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MAX SIGNAL®

Doxycycline ELISA Test Kit Manual

Catalog #: 1083

Reference #: 1083-01

ISO 9001 QUALITY ASSURANCE

This kit is manufactured to the international quality standard ISO 9001:2008.
ISO CI#: SARA-2009-CA-0114-02-A

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GENERAL INFORMATION

Product Description

The *MaxSignal® Doxycycline ELISA Test Kit* is a competitive enzyme immunoassay for the quantitative analysis of doxycycline in honey, milk, urine fish, shrimp and meat. Doxycycline is a broad spectrum antibiotic which is frequently employed in animal production for its excellent antibacterial and pharmacokinetic properties.

The *MaxSignal® Doxycycline ELISA Test Kit* enables international and government regulatory agencies, food manufacturers and processors, as well as quality assurance organizations, to detect doxycycline in honey, milk or meat and to satisfy customer concerns about food safety. The unique features of the kit are:

- High recovery (>80%), rapid (less than 30 minutes), and cost-effective extraction methods
- High sensitivity (0.15 ng/g or ppb) and low detection limit
- A quick ELISA assay (less than 2 hours regardless of number of samples)
- High reproducibility

Procedure Overview

The method is based on a competitive colorimetric ELISA assay. The drug of interest has been coated in the plate wells. During the analysis, sample is added along with the primary antibody specific for the target drug. If the target is present in the sample, it will compete for the antibody, thereby preventing the antibody from binding to the drug attached to the well. 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 substrate, has an inverse relationship with the target concentration in the sample.

Kit Contents, Storage and Shelf Life

The *MaxSignal® Doxycycline 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
Doxycycline-coated Plate	1 x 96-well Plate (8 wells x 12 strips)	2-8°C
Doxycycline Standard Stock (450 ng Powder)	3	-20°C
Empty Vials for Standards:		
Negative control *(white CAP tube)*	1	2-8°C
0.15 ng/mL *(yellow CAP tube)*	1	
0.5 ng/mL *(orange CAP tube)*	1	
1.5 ng/mL *(pink CAP tube)*	1	
4.5 ng/mL *(purple CAP tube)*	1	
13.5 ng/mL *(blue CAP tube)*	1	
Doxycycline Antibody #1	12 mL	2-8°C *
100X HRP-Conjugated Antibody #2	0.3 mL	2-8°C *
Antibody #2 Diluent **	20 mL	2-8°C
20X Wash Solution **	28 mL	2-8°C
Stop Buffer **	14 mL	2-8°C
TMB Substrate**	12 mL	2-8°C
10X Tissue Extraction Buffer (Optional)	10 mL	2-8°C
10X Tissue Balance Buffer (Optional)	10 mL	2-8°C
Standard Diluent	28 mL	2-8°C

***** If you are not planning to use the kit for over 1 month, storing *Doxycycline Antibody #1* and *100X HRP-Conjugated Antibody #2* at -20°C or in a freezer is recommended.

****** Components with the same BIOO part number within their expiration dates are interchangeable among BIOO kits.

Sensitivity (Detection Limit)

Sample Type	**Detection Limit** (ng/g or ppb)
Honey	4
Fish/Shrimp	5
Meat	10
Milk	5
Milk powder	45
Serum	6
Urine	6

Specificity (Cross-Reactivity)

Analytes	Cross-Reactivity (%)
Doxycycline	100.0
Oxytetracycline	>100
Minocycline	79
Demeclocycline	41
Tetracycline	>100
Chlortetracycline	>100

Required Materials Not Provided With the Kit

- Microtiter plate reader (450 nm)
- Vortex mixer, (e.g. Gneie Vortex mixer from VWR)
- 10, 20, 100 and 1000 µL pipettes
- Multi-channel pipette: 50-300 µL (Optional)
- 10 mM PBS buffer: 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

BIOO strongly recommends that you read the following warnings and precautions to ensure your full awareness of ELISA techniques and other details you should pay close attention to when running the assays. More information can also be found in Troubleshooting section. Periodically, optimizations and revisions are made to the kit and manual. Therefore, it is important to follow the protocol coming with the kit. If you need further assistance, you may contact your local distributor or BIOO at techsupport@biooscientific.com.

- The standards contain doxycycline. 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. 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.

