III PRINCIPLES OF THE BIOSOURCE sTNF-RI EASIA ASSAY

The BIOSOURCE sTNF-RI EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on microtiter plate. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of sTNF-RI are used. Antibody-producing cells are immortilized using the myeloma cell fusion method of Kohler and Milstein. A hybridoma cell is produced which secretes specific homogeneous antibodies.
The use of a number of distinct MAbs avoids hyperspecificity and allows high sensitive 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 microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich : coated MAbs 1 - sTNF-RI - MAb 2 - HRP, the microtiter plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme-labelled antibodies are measured through a chromogenic reaction. Chromogenic Solution (TMB+H_2O_2) is added and incubated. The reaction is stopped with the addition of Stop Solution (HCl) and the microtiter plate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance which is proportional to the sTNF-RI concentration. A standard curve is plotted and sTNF-RI concentrations in a sample is determined by interpolation from the standard curve. The use of the EASIA Reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in high sensitivity in the low range and in an extended standard range.

IV REAGENTS PROVIDED

Reagents	96 tests Kit	192 tests Kit	Reconstitution
Microtiter plate with 96 anti-sTNF-RI coated wells	1 x 96 wells	2 x 96 wells	**Ready** for use
Standard 0 ng/ml, in bovine serum and preservatives	1 vial lyophil.	2 vials lyophil.	**Add** ml distilled water (see the volume on the vial label)
Standards 1 to 5 in bovine serum and preservatives : see vial labels for exact concentrations	5 vials lyophil.	5 vials lyophil.	**Add** 0.5 ml distilled water
Anti-sTNF-RI-HRP Conjugate in buffered solution with proteins and preservatives	1 vial 21 ml	2 vials 21 ml	**Ready** for use
Controls 1 and 2 in human plasma with preservatives	2 vials lyophil.	2 vials lyophil.	**Add** 0.5 ml distilled water
Washing Solution Concentrate (buffer with preservatives)	1 vial 10 ml	1 vial 10 ml	**Dilute** 2 ml in 400 ml distilled water or the vial content in 2000 ml distilled water
Chromogen : TMB (Tetrametylbenzidine)	1 vial 25 ml	1 vial 25 ml	**Ready** for use
Stop Solution	1 vial 25 ml	2 vials 25 ml	**Ready** for use

Note : Standard 0 ng/ml is recommended for sample dilutions.

V EQUIPMENT AND SUPPLIES REQUIRED BUT NOT PROVIDED

1. High quality distilled water.
2. Precision pipette : 50 µl, 200 µl, 500 µl, 1 ml and 10 ml.
3. Vortex mixer and magnetic stirrer.
4. Horizontal microtiter plate shaker capable of 700 rpm ± 100 rpm, microtiter plate reader capable of reading at 450 nm and 490 nm, microtiter plate washer.

VI REAGENT PREPARATION

1. **Standards and Controls** : Reconstitute the lyophilized Standards and Controls to the volume specified on the vial label with distilled water. Allow them to remain undisturbed until completely dissolved, then mix well by gentle inversion.
2. **Wash Solution** : Dilute 2 ml of Washing Solution Concentrate in 400 ml distilled water or all the contents of the Washing Solution Concentrate vial in 2000 ml distilled water (use a magnetic stirrer).

VII STORAGE AND SHELF LIFE OF REAGENTS

A. UNOPENED vials
Store the unopened vials at 2°C to 8°C. All kit components are stable until the expiry date printed on the labels.

B. OPENED vials
1. The Conjugate vial must be stored at 2° to 8°C.
2. The reconstituted Standards and Controls are stable for 4 days at 2°C to 8°C. Aliquots held for longer periods of time should be frozen, a maximum of two times, at -20°C (maximum 2 months) or at -70°C for longer storage (until expiration date).
3. Store the unused strips at 2°C to 8°C in the sealed bag containing the dessicant until expiration date.
4. The Wash Solution Concentrate is stable at room temperature until expiration date. In order to avoid washerhead obstructions, it is recommended to prepare a fresh diluted Wash Solution each day.
5. The freshly prepared Chromogenic Solution is stable for a maximum of 15 min. at room temperature and must be discarded afterwards.

