



ELISA PRODUCT INFORMATION & MANUAL

Porcine IL-18/IL-1F4 ELISA Kit (Colorimetric) *NBP1-94192*

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.

Not for diagnostic or therapeutic procedures.

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Novus kits are guaranteed for 6 months from date of receipt

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1 Intended Use

The pig IL-18/IL-1F4 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of pig IL-18/IL-1F4. **The pig IL-18/IL-1F4 ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2 Summary

Interleukin (IL) -18 is a newly discovered Cytokine, structurally similar to IL-1, with profound effects on T-cell activation.

Formerly called interferon (IFN) gamma inducing factor (IGIF), IL-18 is a novel cytokine that plays an important role in the T-cell-helper type 1 (Th1) response, primarily by its ability to induce IFN gamma production in T cells and natural killer (NK) cells.

In terms of structure, IL-18 and IL-1 beta share primary amino acid sequences of the so-called „signature sequence“ motif and are similarly folded as all beta pleated sheet molecules. Also similar to IL-1 beta, IL-18 is synthesized as a biologically inactive precursor molecule lacking a signal peptide which requires cleavage into an active, mature molecule by the intracellular cysteine protease called IL-1 beta-converting enzyme (ICE, caspase-1). Therefore inhibitors of ICE activity may limit the biologic activity of IL-18 and may be useful as Th1 immunosuppressive agents.

The activity of the mature IL-18 is closely related to that of IL-1. IL-18 induces gene expression and synthesis of tumor necrosis factor (TNF), IL-1, Fas Ligand, and several chemokines. IL-18 induces the differentiation of Th1 or Th2 cells depending upon cytokine milieu and genetic background.

The activity of IL-18 is via an IL-18 receptor (IL-18R) complex. This complex is made up of a binding chain termed IL-18R₁, a member of the IL-1 receptor family previously identified as the IL-1 receptor-related protein (IL-1Rrp), and a signaling chain, also a member of the IL-1R family.

The IL-18R complex recruits the IL-1R – activating kinase (IRAK) and TNF-R – associated factor –6 (TRAF-6) with subsequent activation of NF kappa B.

Thus based on primary structure, three-dimensional structure, receptor family, signal transduction pathways and biological effects, IL-18 appears to be a new member of the IL-1 family.

Similar to IL-1, IL-18 participates in both innate and acquired immunity.

Constitutive IL-18 expression is detected from many different cells, including macrophages, keratinocytes, and osteoblasts.

3 Principles of the Test

An anti-pig IL-18/IL-1F4 coating antibody is adsorbed onto microwells.

Pig IL-18/IL-1F4 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-pig IL-18/IL-1F4 antibody is added and binds to pig IL-18/IL-1F4 captured by the first antibody.

Following incubation unbound biotin-conjugated anti-pig IL-18/IL-1F4 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-pig IL-18/IL-1F4 antibody.

Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 1

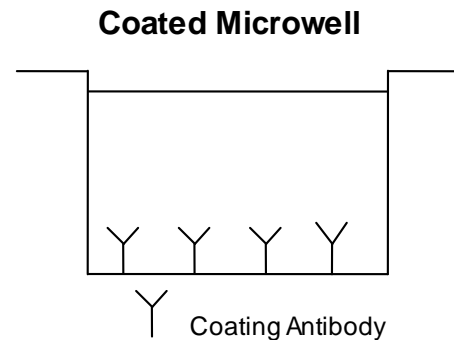


Figure 2

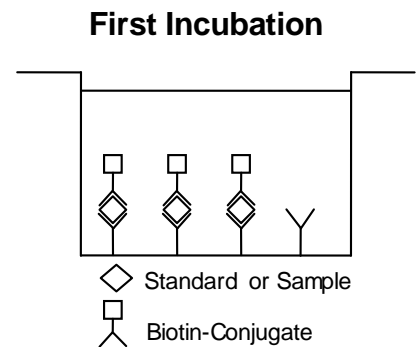


Figure 3

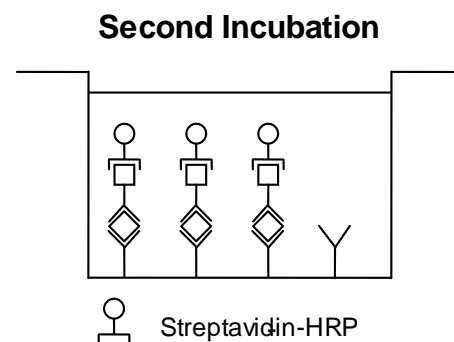
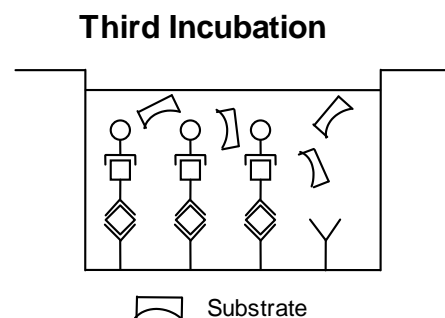
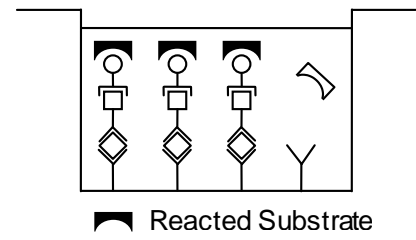


Figure 4



A coloured product is formed in proportion to the amount of pig IL-18/IL-1F4 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 pig IL-18/IL-1F4 standard dilutions and pig IL-18/IL-1F4 sample concentration determined.

Figure 5



4 Reagents Provided

4.1 Reagents for pig IL-18/IL-1F4 ELISA NBP1-94192 (96 tests)

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to pig IL-18/IL-1F4
- 1 vial (70 µl) **Biotin-Conjugate** anti-pig IL-18 monoclonal antibody
- 1 vial (150 µl) **Streptavidin-HRP**
- 2 vials pig IL-18/IL-1F4 **Standard** lyophilized, 5 ng/ml upon reconstitution
- 1 vial (12 ml) **Sample Diluent**
- 1 vial (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20, 10% BSA)
- 1 bottle (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
- 4 **Adhesive Films**

4.2 Reagents for pig IL-18/IL-1F48 ELISA NBP1-94192 (10x96 tests)

- 10 aluminium pouches with a **Microwell Plate coated** with monoclonal antibody to pig IL-18/IL-1F4
- 10 vials (70 µl) **Biotin-Conjugate** anti-pig IL-18 monoclonal antibody
- 10 vials (150 µl) **Streptavidin-HRP**
- 10 vials pig IL-18/IL-1F4 **Standard** lyophilized, 5 ng/ml upon reconstitution
- 7 vials (12 ml) **Sample Diluent**
- 2 vials (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20, 10% BSA)
- 4 bottles (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
- 10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (100 ml) **Stop Solution** (1M Phosphoric acid)
- 20 **Adhesive Films**

5 Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

6 Specimen Collection and Storage Instructions

Cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive pig IL-18/IL-1F4. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 μ l to 1000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ l adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

8 Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9 Preparation of Reagents

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.

If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate (20x)** into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate (20x)** into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

9.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.5 Pig IL-18/IL-1F4 Standard

Reconstitute **pig IL-18/IL-1F4 standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 5000 pg/ml). Allow the reconstituted standard to sit for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded.

Sample Diluent serves as blank.

10 Test Protocol

- a. 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. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- b. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**
- c. **Standard dilution on the microwell plate:**
Add 100 µl of Sample Diluent in duplicate to all **standard wells**. Pipette 100 µl of prepared **standard** (see Preparation of Standard 9.5, concentration = 5000 pg/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 2500 pg/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 6). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of pig IL-18/IL-1F4 standard dilutions ranging from 2500.0 to 39.1 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 6

