

# Human IL-4 ELISA Kit

Instructions for use

|                    |                         |
|--------------------|-------------------------|
| Catalogue numbers: | 1x48 tests: 950.020.048 |
|                    | 1x96 tests: 950.020.096 |
|                    | 2x96 tests: 950.020.192 |

**For research use only**

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# Human IL-4 ELISA KIT

## 1. Intended use

The Diaclone IL-4 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of IL-4 in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human IL-4.

**This kit has been configured for research only. Not suitable for use in therapeutic procedures.**

## 2. Introduction

### 2.1. Summary

IL-4 is a lymphokine that co-stimulates the proliferation of activated B- and T-cells, augments the cytotoxic activity of lymphocytes and monocytes and enhances the functional activity of myeloid cells (1). Produced by mast cells, T-cells and bone marrow stromal cells, IL-4 regulates the differentiation of naive CD4<sup>+</sup> cells into helper Th2 cells characterized by their cytokine secretion-profile that includes secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 which favour a humoral immune response (2,3). In addition, IL-4 can inhibit the proliferation of TNF, IL-1 and IL-6 by macrophages (4,5)

IL-4 induces B-cell class switching to IgE and IgG1 isotypes, and up regulates MHC Class II production and CD23 expression (6)

IL-4 is a 15kDa globular glycoprotein containing 129 amino acid residues. The non glycosylated form of the protein is fully biologically active. (7)

## 2.2. Principle of the method

A capture Antibody highly specific for IL-4 has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of IL-4 in samples and known standards to the capture antibodies is completed and then any excess unbound analyte is removed. During the next incubation period the binding of the biotinylated anti-IL-4 secondary antibody to the analyte occurs. Any excess unbound secondary antibody is then removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of IL-4 present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of IL-4 in any sample tested.

## 3. Reagents provided and reconstitution

| Reagents<br>(Store@2-8°C)                   | Quantity<br>1x48 well kit<br>Cat no.<br>950.020.048 | Quantity<br>1x96 well kit<br>Cat no.<br>950.020.096 | Quantity<br>2x96 well kit<br>Cat no.<br>950.020.192 | Reconstitution                                                                     |
|---------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|------------------------------------------------------------------------------------|
| 96 well microtitre<br>strip plate           | 1/2                                                 | 1                                                   | 2                                                   | Ready to use (Pre-coated)                                                          |
| Plastic plate covers                        | 2                                                   | 2                                                   | 4                                                   | n/a                                                                                |
| Standard: 35pg/ml                           | 1                                                   | 2                                                   | 4                                                   | Reconstitute as directed on the vial (see reagent<br>preparation, section 8)       |
| Standard Diluent<br>(Buffer)                | 1 (25ml)                                            | 1 (25ml)                                            | 1 (25ml)                                            | 10x Concentrate, dilute in distilled water (see<br>reagent preparation, section 8) |
| Standard Diluent<br>(Human serum)           | 1 (7ml)                                             | 1 (7ml)                                             | 2 (7ml)                                             | Ready to use                                                                       |
| Control                                     | 1                                                   | 2                                                   | 4                                                   | Reconstitute as directed on the vial (see reagent<br>preparation, section 8)       |
| Biotinylated anti-IL-4                      | 1 (0.4ml)                                           | 1 (0.4ml)                                           | 2 (0.4ml)                                           | Dilute in biotinylated antibody diluent (see<br>reagent preparation, section 8)    |
| Biotinylated Antibody<br>diluent            | 1 (7ml)                                             | 1 (7ml)                                             | 1 (13ml)                                            | Ready to use                                                                       |
| Streptavidin-HRP                            | 1 (5µl)                                             | 2 (5µl)                                             | 4 (5µl)                                             | Add 0.5ml of HRP diluent prior to use (see<br>reagent preparation, section 8)      |
| HRP Diluent                                 | 1 (23ml)                                            | 1 (23ml)                                            | 1 (23ml)                                            | Ready to use                                                                       |
| Wash Buffer                                 | 1 (10ml)                                            | 1 (10ml)                                            | 2 (10ml)                                            | 200x Concentrate dilute in distilled water (see<br>reagent preparation, section 8) |
| TMB Substrate                               | 1 (11ml)                                            | 1 (11ml)                                            | 1 (24ml)                                            | Ready to use                                                                       |
| H <sub>2</sub> SO <sub>4</sub> stop reagent | 1 (11ml)                                            | 1 (11ml)                                            | 2 (11ml)                                            | Ready to use                                                                       |

## 4. Materials required but not provided

- Microtitre plate reader fitted with appropriate filters (450nm required with optional 620nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

