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BIOO SCIENTIFIC
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MAX SIGNAL®

Beta-Agonist ELISA Test Kit Manual

Catalog #: 1009

Reference #: 1009-03

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GENERAL INFORMATION

Product Description

MaxSignal® Beta-Agonist ELISA Test Kit is a competitive enzyme immunoassay for the quantitative analysis of clenbuterol, salbutamol, bitolterol, carbuterol and other related beta-agonists in feed, meat/liver/kidney, milk and urine. Beta-agonists can improve the meat/fat ratio in fattened animals and accelerate livestock growth. They also have a relaxing effect on non-striated musculature and can be used as antiasthmatic and tocolytic agents in human beings. Therefore, excess beta-agonist residues in livestock products may lead to health risks for consumers. This has led to a prohibition of beta-agonist use in food production. The amount of beta-agonist residues can be determined by methods such as HPLC or GC-MS. However, these traditional methods usually require very expensive preparation procedures. Alternatively, enzyme immunoassay (ELISA) can be used as a screening system, which is simple, rapid, sensitive and cost-effective compared with traditional methods.

MaxSignal® Beta-Agonist ELISA Test Kit enables international and government regulatory agencies, food manufacturers and processors, as well as quality assurance organizations, to detect beta-agonist residues in various sample types in response to customer concerns about food safety. The unique features of the kit are:

- Providing a rapid (10 - 30 minutes) and extraction method for various samples with high recovery (75 - 95%).
- High sensitivity (0.05 ng/g or ppb) and low detection limit (0.025 ng/g or ppb for meat/tissue, 0.05 ng/g or ppb for urine and 0.2 ng/g or ppb for feed).
- A quick ELISA assay (less than 1.5 hours regardless of number of samples).
- High reproducibility.

Procedure Overview

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

Kit Contents, Storage and Shelf Life

MaxSignal® Beta-Agonist 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
Beta-Agonist Ab-coated Microtiter Plate	1 x 96-well plate (8 wells x 12 strips)	2-8°C
Clenbuterol Standards:		2-8°C
Negative control *(white cap tube)*	1.8 mL	
0.05 ng/mL *(yellow cap tube)*	1.8 mL	
0.15 ng/mL *(orange cap tube)*	1.8 mL	
0.5 ng/mL *(pink cap tube)*	1.8 mL	
1.5 ng/mL *(purple cap tube)*	1.8 mL	
4.5 ng/mL *(blue cap tube)*	1.8 mL	
10 ng/mL (spiking, optional, *red cap tube)*	1.8 mL	
Clenbuterol-HRP Conjugate	7 mL	2-8°C *
200X Sample Extraction Buffer **	25 mL	2-8°C
20X Wash Solution **	28 mL	2-8°C
Stop Buffer **	20 mL	2-8°C
TMB Substrate **	12 mL	2-8°C
Sample Balance Buffer **	0.5 mL	2-8°C
10X PBS **	25 mL	2-8°C

* If you are not planning to use the kit for over 1 month, store Clenbuterol-HRP Conjugate at -20°C or in a freezer.

** Components with the same BIOO part No's within their expiration dates are interchangeable among BIOO kits.

Sensitivity (Detection Limit)

ß-Agonists	Feed ** (ppb)	Meat/Liver/Kidney * (ppb)	Milk (ppb)	Urine (ppb)
Clenbuterol	0.2	0.025	0.25	0.05
Bitolterol	0.2	0.025	0.25	0.05
Carbuterol	0.2	0.025	0.25	0.05
Salbutamol (Albuterol)	0.2	0.025	0.25	0.05
Cimbuterol	0.2	0.025	0.25	0.05
Mabuterol	0.2	0.025	0.25	0.05
Terbutaline	0.2	0.025	0.25	0.05
Bromobuterol	0.2	0.025	0.25	0.05
Timolol	10.6	1.25	12.6	3.0

* Based on Organic Reagent Extraction Method.

