

## Product Information

### High Sensitivity Aflatoxin M<sub>1</sub> ELISA Kit for milk, milk powder, and cheese

Catalog Number **SE120004**  
Storage Temperature 2–8 °C

## TECHNICAL BULLETIN

### Product Description

Aflatoxins are toxic metabolites that different molds like *Aspergillus flavus* and *Aspergillus parasiticus* produce. Aflatoxins are carcinogenic and can be present as contaminants in grains, nuts, cottonseed, and other materials, e.g. crops, associated with animal feed or human food. In particular, four aflatoxin sub-types, B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> are known to occur as crop contaminants. Aflatoxin B<sub>1</sub> is the most toxic and frequently detected aflatoxin subtype.<sup>1,2</sup>

When animals such as cows consume feed that is contaminated with aflatoxin B<sub>1</sub>, the aflatoxin B<sub>1</sub> is metabolically converted to aflatoxin M<sub>1</sub>. Aflatoxin M<sub>1</sub> is subsequently secreted in the milk of lactating cows. Aflatoxin M<sub>1</sub> has stability against standard milk processing methods such as pasteurization. If aflatoxin M<sub>1</sub> remains present in raw milk, it may thus persist in the final products for human consumption.<sup>3</sup>

The High Sensitivity Aflatoxin M<sub>1</sub> ELISA Kit is a solid-phase competitive enzyme immunoassay. An antibody with a high affinity for aflatoxin M<sub>1</sub> is coated onto polystyrene microwells. Standard or sample is added to the appropriate well. If Aflatoxin M<sub>1</sub> is present it will bind to the coated antibody. Subsequently, aflatoxin bound to horseradish peroxidase (HRP) is added and binds to the antibody not already occupied by aflatoxin M<sub>1</sub> present in the sample or standard. After this incubation period, the contents of the wells are decanted and washed. 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 M<sub>1</sub> in the standard or sample. Therefore, as the concentration of aflatoxin M<sub>1</sub> 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 (OD<sub>450</sub>). The optical densities (OD) of the samples are compared to the OD's of the kit standards, and an interpolated result is determined.

The High Sensitivity Aflatoxin M<sub>1</sub> Assay is intended for the quantitative detection of Aflatoxin M<sub>1</sub> in milk, reconstituted milk powders, and cheese.

### Components

1. Aflatoxin M<sub>1</sub> Antibody-Coated Microplate (961AFLM01M): 96 wells (12 × 8) well holder coated with a mouse anti-aflatoxin monoclonal antibody.
2. Aflatoxin M<sub>1</sub> Standards (963S1AFLM01M): 6 vials, 3.0 mL/vial of Aflatoxin M<sub>1</sub> at the following concentrations: 0.0, 5.0, 10.0, 25.0, 50.0, and 100.0 pg/mL (ppt) in stabilized skim milk.
3. Aflatoxin M<sub>1</sub> HRP-Conjugate (964MAFLM01, Green Cap), 12 mL of HRP conjugated aflatoxin in buffered solution with preservative.
4. Aflatoxin M<sub>1</sub>-Free Skim Milk (927MK001, White Cap): 12 mL of skim milk for preparation of cheese extract
5. TMB Substrate (916T001, Blue Cap): 12 mL of stabilized urea peroxide and 3,3',5,5'-tetramethylbenzidine (TMB).
6. Stop Solution (946P001, Red Cap): 12 mL of Acidic Solution.
7. PBST Wash Buffer Powder (915X001): 1 packet of PBS with 0.05% TWEEN® 20. Bring to 1 liter with distilled water and store refrigerated.

### Reagents and Equipment Required but Not Provided.

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 100-200 µL volumes
3. Distilled or deionized water
4. Absorbent paper towels
5. Graph paper or computer and software for ELISA data analysis
6. Glass tubes
7. Timer

### Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices. Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with aflatoxin M<sub>1</sub>. Wear protective gloves and safety glasses when using this kit.

### Storage/Stability

Store reagents at 2–8 °C, and do not use beyond expiration date(s). Never freeze the kit components.

Bring all reagents to room temperature (19–25 °C) before use.

HRP-labeled conjugate and TMB Substrate are photosensitive and are packaged in protective opaque bottles. Store in the dark and return to storage after use.

Do not return unused reagents back to their original bottles.

Before doing the assay, prepare a waste container as a receptacle for kit waste. Eject contaminated pipette tips and all other related materials into this container. Following completion of the assay, treat the container with sufficient 5-6% sodium hypochlorite (NaOCl) to saturate the container's contents, about 1/10<sup>th</sup> the volume of the container. 5-6% NaOCl will denature the mycotoxins and neutralize the waste, which renders the waste safe for disposal. Invert the container several times to coat all waste thoroughly.

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

### Preparation Instructions

#### Extraction Procedure / Sample Preparation

##### *Raw Milk*

1. The standards are offered in homogenized skim milk. Thus skim milk (milk plasma) is the appropriate sample for the assay.
2. An aliquot of unprocessed raw fatty milk should be placed at refrigerated temperature overnight to allow the fat globules to rise to the surface, in a natural "creaming" effect. Centrifugation at this point is not necessary.

3. If the sample is at ambient temperature or has been mixed in transit, place an aliquot at refrigerated temperature for 1-2 hours. Centrifuge at 2,000 × *g* for 5 minutes to induce separation of the upper fatty layer.
4. Remove the upper fatty layer by aspiration. Use the lower plasma in the assay.

