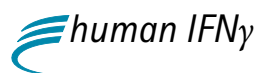


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For in vitro research use only
Storage temperature : 2-8°C

Packaging details :

| | |
|----------|-----------------------------------|
| 62IFNPEB | 384-well low volume plate (20 µL) |
| | 1,000 tests |

www.htrf.com

HTRF® package insert

Document reference : 62IFNPEB rev07 (Sept. 2011)

1. Assay description and intended use

This kit is intended for the quantitative determination of human Interferon gamma.

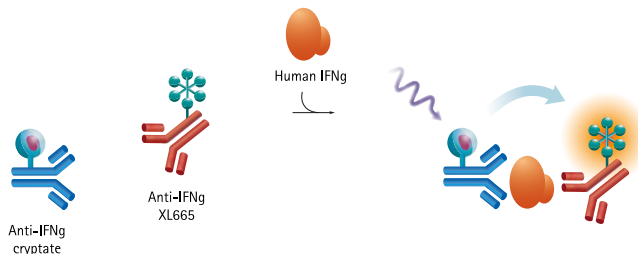
Its principle is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). As shown below, the IFN γ is detected by an anti-IFN γ MAb labeled with XL665, the second MAb is labeled with Cryptate. These mouse MAbs recognize distinct epitopes of human IFN γ . They do not crossreact with IFN γ from other species.

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

The human IFN γ assay can be run under two different protocols:

1. The “supernatant” protocol (see §3), a standard IFN γ 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 IFN γ 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.

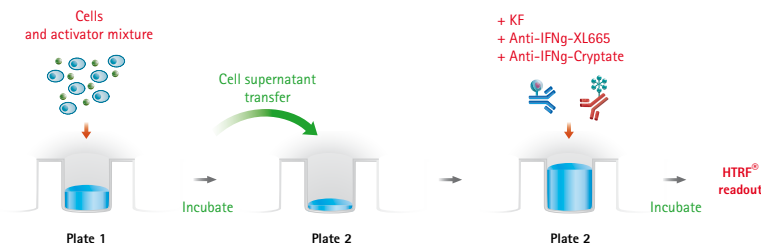


2. Background

IFN γ , also called type II interferon, is a homodimeric glycoprotein of 21-24 kDa. In contrast to type I interferons which are produced by various types of cells, IFN γ production is restricted to T and NK cells. IFN γ synthesis occurs in the cytokine cascade upon T cell signaling by IL-12 and IL-18 from macrophages. During an infection course, IFN γ is secreted by cytotoxic CD8 T cells as well as by a subset of helper T cells, namely Th1 cells. Th1 cells produce IL-2, IFN γ , and TNF β but no IL-4 or IL-10, whereas Th2 cells produce IL-4, IL-5 and IL-10 but little or no IFN γ . IFN γ orientates the immune response towards the cellular inflammatory pathway by enhancing monocytes/macrophages functions such as endocytosis/phagocytosis, inflammatory cytokines production (TNF α , IL-1 β) and by increasing HLA molecules expression. IFN γ also acts on B cells by influencing immunoglobulin heavy chain switching, B cells activation and synergistically with IL-2, promotes B cells proliferation. Finally IFN γ induces T cells differentiation and enhances NK activities. Despite IFN γ displays no molecular homology with type I interferons, it shares anti-viral and anti-proliferative properties.

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 warm up at room temperature for at least 30 mins before reconstitution.

| Supplied reagents | Reagent reconstitution (Stock solutions) | Working solutions |
|--|--|---|
| Anti-IFN γ - Cryptate** | 1 vial Lyophilized* | Add 5 mL of reconstitution buffer to each vial. Mix gently. |
| Anti-IFN γ - XL665 | 1 vial Lyophilized* | |
| IFN γ calibrator. Concentrated recombinant IFN γ . After reconstitution, IFN γ concentration is 6000 pg/mL | 2 vials Lyophilized* | See label indications for reconstitution volume. Mix gently after reconstitution. |
| IFN γ control. Concentrated recombinant IFN γ . See label indications for concentration after reconstitution. | 1 vial Lyophilized* | |
| Reconstitution buffer 50 mM Phosphate buffer, pH 7.0, 0.8M KF | 1 vial See volume on the label | |
| Diluent 50 mM Phosphate buffer, pH 7.0, 0.2 % BSA, 0.02 % NaN ₃ , preservatives | 1 vial of 20 mL | |

* 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 50,000 cps at 620 nm (384-well low volume format), using the reference 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.