## 5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

## 6. Specimen collection, processing & storage

Cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

**Cell culture supernatants:** Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

**Serum:** Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

**Plasma:** EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

**Storage:** If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at -70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

**Recommendation:** Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

## 7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures , e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
- The human serum included in this kit have been tested and found non reactive for HbsAg, anti HIV1 & 2 and anti VHC. Nevertheless, no known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore handling of reagents, serum or plasma specimens should be in accordance with local safety procedures
- Laboratory gloves should be worn at all times
- Avoid any skin contact with H<sub>2</sub>SO<sub>4</sub> and TMB. In case of contact, wash thoroughly with water
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used
- Do not pipette by mouth
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration
- Cover or cap all reagents when not in use
- Do not mix or interchange reagents between different lots
- Do not use reagents beyond the expiration date of the kit
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H<sub>2</sub>SO<sub>4</sub> and substrate solution, avoid pipettes with metal parts
- Use a clean plastic container to prepare the washing solution
- Thoroughly mix the reagents and samples before use by agitation or swirling
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance's within 1 hour after completion of the assay
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
- Follow incubation times described in the assay procedure
- Dispense the TMB solution within 15 min of the washing of the microtitre plate

## 8. Assay Preparation

Bring all reagents to room temperature before use

### 8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard, zero and control should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.

**Example plate layout** (example shown for a 6 point standard curve)

|   | Standards / Controls |      | Sample Wells |   |   |   |   |   |   |    |    |    |
|---|----------------------|------|--------------|---|---|---|---|---|---|----|----|----|
|   | 1                    | 2    | 3            | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 35                   | 35   |              |   |   |   |   |   |   |    |    |    |
| B | 17.5                 | 17.5 |              |   |   |   |   |   |   |    |    |    |
| C | 8.75                 | 8.75 |              |   |   |   |   |   |   |    |    |    |
| D | 4.4                  | 4.4  |              |   |   |   |   |   |   |    |    |    |
| E | 2.1                  | 2.1  |              |   |   |   |   |   |   |    |    |    |
| F | 1                    | 1    |              |   |   |   |   |   |   |    |    |    |
| G | zero                 | zero |              |   |   |   |   |   |   |    |    |    |
| H | Ctrl                 | Ctrl |              |   |   |   |   |   |   |    |    |    |

*All remaining empty wells can be used to test samples in duplicate*

### 8.2. Preparation of Wash Buffer

Dilute the (200x) wash buffer concentrate 200 fold with distilled water to give a 1x working solution. Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2°-25°C.

### 8.3. Preparation of Standard Diluent Buffer

Add the contents of the vial (10x concentrate) to 225ml of distilled water before use.

This Solution can be stored at 2-8°C for up to 1 week.

## 8.4. Preparation of Standard

Depending on the type of samples you are assaying, the kit may include two standard diluents. Because biological fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine you want to measure, you should reconstitute standard vials with the most appropriate Standard Diluent.

For **serum and plasma** samples: use Standard Diluent - human serum

For **cell culture supernatants**: use Standard Diluent Buffer

Standard vials must be reconstituted with the volume of standard diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 35pg/ml of IL-4. Mix the reconstituted standard gently by repeated aspirations/ejections. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 35 to 1pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to well's A1 and A2, which provides the highest concentration standard at 35pg/ml
- Add 100µl of appropriate standard diluent to the remaining standard wells B1 and B2 to F1 and F2
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 35pg/ml to 1pg/ml
- Discard 100µl from the final wells of the standard curve (F1 and F2)

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

## 8.5. Preparation of Controls

Freeze-dried control vials should also be reconstituted with the most appropriate Standard Diluent to your samples.

For **serum and plasma samples**: use Standard Diluent - human serum

For **cells culture supernatants**: use Standard Diluent Buffer

The supplied Controls must be reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution at the concentration stated on the vial. Do not store after use.

## 8.6. Preparation of Biotinylated anti-IL-4

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-IL-4 with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

| Number of wells required | Biotinylated Antibody ( $\mu$ l) | Biotinylated Antibody Diluent ( $\mu$ l) |
|--------------------------|----------------------------------|------------------------------------------|
| 16                       | 40                               | 1060                                     |
| 24                       | 60                               | 1590                                     |
| 32                       | 80                               | 2120                                     |
| 48                       | 120                              | 3180                                     |
| 96                       | 240                              | 6360                                     |

## 8.7. Preparation of Streptavidin-HRP

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom.

