Swine IFN-γ ELISA Kit

Catalog Number
EK0530

For the quantitative determination of swine γ-interferon (IFN-γ) concentrations in cell culture supernates, serum, and plasma.

This instruction must be read in its entirety before using this product.

For research use only. Not for use in diagnostic procedures.

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PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IFN- has been pre-coated onto a microplate. Standard, control, or sample and the working solution of Biotin-Conjugate are pipetted into the wells. Following incubation and wash steps，any IFN- present is bound by the immobilized antibody and the detection antibody specific for IFN- is binds to the combination of capture antibody- IFN- in sample. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Following incubation and wash steps a substrate is added. A coloured product is formed in proportion to the amount of IFN- present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven IFN- standard dilutions and IFN- sample concentration determined.

DETECTION RANGE

15.6 pg/ml - 1000 pg/ml

SENSITIVITY

The minimum detectable dose was 7pg/mL.

SPECIFICITY

This assay recognizes both recombinant and natural porcine IFN-. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5T and RD6-3 and assayed for cross-reactivity. Preparations of the following factors at the same concentrations in a mid-range porcine IFN- control were assayed for interference. No significant cross-reactivity or interference was observed.

Table 1: Factors assayed for cross-reactivity

Recombinant human	**Recombinant mouse**	**Recombinant porcine**
IFN- □	IFN- βα	IL-1
IFN- R1	IFN- R1	IL-1β
IFN- R2	IFN- R2	IL-2
		IL-4
		TNF-

PRECISION

The coefficient of variation of both intra-assay and inter-assay were less than 10%.

MATERIALS PROVIDED

1. Aluminium pouches with a Microwell Plate coated with antibody to swine IFN- (8×12)
2. 2 vials swine IFN- Standard lyophilized, 4000 pg/ml upon reconstitution
3. 2 vials concentrated Biotin-Conjugate anti-swine IFN- antibody
4. 2 vials Streptavidin-HRP solution,
5. 1 bottle Standard /sample Diluent
6. 1 bottle Biotin-Conjugate antibody Diluent
7. 1 bottle Streptavidin-HRP Diluent
8. 1 bottle Wash Buffer Concentrate 20x (PBS with 1% Tween-20)
9. 1 vial Substrate Solution
10. 1 vial Stop Solution
11. 4 pieces Adhesive Films
12. package insert

STORAGE

Table2: Storage of the kit

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8℃. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened/ Reconstituted Reagents</td><td>Standard /sample Diluent</td><td rowspan="8">May be stored for up to 1 month at 2 - 8℃.**</td></tr>
<tr><td>Concentrated Biotin-Conjugate</td></tr>
<tr><td>Streptavidin-HRP solution</td></tr>
<tr><td>Biotin-Conjugate antibody Diluent</td></tr>
<tr><td>Streptavidin-HRP Diluent</td></tr>
<tr><td>Wash Buffer Concentrate 20x</td></tr>
<tr><td>Substrate Solution</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

	Standard	Aliquot and store for up to 1 month at ≤20℃. Avoid repeated freeze-thaw cycles. Diluted standard shall not be reused.
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 – 8℃.**

**Provided this is within the expiration date of the kit.

THE REQUIRED ITEMS (not provided, but can help to buy):

1. Microplate reader (450nm).
2. Micro-pipette and tips: 0.5-10, 2-20, 20-200, 200-1000ul.
3. 37 ℃ incubator, double-distilled water or deionized water, coordinate paper, graduated cylinder.

PRECAUTIONS FOR USE

1. Store kit regents between 2℃and 8℃. After use all reagents should be immediately returned to cold storage(2℃ to 8℃).
2. Please perform simple centrifugation to collect the liquid before use.
3. To avoid cross contamination, please use disposable pipette tips.
4. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material. Avoid contact of skin or mucous membranes with kit reagents or specimens. In the case of contact with skin or eyes wash immediately with water.
5. Use clean, dedicated reagent trays for dispensing the washing liquid, conjugate and substrate reagent. Mix all reagents and samples well before use.
6. After washing microtiter plate should be fully pat dried. Do not use absorbent paper directly into the enzyme reaction wells.
7. Do not mix or substitute reagents with those from other lots or other

sources. Do not use kit reagents beyond expiration date on label.

