Human sTNFR1 EIA

Enzyme Immunoassay for the quantitative determination of human soluble Tumor Necrosis Factor Receptor 1 (sTNFR1)

Instructions for Use

REF **BIO94**

96 determinations

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INTENDED USE

The EKF Diagnostics Human sTNFR1 EIA is an enzyme-linked immunoassay for the quantitative determination of human soluble Tumor Necrosis Factor Receptor 1 (sTNFR1) in human serum and plasma samples. For in vitro diagnostic use.

BACKGROUND

Tumor necrosis factor (TNF, TNF-α, cachectin) is a pleiotropic cytokine which is a major mediator of immunological and inflammatory processes. TNF is synthesised primarily by activated monocytes, macrophages and T-lymphocyte subsets as a type II transmembrane protein arranged in stable homotrimers and is released into the circulation through proteolytic cleavage[1-3]. TNF exerts its biological effects on cell function through interaction with specific high affinity receptors expressed on the cell surface. Two distinct TNF receptors have been described that display high affinity for TNF, namely Tumor Necrosis Factor Receptor 1 (TNFR1, p55, p60, htr antigen, TNFRSF1A, CD120a) and Tumor Necrosis Factor Receptor 2 (TNFR2, p75, p80, utr antigen, TNFRSF1B, CD120b) [4-7]. TNFR1 is constitutively expressed at low levels on almost all cell types, whereas expression of TNFR2 is highly regulated and restricted primarily to cells of the immune system, endothelial cells and neurological tissue[1]. TNFR1 and TNFR2 share only a low degree of homology in their surface exposed TNF-binding domains (28% identity) while their intracellular signalling domains show no notable similarity[8]. TNFR1 appears to be the key mediator of TNF signalling in the vast majority of cells[1,9].

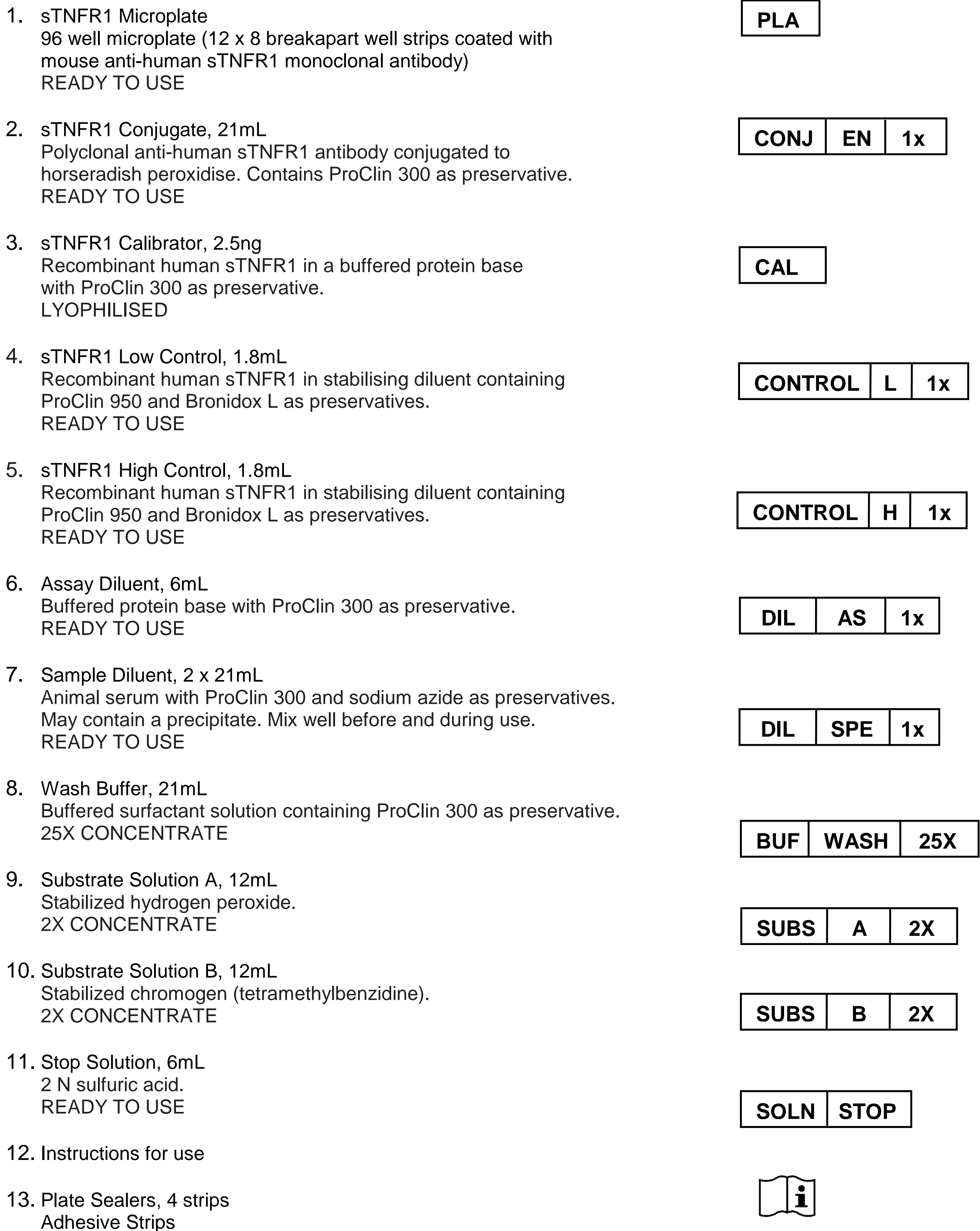
Circulating soluble TNF receptors (sTNFR) are generated through proteolytic cleavage of the surface exposed TNF-binding ectodomains[3]. The full-length 55kDa TNFR1 receptor can also be shed into the circulation through release in exosome-like vesicles[10]. The exact physiological role of circulating sTNFRs is not well established, although a number of roles for circulating sTNFRs have been postulated; 1) sTNFRs may compete with the cell surface receptors for ligand binding and thus block TNF activity; 2) shedding of sTNFRs may act to desensitise cells from the effects of TNF; 3) sTNFRs may act to stabilise to bioactivity of circulating TNF[11].

