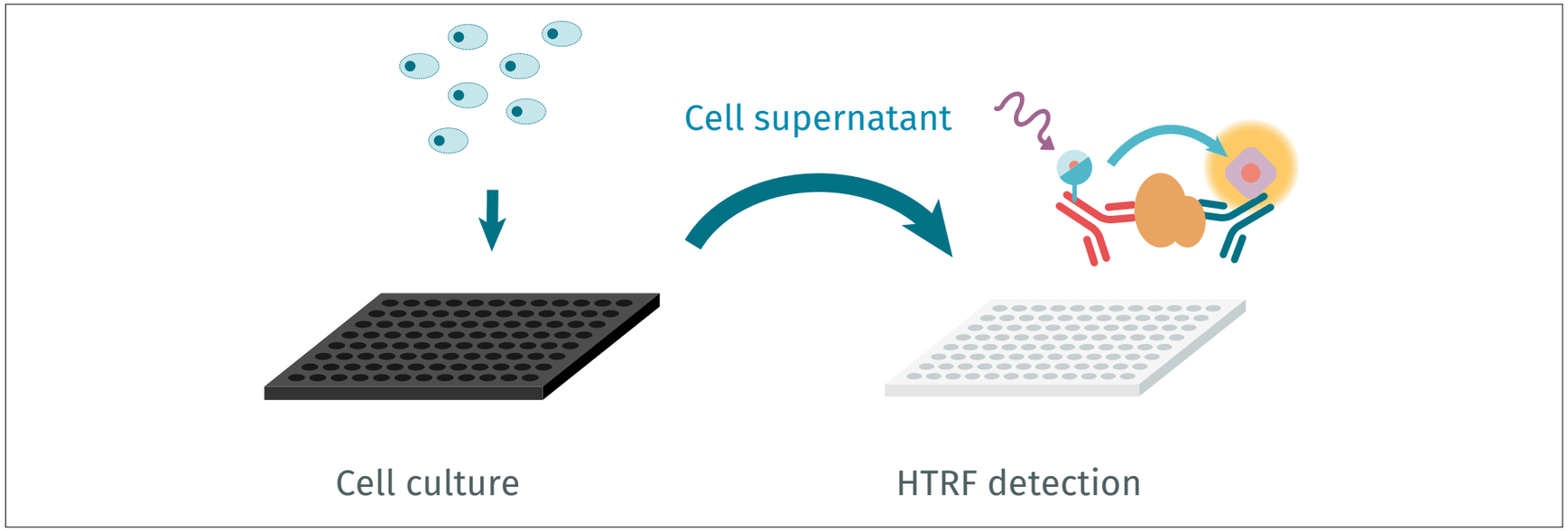
# HTRF cytokine assays

Guide to success with HTRF®

# ASSAY DESCRIPTION
# Assay principle

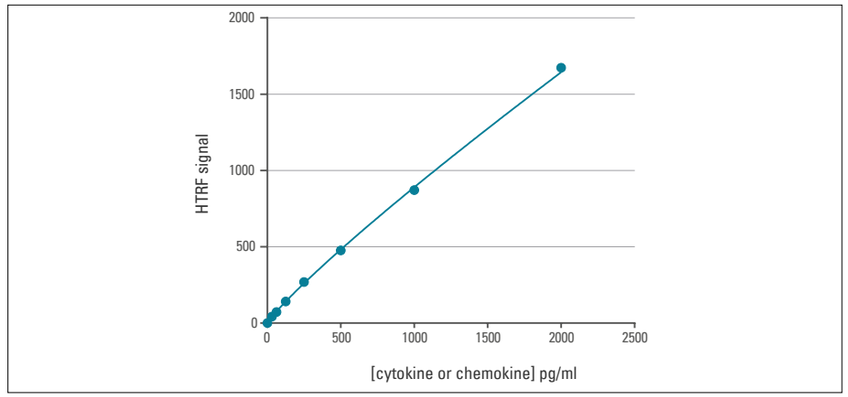



Secreted cytokines are released after cell treatment.