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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.

1. **Preparation of 1X Tissue Extraction Buffer**
   Mix 1 volume of 10X Tissue Extraction Buffer with 9 volumes of distilled water.

2. **Preparation of 1X Tissue Balance Buffer**
   Mix 1 volume of 10X Tissue Balance Buffer with 9 volumes of distilled water.

Honey

1. Weigh out 0.5 g of honey in a screw-top glass vial (50 mL)
2. Add 12 mL 10 mM PBS buffer, pH 7.4.
3. Put the solution in an ultrasonic bath for 5 min and vortex for 2 min; alternatively the sample can be mix vigorously for 10 minutes in a multi-tube vortexer or shaker.
4. Use 75 µL of the sample for the assay.

Note: *Dilution factor: 25.*

Fish/Shrimp

1. Add 3 mL of 1X Tissue Extraction Buffer to 1 g of homogenized fish or shrimp, vortex for 10 minutes in a multi-tube vortexer or shake 30 minutes on a shaker.
2. Centrifuge for 10 min at 4000 rpm.
3. Transfer 200 µL of supernatant to a new tube containing 800 µL of 10 mM PBS buffer, pH 7.4. Vortex for 30 seconds.
4. Use 75 µL per well in the assay.

Note: *Dilution factor: 20 (detection range: 3-270 ppb).*

Meat

1. Add 3 mL of 1X Tissue Extraction Buffer to 1 g of homogenized meat, vortex for 10 minutes in a multi-tube vortexer or shake 30 minutes on a shaker.
2. Centrifuge for 10 min at 4000 rpm.

3. Transfer 200 µL of supernatant to a new tube containing 800 µL of 1X Tissue Balance Vortex for 30 seconds.
4. Use 75 µL per well in the assay.

Note: *Dilution factor: 20 (detection range: 3-270 ppb).*

Milk

1. For regular milk with fat, centrifuge 1.5 mL of the cold milk sample (~*10 ℃ / 50 ℉*) at 10000 rpm for 10 minutes. Discard the upper fat layer. (☝ *For fat-free milk, skip this step*).
2. Take 100 µL of the milk sample and add 0.9 mL of 10 mM PBS buffer, pH 7.4. Vortex the tube for 30 seconds.
3. Use 75 µL of the sample for the assay.

Note: *Dilution factor: 10.*

Milk Powder

1. Weigh out 1 g of milk powder in a centrifugal glass vial, add 9 mL of distilled water and dissolve by shaking.
2. Centrifuge at 10000 rpm for 10 minutes, discard the upper lipid layer.
3. Take 100 µL of the milk sample and add 0.9 mL of 10 mM PBS buffer, pH 7.4. Vortex the tube for 30 seconds.
4. Use 75 µL of the sample for the assay.

Note: *Dilution factor: 90*

Serum

1. Centrifuge 0.2 mL of the serum sample at 4,000 rpm for 5 minutes.
2. Take 0.1 mL of supernatant, add 3.9 mL of 10 mM PBS buffer, pH 7.4.
3. Vortex for 1 minute.
4. Use 75 µL of the sample for the assay.

Note: *Dilution factor: 40.*

Urine

5. Centrifuge 1.5 mL of the urine sample at 4,000 rpm for 5 minutes.
6. Take 0.5 mL of supernatant, add 19.5 mL of 10 mM PBS buffer, pH 7.4.
7. Vortex for 1 minute.
8. Use 75 µL of the sample for the assay.

Note: *Dilution factor: 40.*

Preparation protocols for samples other than above can be made available upon request. Please contact your local distributor or write us at foodfeedsafety@biooscientific.com.

DOXYCYCLINE 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. 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.

1. **Preparation of Doxycycline Work Standards**
   The Doxycycline standard is provided as 450 ng stock. Add 1.5 mL of Standard Diluent to the stock vial and mix by vortexing for 1 minute and leave at room temperature for at least 30 minutes to obtain 300 ppb stock vial. Mix the stock vial again by shaking up and down 10 times before use. To make work standards, serially dilute the 300 ppb standard stock vial with Standard Diluent, mix well by vortexing each work standard tube for 30 seconds.

