Estimation of Aflatoxins in peanuts by Indirect Competitive ELISA

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Enzyme-linked Immunosorbent Assays (ELISA)

The basic principle of Enzyme-linked immunosorbent assays (ELISA) lies in immobilizing the antigen onto a solid surface, or a capturing antigen by specific antibodies, and probing with specific immunoglobulins carrying an enzyme label. The enzyme retained in the case of positive reaction, is detected by adding the suitable substrate. The enzyme converts substrate to a product which can be easily recognized by its colour. ELISA tests rely on evaluation of a visible antigen-antibody precipitate.

Two types of ELISA have been used for the analysis of aflatoxins (1) direct ELISA, and (2) indirect ELISA. Both types are heterogeneous competitive assays. Direct competitive ELISA involves the use of an aflatoxin-enzyme conjugate, while indirect competitive ELISA involves the use of a protein aflatoxin conjugate and a secondary antibody such as goat anti rabbit IgG to which an enzyme has been conjugated.

Direct competitive ELISA

In this assay, a specific antibody is first coated to a solid phase such as a microtiter plate. The samples extract or standard toxin is generally incubated simultaneously with enzyme conjugate or separately incubated in two steps. After appropriate washings, the amount of enzyme bound to the plate is determined by incubation with a specific substrate solution. The resulting colour is then measured spectrophotometrically or by visual comparison with standards. Since this assay is based on competition for antibody binding sites, free toxin concentration is inversely related to antibody-bound enzyme conjugate.

Indirect competitive ELISA

In this procedure, aflatoxin-protein conjugate (BSA- aflatoxin B1) is coated onto the microtiter plate. Sample or standard aflatoxin is added to the wells followed by an aliquot of anti aflatoxin antibody. The amount of antibody bound to the plate is detected by the addition of goat anti rabbit IgG conjugated to alkaline phosphatase (ALP) followed by reaction with p-nitro phenyl phosphate to give a colored product. The toxin is determined by comparing with a standard curve from known toxin concentrations.

The sensitivity of indirect ELISA is comparable to that of direct ELISA. Because only small amount of antibody are required for indirect ELISA, this method is used not only for toxin analysis, but also to monitor the antibody titers of hybridoma culture fluids for the screening of monoclonal antibody producing cells.

Extraction of aflatoxin from peanut seed

Take 200 g peanut kernel sample and make it powder using a blender. Use 20 g sub-sample for toxin extraction with 100 ml of a solvent containing 70 ml methanol + 30 ml water + 0.5 g KCl and blended for 2 minutes. This is followed by shaking in a rotary shaker for 30 minutes at 250 rpm. The extract then filtered through Whatman No. 4 filter papers and diluted to 1:10 with 0.2% bovine serum albumin (BSA) prepared in 0.05M PBS-Tween, pH 7.4 (PBST-BSA). Depending on the concentration of aflatoxin, the sample can be either used directly or diluted further at 10 fold intervals, prior to analysis by ELISA.

Protocol for indirect competitive ELISA

Prior to utilizing this procedure concentrations of various reagents required to give optimum results needs to be determined. These include the concentrations of AFB_1-BSA and dilution of polyclonal antiserum and goat antirabbit IgG's labeled with alkaline phosphatase. Coat the ELISA plates with 150 µl/well of AFB_1-BSA at a concentration of 100 ng/ml prepared in carbonate coating buffer. At each step incubate the plates at 37 °C followed by three washes with PBS-Tween. In the second step treat the plates with PBST-BSA, followed by competition step in which standard or samples and antibodies are mixed in the plate. For competition step, prepare AFB_1 standards ranging from 0.1 to 25 ng/ml in extracts (10%) from peanut not containing any aflatoxin. Peanut kernels (25 g) free of aflatoxin, as determined by HPLC is taken, powdered and extracted with 125 ml of 70% methanol containing 0.5% KCl. The extract is then filtered and diluted to 1:10 (= 10% extract) in PBST-BSA. This is used as a diluent for preparing aflatoxin standards. Use concentrations of the standards starting from 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.097 ng/ml and duplicate each concentration in two wells. Similarly, duplicate each test sample in two wells. Mix 100 µl of each of the test diluted sample extract or standards with 50 µl of antiserum diluted to 1:60,000 in 0.2% PBST-BSA. After this step, add alkaline phosphatase labeled goat antirabbit IgG conjugate diluted to 1:2000 in PBST-BSA. Then add substrate p-nitrophenyl phosphate prepared in 10% diethanolamine. Incubate the plates at room temperature and then

read in an ELISA reader. Allow a maximum interval of 2 hours until optical densities from wells not containing any toxin reached 1.5 to 2.0 OD units at 405 nm. Draw regression curve using for Log_{10} values of concentration for aflatoxin standards plotted on the Y-axis and optical density values plotted on the X-axis. Determine aflatoxin concentration in the sample extract using the formula: AFB_1 concentration (ng/ml) in sample extract * times dilution with buffer * extract solvent volume used (ml) ÷ sample weight

