Assay procedure for

PeliKine compact™ ELISA kit
(288 tests)

Research Use Only

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This assay procedure is suitable for the following products

M1910 PeliKine compact™ human IL-10 ELISA kit
M1913 PeliKine compact™ human IL-13 ELISA kit
M1914 PeliKine compact™ human IL-4 ELISA kit
M1916 PeliKine compact™ human IL-6 ELISA kit
M1918 PeliKine compact™ human IL-8 ELISA kit
M1923 PeliKine compact™ human TNF-α ELISA kit
M1926 PeliKine compact™ soluble human IL-6 receptor ELISA kit
M1933 PeliKine compact™ human IFN-γ ELISA kit
M1934 PeliKine compact™ human IL-1β ELISA kit

Please note: This assay procedure comes with a product specification sheet.
I. PRINCIPLE OF THE TEST

The PeliKine compact™ ELISA kit is a "sandwich-type" of enzyme immunoassay in which a monoclonal antibody has been precoated to polystyrene microtiter wells. The target cytokine, present in a measured volume of sample or standard, is bound by the antibody on the microtiter plate. Non-bound material is then removed by washing. Subsequently, a biotinylated antibody is added. This antibody binds to the cytokine-antibody complex present in the microtiter well. Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidine, which binds onto the biotinylated side of the cytokine sandwich. After removal of non-bound HRP conjugate by washing, substrate solution is added to the wells. A coloured product is formed in proportion to the amount of cytokine 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 cytokine can be determined by interpolation with the standard curve. A schematic representation of this protocol and a proposed plate plan are given on the last pages of this package insert.

The PeliKine compact™ ELISA kit is intended for research purposes only.

II. STORAGE AND STABILITY

The PeliKine compact™ 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.

III. CONTENTS OF THE KIT

See the added specification sheet.

IV. PRECAUTIONS FOR USE

1) Only use the reagents and microtiter plates supplied with the kit, do not mix reagents from different kit lots.
2) Handle all plasma and serum samples with care to prevent transmission of blood-borne infections.
3) Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.
4) All reagents contain merthiolate (0.001 % w/v).
5) Mix all reagents thoroughly before use (without foaming).
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.
V. ADDITIONAL INFORMATION

Additional materials required

- Pipetting devices for accurate delivery of 1-10 µL, 50 µL, 100 µL 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.

Additional buffers and solutions required

Note: Instead of making all the buffers and solutions by yourself, you can also use our PeliKine Toolset (Ref M1980).

Coating buffer: 0.1 M Carbonate/bicarbonate buffer pH 9.6

Solution A: 1.24 g Na₂CO₃.H₂O in 100 mL distilled water
Solution B: 1.68 g NaHCO₃ 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 Na₂HPO₄.2H₂O
6 g NaH₂PO₄.2H₂O
164 g NaCl
in 900 mL distilled water

(intensive stirring and some heating will speed up dissolution).
Bring the temperature of the solution back to 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 (NaN₃) 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 in a waterbath to dissolve the precipitate.
Washing buffer: PBS with 0.005% TWEEN20

- Make 1 liter of working-strength PBS by diluting 50 mL of the PBS stock solution (see above) 20 fold with distilled water.
- Add 50 µL 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 (CH₃COONa·3H₂O) 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 18-25°C and should be protected against light.

Hydrogen peroxide stock solution: 3% H₂O₂ 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 µL TMB stock solution
  - 12 µL H₂O₂ stock solution

- The substrate solution should be prepared just before use and has to be at 18-25°C for optimal reproducible results, and should be protected from prolonged exposure to light.
**VI. ASSAY PROCEDURE**

**Day 0**
- Bring coating antibody to 18-25°C.
- Prepare coating buffer as described in chapter V.

1. **MICROTITER PLATES Coating**
   - The kit contains three micotiter plates for 96 tests each, including the standard curve samples, and one red capped vial with coating antibody.

   Prepare coating buffer as described in chapter V.
   - Per microtiter plate add 120 µL of coating antibody (red-capped vial) to 12 mL coating buffer. Add 100 µL to all wells, cover microtiter plate(s) with lid and **incubate overnight at 18-25°C**.

**Day 1**
- **BRING ALL REAGENTS TO 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.
- For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this package insert.

2. **WASHING PROCEDURE (PBS only)**

   Prepare working-strength PBS (1:20 dilution of stock PBS as described in chapter V). 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.
3. BLOCKING PROCEDURE

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

Per microtiter plate add 500 µL blocking reagent to 25 mL working-strength PBS (1:20 dilution of stock PBS as described in chapter V). 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 18-25°C**.

4. HPE-DILUTION BUFFER

The kit contains one bottle with 5-fold concentrated HPE-dilution buffer. For optimal assay results, dilute samples and standard in working-strength HPE-dilution buffer.

Per microtiter plate add 15 mL concentrated HPE-dilution buffer to 60 mL distilled water to prepare a working-strength HPE-dilution buffer. The opalescent working-strength HPE-dilution buffer can be stored for up to one week at 2-8°C.

**Note:** In the concentrated buffer salt crystals may appear, before preparing the working-strength buffer, first warm the concentrated buffer BRIEFLY to 37°C in a waterbath to dissolve the precipitate.

5. STANDARD

Standard curve preparation

The kit contains one or two black-capped vial(s) with standard (see back of the added specification sheet).

Prepare the standard curve as described on the back of the added specification sheet.

Avoid repeated freeze-thawing of the standard, although experimental data have shown that up to 3 freeze-thaw cycles have no effect on the cytokine levels of the standard. Thaw the standard in tap water (18-25°C) and do not use 37°C or 56°C water baths for this purpose. After use the standard should be stored at -18 to -32 °C.