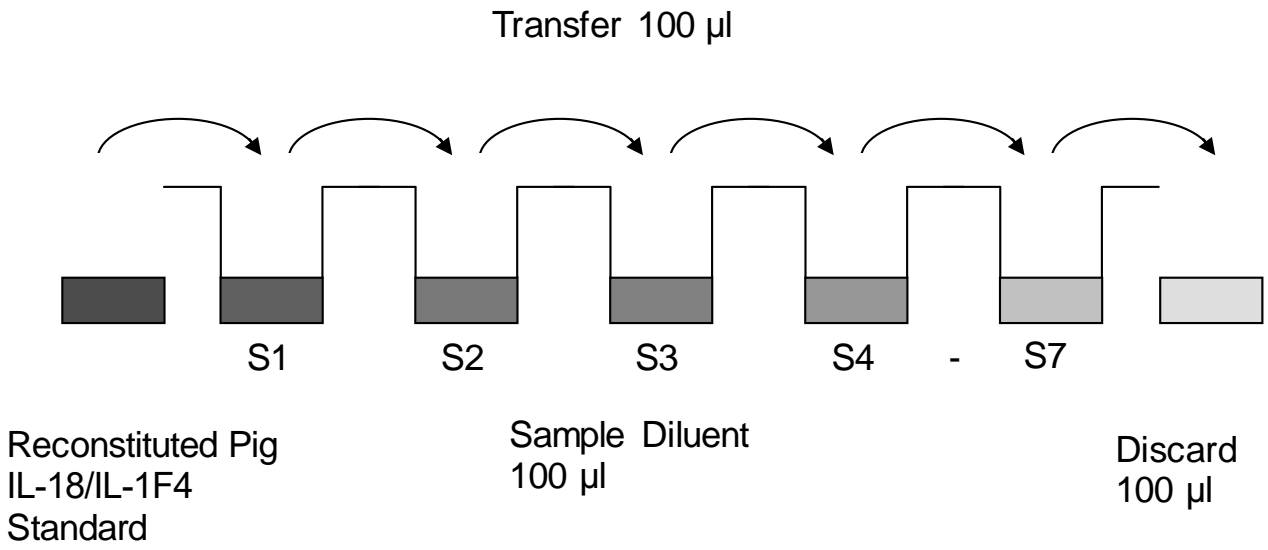


Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (2500.0 pg/ml)	Standard 1 (2500.0 pg/ml)	Sample 1	Sample 1
B	Standard 2 (1250.0 pg/ml)	Standard 2 (1250.0 pg/ml)	Sample 2	Sample 2
C	Standard 3 (625.0 pg/ml)	Standard 3 (625.0 pg/ml)	Sample 3	Sample 3
D	Standard 4 (312.5 pg/ml)	Standard 4 (312.5 pg/ml)	Sample 4	Sample 4
E	Standard 5 (156.3 pg/ml)	Standard 5 (156.3 pg/ml)	Sample 5	Sample 5
F	Standard 6 (78.1 pg/ml)	Standard 6 (78.1 pg/ml)	Sample 6	Sample 6
G	Standard 7 (39.1 pg/ml)	Standard 7 (39.1 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- d. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
- e. Add 50 µl of **Sample Diluent** to the **sample wells**.
- f. Add 50 µl of each **sample** in duplicate to the **sample wells**.
- g. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).
- h. Add 50 µl of **Biotin-Conjugate** to all wells.
- i. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 400 rpm.
- j. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.4).
- k. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point b. of the test protocol. Proceed immediately to the next step.
- l. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- m. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 400 rpm.
- n. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point b. of the test protocol. Proceed immediately to the next step.
- o. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- p. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The

substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

- q. Stop the enzyme reaction by quickly pipetting 100 μ l of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- r. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

11 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the pig IL-18/IL-1F4 concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating pig IL-18/IL-1F4 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding pig IL-18/IL-1F4 concentration.
- **If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low pig IL-18/IL-1F4 levels. Such samples require further external predilution according to expected pig IL-18/IL-1F4 values with Sample Diluent in order to precisely quantitate the actual pig IL-18/IL-1F4 level.**
- It is suggested that each testing facility establishes a control sample of known pig IL-18/IL-1F4 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 7. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 7

Representative standard curve for pig IL-18/IL-1F4 ELISA. Pig IL-18/IL-1F4 was diluted in serial 2-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.