sTNFR1 can be detected in serum and plasma samples in healthy individuals by immunoassay and sTNFR1 levels appear to be stable over time within individuals[12-13]. Elevations in circulating sTNFR1 have been reported in a wide variety of clinical conditions including cancer[14-16], congestive heart failure[17], rheumatoid arthritis[18-20], infection[21-23] and diabetic nephropathy[24-25]. Elevations in circulating sTNFR1 concentrations were strongly associated with the subsequent development of advanced renal disease in type I / type 2 diabetic patients[24-28].

ASSAY PRINCIPLE

The EKF Diagnostics Human sTNFR1 EIA is a 4.5 hour solid phase quantitative sandwich enzyme immunoassay. The test procedure is based on the sequential addition of sample, polyclonal anti-human sTNFR1 antibody-enzyme conjugate and colorimetric substrate to microplate wells coated with anti-human sTNFR1 monoclonal antibody. The resultant colour intensity is proportional to the concentration of sTNFR1 in the test sample. The assay is calibrated against a highly-purified *E. coli*-expressed recombinant human sTNFR1 protein corresponding to the N-terminal ectodomain. The calibration curve range is 7.8 – 500pg/mL; this corresponds to serum / plasma sTNFR1 concentrations of 78 – 5000pg/mL for samples assayed at 10-fold dilution. The assay range can be increased by increasing sample dilution.

