Medical & Drug ● IVD & POCT ● Food & Safety

Nitrofurantoin(AHD) ELISA Test Kit Manual

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➢ GENERAL INFORMATION

Product Description

REAGEN® Nitrofurantoin (AHD) ELISA Test Kit provides a competitive enzyme immunoassay for the quantitative analysis of nitrofurantoin in fish, shrimp, meat (chicken ,beef, pork and hepar), eggs, honey.

The unique features of the kit are:

- Rapid (4 hours), high recovery (80% - 105%), and cost-effective extraction methods for various samples.
- High sensitivity (0.03 ng/g or ppb) and low detection limit (0.06 ng/g or ppb) for various samples.
- High reproducibility.
- A quick ELISA assay (less than 1 hours regardless of number of samples).

Procedure Overview

The method is based on a competitive colorimetric ELISA assay. The AHD-OVA has been coated in the plate wells. During the analysis, sample and the AHD antibody are added along with secondary antibody, tagged with a peroxidase enzyme. If the AHD residue is present in the sample, it will compete for the AHD antibody, thereby preventing the antibody from binding to the AHD-OVA attached to the well. The resulting color intensity, after addition of the HRP substrate (TMB), has an inverse relationship with the AHD residue concentration in the sample.

Kit Contents, Storage and Shelf Life

REAGEN® Nitrofurantoin (AHD) 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
AHD -Coated Plate	1 x 96well plate (8 wells x 12strips)	2-8°C
AHD Standards: Negative control (white cap tube) 0.03 ng/mL (yellow cap tube) 0.1 ng/mL (orange cap tube) 0.4 ng/mL (pink cap tube) 1 ng/mL (purple cap tube) 4 ng/mL (blue cap tube) 10 ng/mL(spiking,optional, red cap tube)	 1.0 mL 1.0 mL 1.0 mL 1.0 mL 1.0 mL 1.0 mL 1.0 mL	 2-8°C 2-8°C
AHD Antibody (Antibody#1)	8 mL	2-8°C
HRP-Conjugated Antibody #2	6 mL	

10X Sample Extraction Buffer	25 mL	
20X Wash Solution	30 mL	
Stop Buffer	12 mL	
TMB Substrate	12 mL	
50 mM 2-Nitrobenzaldehyde	2.0 mL	

* If you are not planning to use the kit for over 1 month, store 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)
Fish/Shrimp/Meat	0.06
Meat (chicken,beef,pork and hepar)	0.06
Egg/Egg Powder	0.06
Honey	0.06

Specificity (Cross-Reactivity)

Analytes	**Cross-Reactivity (%)**
AHD	100
AOZ	<0.04
SEM	< 0.06
AMOZ	< 0.05

Required Materials Not Provided With the Kit

- Microtiter plate reader (450 nm)
- Incubator
- Tissue Mixer (e.g. Omni TissueMaster Homogenizer)
- Rotary evaporator or nitrogen gas
- Vortex mixer (e.g. Gneie Vortex mixer from VWR)
- 10, 20, 100 and 1000 µL pipettes
- Multi-channel pipette: 50-300 µL (Optional)
- Ethyl acetate
- n-Hexane (or n-Heptane)
- 0.1M K_2HPO_4
- 1M NaOH
- 1M HCl

Warnings and Precautions

- The standards contain Nitrofurantoin. Handle with particular care.
- Do not intermix reagents from different kits or lots except for components with the same part No's within their expiration dates. ANTIBODIES AND PLATES ARE KIT- AND LOT-SPECIFIC.
- 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 1X Sample Extraction Buffer:

Mix 1 volume of 10X Sample Extraction Buffer with 9 volumes of distilled water.

Fish

- Mix 1 g of the homogenized sample with 0.5 mL of 1X Sample Extraction Buffer, 3.5 mL of distilled water, 0.5 mL of 1 M HCl and 20 µL of 50 mM 2-Nitrobenzaldehyde by vortexing for 30 seconds.
- Incubate at 50°C -55°C for 3 hours. Vortex the sample for 5 seconds every hour during the incubation.
- Add 5 mL of 0.1 M K_2HPO_4, 0.4 mL of 1 M NaOH and 6 mL of ethyl acetate, vortex for 2 minutes.
- Centrifuge at 4,000 x g for 5 minutes at room temperature (20 – 25°C).
- Transfer 3 mL of the ethyl acetate supernatant (corresponding to 0.5 g of the original sample) into a new vial (✐ Avoid the lower aqueous layer! If contaminated with lower layer, centrifuge the extracted ethyl acetate for 5 minutes at 4,000 x g again and get the upper organic layer).In case emulsion happened and the upper ethyl acetate layer was less than 3 mL,incubate the sample in water bath for 3 minutes at 85°C.Use a rotary evaporator to dry the sample in a 60-70°C water bath under reduced pressure. Alternatively,the sample can be dried by blowing nitrogen gas in a 60-70°C water bath.
- Dissolve the dried residue in 1 mL of n-hexane (or n-heptane).
- Add 1 mL of **1X** Sample Extraction Buffer, vortex the sample for 2 minutes.
- Centrifuge the sample at 4,000 x g for 10 minutes at room temperature (20 – 25°C / 68 – 77°F).
- Use 30µL of the lower aqueous layer per well for the assay.

