
Human Thrombopoietin, TPO ELISA kit

Catalog No.E0135h

(96 tests)

Operating instruction

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PLEASE READ THROUGH ENTIRE PROCEDURE BEFORE BEGINNING!**

Intended use

This immunoassay kit allows for the specific measurement of human Thrombopoietin, TPO concentrations in cell culture supernates, serum and plasma.

Introduction

Thrombopoietin (Tpo), also known as megakaryocyte growth and development factor (MGDF), the ligand for the receptor encoded by the c-Mpl proto-oncogene, is a key regulator of megakaryocytopoiesis and thrombopoiesis in vitro and in vivo. Tpo has been purified and cloned from several species including mouse, mouse, rat, and dog. The proteins from the various species are highly conserved, exhibiting from 69-75% sequence identity at the amino acid sequence level. The mouse Tpo cDNA sequence encodes a 356 amino acid residue protein with a 21 amino acid residue signal peptide that is cleaved to yield the 335 amino acid residue mature protein. Mature Tpo can be divided into two domains: the amino-terminal half with homology to erythropoietin (Epo) and the carboxy-terminal half rich in serine, threonine and proline residues and containing seven potential N-linked glycosylation sites. The Epo-domain of mouse Tpo shows approximately 21% amino acid sequence identity to mouse Epo and has been shown to activate the c-Mpl receptor. The carboxy terminus domain of Tpo has been shown to regulate the specific activity and circulating half-life of Tpo. The carboxy-terminal may also have a role in promoting the efficient biosynthesis and secretion of Tpo. The cDNA for a variant form of Tpo (Tpo-2) with a four amino acid residue deletion within the Epo homology domain has been isolated. Tpo-2 is very poorly secreted and lacks Tpo activity. Tpo mRNA is expressed predominantly in adult and fetal liver, kidney and bone marrow stromal cells.

Test principle

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for TPO has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TPO present is bound by the immobilized antibody. An enzyme-linked antibody specific for TPO is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TPO bound in the initial step. The color development is stopped and the intensity of the color is measured.

Materials and components

Reagent	Quantity
Assay plate	1
Standard	2
Sample Diluent	1 x 20ml
Assay Diluent A	1 x 10ml
Assay Diluent B	1 x 10ml
Detection Reagent A	1 x 120ul
Detection Reagent B	1 x 120ul
Wash Buffer (25 x concentrate)	1 x 30ml
Substrate	1 x 10ml
Stop Solution	1 x 10ml

Sample collection and storage

Cell culture supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at -20° C.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 x g at $2 - 8^{\circ}$ C within 30 minutes of collection. Store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Note: Citrate plasma has not been validated for use in this assay.

Limitations of the procedure

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1. The kit should not be used beyond the expiration date on the kit label.
2. Do not mix or substitute reagents with those from other lots or sources.
3. If samples generate values higher than the highest standard, further dilute the samples with the Assay Diluent and repeat the assay. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
4. This assay is designed to eliminate interference by soluble receptors, ligands, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

Reagent preparation

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 ml of Wash Buffer.

Standard - Reconstitute the **Standard** with 1.0 mL of **Sample Diluent**. This reconstitution produces a stock solution of 2,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The diluted standard serves as the high

standard (1,000 pg/mL). The **Sample Diluent** serves as the zero standard (0 pg/mL).

Detection Reagent A and B - Dilute to the working concentration specified on the vial label using **Assay Diluent A and B** (1:100), respectively.

Assay procedure

Allow all reagents to reach room temperature. Arrange and label required number of strips.

1. Prepare all reagents, working standards and samples as directed in the previous sections.
2. Add 100 uL of **Standard**, Control, or sample* per well. Cover with the adhesive strip. Incubate for 2 hours at 37° C.
3. Remove the liquid of each well, don't wash.
4. Add 100 uL of **Detection Reagent A** to each well. Incubate for 1 hour at 37°C. **Detection Reagent A** may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.
5. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (350 uL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 uL of **Detection Reagent B** to each well. Cover with a new adhesive strip. Incubate for 1 hours at 37° C.
7. Repeat the aspiration/wash as in step 5.
8. Add 90 uL of **Substrate Solution** to each well. Incubate for 30 minutes at room temperature. Protect from light.
9. Add 50 uL of **Stop Solution** to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

Specificity

This assay recognizes recombinant and natural human TPO. No significant cross-reactivity or interference was observed.

Sensitivity

The minimum detectable dose of human TPO is typically less than 3.9 pg/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest detectable concentration that could be differentiated from zero.

Detection Range

15.6-1,000 pg/mL. The assay range was estimated by calculating the coefficient of variation (CV) of each standard constructing five independent standard curves. The standard curve concentrations used for the ELISA's were 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL.

Important Note:

1. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
2. It is recommended that no more than 32 wells be used for each assay run if manual pipetting is used since pipetting of all standards, specimens and controls should be completed within 5 minutes. A full plate of 96 wells may be used if automated pipetting is available.
3. Duplication of all standards and specimens, although not required, is recommended.
4. When mixing or reconstituting protein solutions, always avoid foaming.
5. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
6. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

Calculation of results

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the TPO concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Storage of test kits and instrumentation

1. Unopened test kits should be stored at 2-8°C upon receipt and the microtiter plate should be kept in a sealed bag with desiccants to minimize exposure to damp air. The test kit may be used throughout the expiration date of the kit (six months from the date of manufacture). Refer to the package label for the expiration date.
2. Opened test kits will remain stable until the expiring date shown, provided it is stored as prescribed above.
3. A microtiter plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 OD or greater at 450nm wavelength is acceptable for use in absorbance measurement.

Precaution

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.