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Human IL-21 ELISA Kit

Catalog No: CKR062 **Size:** 5 x 96 tests

Range:	5 - 320 pg/ml
Sensitivity:	5 pg/ml
Specificity:	Native and Recombinant Human IL-21
Calibration:	Recombinant Human IL-21
Type of sample:	Cell culture supernatant

Introduction

Cytokines and chemokines are a group of signaling proteins critically involved in various physiological processes such as immune regulation, cell differentiation, cell proliferation, and chemotaxis. They are produced by a variety of different cell types in many vertebrate species and are active at very low concentrations mostly in the picogram to femtogram range. Cytokines are usually produced transiently and locally, acting in a paracrine or autocrine manner and interact with high affinity cell surface receptors specific for each cytokine or cytokine group. Chemokines are a family of small cytokines (with four conserved cysteine residues) that can mediate chemoattraction between cells. Some chemokines are homeostatic in nature and are constitutively produced and secreted, whereas others are considered inflammatory and are only produced by cells during infection or a pro-inflammatory stimulus. Another important group of signaling proteins involved in the immune system includes perforin and granzymes (serine proteases), which are linked with cell apoptosis.

The ELISA assay is a simple and sensitive sandwich immunoassay for the determination of cytokine, chemokine and granzyme levels in biological fluids such as cell culture supernatant, plasma or serum. The assay utilizes a coating antibody specific for the analyte of interest (e.g. cytokine, chemokine or granzyme) coated on the wells of a 96-well microtiter plate. The wells are washed and blocked. Standards and samples are added to the wells and any analyte present binds to the immobilized coating antibody. After washing off excess/unbound materials, the bound analyte is allowed to associate with a biotinylated detection antibody. The wells are washed again and a streptavidin-HRP (SPP) conjugate is added to the antibody-antigen-antibody complex.

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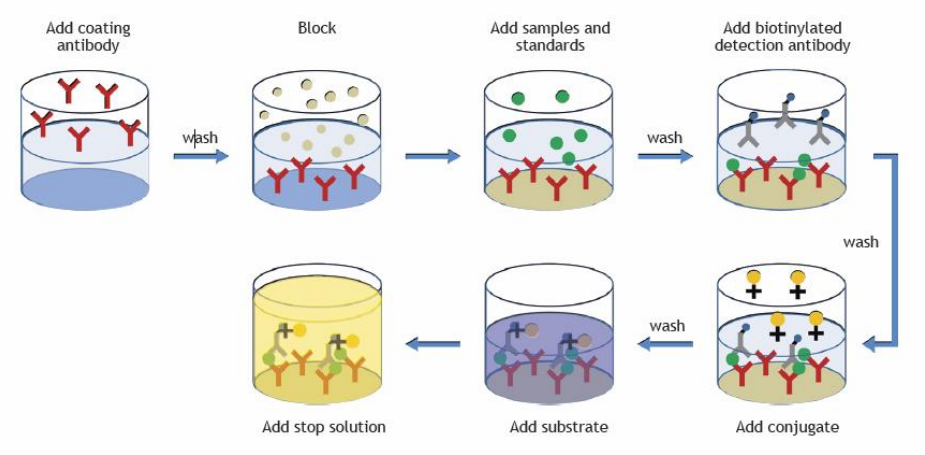


After another wash, a chromogenic substrate (TMB) is introduced, which produces a blue-colored product of which the intensity is related to the amount of analyte in the sample. The optical density (OD) can now be read at 370 or 655 nm (not recommended as the OD will change over time). A sulfuric acid solution is added to stop the enzymatic reaction (changing the color to yellow) and OD is read at 450 nm.

Reagents and materials supplied with the kit:

Items	Quantity (5 Plates)	Storage conditions
Coating antibody (lyophilized)	1 vial	4°C
Standard (lyophilized)	5 vials	4°C
Biotinylated detection antibody (lyophilized)	1 vial	4°C
SPP conjugate (Streptavidin-HRP polymer) (lyophilized)	1 vial	-20°C to -80°C in the dark
BSA Stock solution (10%)	2 vials (2 x 12 ml)	4°C
Cytokine stabilization buffer (CSB)*	1 vial (5 ml)	4°C
Tween-20	1 vial (5 ml)	Room temperature in the dark
TMB substrate solution	2 vials (2 x 30 ml)	4°C in the dark
Stop solution (0.175 M H_2SO_4)	2 vials (2 x 30 ml)	4°C
ELISA plates (not pre-coated)	8	Room temperature
Adhesive cover slips	10	Room temperature

* For use with serum and plasma samples only, see section “Preparation of Samples”.

