DRG® LIF / HILDA (EIA-3878)

REVISED 15 JUNE 2007 **RUO**

NAME AND INTENDED USE

The DRG LIF/HILDA ELISA is for the quantitative determination of human Leukemia Inhibitor Factor/Human Interleukin for DA cells (LIF/HILDA) in serum, plasma, cell culture medium or other biological fluids. This kit has been configured for research use only and is not to be used in diagnostic procedures.

CLINICAL RELEVANCE

LIF/HILDA (Leukemia Inhibitory Factor/Human Interleukin for DA cells) is a 32-45-kDa glycoprotein produced by activated lymphocytes and macrophages. LIF/HILDA has pleiotropic functions, which are as yet not completely characterized. It inhibits embryonic stem (ES) cell differentiation, lipoprotein lipase activity in adipocyte, bovine aortic endothelial cell growth, induced cholinergic nerve differentiation and acute phase protein production by hepatocytes. This cytokine has also an effect on bone metabolism and muscle cells, is able to sustain the proliferation of IL-3 dependent cell lines and induces the macrophagic differentiation of the M1 Leukemic cell lines. In man, LIF/HILDA can substitute for IL-6 and G-CSF to induce undifferentiated blastic colonies from bone marrow progenitors in synergy with IL-3. LIF/HILDA has been found in synovial fluid of rheumatoid arthritic but not osteoarthritic patients, its role in the pathogenesis of that pathology being suggested; presence of LIF/HILDA have been shown in urine but not plasma of kidney graft recipients during acute rejection episodes; and finally, mRNA-LIF/HILDA expression in the uterine endometrial glands, specifically on day 4, of pregnant mice has been shown, suggesting a regulatory role of the cytokine on blastocyst growth and implantation.

PRINCIPLE OF THE TEST

The LIF/HILDA ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay 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 LIF/HILDA is used. Antibody-producing cells are immortalized 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 LIF/HILDA 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 – LIF/HILDA - MAb 2 - HRP, the microtiter plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme-labeled 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 colorimetrically by measuring the absorbance, which is proportional to the LIF/HILDA concentration. A standard curve is plotted and LIF/HILDA concentration in a sample is determined by interpolation from the standard curve. The use of the 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.

WARNINGS AND PRECAUTIONS FOR USERS

GENERAL REMARKS

- Test methods are not available which can offer complete assurance that Hepatitis B virus, Human Immunodeficiency Virus (HIV/HTLV-III/LAV), or other infectious agents are absent from the reagents in this kit. Therefore, all human blood products, including patient samples, should be considered potentially infectious. Handling and disposal should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation, where it exists (e.g., USA Center for Disease Control/National Institute of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories," 1984).
- Avoid contact with Stop Solution - H_2SO_4. It may cause skin irritation and burns.
- Replace caps on reagents immediately. Do not switch caps.

- Solutions containing additives or preservatives, such as sodium azide, should not be used in the enzyme reaction.
- Do not pipette reagents by mouth.
- For Research Use Only.
- Do not mix or use components from kits with different lot numbers.

REMARKS FOR THE TEST

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination. For the dispensing of the Substrate Solution and the Stop Solution avoid pipettes with metal parts.
- Pipette standards and samples onto the bottom of the well. For pipetting of Conjugate and Stop Solution it is recommended to hold the pipette in a vertical position above the well and dispense the correspondent solution into the centre of the well so that a complete mixing of Conjugate with sample or standard and of the Stop solution with the Substrate Solution is achieved. Vortex-mixing or shaking of wells after the pipetting step is not required.
- Before starting the assay, it is recommended that all reagents be ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- The Substrate Solution should be colourless or slightly blue or green. If the solution is dark blue the reagent is unusable and must be discarded.
- During incubation with Substrate Solution avoid direct sunlight on the microtiter plate.

KIT COMPONENTS

Reagents	96-Tests Kit	192 Tests-Kit	Reconstitution
1 microtiter plate with 96 LIF/HILDA	1 x 96 wells	2 x 96	Ready for use
Standard 0 in human plasma with preservatives	1 vial lyophil.	2 vials lyophil.	Add distilled water (see exact volume on the vial label)
Standards 1 to 5 in human serum with preservatives: see vial label for exact concentrations	5 vials lyophil.	5 vials lyophil.	Add 1 ml distilled water
Solution A (human plasma with preservatives) for cell culture or urine	2 vials lyophil.	3 vials lyoph.	Add distilled water (see vial label for exact reconstitution volume)
Solution B (buffer with preservatives) for serum/plasma	1 vial 11 ml	2 vials 11 ml	Ready for use
Anti-LIF/HILDA-HRP Conjugate in a buffered solution with proteins and preservatives	1 vial 6 ml	2 vials 6 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 in 400 ml distilled water or the vial contents in 2000 ml distilled water
Concentrated Chromogen: TMB (Tetramethylbenzindine) in DMF	1 vial 1 ml	1 vial 1 ml	Pipette 0.2 ml into 1 vial of Substrate Buffer
Substrate Buffer: H_2O_2 in acetate/citrate buffer	3 vials 21 ml/vial	3 vials 21 ml/vial	Ready for use
Stop Solution: H_2SO_4 1.8 N	1 vial 6 ml	1 vial 11 ml	Ready for use

Note: 1 pg of the standard preparation is equivalent to 0.02 mIU NIBSC 91/602.

