



ELISA antibody pair

Technical Data Sheet 10-plate format

Version 060905

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

Introduction

The usefulness of ELISAs (enzyme-linked immunosorbent assays) in cytokine biology is evident from the many reports published on this subject. The assay is based on the use of a monoclonal /monoclonal or monoclonal/polyclonal antibody pair that bind with high affinity to different sites on the cytokine molecule. One of the antibodies is immobilized to the wells of a 96-well microtiter plate. This so-called capture or coating antibody functions to selectively immobilize the cytokine from crude protein preparations. The second antibody (detection antibody) is labeled with biotin and recognizes a different site on the cytokine molecule. The biotin molecules give the antibody the ability to interact with streptavidin molecules. By using HRPO (horseradish peroxidase)-labeled streptavidin, the cytokine can now quantitatively be determined by enzymatic conversion of a HRPO-specific substrate to a colored product. Since many biotin molecules can be coupled to the detector antibody, several HRPO molecules can be bound. The enzyme activity is subsequently further increased by using complexes (polymers) of HRPO-streptavidin. This results in an extreme sensitive assay with a detection limit within the low picogram range (< 5 pg/ml).

Contents

- 2 vials with unlabelled anti-cytokine coating antibody, sterile and supplied in lyophilized form, each vial contains sufficient material for five 96-well ELISA plates.
- 2 vials with biotinylated anti-cytokine detection antibody, sterile and supplied in lyophilized form, each vial contains sufficient material for five 96-well ELISA plates.
- 5 vials with cytokine standards, sterile and supplied in lyophilized form.

Required materials and reagents

- PB stock: dissolve 96.0 g Na₂HPO₄·2H₂O plus 17.5 g KH₂PO₄ in 1.0 ltr distilled water and adjust pH to 7.4
- Sterile distilled water
- 2 M H₂SO₄
- Bovine serum albumin (BSA; ELISA grade)
- 96-well ELISA plates. Plates from Greiner Bio-one are recommended (Greiner Bio-one cat.nr.655092 or U-CyTech cat.nr. ACT361)
- Adhesive cover slips (Greiner Bio-one cat.nr. 676001)
- Pipetting devices for the accurate delivery of volumes required for assay performance
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc)
- Reading device for microtiter plates set at 370, 450 or 655 nm
- Tween-20 detergent
- Coloring system (e.g. HRPO-labeled streptavidin + tetramethylbenzidine (TMB)). Reagents from U-CyTech (cat.nr. ACT357, ACT367 and ACT369) are recommended.

Working solutions

- PBS: 10 ml PB stock solution and 8.8 g NaCl added to 1.0 ltr distilled water
- PBST (= wash buffer): 0.5 ml Tween-20 added to 1 ltr PBS
- PBST-B : PBST supplemented with 0.5% (w/v) BSA
- Blocking buffer: PBS supplemented with 1% (w/v) BSA

ELISA protocol

Coating antibody

Reconstitute the contents of the vial in 250 µl distilled water and dilute 100-fold in PBS. Bring 50 µl in the wells of an ELISA plate and fill up to 100 µl with PBS. Seal the plate with an adhesive cover slip and incubate overnight at 4°C.

Blocking

Remove coating antibody solution and wash the wells at least 6 times with PBST. Add 200 µl blocking buffer to each well. Seal the plate with an adhesive cover slip and incubate 1 h at 37°C or 2 h at room temperature.

Samples and standards

Remove blocking buffer by a vigorous 'shake-out' action but do not wash. Dilute samples and standards in PBST-B (see 'Cytokine standards'). Add 100 µl/well. Seal the plate and incubate for 2 h at 37°C. Wash six times with PBST.

Detection antibody

Reconstitute the contents of the vial in 500 µl distilled water and dilute 100-fold in PBST-B. Add 100 µl to each well. Seal the plate and incubate 1 h at 37°C or overnight at 4°C. Wash six times with PBST.

Enzyme conjugate

Dilute an appropriate enzyme conjugate preparation (e.g. HRPO-streptavidin or other enzyme conjugate) to its pre-titered optimal concentration in PBST-B. Add 100 µl/well. Seal the plate and incubate for 1 h at 37°C. Wash six times with PBST.

Substrate

When HRPO-streptavidin is used, the following substrate solution should be prepared. Dissolve one TMB tablet (U-CyTech cat.nr. ACT367 or Sigma cat.nr. T-5525) in 1.0 ml dimethylsulfoxide (DMSO) and add this to 10 ml substrate buffer (Phosphate-Citrate buffer containing sodium perborate (U-CyTech cat.nr. ACT369 or Sigma cat.nr. P-4922)). Immediately dispense 100 µl into each well and incubate at room temperature (15-30 minutes). In case of a positive reaction, the colorless solution will become blue that can be read at 370 nm or 655 nm. The reaction can be stopped by adding 50 µl 2M H₂SO₄ (resulting in a yellow color that can be read at 450 nm).

Cytokine standards

For maximum recovery, the vial with lyophilized cytokine standards should be reconstituted in distilled water (volume indicated on the vial) and allowed to stand for 1 minute at room temperature. Thereafter, the reconstituted cytokine standard should be used immediately (preferentially within one hour). For measuring cytokines in cell culture supernatant, test samples and the reconstituted cytokine standard solution should be diluted in PBST-B. However, when measuring cytokines in serum or plasma, the standard diluent should preferentially be control serum or plasma originating from the same species.

Storage antibodies and standards

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 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.

The vials with lyophilized cytokine standard can be safely stored in a refrigerator until time of use. Use the vials with cytokine standard only once.

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, then aspirate the liquid. 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 whether the substrate solution was properly prepared (pH should be between 5.0 and 5.5)
- verify whether the antibodies, enzyme conjugate and standard preparations were properly diluted

Avoid sodium azide in wash buffers and diluents, as this is an inhibitor of peroxidase activity.