Flow diagram of the protocol

SAMPLE EXTRACTION

Take 100g kernel and grind in a blender to a fine powder

Take 20g of above and add 100ml of 70% methanol and blend in a Mixer

Transfer the content in a conical flask and shake at 300 rpm for 30 minute

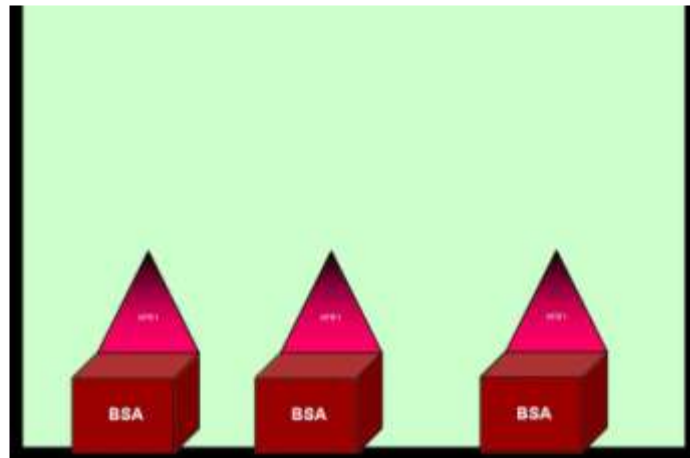
Filter through Whatman No.41 filter paper and dilute the extract in PBS- Tween (1:10)

COATING

Prepare AFB1-BSA conjugate in carbonate coating buffer at 100 ng/ml
(1µl AFB_{1-} BSA in 10 ml of carbonate coating buffer and vortex)

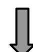
Dispense 150 µl of diluted AFB1-BSA to each well of microtitre plates (ELISA Plates)

Incubate at either 4^0C for overnight or at 37^0C for 2 hours

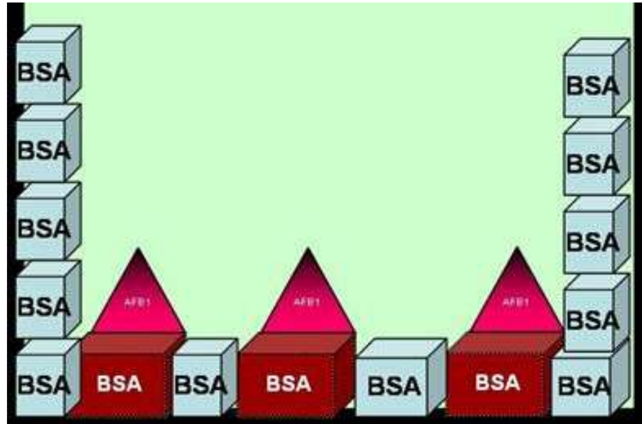


Wash the plates thrice with PBS-Tween, allowing 3 minutes for each wash

BLOCKING

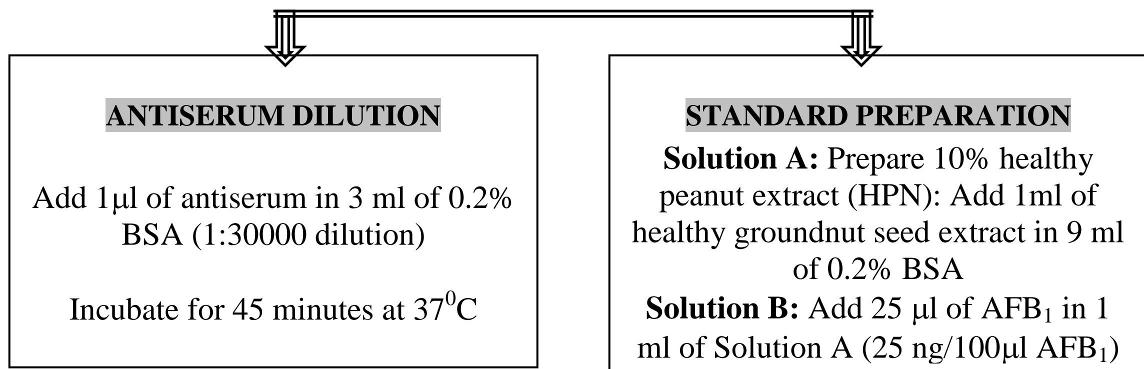


Add 200 µl of 0.2 % BSA prepared in PBS-T (2mg/ml) in each well and incubate at 37^0C for 1 hour