COMPONENTS



Component	Symbol
1. sTNFR1 Microplate 96 well microplate (12 x 8 breakapart well strips coated with mouse anti-human sTNFR1 monoclonal antibody) READY TO USE	PLA
2. sTNFR1 Conjugate, 21mL Polyclonal anti-human sTNFR1 antibody conjugated to horseradish peroxidise. Contains ProClin 300 as preservative. READY TO USE	CONJ | EN | 1x
3. sTNFR1 Calibrator, 2.5ng Recombinant human sTNFR1 in a buffered protein base with ProClin 300 as preservative. LYOPHILISED	CAL
4. sTNFR1 Low Control, 1.8mL Recombinant human sTNFR1 in stabilising diluent containing ProClin 950 and Bronidox L as preservatives. READY TO USE	CONTROL | L | 1x
5. sTNFR1 High Control, 1.8mL Recombinant human sTNFR1 in stabilising diluent containing ProClin 950 and Bronidox L as preservatives. READY TO USE	CONTROL | H | 1x
6. Assay Diluent, 6mL Buffered protein base with ProClin 300 as preservative. READY TO USE	DIL | AS | 1x
7. Sample Diluent, 2 x 21mL Animal serum with ProClin 300 and sodium azide as preservatives. May contain a precipitate. Mix well before and during use. READY TO USE	DIL | SPE | 1x
8. Wash Buffer, 21mL Buffered surfactant solution containing ProClin 300 as preservative. 25X CONCENTRATE	BUF | WASH | 25X
9. Substrate Solution A, 12mL Stabilized hydrogen peroxide. 2X CONCENTRATE	SUBS | A | 2X
10. Substrate Solution B, 12mL Stabilized chromogen (tetramethylbenzidine). 2X CONCENTRATE	SUBS | B | 2X
11. Stop Solution, 6mL 2 N sulfuric acid. READY TO USE	SOLN | STOP
12. Instructions for use	
13. Plate Sealers, 4 strips Adhesive Strips	

PRECAUTIONS

SAFETY

- The EKF Diagnostics BIO94 Human sTNFR1 EIA is intended for use by qualified laboratory staff only.
- The Stop Solution contains sulphuric acid, which is corrosive. Avoid contact with the skin and eyes. If contact occurs, rinse off immediately with water and seek medical advice.
- Substrate solution B contains tetramethylbenzidine (TMB), which may irritate the skin and mucous membranes. Any substrate which comes into contact with the skin must be rinsed off immediately with water.
- Certain components contain ProClin 300 or ProClin 950 as preservatives, which are classified as per applicable European Community (EC) directives as corrosive, harmful, dangerous for the environment and an irritant. Avoid contact with the skin and eyes. Rinse immediately with large volumes of water if contact occurs.
- Sample Diluent solution contains sodium azide, which may form potentially explosive metal azides with lead and copper plumbing. For disposal, reagents should be flushed with large volumes of water to prevent azide build up.
- Dispose of all clinical specimens, infected or potentially infected material in accordance with good laboratory practice. All such materials should be handled and disposed of as though potentially infectious.
- Residues of chemicals, preparations and kit components are generally considered as hazardous waste. All such materials should be disposed of in accordance with established safety procedures.
- Expired unused kit components are generally considered as hazardous waste and should be disposed of in accordance with established safety procedures.
- Wear protective clothing, disposable latex gloves and eye protection while handling specimens and performing the assay. Wash hands thoroughly when finished.
- Do not pipette materials by the mouth and never eat or drink at the laboratory workbench.

PROCEDURAL

- Do not use kit or individual reagents beyond their expiration date.
- Do not mix or substitute reagents from different kit lot numbers.
- Deviation from the protocol provided may cause erroneous results.
- Performing the assay outside the time and temperature ranges provided may produce invalid results.
- The assay is destructive; do not attempt to re-use microplate strips.
- Do not allow the wells to dry at any stage during the assay procedure.
- To avoid cross-contamination, change pipette tips between additions of each calibrator level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Do not use reagents that are cloudy or that have precipitated out of solution.
- High quality distilled or deionised water is required for preparation of Wash Buffer. The use of poor quality or contaminated water may lead to background colour in the assay.
- Allow all reagents to come to room temperature (20-25°C) and mix well prior to use.
- Avoid leaving reagents in direct sunlight and/or above 2-8°C for extended periods.
- Always use clean, preferably disposable, glassware for all reagent preparation.
- Ensure that the upper surface of the wells is free of droplets before adding the next reagent. Drops should be gently blotted dry on completion of the wash step.
- Ensure that the bottom surface of the plate is clean and dry before reading.
- Before commencing the assay, an identification and distribution plan should be established.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.