##### *Homogenized Milk*

1. Homogenized skim milk should be used directly in the assay.
2. Due to the stabilization of the fat globules induced by the homogenizing process, they are difficult to eliminate, even by high-speed centrifugation, in order to create a plasma from homogenized fatty milk. Therefore, use homogenized fatty milk directly in the assay.

##### *Milk Powder*

Reconstitute milk powder samples according to the manufacturer's instructions.

- If the reconstituted milk powder has high fat content, e.g. like raw milk, then treat the reconstituted milk powder using the "Raw Milk" procedure.
- If the reconstituted milk powder is more like skim milk, then treat the reconstituted milk powder in the manner of "Homogenized Milk" samples.

##### *Cheese*

1. One gram of finely grated or otherwise macerated cheese is mixed with 5 mL of absolute methanol in a capped tube, and mixed for 5 minutes. The tube is clarified by centrifugation (5,000 × *g* for 5 minutes) and the supernatant removed.
2. 0.5 mL of this supernatant is transferred to a glass tube. The methanol is evaporated by a stream of air. (Better recovery is obtained with nitrogen gas.) This procedure results in the deposition of a semisolid viscous material on the inside of the tube. Add 0.5 mL of the provided blank skim milk to the tube and vortex vigorously for 1 minute. Allow the tube to stand for a further 5 minutes. Use 2 × 200 μL of this milk extract in the assay.

### Procedure

1. Reconstitute the PBST buffer into 1 L of distilled water. The remaining content packets may be washed out with a gentle stream of distilled water. Store the reconstituted PBST buffer refrigerated, when not in use.
2. Remove and place the required number of wells for the number of standards and samples to be tested in a microwell holder.
3. Return unused wells to the pouch and re-seal with tape to avoid moisture entry.
4. Using a fresh pipette tip for each, dispense 200  $\mu$ L aliquots of standards and samples into the appropriate wells in duplicate.
5. Cover the plate with sealing tape to avoid evaporation and protect from excess UV light.
6. Incubate at ambient temperature (19–25 °C) for 2 hours.
7. Discard the contents of the wells into an appropriate receptacle. Wash the wells with the PBST wash buffer solution, either from a wash bottle or a multi-channel pipette. Immediately discard the washings into an appropriate receptacle. Repeat for a total of three washings. Tap the wells face down on a layer of absorbent paper to remove residual wash buffer.
8. Add 100  $\mu$ L of the HRP-Conjugate to each well.
9. Re-seal the plate and incubate at ambient temperature for 15 minutes.
10. Repeat step 7.
11. Add 100  $\mu$ L of enzyme substrate (TMB) to each well and incubate for 15 minutes. Cover to avoid direct light.
12. Stop the reaction by adding 100  $\mu$ L of Stop Solution. The blue color should change to yellow.
13. Read and record the optical density (OD) of each microwell with a microwell plate reader using a 450 nm filter.

### Alternative Incubation Procedure

- The first incubation period (step 6 in the Procedure), with the samples and the standards, may alternatively be done overnight at refrigerated temperature (4-8 °C).
- This can potentially result in improved inhibition of zero binding for samples of  $\geq 5$  ppt (5 ng/L).
- If this alternative refrigerated temperature incubation is chosen, the subsequent incubation steps with HRP-conjugate (step 9 in the Procedure) and TMB (step 11 in the Procedure) should still be performed at ambient temperature.

### 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 standard against the aflatoxin M<sub>1</sub> content of the standard. Unknowns are measured by interpolation from the standard curve.

The mean values of the absorbance values obtained for the standards and for the samples are divided by the absorbance value of the zero standard, and then multiplied by 100. The zero standard thus is made equal to 100%. The absorbance values of other standards and samples are quoted in percentages of this value.

To obtain the aflatoxin M<sub>1</sub> concentration in ng/L actually present in a sample, the concentration read from the calibration curve must be further multiplied by the corresponding dilution factor. This is 1 for milk samples and 5 for cheese samples.

#### Reproducibility

Intra-Assay: CV <5%

Inter-Assay: CV <8%

#### Recovery

Recovery was determined after spiking aflatoxin M<sub>1</sub> into milk and cheese samples. Sample mean recovery data are as follows:

Sample Type (spiked aflatoxin M <sub>1</sub> amount)	% Recovery
Skim Milk (50 ppt)	100
1% Fat Homogenized Milk (50 ppt)	93
Full Fat Homogenized Milk (50 ppt)	92
Parmesan cheese (finely grated; 10 pg)	60.5

#### Limit of Detection (LOD) and Sensitivity

Limit of detection (LOD) is defined as 2 standard deviations below the mean OD of multiple determinations of zero binding. This kit has been tested to have a sensitivity of 2 ppt.

#### **Specificity**

This assay will cross-react with aflatoxin analogs as follows: B<sub>1</sub>: 100%, B<sub>2</sub>: 77%, G<sub>1</sub>: 64%, G<sub>2</sub>: 25%.

**References**

1. Klich, M.A., Environmental and developmental factors influencing by *Aspergillus flavus* and *Aspergillus parasiticus*. *Mycoscience*, **48(2)**, 71-80 (2007).
2. Williams, J.H. *et al.*, Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.*, **80(5)**, 1106-1122 (2004).
3. Ismail, A. *et al.*, Aflatoxin M1: Prevalence and decontamination strategies in milk and milk products. *Crit. Rev. Microbiol.*, **42(3)**, 418-427 (2016).

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