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Human IL6 20,000 tests

www.htrf.com

HTRF® package insert

Document reference : 62IL6PEC rev03 (July 2008)

For in vitro research use only
Storage temperature : 2-8°C

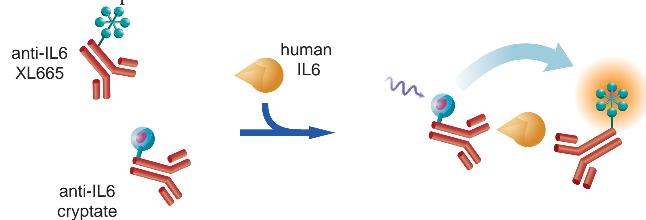
Packaging details :

	384 low-volume plate (20 µL)
62IL6PEC	20,000 tests

1. Assay description and intended use

This kit is intended for the quantitative determination of human Interleukin-6 in cell culture supernatant.

The principle of this kit is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). As shown alongside, IL6 is detected by anti-IL6 MAb labeled with XL665, the second MAb being labeled with Cryptate. These mouse MAbs recognize distinct epitopes of human IL6. They recognize both the natural and recombinant human IL6 forms. They do not cross react with other cytokines such as IL1β, TNFα, IFNγ, IL2 and IL12.



Specific signal (i.e. energy transfer) is proportional to the concentration of IL6 in the sample or standard.

The human IL6 assay can be run under two different protocols:

1. The "supernatant" protocol (see §3), a standard IL6 assessment in cell supernatant : i) cells are stimulated in a regular cell culture plate, and ii) cell supernatant is then transferred to the assay plate.
2. The "cell-based" protocol (see §4) is carried out in a single plate and allows the quantification of IL6 directly on stimulated cells, without any transfer steps.

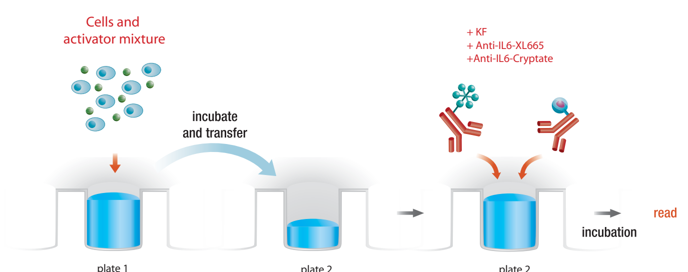
CAUTION ! Reagent reconstitution differs between the two protocols. Make sure to reconstitute the conjugates according to the chosen protocol's specifications.

2. Background

IL6 is a prototypic pleiotropic cytokine produced by a variety of cells, and acts on a wide range of tissues far beyond the immune system. It is involved in the regulation of the immune response, hematopoiesis, acute phase reaction and also plays a central role in the host defense mechanism. IL6 is a single chain protein of MW 21 to 28 kDa, depending on the cellular source. This heterogeneity is due to post-translational modifications such as N and O glycosylations. Human IL6 cDNA predicts a precursor of 212 residues, whereas the secreted form is composed of 184 amino acids after cleavage of the single peptide. IL6 is usually not produced constitutively but is induced by other cytokines such as IL1 and TNFα. Elevated levels of IL6 have been measured in patients with acute bacterial or viral infections, and autoimmune and neoplastic diseases.

3. Supernatant assay protocol

The supernatant assay protocol must be run in two distinct microplates : i) a culture plate for cell stimulation and ii) an assay plate in which cytokine detection is carried out.



3.1. Supplied reagents and reconstitution

Allow the reagents to come to room temperature for at least 30 minutes before reconstitution.

Supplied reagents	Reagent reconstitution (stock solutions)*		Working solutions
Anti-IL6-Cryptate** 1 vial Lyophilized*	Reconstitute each vial with 5 mL of distilled water. Mix gently.	⇨	For each vial dilute 1 volume of reconstituted reagent in 19 volumes of reconstitution buffer (e.g. for 10,000 tests: 2.5 mL of reconstituted reagent + 47.5 mL of reconstitution buffer).
Anti-IL6-XL665 1 vial Lyophilized*			
IL6 calibrator. Concentrated recombinant IL6. 1 vial Lyophilized*	See label indications for reconstitution volume and concentration. Mix gently after reconstitution	⇨	See calibration curve preparation for further dilution
IL6 control. Concentrated recombinant IL6. 1 vial Lyophilized*		⇨	To be used directly after reconstitution

Reagent
Reconstitution buffer (200 mL) 50 mM Phosphate buffer, pH 7.0, 0.8M KF, 0.2 % BSA
Diluent (20 mL) 50 mM Phosphate buffer, pH 7.0, 0.2 % BSA, 0.02 % NaN_3, preservatives

Note : Supplementary IL6 maximum calibrator (ref 62IL6CDA) and diluent (ref 62DL1DDD) can be obtained separately on request.

