3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone (800) 325-5832 (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

sTNF-RI ELISA, Human

Product Number **CS0680**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

sTNF-RI ELISA, Human is a solid phase enzyme amplified sensitivity immunoassay performed on a multiwell plate. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of sTNF-RI (Soluble Tumor Necrosis Factor-Receptor I) is used. Antibodies producing cells are immortalized using the myeloma cell fusion method of Kohler and Milstein. A hybridoma cell is produced which secretes specific homogenous antibodies. The use of a number of distinct MAbs results in high sensitivity assays with extended standard range and short incubation time.

Standards or samples containing sTNF-RI react with capture monoclonal antibodies (MAbs 1) coated on the multiwells and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). During an incubation period a sandwich containing: coated MAbs 1 - sTNF-RI - MAb 2 – HRP is formed. The multiwell plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme-labelled antibodies are measured through a chromogenic reaction. Stabilized chromogen solution (TMB + H_2O_2) is added and incubated. The reaction is stopped with the addition of Stop Solution. The amount of substrate turnover is determined colorimetrically by measuring the absorbance, which is proportional to the sTNF-RI concentration. A standard curve is plotted and sTNF-RI concentration in a sample is determined by interpolation from the standard curve. We recommend the use of a plate reader with a linearity up to 3 OD units and a data reduction method which result in high sensitivity in the low range and in an extended standard range**.**

Tumor necrosis factor-α (TNF-α), a potent cytokine, elicits a broad spectrum of biologic responses that are mediated by binding to a cell surface receptor. Two distinct TNF-binding proteins bind TNF-α and TNF-β specifically and with high affinity. sTNF-RI, also named TBPI or p55, is one of the two receptors present at the surface of many cells. Different processes modulate their presence. IL-2 and the activation of T-lymphocytes increase the presence of both TNF-RI and RII. An increase is also noticed during the maturation of the macrophages or in the presence of protein kinase activators. TNF-Rs decrease in the presence of H_2O_2, epinephrine, insulin and somatostatin. The two receptors of TNF are able to bind TNF-a and TNF-ß. The MW of TNF-RI is about 55 kDa that suggests an important glycosylation. The proliferation of circulating human mononuclear cells under the influence of PHA involves the participation of the two receptors.

Soluble forms of the receptors are shedded from the cell membrane and are present in urine, plasma or culture supernatants. Their presence has been proved in the serum of cancer patients, in chronic renal deficiency and in the broncho-alveolar lavage of patients suffering from ARDS. The soluble receptors for TNF are also putative markers of disease progression in HIV infection. sTNF-R correlates also with parasitemia and disease severity in human malaria. These forms bind perfectly to TNF and, in high concentration, inhibit the biological activity of TNF. Under some conditions, these soluble forms are able to protect TNF and increase it's half live.

Reagents

- **Monoclonal-Anti-sTNF-RI-Coated 96 well plate, 1EA, Product No. T 4575 -** A plate using break-apart strips coated with monoclonal antibodies (MAbs 1).
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**
- **sTNF-RI Standard, 0 ng/mL, lyophilized, 1 VL, Product No. S 8694 –** contains BSA and preservative. Recommended for sample dilution.

- **sTNF-RI Standard 1, lyophilized, 1 VL, Product No. S 5195-** recombinant, expressed in *E. coli,* in bovine serum with preservatives
- **sTNF-RI Standard 2, lyophilized, 1 VL, Product No. S 5320-** recombinant, expressed in *E. coli,* in bovine serum with preservatives
- **sTNF-RI Standard 3, lyophilized, 1 VL, Product No. S 5445-** recombinant, expressed in *E. coli,* in bovine serum with preservatives
- **sTNF-RI Standard 4, lyophilized, 1 VL, Product No. S 5570-** recombinant, expressed in *E. coli,* in bovine serum with preservatives
- **sTNF-RI Standard 5, lyophilized, 1 VL, Product No. S 5695-** recombinant, expressed in *E. coli,* in bovine serum with preservatives
- **Control 1, lyophilized, 1 VL, Product No. C 1866-** in human plasma, contains preservative
- **Control 2, lyophilized, 1 VL, Product No. C 1991-** in human plasma, contains preservative
- **Monoclonal Anti-sTNF-RI-HRP Conjugate, 21 mL, Product No. T 4700 –** in buffered solution with preservative
- **Wash Buffer Concentrate (200X), 10 mL, Product No. W 3890**
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.

Note: See vial labels for exact concentrations and reconstitution volumes of standards and controls

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

The human blood components included in this kit have been tested by European approved and USA FDA approved methods and found negative for HBsAg, anti-HCV and anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum, or plasma specimens should be in accordance with appropriate safety procedures.

