Medical & Drug ● IVD & POCT ● Food & Safety

Aflatoxin B1 ELISA Test Kit Manual

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➢ GENERAL INFORMATION

Product Description

REAGEN® Aflatoxin B1 ELISA Test Kit is a competitive enzyme immunoassay for the quantitative analysis of Aflatoxin B1 in cereals, feed, milk, milk powder,enzyme,cookies, soy sauce, vinegar, oils, peanuts .The unique features of the kit are:

- High recovery (75-125%),rapid(10-40minutes), cost-effective extraction methods.
- High sensitivity (0.02 ng/g or ppb).
- High reproducibility.
- A quick ELISA assay (only 1 hour regardless of number of samples).

Procedure Overview

REAGEN® The method is based on a competitive colorimetric ELISA assay. The Aflatoxin B1-BSA has been coated in the plate wells. During the analysis, sample and Aflatoxin B1 antibody (Antibody#1) and HRP-Conjugate (HRP-Conjugated Antibody #2) are added to the wells for incubation. If the Aflatoxin B1 residue is present in the sample, it will compete with the Aflatoxin B1 on the plate wells for the Aflatoxin B1 antibody, The secondary antibody, tagged with a peroxidase enzyme, targets the primary antibody that is complexed to the drug coated on the plate wells. The resulting color intensity, after addition of the HRP substrate (TMB), has an inverse relationship with the Aflatoxin B1 residue concentration in the sample.

Kit Contents, Storage and Shelf Life

REAGEN® Aflatoxin B1 ELISA Test Kit has the Aflatoxin B1acity for 96 determinations or testing of 42 samples in duplicate (assuming 12 wells for standards). Return any unused microwells to the foil bag and reseal them with the desiccant provided in the original package. Store the kit at 2-8℃ *. The shelf life is 12 months when the kit is properly stored.

Kit Contents	Amount	Storage
Aflatoxin B1-BSA-Coated Plate	1x 96-well Plate (8 wells x 12 strips)	2-8°C
Aflatoxin B1 Standards: Negative control (white cap tube) 0.02ng/mL (yellow cap tube) 0.06 ng/mL (orange cap tube) 0.2 ng/mL (pink cap tube) 0.6 ng/mL (purple cap tube) 1.5 ng/mL (blue cap tube)	 1.0 mL 1.0.mL 1.0 mL 1.0 mL 1.0 mL 1.0 mL	
Antibody #1	6 mL	2-8°C
HRP-Conjugated Antibody #2	6 mL	
20X Wash Solution	30 mL	
10 X Sample Dilution Buffer	25mL	

Stop Buffer	12 mL	
TMB Substrate	12 mL	

Sensitivity (Detection Limit)

Sample Type	**Detection Limit** (ng/g or ppb)
Milk/Milk Powder，Enzyme	0.04/0.1
Cereal/Feed/Seed/Cookies	1.0
Vegatable/bean/peanut/oils	0.2
Chili powder/Soy sauce/vinegar	0.5

Specificity (Cross-Reactivity)

Analytes	**Cross-Reactivity** (%)
Aflatoxin B1	100.0
Aflatoxin B2	49
Aflatoxin G1	37
Aflatoxin G2	8

Required Materials Not Provided With the Kit

- Microtiter plate reader (450 nm)
- Incubator
- Tissue Mixer (e.g. Omni TissueMaster Homogenizer)
- Vortex mixer (e.g. Gneie Vortex mixer from VWR)
- 10, 20, 100 and 1000 µL pipettes
- Multi-channel pipette: 50-300 µL (Optional)
- Methanol

Warnings and Precautions

- The standards contain Aflatoxin B1 . Handle with particular care.
- Do not use the kit past the expiration date.
- Do not intermix reagents from different kits or lots except for components with the same part No's within their expiration dates.
- Try to maintain a laboratory temperature of 20–25°C (68°–77°F). Avoid running assays under or near air vents, as this may cause excessive cooling, heating and/or evaporation. Also, do not run assays in direct sunlight, as this may cause excessive heat and evaporation. Cold bench tops should be avoided by placing several layers of paper towel or some other insulation material under the assay plates during incubation.
- Make sure you are using only distilled or deionized water since water quality is very important.
- When pipetting samples or reagents into an empty microtiter plate, place the pipette tips in the

lower corner of the well, making contact with the plastic.

