III PRINCIPLES OF THE BIOSOURCE IL-1ra CYTOSCREEN ASSAY

The BIOSOURCE IL-1ra is a solid phase performed on microtiter plate. Standards or samples containing IL-1ra react with capture monoclonal antibody (Mab 1) coated on the microtiter well and with a biotinylated monoclonal antibody (Mab 2). After an incubation period allowing the formation of a sandwich : coated Mab 1 - IL-1ra - Mab 2 - Biotin, the microtiter plate is washed to remove unbound biotinylated antibodies. Streptavidin-Peroxidase is added and this binds to the biotinylated antibody. After incubation, the unbound enzyme is removed by washing and a substrate solution is added. The reaction is stopped with the addition of Stop Solution and the microtiter plate is then read at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is proportional to the IL-1ra concentration. A standard curve is plotted and IL-1ra concentrations in a sample is determined by interpolation from the standard curve.

IV REAGENTS PROVIDED

Reagents	96 Tests Kit	192 Tests Kit	Reconstitution
Microtiter plate with 96 anti-IL-1ra coated wells	1 x 96 wells	2 x 96 wells	**Ready** for use
Standard in human serum with preservatives	2 vials lyophil.	4 vials lyophil.	**See** vial label for reconstitution
Standard Diluent (plasma with preservatives)	1 vial 25 ml	2 vials 25 ml	**Ready** for use
Incubation Buffer (buffer with preservatives)	1 vial 25 ml	1 vial 25 ml	**Ready** for use
Anti-IL-1ra-Biotin Conjugate in buffered solution with proteins and preservatives	1 vial 6 ml	2 vials 6 ml	**Ready** for use
Streptavidin-HRP diluent with preservatives	1 vial 25 ml	1 vial 25 ml	**Ready** for use
Controls 1 and 2 in human plasma with preservatives	2 vials lyophil.	2 vials lyophil.	**Add** 2 ml distilled water
Washing Solution Concentrate (25 X)	1 vial 100 ml	1 vial 100 ml	**Dilute** 100 ml in 2400 ml distilled water
Streptavidin-HRP (100 x concentrated)	1 vial 0.125 ml	2 vials 0.125 ml	**See** the table (VII.2)
Chromogen : TMB	1 vial 25 ml	1 vial 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 1 nIU NIBSC 92/644.

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 (TMB). 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, 250 µl, 1 ml and 10 ml.
3. Vortex mixer and magnetic stirrer.
4. RPMI

VII REAGENT PREPARATION

1. **Controls** : Reconstitute the 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. **Streptavidin-HRP Dilution (100 x concentrated)** : Following the number of wells to be used, dilute the concentrated conjugate with the Streptavidin-HRP diluent in a clean glass vial: see below table for the volumes to pipette. Extemporaneous preparation is recommended.

TABLE STREPTAVIDIN-HRP DILUTION

Number of wells	Streptavidin-HRP	Streptavidin HRP diluent
16	20 µl	2 ml
32	40 µl	4 ml
48	60 µl	6 ml
96	110 µl	11 ml
192	220 µl	22 ml

3. **Wash Solution** : Dilute 100 ml of Washing Solution Concentrate in 2400 ml distilled water.
4. **Reconstitution and dilutions of the standard** :

Note : Either Glass or plastic tubes may be used to standard dilutions.

- Reconstitute standard to 10000 pg/ml with standard Diluent Buffer. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution.
- Dilute the reconstituted standard to obtain a concentration of 2000 pg/ml. Mix.
- Add 400 µl of Standard Diluent Buffer to each of 6 tubes labelled 1000, 500, 250, 125, 62.5 and 31.3 pg/ml.
- Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

DILUTION TABLE

Standard	Add :	Into :
2000 pg/ml	-	-
1000 pg/ml	400 µl of 2000 pg/ml	400 µl of Standard Diluent
500 pg/ml	400 µl of 1000 pg/ml	400 µl of Standard Diluent
250 pg/ml	400 µl of 500 pg/ml	400 µl of Standard Diluent
125 pg/ml	400 µl of 250 pg/ml	400 µl of Standard Diluent
62.5 pg/ml	400 µl of 125 pg/ml	400 µl of Standard Diluent
31.3 pg/ml	400 µl of 62.5 pg/ml	400 µl of Standard Diluent
0 pg/ml	400 µl of Standard Diluent	-

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 Biotin Conjugate vial must be stored at 2° to 8°C.
2. The reconstituted 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) of the kit.
3. Discard all remaining reconstituted and diluted standards after completing the assay.
4. Store the unused Streptavidin-HRP and Standard Diluent at 4°C until expiration date of the kit.
5. Store the unused strips at 2°C to 8°C in the sealed bag containing the desiccant until expiration date.
6. The Washing Solution Concentrate is stable at room temperature until expiration date. The diluted buffer should be used within 14 days.