Wash the plates thrice with PBS-Tween, allowing 3 minutes for each wash

Prepare suitable dilution of Antiserum and Aflatoxin standards

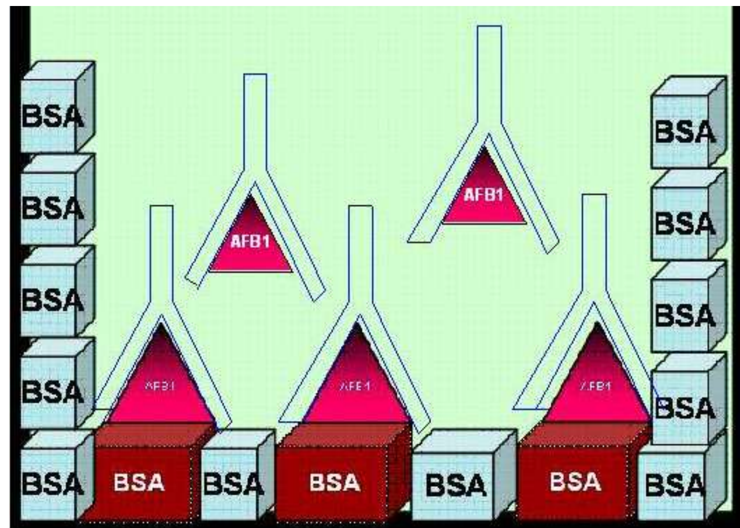


COMPETITION

Load Aflatoxin B_1 Standard, Healthy Peanut Extract and Test samples in wells of ELISA plates as per the template

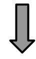
	1	2	3	4	5	6	7	8	9	10	11	12
A					**L E A**	**V E**	**B L A**	**NK**				
B		100µl Sol B	100µl B + 100µl A	100µl A	100µl A	100µl A	100µl A	100µl A	100µl A	100µl A	100µl A	
C	**L E A**	100µl Sol B	100µl B + 100µl A	100µl A	100µl A	100µl A	100µl A	100µl A	100µl A	100µl A	100µl A	**L E A**
D	**V E**	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	**V E**
E	**B L A**	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	**B L A**
F	**N K**	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	**N K**
G		90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	90µl BSA + 10µl sample	
H					**L E A**	**V E**	**B L A**	**NK**				

Add 50 µl of Antiserum in each well and incubate at 37 °C for 1 hour (Total volume in each well: 150 µl)

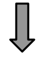


Wash the plates thrice with PBS-Tween, allowing 3 minutes for each wash

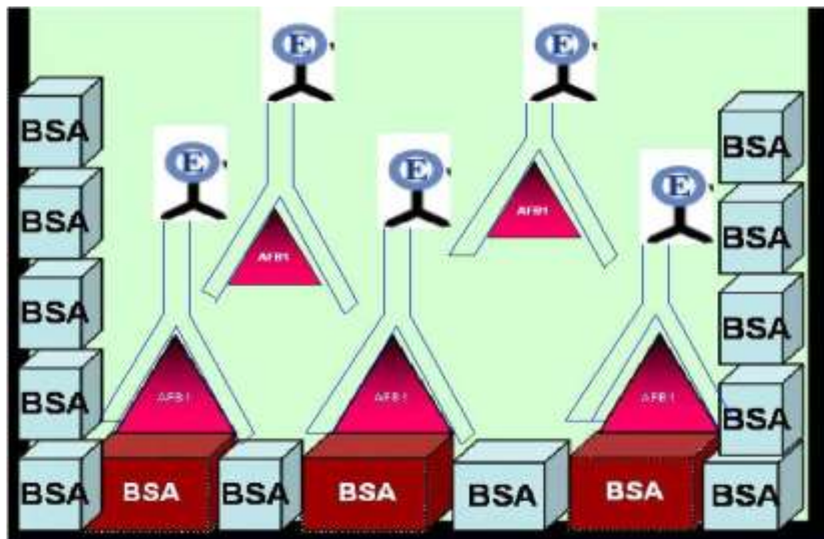
ADDITION OF ENZYME LABELED ANTIBODY



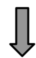
Prepare 1:1000 dilution of goat anti-rabbit IgG labeled with alkaline phosphatase in PBS-Tween containing 0.2% BSA (10 µl of IgG + 10 ml 0.2% BSA)