- Substrate Solution should remain colourless until added to the plate. Keep Substrate Solution protected from light and use within 15 minutes of preparation. Substrate Solution should change from colourless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The colour developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in colour indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution. Gently tap the side of the microplate until all green colouration has changed to yellow, taking care not to splash the contents of the wells.
- If samples generate absorbance values higher than the highest calibrator, further dilute the samples in Sample Diluent and repeat the assay.
- Variations in operator, pipetting technique, washing technique, incubation time, incubation temperature and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

STABILITY AND STORAGE

1. Unopened kit reagents should be stored at 2-8°C and are stable as supplied until the expiry date shown.
2. Opened reagents, prepared 1x Wash Buffer and reconstituted Calibrator may be stored for up to 1 month at 2-8°C, provided this is within the expiration of the kit. Return unused microplate wells to the foil pouch together with desiccants, reseal and store at 2-8°C.
3. Substrate Solution A and Substrate Solution B should only be mixed in the required volumes and used within 15 minutes of preparation. Do not store the prepared ready-to-use substrate solution.

ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 540 nm or 570 nm if available.
2. Micropipettes and a multichannel pipette.
3. Microassay strip washing system (manifold dispenser or automated microplate washer).
4. Deionised or distilled water.
5. Timer.
6. Reagent reservoirs.
7. 500mL graduated cylinder.
8. Test tubes for dilution of calibrators and test samples.
9. Incubator (20 – 25ºC).
10. Vortex.

PREPARATION OF REAGENTS

Bring all reagents to room temperature before use.

1. **1X Wash Buffer**

   If crystals have formed in the Wash Buffer Concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20mL of Wash Buffer Concentrate to a clean graduated cylinder and add deionized or distilled water to a final volume of

500mL to prepare 1X Wash Buffer. Prepare only the volume of 1X Wash Buffer required for the assay, each microplate strip requires approximately 40mL of 1X Wash Buffer.

2. **Substrate Solution**

   Substrate Solutions A and B should be mixed together in equal volumes and used within 15 minutes of preparation. Protect from light. Prepare only the required volume of substrate solution for the number of microplate strips being assayed, 2mL of mixed substrate solution is required per microplate strip. Do not store mixed substrate solution.

3. **sTNFR1 Calibrators**

   Reconstitute the lyophilised sTNFR1 Calibrator with 5.0mL Sample Diluent. This reconstitution produces a calibrator solution of 500pg/mL sTNFR1. Allow the reconstituted calibrator to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

   To prepare sTNFR1 calibrators at 7.8 – 250pg/mL, pipette 500µL Sample Diluent into labelled tubes. Serially dilute the 500pg/mL sTNFR1 calibrator solution in 500µL volumes to produce a dilution series (see Figure 1). Mix each tube thoroughly before the next dilution step and change pipette tips between additions to prevent sample carryover. Sample Diluent serves as the zero calibrator (0 pg/mL).

