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IFNG
Monkey Interferon gamma ELISA Kit

Catalog No: CKM001 **Size:** 5 x 96 tests

Assay range:	5-320 pg/ml
Sensitivity:	2 pg/ml
Specificity:	African green monkey Baboon Cynomolgus monkey Marmoset Pig-tailed macaque Rhesus macaque
Calibration:	Recombinant rhesus macaque IFN-gamma
Type of sample:	Plasma, serum, culture supernatant

Introduction

Cytokines are a group of regulatory proteins critically involved in many physiological processes such as immune recognition, cell differentiation and cell proliferation. They have been identified in many vertebrate species and are produced by a variety of different cell types. Cytokines are usually produced transiently and locally, acting in a paracrine or autocrine manner. They interact with high affinity cell surface receptors specific for each cytokine or cytokine group and are active at very low concentrations mostly in the picogram range.

It is well known now that the type of an antigen-specific immune response largely depends on the selection or preferential activation of defined CD_4^+ T-cell subsets (i.e. T_h1 and T_h2). Activation of these subsets is characterized by the secretion of distinct patterns of cytokines. T_h1, but not T_h2 cells, primarily secrete IL2 and IFN-gamma while T_h2, but not T_h1 cells, produce IL4, IL5, IL6, IL10 and IL13. Other cytokines, such as TNF-alpha and GM-CSF are produced by both T_h subsets. In addition, the production of IL12 and IL10, produced by antigen presenting cells (APC) such as macrophages and dendritic cells, critically contributes to the preferential expansion of T_h1- or T_h2 -type cells. For instance, early production of IL12 is considered essential for the development of T_h1 cells. On the other hand, the absence or low concentrations of IL12 and IFN-gamma in the early phase of an immune response and concomitant production of IL4 by cells of the mast cell/basophil lineage or T-cells themselves is known to favor the development of T_h2 cells. In addition to their regulatory effects on T_h subset differentiation, the cytokines released by the two types of T_h cells also produce distinct effector functions. For instance, IL4 and IFN-gamma have differential or antagonistic activities on immunoglobulin isotype selection or Major Histocompatibility Complex (MHC) class II expression. Therefore, the properties of an immune response can be best studied by determining the amounts of cytokines produced by the responding T-cells and APC.

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Reagents and materials supplied with the kit:

Items	Quantity (5 Plates)	Storage conditions
Coating antibodies	1 vial	2-4°C
Cytokine standard	5 vials	2-4°C
Biotinylated detection antibodies	1 vial	2-4°C
Streptavidin-HRP polymer (SPP)	1 vial	≤-20°C
TMB substrate tablets	5	2-4°C
Substrate buffer capsules	5	room temperature
BSA stock solution (10%)	2 vials (24 ml)	2-4°C
Cytokine stabilization buffer (CSB*)	1 vial (5 ml)	2-4°C
Tween-20	1 vial (5 ml)	room temperature
ELISA plates (not pre-coated)	8	room temperature
Adhesive cover slips	10	room temperature

* For serum and plasma samples only; see under Test samples and standards.

Hazard Information:

Tetramethylbenzidine (TMB) substrate tablets are classified as irritant according to Directive 67/548/EC and its amendments.
Risk phrase: R36 Safety phrase: S26
In case of contact with eyes, wash with plenty of water for at least 15 minutes. Assure adequate flushing by separating the eyelids and seek medical advice immediately. Upon ingestion or contact with skin, rinse mouth (if person is conscious) or wash skin with soap and water and remove contaminated clothing and shoes.
Other kit components are not classified as dangerous according to Directive 67/548/EC or 1999/45/EC and their amendments.

Storage of Kit Reagents

- The vials with lyophilized coating antibodies and biotinylated detection antibodies can be safely stored at 2-4°C until the expiration date (indicated on the vials). After reconstitution, the antibodies remain fully active for at least 6 months at 2-4°C when kept sterile. However, it is strongly recommended to divide the reconstituted antibody solutions into small aliquots for single use. These aliquots should be stored at ≤-20°C. Under these conditions the antibodies are stable for at least one year.