* All Lyophilized reagents must be reconstitued with distilled water
All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing BSA protease free and stabilizers.
** The Cryptate conjugate concentration was optimized for a maximum assay sensitivity and to ensure an average counting of 40,000 cps at 620 nm (384-well low volume format), using the reference PHERAstar Plus reader (BMG LABTECH).

Precaution : HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the XL665 and Cryptate-conjugates will impair the assay's quality.

3.2. Reagent storage and stability

All reagents should be stored at 2-8°C until reconstituted. Under proper storage conditions, they are stable until the expiry date indicated on the labels.

Reconstituted reagents are stable for four days at 4°C. They can be refrozen (at -80°C) and thawed at least one more time.

3.3. Calibration curve preparation

Reconstitute the maximum calibrator according to the indications printed on the label and follow the dilution sequence shown in the following table to draw up the calibration curve. Dilution must be carried out with the diluent (or with freshly made PO_4 50 mM, BSA 0.1% pH7).

Calibrator	Calibrator concentration in pg/mL	Preparation
Cal 6	2500	120 µL reconstituted calibrator + 480 µL diluent
Cal 5	1250	300 µL Cal 6 + 300 µL diluent
Cal 4	625	300 µL Cal 5 + 300 µL diluent
Cal 3	312.5	300 µL Cal 4 + 300 µL diluent
Cal 2	156.2	300 µL Cal 3 + 300 µL diluent
Cal 1	78.12	300 µL Cal 2 + 300 µL diluent
Cal 0	0	300 µL diluent

3.4. Sample preparation

Dilute all samples to be assayed with the diluent (or with freshly made PO4 50 mM, BSA 0.1% pH7). Consecutive dilutions should be made within the 0-2500 pg/mL range (working solution).

3.5. Assay protocol for 384-well low volume plate (20 µL)

Dispense the reagents in the following order :

- 10 µL standard or sample *
- 5 µL anti-IL6 XL665
- 5 µL anti-IL6 Cryptate

* For negative control, replace the first reagent by 10 µL of diluent.

- Cover the plate with a plate sealer and incubate at room temperature for 3 hours.
- Remove the plate sealer and read on a compatible HTRF® reader (more information about compatible reader at htrf-assays.com/readers).

3.6. Data reduction and example of a calibration curve

This data should not be substituted for that obtained in the laboratory

	A (665nm)	B (620nm)	Ratio (1)	Mean Ratio (2)	CV % (3)	Delta F % (4)
Negative control	1626 1644	42218 42977	385 383	384	0.5	
[calibrator] pg/mL						
78.12	1890 1846	40922 39713	462 465	463	0.5	21
156.25	2184 2206	41928 41650	521 530	525	1.2	37
312.5	2736 2848	41750 40756	655 699	677	4.5	76
625	4129 4152	43164 42439	957 978	967	1.6	152
1250	6888 6625	44272 42689	1556 1552	1554	0.2	305
2500	11436 11338	40557 42963	2820 2639	2729	4.7	611
Control	5328 5983	40129 44242	1328 1352	1340	1.3	249

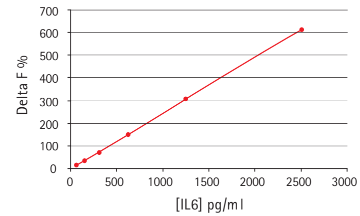
1. $\text{Ratio} = \frac{A_{665nm}}{B_{620nm}} \times 10^4$

2. $\text{Mean Ratio} = \frac{\Sigma \text{ratios}}{2}$

3. $\text{CV} = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$

4. $\text{Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} \times 100$

(Ratio_{neg} = negative control)



3.7. Analytical characteristics

Detection limit (dose of mean zero + 2 SD)	Hook effect	Linear range
< 20 pg/mL	> 100,000 pg/mL	up to 12,500 pg/mL

This immunoassay is calibrated against the NIBSC/WHO standard IL6 89/548. To convert sample values obtained with HTRF® IL6 kit to equivalent NIBSC units, use the equation below :

NIBSC/WHO (89/548) equivalent value (U/mL) = 0.1 x IL6 value (pg/mL)

3.8. Assay flexibility and miniaturization

When used as suggested, the kit will provide sufficient reagents for 20,000 tests using a 384- well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

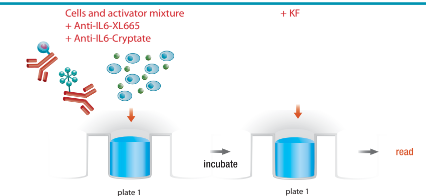
To move to other plate formats (96 half-well or 1536-well) and final volumes (100 µL to less than 10 µL), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20 µL final assay volume. For instance, in the case of the 1536-well format in 10 µL final volume, half as much material per well is used, thereby allowing 40,000 tests to be run. The performances of the HTRF® assay remain the same whatever the level of miniaturization.