Dilute the 5 $\mu$ l vial with 0.5ml of HRP diluent **immediately before use**. Do-not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

| Number of wells required | Streptavidin-HRP ( $\mu$ l) | Streptavidin-HRP Diluent (ml) |
|--------------------------|-----------------------------|-------------------------------|
| 16                       | 30                          | 2                             |
| 24                       | 45                          | 3                             |
| 32                       | 60                          | 4                             |
| 48                       | 75                          | 5                             |
| 96                       | 150                         | 10                            |

## 9. Method

We strongly recommend that every vial is mixed without foaming prior to use.

Prepare all reagents as shown in section 8.

Note: final preparation of Biotinylated Secondary Antibody (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

| Assay Step                                                                                                                                                                                                                    |            | Details                                                                                                                                                                                                                                          |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1.                                                                                                                                                                                                                            | Addition   | <b>Prepare Standard curve</b> as shown in section 8.4                                                                                                                                                                                            |
| 2.                                                                                                                                                                                                                            | Addition   | Add 100µl of each, <b>Sample and zero (appropriate standard diluent)</b> in duplicate to appropriate number of wells                                                                                                                             |
| 3.                                                                                                                                                                                                                            | Incubation | Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for <b>2 hour(s)</b>                                                                                                                                              |
| 4.                                                                                                                                                                                                                            | Wash       | Remove the cover and wash the plate as follows:<br>a) Aspirate the liquid from each well<br>b) Dispense 0.3 ml of <b>1x washing solution</b> into each well<br>c) Aspirate the contents of each well<br>d) Repeat step b and c another two times |
| 5.                                                                                                                                                                                                                            | Addition   | Add 50µl of diluted <b>biotinylated anti-IL-4</b> to all wells                                                                                                                                                                                   |
| 6.                                                                                                                                                                                                                            | Incubation | Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for <b>1 hour(s)</b>                                                                                                                                              |
| 7.                                                                                                                                                                                                                            | Wash       | Repeat wash step 4.                                                                                                                                                                                                                              |
| 8.                                                                                                                                                                                                                            | Addition   | Add 100µl of <b>Streptavidin-HRP</b> solution into all wells                                                                                                                                                                                     |
| 9.                                                                                                                                                                                                                            | Incubation | Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for <b>30 min</b>                                                                                                                                                 |
| 10.                                                                                                                                                                                                                           | Wash       | Repeat wash step 4.                                                                                                                                                                                                                              |
| 11.                                                                                                                                                                                                                           | Addition   | Add 100µl of ready-to-use <b>TMB Substrate Solution</b> into all wells                                                                                                                                                                           |
| 12.                                                                                                                                                                                                                           | Incubation | Incubate in the dark for <b>12-15 minutes*</b> at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.                                                                                                      |
| 13.                                                                                                                                                                                                                           | Addition   | Add 100µl of <b>H<sub>2</sub>SO<sub>4</sub>:Stop Reagent</b> into all wells                                                                                                                                                                      |
| <b>Read the absorbance</b> value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable). |            |                                                                                                                                                                                                                                                  |

*\*Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*

## 10. Data Analysis

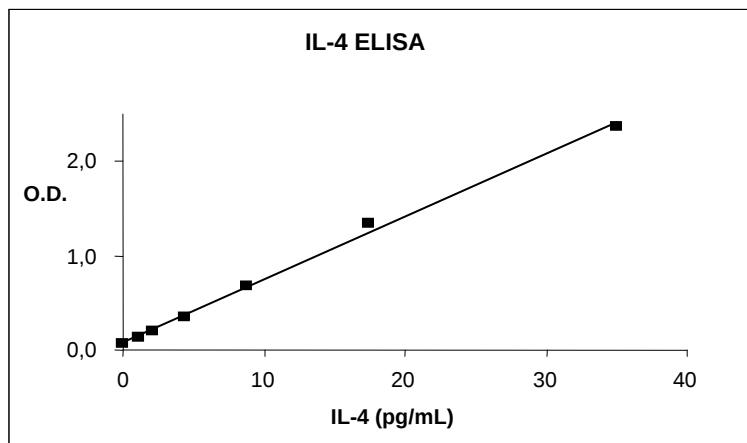
Calculate the average absorbance values for each set of duplicate standards, controls and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IL-4 standard concentration on the horizontal axis.

The amount of IL-4 in each sample is determined by extrapolating OD values against IL-4 standard concentrations using the standard curve.