HTRF cytokine assays are based on sandwich immunoassays including:

- One antibody labeled with the **Donor** fluorophore
- One antibody labeled with the **Acceptor** fluorophore

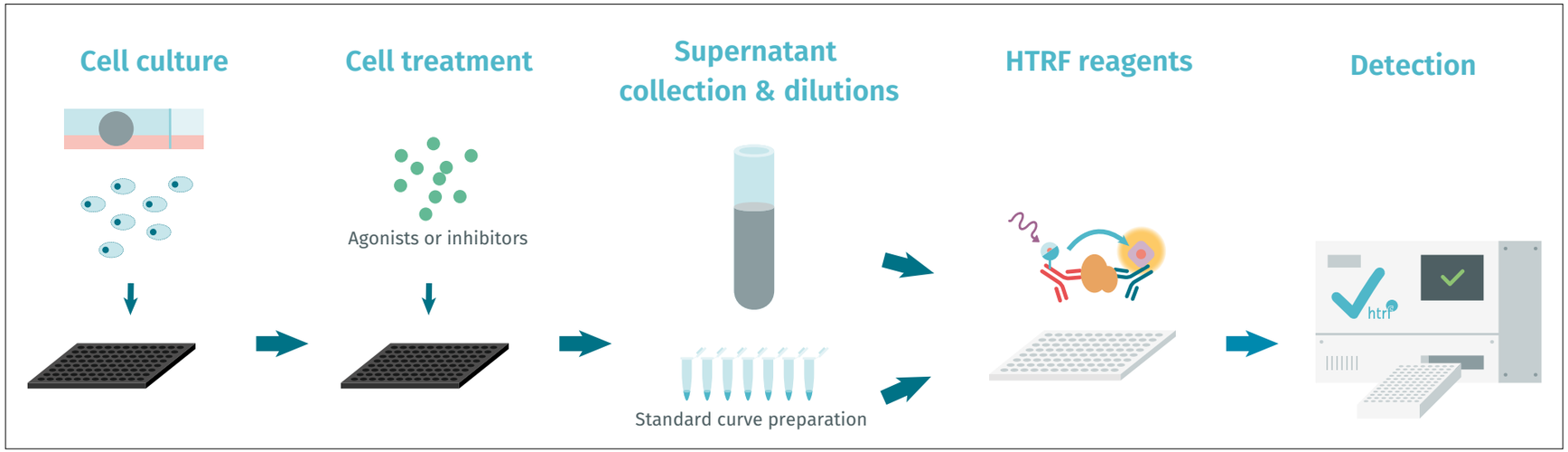
Because of the sandwich format of HTRF cytokine assays, the HTRF signal increases proportionally to the concentration of the cytokine of interest.






## TABLE OF CONTENTS

# ASSAY DESCRIPTION
# Assay workflow




## 5 MAIN STEPS COMMON TO ALL HTRF CYTOKINE ASSAY PROTOCOLS

- Cell culture: cells are usually plated in 96 well plates. Of note, other types of cell culture formats can also be used (see p12)
- Cell treatment using agonists, antagonists or any other class of modulators
- Cell supernatant collection and dilutions (if necessary) & Standard curve preparation
- HTRF reagents: addition and incubation according to the protocol provided
- HTRF detection step, after the addition of HTRF reagents, on an HTRF compatible plate reader



## TABLE OF CONTENTS

# ASSAY DESCRIPTION
# Assay reagents

The HTRF reagents provided are:

**HTRF Detection antibodies****

Donor-antibody (20X)
Acceptor-antibody (20X)

**Buffers**

Diluent
Detection buffer (ready to use)

**Standard protein**

**Package insert**

All HTRF cytokine kits are provided frozen, except for the protein standard which is lyophilized.

All HTRF cytokine assays are available in 500 and 10,000 tests. Some references are also available in 96 tests.

All reagents needed are supplied in each kit, except the detection microplates*.

Note that HTRF standards are also available separately.

* A Revvity Low Volume microplate is included in 96-test cytokine assays.

** The concentration of each detection antibody has been carefuly optimized. Changing the dilution factor will impair the performances of the assay.

Please refer to our website (www.Revvity.com/readers) for the references of commercial HTRF compatible microplates.



## TABLE OF CONTENTS

# ASSAY DESCRIPTION
# Definitions

Like any immunoassay, the performance of an HTRF cytokine assay is associated with a Limit of Detection and a Limit of Quantification. Their definitions as well as their methods of determination are explained below.

## Limit of Detection (LoD or analytical sensitivity)*

Also called “lower limit of detection”, “minimum detectable concentration”, or “sensitivity”.

The LoD corresponds to the lowest amount of analyte in a sample that can be discriminated from the background. This value refers to the analytical sensitivity of an assay and is dependent on the technology used for detection.

