Human IL-6 ELISA

Interleukin-6

Cat.No. BI-IL6
12x8 Tests

IMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF HUMAN INTERLEUKIN-6 (IL-6) IN SERUM, PLASMA, CELL CULTURE SUPERNATANTS, AND URINE

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

This kit was developed and manufactured by:

BIOMEDICA

This package insert must be read entirely before using this product.

Detailed information on the human IL-6 ELISA, e.g. assay validation data, sample matrix comparisons, and stability data is available on our website.

Related Products

- Human VEGF ELISA (#BI-VEGF)
- Human Angiopoietin-2 ELISA (#BI-ANG2)
- Total soluble Neuropilin-1 ELISA (#BI-20409)

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INTRODUCTION

IL-6 PROTEIN

Interleukin-6 (IL-6), also known as B-cell stimulatory factor 2 (BSF-2), CTL differentiation factor (CDF), Hybridoma growth factor or Interferon beta-2 (IFN-beta-2), was successfully cloned by Hirano et al. in 1986 (Hirano T et al.). The gene is mapped at chromosome 7p21. IL-6 protein is built up by 183 amino acids and has a calculated molecular weight of 20.8 kDa. It is a pleiotropic, alpha helical protein that is composed of a four-helix bundle (Somers W et al.). It shares 39% sequence identity with mouse and 40% with rat IL-6. IL-6 is phosphorylated at amino acid 81 and it is variably glycosylated by N-linked glycosylation. IL-6 belongs to the IL-6/GCSF/MGF protein family (Rose-John S et al.) whose members share a common use of the gp130 receptor subunit. IL-6 isoforms, with internal deletions, are generated by alternative splicing. The principal cell sources for IL-6 are mononuclear phagocytes, vascular endothelial cells, fibroblasts or other cells. IL-6 is the ligand for the Interleukin-6 receptor α (IL-6Rα) (Schwantner A et al.) that occurs membrane-bound, but that may also circulate as soluble form generated by alternative splicing or proteolytic cleavage. To induce signaling, IL-6 first forms a complex with the non-signaling IL-6Rα. Subsequent binding to the signal transducing subunit gp130 leads to dimerization of gp130 and finally to the formation of the hexameric signaling complex (Boulanger MJ et al.). Complexes of IL-6 and soluble IL-6Rα may elicit responses in cells lacking the membrane-bound IL-6Rα but expressing the ubiquitous gp130 coreceptor. This process is known as trans-signaling, it enlarges the spectrum of target cells responding to IL-6 (Mihara M et al.).

IL-6 FUNCTION

IL-6 is immediately produced in response to infections or tissue injury, and it plays a major role in host defense. After synthesis the principal cellular targets of IL-6 are liver cells where IL-6 leads to the synthesis of acute phase proteins, B cells where proliferation of antibody producing cells is induced, or T cells where differentiation is induced. Signaling is induced by homodimerization of the receptor complex upon IL-6 binding, and subsequent activation of Janus kinases that then phosphorylate tyrosine residues in the cytoplasmic domain of gp130. Two main pathways are activated in the signaling event: the MAPK and the JAK/STAT pathway. IL-6 expression is tightly regulated, and mis-regulation contributes to chronic inflammation and autoimmunity. IL-6 plays an important role in acute phase reaction, inflammation, hematopoiesis, bone metabolism, and cancer progression (Ramadori G et al., Tanaka T et al., Hou T et al.). Increased IL-6 levels were observed in inflammatory conditions like rheumatoid arthritis, systemic juvenile idiopathic arthritis, castleman's disease, or sepsis. In this context, pro-inflammatory activities seem to depend mainly on IL-6 trans-signaling via sIL-6Rα. IL-6 also has anti-inflammatory activities that depend on membrane-bound IL-6Rα (Jones SA et al., Calabrese LH et al., Schmidt-Arras D et al.). In healthy individuals, IL-6 levels in the blood are reported in the single-digit pg/ml range. However, during inflammatory states IL-6 levels can increase several thousand-fold.

