Assay procedure for

Etanercept level ELISA

An enzyme immunoassay for the quantitative determination of Etanercept drug levels

Four 96-wells plates (384 tests)

Research Use Only
I. INTRODUCTION
The therapeutic fusion protein Etanercept affects tumour necrosis factor alpha (TNF) and is frequently administered to patients that suffer from rheumatic arthritis, intestinal disorders, dermatological diseases and cancer. TNF plays an important role in inflammation; it causes for example pain, swollen joints and stiffness in rheumatoid arthritis patients. Inhibition of TNF is therefore believed to relieve some of these symptoms and subsequently to improve quality of life of patients.

Identification of drug levels can be important for patient adjusted treatment schedules. In addition, low drug levels may be a sign of ineffectiveness of Etanercept before rebound of clinical symptoms. Alternatively, it is proposed that in patients that respond well to Etanercept, the dosing of Etanercept can be reduced according to serum concentrations. Drug level tests can therefore help to adapt patient medication or to switch to an alternative TNF inhibitor.

Plasma and serum levels of TNF inhibitors are variable between patients, and correlate to the clinical symptoms in patients. In less than 2% of patients treated with Etanercept, anti-drug antibodies were found.

This Etanercept level ELISA has been developed for fast, reproducible and specific quantification of Etanercept concentrations in plasma and serum.

II. TECHNICAL PRINCIPLE
The Etanercept level ELISA is a "sandwich-type" of enzyme immunoassay in which TNF is captured by monoclonal antibodies which are coated to polystyrene microtiter wells. The Etanercept, present in a measured volume of sample or standard, binds to the TNF on the microtiter plate. Non-bound material is then removed by washing. Subsequently, a biotinylated anti-drug antibody is added. This antibody binds to the Etanercept/TNF/anti-TNF 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 anti-drug antibody. 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 Etanercept 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 Etanercept can be determined by interpolation with the standard curve.

Note: The Etanercept level ELISA is intended for research purposes only.

III. STORAGE AND STABILITY
The Etanercept level ELISA 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

<table>
<thead>
<tr>
<th>Quantity</th>
<th>REF</th>
<th>Kit component</th>
<th>Concentration</th>
<th>Volume</th>
<th>Cap colour</th>
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<td>1 vial</td>
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<td>coating antibody</td>
<td>1000-fold</td>
<td>60 µL</td>
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<td>1 vial</td>
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<td>1 vial</td>
<td>M188704</td>
<td>biotinylated antibody</td>
<td>1000-fold</td>
<td>60 µL</td>
<td>yellow</td>
</tr>
<tr>
<td>1 vial</td>
<td>M1942</td>
<td>streptavidin-poly-HRP</td>
<td>10,000-fold</td>
<td>20 µL</td>
<td>brown</td>
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<tr>
<td>1 bottle</td>
<td>M1940</td>
<td>HPE-dilution buffer</td>
<td>5-fold</td>
<td>55 mL</td>
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</tbody>
</table>

Note: The kit does not contain the Etanercept standard, a protocol is supplied how to prepare the Etanercept standard curve from an Etanercept syringe of 50 mg/mL.
V. TEST SAMPLE HANDLING

- Serum and EDTA anti-coagulated plasma are suitable for use in the assay. Samples should be taken within 24 hours before the drug is injected. The indicated expected levels reflect the through level of the patient.
- Separate plasma or serum from the blood cells within 4 hours after collection.
- If samples are not analysed within 24 hours, the samples should be stored frozen (&lt;-18°C, preferably &lt;-70°C). Aliquot samples to avoid excessive freeze-thaw cycles.
- **Fresh samples:** If samples are to be run within 24 hours, they may be stored at 2-8°C.
- **Frozen samples:** 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.

**CAUTION: DO NOT** use grossly haemolysed or lipaemic specimens.

VI. ADDITIONAL MATERIALS REQUIRED

1. **Materials**
   - Pipettes for accurate delivery of 1-10 µL, 50 µL, 100 µL and 1 mL volumes.
   - Polystyrene 96-wells microtiter plates (we recommend 96-wells ELISA plates Nunc Maxisorp®).
   - Beakers, flasks, cylinders necessary for preparation of reagents.
   - Microtiter plate reader 450 nm. We advice a reader with a dynamic range 0 – 6.0 OD.
   - One vial with Etanercept (50 mg/mL or a different concentration) within the expiry date.