Warnings and Precautions

- This kit is designed for research use only, and not for use in diagnostic or therapeutic procedures.
- When cytokine, chemokine or granzyme levels are determined in blood components or other biological materials, then please note that all these materials should be considered as potentially infectious and handled with the usual precautions under Bio-Hazard conditions. Follow universal precautions such as established by

the US government agencies, Centers for Disease Control and Prevention and Occupational Safety and Health Administration, when handling and disposing of (potentially) infectious waste.

Hazard Information:

TMB (tetramethylbenzidine) is irritating to eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water for at least 15 minutes, assuring adequate flushing by separating eyelids, and seek medical help. In case of ingestion or contact with skin, rinse mouth (if person is conscious) or wash skin with soap and water, remove contaminated clothing and shoes, and seek medical help.

Storage and Stability:

- The vials with lyophilized coating and biotinylated detection antibody can be safely stored at 4ºC until the expiration date. After reconstitution, the antibodies are stable for at least 12 months at 4ºC when kept sterile. However, it is recommended to divide the reconstituted antibody solutions into small aliquots for single use. These aliquots should be stored at -20ºC to -80ºC (stable for at least two years).
- The vials with lyophilized standards can be safely stored at 4ºC until the expiration date. These vials are for single use only.
- The vial with lyophilized SPP conjugate is stable until the expiration date when stored at -20ºC to -80ºC in the dark. After reconstitution, the reagent is stable for at least 2 months at 4ºC when kept sterile and protected from light. However, it is strongly recommended to divide the solution into small aliquots for single use. These aliquots should be stored at -20ºC to -80ºC in the dark (stable for at least one year).
- The ready-to-use TMB substrate solution should be stored at 4ºC and is stable until the expiration date. Avoid exposure to light, heat and contamination with metal ions or peroxidase.
- The vials with BSA stock solution and Cytokine stabilization buffer can be safely stored at 4ºC until the expiration date. After opening, these solutions are stable for at least 6 months when kept sterile.
- Tween-20 should be stored at room temperature (RT) and is stable until the expiration date.
- The ready-to-use Stop solution can safely be stored at 4ºC and is stable until the expiration date.

Materials/reagents required but not provided:

- PBS, pH 7.4.
- Sterile distilled water.
- Pipetting devices for the accurate delivery of volume required for the assay performance.
- Tubes and containers/plates to make solutions.
- 37°C incubator.
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc).
- Reading device for microtiter-plate set to 370, 450 and/or 655 nm.

Specimen Collection and Handling:

Specimens should be clear, non-hemolyzed and non-lipemic. Excessive hemolysis and the presence of large clots or microbial growth in the sample may interfere with the performance of the test.

- **Cell culture supernatant:** remove any particulate matter by centrifugation.
- **Serum:** use a clot tube and allow sample to clot for 30-45 min at RT, then centrifuge for 10-15 min at 1,000 to 2,000 x g (RT) and collect serum immediately.
- **Plasma:** collect plasma by using anticoagulant, such as EDTA or heparin. Mix well immediately after collection. Centrifuge for 10-15 min at 1,000-2,000 x g at RT and collect plasma.

Samples should be aliquoted and stored frozen at -20ºC to -80ºC to prevent cytokine degradation. If samples are run within 24 hours, they may be stored at 2-8 ºC. Avoid repeated freeze-thaw cycles. Do not heat serum or plasma samples. Prior to assay, frozen samples should be completely thawed and mixed well.

Note: Specimen collection from humans and non-human primates should be carried out in accordance with the Clinical and Laboratory Standards Institute document M29-T2, "Protection of laboratory workers from infectious diseases transmitted by blood and tissue".

Preparation of Solutions and Reagents:

PBS

- PB stock: dissolve 96.0 g of $Na_2HPO_4 \cdot 2H_2O$ plus 17.5 g of KH_2PO_4 in 1 L distilled water, adjust pH to 7.4 and filter solution (0.2 µm). Store solution at RT. The PB stock is stable for at least 6 months when kept sterile.
- PBS: add 10 ml of the PB stock and 8.8 g of NaCl to 1 L distilled water. It is strongly recommended to prepare PBS freshly each day. Alternatively, when PBS is prepared in advance, the solution should be filter sterilized (0.2 µm) or autoclaved.