INTRODUCTION CONTINUED

Targeting of the IL-6 pathway has led to innovative therapeutic approaches for various rheumatic diseases, such as rheumatoid arthritis, juvenile idiopathic arthritis, adult-onset Still's disease, giant cell arteritis and Takayasu arteritis, as well as other conditions such as Castleman disease and cytokine release syndrome. Targeting this pathway has also identified avenues for potential expansion into several other indications, such as uveitis, neuromyelitis optica and, most recently, COVID-19 pneumonia (Choy et al.).

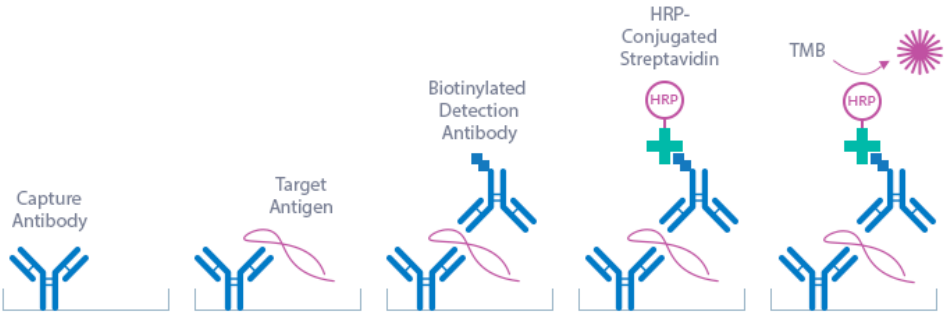
AREAS OF INTEREST

- Inflammation
- Rheumatoid arthritis
- Systemic juvenile idiopathic arthritis
- Castleman´s disease
- Sepsis
- Cancer
- Bone metabolism
- Cardiovascular disease
- Metabolic syndrome
- Covid-19

ASSAY PRINCIPLE

The Biomedica human Interleukin-6 ELISA (IL-6) ELISA kit is a sandwich enzyme immunoassay that has been optimized and fully validated for the quantitative determination of human IL-6 in serum, EDTA-plasma, citrate plasma, and heparin plasma. Validation experiments have been performed according to international quality guidelines (ICH/ FDA/ EMEA).
Cell culture supernatant and urine samples are compatible with this ELISA (data download: www.bmgrp.com). The IL-6 ELISA assay recognizes both natural and recombinant human IL-6. The assay employs highly purified epitope mapped antibodies as well as human serum-based standards and controls.

The figure below explains the principle of the human IL-6 sandwich ELISA:



In a first step, STD/sample/CTRL are pipetted into the wells, which are pre-coated with the recombinant anti-human IL-6 antibody. Any soluble IL-6 present in the STD/sample/CTRL binds to the pre-coated anti-IL-6 antibody in the well. After incubation, a washing step is applied where all non-specific unbound material is removed. In a next step, the biotinylated anti-IL-6 antibody (AB) is pipetted into the wells and reacts with the IL-6 present in the sample, forming a sandwich.

Next, all unbound antibody is removed during another washing step. In the next step, the conjugate (streptavidin-HRPO) is added and reacts with the biotinylated anti-IL-6 antibody. After another washing step, the substrate (tetramethylbenzidine; TMB) is pipetted into the wells. The enzyme catalysed color change of the substrate is directly proportional to the amount of IL-6 present in the sample. This color change is detectable with a standard microtiter plate ELISA reader.

A dose response curve of the absorbance (optical density, OD at 450 nm) versus standard concentration is generated, using the values obtained from the standards. The concentration of soluble IL-6 in the sample is determined directly from the dose response curve.

ELISA KIT COMPONENTS

All reagents supplied in the human IL-6 ELISA kit are stable at 2-8°C until the expiry date stated on the label of each reagent.