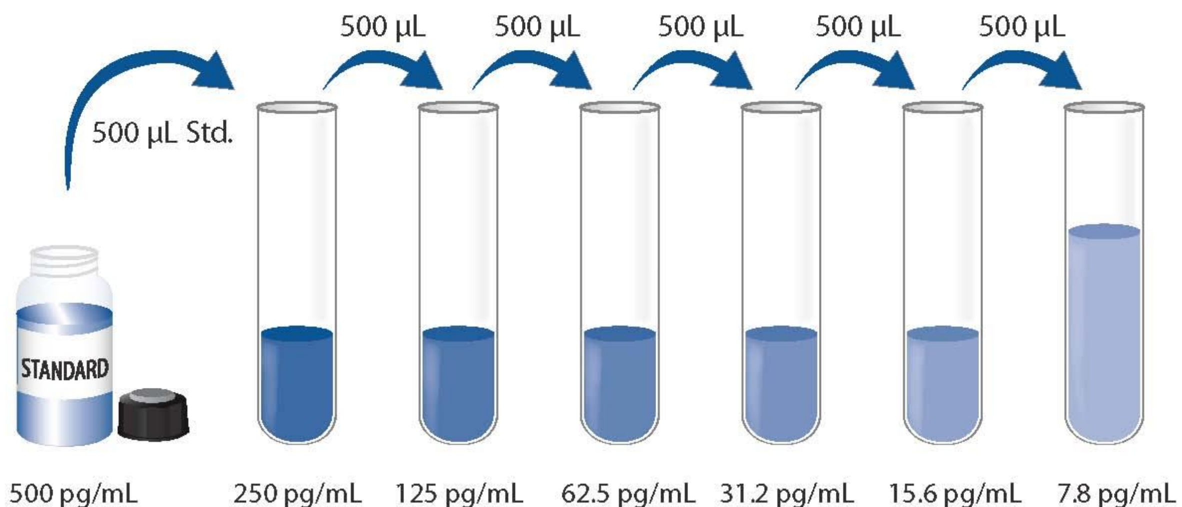


Figure 1 *Schematic representation of sTNFR1 calibrator preparation*

SAMPLE COLLECTION, HANDLING AND STORAGE

Serum, heparin plasma and EDTA plasma samples can be used on the Human sTNFR1 EIA.

Collect blood samples observing routine precautions for venipuncture using appropriate blood collection tubes. Follow the manufacturers' instructions for sample preparation. It is important when collecting plasma samples to invert the blood collection tubes immediately after sample collection to ensure complete mixing with the anticoagulant. Remove serum or plasma to a clean polypropylene tube.

If samples are to be tested within 48 hours they may be stored at 2-8°C. For longer storage, aliquot and store samples at ≤ -20°C. Allow samples to thaw at ambient temperature and briefly

mix by inversion or using a vortex before use.

The mean recovery of sTNFR1 in serum, EDTA plasma and heparin plasma sample pools containing ~4000pg/mL sTNFR1 was 93% after 3 freeze-thaw cycles (range 86.5 – 97.5%) indicating that sTNFR1 is relatively stable to multiple freeze-thaw cycles. Nevertheless, it is recommended to minimise the number of freeze-thaw events that samples are subjected to.

SAMPLE PREPARATION

Serum and plasma samples require **at least** 10-fold dilution in Sample Diluent.

Dilute samples 10-fold by adding 50µL sample to 450µL Sample Diluent and mix well using a vortex. Samples containing high levels of sTNFR1 will require increased dilution, for example a 20-fold dilution (25µL sample added to 475µL Sample Diluent).

sTNFR1 Low Control and sTNFR1 High Control solutions are ready to use and do not require dilution.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all calibrators, samples, and controls be assayed in duplicate. A calibration curve must be assayed for each set of samples tested.

1. Prepare wash buffer, sTNFR1 calibrators and diluted test samples as described in ‘Preparation of Reagents’ and ‘Sample Preparation’ sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Using a multichannel pipette, add **50µL** Assay Diluent to each assay well to be used.
4. Add sTNFR1 calibrators (0 – 500pg/mL), sTNFR1 Low Control, sTNFR1 High Control and diluted test samples (**200µL** per well) to the microplate in duplicate and cover with an adhesive strip. Incubate the plate in a room temperature incubator (20 - 25ºC) without shaking for 2 hours.
5. Remove the adhesive cover and aspirate the well contents. Wash each strip 3 times with 1X Wash Buffer (400µL per well) using a multichannel pipette, manifold dispenser or automated plate washer. Complete removal of well contents at each wash step is essential to good performance. After the last wash cycle, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Using a multichannel pipette, add **200µL** sTNFR1 Enzyme Conjugate to each well. Cover with a new adhesive strip and incubate in a room temperature incubator (20-25ºC) without shaking for 2 hours.
7. Repeat the aspiration/wash as detailed in step 5.
8. Prepare the required volume of Substrate Solution as described in ‘Preparation of Reagents’ by mixing Substrate Solution A and B in equal volumes.
9. Using a multichannel pipette, add **200µL** of prepared Substrate Solution to each well and cover with a new adhesive strip. Incubate in a room temperature incubator (20-25ºC) without shaking for 20 minutes. **Protect from light.**
10. Add **50µL** Stop Solution to each well. If the colour in the well is green or the colour change does not appear uniform, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes using a microplate reader with wavelength set to 450 nm. If available, determine the optical density at reference wavelength set at 540nm or 570nm.