  Note: Dilution factor: 2. To avoid high background, it is recommended that a solvent blank sample be prepared in parallel, starting with the reduction of 3 mL of ethyl acetate to dryness and continue with the rest extraction procedure. Subtract the result of the solvent control from the sample results.

Shrimp /Meat/Hepar

- Mix 1 g of the homogenized sample with 0.5 mL 1X Sample Extraction Buffer, 3.5 mL of distilled water, 0.5 mL of 1 M HCl and 20 µL of 50 mM 2-Nitrobenzaldehyde by vortexing for 30 seconds.
- Incubate at50°C -55°C for 3 hours. Vortex the sample for 5 seconds every hour during the incubation.

- Add 5 mL of 0.1 M K_2HPO_4, 0.4 mL of 1 M NaOH and 6 mL of ethyl acetate, vortex for 5 minutes.
- Centrifuge at 4,000 x g for 5 minutes at room temperature (20 – 25°C / 68 – 77°F).
- Transfer 3.0 mL of the ethyl acetate supernatant (corresponding to 0.5 g of the original egg sample) into a new vial (✍ Avoid the lower aqueous layer! If contaminated with lower layer, centrifuge the extracted ethyl acetate for 5 minutes at 4,000 x g again and get the upper organic layer). Use a rotary evaporator to dry the sample in a 60-70 °C water bath under reduced pressure. Alternatively, the sample can be dried by blowing nitrogen gas in a 60-70°C water bath.
- Dissolve the dried residue in 1 mL of n-hexane (or n-heptane).
- Add 1 mL of **1X** Sample Extraction Buffer and vortex the sample for 2 minutes.
- Centrifuge the sample at 4,000 x g for 10 minutes at room temperature (20 – 25 °C / 68 – 77°F).
- Use 30 µL of the lower aqueous layer per well for the assay.
  Note: Dilution factor: 2. To avoid high background, it is recommended that a solvent blank sample be prepared in parallel, starting with the reduction of 3 mL of ethyl acetate to dryness and continue with the rest procedure. Subtract the result of the solvent control from the sample results.

Egg

- Mix 1 g of the egg sample with 0.5 mL of 1X Sample Extraction Buffer, 3.5 mL of distilled water, 0.5 mL of 1 M HCl and 20 µL of 50 mM 2-Nitrobenzaldehyde by vortexing for 2 minutes.
- Incubate at 50°C -55°C for 3 hours. Vortex the sample for 5 seconds every hour during the incubation.
- Add 5 mL of 0.1 M K_2HPO_4, 0.4 mL of 1 M NaOH and 6 mL of ethyl acetate, vortex 5 minutes at max speed.
- Centrifuge at 4,000 x g for 10 minutes at room temperature (20 – 25°C / 68 – 77°F).
- Transfer 3.0 mL of the ethyl acetate supernatant (corresponding to 0.5 g of the original honey sample) into a new vial (✍ Avoid the lower aqueous layer! If contaminated with lower layer, centrifuge the extracted ethyl acetate for 5 minutes at 4,000 x g again and get the upper organic layer). Use a rotary evaporator to dry the sample in a 60-70 °C water bath under reduced pressure. Alternatively, the sample can be dried by blowing nitrogen gas in a 60-70°C water bath.
- Dissolve the dried residue in 1 mL of n-hexane (or n-heptane).
- Add 1 mL of **1X** Sample Extraction Buffer and vortex for 2 minutes.
- Centrifuge the sample at 4,000 x g for 10 minutes at room temperature (20 – 25 °C / 68 – 77°F).
- Use 30 µL of the lower aqueous layer for the assay.
  Note: Dilution factor: 2.