CONTENT	DESCRIPTION	QUANTITY
PLATE	Microtiter strips coated with recombinant IL-6 antibody specific for human IL-6 in strip holder packed in an aluminum bag with desiccant	12 x 8 tests
WASHBUF	20x wash buffer concentrate, transparent cap	1 x 50 ml
ASYBUF	Assay buffer, **red** cap, ready to use	1 x 10 ml
STD	Recombinant IL-6 standards (0 / 3.125 / 6.25 / 12.5 / 25 / 50 / 100 / 200 pg/ml), human serum based, white caps, lyophilized	8 vials
CTRL	Control A and B, human serum based, **yellow** cap, lyophilized, exact concentration is stated on labels	2 vials
AB	Polyclonal IL-6 antibody specific for human IL-6, biotin-labeled, **green** cap, ready to use	1 x 13 ml
CONJ	Conjugate (streptavidin-HRPO), **brown** cap, ready to use	1 x 13 ml
SUB	Substrate (TMB solution), **blue** cap, ready to use	1 x 13 ml
STOP	STOP solution, white cap, ready to use	1 x 7 ml

ADDITIONAL KIT COMPONENTS

Four self-adhesive plastic films
Quality control protocol
Instruction for use
Plate layout sheet

OTHER SUPPLIES REQUIRED

Precision and multichannel pipettes calibrated to deliver 50 µl, 100 µl, 300 µl, 500 µl, and disposable tips.
Distilled or deionized water.
A plate washer is recommended for washing. Alternatively use a multichannel pipette or manifold dispenser.
A microplate reader capable of measuring absorbance at 450 nm (optionally with a correction wavelength at 630 nm).
Software for the calculation of results or, alternatively, graph paper.

SAMPLE COLLECTION AND STORAGE

Serum, plasma (EDTA, citrate, heparin), cell culture supernatants, and urine samples are suitable for use in this assay. Do not change sample type during studies. The sample collection and storage conditions listed are intended as general guidelines.

SERUM & PLASMA

Collect venous blood samples by using standardized blood collection tubes. Perform plasma or serum separation by centrifugation according to supplier's instructions of the blood collection devices. Assay the acquired samples immediately or aliquot and store at -25°C or lower. Lipemic or hemolyzed samples may give erroneous results. Samples are stable for up to five freeze-thaw cycles.

CELL CULTURE SUPERNATANT

Cell culture supernatants should contain at least 1% fetal bovine serum for stability of the IL-6. Remove particles by centrifugation and assay immediately or aliquot and store samples at -25°C or lower. Avoid repeated freeze-thaw cycles.

URINE

Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particles, assay immediately or aliquot and store at -25°C or lower. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

WASH BUFFER

1.	Bring the WASHBUF concentrate to room temperature. Crystals in the buffer concentrate will dissolve at room temperature (18-26°C).
2.	Dilute the WASHBUF concentrate 1:20, e.g., 50 ml WASHBUF + 950 ml distilled or deionized water. Only use diluted WASHBUF when performing the assay.

The diluted WASHBUF is stable up to one month at 4°C (2-8°C).

STANDARDS & CONTROLS FOR SERUM, PLASMA, CELL CULTURE SUPERNATANTS, AND URINE MEASUREMENTS

1.	Pipette **500 µl** of distilled or deionized water into each standard (STD) and control (CTRL) vial. The exact concentration is printed on the label of each vial.
2.	Leave at room temperature (18-26°C) for 15 min. Vortex gently.

Reconstituted STDs and CTRLs are stable at -25°C or lower until the expiry date stated on the label. STDs and CTRLs are stable for up to five freeze-thaw cycles.

The standards and controls provided in the kit are suitable for all sample types.

However, for measurement of IL-6 in cell culture supernatants, the STDs and CTRLs can also be resuspended in cell culture medium (containing additives) to improve matrix comparison.

ASSAY PROTOCOL

Read the entire instructions for use before beginning the assay.

