



## **PeliKine Compactä human IL-10 ELISA kit**

288 tests

An enzyme immunoassay  
for the quantitative determination of human IL-10

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## I. INTRODUCTION

The initial discovery that led to the characterization and cDNA cloning of Interleukin 10 (IL-10) was the demonstration that supernatants from activated T-cells could inhibit the secretion of cytokines by TH1 cell clones [1,2]. Later it was found that IL-10, also known as cytokine synthesis inhibitory factor (CSIF) is an immunoregulatory protein produced by a number of cell lineages, like TH2 cells, B-cells and activated monocytes [1,3-8]. As an immune system down-regulator, IL-10 inhibits the synthesis of several cytokines that are normally secreted by both human and mouse monocytes/macrophages in response to activation by LPS. These cytokines include IL-1, GM-CSF, TNF, IL-6, IL-8, IL-10 and IL-12 [1,6,8]. IL-10 is secreted by activated macrophages, and this is also inhibited by IL-10 [6]. The secretion of IL-10 is relatively late compared with other cytokines, which may explain why macrophages are able to secrete substantial amounts of various cytokines before IL-10 inhibition occurs. IFN $\gamma$  also inhibits macrophage secretion of IL-10 [9], resulting in direct cross-inhibition of IL-10 and IFN $\gamma$ . Nevertheless, IL-10 has also been shown to have up-regulating capacities, including the enhancement of *in vitro* proliferation of IL-2 and/or IL-4 induced mouse thymocytes, the induction of differentiation and proliferation of activated B-cell lines into antibody secreting lines [11], supporting the growth of mast cell lines [12] and induction of class II MHC antigen expression on B-cells [11,12].

Bioassays for the quantification of IL-10, based on the inhibition of IFN $\gamma$  and other cytokines produced by TH1 cells in response to stimulation by antigen have been used for several years. These bioassays, although sensitive, are time consuming and susceptible to interference by other substances.

The Pelikine compact™ human IL-10 ELISA kit [10] has been developed for faster, more reproducible and specific quantification of human IL-10 in serum, plasma and other body fluids, as well as in cell-culture supernatant.

## II. PRINCIPLE OF THE TEST

The Pelikine compact™ human IL-10 ELISA kit is a "sandwich-type" of enzyme immunoassay in which a monoclonal anti human IL-10 antibody is bound onto polystyrene microtiter wells. Human IL-10, present in a measured volume of sample or standard is captured by the antibody on the microtiter plate, and non-bound material is removed by washing. Subsequently, a biotinylated second monoclonal antibody to human IL-10 is added. This antibody binds to the IL-10 antibody complex present in the microtiter well. Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the IL-10 sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution is added to the wells. A coloured product is formed in proportion to the amount of IL-10 present in the sample or standard. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microtiter plate reader. From the absorbance of samples and those of a standard curve, the concentration of IL-10 can be determined by interpolation with the standard curve.

### III. STORAGE AND STABILITY

The PeliKine compact™ human IL-10 ELISA kit should be stored at -18°C to -32°C. The performance of the kit is guaranteed until the expiration date shown on the case label.

### IV. CONTENTS OF THE KIT

The PeliKine compact™ human IL-10 ELISA kit contains material sufficient for 288 tests, including standard curve samples. The reagents provided are:

Quantity	Kit component		Volume	Cap colour
1 vial	coating antibody	100-fold concentrated	375 $\mu$ l	red
1 vial	blocking reagent	50-fold concentrated	2 ml	transparent
2 vial	IL-10 standard (lyophilized)	4500 pg/ml	1000 $\mu$ l	-
1 vial	biotinylated antibody	100-fold concentrated	375 $\mu$ l	yellow
1 vial	streptavidin-poly-HRP conjugate	10,000-fold concentrated	20 $\mu$ l	brown
1 bottle	dilution buffer	5- fold concentrated	60 ml	
3 pcs	microtiter plate + lid	-	-	
10 pcs	plate seals	-	-	

### V. PRECAUTIONS FOR USE

- 1) The PeliKine compact™ human IL-10 ELISA kit is intended *for research purposes only*.
- 2) Only use the reagents and microtiter plates supplied with the kit, do not mix reagents from different kit lots.
- 3) Handle all plasma and serum samples with care to prevent transmission of blood-borne infections.
- 4) Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.
- 5) All reagents contain thiomersal (0.001 % w/v) and may be toxic upon ingestion, inhalation or skin contact. Avoid contact of skin, eyes or clothing with dilution, washing or substrate buffer. In case of contact, wash skin or eyes with water and consult a physician.
- 6) Centrifuge all vials before use (1 minute 3000 x g).
- 7) With the exception of the substrate blank wells, do not allow wells to stand uncovered or dry for extended periods between incubation steps.

