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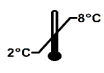
Please use only the valid version of the Instructions for Use provided with the kit

Instructions for use

TNF-a-ELISA

REF **IL E-3100R**



RUO For research use only – Not for use in diagnostic procedures

TNF-α-ELISA

1. INTENDED USE

Immunoenzymetric assay for the quantitative measurement of human Tumor Necrosis Factor α (TNF-α) in serum.

2. BACKGROUND

Human Tumor Necrosis Factor Alpha (TNF-α) also named cachectin, is a 157 A.A. unglycosylated polypeptide cytokine mainly produced by activated macrophages (monocytes). Lipopolysaccharide (LPS), the cell-wall component of gram-negative bacteria (endotoxin), is a potent stimulus for TNF-α production by macrophages and TNF-α is an important mediator of the well-known in vivo effects of LPS such as tumour hemorrhagic necrosis, fever, shock and activation of neutrophils. The various biological activities of TNF-α may be classified as:

- *Antitumoral and growth regulatory activities:* TNF-α displays a selective toxicity for tumor and virus-infected cells. Conversely, it is angiogenic and stimulates the growth of cultured fibroblasts.
- *Immunomodulatory and proinflammatory activities:* TNF-α activates macrophages, neutrophils and eosinophils, as well as endothelial cells (which display procoagulant activity). It regulates the production of antibodies by B cells and stimulates cytotoxic T cells. It induces the production of several other inflammatory mediators such as IL-1, IL-6, colony stimulating factors, prostaglandins, platelet-activating factor (PAF), collagenases, etc.
- *Metabolic activities:* TNF-α strongly inhibits lipoprotein lipase and adipocyte gene expression.

3. PRINCIPLES OF THE METHOD

The TNF-α-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of TNF-α. Calibrators and samples react with the capture monoclonal antibody (MAb 1) coated on 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 MAb 1 – human TNF-α – MAb 2 – HRP, the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB) is added and incubated. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is proportional to the TNF-α concentration.
A calibration curve is plotted and TNF-α concentration in samples is determined by interpolation from the calibration curve. The use of the ELISA reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in a high sensitivity in the low range and in an extended calibration range.

4. REAGENTS PROVIDED

IL E-3131	**MTP 96**	**Microtiterplate** – **Ready** for use
Content:	Microtiterplate with 96 anti TNF-α (monoclonal antibodies) coated wells; 96 wells	
IL E-3140	**CONJUGATE-CONC**	**Conjugate**
Content:	Conjugate: HRP labelled anti-TNF-α (monoclonal antibodies) in TRIS-Maleate buffer with bovine serum albumin and thymol	
Volume:	1 x 0.75 ml	
Preparation:	**Add** conjugate buffer (see section 6)	

Calibrators and **Controls** – lyophilized

Cat. no.	Component	Calibrator/Control	
IL E-3101	**CAL 0**	**Calibrator 0**	2 vials
IL E-3102	**CAL 1**	**Calibrator 1**	1 vial
IL E-3103	**CAL 2**	**Calibrator 2**	1 vial
IL E-3104	**CAL 3**	**Calibrator 3**	1 vial
IL E-3105	**CAL 4**	**Calibrator 4**	1 vial
IL E-3106	**CAL 5**	**Calibrator 5**	1 vial
IL E-3151	**CONTROL 1**	**Control 1**	1 vial
IL E-3152	**CONTROL 2**	**Control 2**	1 vial

Content:	Calibrators (**see exact values on vial labels**) in human plasma, benzamidin and thymol / Controls in human plasma and thymol
Preparation:	Calibrator 0: **Add** distilled water (see on the label for the exact volume)
	Calibrator 1 – 5 / Controls 1 + 2: **Add** 2 ml distilled water