MATERIALS REQUIRED BUT NOT SUPPLIED

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

SPECIMEN COLLECTION AND PREPARATION OF SAMPLES

SPECIMEN COLLECTION AND PREPARATION

- The LIF/HILDA kit may be used to measure LIF-HILDA 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.
- 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 LIF/HILDA production by blood cells and thus falsely increase plasma LIF/HILDA values.
- Serum must be removed as soon as possible from the clot of red cells after clotting and centrifugation, and kept at 4°C.
- 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.
- These recommendations are also valuable for other biological fluids (urine, etc.)

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).

SAMPLE DILUTION

If samples generate values higher than the highest standard, dilute the sample with the appropriate solution (see below) and repeat the assay. Serum and plasma: dilute with Solution A. Cell culture supernatant and urine: dilute with Solution B or the cell culture medium used.

ASSAY PROCEDURE

GENERAL REMARKS

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use. A standard curve must be run with each assay. It is recommended that all standards, controls and samples be run in duplicate. Because Stabilized Chromogen is light sensitive, avoid prolonged exposure to light. Also avoid contact between Stabilized Chromogen and metal, or color may develop. 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. Use a clean plastic container to prepare the

Wash Solution. The Chromogenic Solution should be colorless. If a blue color develops within a few minutes after preparation, this indicates that the reagent is unusable, and most be discarded. Dispense the Chromogenic Solution within 15 minutes of preparation following the washing of the microtiter plate:

- Respect the incubation times described in the assay procedure.
- Preparation, Reconstitution and Storage of Reagents

CONJUGATE
The vial must be stored at 2° to 8°C. Unused strips: Store at 2°C to 8°C in the sealed bag containing the desiccant until expiration date.

STANDARDS, CONTROLS AND SOLUTION A
Reconstitute the Iyophilized 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.
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).

WASH SOLUTION
Dilute 2 ml of Wash Solution Concentrate in 400 ml distilled water or all the contents of the Wash Solution Concentrate vial in 2000 ml distilled water (use a magnetic stirrer). 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.

CHROMOGENIC SOLUTION
Pipette 0.2 ml of the Concentrated Chromogen (TMB) into one of the vials of Substrate Buffer (H_2O_2 in acetate/citrate buffer). Extemporaneous preparation is necessary. Use only at room temperature. Chromogenic Solution is stable for a maximum of 15 minutes Avoid direct exposure to sunlight. The freshly prepared Chromogenic Solution is stable for a maximum of 15 minutes at room temperature and must be discarded afterwards.

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.

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 100 µI of Solution B into the appropriate wells foreseen for the Standards and Controls.
4) Pipette 100 µl of Solution B into the appropriate wells for serum/plasma samples, or, pipette 100 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) Pipette 50 µI of anti-LIF/HILDA Conjugate into all the wells.
7) Incubate for 4 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm. The use of the shaker is recommended.
8) Aspirate the liquid from each well;
9) Wash the plate three times by: Dispensing of 0.4 ml of Wash Solution into each well, aspirating the content of each well,
10) Pipette 200 µI of freshly prepared Chromogenic Solution into each weIl within 15 minutes following the washing step.
11) Incubate the plate for 30 minutes at room temperature on an horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight.
12) Pipette 50 µI of Stop Solution into each well.
13) Read absorbances at 450 nm and 490 nm (reference filter: 630 or 650 nm) within 3 hours and calculate the results as described in next section

CALCULATION OF THE RESULTS

Reading the plate with the Reader. Read the plate according to the instructions of the ELISA Reader and ELISA Software.

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 LIF/HILDA 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.

EXAMPLE OF A TYPICAL REFERENCE CURVE

The following data are for demonstration purpose only and cannot be used in place of data generated at the time of assay. Using the Reader and the Software provides these data.

LIF/HILDA		Polychromatic model (OD units)
Standard	0 pg/ml	0.070
	300 pg/ml	0.165
	1000 pg/ml	0.454
	1800 pg/ml	0.994
	3500 pg/ml	2.130
	9000 pg/ml	4.246

QUALITY CONTROL

- The two Controls provided in the kit can he 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 Iaboratory 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. lt 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.

EXPECTED VALUES (REFERENCE INTERVAL)

At the present stage of our studies, only preliminary results can be provided and we thus recommend that each laboratory establish its own normal values. For guidance, the mean of 276 normal plasma was 20 pg/ml (SD = 25), ranging between 0 pg/ml and 300 pg/ml. This study was performed with samples collected in strict sampling condition. The level of LIF/HILDA in serum samples can be much higher if strict conditions are not taken.