**VI. ADDITIONAL BUFFERS & SOLUTIONS REQUIRED**

Coating buffer: 0.1 M Carbonate/bicarbonate buffer pH 9.6

Solution A: 1.24 g  $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$  in 100 ml distilled water

Solution B: 1.68 g  $\text{NaHCO}_3$  in 200 ml distilled water

Take 70 ml of solution A, and add solution B until the pH is 9.6 (approximately 175 ml of solution B required)

The prepared buffer can be stored up to one week at 2-8°C.

PBS stock solution [20 x]: 0.2 M Phosphate Buffered Saline (PBS)

Dissolve	32 g	$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$
	6 g	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
	164 g	$\text{NaCl}$

in 900 ml distilled water

(intensive stirring and some heating will speed dissolution).

Bring the temperature of the solution back to room temperature (18-25°C) and check pH; if necessary adjust pH to 6.8 - 6.9 with concentrated HCl or NaOH, and add distilled water to a volume of 1 liter (when diluted 20 times the obtained buffer will have a pH of 7.2 - 7.4).

Add 20 mg thiomersal as preservative. Do not use sodium azide ( $\text{NaN}_3$ ) since this preservative reduces the quality of the enzymatic label.

The prepared buffer can be stored up to three months at 2-8°C.

Note: in the concentrated buffer salt crystals may appear when stored at 2-8°C. Before preparing the working-strength buffer, first warm the concentrated buffer BRIEFLY to 37°C to dissolve the precipitate.

Washing buffer: PBS with 0.005% TWEEN20

Make 1 liter of working-strength PBS by diluting the PBS stock solution (see above) 20 fold with distilled water.

Add 50  $\mu\text{l}$  TWEEN 20.

The prepared buffer can be stored up to one month at 2-8°C.

Substrate buffer: 0.11 M acetate buffer pH 5.5

Dissolve 15.0 g sodium-acetate (  $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$  ) in 800 ml distilled water. Adjust pH to 5.5 with glacial acetic acid, add distilled water to a volume of liter. Do not add any preservative (e.g. merthiolate, sodium azide) since this may affect the quality of the enzymatic colour development.

The prepared buffer can be stored up to two weeks at 2-8°C.

3,5,3',5'-tetramethylbenzidine (TMB) stock solution: 6 mg/ml TMB in DMSO

Dissolve 30 mg 3,5,3',5'-tetramethylbenzidine (TMB) in 5 ml dimethylsulfoxide (DMSO). The prepared stock solution can be stored up to 1 month at room temperature (18-25°C) and protected against light.

Hydrogen peroxide stock solution: 3%  $\text{H}_2\text{O}_2$  solution in distilled water.

The prepared stock solution can be stored up to one month at 2-8°C.

Substrate solution:

For each plate mix the following reagents:

12 ml substrate buffer  
200  $\mu\text{l}$  TMB stock solution  
12  $\mu\text{l}$   $\text{H}_2\text{O}_2$  stock solution

**The substrate solution should be prepared just before use and has to be at room temperature (18-25°C) for optimal reproducible results.**

Stop solution: 1.8 M  $\text{H}_2\text{SO}_4$  solution in distilled water.

## VII. ADDITIONAL INFORMATION

### Additional materials required

- Pipetting devices for accurate delivery of 1-10  $\mu$ l, 50  $\mu$ l, 100  $\mu$ l and 1 ml volumes.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Device for delivery of washing buffer (wash bottle / automated plate washer).
- Microtiter plate reader.

### Sensitivity

MEAN calculated zero signal + 3 SD : 1 – 3 pg/ml (shake – static incubation)  
2x (MEAN calculated zero signal) : 3 – 5 pg/ml (shake – static incubation)

Note: the sensitivity is dependent of the type and quality of enzymatic substrate.