IX SPECIMEN COLLECTION, PREPARATION, STORAGE AND DILUTION

A. Specimen Collection and preparation

1. The BioSource IL-1ra Cytoscreen kit may be used to measure IL-1ra in serum, plasma, urine, 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-1ra production by blood cells and thus falsely increase plasma IL-1ra 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 for maximum one day.
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.).

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 last standard point, dilute the sample with the appropriate solution (see below). However, urines, amniotic fluids and synovial fluids must always be diluted (see below).

1 **Serum and plasma** : dilute with Standard Diluent.
2. **Cell culture supernatant** : dilute with RPMI
3. **Urine** : dilute at least 1/10 + FSC 10%

X BIOSOURCE IL-1ra CYTOSCREEN 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. During incubation with Chromogenic Solution, avoid direct sunlight on the microtiter plate.
12. 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 dessicant and stored at 2-8°C.
2. Secure the strips into the holding frame.
3. **Pipette 100 µl of Incubation Buffer** into the appropriate wells for the : Standards, Controls, Serum/plasma samples.
4. **Pipette 100 µl of Standard Diluent** into the appropriate wells for **cell culture supernatant and diluted urine samples**.
5. **Pipette 100 µl of each Standard, Control, or Serum Sample** into the appropriate wells.
6. **Pipette 50 µl of Biotin conjugate** into all the wells.
7. **Incubate** for **2 hours** at room temperature ;
8. **Aspirate** the liquid from each well ;
9. **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.
10. **Pipette 100 µl of diluted Streptavidin-HRP conjugate** into all the wells.
11. **Incubate** for **60 min.** at room temperature ;
12. **Aspirate** the liquid from each well ;
13. **Wash** the plate four times by :
    a) dispensing of 0.4 ml of BioSource Wash Solution into each well ;
    b) aspirating the content of each well.
14. **Pipette 100 µl of Chromogenic Solution (TMB)** into all the wells.
15. **Incubate** the plate for **30 min.** at room temperature, avoiding direct sunlight.
16. **Pipette 100 µl of Stop Solution** into each well.
17. **Read** the absorbance within 1 hour and calculate the results as described in section XI.

XI CALCULATION OF ANALYTICAL RESULTS

A. Reading the plate with an equipment capable to record an optical density of 3.0 or more

- Read the microtiter plate at 450 nm (reference filter : 630 or 650 nm).
- Construct a standard curve by plotting 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 ligne.
- Determine IL-1ra concentrations of Samples or Controls.

B. Reading the plate with an equipment capable to record an optical density lower than 3

Read the microtiter plate at 490 nm (reference filter : 630 or 650 nm).
It will result in a decrease of the OD units when compared to ODs read at 450 (as shown on the table hereafter). Nevertheless, results remain quite similar.

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 reader and the BioSource ELISA$^{AID™}$ software.

IL-1ra Cytoscreen		Reading 450 nm (OD Units)	Reading 490 nm (OD Units)
Standard	0 pg/ml	0.117	0.023
	31.25 pg/ml	0.217	0.041
	62.5 pg/ml	0.413	0.073
	125 pg/ml	0.602	0.122
	250 pg/ml	1.029	0.190
	500 pg/ml	1.653	0.300
	1000 pg/ml	2.715	0.495
	2000 pg/ml	3.473	0.663
Sample 1	(pg/ml)	160	160
Sample 2	(pg/ml)	257	257

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 studies, only preliminary results can be provided and we thus recommend that each laboratory establishes its own normal values. For guidance, see table below :

Type of sample	n	Mean ± SD (pg/ml)	Range (pg/ml)
EDTA plasma	27	124 ± 91	41 - 493
Heparin plasma	14	124 ± 128	9 - 324
Serum	14	119 ± 71	39 - 279

This study was performed with samples collected in strict sampling conditions.