Wash buffer

- PBS containing 0.05% Tween-20 (add 0.5 ml of Tween-20 to 1 L PBS). The volume is dependent on the washing procedure (manual or automatic washing). For directions, see below.

Blocking buffer

- PBS containing 1% BSA. For one ELISA plate, 2 ml BSA stock solution (10%) is gently but thoroughly mixed with 18 ml PBS.

Dilution buffer

- PBS containing 0.5% BSA and 0.05% Tween-20. You can prepare this buffer at once for 5 ELISA plates with at least 250 ml PBS under sterile conditions. Add 12.5 ml of BSA stock solution (10%) and 125 µl of Tween-20 to 250 ml PBS, mix gently and store at 4ºC. This solution will be stable for at least one month when kept sterile.
- For one ELISA plate, you will need 20 ml of Dilution buffer for detection and conjugate solutions, and at least 20 ml for standards and samples (this volume will depend on the number of sample dilutions).

Coating antibody

- Reconstitute the lyophilized antibody by injecting 250 µl of sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 min at RT. Avoid vigorous shaking.
- For one ELISA plate: 50 µl is gently but thoroughly mixed with 5 ml PBS.
- **Note:** Do not use commercially available PBS **tablets** for the preparation of the coating solution (the filler in the tablets interferes with the coating process).

Standard

- For maximum recovery, the vial with lyophilized standard should be reconstituted in 500 µl sterile distilled water, gently mixed for approximately 15 seconds, and allowed to stand for 5 min at RT. Avoid vigorous shaking. Thereafter, the reconstituted standard (stock solution) is placed on melting ice and is immediately diluted as indicated below (preferentially within one hour).
- Note: The quantity (expressed in ng/vial) of the Standard is indicated on the vial and is variable for each kit and batch. After reconstitution, the concentration can be calculated as follows: divide the quantity (indicated on the vial) by the volume used for reconstitution. For example, the concentration of a standard containing 5 ng/vial will be 10 ng/ml (= 10,000 pg/ml) after reconstitution in 0.5 ml distilled water.

Biotinylated detection antibody

- Reconstitute the lyophilized antibody by injecting 500 µl of sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 min at RT. Avoid vigorous shaking.
- For one ELISA plate: 100 µl is gently and thoroughly mixed with 10 ml Dilution buffer.

SPP conjugate

- Reconstitute the contents of the vial by injecting 500 µl of sterile distilled water into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 5 min at 4 ºC in the dark. Avoid vigorous shaking.
- For one ELISA plate: 100 µl is gently and thoroughly mixed with 10 ml Dilution buffer.

TMB substrate solution (ready-to-use)

- Bring TMB substrate solution to RT prior to use.

Stop solution (ready-to-use)

- Bring Stop solution to RT prior to use.

Sample Preparation

- Dilute samples in Dilution buffer (at least 1:1). We recommend analyzing a series of dilutions of the sample to ensure that sample measurements fall within the standard curve range (see also section "Interpretation of Results").
- When measuring cytokines in serum or plasma, add 1/20 volume of CSB (ready-to-use) to **pure** serum or plasma sample (CSB is not required for other samples such as cell culture supernatant) before further dilution in Dilution buffer. CSB inhibits the degradation of cytokines.
- It is recommended to analyze samples in triplicate.

