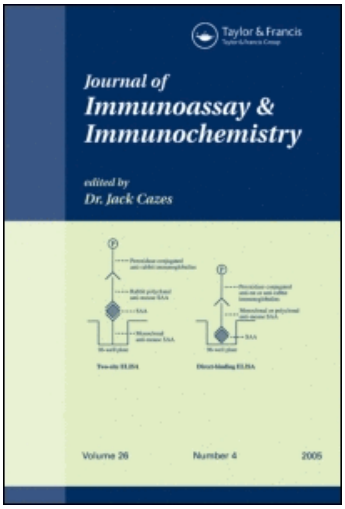

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Non-Specific Serum Binding to Streptavidin in a Biotinylated Peptide Based Enzyme Immunoassay

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To cite this Article Norton, Robert , Heuzenroeder, Michael and Manning, Paul A.(1996) 'Non-Specific Serum Binding to Streptavidin in a Biotinylated Peptide Based Enzyme Immunoassay', Journal of Immunoassay and Immunochemistry, 17: 3, 195 — 204
To link to this Article: DOI: 10.1080/01971529608005788
URL: http://dx.doi.org/10.1080/01971529608005788

JOURNAL OF IMMUNOASSAY, 17(3), 195-204 (1996)

NON-SPECIFIC SERUM BINDING TO STREPTAVIDIN IN A BIOTINYLATED PEPTIDE BASED ENZYME IMMUNOASSAY

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ABSTRACT

Streptavidin coated microtitre wells can be used to provide attachment to biotinylated peptides, regardless of charge and size, for use in an enzyme immunoassay. Considerable non-specific human serum binding to streptavidin at the recommended concentration was noted. The level of binding varied considerably with serum samples and was unrelated to previous streptococcal disease. Strategies used to reduce this phenomenon included pre-absorption of serum with streptavidin and reducing the streptavidin concentration in the wells. Significant reduction in binding was found with a reduction in streptavidin concentration from the recommended concentration of 0.5 ug/well to 0.01 ug/well.

(Key words: Streptavidin, enzyme immunoasay, peptide)

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INTRODUCTION

Epitope analysis using synthetic peptides is a well established technique. It has been used for a variety of purposes ranging from diagnostic serology to vaccine synthesis(1).

Synthetic peptide antigens which are hydrophilic and contain less than 20 amino-acids, are difficult to immobilize on plastic surfaces in sufficiently reliable quantities for a direct enzyme immunoassay (EIA) (2). The binding of these peptides to microtiter plates has been achieved by the use of peptide - polymer rod combinations (1), conjugation to carrier proteins with covalent binding to chemically activated surfaces (6), the use of high pH carbonate buffers (7) and the capture of biotinylated peptides by immobilized avidin or streptavidin (2, 3).

The attachment of biotin to synthetic peptides is done by specific introduction at the amino terminus after solid - phase synthesis (4). Antibody detection by enzyme immunoassay can be enhanced by the use of the biotin - avidin system. In this, microtiter plates are coated with avidin or streptavidin and biotinylated peptides are captured by the immobilised avidin or streptavidin. Amplification occurs because each avidin molecule has several biotin binding sites (2).

A recognised problem with using avidin to coat plates is the higher background absorbance readings (3). Streptavidin (from *Streptomyces avidinii*) has been suggested as an alternative (3). One mole of streptavidin is reported to bind four moles of biotin. Alternatively, one unit of active streptavidin will bind 1.0 ug of biotin (5).

The concentration of streptavidin recommended for coating plates is 5 ug/ml or 0.5 ug/well (3).

The C-terminal region of M24 protein of *Streptococcus pyogenes* has previously been described as being of possible importance in the pathogenesis of rheumatic fever (7).
Biotinylated peptides based on this region of M24 protein, were used in an EIA to map linear epitopes reactive with sera from subjects with rheumatic fever or rheumatic heart disease. Considerable variation in human serum non - specific binding to streptavidin was noted, resulting in high absorbance values. This was unrelated to the peptide used (Norton R. et al, Adelaide, 1995;unpublished observation).

This study looks at strategies to reduce this binding. These include pre-absorption of serum with streptavidin and reducing streptavidin concentration in the wells.

MATERIALS AND METHODS

Streptavidin:
Streptavidin (Sigma) stock solution was made up from affinity purified, lyophilized powder to a concentration of 1 mg/ml in distilled water. This gave approximately 14 units streptavidin activity per mg of protein (5).

Sera:
A total of eight sera from Aboriginal adults were used in the EIA

described below. Of these, two were from subjects with clinically proven acute rheumatic fever based on the Revised Jones criteria (9). Two were from subjects with proven rheumatic heart disease based on clinical, echocardiographic and histological evidence. These were patients who had valve replacements done for rheumatic valvular heart disease. There were four controls and these were matched by age, sex and area of origin. All controls were examined to exclude rheumatic fever or rheumatic heart disease.

