XIV PERFORMANCE CHARACTERISTICS

1. **Minimum Detectable Concentration (MDC).**
The MDC is estimated to be 2 pg/ml and is defined as the IL-6 concentration corresponding to the average OD of 20 replicates of the zero standard + 2 standard deviations.

2. **Precision**

INTRA-ASSAY				INTER-ASSAY (day-to-day)			
Sample	n	<X> ± SD (pg/ml)	CV %	Sample	n	<X> ± SD (pg/ml)	CV %
Serum 1	24	75.6 ± 6.1	5.6	Serum 1	4	70.7 ± 5.3	7.5
Serum 2	22	205.4 ± 10.1	4.7	Serum 2	6	194.9 ± 4.3	2.2

3. **Specificity**
No significant cross-reaction was observed in presence of 50 ng of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-7, IL-8, IL-10, GM-CSF, IFN-α, IFN-γ, LIF, MIP-1α, MIP-1β, MCP-1, OSM, RANTES, TGF-β, TNF-α and TNF-β. A very tenuous cross-reaction (0.06%) is observed with G-CSF.

4. **Interference with the soluble Receptors (sIL6R and sgp-130)**
To check the absence of any interference of the sIL6 Receptor and sgp-130 on the assay, a recovery has been performed in presence of high concentration of sIL6R, sgp-130 and the mix of the two receptors.

IL6 added (pg/ml)	IL6 measured with 100 ng/ml of sIL6R	IL6 measured with 100 ng/ml of sgp-130	IL6 measured with 100 ng/ml of sIL6R and 100 ng/ml of sgp-130
0	0	0	0
207	209	202	203
846	875	850	869
1766	1890	1766	1732

No interference was observed.

5. **Accuracy**

RECOVERY				DILUTION TEST			
Sample	Added IL-6 (pg/ml)	Recovered IL-6 (pg/ml)	Recovery (%)	Sample	Dilution	Theor. conc. (pg/ml)	Meas. conc. (pg/ml)
Serum 1	1066	1035	97.1	Serum	1/1	966	-
	547	541	98.9		1/2	483	478
	228	234	102.6		1/4	241.5	247
					1/8	120.8	130
Serum 2	1066	1110	104.1		1/16	60.4	54.1
	547	531	97.1		1/32	30	23.3
	228	250	109.7				
Plasma 1	804	812	101	Plasma	1/1	835.9	-
	409	419	102		1/2	418	411.9
	214	211	99		1/4	209	212.2
					1/8	104.5	106.7
Plasma 2	804	813	101		1/16	52.2	47.9
	409	407	100				
	214	185	86				
Culture Medium	772	709	91	Culture Medium	1/1	763	-
	347	384	109		1/2	381.5	363
	166	188	110		1/4	190.8	178
					1/8	95.4	87
					1/16	47.7	41

XV LITERATURE REFERENCES

1. HOUSSIAU F.A. et al., (1988)
IL-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides.
Arth. Rheum., 31:784-788.

2. MOSCOVITZ H. et al., (1994)
Plasma cytokine determination in emergency department patients as predictor of bacteremia and infectious disease severity.
Critical care Medicine, 22:1102-1107.

3. SAKAMOTO K. et al., (1994)
Elevation of circulating interleukin 6 after surgery : factors influencing the serum level.
Cytokine, 6:181-186.

4. KITA Y. et al., (1994)
Evaluation of sequential serum interleukin-6 levels in liver allograft recipients.
Transplantation, 57:1037-1041.

5. LE MOINE O. et al., (1994)
Interleukin-6 : an early marker of bacterial infection in decompensated cirrhosis.
J. of Hepatology, 20:819-824.

XVI SUMMARY OF ASSAY PROCEDURE

	Standards (µl)	Serum/ plasma samples (µl)	Culture supernatant/urine (µl)
Solution B	50	50	-
Solution A	-	-	50
Standards (0-5), controls	100	-	-
Serum/plasma samples	-	100	-
Culture supernatants/urines	-	-	100
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			
Anti-IL-6-HRP Conjugate	100	100	100
Solution A	50	50	50
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			
Chromogen	200	200	200
Incubate 15 min. at R.T. with continuous shaking			
Stop Solution	100	100	100
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).			

