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BIO-X ELISA KIT FOR THE QUANTITATIVE DETERMINATION OF BOVINE GAMMA INTERFERON (BIO K 093)

(Quantitative sandwich test)

KIT FOR THE QUANTITATIVE DETERMINATION OF BOVINE GAMMA INTERFERON BY ELISA

I - PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by a specific monoclonal antibody for the bovine gamma Interferon. This antibody allows a specific capture of the bovine gamma Interferon which is present in the samples (plasma or serum). The entire plate has been sensitized with this antibody.
Samples are applied without dilution and incubated on the microplate for 60 minutes at room temperature. A reference curve must be prepared by dilution of the standard purchased with the kit. The standard is a supernatant of bovine lymphocytes stimulated "in vitro" by concanavalin A. As no international standard exists for gamma interferon, the standard of the kit is calibrated in arbitrary units.
After this first incubation step, the plate is washed and incubated for 60 minutes with the conjugate - a peroxidase labelled anti-bovine gamma Interferon specific antibody. After this second incubation, the plate is washed again and the enzyme substrate (hydrogen peroxide) and the chromogen (tetramethyl benzidine TMB) are added. This chromogen has the advantage of being more sensitive than the other peroxidase chromogens and of not being carcinogenic.
Enzymatic reaction can be stopped by acidification and the resulting optical density at 450 nm can be recorded using a photometer.
The optical density obtained for unknown samples is reported on the calibration curve in order to calculate the IFN concentration.

II - COMPOSITION OF THE KIT

- **Microplates**: Two 96-well microtitration plates. The whole plate is sensitised by anti-Bovine gamma Interferon specific antibodies.
- **Washing solution**: One 100- ml bottle of 20x concentrated washing solution. The solution crystallises spontaneously at low temperatures. If only part of the solution is to be used, bring the bottle to room temperature until disappearance of all crystals. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water. Store the diluted solution at 4°C.
- **Dilution buffer**: One 50-ml bottle of 5x concentrated buffer for diluting of standard and conjugate. Dilute this concentrated dilution buffer 1:5 with distilled or demineralised water. Store the diluted solution at 4°C. If a deposit forms at the bottom of the container filter the solution on Whatman filter paper.

- **Conjugate**: One bottle of anti-bovine gamma Interferon-peroxidase conjugate. The conjugate will keep at 4°C.
- **Bovine gamma Interferon standard**: 2 bottles containing the Bovine gIFN standard.
- **Chromogen solution**: One 2-ml drop-dispenser bottle of the chromogen tetramethylbenzidine. Store at 4°C.
- **Substrate solution**: One 30-ml bottle of the hydrogen peroxide substrate solution. Store this reagent at 4°C.
- **Stopping solution**: One 15-ml bottle of the 1 M phosphoric acid stop solution.

III - PRECAUTIONS FOR USE

- This test may be used for in vitro diagnosis only.
- The reagents must be kept at 4°C to 8°C. The conjugate must be kept at 4°C. The standard solution of gIFN must be kept at -20°C once reconstituted. The standard solution of gIFN must be prepared precisely. The dilutions of the standard must be prepared precisely. It is recommended to use precision equipment such as Hamilton seringes rather than pipettes with plastic tips. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been stored under the conditions described in this insert.
- Do not use reagents from other kits.
- The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
- Discard all solutions contaminated with bacteria or fungi.
- Some of the bottles contain merthiolate or thimerosal. This substance is toxic if it is inhaled or if it comes in contact with the skin. Take the usual precautions in handling these bottles.
- The stop solution contains 1 M phosphoric acid. Handle carefully.