All reagents and samples must be at room temperature (18-26°C) before use in the assay.

Mix samples gently to ensure the samples are homogenous. We recommend performing duplicate measurements for all samples, standards and controls.

Mark position for STD/CTRL/SAMPLE (standard/control/sample) on the plate layout sheet.

Take microtiter strips out of the aluminum bag. Store unused strips with desiccant at 4°C (2-8°C) in the aluminum bag. Strips are stable until the expiry date stated on the label.

1.	Add **100 µl** of STD/SAMPLE/CTRL (Standard/Sample/Control) in duplicate into respective well. Swirl gently.
2.	Cover tightly and incubate for **2 hours** at room temperature (18-26°C).
3.	Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
4.	Add **100 µl** AB (biotinylated anti-IL-6 antibody, **green** cap) into each well. Swirl gently.
5.	Cover tightly and incubate for **1 hour** at room temperature (18-26°C).
6.	Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
7.	Add **100 µl** CONJ (Conjugate, **brown** cap) into each well. Swirl gently.
8.	Cover tightly and incubate for **1 hour** at room temperature (18-26°C).
9.	Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
10.	Add **100 µl** SUB (Substrate, **blue** cap) into each well. Swirl gently.
11.	Incubate for **30 min** at room temperature (18-26°C), in the dark.
12.	Add **50 µl** STOP (Stop solution, white cap) into each well. Swirl gently.
13.	Measure absorbance immediately at 450 nm with reference 630 nm, if available.

PRECAUTIONS

Do not pipette by mouth.
Do not eat, drink, smoke or apply cosmetics where reagents are used.
Refer to the Material Safety Data Sheet (MSDS) available for download at www.bmgrp.com.
All test components of human origin were tested against HIV-Ab, HCV-Ab, and HBsAg and were found negative. Nevertheless, they should be handled and disposed of as if they were infectious.
Avoid all contact with reagents by using protective gloves, clothing and eye protection.
Sulfuric acid contained in the STOP solution may cause irritations to eyes and skin. Avoid contact with skin and mucous membrane. Flush with water if contact occurs!
Liquid reagents in this assay contain ≤0.1% Proclin 950 as a preservative. Proclin 950 is not toxic in concentrations used in this kit but may cause allergic skin reactions – avoid contact with skin or eyes.

TECHNICAL HINTS

Do not mix or substitute reagents with those from other lots or sources.
Do not mix stoppers and caps from different reagents or use reagents between lots.
Do not use reagents beyond the expiration date.
Protect reagents from direct sunlight.
Substrate solution should remain colorless until added to the plate.
Properly seal plates with the self-adhesive films during incubation steps to ensure accurate results.
Avoid foaming when mixing reagents.

CALCULATION OF RESULTS

Construct a standard curve from the absorbance read-outs of the standards using commercially available software capable of generating a four-parameter logistic (4-PL) fit. Alternatively, plot the standards' concentration on the x-axis against the mean absorbance for each standard on the y-axis and draw a best fit curve through the points on the graph. Curve fitting algorithms other than 4-PL have not been validated and will need to be evaluated by the user.

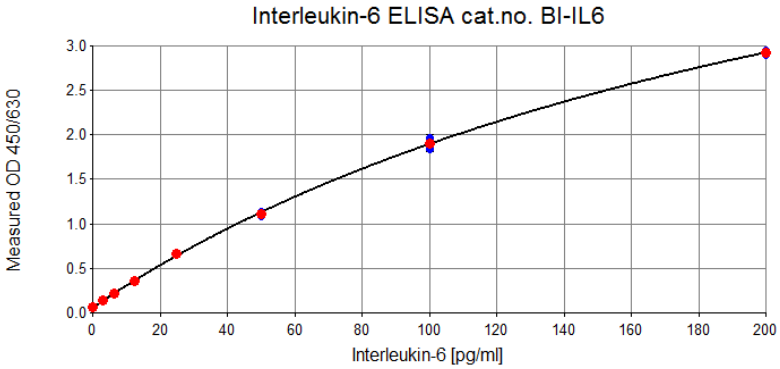
Obtain sample concentrations from the standard curve. If required, pg/ml can be converted into pmol/l by applying a conversion factor (1 pg/ml = 0.048 pmol/l, MW: 20.8 kDa).