6. SAMPLES

Serum, EDTA-anti-coagulated plasmas, and culture fluids are suitable for use in the assay (caution: separate plasma/serum and blood cells within 4 hours after collection, non-separated samples must be kept at temperatures from 2-8°C). Do not use grossly haemolyzed or lipemic specimens. 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, preferably < -70°C), aliquot samples to prevent excessive freeze-thaw cycles. 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 water baths for this purpose.

The recommended dilution of the test samples is described on the back of the added specification sheet.
7. FIRST WASH STEP (Washing buffer)

Prepare washing buffer as described in chapter V.

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

8. FIRST INCUBATION STEP (Standards and samples)

Leaving the substrate blank wells empty, transfer 100 µL 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 18-25°C.

Just before washing, prepare next incubation reagent as described in point 10.

9. SECOND WASH STEP

Aspirate supernatant from wells and wash the microtiter plate as described in point 7.

10. SECOND INCUBATION STEP (biotinylated antibody)

The kit contains one yellow-capped vial with biotinylated antibody.

Per microtiter plate add 120 µL biotinylated antibody to 12 mL working-strength HPE-dilution buffer. Leaving the substrate blank wells empty, add 100 µL 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 18-25°C.

Just before washing prepare next incubation reagents as described in point 12.

11. THIRD WASH STEP

Aspirate supernatant from wells and wash the microtiter plate as described in point 7.
12. THIRD INCUBATION STEP

The kit contains one brown-capped vial of concentrated streptavidin-HRP conjugate, which must be stored at –18°C to –32°C to maintain maximal stability. The contents of the vial will not be frozen at this temperature.

Per microtiter plate, add 3 µL streptavidin-HRP conjugate to 30 mL of working-strength HPE-dilution buffer just before use. Do not prepare in advance of assay.

Leaving the substrate blank wells empty, add 100 µL of the diluted 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 18-25°C.

Just before washing prepare next incubation reagents as described in points 14 and 15.

13. FOURTH WASH STEP

Aspirate supernatant from wells and wash the microtiter plate as described in point 7.

14. FOURTH INCUBATION STEP

Approximately 10 minutes before use, prepare the substrate solution as described in chapter V. The substrate must be at 18-25°C and must be protected from exposure to light.

Add 100 µL 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 18-25°C in the dark.

Do not cover the plate with aluminum foil.

Note: Colour development and sensitivity are dependent on the type and quality of the enzymatic substrate. The data shown in the added specification sheet are obtained by using the PeliKine Toolset.

15. STOP ENZYMATIC REACTION

Use the prepared stop solution of 1.8 M H₂SO₄ (as described in chapter V)

Add 100 µL of stop solution to all wells.

After stopping the colour is stable for maximally 30 minutes.
16. PLATE READ-OUT

Read at 450 nm in an ELISA reader.

VII. 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 cytokine concentration (abscissa) on log-linear paper and draw the best fitting curve. An example of a standard curve is given on the added specification sheet.

**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 cytokine concentration from the horizontal axis. Multiply the obtained cytokine concentration with the dilution factor of the sample and record this figure.

A computer program to calculate ELISA results (developed by Mr. E.J. Nieuwenhuys, Sanquin Amsterdam) is available free of charge to our kit users. This program can be downloaded from internet (see link at www.sanquinreagents.com) [http://www.xs4all.nl/~ednieuw/Calibration/Logit/logit.htm](http://www.xs4all.nl/~ednieuw/Calibration/Logit/logit.htm)

VIII. 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 VI), but perform all incubations of day 1 at 18-25°C, but on a horizontal plate shaker at 700 ± 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.
### IX. PROPOSED PLATE PLAN

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**Key:**
- **B**: substrate blank
- **S1-S8**: standard curve (see specification sheet)
- Empty: samples
X. Protocol summary and checklist

Day 0:
- Bring coating antibody to 18-25°C.
- Prepare coating buffer.
- Dilute coating antibody 1:100 in coating buffer, add 100 µL to all wells, cover the plate(s) and incubate overnight at 18-25°C.

Day 1:
- Bring all reagents, with the exception of streptavidin-HRP, to 18-25°C.
- Prepare blocking buffer.
- Wash the plate(s) five times with PBS.
- Add 200 µL blocking buffer to all wells and incubate for one hour at 18-25°C.
- Prepare working-strength HPE-dilution buffer.
- Prepare standard and sample dilutions.
- Prepare washing buffer.
- Wash the plate five times with washing buffer.
- Leaving the substrate blank wells empty, add 100 µL of standard and sample dilutions to the appropriate wells, cover the plate and incubate for one hour at 18-25°C.
- Dilute biotinylated antibody 1:100 in working-strength HPE-dilution buffer.
- Wash the plate five times with washing buffer.
- Leaving the substrate blank wells empty, add 100 µL of the diluted biotinylated antibody to all wells, cover the plate and incubate for one hour at 18-25°C.
- Dilute the streptavidin-HRP conjugate 1:10,000 in working-strength HPE-dilution buffer.
- Wash the plate five times with washing buffer.
- Leaving the substrate blank wells empty, add 100 µL of the streptavidin-HRP conjugate to all wells, cover the plate and incubate for 30 minutes at 18-25°C.
- Prepare substrate and stop solution.
- Wash the plate five times with washing buffer.
- Add 100 µL substrate solution to all wells, including the substrate blank wells, incubate for 30 minutes at 18-25°C in the dark.
- Add 100 µL stop solution to all wells and read the plate at 450 nm.
- Calculate the amount of cytokine in the samples.