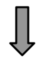
Add 150 µl in each well and incubate for one hour at 37^0C



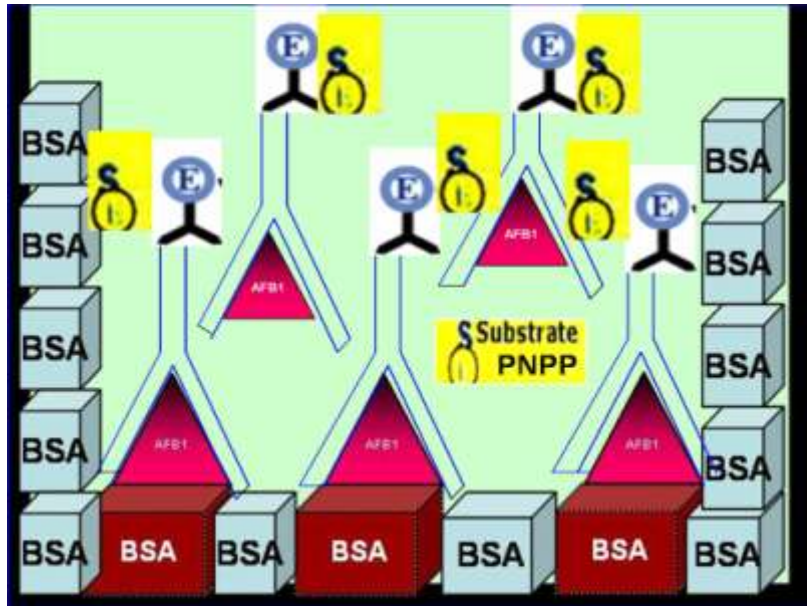
Wash the plates thrice with PBS-Tween, allowing 3 minutes for each wash



ADDITION OF SUBSTRATE



Add 150 µl of substrate [15 mg ρ-nitrophenyl phosphate (pnpp) in 30 ml of 10% Diethanolamine pH 9.8 (DEA)] in each well and incubate for 1 hr at room temperature in dark



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MEASURING ABSORBANCE AND CALCULATION OF TOXIN CONCENTRATION

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Measure absorbance at 405 nm in an ELISA Reader

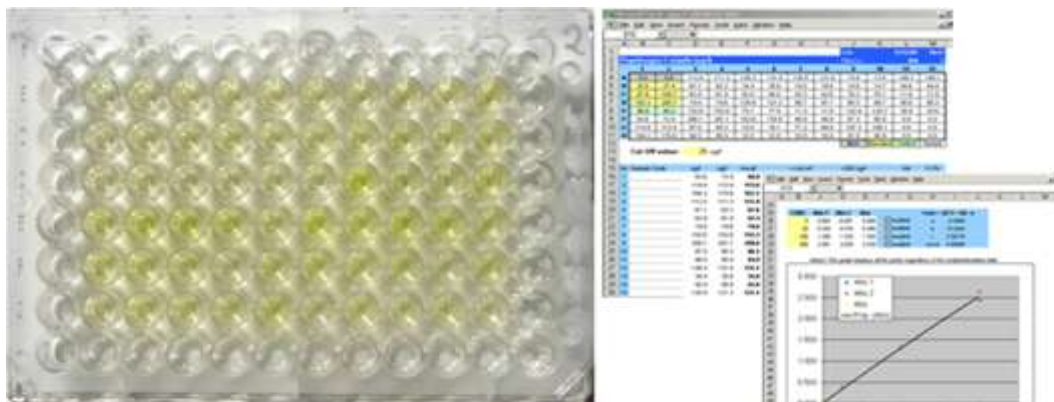
AFB1 (µg/kg): $\frac{A \times D \times E}{G}$

A = AFB1 concentration in diluted sample extract

D = Times dilution with buffer

E = Extraction solvent volume used (ml)

G = Sample weight (g)



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