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## HTRF® package insert

Document reference : 62IL8PEC rev09 (September 2009)

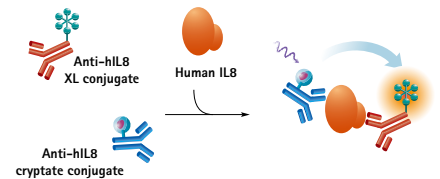
For in vitro research use only  
 Reagent storage temperature except conjugates : 2-8°C  
 Conjugate storage temperature : -80°C  
 Packaging details :

62IL8PEC	384-well low volume plate (20 µL)
	20,000 tests

## 1. Assay description and intended use

This assay is intended to be used for the quantitative determination of IL-8 in buffered solution or cell-culture supernatants.

Anti-cytokine antibodies are respectively labeled with europium Cryptate and XL665. With their binding to IL-8 molecules, the two antibodies come into close proximity, allowing FRET to occur between the europium Cryptate and the allophycocyanin. This FRET increases proportionally to IL-8 concentrations.



## 2. Background

Interleukin-8 (IL-8) belongs to the CXC family type of chemokines. It is a non-glycosylated protein of 8 kDa found in at least under four variants of 79, 77, 72, and 69 amino acids which differ in the length of their N-terminal. They all originate from the proteases digestion of a precursor of 99 residues (1, 2, 3, 4). IL-8 is found in solution either as a monomer or a dimer. The two disulphide bonds (Cys-7/Cys-34 ; Cys-9/Cys-50) keep the two amino-terminal regions together which is essential for the biological activity of the molecule .

Exogenous stimuli such as LPS, but also IL-1β, TNFα and TNFβ induce the secretion of IL-8 by several cell types including monocytes, endothelial and epithelial cells, dermal fibroblasts, keratinocytes, neutrophils, hepatocytes, synovial cells, astrocytes and T lymphocytes.

IL-8 has two receptors CXCR1 and CXCR2, both members of the GPCR family. Receptor density ranges from 300/cell on T cells and goes up to 20000/cell on neutrophils .

IL-8 is chemotactic for a broad range of immunocompetent cells. It enhances the metabolism of reactive oxygen species, the release of enzymes from granules, and increases the expression of adhesion molecules .

IL-8 may be of clinical relevance in psoriasis, as elevated concentrations are observed in psoriatic scales. IL-8 is also a marker of several inflammatory processes and may participate in the pathogenesis of rheumatoid arthritis, since excessive amounts of this factor are found in synovial fluids. The activation of neutrophils may enhance the migration of cells into the capillaries of the joints .

## 3. Protocol

### 3.1. Supplied reagents, reconstitution and dilution

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

Supplied reagents	Reagent reconstitution (stock solutions)	Working solutions
Anti- IL8 - Cryptate Conjugate**	1 vial of 1 mL (frozen)	⇒ Take 1 mL of stock solution and add it to 99 mL of reconstitution buffer supplied. Mix gently.
Anti- IL8- XL665 Conjugate	1 vial of 1 mL (frozen)	⇒ Take 1 mL of stock solution and add it to 99 mL of reconstitution buffer supplied. Mix gently.
IL8 calibrator	2 vials lyophilized*	⇒ See label indications for reconstitution volume. Mix gently after reconstitution.
Reconstitution buffer (50 mM PO4- 0.8 M KF - 0.2% BSA pH7.0)	1 vial of 200 mL	
Diluent (50 mM PO4- 0.2% BSA pH7.0 - preservatives-NaN3)	1 vial of 20 mL	

\* All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing protease free BSA 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

Lyophilized calibrator should be stored at 2-8°C until reconstituted. IL8 is not stable once reconstituted. Always use fresh made IL8 solutions and discard unused reagent. Two vials of maximum calibrator are supplied with the kit. Extra vials can be ordered separately (réf 62IL8CDA).

Frozen conjugates should be stored at -80°C until use. Once thawed and diluted, unused conjugates are stable one week at 4°C. They can be refrozen (-80°C) and thawed once.

### 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. For greater relevance of results, dilution must be carried out with the medium used for samples preparation (i.e. **culture medium**, diluent or with freshly made PO4 50 mM, BSA 0.2% pH7...)

Calibrator	Working concentration in pg/mL	Preparation
Cal 7 (max calibrator)	2000	200 µL reconstituted calibrator + 800 µL diluent
Cal 6	1000	500 µL Cal 7 + 500 µL diluent
Cal 5	500	500 µL Cal 6 + 500 µL diluent
Cal 4	200	400 µL Cal 5 + 600 µL diluent
Cal 3	100	500 µL Cal 4 + 500 µL diluent
Cal 2	50	500 µL Cal 3 + 500 µL diluent
Cal 1	20	400 µL Cal 2 + 600 µL diluent

### 3.4. Sample preparation

If necessary, dilute all samples to be assayed with the diluent or the more appropriate buffer i.e. culture medium, buffered solution.