Assay components	Volume proportion	Assay format		
		1536-well (10 µL)	**384-well low volume (20 µL)**	**96 half-well (100 µL)**
Sample	2 volumes	5 µL	10 µL	50 µL
XL665 conjugate	1 volume	2.5 µL	5 µL	25 µL
Cryptate conjugate	1 volume	2.5 µL	5 µL	25 µL
	Bulk size	40,000 tests	20,000 tests	4,000 tests

Plate references : 96 half-well plate (Costar # 3694 or equivalent) - 384 well low volume plate (Greiner # 784076) - 1536 well (Greiner # 782086).

4. Cell-based assay protocol

In this protocol, reagent reconstitution and distribution have been modified in order to allow the direct measurement of IL6 on stimulated cells. This protocol is particularly well adapted to primary and secondary screening.



4.1. Reagent reconstitution

Reagent		Instructions
Anti- IL6 - Cryptate Anti- IL6 - XL665	⇨	Reconstitute each vial with 5 mL of distilled water. Mix gently. Dilute 1 volume of reconstituted reagent in 7 volumes of culture medium (i.e. for 10,000 tests : 2.5mL of reconstitution reagent+17.5 mL of culture medium).
IL6 calibrator IL6 control	⇨	• **Calculate the reconstitution volume by dividing the reconstitution volume indicated on the label of the vials by 1.67** (i.e. if 500 µL is printed on the calibrator label, the reconstitution volume will be 299.4 µL). • Use culture medium NOT WATER to reconstitute both calibrator and control.
Reconstitution buffer 50 mM Phosphate buffer, pH 7.0, 0.8M KF, 0.2% BSA	⇨	Ready to use : in the cell-based assay, the reconstitution buffer is not used for conjugate reconstitution. It is added as KF buffer at the end of the incubation to allow HTRF® readout.

4.2. Reagent storage and stability

Refer to § 3.2.

4.3. Calibration curve preparation

Reconstitute the maximum calibrator with the culture medium volume indicated on the label, divided by 1.67. Follow the same dilution sequence as shown in the table in § 3.3., **replacing the diluent by culture medium**.

4.4. Sample preparation

Cell density optimization is a key step in the IL6 cell-based assay. Typically, the level of IL6 produced by cells must fall within the 100-2500 pg/mL range.

Optimization consists of testing a broad range of cell concentrations (e.g. between 100 and 20,000 cells per well) in the presence or the absence of a direct activator of IL6 production, e.g. LPS at 10 µg/mL.

In practice, resuspend the cells in the culture medium supplemented with the activator(s) or inhibitor(s) so that the desired number of cells will be dispensed under 6 µL.

4.5. Cell-based assay protocol for 384-well low volume plates (20 µL)

Dispense the reagents in the following order:

- 6 µL standard or cell suspension
- 2 µL anti-IL6 Cryptate
- 2 µL anti-IL6 XL665

* For negative control, replace the first reagent by 6 µL of culture medium.

- Incubate the plate for 18 to 24 hours at 37°C in a CO_2 incubator with a fully humidified atmosphere.
- Add 10 µL of reconstitution buffer.
- Read on a compatible HTRF® reader (more information about compatible reader at htrf-assays.com/readers).

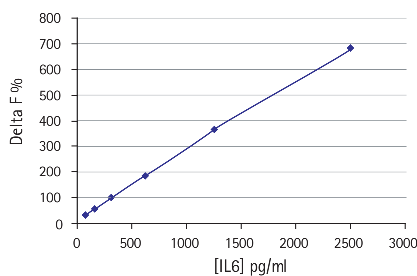
4.6. Case study : IL6 quantification in peripheral blood mononucleated cells (PBMC)

• PBMC were separated on a ficoll density gradient (d=1.077) (Sigma). Cells were washed twice and viability was evaluated by trypan blue dye exclusion. Cells were then resuspended in RPMI1640 culture medium complemented with 10% heat inactivated calf serum, and stimulated with 10 µg/mL of LPS.

• A graduated number of cells per well were distributed in 6 µL of culture medium including the LPS activator, according to the cell-based assay protocol described above.

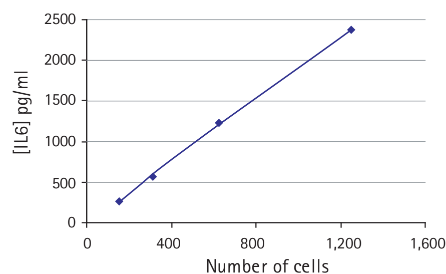
IL6 calibration curve in culture medium

[IL6] pg/mL	Delta F % (Overnight incubation, readout after KF addition)
78.1	30
156.2	55
312.5	101
625	185
1250	366
2500	684



IL6 quantification in PBMC

Number of cells / well	[IL6] pg/mL
1,250	2374.3
625	1231.7
300	561.2
150	260.5



The data shows that the co-incubation of the stimulated cells with the labeled antibody pair does not alter HTRF® readout or assay performances. IL6 quantification is proportional to the number of cells per well. In this example, the cell density selected for a secondary screen to determine inhibitors of IL6 production would be 1,250 cells/well. In all instances, cell density should be carefully optimized, as the secretion of IL6 may vary between cell types.

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