



Manufactured By: U-CyTech Biosciences

Instruction Manual ELISA kit



5-plate format



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



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This ELISA manual applies for the following U-CyTech ELISA kits

(please find below the catalogue number of the ELISA kit)

Analyte	Species				
	Human	Old World Monkey	New World Monkey	Mouse	Rat
IFN- γ	ACT201	ACT141	ACT340	ACT301	ACT071
IL-1B	ACT213	ACT139			
IL-2	ACT202	CT142		ACT309	
IL-4	ACT203	ACT143		ACT306	ACT073
IL-5	ACT204	ACT144		ACT308	
IL-6	ACT205	CT145		ACT299	
IL-8	ACT212	ACT151			
IL-10	ACT206	ACT146		ACT307	
IL-12/23p40		ACT149			
IL-12p70	ACT210				
IL-13	ACT208	ACT147	ACT341		
IL-17	ACT516	ACT501			
IL-23	ACT517	ACT502			
G-CSF	ACT390				
GM-CSF	CT200	ACT140			
Granzyme B	ACT211				
Perforin	ACT391	CT154			
TNF- α	ACT209	ACT148	ACT342	ACT303	ACT075

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Abbreviations

APC	Antigen presenting cells
BSA	Bovine serum albumin
CD	Cluster of differentiation
CSB	Cytokine stabilization buffer
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
IFN	Interferon
IL	Interleukin
L	Liter
MHC	Major histocompatibility complex
OD	Optical density
PB	Phosphate buffer
PBS	Phosphate buffered saline
PBST	PBS containing 0.05% Tween-20
PBST-B	PBST containing 0.5% bovine serum albumin
RT	Room temperature
SPP	Streptavidin-HRP polymer
Th	T helper subset
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor

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 CD₄⁺ T cell subsets (i.e. Th1 and Th2). Activation of these subsets is characterized by the secretion of distinct patterns of cytokines. Th1, but not Th2 cells, primarily secrete IL-2 and IFN- γ while Th2, but not Th1 cells, produce IL-4, IL-5, IL-6, IL-10 and IL-13. Other cytokines, such as TNF- α and GM-CSF are produced by both Th subsets. In addition, the production of IL-12 and IL-10, produced by antigen presenting cells (APC) such as macrophages and dendritic cells, critically contributes to the preferential expansion of Th1- or Th2-type of cells. For instance, early production of IL-12 is considered essential for the development of Th1 cells. On the other hand, the absence or low concentrations of IL-12 and IFN- γ in the early phase of an immune response and concomitant production of IL-4 by cells of the mastcell/basophil lineage or T cells themselves is known to favor the development of Th2 cells. In addition to their regulatory effects on Th subset differentiation, the cytokines released by the two types of Th cells also produce distinct effector functions. For instance, IL-4 and IFN- γ have differential or antagonistic activities on immunoglobulin isotype selection or 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.

Contents of the kit

Items	Quantity (5-plate format)	Storage conditions
Coating antibodies	1 vial	4°C (39°F)
Cytokine standard	5 vials	4°C (39°F)
Biotinylated detector antibodies	1 vial	4°C (39°F)
SPP conjugate (Streptavidin-HRP polymer)	1 vial	≤ -20°C (-4°F)
TMB substrate tablets	5	4°C (39°F)
Substrate buffer capsules	5	RT*
BSA stock solution (10%)	2 vials (24 ml)	4°C (39°F)
Cytokine stabilization buffer (CSB)**	1 vial (5 ml)	4°C (39°F)
Tween-20	1 vial (5 ml)	RT*
ELISA plates	8	RT*
Adhesive cover slips	10	RT*

* Room temperature

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



Hazard information

Warning:

TMB (Tetramethylbenzidine) substrate tablets are classified as irritant to eyes according to Regulation (EC) no. 1272/2008 and Directive 67/548/EC and its amendments.

Hazard statement: H319: Causes serious eye irritation

Precaution statements: P305 + P351 + P338:

IF IN EYES: Rinse continuously with water for several minutes.
Remove contact lenses if present and easy to do. Continue rinsing.

Hazard symbol: Xi Risk phrase: R36: Irritating to eyes

Safety phrase: S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

Other kit components are not classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EC or 1999/45/EC and their amendments.

Please find the Material Safety Data Sheet on www.ucytech.com/manuals.

Materials and reagents required but not provided

- PB stock: dissolve 96.0 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ plus 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 wavelength set to 370, 450 and/or 655 nm

Working solutions

- PBS: add 10 ml PB stock and 8.8 g NaCl to 1.0 L distilled water. Adjust pH to 7.4. Alternatively, use commercially available liquid PBS from Invitrogen or other suppliers.
- Do not use commercially available PBS tablets for the preparation of the coating solution (the filler in the tablets interferes with the coating process).
- PBST: 0.5 ml Tween-20 dissolved in 1 L PBS.
- 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: the content of one capsule is dissolved 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

Coating antibodies

- Reconstitute the lyophilized antibodies 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 1 minute at room temperature. Avoid vigorous shaking. To coat 96 wells of an ELISA plate 50 µl is pipetted out of the vial (or use a frozen aliquot of 50 µl; see "*Storage kit reagents*") and added to 5 ml PBS. Mix gently.
- Add 50 µl of diluted antibody solution to each well of the ELISA plate and fill up to 100 µl with PBS.
- Seal the plate to prevent evaporation. Incubate overnight at 4°C or alternatively 1 to 2 hours at 37°C.