Honey

- Mix 1 g of the honey sample with 0.5 mL of 1X Sample Extraction Buffer, 3.5 mL of distilled water, 0.5 mL of 1 M HCl and 40 µL of 50 mM 2-Nitrobenzaldehyde by vortexing for 1 minute
- Take 1 mL of the milk sample and mix with 0.5 mL of 1X Sample Extraction Buffer, 3.5 mL of distilled water, 0.5 mL of 1 M HCl and 40 µL of 50 mM 2-Nitrobenzaldehyde by vortexing for 1 min.Incubate at 50°C -55°C C for 3 hours.
- Add 5 mL of 0.1 M K_2HPO_4, 0.4 mL of 1 M NaOH and 6 mL of ethyl acetate, vortex 5 minutes at max speed.
- Centrifuge at 4,000 x g for 10 minutes at room temperature (20 – 25°C / 68 – 77°F).
- Transfer 3.0 mL of the ethyl acetate supernatant (corresponding to 0.5 g of the original milk sample) into a new vial (✍ Avoid the lower aqueous layer! If contaminated with lower layer, centrifuge the extracted ethyl acetate for 5 minutes at 4,000 x g again and get the upper organic layer). Use a rotary evaporator to dry the sample in a 60-70 ° C water bath under reduced pressure. Alternatively, the sample can be dried by blowing nitrogen gas in a 60-70°C water bath.
- Dissolve the dried residue in 1 mL of n-hexane (or n-heptane).
- Add 1 mL of 1X Sample Extraction Buffer and vortex for 2 minutes.
- Centrifuge the sample at 4,000 x g for 10 minutes at temperature (20–25°C/68– 77°F).
- Use 30 µL of the lower aqueous layer for the assay.

  Note: Dilution factor: 2.

****Alternative Step 1 and 2 (or step 2 and 3)**

1. Mix the suggested amount of sample (homogenize if necessary) with 4 mL of distilled water, 0.5 mL of 1 M HCl and 20 µL of 50 mM 2-Nitrobenzaldehyde by vortexing for 30 seconds.
2. Incubate at 37°C for 16 hours.

➢ NITROFURANTOIN(AHD) 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
AHD antibody	70 µL	1.68 mL
HRP Conjugate	50 µL	1.2 mL
1X Wash Solution	1.0 mL	24 mL
Stop Buffer	100 µL	2.4 mL
TMB Substrate	100 µL	2.4 mL

1. Add 30 µL of each AHD Standards in duplicate into different wells (✌ Add standards to plate only in the order from low concentration to high concentration).
2. Add 30 µL of each sample in duplicate into different sample wells.
3. Add 50 µL of HRP Conjugate and 70 µL of AHD antibody ,then mix the well by gently rocking the plate manually.
4. Incubate the plate for 30 minutes at room temperature (25°C /77°F). ⏲
5. Wash plate 4 times with 250 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 dry between working steps).
6. Add 100 µL 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).
7. After incubating for 15 minutes at room temperature (25°C / 77°F), add 100 µL of Stop Buffer to stop the enzyme reaction.
8. 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).

Nitrofurantoin(AHD) Concentration Calculations

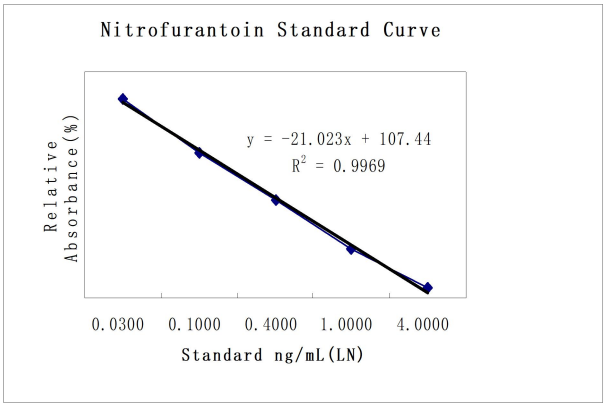
A standard curve can be constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its concentration in ng/mL on a logarithmic curve.

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

Use the mean relative absorbance values for each sample to determine the corresponding concentration of the tested drug in ng/mL from the standard curve.
The following figure is a typical nitrofurantoin (AHD) standard curve. The sample detection and quantification limit are calculated as below.

Sample detection limit = (0.03ng/g or ppb) x (dilution factor)
Sample quantification limit = (0.1 ng/g or ppb) x (dilution factor)

For example, the dilution factor for meat sample is 2.0, therefore, the detection limit for meat sample is 0.06ng/g or ppb (0.03 ng/g x dilution factor 2.0) and the quantification limit is 0.2ng/g or ppb (0.1ng/g x dilution factor 2.0).



➢ 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.