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

Dispense the reagents in the following order :

- 10 µL sample or calibrator\*
- 5 µL anti-IL8-XL conjugate
- 5 µL anti-IL8-Cryptate conjugate

\* For negative control, replace the calibrator by 10 µL of diluent.

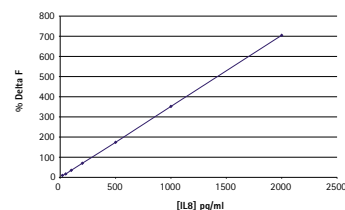
- Cover the plate with a plate sealer and incubate at room temperature for 2 hours.
- Remove the plate sealer and read on a compatible HTRF® reader (more information about compatible reader at [htrf.com/readers](http://htrf.com/readers)).

### 3.6. Data reduction and example of a calibration curve

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

[IL-8] pg/mL initial	A (665nm)	B (620nm)	Ratio (1)	Mean Ratio (2)	CV % (3)	Delta F % (4)
0	1318	35567	371	374	1.2	
	1358	36031	377			
20	1394	35221	396	397	0.3	6
	1738	43727	397			
50	1578	34603	456	456	0.0	22
	1503	32964	456			
100	1706	33509	509	516	1.9	38
	1729	33070	523			
200	2220	31276	710	675	7.3	81
	2190	34228	640			
500	3878	35271	1099	1087	1.6	191
	3629	33782	1074			
1000	6041	33998	1777	1750	2.2	368
	6119	35515	1723			
2000	10366	34631	2993	2986	0.4	699
	10173	34162	2978			

- Ratio =  $\frac{A_{665nm}}{B_{620nm}} \times 10^4$
- Mean Ratio =  $\frac{\sum \text{ratios}}{2}$
- CV =  $\frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$
- Delta F =  $\frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} \times 100$   
(Ratio<sub>neg</sub> = negative control)



Delta F obtained for samples can be reported on the calibration curve to deduce respective IL-8 concentration.

### 3.7. Kit performances and specificity

No cross reactions were observed with IL-1β, IL-2, IL-6, IL-10, IL-12, TNFα, IFNγ tested at concentrations up to 100 ng/mL.

The sensitivity threshold of the assay is 15 pg/mL IL-8.

## 4. Cell-based assay protocol

In this protocol, reagent reconstitution and distribution have been modified in order to allow the direct measurement of IL-8 on stimulated cells. This protocol is particularly well adapted to secondary screening.

### 4.1. Reagent reconstitution and dilution

Supplied reagents	Reagent reconstitution (stock solutions)	Reagents preparation
Anti-IL-8 - Cryptate conjugate	1 vial of 1 mL (frozen)	⇒ Take 1 mL of stock solution and add it to 39 mL of <b>culture medium</b> . Mix gently.
Anti-IL-8- XL665 conjugate	1 vial of 1 mL (frozen)	⇒ Take 1 mL of stock solution and add it to 39 mL of <b>culture medium</b> . Mix gently.
IL-8 calibrator	2 vials lyophilized*	⇒ <b>Calculate the reconstitution volume for by dividing the reconstitution volume indicated on the labels of the vials by 1.67</b> (i.e. if 500 µL is printed on the calibrator label, the reconstitution volume will be 299.4 µL). Mix gently. See calibration curve preparation below.

\* All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing protease free BSA and stabilizers.

### 4.2. Reagent storage and stability

Refer to § 3.2.

### 4.3. Calibration curve preparation

Reconstitute the maximum calibrator as indicated above with culture medium. 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 IL-8 cell-based assay. Typically, the level of IL-8 produced by cells must fall within the 30-1000 pg/mL range.

Optimization consists of testing a broad range of cell concentrations (e.g. between 200 and 25,000 cells per well) in the presence or the absence of a direct activator of IL-8 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 final)

Dispense the reagents in the following order:

- 6 µL standard or cell suspension\*
- 2 µL anti-IL-8 Cryptate
- 2 µL anti-IL-8 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<sub>2</sub> incubator with a fully humidified atmosphere.
- Add 10 µL of reconstitution buffer to all wells.
- Read on a compatible HTRF® reader (more information about compatible reader at [htrf.com/readers](http://htrf.com/readers)).

### 4.6. Reagents storage

Conjugates should be stored at -80°C until used.

Reconstitution buffer, diluent and calibrator should be stored at 2-8°C.