- Incubations of assay plates should be timed as precisely as possible. Be consistent when adding standards to the assay plate. Add your standards first and then your samples.
- Add standards to plate only in the order from low concentration to high concentration as this will minimize the risk of compromising the standard curve.
- Always refrigerate plates in sealed bags with a desiccant to maintain stability. Prevent condensation from forming on plates by allowing them equilibrate to room temperature (20 – 25℃ / 68 – 77°F) while in the packaging.

➢ SAMPLE PREPARATION

Be sure samples are properly stored. In general, samples should be refrigerated at 2-4°C for no more than 1-2 days. Freeze samples to a minimum of -20°C if they need to be stored for a longer period. Frozen samples can be thawed at room temps (20 – 25℃ / 68 – 77°F) or in a refrigerator before use.

1. **Preparation of 1X Wash Solution:**
   Mix 1 volume of 20X Wash Buffer concentrate with 19 volumes of distilled water.

2. **Preparation of 70% Methanol:**
   Mix 7 volumes of 100% Methanol with 3 volumes of distilled water.

3. **Preparation of Solution A:**
   Mix 3 volumes of 100% Methanol with 7 volumes of 1X Wash Solution.

4. **Preparation of 1X Sample Dilution Buffer:**
   Mix 1 volume of 10X Sample Dilution Buffer with 9 volumes of distilled water.

5. **Preparation of 30% Methanol:**
   Mix 3 volumes of 100% Methanol with 7 volumes of distilled water.

When preparing solutions, vortex vigorously for at least one minute in an air-tight container.

Milk

1) To 1 mL of milk, add 1 mL of **Solution A**.
2) Vortex vigorously for 1 minute.
3) Centrifuge for 5 minutes at 4,000 x *g* at room temperature (*20 – 25 ℃* / 68 – *77*°F).
4) Use 50 µL of the sample per well for the assay, avoiding any fat that may have formed on top of the sample.

Note: *Dilution factor = 2*

Milk Powder/ Enzyme

1) To 1 g of milk powder in a vial, add 2mL of **70% Methanol**. Vortex vigorously for 5 minutes to obtain a homogeneous suspension.
2) Centrifuge the samples for 5 minutes at 4,000 x *g* at room temperature (*20 – 25 ℃* / 68 – *77*°F).
3) Dilute 200 µL of the obtained supernatant with 300 µL of distilled water.
   Vortex for 1 minute.
4) Use 50 µL of the diluted sample per well in the test.

Note: *Dilution factor = 5*

Cereal, Feed, Seed ,Cookies

1) Grind and mix a representative sample (according to accepted sampling techniques).

2) Weigh out 1 g of the ground sample and place into a suitable container.
3) Add 10 mL of **70% Methanol** and vortex vigorously for 3 minutes manually or 10 minutes using a multi-vortexer.
4) Centrifuge the samples for 5 minutes at 4,000 x *g* at room temperature (*20 – 25 ℃* / 68 – *77*°F). Dilute 200 µL of the obtained supernatant with 800 µL of **1X Sample Dilution Buffer**. Vortex for 1 minute.
5) Use 50 µL of the diluted sample per well in the test.
   Note: *Dilution Factor = 50*

**The sample size may be increased if required, but the volume of methanol/water must be changed accordingly, for example, if a 10 g sample is to be used, 100 mL of 70% methanol should be used. If the Aflatoxin B1 concentration is expected to be high, the sample can be further diluted with 14% Methanol in 1XSample Dilution Buffer.*