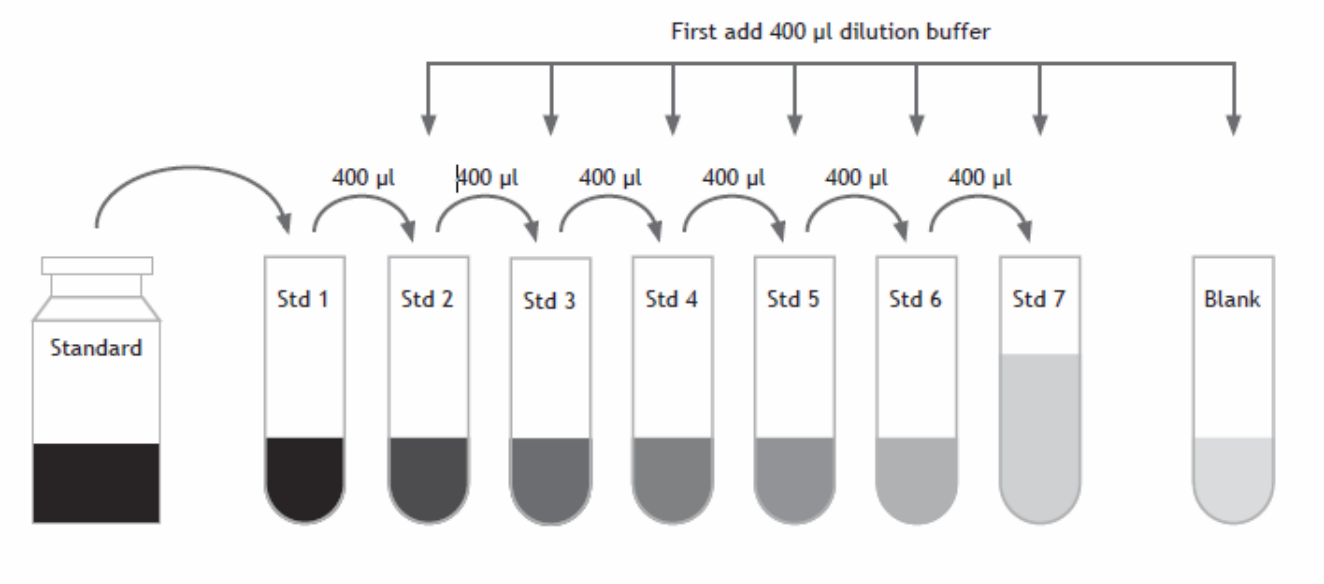
Preparing the Standards

With a standard curve, the analyte concentrations in the unknown samples can be determined. The standard curve is generated from the data of 7 two-fold serial dilutions (Std 1-7) of the Standard. The recommended assay range for each specific ELISA kit can be seen in the Specifications Table at the top of the first page. It is recommended to test the standard dilutions (Std 1-7) in triplicate.

- Take 8 tubes and add 400 µl Dilution buffer to 7 of these tubes (Std 2 to Std 7 and Blank).
- Prepare in the remaining tube (Std 1) the highest concentration to be used in the standard curve (see Specifications Table) by mixing an appropriate volume of Standard with Dilution buffer. The final volume of Std 1 should be 800 µl. Allow the mixture to stand for at least 15 seconds before using in further dilutions.
- Perform two-fold serial dilutions: transfer 400 µl diluted standard from Std 1 to the next tube (Std 2), mix well and repeat this step until Std 7 (see figure on next page).

Notes:

- If less than 10 µl of the Standard is needed to make Std 1, it is recommended to dilute the Standard 10 times in Dilution buffer (mix 10 µl Standard with 90 µl Dilution buffer) and use this to make Std 1.
- A standard curve, including blank, should be run on each ELISA plate.
- Use vials with Standard only once.
- It is recommended to test standard dilutions in triplicate.
- Standard dilutions should be used as soon as possible (preferentially within one hour).



Directions for washing

- Incomplete washing of the wells will adversely affect the assay. All washing steps should be performed with Wash buffer.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering the tip of an aspiration device into each well (without touching the bottom). After aspiration, fill the wells with at least 250 µl Wash buffer and then aspirate the liquid. Repeat these steps at least six times. After washing, the plate is inverted and tapped dry on absorbent tissue paper. Alternatively, the Wash buffer may be put into a squirt bottle. If a squirt bottle is used, empty the wells and flood the plate with Wash buffer, completely filling all wells. Repeat these steps at least six times. After washing, the plate is inverted and tapped dry on absorbent tissue paper.
- When using an automated washing device, follow operating instructions carefully.

Assay procedure

Note: All solutions should reach RT prior to use.

1. Add 50 µl of diluted coating antibody solution to each well of the ELISA plate and fill up to 100 µl with PBS. Seal the plate to prevent evaporation.

2. Incubate overnight at 4ºC (or alternatively 2 hours at 37ºC).

3. Remove the coating antibody solution and wash the wells at least 6 times with Wash buffer.

4. Add 200 µl of Blocking buffer to each well.

5. Seal the plate and incubate for 1 hour at 37ºC.

6. Remove the Blocking buffer (do not wash the wells).

7. Add 100 µl of diluted standards, blank, or samples to each well.

8. Seal the plate and incubate for 2 hours at 37ºC (or alternatively overnight at 4ºC).

9. Remove standards/samples and wash the wells at least 6 times with Wash buffer.

10. Add 100 µl of diluted detector antibody solution to each well.

11. Seal the plate and incubate for 1 hour at 37ºC.

12. Remove detection antibody solution and wash the wells at least 6 times with Wash buffer.

13. Add 100 µl of diluted SPP conjugate to each well.

14. Seal the plate and incubate for 1 hour at 37ºC.

15. Remove SPP conjugate and wash the wells at least 6 times with Wash buffer.

16. Add 100 µl of TMB substrate solution into each well.

17. Leave the plate for 20 min at RT in the dark. (The substrate produces a soluble end product that is blue in color and can be read at 370 or 655 nm.)

