ELISA

An inexpensive and highly precise tool for estimation of aflatoxins

RESEARCH PROGRAM ON
Agriculture for Nutrition and Health
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Aflatoxins: Agricultural products are often at risk of fungal invasion that can produce toxic metabolites called "mycotoxins". Among these, aflatoxins are of economic importance because of their influence on the health of human beings and livestock, and on the marketability of agricultural products. It is essential to analyze food products to ensure their safety. In most developing countries limited or no facilities exist for monitoring these toxins in foods and feeds. They are based on physicochemical methods such as thin layer chromatography (TLC), and high performance liquid chromatography (HPLC). However, these are very expensive and may not be available everywhere.

Immunological methods are preferred over analytical methods because of their simplicity and cost-effectiveness. ICRISAT, in collaboration with the Scottish Crop Research Institute (SCRI), produced high quality monoclonal and polyclonal antibodies for aflatoxins, and developed methodologies to use antibodies for aflatoxin estimation by the enzyme-linked immunosorbent assay (ELISA) in different agricultural commodities. The results were comparable with those of HPLC. Costs for performing this test procedure were compared with those of TLC and HPLC and was found to be the least expensive.

The method developed is simple, robust, versatile and cost-effective. Constant monitoring of food and feed will contribute to the improvement of human and livestock health, and will also enhance the export potential leading to increased income for poor farmers in developing countries. In recent years ICRISAT has further simplified the technique and produced both polyclonal and monoclonal antibodies for several mycotoxins.

Procedures

Sampling is a critical step in the process of estimating the presence of aflatoxin in food and feed. It is advisable to adopt the standard sampling procedure recommended by FAO and the EU.

Sample extraction (eg, groundnut): Powder 100 g of groundnut kernels using a blender. Take 20 g of this powder, add 100 ml 70% methanol (v/v- 70 ml absolute methanol in 30 ml distilled water) containing 0.5% KCl and blend them until the mixture is thoroughly homogenized. Transfer the extract to a conical flask, seal it with parafilm and shake it for 30 minutes at 300 rpm in a mechanical shaker. Filter the extract through Whatman No. 4 filter paper, and store the filterate at 4°C till needed for analysis. In the same way, prepare a toxin-free sample (healthy groundnut - HGN) extract, which will be used for dilution of standards as well as a negative control.

ELISA test: ICRISAT developed two types of ELISAs for the analysis of aflatoxins: (i) indirect competitive ELISA and (ii) direct competitive ELISA. Both types are heterogeneous competitive assays that involve the separation of free (un-reacted) toxin in liquid phase from the bound toxin in solid phase.

The basic principle of ELISA lies in trapping the antigen on a solid surface, or capturing the antigen with specific antibodies, and probing with specific immunoglobulins carrying an enzyme label. The enzyme, retained in the case of a positive reaction is detected by adding a suitable substrate. The enzyme converts the substrate to a product that can be easily recognized by its color. Moreover, both ELISAs were developed using alkaline phosphatase, pencillinase, horseradish peroxidase enzyme systems separately, and they do not significantly differ from each other.

Equipment: ELISA reader, incubator, orbital shaker, ELISA plate shaker, a set of micro-pipettes including 12 channel one, balance, pH meter, fume hood, vortex mix, waring blender with mini jars, ELISA plates (Nunc, Maxisorp).

Preparation of reagents

Aflatoxin B_1-BSA conjugate (AFB_1-BSA): Dissolve 1 mg AFB_1-BSA (Sigma 6655) in 1 ml sterile distilled water.

Carbonate buffer (coating buffer) Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, distilled water 1.0 L, pH of buffer should be 9.6 (No need to adjust the pH).

Phosphate buffered saline with Tween (PBS T): Na_2HP0_4 2.38 g, KH_2P0_4 0.4 g, KCl 0.4 g, NaCl 16.0 g, Tween 20: 1 mL, distilled water 2 L.

PBST-BSA: Dissolve 200 mg bovine serum albumin (BSA) (Sigma A 6793) in 100 ml PBS-T.

Substrate buffer for alkaline phosphatase system: It is preferable to use the p nitrophenyl

phosphate (pNPP) chemical in tablet form (available in 5, 15 or 20 mg tablets). Prepare 10% diethanolamine (v/v) in distilled water, adjust pH to 9.8 with concentrated HCl. Prepare 0.5 mg ml^{-1} pNPP in 10% diethanolamine, pH 9.8 (30 ml solution is required for each 15 mg tablet).

