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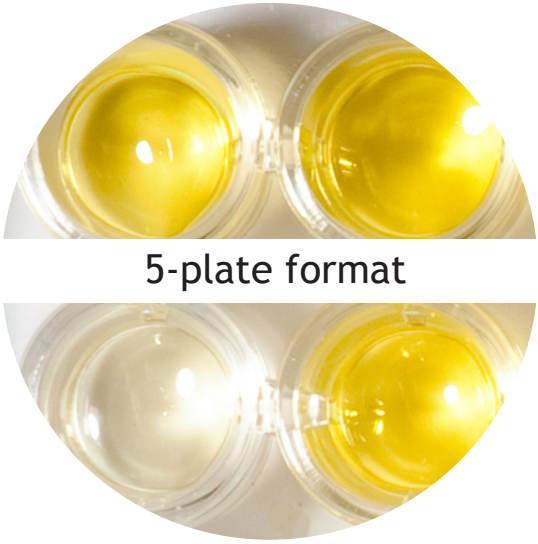
Instruction Manual ELISA kit



5-plate format

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

Contents

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
MCP	Monocyte Chemoattractant Protein
min	minute(s)
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical density
PB	Phosphate buffer
PBS	Phosphate buffered saline
RT	Room temperature (temperature between 20 °C and 26 °C)
SPP	Streptavidin-HRP
Std	Standard dilution
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor

This manual applies to the following U-CyTech ELISA kits

	Human	Old World Monkey	New World Monkey	Mouse	Rat
IFN-γ	CT201A	CT141A	CT340A	CT301A	CT071A
IL-1β	CT526A	CT139A			
IL-2	CT202A	CT142A	CT344A	CT309A	
IL-4	CT203A	CT143A		CT306A	CT073A
IL-5	CT204A	CT144A		CT296A	
IL-6	CT205A	CT145A	CT346A	CT299A	
IL-7	CT523A				
IL-8	CT212A	CT151A			
IL-10	CT206A	CT146A		CT307A	
IL-12/23p40		CT149A	CT345A		
IL-12p70	CT210A				
IL-13	CT208A	CT147A	CT341A		
IL-17A	CT516A	CT501A	CT343A		
IL-17F	CT518A	CT503A			
IL-23	CT517A	CT502A			
IL-27	CT524A				
IL-29	CT525A				
IL-31	CT520A				
IL-33	CT519A				
IP-10	CT522A	CT157A			
Angiopoietin-2	CT527A	CT158A			
G-CSF	CT389A	CT155A			
GM-CSF	CT200A	CT140A			
Granzyme B	CT211A				
MCP-1	CT521A	CT156A			
MICB	CT528A				
Perforin	CT391A	CT154A			
VEGF-A	CT529A				
TNF-α	CT209A	CT148A	CT342A	CT303A	CT075A

Overview of catalogue numbers of U-CyTech ELISA kits.

Introduction

Cytokines, chemokines and granzymes are a group of signaling proteins that affect the behavior of cells when released. They are critically involved in various physiological processes such as immune regulation, cell differentiation, cell proliferation, chemotaxis and cell apoptosis. These signaling proteins 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.

The enzyme-linked immunosorbent assay (ELISA) is one of the primary and most popular methods to detect and measure these signaling proteins. The ELISA test is rapid, simple to perform and is one of the most sensitive and reliable technologies available.

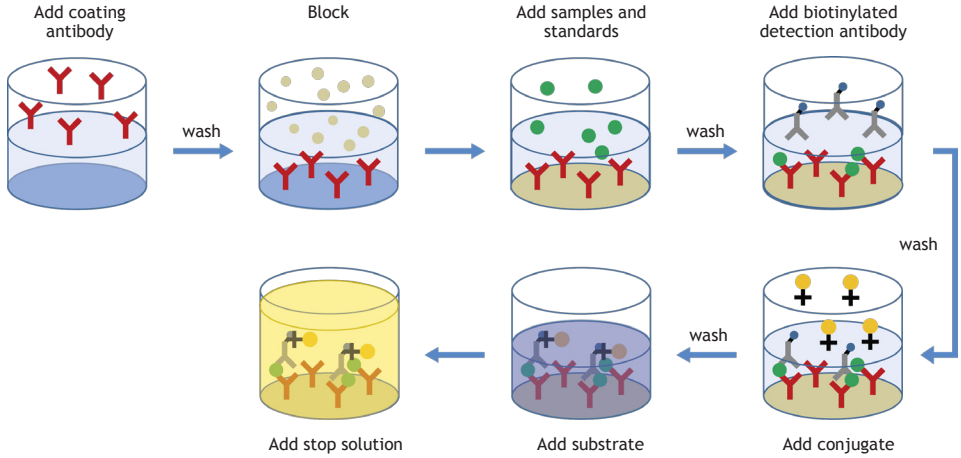
U-CyTech has developed various high-quality ELISA kits for the detection of cytokines, chemokines and granzymes for human, monkey (including macaques and marmoset), mouse and rat. These assays are widely applied in different research fields, including cancer research, vaccine development, infectious diseases, autoimmune diseases, organ transplantation and parasitology. Hundreds of peer-reviewed publications describe the successful use of U-CyTech's ELISA systems in different biological fluids.