Blocking

- Prepare blocking buffer (see "Working solutions").
- Remove the coating antibody solution and wash the wells at least six times with PBST.
- Add 200 µl of blocking buffer to each well.
- Seal the plate and incubate at 37°C for 1 hour.

Test samples and standards

- 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 an appropriate diluent (see "*Cytokine standards*").
- Remove the blocking buffer but do not wash.
- Add 100 µl of diluted standard and test samples to each well.
- Seal the plate and incubate at 37°C for 2 hours or overnight at 4°C.

Biotinylated detection antibodies

- Reconstitute the lyophilized antibodies by injecting 0.5 ml of 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. For one ELISA plate, 100 µl is pipetted out of the vial (or use a frozen aliquot of 100 µl; see "*Storage kit reagents*") and added to 10 ml PBST-B. Mix gently.
- Remove test samples/standards and wash the wells at least six times with PBST.
- Add 100 µl of diluted antibody solution to each well.
- Seal the plate and incubate at 37°C for 1 hour.

SPP conjugate

- Reconstitute the contents of the vial by injecting 0.5 ml of 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. For one ELISA plate, 100 µl is pipetted out of the vial (or use a frozen aliquot of 100 µl; see "Storage kit reagents") and added to 10 ml PBST-B. Mix gently.
- Remove detector antibody solution and wash the wells at least six times with PBST.
- Add 100 µl of diluted SPP conjugate to each well.
- Seal the plate and incubate at 37°C for 1 hour.

Substrate

- For one ELISA plate, dissolve one TMB tablet in 1.0 ml DMSO (vortex at high speed for 5 minutes for complete dissolution) and then add 10 ml substrate buffer (see "Working solutions"). Mix thoroughly (use substrate solution within 30 minutes).
- Remove SPP conjugate and wash the wells at least six times with PBST.
- Dispense 100 µl substrate solution into each well. Leave the plate at room temperature (color development between 10 and 30 minutes). The 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 2 M H₂SO₄ (resulting in a yellow solution which can be read at 450 nm).

Cytokine standards

For maximum recovery, the vial with lyophilized cytokine standard should be reconstituted in 0.5 ml distilled water and allowed to stand for 1 minute at room temperature. Thereafter, the reconstituted cytokine standard (stock solution) is placed on melting ice and is immediately diluted as indicated below (preferentially 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 0-4°C until use in the ELISA.

The total amount of 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. when amount on label is 4.8 ng/vial; after reconstitution, the concentration becomes 9.6 ng/ml = 9600 pg/ml].

The standard stock solution is diluted in PBST-B to the highest concentration cytokine to be used in the standard range). The recommended standard range is mentioned in the typical data of the specific ELISA kit (see www.ucytech.com/manuals). In this example 320 pg/ml is used as highest cytokine concentration in the standard range.

The linear region of the 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 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: In general, the plotted standard curve is not completely linear. Regularly, the linear portion falls between 5 and 40 pg/ml (this may vary for each curve). When computer-based curve-fitting statistical software is employed, choose a linear regression curve for at least 3 concentrations in the linear part.

Note 3: It is recommended to test each test sample and the standard curve in duplicate or triplicate.

Note 4: 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 preferentially be control serum or plasma originating from the same species.

Storage kit reagents

The vials with lyophilized coating antibodies and biotinylated detector antibodies can be safely stored in a refrigerator for a defined length of time (expiry date indicated on the vial). After reconstitution, the antibodies remain fully active for minimal 6 months at 4°C (39°F) 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 $\leq -20^{\circ}\text{C}$. Under these conditions the antibodies are stable for at least one year.

Upon arrival, the vial with lyophilized SPP conjugate should be stored at $\leq -20^{\circ}\text{C}$. Storage of the vial at room temperature or at 4°C for several months may lead to lower OD readings in the ELISA. After reconstitution, the SPP solution is stable for 2 months at 4°C but rapidly loses activity when stored at room temperature. It is strongly recommended that after reconstitution, the solution is immediately divided into small aliquots for single use and stored at $\leq -20^{\circ}\text{C}$. Under these conditions SPP is stable for minimal 12 months.

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, the plate is inverted and tapped 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, the plate is inverted and tapped dry on absorbent paper.
- If using an automated washing device, the operating instructions should carefully be followed.

Trouble shooting

- Poor consistency of replicates can be overcome by increasing the stringency of washes particularly after the incubation step with detector antibody.
- High values of the blank control (optical density > 0.2) can be overcome by shortening the incubation time with the substrate solution or is 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.

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