REAGEN

Medical & Drug ● IVD & POCT ● Food & Safety

Ochratoxin A ELISA Test Kit Manual

REAGEN LLC

Add.: 102CommerceDr.Unit8, Moorestown, NJ08057
Tel.: 856-727-0250 Fax.: 856-727-0251
E-mail: reagenkits@gmail.com reagenllc@gmail.com
Web.: www.reagen.us

TABLE OF CONTENTS

➢ GENERAL INFORMATION

Product Description

REAGEN® Ochratoxin A ELISA Test Kit is a competitive enzyme immunoassay for the quantitative analysis of Ochratoxin A in cereals, dried meat/fish, feed, milk,urine, serum, seed, pepper, paprika, curry and nutmeg eng.

The unique features of the kit are:

1. High recovery (>80%),cost-effective extraction methods.
2. High sensitivity (0.1 ng/g or ppb).
3. High reproducibility.
4. A quick ELISA assay (less than 1 hours regardless of number of samples).

Procedure Overview

REAGEN® Ochratoxin A ELISA Test Kit is based on a competitive colorimetric ELISA assay. The toxin of interest has been coated in the plate wells. During the analysis, sample is added along with the primary antibody specific for the target toxin. If the target is present in the sample, it will compete for the antibody, thereby preventing the antibody from binding to the toxin attached to the well. The second antibody, tagged with a peroxidase enzyme, targets the primary antibody that is complexed to the toxin coated on the plate wells. The resulting color intensity, after addition of substrate, has an inverse relationship with the target concentration in the sample.

Kit Contents, Storage and Shelf Life

REAGEN® Ochratoxin A ELISA Test Kit has the capacity 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°C *. The shelf life is 12 months when the kit is properly stored.

Kit Contents	Amount	Storage
Ochratoxin A Coated Plate	1x 96-well Plate (8 wells x 12 strips)	2-8°C
Ochratoxin A Standards: Negative control (white cap tube) 0.1ng/mL (yellow cap tube) 0.3ng/mL (orange cap tube) 0.9 ng/mL (pink cap tube) 2.7ng/mL (purple cap tube) 8.1ng/mL (blue cap tube)	 1.0 mL 1.0 mL 1.0 mL 1.0 mL 1.0 mL 1.0 mL	2-8°C
Ochratoxin A Antibody #1	5.5ml	2-8°C
HRP Conjugated Antibody #2	11mL	
20X Wash Solution	40 mL	
Stop Buffer	6 mL	
TMB Substrate	12 mL	

* If you are not planning to use the kit for over 3 months, store Ochratoxin A Antibody #1 and HRP-Conjugated Antibody #2 at -20°C or in a freezer.

Sensitivity (Detection Limit)

Sample Type	Detection Limit (ng/g or ppb)
Dried Meat/Fish, Seed/Cereals	1
Feed	2
Milk	1
Pepper/Paprika/Curry/Nutmeg Eng	1
Urine/Serum	0.5

Specificity (Cross-Reactivity)

Analytes	Cross-Reactivity (%)
Ochratoxin A	100

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
- $NaHCO_3$
- 10 mM PBS buffer (1X PBS): 0.24 g KH_2PO_4 + 1.44 g Na_2HPO_4 + 8 g NaCl, + 0.2 g KCl, adjust pH to 7.4 with NaOH, fill up to 1000 mL with distilled water

Warnings and Precautions

- The standards contain Ochratoxin A . 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°C / 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°C / 68 – 77°F) or in a refrigerator before use.

Preparation of 70% methanol
V methanol: V water = 7:3 (7 parts methanol plus 3 parts deionized water).

Preparation of 0.1M $NaHCO_3$ solution
Weigh 4.2g of $NaHCO_3$, add deionized water to mix and dissolve, and dilute to 500mL.

Dried Meat/Fish/Seed

1. Grind and mix a representative sample(according to accepted sample techniques).
2. Weight out 5 g of the ground sample and place into a suitable container .
3. Add 25ml 70% methanol and shake for 20 minutes with a shaker or multi-tube vortexer.
4. Centrifuger sample for 10 minutes at 4000 rpm.
5. Dilute 1 ml of the obtained supernatant with 1 ml of distilled or deionized water.
6. Use 50µL of the diluted supernatant per well in the test.

Note: Dilution factor: 10.
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, 50 mL of 70% methanol should be used. If the Ochratoxin A concentration is expected to be higher (10-50 ppb), Step 5 above can be changed as following: "Dilute 100 µL of the obtained supernatant with 1.9 mL of 35% methanol in dH2O." The dilution factor will be 100.