Please, find references of studies in different research areas using our ELISA kits on: www.ucytech.com/ELISA of www.ucytech.com/references.

Principle of the test

U-CyTech's ELISA kits are simple and sensitive sandwich immunoassays for the determination of cytokine, chemokine and granzyme levels in biological fluids such as cell culture supernatant, plasma or serum.

The assays utilize 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 the excess and 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. 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. A sulfuric acid solution is added to stop the enzymatic reaction (changing the color to yellow) and OD is read at 450 nm.



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

All kit components are not classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EEC or 1999/45/EC and their amendments.
Please find the Material Safety Data Sheet on www.ucytech.com/manuals.

Contents of the kit

Items	Quantity (5-plate format)	Storage conditions
Coating antibody*	1 vial	4 °C
Standard*	5 vials	4 °C
Biotinylated detection antibody*	1 vial	4 °C
SPP conjugate*	1 vial	≤-20 °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)	RT in the dark
TMB substrate solution	2 vials (2x 30 ml)	4 °C in the dark
Stop solution (0.175 M H_2SO_4)	2 vials (2x 30 ml)	4 °C
ELISA plates	8 plates	RT
Adhesive cover slips	10 slips	RT

* Lyophilized.
** For use in serum and plasma samples only, see section “Sample preparation” on page 11.

Storage and stability

Coating antibody and biotinylated detection antibody
The vials with lyophilized coating and biotinylated detection antibody can be safely stored at 4 ºC until the expiry date (indicated on the vials). After reconstitution, the antibodies are stable for at least 12 months at 4 ºC 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 ≤-20 ºC (stable for at least two years).

Standard
The vials with lyophilized standard can be safely stored at 4 ºC until the expiry date (indicated on the vials). These vials are for single use only.

SPP conjugate
The vial with lyophilized SPP conjugate is stable until the expiry date (indicated on the vial) when stored at ≤-20 ºC in the dark. After reconstitution, the reagent is stable for at least 2 months at 4 ºC 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 ≤-20 ºC in the dark (stable for at least one year).

TMB substrate solution
The ready-to-use TMB substrate solution should be stored at 4 ºC and is stable until the expiry date (indicated on the vial). Avoid exposure to light, heat and contamination with metal ions or peroxidase.

BSA stock solution and Cytokine stabilization buffer
The vials with BSA stock solution and Cytokine stabilization buffer can be safely stored at 4 ºC until the expiry date (indicated on the vial). After opening, these solutions are stable for at least 6 months when kept sterile.

Tween-20
Tween-20 can safely be stored at RT and is stable until the expiry date (indicated on the vial).

Stop solution
The ready-to-use Stop solution can safely be stored at 4 ºC and is stable until the expiry date (indicated on the vial).

Materials and equipment (required but not provided)

- PBS (pH 7.4; ingredients: $Na_2HPO_4 \cdot 2H_2O$, KH_2PO_4, NaCl and distilled water). Alternatively, use commercially available liquid PBS (pH 7.4) from Life Technologies (cat. no. 10010-015) or other suppliers.
- Sterile distilled water.
- Pipetting devices for the accurate delivery of volume required for the assay performance.
- Tubes and containers/plates to make the solutions.
- 37 ºC incubator.
- Plate washer: automated or manual (squirt bottle, manifold dispenser).
- Reading device for microtiter-plate (wavelength set to 370, 450 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 1,000-2,000 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 (RT) and collect plasma.

Samples should be aliquoted and stored frozen at ≤-20 ºC 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 NCCLS document M29-T2, "Protection of laboratory workers from infectious diseases transmitted by blood and tissue".

Preparation 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 filtrate solution (0.2 µm). Store solution at RT (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 depending on the washing procedure (manual or automatic washing).

Blocking buffer

PBS containing 1% BSA. For one ELISA plate: mix 2 ml BSA stock solution (10%) gently but thoroughly 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 by making at least 250 ml 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 4 °C. This solution will be stable for at least one month when kept sterile.