Vegatable/bean/peanut/oils

1) Weign out 1g of the oil sample, add 5 ml of **30% Methanol**
2) Vortex vigo- rously for 2 minutes t maximum speed manually, then add 3 ml of n-hexane and Vortex vigo- rously for 2 minutes t maximum speed manually.
3) Centrifuge the samples for 5 minutes at 4,000 x *g* at room temperature (*20 – 25 ℃* / 68 – *77*°F).
4) Dilute 500 µL of the lower aqueous layer with 500 µL of **1X Sample Dilution Buffer**. Vortex for 1 minute.
5) Use 50 µL of the diluted sample per well in the test.
   Note: *Dilution factor: 10*

Soy sauce/vinegar

1) Weigh out 1mL of the ground sample and place into a suitable container.
2) Add 4mL of **70% Methanol** and vortex vigorously for 3 minutes manually or 10 minutes using a multi-vortexer.
3) Centrifuge the samples for 5 minutes at 4,000 x *g* at room temperature (*20 – 25 ℃* / 68 – *77*°F). Dilute 200 µL of the obtained supernatant with 800 µL of distilled water. Vortex for 1 minute,test the pH, the acid is adjusted to 6.8-7.2 with 1M sodium hydroxide; the alkaline is adjusted to 6.8-7.2 with 1M hydrochloric acid.
4) Use 50 µL of the diluted sample per well in the test.
   Note: *Dilution Factor = 25*

Chili powder

1) Weigh out 1mL of the ground sample and place into a suitable container.
2) Add 5mL of **70% Methanol** and vortex vigorously for 3 minutes manually or 10 minutes using a multi-vortexer.
3) Centrifuge the samples for 5 minutes at 4,000 x *g* at room temperature (*20 – 25 ℃* / 68 – *77*°F). Dilute 200 µL of the obtained supernatant with 800 µL of **1X Sample Dilution Buffer**. Vortex for 1 minute.
4) Use 50 µL of the diluted sample per well in the test.
   Note: *Dilution Factor = 25*

➢ AFLATOXIN B1 ELISA TEST KIT PROTOCOL

Reagent Preparation

IMPORTANT: All reagents should be brought up to room temperature before use (1 – 2 hours at 20 – 25℃ / 68 – 77°F); Make sure you read "Warnings and Precautions" section on page 3. Solutions should be prepared just prior to ELISA test. All reagents should be mixed by gently inverting or swirling prior to use. Prepare volumes that are needed for the number of wells being run. Do not return the reagents to the original stock tubes/bottles. Using disposable reservoirs when handling reagents can minimize the risk of contamination and is recommended.

ELISA Testing Protocol

Label the individual strips that will be used and aliquot reagents as the following example:

Component	Volume per Reaction	24 Reactions
Antibody #1	50 µL	1.2 mL
HRP-Conjugated Antibody #2	50 µL	1.2 mL
1X Wash Solution	1.0 mL	24mL
Stop Buffer	100 µL	2.4 mL
TMB Substrate	100 µL	2.4 mL

1. Add 50µL of each Aflatoxin B1 Standards in duplicate into different wells (✌ Add standards to plate only in the order from low concentration to high concentration).
2. Add 50µL of each sample in duplicate into different sample wells.
3. Add 50 µL HRP-Conjugated Antibody #2 to each well .
4. Add 50 µL Antibody #1 to each well and mix well by gently rocking the plate manually for 1 minute.
5. Incubate the plate for 15 minutes at room temperature (20 – 25℃ / 68 – 77°F).
6. Wash the plate 4 times with 250µL of 1X Wash Solution.After the last wash, invert the plate and gently tap the plate dry on paper towels(Perform the next step immediately after plate washings.Do not allow the plate to air dry between working steps).
7. Add 100µL of TMB substrate. Time the reaction immediately after adding the substrate. Mix the solution by gently rocking the plate manually for 1 minute while incubating. (Do not put any substrate back to the original container to avoid any potential contamination. Any substrate solution exhibiting coloration is indicative of deterioration and should be discarded. Covering the microtiter plate while incubating is recommended).
8. After incubation for 10 minutes in room temperature, add 100 µL of Stop Buffer to stop the enzyme reaction.
9. Read the plate as soon as possible following the addition of Stop Buffer on a plate reader with 450 nm wavelength (Before reading, use a lint-free wipe on the bottom of the plate to ensure no moisture or fingerprints interfere with the readings).