8. Each sample, standard, blank and optional control samples should be assayed in duplicate or triplicate.
9. Adequate mixing is very important for good result. Use a mini-vortexer at the lowest frequency or Shake by hand at 10min interval when there is no vortexer.
10. Avoid microtiter plates drying during the operation.
11. Dilute samples at the appropriate multiple, and make the sample values fall within the standard curve. If samples generate values higher than the highest standard, dilute the samples and repeat the assay.
12. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time and temperature, and kit age can cause variation in binding.
13. This method can effectively eliminate the interference of the soluble receptors, binding proteins and other factors in biological samples.

SAMPLE COLLECTION AND STORAGE

1. **Cell Culture Supernates** - Remove particulates by centrifugation.
2. **Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum, avoid hemolysis and high blood lipid samples.
3. **Plasma** - Recommended EDTA as an anticoagulant in plasma. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection.
4. Assay immediately or aliquot and store samples at -20℃. Avoid repeated freeze-thaw cycles.
5. Dilute samples at the appropriate multiple (recommended to do pre-test to determine the dilution factor).
   Note: The normal swine serum or plasma samples are suggested to make a 1:2 dilution.

REAGENT PREPARATION

1. Bring all reagents to room temperature before use.
2. **Wash Buffer** - Dilute 10mL of Wash Buffer Concentrate into deionized or distilled water to prepare 200mL of Wash Buffer. If crystals have

formed in the concentrate Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.

3. **Standard** - Reconstitute the Standard with 1.0mL of Standard /sample Diluent. This reconstitution produces a stock solution of 4000 pg /mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

   Pipette 250μL of Standard/sample Diluent into the 1000 pg/mL tube and the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 1000 pg/mL standard serves as the high standard. The Standard/ sample Diluent serves as the zero standard (0 pg/mL).

   If you do not run out of re-melting standard, store it at -20℃. Diluted standard shall not be reused.

4. Working solution of Biotin-Conjugate anti-swine IFN- antibody: Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with the Biotin-Conjugate antibody Diluent in a clean plastic tube.

   The working solution should be used within one day after dilution.

5. Working solution of Streptavidin-HRP: Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with the Streptavidin-HRP Diluent in a clean plastic tube.

   The working solution should be used within one day after dilution.

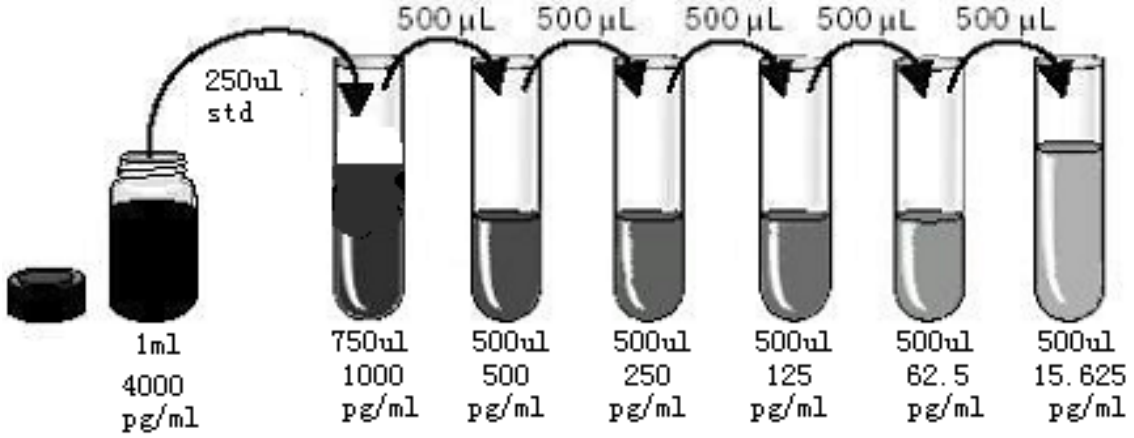

Figure 1: Preparation of IFN-• standard dilutions

GENERAL ELISA PROTOCOL

1. Prepare all reagents and working standards as directed in the previous

sections.