Samples with analyte concentrations outside of the calibration range of the assay (200 pg/ml) should be diluted with assay buffer. The kit includes sufficient volume to dilute 80 samples (1+1). Additional assay buffer can be ordered separately (cat# BI-IL6-ASYBUF).

Concentrations of high-measuring samples that have been diluted during sample preparation must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve and the displayed OD values are for demonstration only. A standard curve should be generated for each assay run.



STANDARD	IL-6 pg/ml	OD			CV%
		#1	#2	AVERAGE	
STD1	0	0.056	0.059	0.058	4
STD2	3.12	0.140	0.129	0.135	6
STD3	6.25	0.216	0.207	0.212	3
STD4	12.5	0.354	0.366	0.360	2
STD5	25	0.661	0.660	0.661	0
STD6	50	1.090	1.138	1.114	3
STD7	100	1.970	1.835	1.903	5
STD8	200	2.951	2.894	2.923	1

The quality control protocol supplied with the kit shows the results of the final release QC for each kit at the production date. ODs obtained by customers may differ due to various influences including a normal decrease of signal intensity throughout shelf life. However, this does not affect the validity of the results provided an OD of 1.50 or higher is obtained for the standard with the highest concentration, and the measured control values fall into their target ranges (see labels).

ASSAY CHARACTERISTICS OVERVIEW

Method	Sandwich ELISA, HRPO/TMB, 12x8-well detachable strips			
Sample type(s)	Serum, plasma (EDTA, citrate, heparin), cell culture supernatants, urine			
Sample volume	100 µl sample / well			
Standard range	0 – 200 pg/ml (0 / 3.125 / 6.25 / 12.5 / 25 / 50 / 100 / 200)			
Sensitivity	LOD: 0.28 pg/ml; LLOQ: 0.78 pg/ml *(measurable concentrations in serum AND plasma samples)*			
Assay time	2 h / 1 h / 1 h / 30 min			
Precision		**n**	**Average % CV**	
	Within-run	3	≤7	
	In-between-run	*in progress*		
Accuracy (Spike/Recovery of recombinant human IL-6)		**n**	**Average % recovery**	
			+100 pg/ml	**+50 pg/ml**
	Serum	6	113	112
	EDTA plasma	6	111	109
	Citrate plasma	2	111	99
	Heparin plasma	2	107	103
	Cell culture	3	n.d.	97
	Urine	5	n.d.	102
Parallelism of endogenous human IL-6		**n**	**Average % of expected dilution**	
			1+1	**1+3**	**1+7**
	Serum	5	93	90	89
	EDTA plasma	5	99	96	92
	Citrate plasma	2	103	100	99
	Heparin plasma	2	104	90	89
	Cell culture	2	95	102	98
	Urine	2	96	108	108
Specificity	This assay recognizes recombinant and endogenous (natural) human IL-6.			
Use	Research use only.			
Values of apparently healthy donors		**n**	**Median /Range IL-6 (pg/ml)**	
	Serum	48	1.50 (0.30-4.36)	
	EDTA plasma	26	0.98 (0.01-2.69)	
	Citrate plasma	14	0.71 (0.01-2.10)	
	Heparin plasma	11	0.60 (0-2.41)	
	Urine	4	0.77 (0-1.5)	

n.d.: not determined

PRECISION

WITHIN-RUN PRECISION

Within-run precision was tested by measuring two samples of known concentrations three times within one IL-6 ELISA lot by one operator.