The two conjugates are stable for one week at 4°C and can be refrozen (at -80°C) and thawed one more time. IFN γ is not stable once reconstituted. Always use fresh made IFN γ solutions and discard unused reagent. Two vials are supplied with the kit. Extra vials can be ordered separately (ref.: 62IFNCDA).

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 constitute the calibration curve. Dilution must be carried out with the diluent (or with freshly made PO₄ 50 mM, BSA 0.1% pH7).

| Calibrator | Preparation | Calibrator concentration in pg/mL |
|------------|---|-----------------------------------|
| Cal 7 | 150 μ L max. calibrator + 450 μ L diluent | 1500 |
| Cal 6 | 300 μ L Cal 7 + 300 μ L diluent | 750 |
| Cal 5 | 300 μ L Cal 6 + 300 μ L diluent | 375 |
| Cal 4 | 300 μ L Cal 5 + 300 μ L diluent | 187,5 |
| Cal 3 | 300 μ L Cal 4 + 300 μ L diluent | 93,75 |
| Cal 2 | 300 μ L Cal 3 + 300 μ L diluent | 46,87 |
| Cal 1 | 300 μ L Cal 2 + 300 μ L diluent | 23,4 |
| Cal 0 | 300 μ L diluent | 0 |

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 to 1500 pg/mL range (working solution).

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

- Dispense the reagents in the following order :
- 10 μ L standard or sample *
- 5 μ L anti-IFN γ Cryptate
- 5 μ L anti-IFN γ XL665

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

- Cover the plate with a plate sealer and incubate at room temperature for 18 - 20 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 | 2081 1939 | 42102 42075 | 494 461 | 478 | 4.9% | |
| [IFN γ] pg/mL | | | | | | |
| 23.4 | 2320 2406 | 42875 43671 | 541 551 | 546 | 1.3% | 14 |
| 46.9 | 2589 2729 | 42354 43645 | 611 625 | 618 | 1.6% | 29 |
| 93.8 | 3479 3454 | 44841 43898 | 776 787 | 781 | 1.0% | 64 |
| 187.5 | 4749 4719 | 43099 43837 | 1102 1077 | 1089 | 1.6% | 128 |
| 375 | 7022 6649 | 42486 41762 | 1653 1592 | 1622 | 2.6% | 240 |
| 750 | 11580 12210 | 42401 42746 | 2731 2856 | 2794 | 3.2% | 485 |
| 1500 | 20904 20934 | 42094 42945 | 4966 4875 | 4920 | 1.3% | 930 |
| IFN γ control | 5601 5704 | 43889 44189 | 1276 1291 | 1284 | 0.8% | 169 |

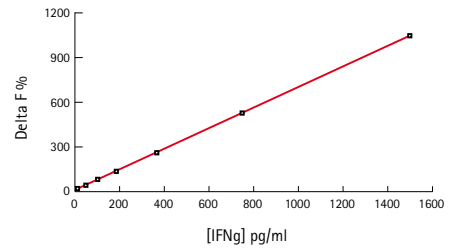
$$1. \text{ Ratio} = \frac{A_{665\text{nm}}}{B_{620\text{nm}}} \times 10^4$$

$$2. \text{ Mean Ratio} = \frac{\sum \text{Ratios}}{2}$$

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

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

(Ratio_{neg} = negative control)



3.7. Analytical characteristics

| Detection limit (dose of mean zero + 2 SD) | Hook effect | Linear range |
|--|--------------|-------------------|
| < 10 pg/mL | 90,000 pg/mL | up to 5,000 pg/mL |

This immunoassay is calibrated against the NIBSC/WHO standard IFN γ 82/587. To convert sample values obtained with HTRF® IFN γ kit to equivalent NIBSC units, use the equation below :

$$\text{NIBSC/WHO (82/587) equivalent value (U/mL)} = 0,019 \times \text{IFN}\gamma \text{ value (pg/mL)}$$

3.8 Assay flexibility and miniaturization

When used as suggested, the kit will provide sufficient reagents for 1,000 tests using using a 384- well low volume plate in 20 μ L final assay volume (HTRF® packaged basis).