** Based on Rapid Extraction Method.

Specificity (Cross-Reactivity)

Beta-Agonists	Cross-Reactivity (%)
Clenbuterol	100
Bitolterol	100
Carbuterol	100
Salbutamol	100
Cimbuterol	100
Mabuterol	100
Terbutaline	100
Bromobuterol	100
Timolol	10
Metaproterenol	1.0
Salmeterol	<0.01
Fenoterol	<0.01
Ractopamine	<0.01

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*)

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 clenbuterol. 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. CLENBUTEROL-HRP CONJUGATES 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 temperature (20 – 25°C / 68 – 77°F) or in a refrigerator before use. Preparation protocols for samples other than below can be made available upon request. Please contact your local distributor or write us at foodfeedsafety@biooscientific.com.

1. Preparation of 1X Sample Extraction
   Mix 1 volume of 200X Sample Extraction Buffer with 199 volumes of distilled water.

2. Preparation of 1X PBS
   Mix 1 volume of the 10X PBS with 9 volumes of distilled water.

Feed

*Rapid Extraction Method**
(*This procedure is not recommended for tests on compound feed)

1. To a reasonable amount of feed sample, add 4 times the amount of 1X Sample Extraction Buffer (e.g. 2 g of sample + 8 mL of buffer); homogenize the sample with a suitable mixer.
2. Take out 1.5 mL of the homogenized sample and centrifuge 5 minutes at 4,000 x *g* at room temperature (*20 – 25°C / 68 – 77°F*).
3. Carefully transfer 0.5 mL of the supernatant to a tube.
4. Incubate the supernatant at 75°C for 5 minutes and vortex 1 minute at maximum speed.
5. Centrifuge for 5 minutes at 4,000 x *g* at room temperature (*20 – 25°C / 68 – 77°F*).
6. Transfer 0.3 mL of the supernatant to a new tube, add 7.5 µL of Sample Balance Buffer to the tube and mix well.
7. Use 100 µL per well for the assay.

Note: *Dilution factor: 4.*

*Organic Reagent Extraction***
(**This procedure works for both general animal feed and compound feed)

1. Homogenize the feed sample with a suitable mixer.
2. Add 8 mL of acetonitrile and 1 mL of ethyl acetate to 1.5 g of the homogenized sample. Vortex for 3 minutes at maximum speed.
3. Centrifuge for 5 minutes at 4,000 x *g* at room temperature (*20 – 25°C / 68 – 77°F*), transfer 6 mL of the supernatant to a new tube.
4. 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.
5. Add 1 mL of hexane to dissolve the sample and then add 1 mL of 1X PBS, vortex for 1 minute at maximum speed.
6. Centrifuge the sample for 5 minutes at 4,000 x *g*.
7. Use 100 µL of the lower aqueous layer for the assay.

Note: *Dilution factor: 1.*

Meat/Liver/Kidney

Rapid Extraction Method

1. Remove fat from meat, liver or kidney. Homogenize the sample with a suitable mixer.
2. Add 1 mL of 1X Sample Extraction Buffer to 0.5 g of the homogenized sample. Vortex for 3 minutes at maximum speed or rotorack for 20 minutes (*e.g. VWR Tube Rotator*).
3. Centrifuge for 5 minutes at 4,000 x *g* at room temperature (*20 – 25°C / 68 – 77°F*), transfer 0.5 mL of the supernatant to a new tube (✌ *Avoid touching the fatty layer!*).
4. Incubate the supernatant at 75°C for 5 minutes and vortex 1 minute at maximum speed.
5. Centrifuge for 5 minutes at 4,000 x *g* at room temperature (*20 – 25°C / 68 – 77°F*).
6. Transfer 0.3 mL of the supernatant to a new tube (✌*Avoid touching the fatty layer!*), add 7.5 µL of Sample Balance Buffer and mix well.
7. Use 100 µL for assay.