IN-BETWEEN-RUN PRECISION *in progress (i.p.)*

Within-run (n=3)	Sample 1	Sample 2	In-between-run	Sample 1	Sample 2
Mean (pg/ml)	6.5	50.5	Mean (pg/ml)	i.p.	i.p.
SD (pg/ml)	0.5	0.7	SD (pg/ml)	i.p.	i.p.
CV (%)	7	1	CV (%)	i.p.	i.p.

SENSITIVITY

LOWER LIMIT OF DETECTION (LOD) & LOWER LIMIT OF QUANTIFICATION (LLOQ)

The LOD is defined as the mean back-calculated concentration of standard 1 (0 pg/ml of IL-6, five independent measurements) plus three times the standard deviation of the measurements.

The LLOQ is defined as the lowest concentration at which an analyte can be accurately quantified. To determine the LLOQ, standard 2, i.e., the lowest standard containing human IL-6 is diluted, measured five times and its concentration back calculated.

The following values were determined for the human IL-6 ELISA:

LOD	0.28 pg/ml
LLOQ	0.78 pg/ml

CALIBRATION

The human Interleukin-6 (IL-6) ELISA kit is calibrated against a highly purified recombinant human IL-6 protein (expressed in human embryonic kidney cell, HEK-293).

The human serum based calibrator is provided in eight lyophilized glass vials in the following concentrations: 0 / 3.125 / 6.25 / 12.5 / 25 / 50 / 100 / 200 pg/ml.

CALIBRATION using WHO standard

The WHO reference reagent IL-6/NIBSC code 89/548 (recombinant DNA, human sequence) was analysed in this human IL-6 ELISA kit.

The equation below can be used to convert the sample values obtained with this kit to approximate WHO/IL-6 /NIBSC 89/548 units:

WHO/NIBSC (89/548) reference (IU/ml) = 0.08 BI-IL6 value (pg/ml).

SAMPLE VALUES

SERUM/PLASMA

IL-6 was measured in samples from apparently healthy donors (no medical histories were available).

		IL-6 [pg/ml]			
Sample Matrix	**n**	**Mean**	**Range**	**Median**	**% Detectable**
Serum	48	1.73	0.30 – 4.36	1.50	100
EDTA plasma	26	1.01	0.01 – 2.69	0.98	100
Citrate plasma	14	1.86	0.01 – 2.10	0.71	100
Heparin plasma	11	1.52	0.00 – 2.41	0.60	91

It is recommended to establish the normal range for each laboratory.

CELL CULTURE SUPERNATANTS (CCS)

Two human breast cancer cell lines MDA-MB-231, MCF-7 and a human macrophage cell line 4TL9.R were cultured in DMEM/Ham's F12 and RPMI, respectively and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 for 48 hours. Aliquots of the cell culture supernatants were removed, centrifuged to remove particles, and assayed for levels of human IL-6.

Sample Matrix CCS	**IL-6 [pg/ml]**
CCS - MDA-MB-231	146.6
CCS - MCF-7	18.5
CCS - 4TL9.R	0.4
DMEM-F-12 (with supplements)	0.0
RPMI (with supplements)	0.0

URINE

Nine human urine samples from several donors (apparently healthy and diseased) were measured with this assay and showed IL-6 concentrations between 0 – 122.6 pg/ml.

For more information please visit our website www.bmgrp.com.

SPECIFICITY

This human IL-6 ELISA recognizes recombinant and endogenous (natural) human IL-6 and detects free circulating IL-6 as well as receptor-bound IL-6.

CROSS REACTIVITY with non-human samples

This human IL-6 ELISA kit cannot be used for the detection of IL-6 in rat, mouse, or porcine samples.