Preparation Instructions

Sample Preparation

- The immunoassay measures sTNF-RI in serum, plasma, cell culture supernatants and other biological fluids.
- Peripheral blood mononuclear cells may be isolated by accepted methods. However, one should avoid unintentional stimulation of cells by the procedure. The use of pyrogen-free reagents and appropriate controls is mandatory.
- Sampling conditions can affect values measured in serum or plasma, therefore, strict precautions have to be taken during sampling to avoid impurities contained in sampling materials that would stimulate sTNF-RI production by blood cells and thus falsely increase plasma sTNF-RI values.
- Remove serum from the clot after centrifugation as soon as possible and store at 2-8 °C.
- Plasma can be collected in pyrogen-free sterile EDTA or heparin tubes and separated immediately after centrifugation.
- Batches of heparin are often contaminated with pyrogen. Test each batch of heparin to avoid unintentional stimulation of blood cells. Other substances in the tube must be also pyrogen-free.
- These recommendations are also valuable for other biological fluids (cell culture supernatant, etc.).
- Serum and plasma samples may be stored at –20 °C for up to 2 months and at –70 °C for a maximum storage of one year.
- Samples with low protein levels (cell culture supernatants) should be stored at –70 °C.
- If the samples generate values higher than the highest standard, dilute with Standard 0.

Reagent Preparation

Standards and Controls

1. Reconstitute the lyophilized *Standards* and *Controls* with distilled water. Refer to vial label for reconstitution volume and instructions.
2. Allow to remain undisturbed until completely dissolved, and then mix well by gentle inversion.
3. The reconstituted *Standards and Controls* are stable for 4 days at 2-8 °C.
4. For long-term storage, aliquots should be frozen at –20 °C (maximum 2 months) or at –70 °C until expiration date.

Wash Buffer

1. Dilute 2 mL of *Wash Buffer Concentrate* in 400 mL distilled water or all the contents of the *Wash Buffer Concentrate* vial in 2000 mL distilled water (use a magnetic stirrer).
2. The *Wash Buffer Concentrate* is stable at room temperature until expiration date.
3. In order to avoid precipitation, it is recommended to prepare a fresh diluted Wash Buffer each day.

Storage/Stability

All components of this kit are stable at 2-8 °C. Any unused reconstituted standard or control should be discarded or frozen at –70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8°C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- The freshly prepared Chromogenic Solution is stable for a maximum of 15 min. at room temperature and must be discarded afterwards
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 3 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle three times, blotting as dry as possible after the 3rd wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

sTNF-RI Assay Summary

1) 50 µL of Standards, Controls or Samples
200 µL Monoclonal Anti-sTNF-RI-HRP Conjugate

Incubate 1 hour at RT on shaker
aspirate and wash 3X

2) Add 50 µL Stabilized Chromogen

Incubate 15 minutes at RT on shaker

3) Add 200 µL of Stop Solution
Read at 450nm

Total Assay Time - 1.5 hours

- Determine the number of wells for the assay run, including: 2 zero standard wells, 10 standard wells, 2 control 1 and 2 Control 2 wells.
- Add 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch.

1st incubation

a Add 50 µL Standard 0, Standards 1 to 5, Control 1 and Control 2 to designated wells.
b Add 50 µL of each sample to be tested to the appropriate duplicate wells.
c Add 200 µL Monoclonal Anti-sTNF-RI-HRP Conjugate to all wells.
d Tap gently on the plate to mix, cover with Plate Cover and incubate one hour at room temperature. on a horizontal shaker set at 700 ± 100 rpm
e Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 3 times following washing instructions.

Substrate incubation

a Add 50 µL of Stabilized Chromogen into all wells.
b Incubate the plate for 15 min**.** at room temperature on a horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight.

Stop reaction

a Add 200 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Read the absorbance of the entire plate at 450 nm within 3 hours after addition of Stop Solution

Results

1. If a software program is not readily available, the concentrations of sTNF-RI may be calculated manually.
2. Calculate the Average OD (average reading of 2 wells) for each standard dilution and samples
3. On graph paper plot the Average OD of standard dilutions against the standard concentration (ng/mL) of sTNF-RI. Draw the best curve through these points to construct the standard curve.
4. The sTNF-RI concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by dilution factor of each sample.

Samples producing signals higher than the 47 ng/mL standard should be further diluted and assayed again

Product Profile

Typical Results

The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Standard ng/mL	**OD 450 nm**
0	0.028
1	0.251
2.5	0.702
8	1.872
22	3.689
47	5.198

Expected Range

At the present stage only preliminary results can be provided. Each laboratory should establish its own normal values. For guidance, the mean of 129 normal plasmas was 1.2 ng/mL (SD = 0.6), ranging between 0.3 ng/mL and 2.9 ng/mL. This study was performed with samples collected under strict sampling conditions.