The LoD of HTRF cytokine assays was determined by adding two standard deviations to the mean HTRF Ratio value of twenty-four standard zero replicates and calculating the corresponding concentration.

## Limit of Quantitation (LoQ or functional sensitivity)*

The lowest amount of analyte in a sample which can be quantitatively determined with precision and accuracy.

The LoQ of HTRF cytokine assays represents the concentration of analyte corresponding to an HTRF Signal over Background equal to 1.2.

## Hook effect

The hook effect is a state of antigen excess relative to the antibody probes, resulting in falsely lowered values. If overlooked, a significantly lower value can be reported and lead to misinterpretation of results (for more information, see next page).

* Adapted from Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline EP-17A



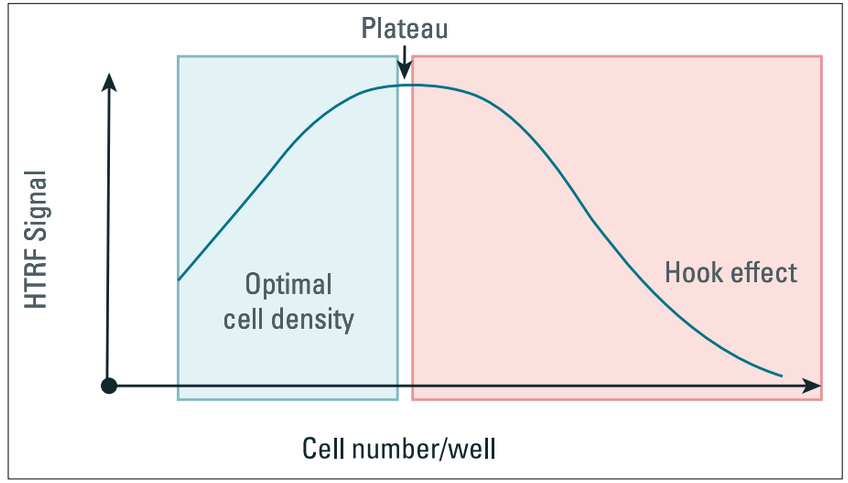
## TABLE OF CONTENTS

# ASSAY DESCRIPTION
# Hook effect

Like any sandwich immunoassay, the signal increases proportionally to the analyte being quantified until a plateau is reached. If the amount of cytokine is significantly higher than the detection reagents present in the well, the signal goes down.

This situation is known as the hook effect.

The experimental conditions (cell density, dilution of cell supernatant) must be carefully optimized for each cytokine kit, each cell-based model, or even each other parameter (compound nature, time of treatment…) in order to avoid the hook effect.



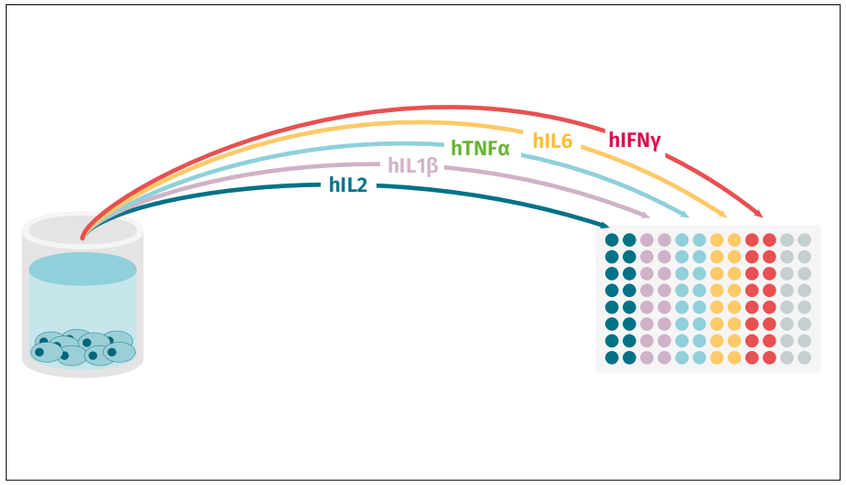

## TABLE OF CONTENTS

# ASSAY FORMAT
# Cell culture format

The standard protocol provided in the HTRF cytokine package insert describes the cell seeding step in a 96 well clear-bottom microplate.

However upscaling the cellular steps in order to generate a larger amount of biological material (e.g. cell supernatant) is possible.