2. **Buffers and solutions**

   **PBS stock solution** (20 x): 0.2 M Phosphate Buffered Saline (PBS)
   
   Dissolve in 900 mL distilled water
   
   32 g Na$_2$HPO$_4$·2H$_2$O
   
   6 g NaH$_2$PO$_4$·2H$_2$O
   
   164 g NaCl
   
   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 (NaN$_3$) since this preservative reduces the quality of the enzymatic reaction.

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

   **DO NOT use PBS tablets!**

   **Washing buffer:** PBS with 0.005% TWEEN 20

   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 (CH$_3$COONa·3H$_2$O) in 800 mL distilled water. Adjust pH to 5.5 with glacial acetic acid, add distilled water to a volume of 1 liter.

   Do not add any preservative 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.
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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.

Stop solution:

1.8 M H₂SO₄ solution in distilled water.

3. Notes
- In the concentrated PBS buffer salt crystals may appear, before preparing the working-strength buffer, first warm the concentrated buffer briefly to 37°C in a water bath to dissolve the precipitate.
- Avoid repeated freeze-thawing of the TNF and antibodies. Thaw the samples in tap water (18-25°C) and do not use 37°C or 56°C water baths for this purpose.
- Use calibrated pipettes.
- Prepare dilutions in tubes or in the U-shape microtiter plate.
- Prevent wells to stay dry.

VII. ASSAY PROTOCOL

1. REAGENTS
- All reagents have to be at room temperature (RT:18-25°C) before use, with the exception of the streptavidin-HRP which has to be kept at -18°C to -32°C to ensure stability.
- All steps should be performed at RT.
- In all steps, a volume of 100 µL/well should be used.
- All incubations, except for the coating step, should be performed in HPE buffer.
- All incubation steps, except for the coating step, should be performed on a horizontal plate shaker (maximal size 30x30 cm) 200 ± 100 rpm.
- Before incubation, 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.

2. WASHING
- Aspirate supernatants from wells and completely fill the wells (> 250 µL) with washing buffer.
- Aspirate washing buffer and completely fill the wells with washing buffer. Repeat this four times.

After the final aspiration the wells should be dry!
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3. PROCEDURE

Day 0
- Prepare the PBS as described in chapter VI.
- Dilute the coating antibody (anti-TNF antibody) 1:1000 in PBS.
- Add 100 µL to all wells, cover microtiter plate(s) with lid and incubate overnight at RT.

Day 1
- At the start of the day, bring all reagents to 18 to 25°C, with the exception of the streptavidin-HRP which has to be kept at -18°C to -32°C at all times to ensure stability.
- Prepare the following solutions.

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 (also called: HPE buffer).

Per microtiter plate add 13 mL 5-fold concentrated HPE buffer to 52 mL distilled water to prepare HPE buffer.

The opalescent HPE buffer can be stored for one week at 2-8°C.

Etanercept standard of 50 ng/mL in HPE buffer:
Dilute the Etanercept from the vial (for this example we assume a concentration of 50 mg/mL of Etanercept. Etanercept may come in different concentrations. Adjust the dilutions of your standard accordingly) directly into HPE buffer in 3 steps as described below.

Step 1: Take 10 µL of the drug and dilute the drug into 990 µL HPE buffer (= 500 µg/mL)
Step 2: Take 10 µL of the solution from step 1 and dilute the drug into 990 µL HPE buffer (= 5 µg/mL)
Step 3: Take 10 µL of the solution from step 2 and dilute the drug into 990 µL HPE buffer (= 50 ng/mL)

The dilutions in each step can be stored for one week at 2-8°C.

IMPORTANT:
Use calibrated pipettes and prepare the Etanercept dilutions in tubes. Close the tubes after each step and mix the dilutions by GENTLY vortexing for ~1 minute. Prevent foam formation.

