15.2 Estimation of Aflatoxin B_1 - Elisa kit method

Intended use

The HELICA Low Matrix Aflatoxin Assay is a competitive enzyme-linked immunoassay for the quantitative detection of aflatoxins B_1, B_2, G_1 and G_2 in grains, nuts, cotton seed, cereals and all commodities which are difficult to measure due to high matrix effect such as silage and most spices.

Principle

The HELICA Low Matrix Total Aflatoxin Assay is a solid phase direct competitive enzyme immunoassay. An aflatoxin specific antibody optimized to cross react with all four subtypes of aflatoxin is coated to a polystyrene micro well. Toxins are extracted from a ground sample with either 80% methanol or 80% acetonitrile and after dilution, added to the appropriate well. If aflatoxin is present, it will bind to the coated antibody. Subsequently, aflatoxin bound to horse-radish peroxidase (HRP) is added and binds to the

antibody not already occupied by aflatoxin present in the sample or standard. After this incubation period, the contents of the wells are decanted, washed and an HRP substrate is added which develops a blue color in the presence of enzyme. The intensity of the color is directly proportional to the amount of bound conjugate and inversely proportional to the amount of aflatoxin in the standard or sample. Therefore, as the concentration of aflatoxin in the sample or standard increases, the intensity of the blue color will decrease. An acidic solution is added which changes the chromogen color from blue to yellow. The micro wells are measured optically by micro plate reader with an absorbance filter of 450 nm (Fig. 15.2). The optical densities of the samples are compared to the OD's of the kit standards and a result is determined by interpolation from the standard curve.

Fig. 15.2 Elisa reader

Reagents Provided

1 pouch: Antibody coated micro wells	- 96 wells (12 eight well strips) in a micro well holder coated with a mouse anti-aflatoxin monoclonal antibody
1 plate: Mixing wells (red)	- 96 non-coated wells (12 eight well strips) in a micro well holder.
6 vials: Aflatoxin standards	- 1.5 ml/vial of aflatoxin at the following concentrations: 0.0, 0.02, 0.05, 0.1, 0.2, and 0.4 ng/ml in aqueous solution
1 bottle: Aflatoxin HRP-conjugate	- 12 ml of aflatoxin B_1, conjugated to peroxidase in buffer with preservative
2 bottles: Assay Diluent	- 2 x 12 ml propriety assay diluent
1 bottle: Substrate Solution	- 12 ml stabilized tetramethylbenzidine (TMB)
1 bottle: Stop Solution	- 12 ml acidic solution
1 pouch: Washing Buffer	- PBS with 0.05% Tween20, bring to1 litre with distilled water and store refrigerated

Materials required

Extraction procedure

- Grinder: Sufficient to render sample to particle size of fine instant coffee.
- Collection container: Minimum 125 ml capacity
- Balance: 20 g measuring capability
- Graduated cylinder: 100 ml.
- Methanol or acetonitrile: 80 ml reagent grade per sample.
- Distilled or de-ionized water : 20 ml per sample
- Filter paper: Whatman no. 1 or equivalent filter funnel.

Assay procedure

- Pipettor with tips : 100 µl and 200 µl
- Timer
- Wash bottle
- Dilution tubes
- Absorbent paper towels
- Micro plate reader with 450 nm filter

Precautions

1. Bring all reagents to room temperature (19-27ºC) before use.
2. Store reagents at 2 to 8ºC and do not uses beyond expiration date(s). Never freeze kit components.
3. Do not return unused reagents backs into their original bottles. The assay procedure details volumes required.
4. Adhere to all time and temperature conditions stated in the procedure.
5. Never pipette reagents or samples by mouth.
6. Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with aflatoxin. Wear protective gloves and safety glasses when using this kit.
7. Dispose of all materials, containers and devices in the appropriate receptacle after use.

Extraction procedure

Note: The sample must be collected according to established sampling techniques.