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IL E-3141	**CONJUGATE-BUFF**	**Conjugate Buffer** – **Ready** for use
Content:	Conjugate buffer: TRIS-Maleate buffer with bovine serum albumin, EDTA and thymol	
Volume:	1 x 6 ml	
Hazard identification:		
	H315 Causes skin irritation. H319 Causes serious eye irritation.	
IL E-3113	**INC-BUFF**	**Incubation Buffer** – **Ready** for use
Content:	Incubation buffer: TRIS-Maleate buffer with bovine serum albumin, EDTA and thymol	
Volume:	1 x 6 ml	
Hazard identification:		
	H315 Causes skin irritation. H319 Causes serious eye irritation.	
IL E-3030	**WASH-CONC** **200x**	**Wash Solution** – 200x concentrated
Content:	Wash Solution (TRIS-HCl)	
Volume:	1 x 10 ml	
Preparation:	**Dilute** 200x with distilled water (use a magnetic stirrer).	
Hazard identification:		
	H317 May cause an allergic skin reaction.	
IL E-3155	**SUBSTRATE**	**Chromogen TMB** – **Ready** for use
Content:	Chromogen TMB (Tetramethylbenzydine)	
Volume:	1 x 12 ml	
IL E-3080	**STOP-SOLN**	**Stopping Solution** – **Ready** for use
Content:	Stopping solution: HCl 1.0 N	
Volume:	1 x 12 ml	
Hazard identification:		
	H290 May be corrosive to metals.	

Note:
1. Use Calibrator 0 for sample dilutions.
2. 1 pg of the calibrator preparation is equivalent to 40 mIU of the NIBSC IS 87/650.

5. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:
1. High quality distilled water
2. Pipettes for delivery of: 50 µl, 200 µl, 1 ml and 10 ml (the use of accurate pipettes with disposable plastic tips is recommended)
3. Vortex mixer
4. Magnetic stirrer
5. Horizontal microtiterplate shaker capable of 700 rpm ± 100 rpm
6. Washer for microtiterplates
7. Microtiterplate reader capable of reading at 450 nm, 490 nm and 650 nm (in case of polychromatic reading) or capable of reading at 450 nm and 650 nm (bichromatic reading).

6. REAGENT PREPARATION

Calibrators:
Reconstitute Calibrator 0 to the volume specified on the vial label with distilled water and the other calibrators with 2 ml distilled water.

Controls:
Reconstitute the controls with 2 ml distilled water.

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Conjugate Solution:
Following the number of wells to be used, dilute the concentrated conjugate with the conjugate buffer in a clean glass vial: see below table for the volumes to pipette. Extemporaneous preparation is recommended. Diluted conjugate is stable for max. 1 week at 2 – 8 °C.

Table Conjugate Dilution

Number of wells	Concentrated Conjugate	Conjugate Buffer	Working Volume
8	50 µl	500 µl	550 µl
16	100 µl	1000 µl	1100 µl
24	150 µl	1500 µl	1650 µl
32	200 µ	2000 µl	2200 µl
48	300 µl	3000 µl	3300 µl
96	600 µl	6000 µl	6600 µl

Working Wash solution:
Prepare an adequate volume of Working Wash solution by adding 199 volumes of distilled water to 1 volume of Wash Solution (200x). Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.

7. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kits components are stable until the expiry date, indicated on the vial label, if kept at 2 to 8 °C.
- Unused strips must be stored, at 2 – 8 °C, in a sealed bag containing a desiccant until expiration date.
- After reconstitution, calibrators and controls are stable for 4 days at 2 to 8 °C. For longer storage periods, aliquots should be made and kept at -20 °C for maximum 2 months. Avoid successive freeze thaw cycles.
- The concentrated Wash Solution is stable at 18 – 25 °C until expiration date.
- Freshly prepared Working Wash solution should be used on the same day.
- After its first use, the conjugate is stable until expiry date, if kept in the original well-closed vial at 2 to 8 °C.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

8. SPECIMEN COLLECTION AND PREPARATION

- Serum must be removed as soon as possible from the clot of red cells after clotting and centrifugation, and kept at 4 °C. If the samples are not used immediately, they must be kept at -20 °C for maximum 2 months, and at -70 °C for longer storage (maximum one year).
- Avoid subsequent freeze thaw cycles.
- Prior to use, all samples should be at 18 – 25 °C. It is recommended to vortex the samples before use.
- Sampling conditions can affect values, therefore, strict precautions have to be taken during sampling to avoid impurities contained in sampling materials that would stimulate TNF-α production by blood cells and thus falsely increase serum TNF-α values.
- Collection tubes must be pyrogen-free.