This scaling up is of particular interest if multiple cytokines need to be quantified from a single supernatant (for multiparameter analysis, please refer to p29).




| CELL CULTURE MICROPLATE | | |
|---|---|---|
| **Microplate** | **Seeding cell density ($x10^3$ cells/well)** | **Culture medium (µL)** |
| 96 wells | 25-100 | 50-100 |
| 24 wells | 140-550 | 500-1000 |
| 16 wells | 290-1150 | 1000-2000 |
| 6 wells | 700-2800 | 2500-5000 |

This table provides cell density adjustment, as well as cell culture medium volume, for different types of microplates.

## TABLE OF CONTENTS

# ASSAY FORMAT
# HTRF detection format

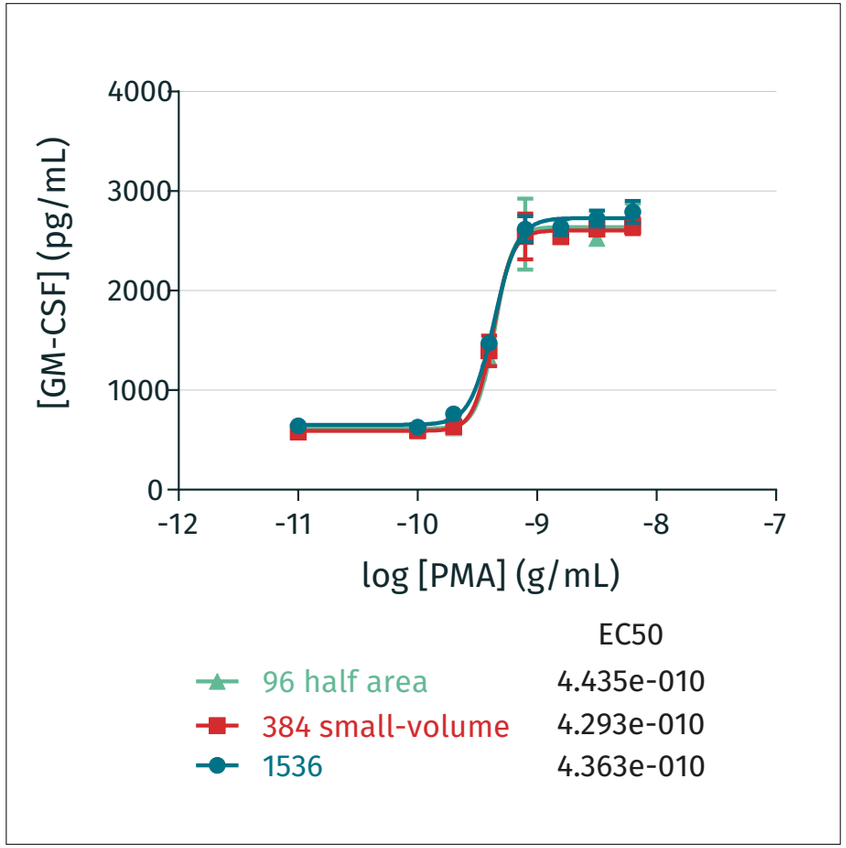
All HTRF cytokine kits are developed in a 20µL final assay volume, corresponding to a 384 low volume plate or a Revvity 96 well plate for detection.

However, the detection format can be up or downscaled to meet your throughput needs, as described in the table below.

The graph shows 3 different detection formats from the same experiment where 100,000 PBMCs were plated into a 96 well culture plate containing 200µL RPMI + 10% FCS. The PBMCs were then incubated with increasing doses of PMA + 1µg/mL Ionomycin for 16 hours at 37°C-5% $CO_2$.