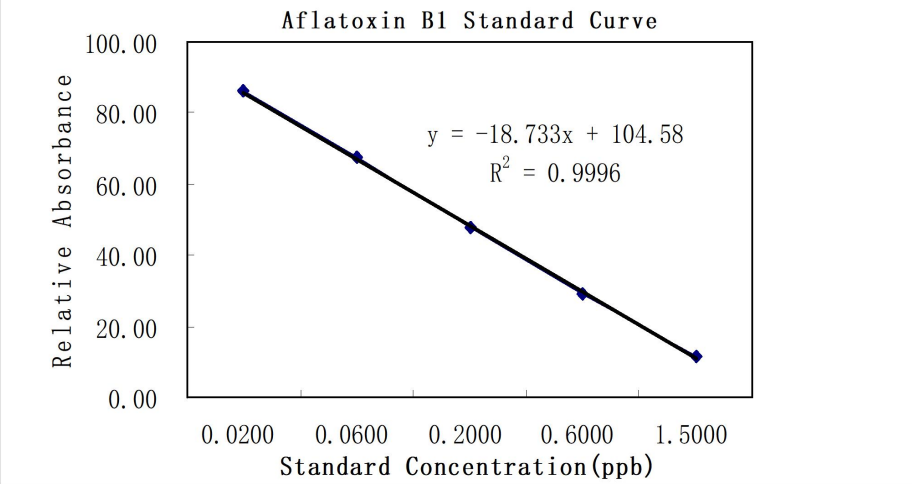
Aflatoxin B1 Concentration Calculations

A standard curve can be constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its concentration in ng/mL on a logarithmic curve.

$$\text{Relative absorbance (\%)} = \frac{\text{absorbance standard (or sample) x 100}}{\text{absorbance zero standard}}$$

When use computing software, recommends Log/Log standard curves for each sample to determine the corresponding concentration of the tested drug in ng/mL.

The following figure is a typical Aflatoxin B1 standard curve



➢ TROUBLESHOOTING

No Color Development or No Signals with Standards

Possible Causes	Recommended Action
Reagents were used in the wrong order or a step was skipped.	Follow the protocol carefully and repeat the assay.
Wrong antibodies were used or antibodies have deteriorated.	Make sure that the antibodies used are the ones that came with the kit. All antibodies are kit- and lot-specific.
TMB substrate has deteriorated.	Use a new set of REAGEN TMB substrate.

Low Optical Density (OD) Readings

Possible Causes	Recommended Action
Reagents were expired or mixed with a different lot number.	Verify the expiration dates and lot numbers.
Wash solution was prepared incorrectly.	Use the wash solution for the kit and that make sure it is prepared correctly.
Too many wash cycles were used.	Make sure to use the number of washes per the protocol instruction.
Incubation times were too short.	Time each plate separately to ensure accurate incubation times, follow protocol.
Lab temperature was too low.	Maintain the lab room temperature within 20°–25°C (68°–77°F). Do not run assays under air conditioning vents or near cold windows.
Reagents and plates were too cold.	Make sure plates and reagents are brought up to room temperature. Keep the kit components out of the kit box for at least 1 hour before starting the assay.
Reader was at wrong wavelength, or reader was malfunctioning.	Make sure the wavelength is 450 nm for the assay and read the plate again. Verify reader calibration and lamp alignment.
Excessive kit stress has occurred.	Check records to see how many times the kit has cycled from the refrigerator. Check to see if the kit was left at extreme temperatures for too long.
Assay plates were compromised.	Always refrigerate plates in sealed bags with a desiccant to maintain stability. Prevent condensation from forming on plates by allowing them equilibrate to room temperature (20 – 25°C / 68 – 77°F) while in the packaging.