VIII SPECIMEN COLLECTION, PREPARATION, STORAGE AND DILUTION

A. Specimen Collection and preparation
1. The BIOSOURCE sTNF-RI EASIA kit may be used to measure sTNF-RI in serum, plasma, cell culture supernatant as well as other biological fluids. Isolation and culture of peripheral blood mononuclear cells may be realized by usual methods. However, one should avoid an unintentional stimulation of the cells by the procedure. The use of pyrogen-free reagents and adequate controls are mandatory.
2. 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.
3. Serum must be removed as soon as possible from the clot of red cells after clotting and centrifugation, and kept at 4°C.
4. Collection tubes must be pyrogen-free. Plasma can be collected on sterile EDTA or heparin tubes (at 4EC) and rapidly separated after centrifugation. However, as batches of heparin are often contaminated with pyrogen, it is recommended to test each batch of heparin to avoid unintentional stimulation of blood cells. Other substances in the tube must be also pyrogen-free.
5. These recommendations are also valuable for other biological fluids (cell culture supernatant, etc.).

B. Storage
Serum/plasma samples must be kept at -20°C for maximum 2 months, and for longer storage (maximum one year) at - 70°C. Samples with low protein levels (e.g. cell culture medium, urine, etc.) should be stored at -70°C (maximum one year).

C. Sample Dilution
If samples generate values higher than the highest standard, dilute the sample with Standard 0.

IX BIOSOURCE sTNF-RI EASIA PROCEDURE

The instructions of the assay procedure must be followed to obtain reliable results.

A. Procedural notes
1. Allow the samples and reagents to equilibrate to room temperature (18°C to 25°C) before commencing the assay. Thoroughly mix the reagents and samples before use by gentle agitation or swirling.
2. Do not use kit components beyond the expiration date.
3. Do not mix materials from different kit lots.
4. Do not mix strips from different plates.
5. Perform Standards,Controls and Unknowns in duplicate. Vertical alignment is recommended.
6. A standard curve should be run with each assay run or each plate run.
7. To avoid drift, the time between pipetting of the first standard and the last sample must be no longer than 30 minutes. Otherwise, results will be affected.
8. Use a clean disposable plastic pipette for each reagent, standard, control or specimen addition in order to avoid cross contamination .
9. For the dispensing of the Chromogenic Solution and Stop Solution avoid pipettes with metal parts.
10. Use a clean plastic container to prepare the Wash Solution.
11. The Chromogenic Solution should be colourless, if not this indicates that the reagent is unusable, and must be discarded. Dispense the Chromogenic Solution within 15 min. following the washing of the microtiter plate.
12. During incubation with Chromogenic Solution, avoid direct sunlight on the microtiter plate.
13. Respect the incubation times described in the assay procedure.

B. Assay Procedure
1. **Select the required number of strips for the run.** The unused strips should be resealed in the bag with desiccant and stored at 2-8°C.
2. **Secure** the strips into the holding frame.
3. **Pipette 50 µl of each Standard, Control, or Sample** into the appropriate wells.
4. **Pipette 200 µl of anti-sTNF-RI Conjugate Solution** into all the wells.
5. **Incubate** for **1 hour** at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
6. **Aspirate** the liquid from each well ;
7. **Wash** the plate three times by :
   a) dispensing of 0.4 ml of Biosource Wash Solution into each well;
   b) aspirating the content of each well.
8. **Pipette 50 µl of Chromogenic Solution** into each well within 15 min. following the washing step.
9. **Incubate** the plate for **15 min.** at room temperature on an horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight.
10. **Pipette 200 µl of Stop Solution** into each well.
11. **Read** absorbances at 450 nm and 490 nm (reference filter : 630 or 650 nm) within 3 hours and calculate the results as described in section XI.

X CALCULATION OF ANALYTICAL RESULTS

A. Reading the plate with the EASIA Reader
. Read the plate according to the instructions of the EASIA Reader and ELISA$^{AID™}$ Software.