CALCULATION OF RESULTS

1. Calculate the average absorbance value at 450nm wavelength for each calibrator, control and test sample. If measured, calculate the average absorbance measured at the reference wavelength (540nm or 570nm) and subtract from absorbance values measured at 450nm wavelength.
2. Calculate the corrected absorbance by subtraction of the average absorbance measured for the 0pg/mL sTNFR1 calibrator from the average absorbance measured for remaining calibrators, controls and test samples.
3. Create a calibration curve by plotting corrected absorbance values (y-axis, logarithmic scale) against sTNFR1 calibrator concentration (x-axis, logarithmic scale) using linear regression analysis, refer to Figure 2 for an example of a typical calibration curve.
4. Determine [sTNFR1] pg/mL for controls and test samples from the calibration curve indicated from the mean corrected absorbance values for each sample.
5. Measured sTNFR1 concentrations for serum and plasma samples must be multiplied by the appropriate sample dilution factor to calculate the actual sTNFR1 concentration.
6. If the mean corrected absorbance for test samples is greater than the highest sTNFR1 calibration standard, the samples should be re-tested at increased dilution; it is not recommended to extrapolate results.
7. The concentrations of the sTNFR1 Low Control and sTNFR1 High Control are read directly from the calibration curve.

QC CRITERIA

Two control solutions (sTNFR1 Low Control and sTNFR1 High Control) are provided with this kit to allow the validity of the test results to be determined. It is recommended that at least one control solution is tested in each assay run performed. Results are considered valid if the value of the control is within the range provided on the inside of the box lid. If the control samples are not within the specified range, the associated test results may be invalid and samples must be re-tested.

LIMITATIONS OF USE

Results must be assessed in conjunction with the subjects clinical profile and other clinical laboratory results.

EXPECTED RANGES

Samples from apparently healthy volunteers were assayed using EKF Diagnostics Human sTNFR1 EIA to determine endogenous levels of sTNFR1. No medical histories were available. These values reflect the donor population used in this study. It is recommended that each laboratory determine expected ranges specific for their study group.

Sample Type	Mean [sTNFR1] pg/mL	Median [sTNFR1] pg/mL	Range [sTNFR1] pg/mL
Serum (n=25)	928	836	503 - 1583
Heparin plasma (n=25)	860	852	596 - 1326
EDTA plasma (n=25)	877	855	568 - 1292

PERFORMANCE CHARACTERISTICS

MEASURING RANGE

The calibration curve range is 7.8 – 500pg/mL, corresponding to concentrations of 78 – 5000pg/mL sTNFR1 in serum and plasma samples assayed at 10-fold dilution. The measuring range can be extended by increasing sample dilution.

PRECISION

Intra-assay Precision (within run precision)

Six samples containing varying levels of sTNFR1 were each tested in sixteen assay wells in a single run using EKF Diagnostics Human sTNFR1 EIA to assess intra-assay precision. The average %CV for all samples was 3.3%.

Sample	n	Final [sTNFR1] pg/mL	SD	%CV
Low Serum	16	1047.0	18.49	1.8
Medium Serum	16	2455.9	48.83	2.0
High Serum	16	3875.8	203.77	5.3
Low Plasma	16	825.4	14.53	1.8
Medium Plasma	16	2318.4	89.17	3.8
High Plasma	16	3770.0	189.06	5.0

Inter-assay Precision (between run precision)

Eight samples were each tested in duplicate across ten separate assays using EKF Diagnostics Human sTNFR1 EIA to assess inter-assay precision. The average %CV for all samples was 5.1%.

Sample	n	Final [sTNFR1] pg/mL	SD	%CV
Low Control	10	29.0	1.28	4.4
Medium Control	10	140.9	5.05	3.6
High Control	10	283.4	15.37	5.4
Low Spike Serum	10	1211.5	82.32	6.8
Medium Spike Serum	10	3671.0	147.79	4.0
High Spike Serum	10	7416.8	312.14	4.2
Native Serum	10	1041.7	54.76	5.3
Native Plasma	10	965.3	64.96	6.7

SPECIFICITY

The EKF Diagnostics Human sTNFR1 EIA is highly specific for human sTNFR1. There was no cross-reactivity observed with related markers of the TNF-pathway (TNF-α, TNF-β or sTNFR2) when assayed at 100ng/mL in Sample Diluent.