### Expected values

IL-10 values in fresh serum and plasma of healthy individuals are below 5 pg/ml.

### Specificity

No crossreactivity was observed with the following recombinant human proteins: IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, sIL-6r (GP80), IL-7, IL-8, IL-9, IL-11, IL-13, Macrophage Colony Stimulating Factor (M-CSF), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF), Leukemia Inhibitory Factor (LIF), RANTES, Stem Cell Factor/ Mast Cell Factor (SCF/MCF), Transforming Growth Factor  $\beta$ -1 (TGF $\beta$ -1), Tumour Necrosis Factor  $\alpha$  (TNF $\alpha$ ), Tumour Necrosis Factor  $\beta$  (TNF $\beta$ /Lymphotoxin), and Interferon  $\gamma$  (IFN $\gamma$ ).

### References

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## VIII. ASSAY PROCEDURE

1. **BRING ALL REAGENT TO ROOM TEMPERATURE (18-25°C)**, with the exception of the streptavidin-HRP conjugate which has to be kept at -18°C to -32°C to ensure stability. Centrifugate all vials before use (1 minute 3000 x g).

**For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this leaflet.**

### 2. DILUTION BUFFER

The kit contains one bottle with 5-fold concentrated dilution buffer.

For optimal assay results, dilute samples and standards in working-strength dilution buffer.

Calculate the quantity of dilution buffer required (approximately 15 ml undiluted buffer per microtiter plate) and prepare a working-strength solution by diluting the opalescent concentrated buffer 5 times in distilled water before use. The working-strength dilution buffer can be stored for up to one week at 2-8°C

### 3. MICROTITER PLATES

#### Coating antibody

#### Coating

The kit contains three micotiter plates for 96 tests each, including the standard curve samples.

Prepare coating buffer as decribed on page 3 of the information leaflet.

Per mivrotiter plate ass 120 µl of coating antibody (red-capped vial) to 12 ml coating buffer. Add 100 µl to all wells, cover micrititer plate(s) with lid and **incubate overnight at room temperature (18-25°C)**.

#### Washing procedure

Prepare working-strength PBS (1:20 dilution of stock PBS as described on page 3 of the information leaflet).

Aspirate supernatants from wells and completely fill the wells (> 300 µl) with working-strength PBS and aspirate. Repeat this four times, after the final aspiration the wells should be dry.

#### Blocking procedure

The kit contains one transparent-capped vial with 2 ml blocking reagent.

Prepare blocking buffer by adding 500 µl blocking reagent to 25 ml working-strength PBS (1:20 dilution of stock PBS as described on page 3 of the information leaflet).

Add 200 µl blocking buffer to all wells, cover microtiter plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 1 hour at room temperature (18-25°C)**.



#### 4. IL-10 STANDARD

#### Standard curve preparation

A recombinant human IL-10 standard has been calibrated against the WHO International Reference Preparation (IL-10 92/516; National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, U.K. 1 WHO Unit = 200 pg IL-10).

The kit contains two lyophilized vial with 4500 pg/ml natural human IL-10.

Reconstitute the lyophilized standard by adding 1000  $\mu$ l of distilled water to the vial. Incubate for 10 minutes at room temperature and mix gently. After reconstitution the standard must be kept cold (2-8°C) and stored frozen after use (<-18°C, preferably <-70°C).

Label 7 tubes, one tube for each dilution: 300, 120, 48, 19.2, 7.7, 3.1, and 1.2 pg/ml. Pipette 420  $\mu$ l of working-strength dilution buffer into the tube labelled 300 pg/ml and 300  $\mu$ l of working-strength dilution buffer into the other tubes. Transfer 30  $\mu$ l of the IL-10 standard (4500 pg/ml) into the first tube labelled 300 pg/ml, mix well and transfer 200  $\mu$ l of this dilution into the second tube labelled 120 pg/ml. Repeat the serial dilution's six more times by adding 200  $\mu$ l of the previous tube of diluted standard to the 300  $\mu$ l of dilution buffer.

The standard curve will contain 300, 120, 48, 19.2, 7.7, 3.1, 1.2 and 0 pg/ml (dilution buffer).

It is recommended to prepare two separate series for each assay.