### Example IL-4 Standard curve

| Standard | IL-4 Conc<br>pg/ml | OD<br>(450nm)<br>mean | CV<br>(%) |
|----------|--------------------|-----------------------|-----------|
| 1        | 35                 | 2.559                 | 2.4       |
| 2        | 17.5               | 1.347                 | 2.7       |
| 3        | 8.75               | 0.672                 | 4         |
| 4        | 4.4                | 0.346                 | 0.2       |
| 5        | 2.1                | 0.204                 | 2.6       |
| 6        | 1                  | 0.139                 | 4.6       |
| zero     | 0                  | 0.066                 | 13.5      |



**Note;** curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

## 11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

## 12. Performance Characteristics

### 12.1. Sensitivity

The sensitivity, minimum detectable dose of IL-4 using this Diaclone IL-4 ELISA kit was found to be **0.7pg/ml**. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 35 times.

### 12.2. Specificity

The assay recognizes both natural and recombinant human IL-4. To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1 $\alpha$ , IL-1 $\beta$ , IL-10 IL-12, IFN $\gamma$ , IL-2, IL-6, TNF $\alpha$ , IL-8 and IL-13).

### 12.3. Precision

#### Intra-assay

Reproducibility within the assay will be evaluated in three independent experiments. Each assay will be carried out with 6 replicates (3 duplicates) of 3 spiked serum or human pooled serum samples and 2 supernatants containing different concentrations of IL-4. **The overall intra-assay coefficient of variation has been calculated to be 3.5% .**

| Session | Sample | Assay 1<br>IL-4 pg/ml | Assay 2<br>IL-4 pg/ml | Assay 3<br>IL-4 pg/ml | Mean<br>IL-4 pg/ml | SD  | CV % |
|---------|--------|-----------------------|-----------------------|-----------------------|--------------------|-----|------|
| 1       | 1      | 30                    | 28                    | 29                    | 29                 | 1.0 | 3.4  |
|         | 2      | 21                    | 20                    | 20                    | 20                 | 0.6 | 2.8  |
|         | 3      | 10                    | 11                    | 11                    | 11                 | 0.6 | 5.4  |
|         | 4      | 30                    | 29                    | 29                    | 29                 | 0.6 | 1.9  |
|         | 5      | 22                    | 21                    | 22                    | 22                 | 0.6 | 2.6  |
| 2       | 1      | 23                    | 23                    | 23                    | 23                 | 0.4 | 1.6  |
|         | 2      | 15                    | 16                    | 16                    | 16                 | 0.2 | 0.9  |
|         | 3      | 10                    | 12                    | 10                    | 11                 | 1.3 | 11.8 |
|         | 4      | 28                    | 28                    | 27                    | 28                 | 0.4 | 1.3  |
|         | 5      | 22                    | 22                    | 22                    | 22                 | 0.4 | 1.8  |
| 3       | 1      | 26                    | 26                    | 24                    | 25                 | 1.2 | 4.6  |
|         | 2      | 18                    | 18                    | 18                    | 18                 | 0   | 0    |
|         | 3      | 11                    | 11                    | 13                    | 12                 | 1.2 | 10   |
|         | 4      | 32                    | 32                    | 31                    | 32                 | 0.6 | 1.8  |
|         | 5      | 24                    | 24                    | 25                    | 24                 | 0.6 | 2.4  |

## Inter-assay

Assay to assay reproducibility within one laboratory will be evaluated in three independent experiments by two technicians. Each assay will be carry out with 6 replicates of 3 spiked serum human pooled serum samples and 2 supernatants containing different concentration of IL-4. **The calculated overall coefficient of variation was 7.3%.**

| Technician      | Session | Sample 1<br>IL-4 pg/ml | Sample2<br>IL-4 pg/ml | Sample 3<br>IL-4 pg/ml | Sample 4<br>IL-4 pg/ml | Sample 5<br>IL-4 pg/ml |    |
|-----------------|---------|------------------------|-----------------------|------------------------|------------------------|------------------------|----|
| A               | 1       | 30                     | 21                    | 10                     | 30                     | 22                     |    |
|                 |         | 28                     | 20                    | 11                     | 29                     | 21                     |    |
|                 |         | 29                     | 20                    | 11                     | 29                     | 22                     |    |
|                 | 2       | 23                     | 16                    | 9                      | 28                     | 22                     |    |
|                 |         | 23                     | 16                    | 12                     | 28                     | 22                     |    |
|                 |         | 23                     | 16                    | 10                     | 27                     | 22                     |    |
|                 | 3       | 26                     | 18                    | 11                     | 32                     | 24                     |    |
|                 |         | 26                     | 18                    | 11                     | 32                     | 24                     |    |
|                 |         | 24                     | 18                    | 13                     | 31                     | 25                     |    |
|                 | B       | 1                      | 24                    | 17                     | 13                     | 28                     | 21 |
|                 |         |                        | 23                    | 15                     | 11                     | 28                     | 22 |
|                 |         |                        | 22                    | 15                     | 10                     | 27                     | 11 |
| 2               |         | 27                     | 18                    | 14                     | 31                     | 23                     |    |
|                 |         | 25                     | 18                    | 13                     | 30                     | 24                     |    |
|                 |         | 24                     | 18                    | 12                     | 29                     | 24                     |    |
| 3               |         | 26                     | 17                    | 12                     | 27                     | 21                     |    |
|                 |         | 24                     | 17                    | 11                     | 27                     | 21                     |    |
|                 |         | 23                     | 16                    | 11                     | 27                     | 23                     |    |
| Mean IL-4 pg/ml |         | 24                     | 17                    | 11                     | 29                     | 23                     |    |
| SD              |         | 1                      | 1                     | 1                      | 2                      | 1                      |    |
| CV %            |         | 6.2                    | 6.6                   | 11.1                   | 6.6                    | 6.3                    |    |