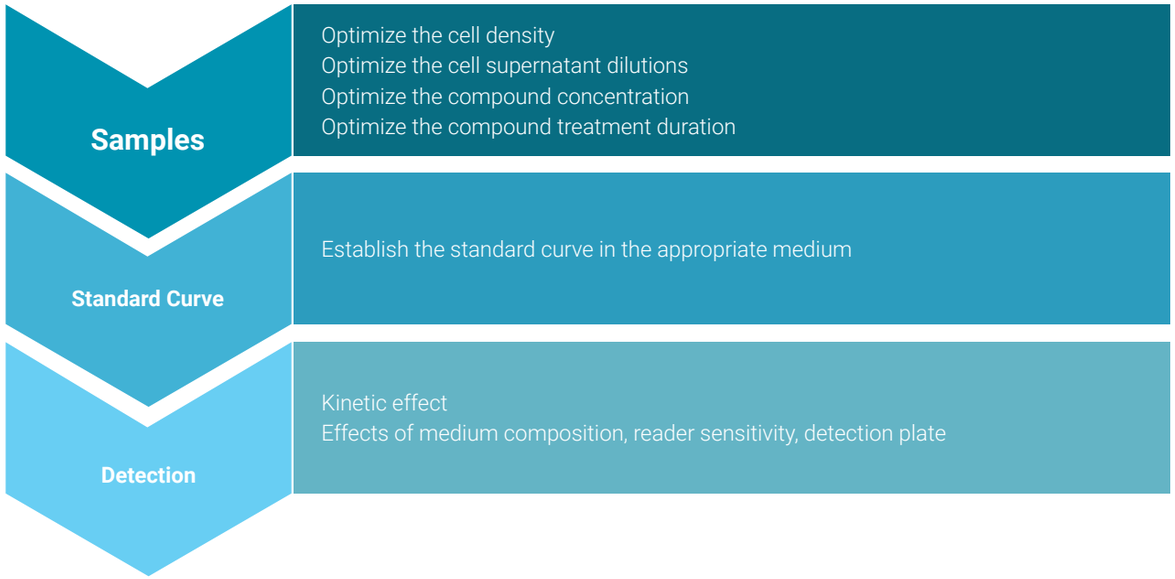
Neat supernatants were transferred into different detection plates, and HTRF human GM-CSF detection reagents were added according to the volumes indicated in the table. The HTRF signal was recorded after 3h incubation.

| Plates | Volume of samples | Volume of HTRF detection reagents |
|---|---|---|
| 96 regular | 160 µL | 40 µL |
| 96 half well | 80 µL | 20 µL |
| 384 regular | 80 µL | 20 µL |
| Revvity 96 low volume | 16 µL | 4 µL |
| 384 small volume | 16 µL | 4 µL |
| 1536 | 8 µL | 2 µL |