Performance characteristics

Minimum Detectable Concentration (MDC).

The MDC is estimated to be 50 pg/ml and is defined as the sTNF-RI concentration corresponding to the average OD of 20 replicates of the zero standard + 2 standard deviations.

Precision

1. Intra-Assay Precision

Samples of known concentration were assayed in replicates of 12 to determine precision within an assay.

	Sample 1	Sample 2
Mean (ng/mL)	0.99	19.11
Standard Deviation (SD)	0.02	1.25
Coefficient of Variation (%)	1.7	6.5

2. Inter-Assay Precision

Samples were assayed 10 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2
Mean (ng/mL)	1.76	26.80
Standard Deviation (SD)	0.10	2.40
Coefficient of Variation (%)	5.7	8.9

Specificity

Cross-reactivity and interference were analysed by the addition of different analytes to sTNF-RI samples and measuring the apparent sTNF-RI concentration

Analyte	Added sTNF-RI 1.62 ng/mL	Added sTNF-RI 5.20 ng/mL	Added sTNF-RI 12.60 ng/mL
sTNF-RII (1500ng/mL)	1.88 ng/mL (116.1 %)	5.65 ng/mL (108.6 %)	14.45 ng/mL (114.7 %)
TNF-α (400 ng/mL)	1.38 ng/mL (85.2 %)	4.80 ng/mL (92.3 %)	12.23 ng/mL (97.1 %)

These results demonstrate that the sTNF-RI ELISA does not cross react with sTNF-RII and that TNF-α does not interfere with the assay.

Linearity of Dilution

Dilution	Serum		
	Measured ng/mL	Expected ng/mL	% Expected
Neat	18.09	18.09	-
1:2	9.55	9.05	105.5
1:4	4.67	4.52	103.3
1:8	2.56	2.26	113.3
1:16	1.44	1.13	127.4
	Plasma		
Neat	23.82	23.82	-
1:2	10.53	11.91	88.4
1:4	4.85	5.96	81.4
1:8	2.63	2.98	88.3
1:16	1.43	1.49	96.0
	Cell Culture Medium		
Neat	33.16	33.16	-
1:2	14.66	16.58	88.4
1:4	8.06	8.29	97.2
1:8	4.39	4.15	105.8
1:16	2.43	2.07	117.4

Recovery

Added sTNF-RI (ng/mL)	Recovered sTNF-RI (ng/mL)	Recovery (%)
Serum		
0	1.27	-
1.72	2.96	98.3
4.38	5.56	98.0
19.62	20.48	97.9
Plasma		
0	1.85	-
2.65	4.11	85.3
6.99	9.92	115.4
30.30	34.37	107.3
Cell Culture Medium		
0	0.04	-
2.52	2.53	98.8
11.45	12.46	108.5
31.12	31.65	101.6

High dose hook effect

Any sample that produces readings above the OD for the highest standard will have to be diluted.

References

1. Scheurich, P., et al., Quantification and characterization of high affinity membrane receptors for tumor necrosis factor on human leukemic cell lines. Int. J. Cancer, **38**, 127-133 (1986).
2. Derre, J. et al., The gene for the type 1 tumor necrosis factor receptor (TNF-R1) is localized on band 12p13. Hum. Genet. **87**, 231-233 (1991).
3. Fuchs, P., et al., Structure of the human TNF receptor 1 (p60) gene (TNFR1) and localization to chromosome 12p13. Genomics **13**, 219-224 (1992).
4. Gray, P., et al., Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein. Proc. Nat. Acad. Sci.,USA, **87**, 7380-7384 (1990).
5. McDermott, M. F., et al., Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. Cell, **97**, 133-144 (1999).

6. Micheau, O. and Tschopp, J., Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell, **114**, 181-190 (2003).
7. Poirier, O., et al., Polymorphism R92Q of the tumor necrosis factor receptor 1 gene is associated with myocardial infarction and carotid intima-media thickness: the ECTIM, AXA, EVA and GENIC studies. Europ. J. Hum. Genet. **12**, 213-219 (2004).
8. Zhang, J. Y., et al., NF-κ-B RelA opposes epidermal proliferation driven by TNFR1 and JNK. Genes Dev. **18**, 17-22 (2004).
9. Bruce, A. J., et al., Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. Nature Med. **2**, 788-794 (1996).

AH,PHC 02-05-1