High Background or High Optical Density (OD) Readings

Possible Causes	Recommended Action
Poor quality water was used in wash solution.	If water quality is questionable, try substituting an alternate distilled water source to prepare the wash solution.
Substrate solution has deteriorated.	Make sure the substrate is colorless prior to addition to the plate.
There was insufficient washing or poor washer performance.	Use the number of washes per the protocol instruction. Make sure that at least 250 µL of wash solution is dispensed per well per wash. Verify the performance of the washer system; have the system repaired if any ports drip, dispense or aspirate poorly.
Reader was malfunctioning or not blanked properly. This is a high possibility if the OD readings were high and the color was light.	Verify the reader's performance using a calibration plate and check the lamp alignment. Verify the blanking procedure, if applicable, and reblank.
Lab temperature was too high.	Maintain the room temperature within 20°–25°C (68°–77°F). Avoid running assays near heat sources or in direct sunlight.
Reagents were intermixed, contaminated or prepared incorrectly.	Ensure that the correct reagents were used, that working solutions were prepared correctly and that contamination has not occurred.

High Intra-Plate Variance

Possible Causes	Recommended Action
Inconsistent time was taken when adding standards, reagents or samples to the assay plate.	Make sure all materials are set up and ready to use. Use a multichannel pipette to add reagents to multiple wells whenever possible. Do not interrupt while adding standards, reagents and samples.
Multichannel pipette was not functioning properly.	Verify pipette calibration and check that tips are on tight. Be sure all channels of the pipette draw and dispense equal volumes.
There was inconsistent washing or washer system malfunctioning.	Check performance of the wash system. Have the system repaired if any ports drip or dispense/aspirate poorly.

High Inter-Plate Variance

Possible Causes	Recommended Action
Inconsistent incubation times occurred from plate to plate.	Time each plate separately to ensure consistent incubation times.
Inconsistent washing occurred from plate to plate.	Make sure to use the number of washes per the protocol instruction. Verify performance of the wash system and have the system repaired if any ports drip or dispense/ aspirate poorly.
Pipette was working improperly.	Check the pipette calibration. Verify that pipette tips are on tight before use and that all channels draw and dispense equal volumes.
Kit plates, reagents, standards and samples were at different temperatures.	Make sure to allow sufficient time for kit plates, reagents, standards and samples come to room temperature (20 – 25°C / 68 – 77°F). Larger volumes will require longer equilibration time. If using a water bath to hasten equilibration, make sure it is maintained at room temperature; do not use a warm water bath to warm reagents, samples and kit standards.
Reagents used were intermixed from different kit lots, or the kits were of different expiration dates.	Carefully label each reagent to make sure the reagents are not intermixed. Kits with different expiration dates might generate different range of OD readings, however, the relative absorbance values may very well be comparable. In general, a value of less than 0.6 in zero standard reading may indicate certain degrees of deterioration of reagents.

One or More of the Standard Curve Points Are Out of Range

Possible Causes	Recommended Action
Standards were added in wrong order or recorded in wrong position.	Follow the protocol and re-run the assay. Make sure the standards are applied and recorded correctly.
Standards were contaminated or intermixed with other standards.	Use a new set of standards. Add standards to plate only in the order from low concentration to high concentration.
There was inconsistent washing or washer system malfunctioning.	Perform washing consistently. Check performance of the wash system. Have the system repaired if any ports drip or dispense/aspirate poorly.
Inconsistent time was taken to add standards and reagents to plate.	Make sure all materials are set up and ready to use. Add standards to plate only in the order from low concentration to high concentration at undisrupted pace. Use a multichannel pipette to add reagents to multiple wells simultaneously.
Multichannel pipette was not functioning properly.	Verify pipette calibration and check that tips are on tight. Be sure all channels of the pipette draw and dispense equal volumes.