## TABLE OF CONTENTS

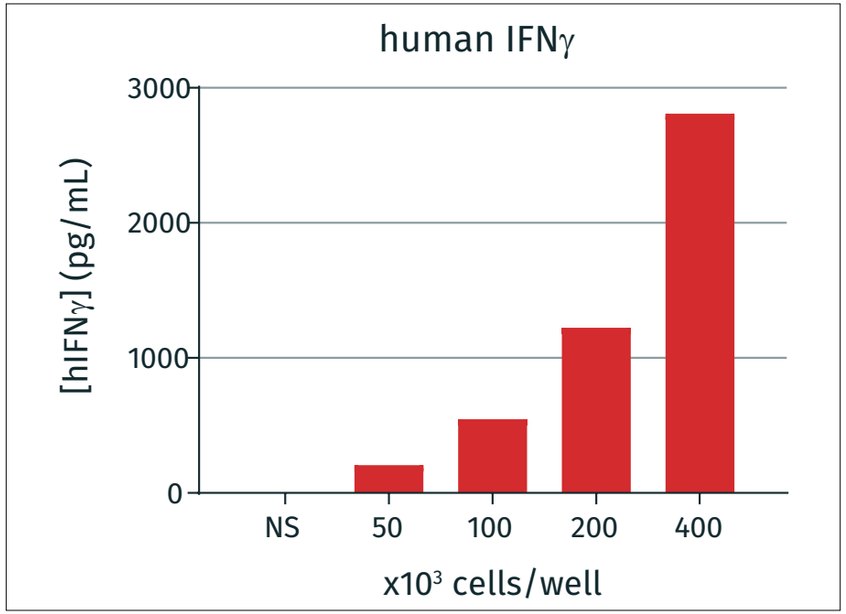
# ASSAY OPTIMIZATION
# Workflow



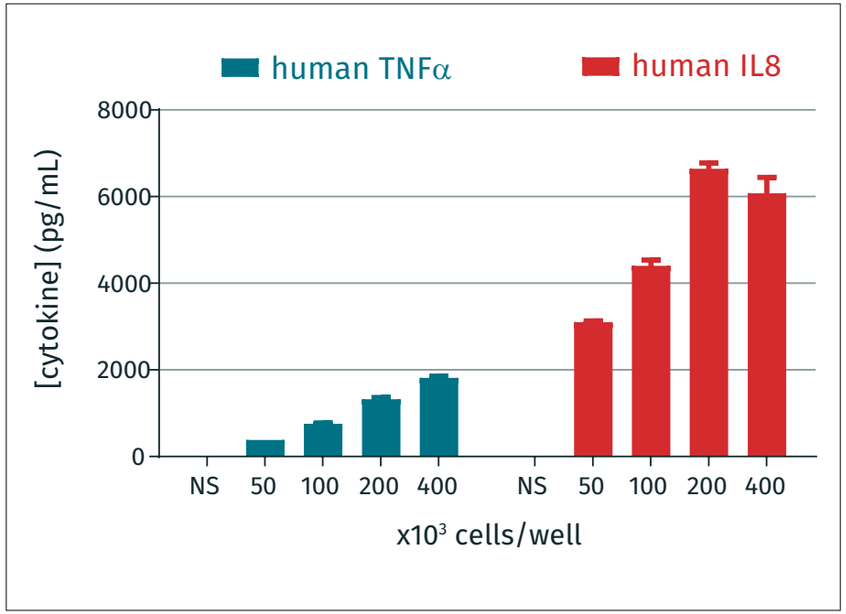

## TABLE OF CONTENTS

# ASSAY OPTIMIZATION
# Cell density optimization

The cell density is a key parameter which must be carefully optimized in order to suit the assay range of each cytokine kit, but also to respect the intrinsic biology of the cellular model.




As illustrated on hIFNγ secretion, PBMCs were seeded at different cell densities ranging from 500 up to 400,000 cells per well into a 96 well microplate, then treated or not (NS) with 50ng/mL PMA + 500ng/mL Ionomycin for 3 hours. Neat cell supernatants were collected and quantified.




For human TNFα and IL8, PBMCs were stimulated or not (NS) with LPS at 0.2 µg/mL for 3 hours.

For a cell density of 400K cells / well, samples for TNFα can be used neat, but samples for IL8 will have to be diluted to avoid the hook effect.