Avoid repeated freeze-thawing of the standard, although experimental data have shown that up to 3 freeze-thaw cycles have no effect on the IL-10 levels of the standard. Thaw the reconstituted standard in tap water (18-25°C), do not use 37°C or 56°C water baths for this purpose.

#### 5. SAMPLES

Serum, EDTA-anti-coagulated plasmas, and culture fluids are suitable for use in the assay. Do not use grossly haemolyzed or lipemic specimens. If rheumatoid factors are expected in serum or plasma samples, it is recommended to add normal mouse serum (CLB product M1250, final concentration in the diluted sample should be 5%) If samples are to be run within 24 hours, they may be stored at 2-8°C; otherwise samples should be stored frozen (<-18°C).

Up to 3 freeze-thaw cycles have no effect on the IL-10 levels of serum or plasma samples. Nonetheless, excessive freeze-thaw cycles should be avoided. Prior to the assay, frozen samples should be thawed **as quickly as possible** in tap water (18-25°C), do not use 37°C or 56°C waterbaths for this purpose.

It is recommended to dilute the test samples at least 1:2 in working-strength dilution buffer. If high levels of IL-10 (outside the standard curve) are expected in the test samples, additional dilutions of sample i.e. 1:10 and 1:50 should also be prepared.

**6. FIRST WASH STEP**

Prepare washing buffer as described on page 3 of this leaflet.

Wash the required microtiter plates five times with washing buffer in a plate washer. In case of manual washing, completely fill the wells ( $> 300 \mu\text{l}$ ) with washing buffer and aspirate, repeat this four times. After the final aspiration the wells should be dry.

**7. FIRST INCUBATION STEP****Standards and samples**

Leaving the substrate blank wells empty, transfer  $100 \mu\text{l}$  of the prepared standards and samples in duplicate into the appropriate wells (see recommended plate plan). Cover plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well and incubate for 1 hour at room temperature ( $18\text{-}25^\circ\text{C}$ ).

**8. SECOND WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate(s) as described in point 6 above.

**9. SECOND INCUBATION STEP****biotinylated antibody**

The kit contains one yellow-capped vial with biotinylated antibody

Per microtiter plate, add  $120 \mu\text{l}$  biotinylated antibody to 12 ml working-strength dilution buffer just before use.

Leaving the substrate blank wells empty, add  $100 \mu\text{l}$  of diluted biotinylated antibody to all wells.

Cover plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well and incubate for 1 hour at room temperature ( $18\text{-}25^\circ\text{C}$ ).

**10. WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate(s) as described in point 6 above.

**11. THIRD INCUBATION STEP****Streptavidin-HRP conjugate**

The kit contains one brown vial of concentrated streptavidin-HRP conjugate, which must be stored at  $-18^{\circ}\text{C}$  to  $-32^{\circ}\text{C}$  to maintain maximal stability. The contents of the vial will not be frozen at this temperature.

Per microtiter plate, add  $3\ \mu\text{l}$  streptavidin-HRP conjugate to 30 ml of working-strength dilution buffer just before use. Do not prepare in advance of assay.

Leaving the substrate blank wells empty, add  $100\ \mu\text{l}$  of streptavidin-HRP conjugate to all wells.

Cover the microtiter plate(s) with adhesive seal, agitate the microtiter plate by tapping the edge of the frame for a few seconds to mix contents of each well and incubate for 30 minutes at room temperature ( $18-25^{\circ}\text{C}$ ).

**12. FOURTH WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate(s) as described in point 6 above.

**13. FOURTH INCUBATION STEP****Enzymatic colour development**

Approximately 10 minutes before use, prepare the substrate solution as described on page 4 of this leaflet.

**The substrate solution should be at room temperature ( $18-25^{\circ}\text{C}$ ) for optimal reproducible results.**

Add  $100\ \mu\text{l}$  of substrate solution to all wells, **including the substrate blank wells.**

Cover microtiter plate(s) with lid, gently agitate the microtiter plate by tapping the edge of the frame for a few seconds to mix contents of each well and **incubate for 30 minutes at room temperature ( $18-25^{\circ}\text{C}$ ) in the dark.**

*Do not cover the plate with aluminium foil.*

Note: The speed of enzymatic colour development is influenced by many factors including temperature and quality of the used TMB.

#### 14. STOP ENZYMATIC REACTION

Add 100  $\mu$ l of stop solution to all wells.