Cereals

1. Weigh 2g of crushed sample into a 50mL centrifuge tube, add 10ml of 70% methanol, shake for 5 minutes, room temperature 4000 rpm centrifugal for 10 minutes;
2. Take 1mL of supernatant, add 1mL of 0.1M $NaHCO_3$ solution, and shake.
3. Use 50µL of the diluted supernatant per well in the test.

Note: Dilution factor: 10.

Feed

1. Weigh 2g of crushed sample into a 50mL centrifuge tube, add 20ml of 70% methanol,

shake for 5 minutes, room temperature 4000 rpm centrifugal for 10 minutes;
2. Take 1mL of supernatant, add 1mL of 0.1M $NaHCO_3$ solution, and shake.
3. Use 50μL of the diluted supernatant per well in the test.

Note: Dilution factor:20.

Milk

1. For fat-free milk, dilute the milk sample with 35% methanol in (1:9). (e.g. 20μL of milk +1 80μL of 35% methanol). Take 50 μL of the diluted sample per well for the assay.
2. For the regular milk with fat, centrifuge the milk sample at 4,000 x g for 5 minutes, discard the upper fat layer. Dilute the samples with 35% methanol (1:9). (e.g. 20μL of milk +180μL of 35% methanol). Take 50 μL of the diluted sample per well for the assay.

Note: Dilution factor: 10.

Pepper/Paprika/Curry/Nutmeg Eng

1. Grind and mix a representative sample (according to accepted sampling techniques).
2. Weigh out 0.5 g of the ground sample and place into a suitable container.
3. Add 2.5 mL of 0.1 M $NaHCO_3$ solution and shake for 20 minutes with a shaker.
4. Centrifuge sample for 10 minutes at 4,000 rpm.
5. Dilute 500 μL of the obtained supernatant with 350 μL methanol (100%) and 150 μL 1 X PBS.
6. Mix well. Use 50 μL of the diluted supernatant per well in the test.

Note: Dilution factor: 10

Urine/Serum

1. Add 500 μL of dichloromethane and 25 μL of 1M $NaHCO_3$ solution to 500 μL of urine or serum sample.
2. Vortex for 1 minute vigorously, centrifuge for 5 minutes at 4,000 rpm.
3. Transfer 200 μL of the top aqueous layer to a new tube. Add 450 μL 1 X PBS and 350 μL methanol (100%).
4. Vortex for 30 seconds, centrifuge for 5 minutes at 4,000 rpm.
5. Use 50 μL of the diluted sample per well in the test.

Note: Dilution factor: 5

➢ OCHRATOXIN A ELISA TEST KIT PROTOCOL

Reagent Preparation

IMPORTANT: All reagents should be brought up to room temperature before use (1 – 2 hours at 20 – 25°C / 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.

Preparation of 1X Wash Solution

Mix 1 volume of 20X Wash Buffer concentrate with 19 volumes of distilled water.

ELISA Testing Protocol

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

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

1. Add 50µLof each Ochratoxin A 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 µ L Antibody #1 to each well and mmix well by gently rocking the plate manually for 1 minute.
4. Incubate the plate for 30 minutes at 37°C .
5. Wash the plate 5 times with 350µL 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).
6. Add 100 µL of Antibody #2 to each well ,Incubate the plate for 30 minutes at 37°C.
7. Wash the plate 5 times with 350 µL 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).
8. Add 100 µ L 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).

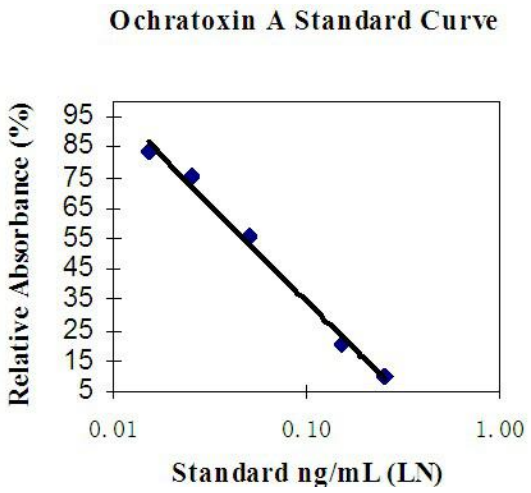
9. After incubation for 15 minutes in room temperature, add 50 µL of Stop Buffer to stop the enzyme reaction.
10. Read the plate as soon as possible following the addition of Stop Buffer on a plate reader with 450 nm wavelength (Before reading, use a lint-free wipe on the bottom of the plate to ensure no moisture or fingerprints interfere with the readings).

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.

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

The following figure is a typical Ochratoxin A 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	Follow the protocol and re-run the assay. Make sure the standards are applied and recorded correctly.

wrong position.	
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.