Work Standards	Doxycycline Source	Volume of Source Doxycycline	Volume of Standard Diluent
13.5 ppb vial	300 ppb stock vial	90 µL	1910 µL
4.5 ppb vial	13.5 ppb vial	500 µL	1000 µL
1.5 ppb vial	4.5 ppb vial	500 µL	1000 µL
0. 5 ppb vial	1.5 ppb vial	500 µL	1000 µL
0.15 ppb vial	0.5 ppb vial	300 µL	700µL
Negative Control vial	N/A	0 µL	500 µL

***Note**: The 300 ppb stock vial and the work standards must be freshly prepared before the ELISA test and these standards can be used within the same day. After use the work standard, empty the standard vials and store them in 4℃. These empty vials can be re-used for next time standard preparation.*

2. **Preparation of 1X Wash Solution**
   Mix 1 volume of the 20X Wash Solution with 19 volumes of distilled water.

3. **Preparation of 1X HRP-Conjugated Antibody #2**
   Mix 1 volume of the 100X HRP-Conjugated Antibody #2 with 99 volumes of Ab#2 Diluent.

ELISA Testing Protocol

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

Component	Volume per Reaction	24 Reactions
Doxycycline Antibody #1	100 µL	2.4 mL
1X HRP-Conjugated Antibody #2	150 µL	3.6 mL
1X Wash Solution	2.5 mL	60 mL
Stop Buffer	100 µL	2.4 mL
TMB Substrate	100 µL	2.4 mL

1. Add 75 µL of each Doxycycline Standard (Negative Control, 0.15, 0.5, 1.5. 4.5, 13.5 ppb) in duplicate into different wells (✐ *Add standards to plate only in the order from low concentration to high concentration*).

2. Add 75 µL of each sample in duplicate into different sample wells.

3. Add 100 µL of Antibody #1 and mix well by gently rocking the plate manually for 1 minute.

4. Incubate the plate for 50 minutes at room temperature (*20 – 25°C / 68 – 77°F*). ⏲

5. Wash the plate 3 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 air dry between working steps*).

6. Add 150 µL of 1X Antibody #2 solution. Incubate the plate for 20 minutes at room temperature (20 – 25°C / 68 – 77°F) (⏲ ✐ *Avoid direct sunlight and cold bench tops during the incubation. Covering the microtiter plate while incubating is recommended*).

7. Wash the plate 3 times with 250 of µL 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 µL of TMB Substrate to each well. 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. Covering the microtiter plate while incubating is recommended*).

9. After incubating for 15 minutes at room temperature (*20 – 25°C / 68 – 77°F*), add 100 µL of Stop Buffer to stop the enzyme reaction.

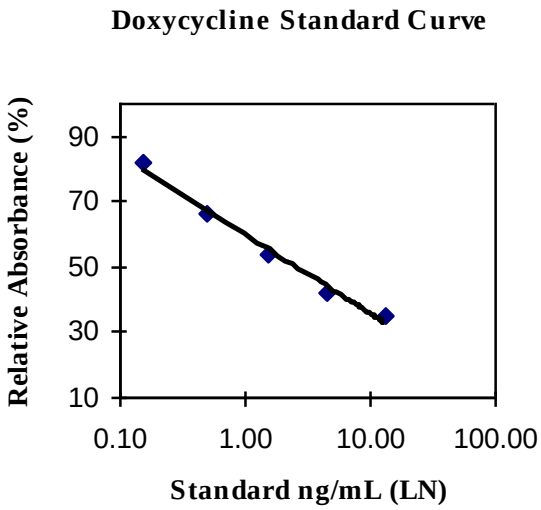
10. Read the plate as soon as possible following the addition of Stop Buffer on a plate reader with 450 nm wavelength (✐ *Before reading, use a lint-free wipe on the bottom of the plate to ensure no moisture or fingerprints interfere with the readings*).

Doxycycline 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. A special program with Excel functionality, the *MaxSignal® ELISA Detection Analysis System*, is available upon request to evaluate the MaxSignal® ELISA test results. Please contact your local distributor or techsupport@biooscientific.com for further information. The following figure is a typical doxycycline 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.	Make sure that the antibodies used are the ones that came with the kit. All antibodies are kit- and lot-specific.
Substrate has deteriorated.	Use a new set of BIOO 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 make sure that 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.

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