2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2-8℃ sealed tightly.
3. Add 100μL of Standard, control, or sample, per well, then add 50 μL of the working solution of Biotin-Conjugate to each well. Cover with the adhesive strip provided and incubate 2 hours at RT. Adequate mixing is very important for good result. Use a mini-vortexer at the lowest frequency.
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (350μL) using a squirt bottle, manifold dispenser or auto-washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 μL of the working solution of Streptavidin-HRP to each well. Cover with a new adhesive strip and incubate for 30 minutes at RT. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 4.
7. Add 100 μL of Substrate Solution to each well. Incubate for 10-20 minutes at RT. Avoid placing the plate in direct light.
8. Add 100 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm.(optionally 630nm as the reference wave length;610-650nm is acceptable)

ASSAY PROCEDURE SUMMARY

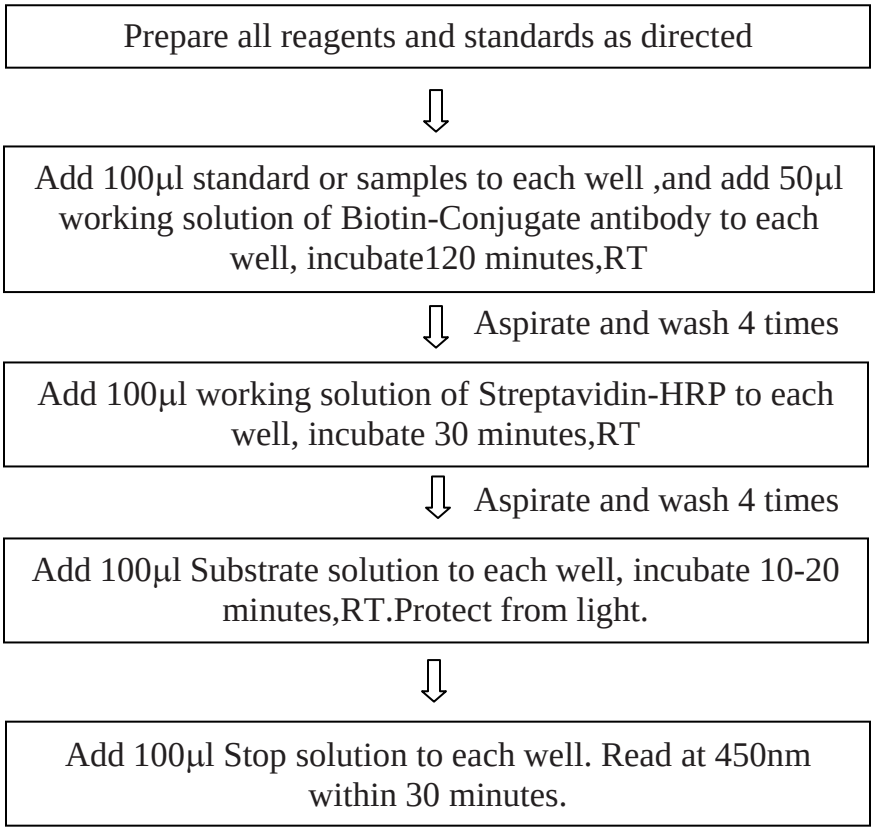

Figure 2: Assay procedure summary

TECHNICAL HINTS

1. When mixing or reconstituting protein solutions, always avoid foaming.
2. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

4. Substrate Solution should remain colorless until added to the plate. Stop Solution should be added to the plate in the same order as the Substrate Solution. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
5. A standard curve should be generated for each set of samples assayed. According to the content of tested factors in the sample, appropriate diluted or concentrated samples, it is best to do pre-experiment.

CALCULATION OF RESULTS

1. Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.
2. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.
3. The data may be linearized by plotting the log of the IFN- concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
4. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Table 3:Typical data using the IFN- ELISA (Measuring wavelength:450nm, Reference wavelength:630nm)

Standared (pg/ml)	OD.	OD.	Average	Corrected
0	0.023	0.024	0.024	——
15.625	0.103	0.101	0.102	0.093
31.25	0.164	0.162	0.163	0.158
62.5	0.304	0.301	0.303	0.296
125	0.585	0.579	0.582	0.564
250	0.946	0.950	0.948	0.999
500	1.587	1.580	1.584	1.541
1000	2.020	2.009	2.015	2.029