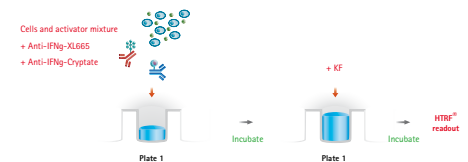
To move to other plate formats (96 half-well or 1536-well) and final volumes (100 μ L to less than 10 μ L), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20 μ L final assay volume. For instance, in the case of the 1536-well format in 10 μ L final volume, half as much material per well is used, thereby allowing 2,000 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 μ L) | 384-well low volume (20 μ L) | 96 half-well (100 μ L) |
| Sample | 2 volumes | 5 μ L | 10 μ L | 50 μ L |
| XL665 conjugate | 1 volume | 2.5 μ L | 5 μ L | 25 μ L |
| Cryptate conjugate | 1 volume | 2.5 μ L | 5 μ L | 25 μ L |
| | | 2,000 tests | 1,000 tests | 200 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 IFN γ on stimulated cells. This protocol is particularly well adapted to secondary screening.



4.1. Reagent reconstitution

| | | |
|------------------------------|---|---|
| Anti-IFN γ - Cryptate | ⇒ | Reconstitute each vial with 2 mL of culture medium . Mix gently. |
| Anti-IFN γ - XL665 | ⇒ | |
| IFN γ calibrator | ⇒ | <ul style="list-style-type: none"> • Calculate the reconstitution volume by dividing the reconstitution volume indicated on the label of the vial 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. |
| IFN γ control | ⇒ | |

Reconstitution buffer
50 mM Phosphate buffer, pH 7.0, 0.8M KF



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 IFN γ cell-based assay. Typically, the level of IFN γ produced by cells must fall within the 100-1500 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 IFN γ production, e.g. 1 ng/mL PMA and 500 ng/mL ionomycin. 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 μ L.

4.5. Cell-based assay protocol for 384-well low volume plate (20 μ L)

Dispense the reagents in the following order:

- 6 μ L standard or cell suspension
- 2 μ L anti-IFN γ Cryptate
- 2 μ L anti-IFN γ XL665

* For negative control, replace the first reagent by 6 μ L 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 μ L of reconstitution buffer.
- Read on a compatible HTRF® reader (more information about compatible reader at htrf-assays.com/readers).

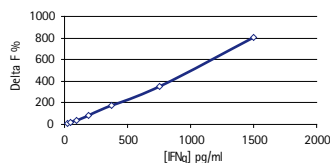
4.6. Case study : IFN γ quantification in peripheral blood mononucleated cells (PBMC)

• 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 1 ng/mL PMA and 500 ng/mL ionomycin.

• A graduated number of cells per well were distributed in 6 μ L of culture medium including PMA and ionomycin activators, according to the cell-based assay protocol described above.

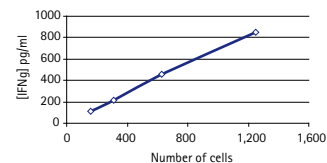
IFN γ calibration curve

| [IFN γ] pg/mL | Delta F % (Overnight incubation, readout after KF addition) |
|-----------------------|---|
| 23.4 | 12 |
| 46.87 | 21 |
| 93.75 | 41 |
| 187.5 | 83 |
| 375 | 180 |
| 750 | 352 |
| 1500 | 805 |



IFN γ quantification in PBMC

| Number of cells / well | [IFN γ] pg/mL |
|------------------------|-----------------------|
| 1,250 | 853.3 |
| 625 | 455.2 |
| 300 | 214.1 |
| 150 | 107.6 |



The data shows that the co-incubation of the stimulated cells with the labeled antibody pair does not alter HTRF® readout or assay performances. IFN γ 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 IFN γ production would be 1,250 cells/well. In all instances, cell density should be carefully optimized, as the secretion of IFN γ may vary between cell types.