BioSource Catalogue Nr : KAC1261/KAC1262	P.I. Number : 1700492	Date of issue : 19 October 2001

Before use, read this Package Insert.

IL-6 EASIA

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

An immunoenzymometric assay for the quantitative measurement of human interleukin 6 (IL-6) in serum, plasma, cell culture medium or other biological fluids.

I GENERAL INFORMATION

A. Proprietary Name : BIOSOURCE IL-6 EASIA kit

B. Catalogue Number : KAC1261: 96 determinations
KAC1262: 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

Human Interleukin 6 (IL-6) is a 184 A.A. polypeptide with potential O and N-glycosylation sites, and a significant homology with G-CSF. It is produced by various cells, including T- and B-cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, astrocytes, bone marrow stroma cells and several tumor cells. It regulates the growth and differentiation of various cell types with major activities on the immune system, hematopoïesis, and inflammation. These multiple actions are integrated within a complex cytokine network, where several cytokines induce (IL-1, TNF, PDGF, IFNs,...) or are induced by IL-6 and the final effects result from either synergistic or antagonistic activities between IL-6 and the other cytokines (IL-1, IL-2, IL-4, IL-5,IFNγ, IL-3, GM-CSF, M-CSF,CSF,...). IL-6 induces final maturation of B-cells into antibody producing cells and is a potent growth factor for myeloma/plasmacytoma cells. It (co-)stimulates T-cell growth and cytotoxic T-cell differentiation. It promotes megakaryocyte development and synergizes with other cytokines to stimulate multipotent hematopoïetic progenitors. It can also induce differentiation and growth inhibition of some leukemia -or non hematopoïetic tumoral cell lines. IL-6 is also a major inducer of the acute phase reactions in response to inflammation or tissue injury. Along with IL-1 and TNF, it induces the synthesis of acute phase proteins (APP) by hepatocytes, each cytokine or combination of cytokines showing a preferential pattern of APP production. IL-6 also interacts with the neuroendocrine system, e.g. by inducing ACTH production. Thus, IL-6 is a pleiotropic cytokine with multiple endocrine, paracrine and possibly autocrine activities in various tissues. Although most normal controls have undetectable levels of IL-6 in their serum, huge quantities of IL-6 are detected in severe inflammatory situations such as septicemia. The elevation of serum IL-6 precedes that of acute phase proteins, e.g. in a postoperative phenomenon, and may thus be a sensitive early parameter to investigate inflammatory conditions.
Serum IL-6 has already been described in association with surgical or traumatic tissue injuries, infectious diseases, auto-immune diseases including arthritis, graft rejection, alcoholic liver cirrhosis, malignancies, etc.

III PRINCIPLES OF THE BIOSOURCE IL-6 EASIA ASSAY

The BIOSOURCE IL-6 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 IL-6 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 IL-6 react with capture monoclonal antibodies (MAbs 1) coated on the microtiter well. After incubation, the occasional excess of antigen is removed by washing. Mab 2, the horseradish peroxidase (HRP)-labelled-antibody, is then added. After an incubation period allowing the formation of a sandwich : coated MAbs 1 - IL-6 - 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 (H_2SO_4) 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 IL-6 concentration. A standard curve is plotted and IL-6 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-IL-6 coated wells	1 x 96 wells	2 x 96 wells	**Ready** for use
Standards 0 to5in human plasma with preservatives : see vial label for exact concentrations	6 vials lyophil.	6 vials lyophil.	**Add** 1 ml distilled water
Solution A (human plasmawith preservatives) for cell culture or urine	3 vials lyophil.	5 vials lyophil.	**Add** distilled water(see the volume on the vial label)
Solution B (buffer with preservatives) : for serum/plasma	1 vial 11 ml	2 vials 11 ml	**Ready** for use
Anti-IL-6-RPConjugate in a buffered solution with proteins and preservatives	1 vial 11 ml	2 vials 11 ml	**Ready** for use
Controls 1 and 2 in human plasma with preservatives	2 vials lyophil.	2 vials lyophil.	**Add** 1 ml distilled water
Washing Solution Concentrate (buffer with preservatives)	1 vial 10 ml	1 vial 10 ml	**Dilute** 2 ml in400 ml distilled water or the vial contents in 2000 ml distilled water
Chromogen : TMB	1 vial 25 ml	2 vials 25 ml	**Ready** for use
Stop Solution	1 vial 25 ml	1 vial 25 ml	**Ready** for use