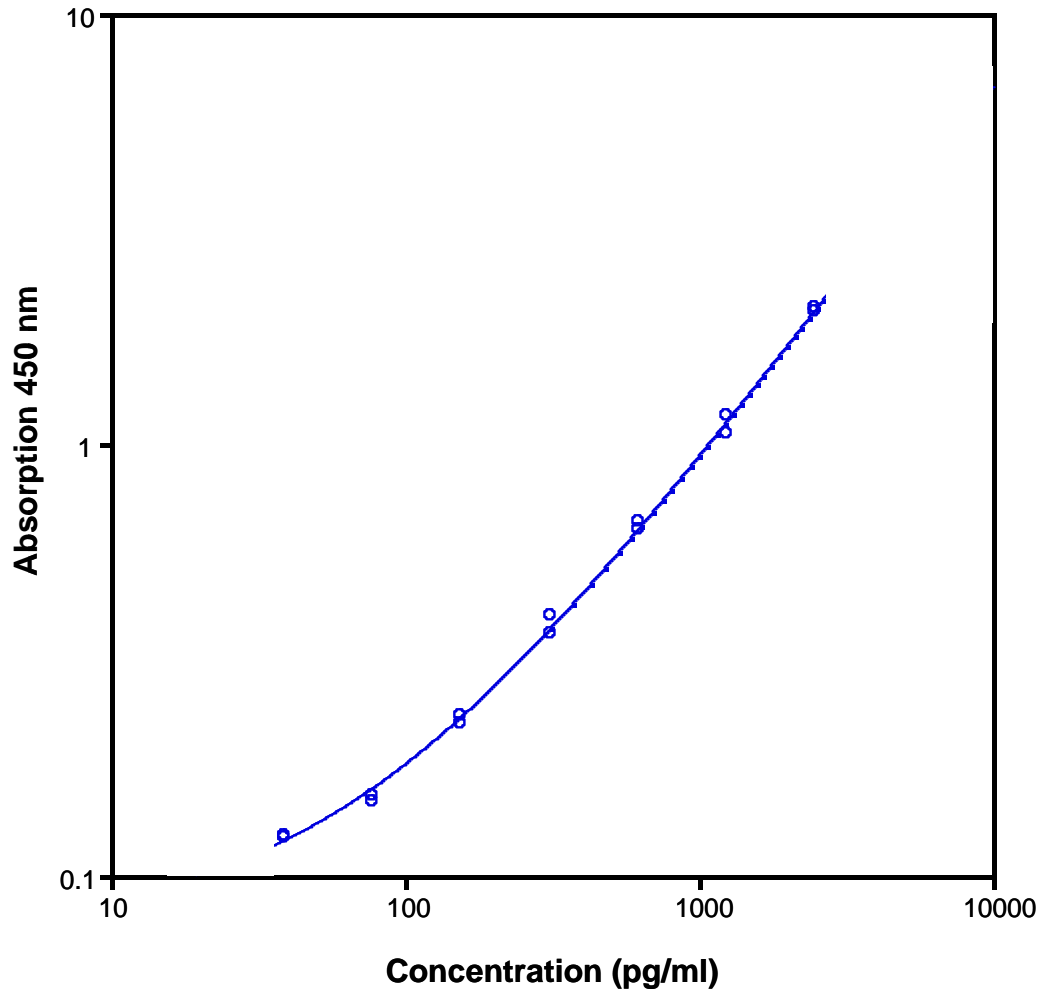


Table 2

Typical data using the pig IL-18/IL-1F4 ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Pig IL-18/IL-1F4 Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	2500.0	2.088 2.035	2.062	1.5
2	1250.0	1.173 1.060	1.117	5.8
3	625.0	0.665 0.636	0.651	2.6
4	312.5	0.400 0.365	0.383	5.3
5	156.3	0.234 0.226	0.231	2.0
6	78.1	0.153 0.148	0.151	1.9
7	39.1	0.124 0.122	0.124	0.9
Blank	0.0	0.080 0.077	0.079	2.7

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

13 Performance Characteristics

13.1 Sensitivity

The limit of detection of pig IL-18/IL-1F4 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 23 pg/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 4 serum samples containing different concentrations of pig IL-18/IL-1F4. 2 standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was 6.8%.

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 4 serum samples containing different concentrations of pig IL-18/IL-1F4. 2 standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was 8.1%.

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of pig IL-18/IL-1F4 into different normal pig serum samples. Recoveries were determined in 3 independent experiments with 6 replicates each. The unspiked serum was used as blank in these experiments. The overall mean recovery was 110%.

13.4 Dilution Parallelism

4 serum samples with different levels of pig IL-18/IL-1F4 were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 91% to 109% with an overall recovery of 96%.

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the pig IL-18/IL-1F4 levels determined. There was no significant loss of pig IL-18/IL-1F4 immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the pig IL-18/IL-1F4 level determined after 24 h. There was no significant loss of pig IL-18/IL-1F4 immunoreactivity detected during storage at -20°C, 2-8°C and RT. A significant loss of pig IL-18/IL-1F4 immunoreactivity (20%) was detected during storage at 37°C after 24 h.

13.6 Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a pig IL-18/IL-1F4 positive serum. There was no crossreactivity detected.

14 Reagent Preparation Summary

14.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate 20x** (50 ml) to 950 ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

14.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate 20x** (5 ml) to 95 ml distilled water.

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

14.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Assay Buffer (1x):

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

14.4 Streptavidin-HRP

Make a 1:100 dilution of **Streptavidin-HRP** in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

14.5 Pig IL-18/IL-1F4 Standard

Reconstitute lyophilized **pig IL-18/IL-1F4 standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

15 Test Protocol Summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
4. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
5. Add 50 µl Sample Diluent to sample wells.
6. Add 50 µl sample in duplicate, to designated sample wells.
7. Prepare Biotin-Conjugate.
8. Add 50 µl Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C).
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 3 times with Wash Buffer.
12. Add 100 µl diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
14. Empty and wash microwell strips 3 times with Wash Buffer.
15. Add 100 µl of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
17. Add 100 µl Stop Solution to all wells.
18. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 2).