For one ELISA plate, 20 ml of Dilution buffer is needed for detection and conjugate solutions, and at least 20 ml for standards and samples (this volume will depend on the number of sample dilutions).

Standard

Reconstitute the lyophilized standard 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. Thereafter, the reconstituted Standard is placed on melting ice and is immediately (preferentially within one hour) diluted as described in “Preparing the standard curve” on page 12.

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.

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).

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 °C 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). It is recommended to analyze a series of dilutions of the sample to ensure that sample measurements fall within the assay range (see also ”Data analysis” on page 13).

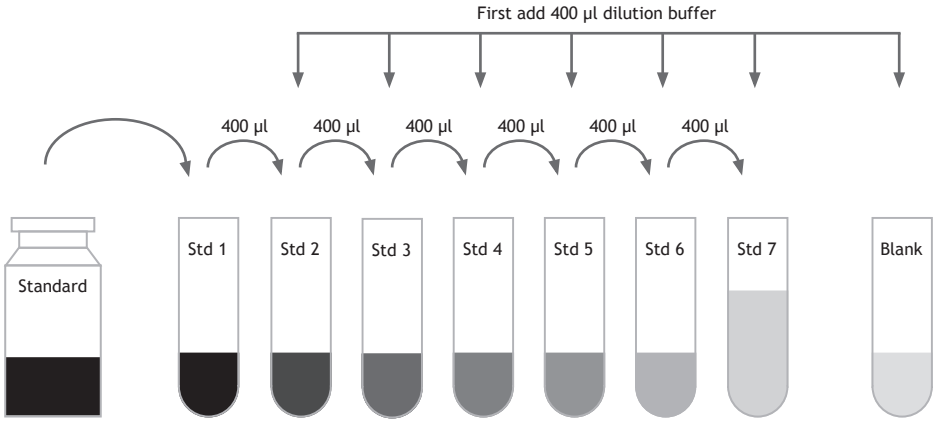
When measuring cytokines in serum or plasma, add 1/20 volume of CSB (ready-to-use) to the pure serum or plasma sample (CSB is not required for other samples such as cell culture supernatant) before further dilution in Dilution buffer. CSB inhibits the degradation of cytokines.

It is recommended to test samples in triplicate.

Preparing the standard curve

With a standard curve the analyte concentration 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 found in the Typical data sheet on www.ucytech.com. 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 till Std 7 and Blank).
- Prepare in the remaining tube (Std 1) the highest concentration to be used in the standard curve (see Typical data sheet) 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.



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).
- Depending on the biological origin of the unknown samples, also other appropriate dilution buffers may be used for the preparation of the standard curve, depleted with the endogenous protein to be quantified (e.g. cell culture medium, serum).

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 by a firm 'shake-out' action 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.

Note: When you have too much airbubbles by using a squirt bottle, you can replace wash buffer by PBS during the last washing step.

- When using an automated washing device, follow operating instructions carefully.

Data analysis

In each experiment a standard curve should 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 dilutions (Std 1-7) minus the mean Blank (see formula below) on the y-axis against the corresponding concentration on the x-axis.

Formula: $OD = \text{mean } OD_{\text{std 1-7/Sample}} - \text{mean } OD_{\text{blank}}$

Most laboratories have (plate reader) software that allows various methods of curve fitting. Since ELISA data are essentially sigmoid 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 concentration 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 should be re-analyzed at a lower dilution. Samples showing an OD that exceeds the highest concentration of the standard curve should be re-analyzed at a higher dilution.

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

Assay procedure

All solutions should be at 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 4 °C (or alternatively 2 hours at 37 °C).
3. Remove coating antibody solution and wash the wells at least six times with Wash buffer.
4. Add 200 µl of Blocking buffer to each well.
5. Seal the plate and incubate for 1 hour at 37 °C.
6. Remove the Blocking buffer (do not wash the wells).
7. Add 100 µl of diluted standard/blank/samples to each well.
8. Seal the plate and incubate for 2 hours at 37 °C (or alternatively overnight at 4 °C).
9. Remove standards/samples and wash the wells at least six times with Wash buffer.
10. Add 100 µl of diluted detection antibody solution to each well.
11. Seal the plate and incubate for 1 hour at 37 °C.
12. Remove detection antibody solution and wash the wells at least six 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 37 °C.
15. Remove SPP conjugate and wash the wells at least six 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.

Note: *The substrate produces a soluble blue end product that can be read at 370 or 655 nm.*

18. After 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.

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.

More information can be found on: www.ucytech.com/FAQs_elisa

Technical assistance

If you require assistance, information or have any questions, please contact our company:

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On our website (www.ucytech.com/manuals) you can find: Manuals, Typical data and MSDS of our ELISA kits.