Note : 1 pg of the standard preparation is equivalent to 100 mIU NIBSC 89/548

V 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 and Chromogen,. 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.

VI EQUIPMENT AND SUPPLIES REQUIRED BUT NOT PROVIDED

1. High quality distilled water.
2. Precision pipette : 50 µl, 100 µl, 200 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.

VII REAGENT PREPARATION

1. **Standards, Controls and Solution A** : Reconstitute the lyophilized Standards, Controls and Solution A 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).

VIII 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, Controls and Solution A 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 desiccant 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.

IX SPECIMEN COLLECTION, PREPARATION, STORAGE AND DILUTION

A. Specimen Collection and preparation

1. The BIOSOURCE IL-6 EASIA kit may be used to measure IL-6 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 IL-6 production by blood cells and thus falsely increase plasma IL-6 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 4°C) 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 (urine, etc.).
6. For biological fluids such as synovial fluids, bile, BALF, CSF, ... : refer to the technical sheet (dilution of biological fluids) in your EASIA Manual.

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 the appropriate solution (see below) and repeat the assay.

1. **Serum and plasma** : dilute with Solution A.
2. **Cell culture supernatant and urine** : dilute with Solution B or the cell culture medium used.

X BIOSOURCE IL-6 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 a blue colour develops within a few minutes after preparation, 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 Solution B** into the appropriate wells foreseen for the Standards and Controls.
4. **Pipette 50 µl of Solution B** into the appropriate wells for **serum/plasma samples**, or , **Pipette 50 µl of Solution A** into the appropriate wells for **cell culture supernatant/urine samples**.
5. **Pipette 100 µl of each Standard, Control, or Sample** into the appropriate wells.
6. **Incubate** for **1 hour** at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
7. **Aspirate** the liquid from each well ;
8. **Wash** the plate three times by :
   a) dispensing of 0.4 ml of Wash Solution into each well ;
   b) aspirating the content of each well.
9. **Pipette 100 µl of anti-IL-6 conjugate** into all the wells.
10. **Pipette 50 µl of Solution A** into each well.
11. **Incubate** for **1 hour** at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
12. **Aspirate** the liquid from each well ;
13. **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.
14. **Pipette 200 µl of Chromogen** into each well within 15 min. following the washing step.
15. **Incubate** the plate for **15 min.** at room temperature on an horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight.
16. **Pipette 100 µl of Stop Solution** into each well.
17. **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.

C. Alternative protocol : 1h incubation

Idem **X.B.**, except for points :

6. **Incubate** for **30 min.** at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
11. **Incubate** for **15 min.** at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.

XI 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 IL-6 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 EASIA reader and the ELISA^{AID} software.

IL-6 EASIA		Normal Protocol (OD units)	Alternative Protocol (OD units)
Standard	0 pg/ml	0.036	0.033
	16 pg/ml	0.102	0.072
	45 pg/ml	0.241	0.145
	147 pg/ml	0.744	0.410
	462 pg/ml	2.237	1.227
	1690 pg/ml	3.878	3.576

XII 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.

XIII 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, 80 normal donor sera were evaluated in this assay : 49 had non-detectable levels of IL-6, 29 present low but detectable levels of IL-6 (range : 3.0 - 8.5 pg/ml), one sample showed 24.5 pg/ml and another 72.3 pg/ml.