Step 1.

Using the normal pin ELISA procedure, carry out a preliminary test(s) of the duplicate set of peptides to determine an antiserum concentration which will give ELISA values for all peptides of less than 2.0 absorbance units. This initial test also establishes the reproducibility of the ELISA signal on duplicate pins. If sufficient experience with the day-to-day variability of ELISA results has been obtained in your laboratories, it would be reasonable to use this data to estimate the standard deviation for repeat (identical) tests on a single pin. Otherwise, it is necessary to carry out a minimum of three serial repeats of the same test, keeping all parameters constant, in order to establish for pin "n" a value for the mean absorbance An, and the standard deviation Sn.

Step 2.

If sufficient antigen is available, prepare two identical antiserum solutions at the dilution established in the preliminary test(s), and to one of these solutions add the antigen to a final concentration of 0.1mg/mL. Incubate both solutions at room temperature for 1h.

Step 3.

Using the pin ELISA procedure, test the antibody solution containing the added antigen ("competed") on one of the sets of pins, and test the antibody solution without added antigen ("uncompeted") on the duplicate set in parallel.

Step 4.

To establish that a significant reduction in ELISA signal has occurred as a result of antigen competition, a significance test should be applied. Since each pin had been tested previously with the same dilution of antibody, an expected absorbance A(n, uncompeted) for each pin ("n") is known. For significant competition to be seen, the observed absorbance A(n, competed) for the pin treated with the competed sample needs to be less than

A(n, uncompeted) - 2Sn, where

2Sn = twice the standard deviation of repeat tests on pin n. We would normally expect the value of Sn to be around 10% of the mean absorbance for that pin, and thus a decrease in ELISA signal of the competed sample of >20%, with no concomitant change in the uncompeted control, would be evidence for competition. A decrease by a higher multiple of Sn would similarly be stronger evidence for competition. To confirm a positive competition result, the competition test can be repeated, this time reversing the roles of the individual pin-peptides, i.e. placing the uncompeted sample on the pin which was treated with the competed sample in the first test, and vice versa.

If there is only one "copy" (pin) of each peptide, it is still possible to carry out a competition test by testing the competed, and control uncompeted samples in serial ELISA's. Single (serial) tests do not have a built-in simultaneous positive control and thus serial tests need to be repeated a larger number of times to show that any competition observed is reproducible.

Discussion

If significant competition is observed for any peptide, then that peptide is operationally identified as a sequential (linear) epitope of the antigen used in the competition test. The epitope identified in this way may be incomplete, as there can be additional residues from the antigen which make contact with the antibody, but they may make little or no contribution to the binding energy. Another possibility, highly unlikely, is that the pin- peptide does not actually represent the cognate site on the protein molecule to which the antibody binds, but is a mimic of an assembled (discontinuous) epitope of the competitor antigen. In effect, such a peptide would be a mimotope [8] occurring naturally within the sequence of the antigen. The competition method described relies on a very low level of contamination of the native antigen solution with denatured antigen [5,6,7]. In addition, the following factors affect the observed level of competition.

1. Affinity

1.1 Competition is affected by the relative affinity of the antibody for peptide, as compared with native antigen. The higher the relative affinity for peptide compared to the whole protein antigen, the more difficult it will be to detect competition.

1.2 The multimeric nature of the pin-peptide surface affects the observed competition. The apparent affinity for pin-bound peptide (by comparison with soluble peptide) is enhanced because each antibody molecule can bind two (or more in the case of IgM) peptide molecules. Thus, even when measuring competition between pin-bound and solution-phase peptide, surprisingly high concentrations of antigen (e.g. the 0.1mg/mL suggested in step 2 of the Procedure) may be needed to see effective competition.

2. Concentration

The result is affected by both the absolute and the relative concentrations of native antigen and of antibody. A single (fixed) amount of antigen was suggested above, mainly because the likely maximum antibody concentration in use is set by the criterion of working with an on-scale ELISA reading. The competition experiment could be expanded into a titration series if there are enough replicate solid phase samples (pins) of the antibody-binding peptide. In the papers which have been critical of direct binding studies with peptide [6,7], comment is usually made that a competition or other test for specificity of the antibodies for the native form of the protein should be carried out to validate the relevance of peptide-binding antibodies. We have always taken the same view [3,4] and we emphasize that initial direct binding studies on pins should be followed up with tests of specificity such as the competition test described here, and preferably also with tests on solution-phase peptides or other forms of the putative epitope. Based on experience with monoclonal antibodies, 5% to 15% of antibodies would be expected to recognise sequential epitopes, the remainder recognising assembled (discontinuous) epitopes.

References

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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