IV – PROCEDURE

1- Bring all the reagents to room temperature at least half an hour before use.
2- Dilute the concentrated washing solution 20 fold in distilled water. Be sure that all crystals have disappeared before dilution.
Dilute the concentrated Dilution buffer 5 fold in distilled water.
Keep these solutions at 4°C when not used.
3- Distribute the unknown samples in duplicate and undiluted on the microplate (100 µl per well). Use 2 wells as blanks (dilution solution).
4- Prepare the calibration curve as follows: resuspend the contents of one bottle of standard in 500 µl of distilled water. Make 7 dilutions (of the order 2) of the standard in the dilution solution. The precision of the ELISA test depends greatly on the careful preparation of the dilutions. In order to obtain reliable results, it is highly recommended to use precision dilution equipment such Hamilton seringes. Distribute the 8 dilutions in duplicate on the microplate (100 µl per well).

	Dilution	Concentration
Stock solution	1/1	100 UA/ml
1° dilution	1/2	50 UA/ml
2° dilution	1/4	25 UA/ml
3° dilution	1/8	12,5 UA/ml
4° dilution	1/16	6,25 UA/ml
5° dilution	1/32	3,12 UA/ml
6° dilution	1/64	1,56 UA/ml
7° dilution	1/128	0,78 UA/ml

5- Incubate the plate at room temperature for 1 hour.
6- Rinse the plate with the washing solution, prepared as instructed in §2, as follows: empty the microplate of its contents by flipping it over sharply over a sink. Tap the microplate upside down against a piece of clean absorbent paper to remove all the liquid. Fill all the used wells with the washing solution using a spray bottle or by plunging the plate in a vessel of the right dimensions, then empty the wells once more by turning the plate over above a sink. Repeat the entire operation twice, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed three times proceed to the next step.
7- Dilute the conjugate 1:50 with the dilution buffer (for example, for one plate dilute 250 µl of the conjugate stock solution in 12.25 ml of diluent). Add 100 µl of the diluted conjugate solution to each well. Incubate at room temperature for 1 hour.
8- Wash the plate as described in §6 above.
9- Prepare 10 ml of indicator solution extemporaneously as follows: Add 12 drops (500 µl) of chromogen to 9.5 ml of the substrate solution (enough for 1 plate). Mix thoroughly, then apply to the plate immediately in volumes of 100 µl per microwell. At the time of distribution of the chromogen-substrate mixture on the plates the solution must be completely colourless. If a blue colour appears at this stage, this solution must be discarded and a new one made up using clean glassware and equipment.
10 - Incubate for 10 minutes at room temperature. This time is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
11 - Add 50 µl of stop solution per microwell.
12 - Read the optical densities in the microwells using a plate reader and a 450 nm filter. Results must be read fairly soon after the stopping solution has been added since the chromogen may cristallize in wells with strong signals and thereby distort the data.

V – INTERPRETING THE RESULTS

In order to calculate the concentrations of gIFN in the unknown samples, it is preferable to use a computer program with curve fitting options of the type Log/Logit or 4 parameter. The Deltasoft program of Biometallics incorporated (P.O. Box 2251 Princeton, NJ USA Tel: 08543 609.275.0133 Fax 609.275.9485) is particularly well adapted to this kit.
If such a program is available, introduce the 8 gIFN concentrations in the standard curve (2 values per dilution).
Indicate what well corresponds to the blanks.
Name each sample and indicate its dilution factor. The program will determine the 4 parameters of the standard curve with its correlation coefficient.
Interpolate the values in order to obtain the concentrations of the unknown samples.

If a program such as Deltasoft is not available, one can determine the concentrations of the unknown samples using a graphic method, as described below.
Use the graph provided with the kit. The bold vertical lines correspond to the concentrations of the standard curve (100 UA/ml - 0,78 UA/ml). Calculate for each of the 8 points of the calibration curve the mean of the optical densities - the mean of the 2 blanks (see example hereunder). Place on each of the 8 vertical lines the values obtained. Draw the curve so that it best fits the 8 experimental points.
For each of the unknown samples, also calculate the mean of the optical densities - the mean of the 2 blanks. Place the values obtained on the ordinate and draw the corresponding horizontal lines. At the point of intersection of the horizontal lines with the curve, draw the vertical lines and determine the concentration of the samples on the abscissa (UA/ml).