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NOTES

SYMBOLS

	Expiry date / Verfallsdatum / Date de péremption / Data di scadenza /Fecha de caducidad / Data de validade / Uiterste gebruiksdatum / Udløbsdato / Utgångsdatum / Termin Ważności / Lejárati idö / Doba exspirácie / Doba exspirace
	Consider instructions for use / Bitte Gebrauchsanweisung beachten / Consultez la notice d'utilisation / Consultare le istruzioni per l'uso / Consulte las instrucciones de utilización / Consulte as instruções de utilização / Raadpleeg de gebruiksaanwijzing / Se brugsanvisningen / Läs anvisningarna före användning / Proszę przeczytać instrukcję wykonania / Vegyük figyelembe a használati utasításban foglaltakat / Postupujte podl'a pokynov na použitie / Postupujte dle návodu k použití
	Lot-Batch Number / Charge-Chargennummer / Lot-Code du lot / Lotto-Numero di lotto / Lote-Código de lote / Lote-Código do lote / Lot-Partijnummer / Lot-Batchkode / Lot-Satskod / Numer serii / Lot-Batch szám / Číslo šarže / Číslo šarže
	Manufactured by / Hergestellt von / Fabriqué par / Prodotto da / Fabricado por / Fabricado por / Vervaardigd door / Fabrikation af / Tillverkad av / Wyprodukowane pr / Gyártotta / Vyrobené / Vyrobeno
	Catalogue Number / Bestellnummer / Numéro de référence / Numero di riferimento / Número de referencia / Número de referência / Referentienummer / Referencenummer / Katalognummer / Numer katalogowy / Katalógusszám / Katalógové číslo / Katalogové číslo
	Store at between / Lagerung bei zwischen / Conserver à entre / Conservare a tra / Conservar a temp. entre / Armazene a entre / Bewaar bij tussen / Opbevares mellem / Förvaras vid / Przechowywać w / Tároljuk ..... között / Skladujte v rozsahu / Skladujte v rozmezí
	Contains sufficient for x tests / Inhalt ausreichend für x Tests / Contient suffisant pour x tests / Contenuto sufficiente per x test / Contiene suficiente para x pruebas / Contém suficiente para x testes / Bevat voldoende voor x bepalingen / Indeholder tilstrækkeligt til x prøver / Innehållet räcker till x analyser / Zawartość na x testów / Tartalma X teszt elvégzésére elegendö / Obsahuje materiál pre x testov / Obsahuje materiál pro x testu

ASSAY PROTOCOL & CHECKLIST - FOR ALL SAMPLE TYPES

Human IL-6 ELISA – Cat.No.: BI-IL6

REAGENT PREPARATION

	Read the entire instruction for use before beginning the assay.
☐	Bring all reagents to room temperature (18-26°C).
☐	Prepare reagents and samples as instructed.
☐	Bring unused and prepared components to the storage temperature mentioned in the package insert.
☐	Take microtiter strips out of the aluminum bag and mark STD, CTRL, and SAMPLE positions on the plate layout sheet.

ASSAY PROCEDURE

☐	1. Add **100 µl of STD/SAMPLE/CTRL** (Standard/Sample/Control) in duplicate into respective well. Swirl gently.
☐	2. Cover tightly and **incubate for 2 hours** at room temperature (18-26°C).
☐	3. Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
☐	4. Add **100 µl** AB (biotinylated anti-IL-6 antibody, **green** cap) into each well. Swirl gently.
☐	5. Cover tightly and **incubate for 1 hour** at room temperature (18-26°C).
☐	6. Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
☐	7. Add **100 µl** CONJ (Conjugate, **brown** cap) into each well. Swirl gently.
☐	8. Cover tightly and **incubate for 1 hour** at room temperature (18-26°C).
☐	9. Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer, transparent cap). After final wash, remove remaining WASHBUF by strongly tapping plate against paper towel.
☐	10. Add **100 µl** SUB (Substrate, **blue** cap) into each well. Swirl gently.
☐	11. **Incubate for 30 min** at room temperature (18-26°C), **in the dark.**
☐	12. Add **50 µl** STOP (Stop solution, white cap) into each well. Swirl gently.
☐	13. Measure absorbance immediately at 450 nm with reference 630 nm, if available.