B. Reading the plate with other equipment
. Read the microtiter plate at 450 nm (reference filter : 630 or 650 nm).
. Construct a standard curve using all standard points for which absorbances are below the limit of linearity of reader used.
. Plot the OD on the ordinate against the standard concentrations on the abscissa using either linear or semi-log graph paper and draw the curve by connecting the plotted points with straight lines.
. Determine sTNF-RI concentrations of Samples or Controls for which absorbance is no greater than those of the last standard plotted at 450 nm.
. If any Control or Sample has an absorbance greater than the absorbance of the last standard read at 450 nm, a second reading at 490 nm (reference filter: 630 or 650 nm) is needed. Proceed as described above to construct a second standard curve at 490 nm using all the standard points. The segment of the curve drawn between the last standard read at 450 nm and the most concentrate standard will be considered at 490 nm. The concentration of Samples and Controls for which absorbance is included in this segment, is read at 490 nm. So, the first reading gives the high sensitivity of the assay and the second reading allows an extended standard range.

Note: The readings at 490 nm are only for off-scale values at 450 nm (above the limit of reader linearity)and should not replace the reading at 450 nm for values below the limit of reader linearity.

C. Example of a typical reference curve
The following data are for demonstration purpose only and can not be used in place of data generated at the time of assay. These data are provided by using the BIOSOURCE EASIA reader and the BIOSOURCE ELISA $^{AID™}$ software.

sTNF-RI EASIA		Polychromatic model (OD units)
Standard	0 ng/ml	0.028
	1 ng/ml	0.251
	2.5 ng/ml	0.702
	8 ng/ml	1.872
	22 ng/ml	3.689
	47 ng/ml	5.198

XI QUALITY CONTROL

- The **two Controls** provided in the kit can be used as internal laboratory controls.

Note: Other controls which contain azide will interfere with the enzymatic reaction and cannot be used.

- Serum or heparin plasma pools as well as stimulated cell culture supernatants can be collected and frozen immediately in aliquot to serve as controls. Repeated freezing and thawing are not permitted.
- **Record keeping** : it is good laboratory practice to record the kit lot numbers and date of reconstitution for the reagents in use.
- **Controls** : it is recommended that Controls be routinely assayed as unknown samples to measure assay variability. It is recommended that quality controls charts be maintained to monitor the performance of the kits. Control ranges are indicated on vial labels. Out of range control results indicate the assay must be repeated. Repeat patient samples may also be used to measure interassay precision.
- **Sample handling** : strictly adhere to the instruction for handling and storage of samples. Standards, Controls, and Unknowns should be run in duplicate. A clean disposable tip should always be used to avoid carryover contamination.
- **Data reduction** : it is good practice to construct a standard curve for each run to check visually the curve fit selected by the computer program.

XII EXPECTED RANGE (Reference Interval)

At the present stage of our studies, only preliminary results can be provided and we thus recommend that each laboratory establishes its own normal values. For guidance, the mean of 129 normal plasma 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 in strict sampling condition.

XIII PERFORMANCE CHARACTERISTICS

A. 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.

B. Precision

	INTRA-ASSAY				INTER-ASSAY (day-to-day)			
Sample	n	<X> ± SD (ng/ml)	CV %	Sample	n	<X> ± SD (ng/ml)	CV %
Serum 1	12	0.99 ± 0.02	1.7	Serum 1	10	1.76 ± 0.10	5.7
Serum 2	18	19.11 ± 1.25	6.5	Serum 2	10	26.80 ± 2.40	8.9

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

Analyte	Results in the presence of 1.62 ng/ml sTNF-RI (Ratio*)	Results in the presence of 5.20 ng/ml sTNF-RI (Ratio*)	Results in the presence of 12.60 ng/ml sTNF-RI (Ratio*)
sTNF-RII (p75) (1500 ng/ml)	1.88 ng/ml (116.1 %)	5.65 ng/ml (108.6 %)	14.45 ng/l (114.7 %)
TNF-a (400 ng/ml)	1.38 ng/ml (85.2 %)	4.80 ng/ml (92.3 %)	12.23 ng/ml (97.1 %)

Ratio* : $\frac{\text{sTNF-RI measured in the presence of analyte x 100}}{\text{sTNF-RI added in the absence of analyte}}$

This demonstrates that the sTNF-RI EASIA does not cross react with sTNF-RII and that TNF-a does not interfere with the assay.