## 12.4. Dilution Parallelism

Four human pooled serum samples with different levels of IL-4 were analysed at different serial two fold dilutions (1:2 To 1:8) with two replicates each. Recoveries ranged from 75 to 121% with an overall **mean recovery of 100%.**

## 12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 concentrations of IL-4 in human serum in 3 separate experiments. Recoveries ranged from 100 to 113% with an overall **mean recovery of 107%**

## **12.6. Stability**

### **Storage Stability**

Aliquots of spiked serum samples were stored at –20°C, 2-8°C, room temperature (RT) and at 37°C and the IL-4 level determined after 24h. There was no significant loss of IL-4 reactivity during storage at RT, 2-8°C and 37°C.

### **Freeze-thaw Stability**

Aliquots of spiked serum were stored frozen at –20°C and thawed up to 5 times and the IL-4 level was determined. There was no significant loss of IL-4 after 5 cycles of freezing and thawing.

## **12.7. Expected serum values**

A panel of 16 human sera was tested for IL-4. All were below the detection level of 0.7pg/ml.

## **12.8. Standard Calibration**

This immunoassay is calibrated against the International Reference Standard NIBSC 88/656. NIBSC 88/656 is quantitated in International Units (IU) and equivalence in ng/ml is indicated. It has been calculated that 1ng correspond to 1ng Diaclone IL-4.

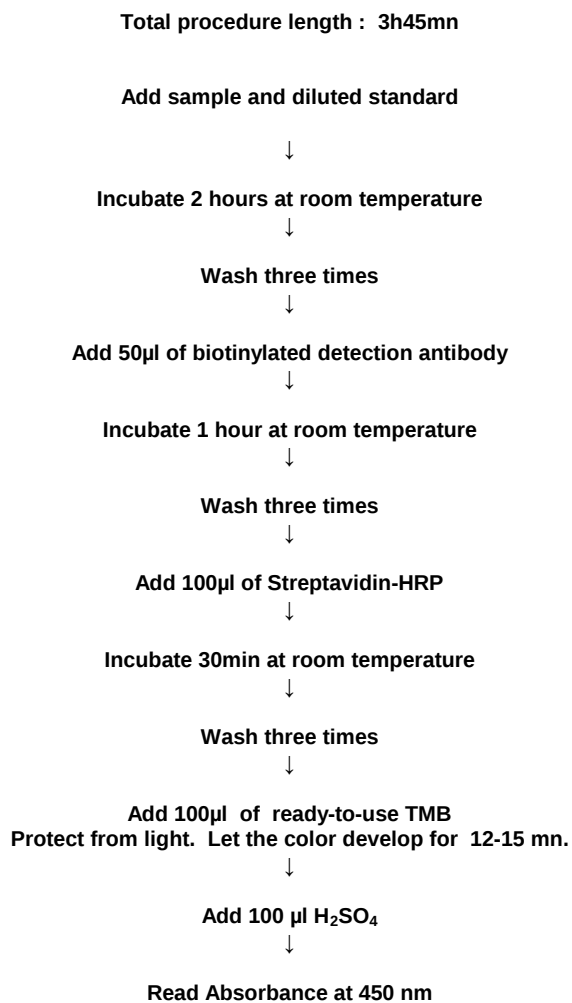
### 13. Bibliography

Howard M., Paul W.E. (1982) lymphokine res. (1-4)  
Paul W. E. , and R. A. Seder. (1994) Cell 76, 241-251.  
Hart P.H., G. F. Vitti, D. R. Burgess, G. A. Whitty, D. S. Piccoli, and J. A. Hamilton. (1989) Proc. Natl. Acad. Sci, USA 86, 3803-3807.  
Lee J. D. , S. G. Swisher, E. H. Minehart, W. H. McBride, and J. S. Economou. (1990) J. Leukocyte Biol. 47, 475-479.  
Coffman R. L., J. Ohara, M. W. Bond, J. Carty, A. Zlotnik, and W. E. Paul. (1986) J. Immunol.141.504-501  
Sideras P. , S. Bergstedt-Lindqvist, H. R. MacDonald, and e. Severison. (1985) Eur. J. Immunol. 15, 586-596.  
Paul, W. E. (1991) Blood 77, 1859-1830.