Peptides

Five sixteen-mer, biotinylated peptides corresponding to part of the C-terminal end of M24 protein were obtained (Chiron Mimotopes). Three of these were noted to be uniformly reactive with sera from Aboriginal adults with rheumatic fever (Peptides 89,102 and 103) and two (Peptides 56 and 80) were uniformly non-reactive (Norton R. et al, Adelaide, 1995;unpublished observation).

The signal sequence was not included. The peptides were dissolved in phosphate-buffered saline (PBS pH7.2) and dimethyl formamide. The working dilution of the peptides was 0.028 mg/ml.

Basic Enzyme Immunoassay Method:

Ninety six well microtitre plates (Nunc Maxisorp) were coated with 100 uL per well of streptavidin at varying concentrations as detailed below. The plates were dried overnight at 37°C then washed with PBS/0.1% Tween 20 (pH 7.2). The wells were blocked with 2% Casein-10 mmol Tris-HCl/PBS (pH 7.0) for 30 minutes and washed. Single peptides were added to each well at this stage if the system was used for

peptide epitope mapping. A final concentration of 0.28 μg peptide /well was used. The plates were put on a shaker for one hour at room temperature.Test sera were either pre-absorbed with streptavidin as described below or used unabsorbed and diluted to 1 in 500 with 0.5% casein -Tris-HCl/PBS. The plates were incubated at 37°C for one hour. Secondary antibody conjugate (Dako rabbit anti-human IgG horseradish peroxidase) at a dilution of 1 in 1000 in 0.5% casein -Tris-HCl/PBS, was added. Substrate (o-phenylenediamine-2HCl) was added and the resulting colour reaction stopped with 1M Sulphuric acid. All washes between steps were done with PBS/0.1% Tween 20 (pH 7.2). Absorbance values were read on a MR 7500 using a test wavelength of 490 nm (A_{490}) and a reference wavelength of 630 nm.

EIA Test Variables:

(1) Varying streptavidin concentration in the wells (Without biotinylated peptides):

Streptavidin was added to the wells at the following concentrations :- 0.5 ug/well (recommended), 0.05 ug/well and 0.01 ug/well. Each of the eight sera were tested.

(2) Pre-absorption of sera with streptavidin (Without biotinylated peptides):

Streptavidin at concentrations of 0 ug/ml, 0.05 ug/ml, 0.1 ug/ml, 0.2 ug/ml, 0.5 ug/ml was added to four sera, with the diluent of 0.5 % casein -Tris-HCl/PBS and incubated at 37°C for 1 hour. The final dilution of sera remained 1 in 500.

(3) A comparison using three different concentrations of streptavidin to coat wells (0.5 ug/well, 0.05 ug/well and 0.01 ug/well) together with the five biotinylated peptides as described earlier, at the recommended concentration of 2.8 ug/ml or 0.28 ug/well (3): Serum from a subject with known rheumatic fever was used.

Data Analysis

One -way analysis of variance (ANOVA) was used to determine if there was a significant variation among the means, of absorbance values for the variables tested.

RESULTS

Results are given as absorbance values (A_{490}) and are presented as graphs in Figures 1,2 and 3.

The effect of varying the streptavidin concentration used in coating wells is shown in Figure 1. A significant difference ($p<0.0001$) in mean absorbance values between the concentrations 0.01 ug/well, 0.05 ug/well and 0.5 ug/well is demonstrated. These were 0.077, 0.9 and 1.225 respectively.

The effect of preabsorbing test sera with varying streptavidin concentrations in an attempt to reduce non-specific binding is shown in Figure 2. The mean absorbance values for each of the streptavidin concentrations used (0 ug/ml, 0.05 ug/ml, 0.1 ug/ml, 0.2 ug/ml, 0.5 ug/ml) were 0.02, 0.02, 0.01, 0.01 and 0.02 respectively

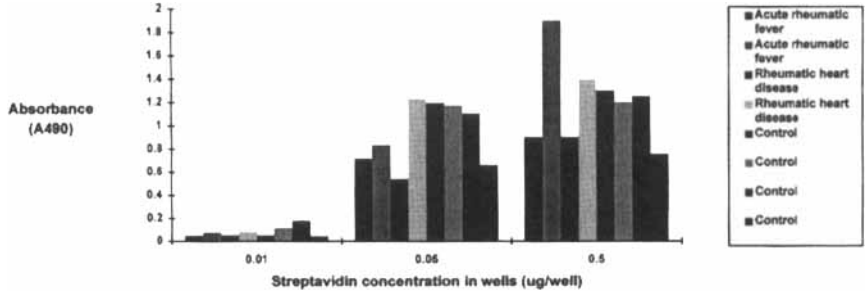

Figure 1 : The effect of varying streptavidin concentration for coating wells in an EIA using 8 different sera