D. Accuracy

	RECOVERY			DILUTION TEST			
Sample	Added sTNF-RI (ng/ml)	Recovered sTNF-RI (ng/ml)	Recovery (%)	Serum Dilution	Theor. conc. (ng/ml)	Meas. conc. (ng/ml)	Recovery (%)
Serum	0	1.27	-	1/1	18.09	18.09	-
	1.72	2.96	98.3	1/2	9.05	9.55	105.5
	4.38	5.56	98.0	1/4	4.52	4.67	103.3
	19.62	20.48	97.9	1/8	2.26	2.56	113.3
				1/16	1.13	1.44	127.4
Plasma	0	1.85	-	1/1	23.82	23.82	-
	2.65	4.11	85.3	1/2	11.91	10.53	88.4
	6.99	9.92	115.5	1/4	5.96	4.85	81.4
	30.30	34.37	107.3	1/8	2.98	2.63	88.3
				1/16	1.49	1.43	96.0
Cell Culture Medium	0	0.04	-	1/1	33.16	33.16	-
	2.52	2.53	98.8	1/2	16.58	14.66	88.4
	11.45	12.46	108.5	1/4	8.29	8.06	97.2
	31.12	31.65	101.6	1/8	4.15	4.39	105.8
				1/16	2.07	2.43	117.4

E. High dose hook-effect
A sample spiked with sTNF-RI up to 4000 ng/ml gives a response higher than that obtained for the last standard point.

XIV PRECAUTIONS AND WARNINGS

1. 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 local safety procedures.
2. Avoid any skin contact with Stop Solution (HCl) and Concentrated Chromogen (TMB), Substrate Buffer, and Chromogenic Solution. In case of contact wash thoroughly with water.
3. Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
4. Do not pipet liquids by mouth.

XV LITERATURE REFERENCES

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Soluble receptors for tumor necrosis factor : a putative marker of disease progression in HIV infection.
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XVI SUMMARY OF ASSAY PROCEDURE

	Standards (µl)	Controls-samples (µl)
Standards (0-5)	50	-
Controls-samples	-	50
Conjugate Anti-sTNF-RI-HRP	200	200
Incubate for 1 hour at R.T. with continuous shaking (700 RPM) **Aspirate** the contents of each well **Wash** 3 times with 0.4 ml of Wash Solution and aspirate		
Chromogenic Solution	50	50
Incubate 15 min. at R.T. with continuous shaking (700 RPM)		
Stop Solution	200	200
Read on a microtiter plate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm) and 490 nm (versus 630 or 650 nm).		

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BioSource Catalogue Nr : KAC1761 - KAC1762	P.I. Number : 1700520	Date of issue : 07 March 2000

Before use, read this Package Insert.

sTNF-RI EASIA

For research use only. Not for use in diagnostic procedures.

An immunoenzymometric assay for the quantitative measurement of human soluble Tumor Necrosis Factor Receptor NE1 (sTNF-RI) in serum, plasma, cell culture medium or other biological fluids.

I GENERAL INFORMATION

A. Proprietary Name : BIOSOURCE sTNF-RI EASIA kit

B. Catalogue Number : KAC1761 : 96 determinations
KAC1762 : 2 x 96 determinations

C. Manufactured by : BioSource Europe S.A.
Rue de l'Industrie, 8 B-1400 Nivelles Belgium.

For technical assistance or ordering information contact :
Telephone numbers : (Voice) +32/67/88.99.00 (Fax) +32/67/88.99.96

II APPLICATION AND INTENDED USE

sTNF-RI, also named TBPI or p55, is one of the two receptors of tumor necrosis factors that are 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 presence of protein kinases activators. On the opposite, TNF-Rs decrease in the presence of H_2O_2, epinephrin, 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, 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 the TNF and, in high concentration, inhibit the biological activity of TNF. In some conditions, these soluble forms are able to protect TNF and increase its half live.