### 14. Diaclone Human IL-4 ELISA references

Bachelet, I. et al.,J Biol Chem.,2006;281(37): 27190-6  
Bachelet, I. et al.,J Immunol.,2005;175(12): 7989-95  
Bessoles, S. et al., J. Leukoc. Biol,2008; 84:224-233.  
Bessoles, S. et al., Eur. J. Immunol.,2009;39(4): 1025-35  
Bittar, M. N. et al.,Asian Cardiovasc Thorac Ann.,2007; 15(3): 214-217.  
Brandt, E. et al.,J Leukoc Biol., 2000;68(1): 125-30  
Britschgi, M. et al., J Clin Invest.,2001;107(11): 1433-41  
Charbonnier, A. S. et al., J Leukoc Biol.,2003; 73(1): 91-9.  
de Nadai, P. et al., J Immunol.,2006;176(10): 6286-93.  
Diel F. et al., Inflammation Research 2003; 52(4): 154-163  
Fahy O. et al., J. immunol., 2002; 168(11): 5912 - 5919  
Hammad H. et al., Blood, 2001; 98(4): 1135 - 1141  
Hatzfeld-Charbonnier, A. S. et al.,J Leukoc Biol.,2007;81: 1179 - 1187  
Iborra, S. et al., Infect Immun.,2005;73(9): 5842-52.  
Jain, S. et al., J. Med. Microbiol.,2009;58(2): 180-184  
Keller, M. et al., J Immunol.,2005;175(11): 7678-86  
Lelievre, E. et al.,Cytokine,1998;10(11): 831-40.  
Naisbitt D.J. et al., J. Pharmacol. Exp. Ther., 2005; 313(3): 1058 - 1065  
Naisbitt, D. J. et al., Mol Pharmacol.2003; 63(3): 732-41.  
Pichavant, M. et al., J Immunol.,2006;177(9): 5912-9.  
Schaerli P. et al., J. immunol., 2004; 173(3): 2151-2158  
Schmitt, C. et al., J Leukoc Biol.,2000; 68(6): 836-44.  
Spanou, Z. et al., J Am Soc Nephrol.,2006; 17 (10):2919-27.  
Tang, Y. et al., Am J Trop Med Hyg.,2008; 79(2): 154-158.  
Tully, G. et al., J Immunol.,2005;174(4): 2174-84.

## 15. Assay Summary



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## 16. International Summaries

### 16.1. French

#### PREPARATION DES REACTIFS : RESUME

|                                                                               |                                                                                                                                                                                                                                |                                           |                                              |
|-------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------|----------------------------------------------|
| <b>1. Tampon de Lavage</b><br>( <i>Washing Buffer</i> )                       | Ajouter 10 ml de <b>Tampon de Lavage concentré</b> ( <i>Washing Buffer Concentrate</i> ) 200 fois (200X) à 1990 ml d'eau distillée                                                                                             |                                           |                                              |
| <b>2 Tampon de Dilution du Standard</b><br>( <i>Standard Diluent Buffer</i> ) | Ajouter 25 ml de <b>Tampon de Dilution du Standard concentré</b> 10 fois ( <i>Standard Diluent Buffer Concentrate 10X</i> ) à 225 ml d'eau distillée                                                                           |                                           |                                              |
| <b>3. Standard IL-4</b><br>( <i>IL-4 Standard</i> )                           | Reconstituer le <b>Standard IL-4</b> ( <i>IL-4 Standard</i> ) en ajoutant la quantité indiquée sur le flacon avec le <b>Tampon approprié</b> (Tampon de Dilution du Standard ou Tampon de Dilution du Standard : Sérum Humain) |                                           |                                              |
| <b>4. Contrôles</b><br>( <i>Controls</i> )                                    | Reconstituer les <b>Contrôles</b> ( <i>Controls</i> ) en ajoutant la quantité indiquée sur le flacon avec le <b>Tampon approprié</b> (Tampon de Dilution du Standard ou Tampon de Dilution du Standard : Sérum Humain)         |                                           |                                              |
| <b>5. Anti-IL-4 Biotinylé</b><br>( <i>Biotinylated anti IL-4</i> )            | Nombre de barrettes                                                                                                                                                                                                            | <b>Anti-IL-4 Biotinylé Concentré (µl)</b> | <b>Diluent de l'Anticorps Biotinylé (µl)</b> |
|                                                                               | 2                                                                                                                                                                                                                              | 40                                        | 1060                                         |
|                                                                               | 3                                                                                                                                                                                                                              | 60                                        | 1590                                         |
|                                                                               | 4                                                                                                                                                                                                                              | 80                                        | 2120                                         |
|                                                                               | 6                                                                                                                                                                                                                              | 120                                       | 3180                                         |
|                                                                               | 12                                                                                                                                                                                                                             | 240                                       | 6360                                         |
| <b>6. Streptavidin-HRP</b><br>( <i>Streptavidin-HRP</i> )                     | Nombre de barrettes                                                                                                                                                                                                            | <b>Streptavidin-HRP pré-diluée (µl)</b>   | <b>Diluent HRP (ml)</b>                      |
|                                                                               | 2                                                                                                                                                                                                                              | 30                                        | 2                                            |
|                                                                               | 3                                                                                                                                                                                                                              | 45                                        | 3                                            |
|                                                                               | 4                                                                                                                                                                                                                              | 60                                        | 4                                            |
|                                                                               | 6                                                                                                                                                                                                                              | 75                                        | 5                                            |
|                                                                               | 12                                                                                                                                                                                                                             | 150                                       | 10                                           |