Just before each washing procedure, prepare the next incubation reagent.
- Take the coated plate(s) of day 0 and wash plate(s) with washing buffer according to the washing procedure.
- Dilute the recombinant TNF 1:1000 in HPE buffer. Add 100 µL of diluted recombinant TNF per well and incubate for 1 hour on a plate shaker (200±100 rpm) at RT.
- Prepare the Etanercept standard of 50 ng/mL in HPE buffer as described.
- The Etanercept standard curve can be prepared by a 2-fold dilution series in HPE buffer of 7 steps starting with 25 ng/mL Etanercept (range standard curve 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4 ng/mL). It is recommended to prepare two separate series for each assay. (See also XI. Proposed plate plan)
- Dilute patient samples 1:100, 1:500, 1:2500 and 1:12500 in HPE buffer. It is recommended to prepare two separate series for each sample.
- Wash plate(s) 5-times with washing buffer.
- Add 100 µL of diluted standard or patient sample per well and incubate for 1 hour on a plate shaker (200±100 rpm) at RT.
- Dilute the biotinylated antibody (rabbit anti-Etanercept antibody) 1:1000 in HPE buffer.
- Wash plate(s) 5-times with washing buffer.
- Add 100 µL of diluted anti-Etanercept antibody per well and incubate for 1 hour on a plate shaker (200±100 rpm) at RT.
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- Dilute the streptavidin-polyHRP 1:10,000 in HPE buffer.
- Wash plate(s) 5-times with washing buffer.
- Add 100 µL of diluted streptavidin-polyHRP per well and incubate 30 minutes on a plate shaker (200 ± 100 rpm) at RT.
- Prepare the substrate solution as described in chapter VI. The substrate must be at 18-25°C and must be protected from exposure to light.
- Wash plate(s) 5-times with washing buffer.
- Add 100 µL of substrate solution per well and wait until the blue colour has developed in the positive wells and the blank is still colourless (average incubation time is 10 ± 3 min, the total time needed for development of the colour is depending on the quality of the substrate solution).
- Stop the reaction by adding 100 µL of 1.8M H₂SO₄ to the coloured substrate solution.
- Measure the plate(s) in an ELISA reader at A450 nm.

Read the plates within 30 minutes after the stop solution is added.

VIII. RESULTS

Every in-house software method for calculation of concentrations may be used. Below, a general method for calculation by hand is described.

Standard curve
Record the absorbance at 450 nm for each well containing standard and calculate the average of the duplicate values. Plot the net average absorbance (ordinate) versus the Etanercept concentration (abscissa) on log-linear scale and draw the best fitting curve.

Samples
Record the absorbance at 450 nm for each well containing a specific sample, and calculate the average of the duplicate values. 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 Etanercept concentration from the horizontal axis. Multiply the obtained Etanercept concentration with the dilution factor of the sample and record this figure.

IX. ASSAY RANGES

Concentrations in individuals treated with Etanercept usually range between 0.8–10 µg/mL. Through samples in patients without antibodies against the drug contain median concentrations of 2 µg/mL (IQR 1-3 µg/mL).

X. SPECIFICITY

No cross reactivity was observed with other plasma proteins or with other TNF inhibitors.
XI. PROPOSED PLATE PLAN

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B: substrate blank  S1-S7: Etanercept standard curve  Empty: samples

XII. RESTRICTIONS

1. User should be trained and familiar with ELISA assays and test procedure.
2. The method described in this manual is specific for manual performance of the test, (fully) automated procedures have to be validated by the user.
3. Grossly haemolysed or lipaemic samples should not be used.
4. Out of range samples should be repeated using different dilutions.
5. Only use the reagents supplied with the kit.
6. Reagents from different batches are not interchangeable; do not mix reagents from different kit lots.
7. Rests of reagents (e.g. dead volume) should not be mixed with contents of freshly opened vials.
8. Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
9. Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.
10. Mix all reagents thoroughly but gently before use (without foaming).
11. Centrifuge all vials before use (1 minute 3000 x g).
12. Do not allow wells to stand uncovered or dry for extended periods between incubation steps.
13. All samples should be considered as potentially infectious. Handle all plasma and serum samples with care to prevent transmission of blood-borne infections.
14. Preservative: merthiolate (0.001% w/v).
15. Use new plate seals for each incubation/fixation step in the ELISA-experiment to avoid cross contamination. Do not use aluminium foil.
16. Use disposable pipette tips for each transfer to avoid cross contamination.
17. The waste-disposal should be performed according to your laboratory regulations.

XIII. REFERENCES