Sample analysis design: Aflatoxin standards and samples should be placed in the plate as given in Figure 1. Standards and samples should be tested in two wells. Occasionally the border wells give non-specific backgrounds, so it is advisable to avoid the border rows. In both ELISAs, the plate should be incubated at 37°C preferably on an ELISA plate shaker.

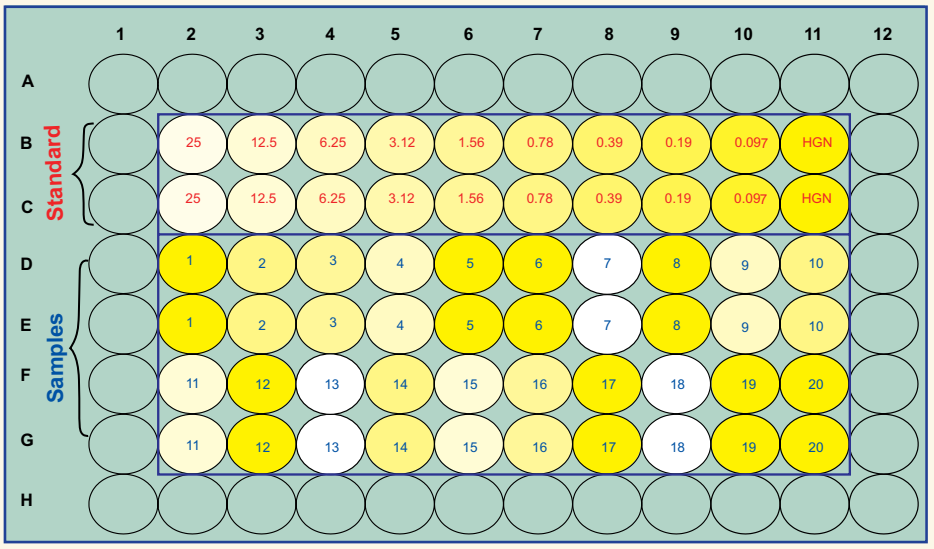
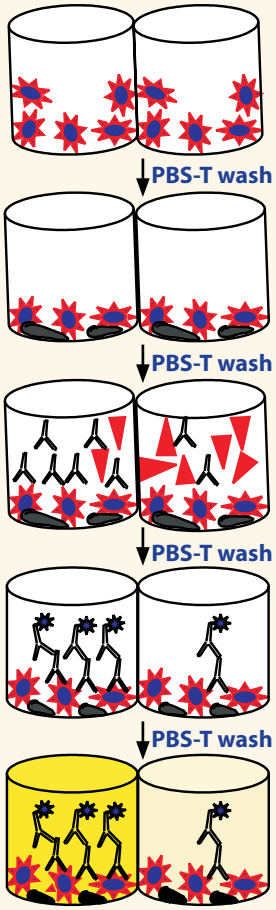


Figure1. ELISA plate design with end color development.

Indirect competitive ELISA protocol



Coating: Prepare AFB_1-BSA in carbonate buffer (100 ng/ml) and add 150 µl in each well. Incubate the plate at 37°C for 1 hour.

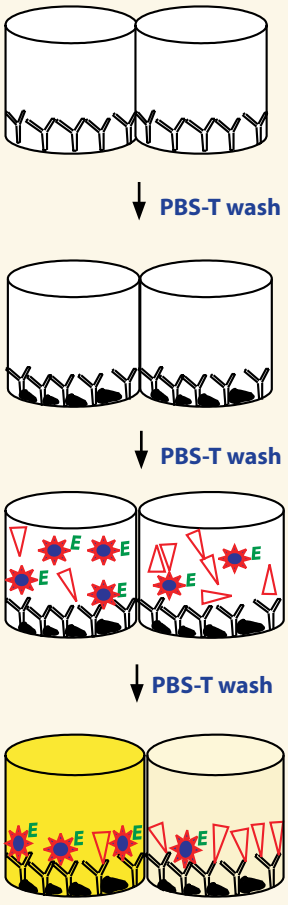
Blocking: Add PBST-BSA and incubate at 37°C for 30 minutes.