XIV PERFORMANCE CHARACTERISTICS

1. **Minimum Detectable Concentration (MDC).**
The MDC is estimated to be 4 pg/ml and is defined as the IL-1ra 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	16	334 ± 16	4.8	Serum 1	13	325 ± 23	7.1
2	16	753 ± 31	4.1	2	12	733 ± 67	9.1

3. **Specificity**
No significant cross-reaction was observed in presence of 200 ng of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, TNF-α, TNF-β, IFN-α, IFN-β, IFN-γ, GM-CSF, OSM , MIP-1α, MIP-1β, LIF, MCP-1, G-CSF,GRO, IP-10, SCF, PDGF, MCP-3, NAP-2 and RANTES. This IL-1ra assay is specific for human natural and recombinant IL-1ra.

4. **Accuracy**

RECOVERY				DILUTION TEST			
Sample	Added IL-1ra pg/ml	Recovered IL-1ra pg/ml	Recovery %	Sample	Dilution	Theor Conc pg/ml	Meas. Conc. pg/ml
Plasma	0	170	-	Plasma	1/2	774	818
	320	485	98		1/4	387	392
	160	339	106		1/8	193	197
	80	250	100		1/16	97	98
					1/32	48	42
Urine	0	-	-	Urine	1/8	703	685
	431	432	100		1/16	352	359
	216	194	90		1/32	176	194
	108	93	86		1/64	88	92
					1/128	44	43
Cell Cult. Med.	0	-	-	Cell Cult. Med.	1/2	793	753
	404	442	109		1/4	396	384
	202	200	99		1/8	198	182
	101	103	102		1/16	99	112
					1/32	50	55

5. **High dose hook-effect**
A sample spiked with IL-1ra up to 0.5 µg/ml gives a response higher than that obtained for the last standard point.

XV LITERATURE REFERENCES

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XVI SUMMARY OF ASSAY PROCEDURE

	Standards (µl)	Serum/ plasma samples (µl)	Culture Supernatant urine (µl)
Incubation Buffer	100	100	-
Standard Diluent	-	-	100
Standards (0-7), Controls	100	-	-
Serum/plasma samples	-	100	-
Culture supernatant/urines	-	-	100
Biotin-conjugate	50	50	50
Incubate for 2 hours at R.T. **Aspirate** the contents of each well **Wash** 3 times with 0.4 ml of Wash Solution and aspirate			
Streptavidin-HRP	100	100	100
Incubate for 60 min. at R.T. **Aspirate** the contents of each well **Wash** 4 times with 0.4 ml of Wash Solution and aspirate			
Chromogenic Solution	100	100	100
Incubate 30 min. at R.T.			
Stop Solution	100	100	100
Read on a microtiter plate reader and record the absorbance of each well at 450 nm.			

BioSource Catalogue Nr : KAC1181 / KAC1182	P.I. Number : 1700557	Date of issue : 07 November 2005

Before use, read this Package Insert.

IL-1ra Cytoscreen

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

An immunoenzymometric assay for the quantitative measurement of human Interleukin-1 receptor antagonist in serum, plasma, cell culture medium or other biological fluids.

I GENERAL INFORMATION

A. Proprietary Name : BIOSOURCE IL-1ra Cytoscreen kit

B. Catalogue Number : KAC1181 : 96 determinations
KAC1182 : 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

Interleukin-1 receptor antagonist is a cytokine which opposes the actions of IL-1α and IL-1β by binding to IL-1 receptors (both type I and II) without inducing signal transduction.
IL-1ra, fully glycosylated form has a molecular weight of 23-25 kd.
IL-1ra is secreted by monocytes/macrophages. It was originally isolated from the urine of patients with monocytic leukaemia.
It is also present in high concentrations in third trimester amniotic fluid, in urine of febrile patients and in synovial fluid of rheumatoid arthritis.
IL-1ra acts as an anti-inflammatory substance and may be useful in the treatment of chronic inflammatory diseases (e.g. rheumatoid arthritis, auto-immune diabetes ...).
In animal, it can also prevent the septic shock observed during gram negative sepsis.