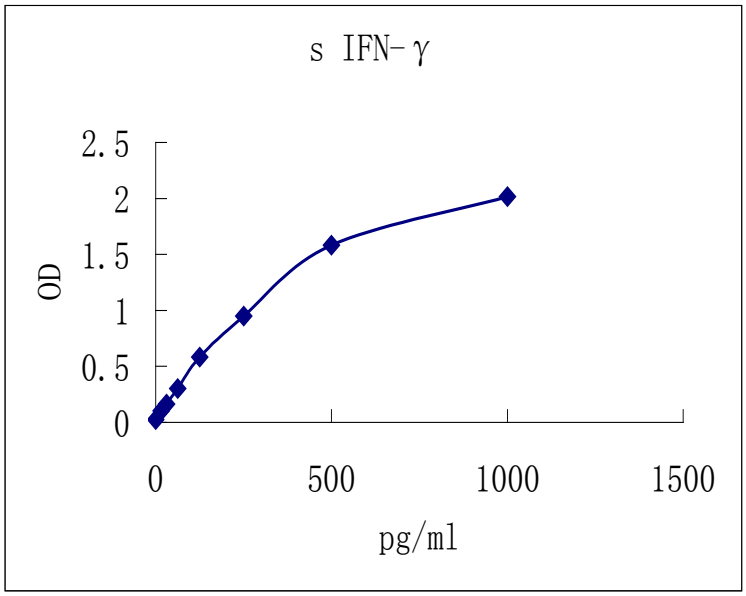

Figure 3:Representative standard curve for IFN- ELISA. IFN- was diluted in serial two-fold steps in Sample Diluent.

Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

BACKGROUND

Interferon-gamma (IFN-γ, also known as type II interferon) is an important immunoregulatory cytokine that was originally identified because of its anti-viral activity (1). It plays key roles in host defense by exerting antiviral, antiproliferative and immunoregulatory activities (2 - 5). On many cell types, IFN-γ induces the production of cytokines and upregulates the expression of various membrane proteins including class I and II MHC antigens, Fc receptors, leukocyte adhesion molecules and B7 family antigens. IFN-γ is a potent activator of macrophage effector functions. It potentiates the secretion of immunoglobulins by B cells, and directs the synthesis of IgG. IFN-γ also influences T-helper cell phenotype development by inhibiting Th2 differentiation and stimulating Th1 development (2 - 5). Finally, IFN- promotes mononuclear cell chemotaxis by inducing the synthesis of CXCL9, CXCL10, CCL2, CCL3, CCL4, and CCL5(3). IFN-γ is produced by a number of cell types, including dendritic epidermal/ T cells (6), keratinocytes (7), peripheral blood T cells (8), mast cells (9), neurons (10),

CD8 T cells (11), macrophages (12), B cells (13), neutrophils (14), NK cells (15), CD4 T cells (16) and testicular spermatids (17). The production of IFN-γ is upregulated synergistically by IL-12, IL-18, IL-23 and IL-27 (18 - 21). Porcine IFN-γ cDNA encodes a 166 amino acid (aa) residue precursor protein with a 20 aa signal sequence that is cleaved to generate a 146 aa residue mature IFN-γ (22, 23). Porcine IFN-γ is presumably a noncovalently linked homodimer (3). In the mature segment, porcine IFN-γ shares 60%, 55%, 41%, 42%, 72%, and 72% aa sequence identity with human (24), guinea pig (25), mouse (26), rat (27), feline (28), and canine (29) IFN-γ, respectively.

The functional IFN-γ receptor complex consists of two distinct subunits (30). The alpha-subunit(IFN-γR1) binds IFN-γ with high affinity and species specificity. The beta-subunit [IFN-γR2, also known as accessory factor-1 (AF-1)] interacts with the IFN- occupied-subunit in a species-specific manner and participates in JAK-STAT mediated signal transduction. Although the functional receptor is suggested to consist of homodimeric IFN-γ in combination with two-chains, and two-chains (30, 31), it has been suggested that additional subunits may be involved (32, 33). Whereas the-chain is expressed constitutively on many cell types, the cellular regulation of the-chain correlates with an IFN-γ responsive state and is tightly regulated (30).

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