## RESUME DU PROTOCOLE OPERATOIRE: durée totale : 3h45mn

1. Ajouter 100 µl de **Tampon de Dilution du Standard** (*Standard Diluent Buffer*) approprié, en duplicate, dans les puits Standards (B1 à F2)
2. Ajouter à la pipette 200 µl de **Standard IL-4** (*IL-4 Standard*) reconstitué dans les puits A1 et A2 puis réaliser des dilutions du Standard allant de 35 à 1 pg/ml en transférant 100 µl d'un puit à l'autre. Jeter les 100 µl des derniers puits (F1 et F2) .
3. Ajouter 100 µl de **Tampon de Dilution du Standard** approprié (*Standard Diluent Buffer ou Standard Diluent : human serum*) en duplicate dans les puits "blancs".
4. Ajouter 100 µl d'**échantillon** (*Sample*), en duplicate, dans les puits désignés et 100 µl de **contrôle** (*control*), en duplicate dans les puits contrôles.
5. Couvrir les barrettes de puits et incuber pendant 2 heures à température ambiante (18-25°C)
6. Vider et laver les puits 3 fois avec le **Tampon de Lavage** (*Washing Buffer*)
7. Préparer l'**anticorps anti IL-4 Biotinylé** (*Biotinylated anti IL-4*)
8. Ajouter 50 µl d'**anticorps anti IL-4 Biotinylé dilué** (*diluted biotinylated anti IL-4*) dans tous les puits.
9. Couvrir les barrettes de puits et incuber pendant 1heure à température ambiante (18-25°C)
10. Vider et laver les puits 3 fois avec le **Tampon de Lavage** (*Washing Buffer*)
11. Préparer la Streptavidin-HRP
12. Ajouter 100 µl de **Streptavidin-HRP diluée** (*diluted HRP-Streptavidin*) dans tous les puits
13. Couvrir les puits et incuber pendant 30 minutes à température ambiante (18°-25°C).
14. Vider et laver les puits 3 fois avec le **Tampon de Lavage** (*Washing Buffer*)
15. Ajouter 100 µl de **solution de TMB** (*TMB solution*) prête à l'emploi dans tous les puits y compris les "blancs".
16. Incuber les barrettes de puits pendant environ 12-15 minutes à température ambiante (18°-25°C) à l'obscurité.
17. Ajouter 100 µl d'**H<sub>2</sub>SO<sub>4</sub>: Solution Stop** (*H<sub>2</sub>SO<sub>4</sub>: Stop Solution*) dans tous les puits y compris les "blancs".
18. Mesurer l'absorbance à la longueur d'onde 450 nm et optionnellement à 620 nm (entre 610 et 650 nm) comme longueur d'onde de référence.

**Remarque:** Les échantillons présentant une valeur de D.O. excédant la gamme de la courbe Standard peuvent résulter à des taux d'IL-4 incorrects. C'est pourquoi, il est recommandé de diluer de tels échantillons avec le **Tampon de Dilution du Standard** (*Standard Diluent Buffer*) afin de quantifier précisément le véritable taux d'IL-4 .