SENSITIVITY

The limit of detection (LoD) of the EKF Diagnostics Human sTNFR1 EIA was estimated from ten separate assays. The LoD was estimated as the back-calculated sTNFR1 concentration corresponding to the mean absorbance of the blank calibrator plus 3 standard deviations. The LoD ranged from 0.9 – 3.1pg/mL with an average of 1.7pg/mL. This corresponds to concentration of 17pg/mL sTNFR1 for serum or plasma samples diluted $^{1}/_{10}$ in Sample Diluent.

LINEARITY

Twenty samples (14 serum, 6 plasma) containing either elevated endogenous sTNFR1 or spiked with recombinant sTNFR1 were serially diluted in Sample Diluent and assayed using EKF

Diagnostics Human sTNFR1 EIA. Samples contained final sTNFR1 concentrations between 800 – 25000pg/mL. Recovery of sTNFR1 upon dilution was calculated as (Measured [sTNFR1] pg/mL / Expected [sTNFR1] pg/mL) x 100. The average sTNFR1 recovery was for all samples across the dilution series was 106.9%, recovery range 90.6 – 118.3%.

SPIKE RECOVERY

Sixty-seven samples (27 serum, 40 plasma) were spiked with varying levels of recombinant sTNFR1 at concentrations between 850 – 12000pg/mL and assayed using EKF Diagnostics Human sTNFR1 EIA. Recovery of spiked sTNFR1 was calculated as (Measured [sTNFR1] pg/mL / Expected [sTNFR1] pg/mL) x 100. The average recovery of spiked sTNFR1 was 102.2%, recovery range 84.9 – 116.4%.

INTERFERENCE

Serum, heparin plasma and EDTA plasma pools were prepared and spiked with recombinant sTNFR1 to a final concentration of ~2000pg/mL. The effect of potentially interfering endogenous substances on sTNFR1 measurement was evaluated by spiking them into the prepared serum and plasma pools and testing on EKF Diagnostics Human sTNFR1 EIA. Interference (% Bias) was calculated as follows:

Interference (% Bias): $\left\{ \frac{\text{[sTNFR1] pg/mL Interferent Spiked Sample}}{\text{[sTNFR1] pg/mL Control Sample (no interferent added)}} \times 100 \right\} - 100$

Test Substance	Test Concentration	Serum (% Bias)	Heparin Plasma (% Bias)	EDTA Plasma (% Bias)
Hemoglobin	200mg/dL	-3.6	+5.3	+0.9
Bilirubin	20mg/dL	-5.4	+4.6	+1.3
Intralipid	1000mg/dL	+5.6	-4.4	+3.2
Glucose	1000mg/dL	+3.3	-4.3	+0.9
Ascorbic Acid	3mg/dL	+7.7	-4.0	-3.1
Creatinine	5mg/dL	-4.6	-7.6	-4.8
TNF-α	30ng/dL	+2.5	-0.3	-3.8
TNF-β	300ng/dL	+6.7	+1.0	+0.5
sTNFR2	4000ng/dL	+5.2	+5.9	+1.6

EXAMPLE OF A CALIBRATION CURVE

Typical calibration curve data obtained using EKF Diagnostics Human sTNFR1 EIA is presented below. The absorbance values of the sTNFR1 calibrators may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique). The data presented below is for demonstration only and cannot be used to derive test results. Calibrators and controls must be prepared and assayed on the same plate for each group of test samples analysed.