**Samples** → **Standard curve** → **Detection**

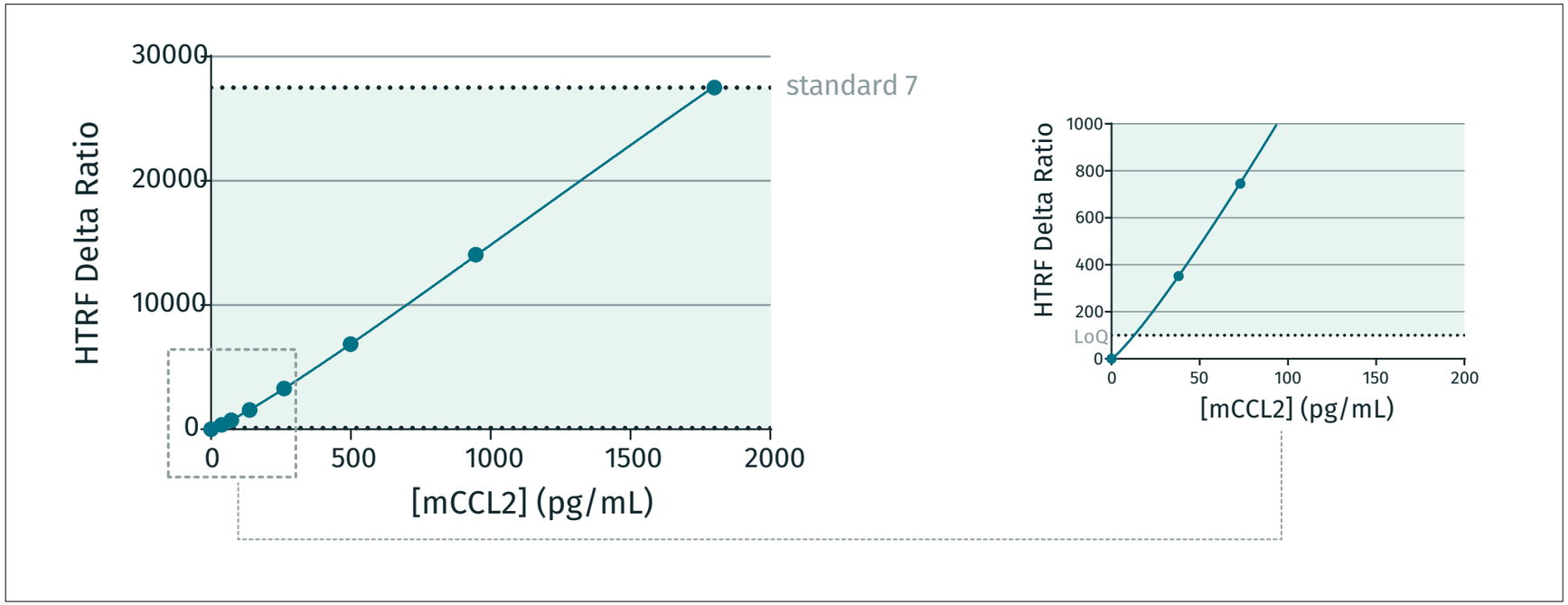


## TABLE OF CONTENTS

## ASSAY OPTIMIZATION

# Cell supernatant dilutions

Performing dilutions of cell supernatant may be a key step to ensure you work within the assay range, defined as the LoQ up to the standard 7, as reported in green on the mouse CCL2 assay below. Note that depending on the HTRF cytokine kit, the LoQ may be less than the first standard.




**Samples** → **Standard curve** → **Detection**



## TABLE OF CONTENTS

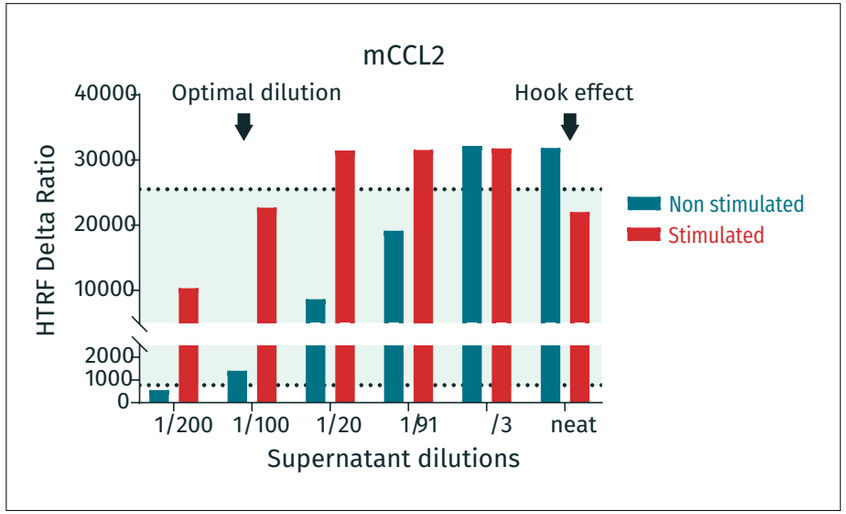
## ASSAY OPTIMIZATION

# Cell supernatant dilutions

Here, 3T3-L1 cells were plated into a 12 well plate at 30,000 cells per well under 1 mL. Cells were grown until confluency, and differentiated in adipocyte (~700,000 cells per well). Differentiated cells were then stimulated for 24h with IL1β and TNFα, both used at 10 ng/mL.

Supernatants were serially diluted as indicated on the graph, and mouse CCL2 quantified by HTRF.

The hook effect can be seen on neat supernatant from the stimulated condition, but the dilution 1/100 represents an optimal condition to quantify both non stimulated and stimulated cases.