## 16.2. Spanish

### PREPARACIÓN DE LOS PRODUCTOS

|                                         |                                                                                                                       |                                         |                                           |
|-----------------------------------------|-----------------------------------------------------------------------------------------------------------------------|-----------------------------------------|-------------------------------------------|
| <b>1. Tampón de Lavado</b>              | Añadir <b>Tampón de Lavado Concentrado</b> 200 X (10 ml) a 1990 ml de agua destilada.                                 |                                         |                                           |
| <b>2. Tampón diluyente del estándar</b> | Añadir <b>Tampón Diluyente del Estándar Concentrado</b> 10 X (25 ml) a 225 ml de agua destilada.                      |                                         |                                           |
| <b>3. Estándar IL-4</b>                 | Reconstituir el <b>Estándar IL-4</b> añadiendo el Diluyente del Estándar apropiado, como indica la etiqueta del vial. |                                         |                                           |
| <b>4. Controles</b>                     | Reconstituir los <b>controles</b> añadiendo el Diluyente del Estándar apropiado, como indica la etiqueta del vial.    |                                         |                                           |
| <b>5. Anti-IL-4 biotinilado</b>         | Número de tiras                                                                                                       | Anticuerpo biotinilado concentrado (µl) | Diluyente del anticuerpo biotinilado (µl) |
|                                         | 2                                                                                                                     | 40                                      | 1,060                                     |
|                                         | 3                                                                                                                     | 60                                      | 1,590                                     |
|                                         | 4                                                                                                                     | 80                                      | 2,120                                     |
|                                         | 6                                                                                                                     | 120                                     | 3,180                                     |
|                                         | 12                                                                                                                    | 240                                     | 6,360                                     |
| <b>6. Estreptavidina-HRP</b>            | Número de tiras                                                                                                       | Estreptavidina-HRP prediluida (µl)      | Diluyente de HRP (ml)                     |
|                                         | 2                                                                                                                     | 30                                      | 2                                         |
|                                         | 3                                                                                                                     | 45                                      | 3                                         |
|                                         | 4                                                                                                                     | 60                                      | 4                                         |
|                                         | 6                                                                                                                     | 75                                      | 5                                         |
|                                         | 12                                                                                                                    | 150                                     | 10                                        |

## RESUMEN DEL PROTOCOLO. El procedimiento total tiene una duración de 3h45min.

1. Añadir 100 µl del **Tampón Diluyente del Estándar** apropiado, por duplicado, a los pocillos designados para el estándar (B1 to F2).
2. Pipetear 200 µl del **Estándar IL-4** reconstituido en los pocillos A1 y A2 y hacer diluciones seriadas del estándar con el rango de concentraciones de 35 al 1 pg/ml, transfiriendo 100 µl de un pocillo al siguiente. Descartar 100 µl de los últimos pocillos.
3. Añadir 100 µl del **Tampón Diluyente del Estándar** apropiado, por duplicado, a los pocillos que van a ser el "blanco".
4. Añadir 100 µl de las muestras, por duplicado, a los pocillos designados para ello, y 100 µl del Control reconstituido, por duplicado, a los pocillos designados como "control".
5. Cubrir la placa e incubar durante 2 horas a temperatura ambiente (18-25°C).
6. Vaciar y lavar la placa 3 veces con **Tampón de Lavado**.
7. Preparar el anticuerpo **Anti-IL-4 Biotinilado**.
8. Añadir 50 µl del **anti-IL-4 Biotinilado** y diluido, a todos los pocillos.
9. Cubrir la placa e incubar durante 1 horas a temperatura ambiente (18-25°C).
10. Vaciar y lavar la placa 3 veces con **Tampón de Lavado**.
11. Preparar la **Estreptavidina-HRP**.
12. Añadir 100 µl de **Estreptavidina-HRP** diluida a todos los pocillos.
13. Cubrir la placa e incubar 30 minutos a temperatura ambiente (18° to 25°C).
14. Vaciar y lavar la placa 3 veces con **Tampón de Lavado**.
15. Añadir 100 µl de solución **TMB preparado para utilizar**, a todos los pocillos, incluidos los pocillos con "blancos".
16. Incubar la placa durante 12-15 minutos a temperatura ambiente (18° to 25°C) y en oscuridad.
17. Añadir 100 µl de H<sub>2</sub>SO<sub>4</sub>: **Solución de Parada**, a todos los pocillos, incluidos los pocillos con los "blancos".
18. Medir la intensidad de color a 450 nm y a 620 nm como longitud de onda de referencia (de 610 nm a 650 nm sería aceptable).

**Nota:** El cálculo de concentraciones de muestras con densidad óptica que supere el rango de la curva estándar, resultaría incorrecto, dando niveles de IL-4 más bajos de lo real. Estas muestras, requerirían ser diluidas con el Tampón de Dilución de Estándar adecuado, para poder precisar la cantidad real de IL-4.