After stopping the colour is stable for maximally 30 minutes.

#### 15. PLATE READ-OUT

Read at 450 nm in an ELISA reader.

### IX. RESULTS

#### **Substrate blank**

- Record the absorbance at 450 nm for the substrate blank wells and average the duplicate values.

#### **Standard curve**

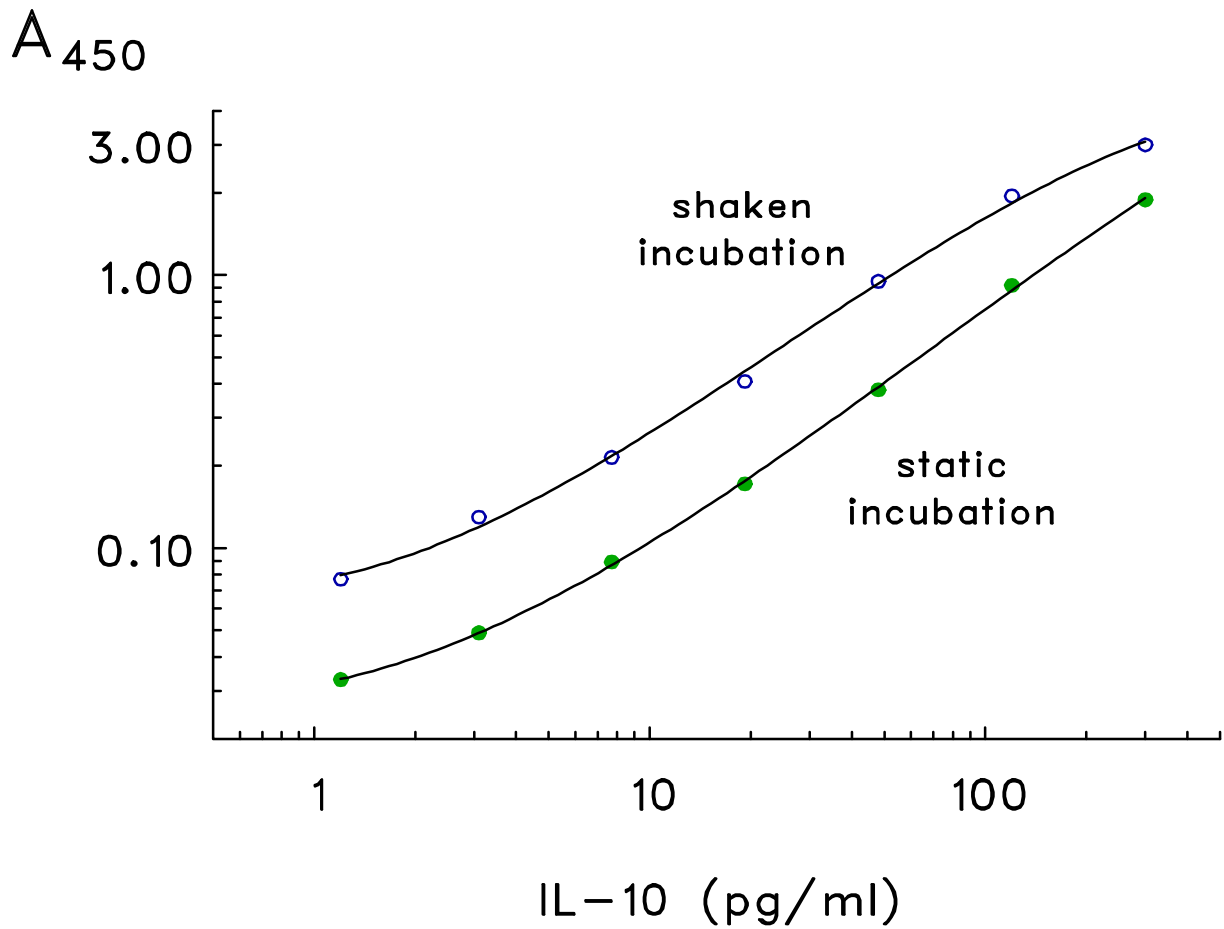
- Record the absorbance at 450 nm for each well containing standard and average the duplicate values.
- Calculate the net average absorbances by subtracting the average of the substrate blank wells.
- Plot the net average absorbances (ordinate) versus the IL-10 concentration in pg/ml (abscissa) on log-linear paper and draw the best fitting curve. An example of a standard curve is given on the next page.