- The lyophilized SPP conjugate should be stored at ≤-20°C. After reconstitution, SPP is stable for at least 2 months at 2-4°C. It rapidly loses activity upon storage at room temperature. It is strongly recommended that after reconstitution, the solution is divided into small aliquots for single use. These aliquots should be stored at ≤-20°C. Under these conditions, SPP is stable for at least 1 year.

Materials/reagents required but not provided:

- Phosphate buffer (PB) stock: dissolve 96.0 g $Na_2HPO_4.2H_2O$ + 17.5 g KH_2PO_4 in 1.0 L distilled water and adjust pH to 7.4.
- Sterile distilled water
- H_2SO_4
- Dimethyl sulfoxide (DMSO)
- Pipetting devices for the accurate delivery of volume required for the assay performance
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.)
- Reading device for microtiter-plate set to 370, 450 and/or 655 nm

Working solutions:

- Phosphate-buffered saline (PBS): add 10 ml PB stock and 8.8 g NaCl to 1 L distilled water. Adjust pH to 7.4. Alternatively, use commercially available liquid PBS.

  Do not use commercially available PBS <u>tablets</u> for the preparation of the coating solution. (The filler in the tablets interferes with the coating process).
- PBS containing 0.05% Tween-20 (PBST): 0.5 ml Tween-20 dissolved in 1 L PBS.
- PBST containing 0.5% BSA (PBST-B): 2 ml BSA stock solution (10%) added to 38 ml PBST.
- Blocking buffer: 2 ml BSA stock solution (10%) added to 18 ml PBS (for 1 ELISA plate).
- Substrate buffer: Dissolve the contents of one capsule in 100 ml distilled water (takes approximately 5 minutes). For optimal performance, the buffer solution should be used within 60 minutes.
- Stopping solution: 2 M H_2SO_4

General procedure

<u>Coating antibody</u>

- Reconstitute the lyophilized antibodies by injecting 250 µl sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 2 minutes at room temperature. Avoid vigorous shaking. To coat 96 wells of an ELISA plate, pipette 50 µl out of the vial (or use a frozen aliquot of 50 µl; see '**Storage of Kit Reagents**') and add to 5 ml PBS. Mix gently.
- Add 50 µl diluted antibody to each well of the ELISA plate and fill up to 100 µl with PBS.
- Seal the plate to prevent evaporation. Incubate overnight at 2-4°C or alternatively 1 to 2 hours at 37°C.

<u>Blocking</u>

- Remove the coating antibody solution and wash the wells at least six times with PBST.
- Add 200 µl blocking buffer.

- Seal the plate and incubate at 37°C for 1 hour.

Test samples and standards

- Remove the blocking buffer but do not wash.
- Add 1/20 volume of CSB to serum or plasma samples but not to other samples such as cell culture supernatants; CSB inhibits the degradation of cytokines in pure serum or plasma.
- Dilute standards and test samples in appropriate diluent (see '**Cytokine standards**').
- Add 100 µl to each well.
- Seal the plate and incubate at 37°C for 2 hours or overnight at 2-4°C.

Biotinylated Detection antibody

- Remove test samples/standards and wash at least six times with PBST.
- Reconstitute the lyophilized antibodies by injecting 0.5 ml sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 2 minutes at room temperature. Avoid vigorous shaking. Pipette 100 µl out of the vial (or use a frozen aliquot of 100 µl; see '**Storage of Kit Reagents**') and add to 10 ml PBST-B. Mix gently.
- Add 100 µl diluted antibody solution to each well.
- Seal the plate and incubate at 37°C for 1 hour.

SPP conjugate

- Remove detection antibody solution and wash at least six times with PBST.
- Reconstitute the contents of the vial by injecting 0.5 ml sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 1 minute at room temperature. Avoid vigorous shaking. Pipette 100 µl out of the vial (or use a frozen aliquot of 100 µl; see '**Storage of Kit Reagents**') and add to 10 ml PBST-B. Mix gently.
- Add 100 µl to each well.
- Seal the plate and incubate at 37°C for 1 hour.