9. PROCEDURE

9.1 Handling notes

- Do not use the kit or components beyond expiry date.
- Do not mix materials from different kit lots.
- Bring all the reagents to 18 – 25 °C prior to use.
- Thoroughly mix all reagents and samples by gentle agitation or swirling.
- Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
- Use a clean plastic container to prepare the Wash Solution.
- In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.
- For the dispensing of the Revelation Solution and the Stop Solution avoid pipettes with metal parts.
- High precision pipettes or automated pipetting equipment will improve the precision.
- Respect the incubation times.
- To avoid drift, the time between pipetting of the first calibrator and the last sample must be limited to the time mentioned in section 12.5 (Time delay).
- Prepare a calibration curve for each run, do not use data from previous runs.
- The Revelation 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 Revelation Solution within 15 minutes following the washing of the microtiterplate.
- During incubation with Revelation Solution, avoid direct sunlight on the microtiterplate.

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9.2 Procedure

1.	Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2 – 8 °C.
2.	Secure the strips into the holding frame.
3.	Pipette 50 µl of incubation buffer into all the wells.
4.	Pipette 200 µl of each Calibrator, Control and Sample into the appropriate wells.
5.	Incubate for 2 hours at 18 – 25 °C on a horizontal shaker set at 700 rpm ± 100 rpm.
6.	Aspirate the liquid from each well.
7.	Wash the plate 3 times by: – Dispensing 0.4 ml of Wash Solution into each well – Aspirating the content of each well
8.	Pipette 100 µl of Calibrator 0 into all the wells.
9.	Pipette 50 µl of anti-TNF-α-HRP conjugate into all the wells.
10.	Incubate for 2 hours at 18 – 25 °C on a horizontal shaker set at 700 rpm ± 100 rpm.
11.	Aspirate the liquid from each well.
12.	Wash the plate 3 times by: – Dispensing 0.4 ml of Wash Solution into each well – Aspirating the content of each well
13.	Pipette 100 µl of the revelation solution into each well within 15 minutes following the washing step.
14.	Incubate the microtiterplate for 30 minutes at 18 – 25 °C on a horizontal shaker set at 700 rpm ± 100 rpm, avoid direct sunlight.
15.	Pipette 100 µl of Stop solution into each well.
16.	Read the absorbencies at 450 nm and 490 nm (reference filter 630 nm or 650 nm) within 30 minutes and calculate the results as described in section 10.

10. CALCULATION OF RESULTS

10.1 Polychromatic Reading

1. In this case, the software will do the data processing.
2. The plate is first read at 450 nm against a reference filter set at 650 nm (or 630 nm).
3. A second reading is performed at 490 nm against the same reference filter.
4. The Software will drive the reader automatically and will integrate both readings into a polychromatic model. This technique can generate OD's up to 10.
5. The principle of polychromatic data processing is as follows:

– Xi = OD at 450 nm
– Yi = OD at 490 nm
– Using a standard unweighted linear regression, the parameters A & B are calculated: Y = A*X + B
– If Xi < 3 OD units, then X calculated = Xi
– If Xi > 3 OD units, then X calculated = (Yi-B)/A
– A 4 parameter logistic curve fitting is used to build up the calibration curve.
– The TNF-α concentration in samples is determined by interpolation on the calibration curve.

10.2 Bichromatic Reading

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. On semi-logarithmic or linear graph paper plot the OD values (ordinate) for each calibrator against the corresponding concentration of TNF-α (abscissa) and draw a calibration curve through the calibrator points by connecting the plotted points with straight lines.
4. Read the concentration for each control and sample by interpolation on the calibration curve.
5. Computer assisted data reduction will simplify these calculations. If automatic result processing is used, a 4 parameter logistic function curve fitting is recommended.