#### **Samples**

- Record the absorbance at 450 nm for each standard well, and average the duplicate values.
- Calculate the net average absorbances by subtracting the average of the substrate blank wells.
- Locate the net average absorbance value found for each sample on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the IL-10 concentration (pg/ml) from the horizontal axis. Multiply the obtained IL-10 concentration with the dilution factor of the sample and record this figure.

### X. INCREASED SENSITIVITY

The assay sensitivity can be increased by a small adaptation of the incubation methodology. Just follow all the instructions as stated in the assay procedure (chapter VIII), but incubate at room temperature (18-25°C) on a horizontal plate shaker at  $700 \pm 100$  rpm. All incubations, including the enzymatic colour development, have to be completed on the shaker, in the same time as stated in the static assay procedure. This will result in an increase in assay sensitivity, with small effects on the background levels (see figure next page).



Typical standard curve for the PeliKine compact™ human IL-10 ELISA kit

	STATIC INCUBATION	SHAKEN INCUBATION
	<b>Calculated mean absorbance at 450 nm</b>	
<b>substrate blank</b>	<b>0</b>	<b>0</b>
<b>0 pg/ml</b>	<b>0.021</b>	<b>0.067</b>
<b>1.2 pg/ml</b>	<b>0.033</b>	<b>0.077</b>
<b>3.1 pg/ml</b>	<b>0.049</b>	<b>0.178</b>
<b>7.7 pg/ml</b>	<b>0.089</b>	<b>0.215</b>
<b>19.2 pg/ml</b>	<b>0.172</b>	<b>0.408</b>
<b>48 pg/ml</b>	<b>0.380</b>	<b>0.950</b>
<b>120 pg/ml</b>	<b>0.917</b>	<b>2.259</b>
<b>300 pg/ml</b>	<b>1.886</b>	<b>&gt; 3.000</b>

**DO NOT USE THESE DATA TO CONSTRUCT A STANDARD CURVE FOR SAMPLE VALUE CALCULATIONS**

Plate plan proposed for the PeliKine compact™ human IL-10 ELISA kit:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	S1	S1										
<b>B</b>	S2	S2										
<b>C</b>	S3	S3										
<b>D</b>	S4	S4										
<b>E</b>	S5	S5										
<b>F</b>	S6	S6										
<b>G</b>	S7	S7										
<b>H</b>	S8	S8									B	B

Key: B: substrate blank

S1-S8: IL-10 standards 0-300 pg/ml

Empty: samples

## Protocol summary and checklist PeliKine compact<sup>®</sup> human IL-10 ELISA kit

### Day 0:

- Bring coating antibody to room temperature (18-25°C).
- Prepare coating buffer.
- Dilute coating antibody 1:100 in coating buffer, add 100  $\mu$ l to all wells, cover the plate(s) and incubate overnight at room temperature.

### Day 1:

- Bring all reagents, with the exception of streptavidin-HRP, to room temperature.
- Prepare blocking buffer.
- Wash the plate(s) five times with PBS.
- Add 200  $\mu$ l blocking buffer to all wells and incubate for one hour at room temperature.
- Prepare standard and sample dilutions.
- Prepare washing buffer.
- Wash the plate(s) five times with washing buffer.
- Leaving the substrate blank wells empty, add 100  $\mu$ l of the diluted biotinylated antibody to all wells, cover the plate(s) and incubate for one hour at room temperature.
- Dilute the streptavidin-HRP conjugate 1:10,000 in dilution buffer.
- Wash the plate(s) five times with washing buffer.
- Leaving the substrate blank wells empty, add 100  $\mu$ l of the streptavidin-HRP conjugate to all wells, cover the plate(s) and incubate for 30 minutes at room temperature.
- Just before use, prepare substrate solution.
- Wash the plate(s) five times with washing buffer.
- Add 100  $\mu$ l substrate solution to all wells, including the substrate blank wells, incubate for 30 minutes at room temperature in the dark.
- Add 100  $\mu$ l stop solution to all wells and read the plate at 450 nm.
- Calculate the amount of IL-10 in the samples.