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PERFORMANCE CHARACTERISTICS
PRECISION

Intra-Assay Precision

Sample	Serum 1	Serum 2
n	20	20
Mean [pg/ml]	1300	2588
S.D. [pg/ml]	65	118
% V	5:0	4:5

SD = Standard Deviation
CV = Coefficient of Variation

Inter-Assay Precision

Sample	Serum 1	Serum 2
n	15	15
Mean [pg/ml]	1426	2130
S.D. [pg/ml]	120	128
%CV	8.4	6.0

SENSITIVITY
The Minimum Detectable Concentration is estimated to be 20 pg/ml and is defined as the LIF/HILDA concentration corresponding to the average OD of 20 replicates of the zero standard + 2 standard deviations.

SPECIFICITY
No significant cross-reaction was observed in presence of 50 ng of IL-1α; IL-1β; IL-2; IL-3, IL-4; IL-4; IL-6; IL-7; IL-8; TNF-α; TNF-β; GM-CSF; OSM; G-CSF; IL-10; MCP-1 and TGF-β. This LIF/HILDA assay is specific for human natural recombinant LIF/HILDA.

ACCURACY

The accuracy of the assay was evaluated by recovery and dilution tests.

RECOVERY				DILUTION TEST			
Sample	**Added LIF/HILDA (pg/ml)**	**Recovery LIF/HILDA (pg/ml)**	**Recovery (%)**	**Sample**	**Dilution**	**Theor. conc. (pg/ml)**	**Meas. Conc. (pg/ml)**
Plasma	670	630	94	Plasma	1/1	5300	5300
	1320	1260	95		1/2	2650	2600
	2630	2520	96		1/4	1325	1300
	5260	5260	100		1/8	662	600
Cell Culture Medium	990	1090	110	Cell Culture Medium	1/1	5580	5580
	1980	2070	105		1/2	2790	2590
	3960	3650	92		1/4	1395	1160
	7920	8000	101		1/8	698	560
Urine	689	550	80	Urine	1/1	5350	5350
	1378	1230	89		1/2	2675	2330
	2755	2550	93		1/4	1338	1200
	5510	5650	103		1/8	669	620

HIGH DOSE HOOK-EFFECT

A sample spiked with LIF/HILDA up to 12.5 µg/ml gives a responsible higher than that obtained for the last standard point.

LIMITATIONS OF PROCEDURE

- Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instruction and with adherence to good laboratory practice.
- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbances.
- This kit is for research use only.
- Not for human therapeutic or diagnostic use.

REFERENCES

1. Hilton D. J., Gough N. M. (1991) Leukemia inhibitory factor: a biological perspective. J. Cell-Biochem., 46(1): 21 – 26.
2. Metcalf D. (1991) The leukemia inhibitory factor (LIF). Int. J. Cell Cloning, 9(2): 95 – 108.
3. Kurzrock R., Estrov Z., Wetzler M., Guttermann J. U., Talpaz M. (1991) LIF: not just a leukemia inhibitory factor. Endocr. Rev., 12(3): 208 – 217.
4. Lotz M., Moats T., Villiger P. M. (1992) Leukemia inhibitory factor is expressed in cartilage and synovium and can contribute to pathogenesis of arthritis. J. Clin. Invest., 90: 888 – 896.
5. Taupin J. L., Morel D., Moreau J. F., Gualde N., Potaux L., Bezian J. H. (1992) LIFZ/HILDA urinary excretion during acute kidney rejection. Transplantation, 53: 655 – 658.
6. Blancho G., Moreau J. F., Anegon I., Soulillou J. P. (1992) HILDA/LIF is present in the urine of rejecting kidney graft recipients. Transpl. Int., 5(1): 57 – 58.
7. Bhatt H., Brunet L. J., Stewart C. L. (1991) Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. Proc. Natl. Acad. Sci. USA, 88(24): 11408 – 11412.

8. De Groote D., Fauchet F., Jadoul M., Dehart I., Raher S., Gevaert Y., Lopez M., Gathy R., Franssen J-D., Radoux D., Franchimont P., Soulillou J. P., Jacques Y., Godard A. (1994) An Elisa for the measurement of human leukemia inhibitory factor in biological fluids and culture supernatants. J. Immunol. Meth., 167: 253 – 261.

SUMMERY OF THE ASSAY PROCEDURE

	Standards [µl]	Serum/ PlasmaSamples [µl]	Culture supernatant/ Urine [µl]
Solution B	100	100	-
Solution A	-	-	100
Standards (0-5), controls	100	-	-
Serum/plasma samples	-	100	-
Culture supernatants/urines	-	-	100
Anti-LIF/HILDA-HRP Conjugate	50	50	50
Incubate for 4 hours 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	200	200	200
Incubate 30 min. at R.T. with continuous shaking			
Stop Solution	50	50	50
Read on a microtiter plate reader and record the absorbance of each well at 450 nm versus 630 of 650 nm) and 490 nm (versus 630 or 650 nm)			