



QUANTITATIVE ASSAY FOR – AFLATOXIN M₁ IN URINE

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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AFLATOXINS

Aflatoxins are toxic and carcinogenic secondary metabolites of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Chronic consumption of aflatoxins has been associated with primary liver cancer¹ and in sufficiently high doses, acute liver failure and death. While their presence in food commodities destined for animals or humans is strictly monitored, accidents and mistakes still occur, particularly in areas of the world with limited technological infrastructure². Lethal outbreaks of aflatoxin poisoning (aflatoxicosis) have been reported recently in Africa⁴ and in the West there has been an outbreak of aflatoxicosis in dogs, linked to contaminated dog food⁵. Aflatoxins have also been considered as a possible agent of bioterrorism^{6,7}. After ingestion of aflatoxin B₁, the most potent and prevalent of the aflatoxins, a portion is converted to the less toxic metabolite aflatoxin M₁ which is excreted in the urine. It has been shown that there is a good correlation between ingested aflatoxin B₁ and the appearance of aflatoxin M₁ in urine. The conversion rate is estimated to be about 2%^{8,9}.

INTENDED USE

The Helica Aflatoxin M₁ in urine assay is an enzyme-linked immunosorbent assay for the quantitative determination of aflatoxin in urine at levels which should be helpful in monitoring populations at risk for acute or chronic aflatoxicosis. The assay has not yet been approved by FDA for diagnostic purposes.

ASSAY PRINCIPLE

The Helica Aflatoxin M₁ in urine assay is a direct enzyme-linked immunosorbent assay in which an antibody with high affinity for aflatoxin M₁ is coated onto polystyrene microwells. After an initial dilution with distilled water, the urine sample is mixed with assay buffer and added to the well. If aflatoxin M₁ is present in the urine 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 the sample. Therefore, as the concentration of aflatoxin in the sample or standard increases, the intensity of the blue color will decrease. An acidic stop solution is added which changes the chromogen color from blue to yellow. The microwells are measured optically by a microplate reader with an absorbance filter of 450 nm (OD₄₅₀). 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.

Reagents Provided

1 pouch: Antibody coated microwells	96 wells (12 eight well strips) in a microwell holder coated with a mouse anti-aflatoxin monoclonal antibody
1 plate: mixing wells (red)	96 non-coated wells (12 eight well strips) in a microwell holder
6 vials: Aflatoxin Standards	1.5 mL/vial of aflatoxin M ₁ at the following concentrations: 0.0, 0.15, 0.40, 0.80, 1.50, and 4.00 ng/mL in stabilized normal human urine
1 bottle: Aflatoxin HRP-conjugate	12 mL of aflatoxin conjugated to peroxidase in buffer with preservative
2 bottles: Assay Buffer	2 × 12 mL propriety assay buffer
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 to 1 liter with distilled water and store refrigerated.

MATERIALS REQUIRED BUT NOT PROVIDED

Variable single and multichannel pipettors with appropriate tips
Dilution tubes
Distilled or deionized water

Wash bottle
Timer
Absorbent paper towels
Microplate reader with 450nm filter

PRECAUTIONS

1. Bring all reagents to room temperature (19° - 27°C) before use.
2. Store reagents at 2° to 8°C, and do not use beyond expiration date(s). Never freeze kit components.
3. Do not return unused reagents back 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. The Stop Solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.
7. 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.
8. Dispose of all materials, containers and devices in the appropriate receptacle after use.
9. HRP-labeled conjugate and TMB-substrate are photosensitive and are packaged in a protective opaque bottle. Store in the dark and return to storage after use.

KIT SAFETY AND WASTE DISPOSAL INSTRUCTIONS

Mycotoxins (aflatoxins, trichothecenes, and others) are well known carcinogens in humans and are thus considered highly toxic. Do not dispose of these materials down the drain. Please note that there is a potential for mycotoxin contamination in or on any of the kit components provided. It is strongly advised that gloves, a lab coat, and safety glasses be worn at all times while handling mycotoxin kits and their respective components.