Substrate

- Remove SPP conjugate and wash at least six times with PBST.
- Dissolve one TMB tablet in 1.0 ml DMSO (vortex at high speed for 5 minutes to complete dissolution) and then add this to 10 ml substrate buffer.
- Mix thoroughly and immediately dispense 100 µl into each well. Leave the plate on the laboratory bench at room temperature (color development between 10 and 30 minutes). This substrate produces a soluble end product that is blue in color and can be read spectrophotometrically at 370 or 655 nm. The reaction can be stopped by adding 50 µl of 2M H_2SO_4 (resulting in a yellow solution that can be read at 450 nm).

Cytokine standards

For maximum recovery, the vial with lyophilized cytokine standards should be reconstituted in 0.5 ml distilled water and allowed to stand for 1 minute at room temperature. Thereafter, the reconstituted cytokine standard should be placed on melting ice and diluted immediately as indicated below (preferably within one hour). Use vials with cytokine standards only once.

Please note that temperature of buffers and standard solution(s) should now be kept at 2-4°C until use in the ELISA.

The total amount of the cytokine standard is indicated on the label of the vial (ng/vial). After reconstitution in 0.5 ml water, the concentration (ng/ml) will become twice the amount on the label (e.g. amount on label is 4.8 ng/vial; after reconstitution, the concentration becomes 9.6 ng/ml = 9600 pg/ml).

Dilute the standard stock solution to 320 pg/ml in PBST-B (highest concentration cytokine to be used in the standard range). The linear region of cytokine standard curve is now obtainable in a series of two-fold dilutions in PBST-B ranging from 320 to 5 pg/ml. Always include a blank control (PBST-B only) in the standard range.

Before establishing the standard curve, the Optical Density (OD) value of the blank control (OD.bl) is subtracted from the measured OD values of the different standard solutions. The standard curve is now plotted as the standard cytokine concentration versus the corresponding (measured) OD value minus OD.bl. In addition, the actual OD values of the test samples are determined by subtracting OD.bl from the measured OD values.

The concentration of the cytokine in the test sample can then be interpolated from the standard curve. It is useful to prepare a series of dilutions of the unknown test sample to assure that the OD will fall in the linear portion of the standard curve.

Note 1: The OD value measured for the blank control (OD.bl) must be below 0.2.

Note 2: For measuring cytokines in cell culture supernatant, samples should be diluted in PBST-B. However, when measuring cytokines in pure serum or plasma, the diluent for the standard and blank control should preferably be control serum or plasma originating from the same species.

Directions for washing

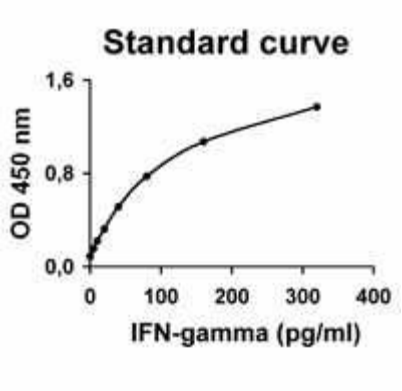
- Incomplete washing will adversely affect the assay. All washing must be performed with wash buffer (PBST).
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into each well. After aspiration, fill the wells with at least 300 µl wash buffer. Let soak for 10 to 20 seconds, then aspirate the liquid. Repeat as directed under '**General procedure**'. After washing, invert the plate and tap dry on absorbent paper.

- Alternatively, the wash buffer may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After washing, invert the plate and tap dry on absorbent paper.
- If using an automated washing device, the operating instructions should be carefully followed.

Troubleshooting

- Poor consistency of replicates can be overcome by increasing the stringency of washes, particularly after the incubation step with detection antibody.
- High values of the blank control (optical density > 0.2) can be overcome by shortening the incubation time with the substrate solution. High OD.bl values may also be caused by improper washing procedures.
- Inconsistent replicates may be due to cross-contamination of wells by improper pipetting procedures.
- If no signal is observed in the wells with the standards:
    - Try a new vial with cytokine standard.
    - Check the pH of the substrate solution (between 5.0 and 5.5).
    - Verify whether the antibody, SPP conjugate and standard preparations were properly diluted.
- Avoid sodium azide in wash buffers and diluents, as this is an inhibitor of peroxidase activity.
- Storage of reconstituted SPP at room temperature for several days can lead to a significant loss of SPP activity and consequently low OD readings.

Typical Data



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