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11. TYPICAL DATA

The following data are for illustration only and should never be used instead of the real time calibration curve.

TNF-α-ELISA		OD units Polychromatic model
Calibrator	0 pg/ml	0.045
	6.8 pg/ml	0.120
	18 pg/ml	0.259
	52 pg/ml	0.619
	176 pg/ml	1.435
	518 pg/ml	3.237

12. PERFORMANCE AND LIMITATIONS

12.1 Detection Limit

Twenty zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations above the average OD at zero binding, was 0.7 pg/ml.

12.2 Specificity

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

12.3 Precision

Intra Assay				Inter Assay			
Serum	N	<X> ± SD (pg/ml)	CV (%)	Serum	N	<X> ± SD (pg/ml)	CV (%)
A	20	91 ± 6	6.6	A	24	122 ± 5	4.5
B	20	526 ± 33	6.3	B	24	431 ± 14	3.3

SD: Standard Deviation; CV: Coefficient of variation

12.4 Accuracy

Recovery Test

Sample	Added TNF-α (pg/ml)	Recovered TNF-α (pg/ml)	Recovery (%)
Serum 1	0	6.2	–
	38.4	43.3	97
	83.9	90.0	100
	188.3	192.5	99
	408.2	376.2	91
Serum 2	0	3.8	–
	38.4	45.5	108
	83.9	91.2	104
	188.3	162.2	84
	408.2	379.2	92

Dilution Test

Sample	Dilution	Theoretical Concent. (pg/ml)	Measured Concent. (pg/ml)
Serum 1	1	–	436.5
	2	218.3	212.4
	4	109.1	104.8
	8	54.6	59.5
	16	27.3	31.7
Serum 2	1	–	420.2
	2	210.1	211.2
	4	105.0	98
	8	52.5	58.3
	16	26.3	30.7

Samples were diluted with Calibrator 0.

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12.5 Time delay between last calibrator and sample dispensing

As shown hereafter, assay results remain accurate even when a sample is dispensed 30 minutes after the calibrators have been added to the coated wells.

	T0	30 min	45 min
SC 1	202	183	222
SC 2	506	520	565

13. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls that contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises.
- It is recommended that controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practise to check visually the curve fit selected by the computer.

14. REFERENCE INTERVALS

These values are given only for guidance; each laboratory should establish its own normal range of values.

For guidance, the results of 30 serum samples from apparently healthy persons with low CRP levels, ranged between 4.6 and 12.4 pg/ml.

15. PRECAUTIONS AND WARNINGS

Safety

For *research* use only.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, 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.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with all reagents, Stop Solution contains HCl. In case of contact, wash thoroughly with water.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

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16. BIBLIOGRAPHY

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17. SUMMARY OF THE PROTOCOL

	Calibrators (µl)	Sample(s) / Controls (µl)
Incubation buffer Calibrators (0 – 5) Samples, Controls	50 200 –	50 – 200
Incubate for 2 hours at 18 – 25 °C with continuous shaking at 700 rpm. Aspirate the contents of each well. Wash 3 times with 400 µl of Wash Solution and aspirate.		
Calibrator 0 Anti-TNF-α-HRP conjugate	100 50	100 50
Incubate for 2 hours at 18 – 25 °C with continuous shaking at 700 rpm. Aspirate the contents of each well. Wash 3 times with 400 µl of Wash Solution and aspirate.		
Chromogenic solution	100	100
Incubate for 30 min at 18 – 25 °C with continuous shaking at 700 rpm.		
Stop Solution	100	100
Read on a microtiterplate reader and record the absorbance of each well at 450 nm (and 490 nm) versus 630 (or 650 nm).		

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Symbols:

+2 +8 °C	Storage temperature		Manufacturer	Σ	Contains sufficient for <n> tests
	Use-by date	LOT	Batch code		
	Consult instructions for use	CONT	Content		
	Caution	REF	Catalogue number		Distributor
	Date of manufacture			RUO	For research use only!