Competition: Prepare AFB_1 standards (25-0.097 ng/ml) in 10% toxin-free extract or PBST-BSA with 7% methanol and add 100 µl/well in rows B-C of the plate. Similarly add 100 µl diluted sample extract (1:10 in PBST-BSA) in rows D-E. Add 50 µl diluted antiserum (1:6000 in PBST-BSA) to all the wells and incubate at 37°C for 1 hour.

Enzyme conjugate: Add 150 µl anti-rabbit-IgG-ALP (1:4000 in PBST-BSA) to all wells and incubate at 37°C for 1 hour.

Substrate: Add pNPP prepared in 10% diethanolamine. Color develops in 20 min. Read the plate in ELISA reader at 405 nm.

Direct competitive ELISA protocol



Coating: Dilute the antiserum (1:2000) in carbonate buffer and add 150 µl in all the wells. Incubate the plate at 37°C for 1 hour.

Blocking: Add PBST-BSA and incubate at 37°C for 30 minutes.

Competition: Prepare AFB_1 standards (25-0.097 ng/ml) in 10% toxin-free extract or PBST-BSA with 7% methanol and add 100 µl/well in rows B-C of the plate. Similarly add 100 µl diluted sample extract (1:10 in PBST-BSA) in rows D-E. Add 50 µl diluted AFB_1-BSA-ALP conjugate (1:3000 in PBST-BSA) to all the wells and incubate at 37°C for 1 hour.

Substrate: Add pNPP prepared in 10% diethanolamine. Color develops in 20 minutes. Read the plate in ELISA reader at 405 nm.

AFB_1-BSA; BSA block; Antibodies; Anti-rabbit-IgG-ALP; Aflatoxin; AFB_1-BSA-Enzyme.

Calculations: Take mean ELISA plate reading values (OD) for each standard and sample. Plot a standard curve by placing AFB_1 standard concentration values on Y axis and respective OD values on X axis (Figure 2) on a semi-log graph paper. Correlate the unknown sample OD values where it touches the standard curve to determine the AFB concentration in the sample and determine the AFB content in the sample using this equation:

AFB_1 content in the sample (µg/kg) = (A*D*E)/G
A = AFB_1 conc. (ng/ml) as determined from standard curve; D = Times sample dilution with buffer; E = Extraction solvent volume in ml (eg, for 20 g sample 100 ml methanol); G = sample weight in grams

Alternatively, use an Excel spreadsheet on the computer to draw a regression curve and using regression equation values, estimate aflatoxin in each sample.

ICRISAT can provide the necessary antibodies, standards, toxin-conjugate, training and help for partners to access this technology.

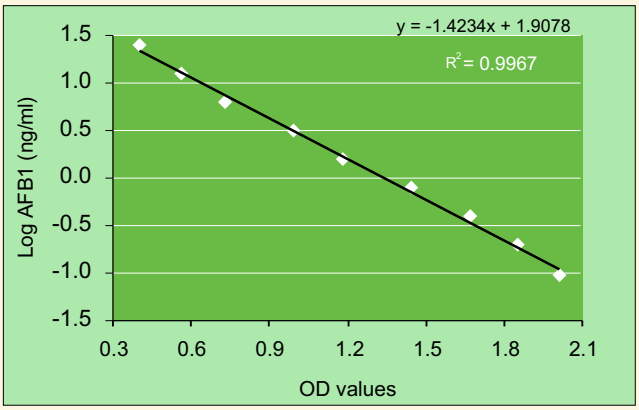


Figure 2: Regression/standard curve for aflatoxin estimation by ELISA.

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About ICRISAT

ICRISAT Science with a human face

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) conducts agricultural research for development in Asia and sub-Saharan Africa with a wide array of partners throughout the world. Over 2 billion people, of whom 644 million are the poorest of the poor, live in the semi-arid tropics, which cover 55 countries. ICRISAT (a non-profit non-political organization) and its partners help empower these poor people to overcome poverty, hunger, malnutrition and a degraded environment through better and more resilient agriculture.

ICRISAT is headquartered in Patancheru near Hyderabad, Andhra Pradesh, India, with two regional hubs and five country offices in sub-Saharan Africa.

ICRISAT is a member of the CGIAR Consortium. The CGIAR is a global research partnership for a food secure future.

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