Note: The ratio of sample to extraction solvent is 1:5 (w/v).

1. Grind a representative sample to the particle size of the fine instant coffee (50% passes through a 20 mesh screen).
2. Prepare extraction solvent (80% methanol of 80% acetonitrile) by adding 20 ml of distilled or de-ionized water to 80 ml methanol or acetonitrile for each sample to be tested.
3. Weigh out a 20 g ground portion of the sample and add 100 ml of the extraction solvent.
4. Mix by shaking in a sealed container or in a blender for a minimum of 2 minutes.
5. Allow the particulate matter to settle, then filter 5-10 ml of the extract through a Whatman no. 1 filter paper (or equivalent) and collect the filtrate to be tested. Alternatively, the sample may be centrifuged to clarify.
6. Dilute an aliquot of the extract 1 in 10 with reconstituted wash buffer.
7. The sample is now ready. The standards required no pre-dilution before use.

Assay procedure

1. Bring all the reagents to room temperature before use. Reconstitute the PBS-Tween packet by washing out the contents with a gentle stream of distilled water into a 1 litre container. Make to 1 litre with distilled water and store refrigerated when not in use.
2. Place one mixing well in a micro well holder for each standard and sample to be tested. Place an equal number of antibody coated micro titre wells in another micro well holder.
3. Dispense 200 µl of the assay diluent into each mixing well.

4. Using a new pipette tip for each add, 100 µl of each standard and prepared sample to appropriate mixing well containing diluent. Mix by priming pipettor at least 3 times.

5. Using a new pipette tip for each, transfer 100 µl of contents from each mixing well to a corresponding. Antibody coated micro titre well. Incubate at room temperature for 30 minutes. The mixing wells contain enough solution to run each standard and/ or sample in duplicate if so desired.

6. Decant the contents from micro wells into a discard basin. Wash the micro wells by filling each with PBS-Tween wash butter, then decanting the wash into a discard basin. Repeat wash for a total of 3 washes.

7. Tap the micro wells (face down) on a layer of absorbent towels to remove residual water.

8. Add 100 µl of conjugate to each antibody coated well and incubate at ambient temperature for 30 minutes.

9. Repeat steps 6 and 7.

10. Measure the required volume of substrate reagent (1 ml/strip or 120 µl well) and place in a separate container. Add µl to each micro well; incubate at room temperature for 10 minutes.

11. Measure the required volume of stop solution (1ml/strip or 120µl /well) and place in a separate container. Add 100 µl in the same sequence and at the same place as the substrate was added.

12. Read the optical density (OD) of each micro well with a micro titre plate reader using a 450 nm filter. Record the optical density (OD) of each micro well.

Note: This assay may be used for detecting aflatoxin in potable water. In this case 100 µl of the sample should be used without pre-dilution with wash buffer.

Interpretation of results

Construct a dose-response curve using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0.0) standard against the aflatoxin content of the standard. Unknowns are measured by interpolation from the standard curve.

The information contained on the label of a standard vial refers to the contents of that vial. However, the sample has been diluted at a 5:1 ratio by extraction solvent and also 10:1 in wash buffer and so the level of aflatoxin shown by the standard must be multiplied by 50 in order to indicate the ng per gram (ppb) of the commodity as follows:

Standard (ng/ml)	**Commodity (ppb)**
0.00	0.0
0.02	1.0
0.05	2.5
0.10	5.0
0.20	10.0
0.40	20.0

The sample dilution results in a standard curve from 1 to 20 ppb. If a sample contains aflatoxin at greater than the highest standard, it should be diluted appropriately in 80% extraction solvent and retested. The extra dilution step should be taken into consideration when expressing the final result.

In the case of potable water there is no pre-dilution so it is measured with a sensitivity equal to the lowest standard which is twenty parts per trillion.

Methanol extract	
ppb	% Recovery
5.1	100
2.5	49
2.7	53
2.7	53
2.9	57