1. Before conducting the assay, prepare a waste container to act as a receptacle for your kit waste. Eject contaminated pipette tips and all other related materials into this container.
2. Once the assay is completed, the container should be treated with a sufficient amount of 5-6% sodium hypochlorite (NaOCl) to saturate the contents of the container (approximately 1/10th the volume of the container). 5-6% NaOCl will denature the mycotoxins and neutralize

the waste, making it safe for disposal. Invert the container several times to thoroughly coat all waste.

3. In the case of an accidental toxin spill, treat the spill surface with 5-6% NaOCl for a minimum of 10 minutes followed by 5% aqueous acetone. Wipe dry with absorbent paper towels.

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-Liter container. Q.S. to 1 Liter with distilled water and store refrigerated when not in use.
2. Remove any debris or precipitate from the urine sample by filtration or centrifugation.
3. Dilute an aliquot of both the urine standards and samples 1:20 with distilled water e.g. 50 μ L plus 950 μ L distilled water.
4. Place one mixing well in a microwell holder for each Standard and Sample to be tested. Place an equal number of Antibody Coated Microtiter Wells in another microwell holder.
5. Dispense 200 μ L of the assay buffer into each mixing well.
6. Using a new pipette tip for each, add 100 μ L of each diluted standard and sample to the appropriate mixing well containing the assay buffer. Mix by priming pipettor at least 3 times.
7. Using a new pipette tip for each, transfer 100 μ L of contents from each mixing well to a corresponding Antibody Coated Microtiter Well. Incubate at room temperature for 1 hour. The mixing wells contain enough solution to run each standard and/or sample in duplicate if so desired.
8. Decant the contents from microwells into a discard basin. Wash the microwells by filling each with PBS-Tween wash buffer, then decanting the wash into a discard basin. Repeat wash for a total of 3 washes.
9. Tap the microwells (face down) on a layer of absorbent towels to remove residual water.
10. Add 100 μ L of conjugate to each antibody coated well and incubate at ambient temperature for 15 minutes.
11. Repeat step 8 for washing procedure.
12. Measure the required volume of Substrate Reagent (1 mL/strip or 120 μ L/well) and place in a separate container. Add 100 μ L to each microwell. Incubate covered from light at room temperature for 15 minutes. Cover to avoid direct light.
13. Measure the required volume of Stop Solution (1 mL/strip or 120 μ L/well) and place in a separate container. Add 100 μ L in the same sequence and at the same pace as the Substrate was added.
14. Read the optical density (OD) of each microwell with a microtiter plate reader using a 450 nm filter within 15 minutes of adding stop solution. Record the optical density (OD) of each microwell.

Note: If more than two strips are used in an assay, the use of a multichannel pipette is recommended for all additions in order to mitigate "beginning to end" variation.

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.

If a sample gives an OD less than the highest standard it should be further diluted in distilled water and re-tested. The extra dilution should be taken into account when calculating the result. Due to the nature of inhibition immunoassays, values derived by extrapolation outside of the measured highest and lowest standards are likely to be erroneous.

Assay Characteristics

Eighteen early morning mid-stream urine samples were collected from male and female subjects ranging in age from 16 – 60 years. The population from which 18 samples were taken were at very low risk for aflatoxin ingestion.

The samples were measured against an arbitrarily chosen urine standard. Mean binding for the 18 samples was 97.5% +/- 1.4% of the zero standard.

Each specimen was spiked with either 0.5 or 2.0 ng/mL aflatoxin M1 in three separate experiments and the recovery measured. Mean recovery is given below:

SPIKE	0.544 ng/mL	1.98 ng/mL
MEAN RECOVERY	96.40%	96.50%
CV	9.40%	9.80%
RECOVERY RANGE	78 - 111%	73 - 109%
	N = 18	N = 18

SELECTED REFERENCES

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