18. After the substrate incubation, do not empty the wells. Stop the reaction by adding 100 µl of Stop solution (resulting in a yellow solution) and read the plate at 450 nm within 30 minutes.

Interpretation of Results

In each experiment, a standard curve needs to be run, consisting of 7 standard dilutions, from which a concentration-response relationship is generated. Construct the standard curve by plotting the mean OD of each standard dilution (Std 1-7) minus the mean Blank (see formula below) on the y-axis against the corresponding concentration on the x-axis.

Formulas: Calculated adjusted Standard OD = mean OD of Standard – mean OD of the Blank for each Standard.

Calculated adjusted Sample OD = mean OD of Sample – mean OD of the Blank for each Sample.

Most laboratories have (plate reader) software that allows various methods of curve fitting. Since ELISA data are essentially sigmoidal rather than linear, we recommend using the 4- or 5-parameter logistic fit for quantitative analysis of the samples. Alternatively, a linear regression curve may be acceptable for the linear portion of the curve consisting of at least 3 concentrations.

After selection of the regression model, the analyte concentrations in unknown samples can be interpolated from the standard curve. The OD value of the sample should fall within the standard curve. Samples showing an OD below the lowest concentration of the standard curve need to be re-analyzed at a lower dilution. Samples showing an OD that exceeds the highest concentration of the standard curve need to be re-analyzed at a higher dilution.

If samples have been diluted, the calculated concentration must be multiplied by the dilution factor.

Troubleshooting

Problem	Possible cause	Solution
Poor consistency of replicates	Inaccurate pipetting	- Ensure accurate pipetting of volume and avoid air bubbles. - Check pipettes.
	Inadequate mixing of reagents	- Mix reagents adequately.
	Inadequate washing	- Increase the stringency of washes (particularly after the 'detection antibody' incubation step). - Check function of the plate washer.
	Evaporation of solutions	- Ensure precise sealing of the plate.
	Non-homogenous samples or with high particulate matter	- Mix samples thoroughly and remove particulates by centrifugation.
OD_{blank} values higher than 0.3	Incubation time of TMB substrate solution is too long	- Shorter incubation time of TMB substrate.
	Incubation temperature of TMB substrate solution is too high	- Decrease incubation temperature of TMB substrate.
	Working solutions were contaminated	- Solutions should be clear and colorless. Use a clean container before addition into wells.
	Detection antibody or conjugate dilution was too concentrated or left too long on the plate	- Ensure proper dilution of detection antibody or conjugate and incubation time.
No signal or low OD values for standards	Improper storage of reconstituted SPP	- Avoid prolonged exposure to light and heat. - Avoid storage at RT.
	Incorrect incubation times or temperature	- Ensure sufficient incubation times. - Reagent solutions should be at RT before use.
	Improper quality or pH of distilled water	- Use distilled water, and not tap water. - Check quality and pH of distilled water.
	Improper antibody, SPP or standard dilution	- Ensure proper dilution of antibody, SPP and standard.
	Degradation of antibodies or SPP	- Follow recommended storage conditions.
	Overly high washing / aspiration pressure from automated plate washer.	- Check function of washing system or utilize manual washing.
	Working solutions contain sodium azide	- Avoid adding sodium azide in solutions, as this is a 'peroxidase activity' inhibitor.
Poor standard curve (linearity and dynamic range)	Improper standard dilutions	- Ensure proper dilution of standards (follow 'two-fold dilutions' guidelines).
	Inaccurate pipetting	- Ensure accurate pipetting of volume. - Check pipettes.

Abbreviations

BSA	Bovine serum albumin
CSB	Cytokine stabilization buffer
ELISA	Enzyme-linked immunosorbent assay
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HRP	Horse Radish Peroxidase
IFN	Interferon
IL	Interleukin
L	Liter
min	minute(s)
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical density
PB	Phosphate buffer
PBS	Phosphate buffered saline
RT	Room temperature
SPP	Streptavidin-HRP
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor

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