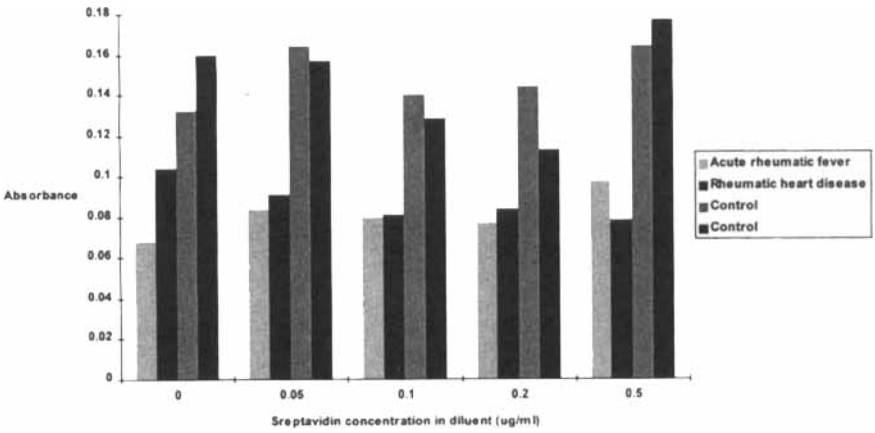

Figure 2: The effect of pre-absorbing test sera (n=4) with varying concentrations of streptavidin

No significant difference was demonstrated between the groups tested (p=0.8934).

In Figure 3, a comparison is made of the use of streptavidin at 0.5 ug/well, 0.05 ug/well and 0.01 ug/well with previously described, reactive and non-reactive biotinylated peptides. As the concentration of

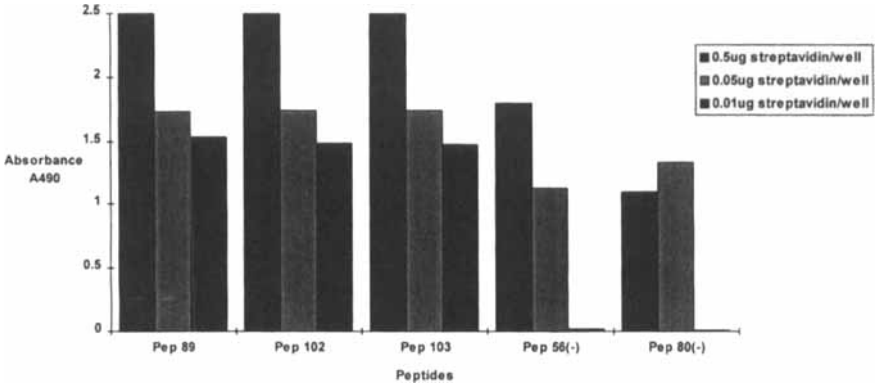

Figure 3: Peptide reactivity using three different concentrations of streptavidin in wells. Serum from a subject with rheumatic fever

streptavidin in the wells is decreased, there is a corresponding fall in absorbance values. This is most evident at the streptavidin concentration of 0.01 ug/well. The non-reactive peptides 56 and 80 had absorbance values of 1.75, 1.1 and 0.05 for the streptavidin well concentrations of 0.5 ug/well, 0.05 ug/well and 0.01 ug/well respectively.

The reactive peptides 89, 102 and 103 remained positive with absorbance values of 2.5, 1.75 and 1.5 for the same streptavidin concentrations.

DISCUSSION

At the higher streptavidin well concentrations of 0.5 ug/well and 0.05 ug/well, considerable non-specific binding occurs. Inter-sera variation in the A_{490} readings at both those concentrations is also noted. Both these effects are not seen at the lower concentration of 0.01 ug/well.

This is confirmed by the relatively high A_{490} values with the negative peptides 56 and 80 using streptavidin well concentrations of 0.5 ug/well and 0.05 ug/well, which reduce to values below 0.1 when a streptavidin well concentration of 0.01 ug/well is used. There is no corresponding fall in A_{490} values with the other peptides which are commonly reactive in rheumatic fever .

Pre-absorbtion of sera with streptavidin is shown here to have no benefit in reducing the non-specific binding.

The presence of high background absorbance values reduces the discriminatory ability of an assay. Using the test and reference wavelengths in this study and if manufacturer recommended streptavidin concentrations of 0.5 ug/well were used, positive sera would have absorbance values reported as being greater than 2.5. This being the upper limit of detection of the colorimeter used. This would reduce the ability to distinguish sera of varying degrees of positivity. The use of streptavidin at a concentration of 0.01 ug/well eliminates non-specific binding without affecting the performance of the assay.

The need for a reliable binding system for peptides, irrespective of size and charge, to microtitre plate wells is recognised (2) and the streptavidin - biotin system is one example of such a system. This study would suggest that significant non-specific serum binding to streptavidin occurs at high streptavidin well concentrations. This can be reduced by titration of the streptavidin concentration in the well with no corresponding reduction in sensitivity of the assay.

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