Note: *Dilution factor: 3. To avoid high background, a known negative sample should be prepared in parallel, subtract the negative sample result from the test sample results.*

Organic Reagent Extraction Method

1. Remove fat from meat, liver or kidney. Homogenize the sample with a suitable mixer.
2. Add 8 mL of acetonitrile and 1 mL of ethyl acetate to 3 g of the homogenized sample. Vortex for 3 minutes at maximum speed.
3. Centrifuge for 5 minutes at 4,000 x *g* at room temperature (*20 – 25°C / 68 – 77°F*), transfer 6 mL of the supernatant to a new tube.
4. 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.
5. Add 1 mL of hexane to dissolve the sample and then add 1 mL of 1X PBS, vortex for 1 minute at maximum speed.
6. Leave the tube open and heat the sample at 85°C for 3 minutes (✌ *This step can be omitted if the lower aqueous layer is enough for the ELISA assay*).
7. Centrifuge the sample for 5 minutes at 4,000 x *g*.
8. Use 100 µL of the lower aqueous layer for the assay.

Note: *Dilution factor: 0.5.*

Milk

1. For fat-free milk, dilute the milk sample with 1X PBS Buffer (1:4) (e.g. 50 µL of milk + 200 µL of buffer). Take 100 µL of diluted sample per well for the assay.
2. For regular milk with fat, centrifuge the milk sample at 4,000 x *g* for 5 minutes, discard the upper fat layer. Dilute the sample with 1X PBS Buffer (1:4) (e. g. 50 µL of milk + 200 µL of buffer). Take 100 µL of the diluted sample per well for the assay.

Note: *Dilution factor: 5.*

Urine

1. Centrifuge 0.5 mL of the urine sample at 4,000 x *g* for 5 minutes.
2. Use 100 µL of the supernatant per well for the assay.

Note: *Dilution factor: 1. To avoid high background, we recommend diluting the sample with 1X PBS 5 fold (1:4).*

BETA-AGONIST 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 4. 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 the 20X Wash Solution 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
Clenbuterol-HRP Conjugate	50 µL	1.2 mL
1X Wash Solution	2.0 mL	48 mL
Stop Buffer	100 µL	2.4 mL
TMB Substrate	100 µL	2.4 mL

1. Add 100 µL of each Clenbuterol Standards in duplicate into different wells (✐ *Add standards to plate only in the order from low concentration to high concentration*).
2. Add 100 µL of each sample in duplicate into different sample wells.
3. Add 50 µL of Clenbuterol-HRP and mix well by gently rocking the plate manually for 1 minute.
4. Incubate the plate for 45 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 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 (*20 – 25°C / 68 – 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*).

Beta-Agonist 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.

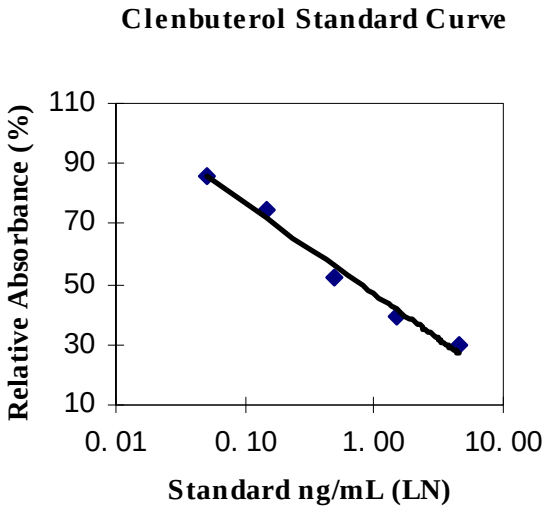
$$\text{Relative absorbance (\%)} = \frac{\text{absorbance standard (or sample)} \times 100}{\text{absorbance zero standard}}$$

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, *MaxSignal® ELISA Analysis Program in Excel*, 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 clenbuterol standard curve. The sample detection limit and quantification limit are calculated as below.

Sample detection limit = (0.05 ng/g or ppb) x (dilution factor)
Sample quantification limit = (0.15 ng/g or ppb) x (dilution factor)

For example, the dilution factor for meat sample is 0.5, therefore, the detection limit for meat sample is 0.025 ng/g or ppb (0.05 ng/g x dilution factor 0.5) and the quantification limit is 0.075 ng/g or ppb (0.15 ng/g x dilution factor 0.5).



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 HRP conjugates were used.	Make sure that the HRP Conjugates used are the ones that came with the kit. All HRP Conjugates are kit- and lot-specific.
TMB substrate has deteriorated.	Use a new set of BIOO 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 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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