Calibrator [sTNFR1] pg/mL	Absorbance ($A_{450} – A_{570}$)	Mean Absorbance	Corrected Absorbance	% CV
0	0.036 0.036	0.036	0.000	0.0
7.8	0.067 0.069	0.068	0.032	2.1
15.6	0.097 0.102	0.100	0.064	3.6
31.2	0.158 0.157	0.158	0.122	0.4

62.5	0.265 0.275	0.270	0.234	2.6
125	0.490 0.478	0.484	0.448	1.8
250	0.882 0.901	0.891	0.855	1.5
500	1.554 1.539	1.547	1.511	0.7

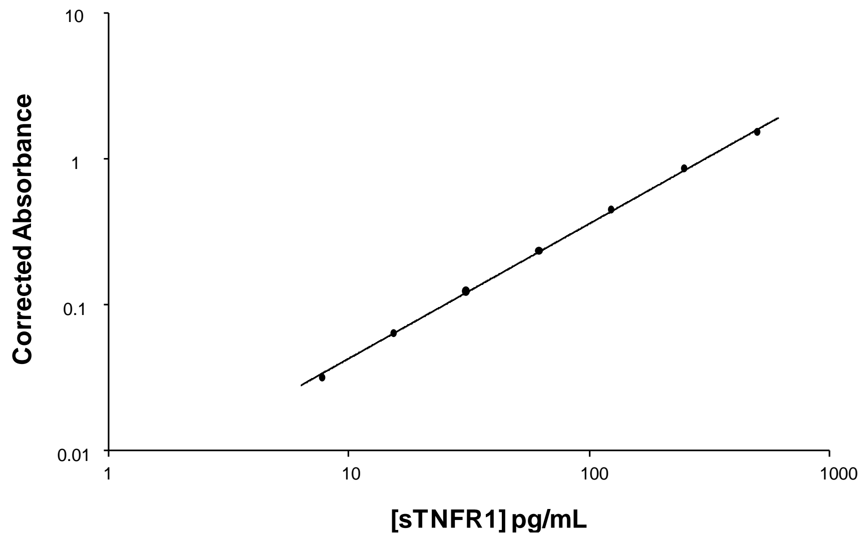


Figure 2: *Representative EKF Diagnostics Human sTNFR1 EIA Calibration Curve*

WARRANTY

The performance data presented here was obtained using the procedure described. Any change or modification of the procedure, not recommended by EKF Diagnostics, may affect the results, in which case EKF Diagnostics disclaims all warranties, expressed, implied or statutory, including implied merchantability and fitness for use. In the case of such an event, EKF Diagnostics shall not be liable for damages, direct or consequential.

INTERPRETATION OF SYMBOLS

For in vitro diagnostic use

Catalogue number

Batch code

Use-by date

Temperature Limit

Consult Instructions for Use

Number of determinations

Control Low Range

Control High Range

Manufacturer EKF Diagnostics Ltd., Unit 9, Trinity Technology and Enterprise Centre, Pearse St., Dublin 2, Ireland

SUMMARY OF ASSAY PROCEDURE

1. **SAMPLE/CALIBRATOR INCUBATION**
   1.1. Prepare all reagents, calibrators and test samples as directed.
   1.2. Remove excess microplate strips from the plate and return to the foil pouch.
   1.3. Add 50µL Assay Diluent to each well.
   1.4. Add 200µL calibrators, controls and diluted samples to each well in duplicate.
   1.5. Cover the microplate with an adhesive strip provided. Incubate at room temperature (20-25˚C) for 2 hours.
   1.6. Aspirate each well and wash with 400µL wash buffer, repeat the process twice for a total of 3 washes. See full assay procedure for correct wash practice.

2. **CONJUGATE INCUBATION**
   2.1 Add 200µL sTNFR1 Conjugate to each well.
   2.2 Cover the microassay plate with a new adhesive strip and incubate at room temperature (20-25°C) for 2 hours.
   2.3 Repeat Step 1.6.

3. **COLOUR DEVELOPMENT**
   3.1. Mix equal volumes of Substrate Solution A and B together and add 200µL of the mixed substrate solution to each well. Cover the microassay plate with a new adhesive strip and incubate at room temperature for 20 minutes. Protect from light.

4. **STOP**
   4.1. Stop the reaction by adding 50µL Stop Solution to each well. Ensure complete mixing of Substrate and Stop Solution.
   4.2. Read the absorbance of the wells within 30 minutes at 450 nm with reference wavelength set to 540 nm or 570nm.

5. **CALCULATE RESULTS**

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