Samples → Standard curve → Detection



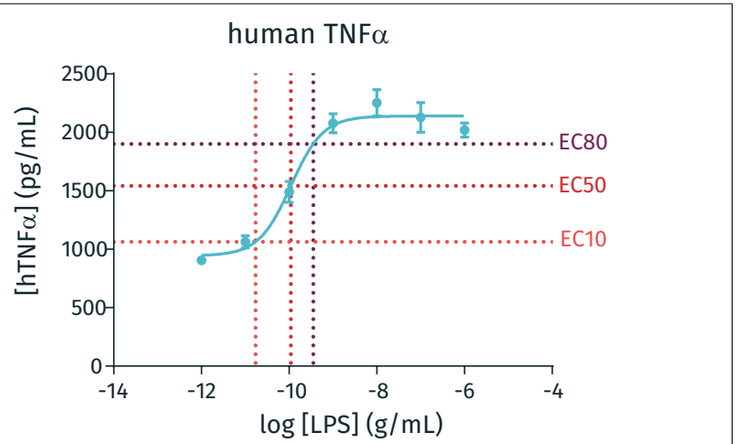
## TABLE OF CONTENTS

# ASSAY OPTIMIZATION
# Cell treatment

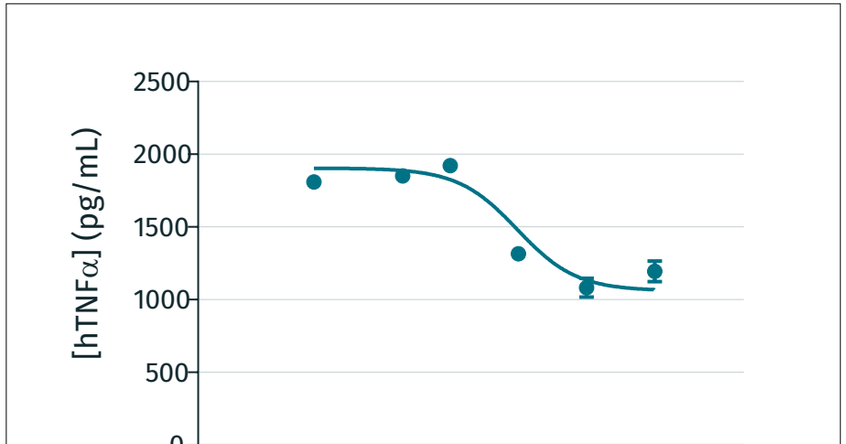
## OPTIMIZATION OF COMPOUND CONCENTRATION

Determination of pharmacological parameters such as IC10 or IC80 may be important to identify new agonists or antagonists.

The graph (left panel) represents a dose response curve of TNFα release after treatment of PBMCs (200,000 cells/well) with increasing concentrations of LPS during an overnight incubation. The EC80 of LPS was determined and used to establish an inhibition curve with the JTE-607 compound, incubated overnight, as shown on the right panel.




| | EC10 | EC50 | EC80 |
|---|---|---|---|
| | -10.766 | -9.958 | -9.449 |
| [LPS]g/mL | 1,7E-11 | 1,1E-10 | 3,5E-10 |




**Samples** → **Standard curve** → **Detection**



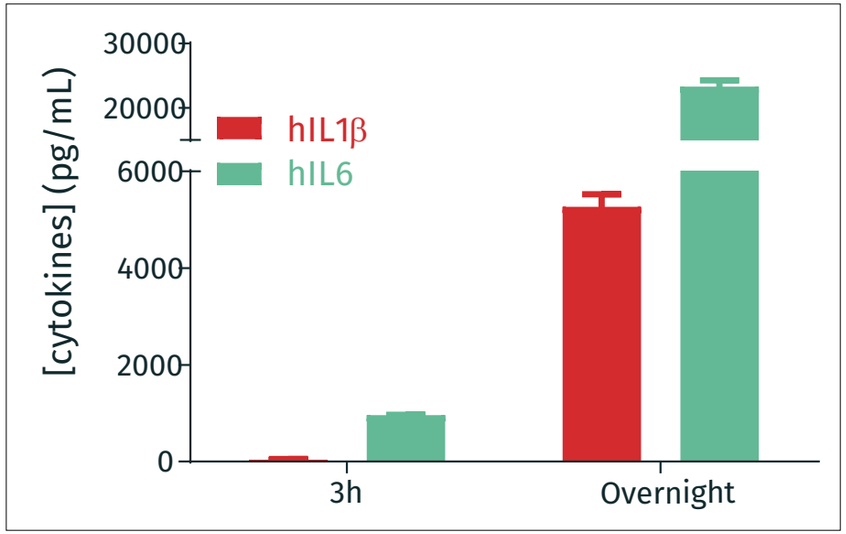
## TABLE OF CONTENTS

# ASSAY OPTIMIZATION
# Cell treatment

## TIME-COURSE OF COMPOUND TREATMENT

Determine the optimal duration of treatment either with agonists or antagonists.

Duration of treatment can be essential in reaching optimal cytokine release. Some cytokines, like IL2, will be released after a short treatment period (2h) while others, like TGFβ, can require much longer (up to 48h).




In this example, PBMCs were dispensed at 100,000 cells per well into a 96 well microplate, then stimulated with LPS at 0.2 µg/mL for 3 hours or overnight (ON). As illustrated here by the secretion level, an ON stimulation enables a stronger IL1β and IL6 release than 3h.

# ASSAY OPTIMIZATION
# Standard curve

## PREPARATION OF THE STANDARDS

### Procedure

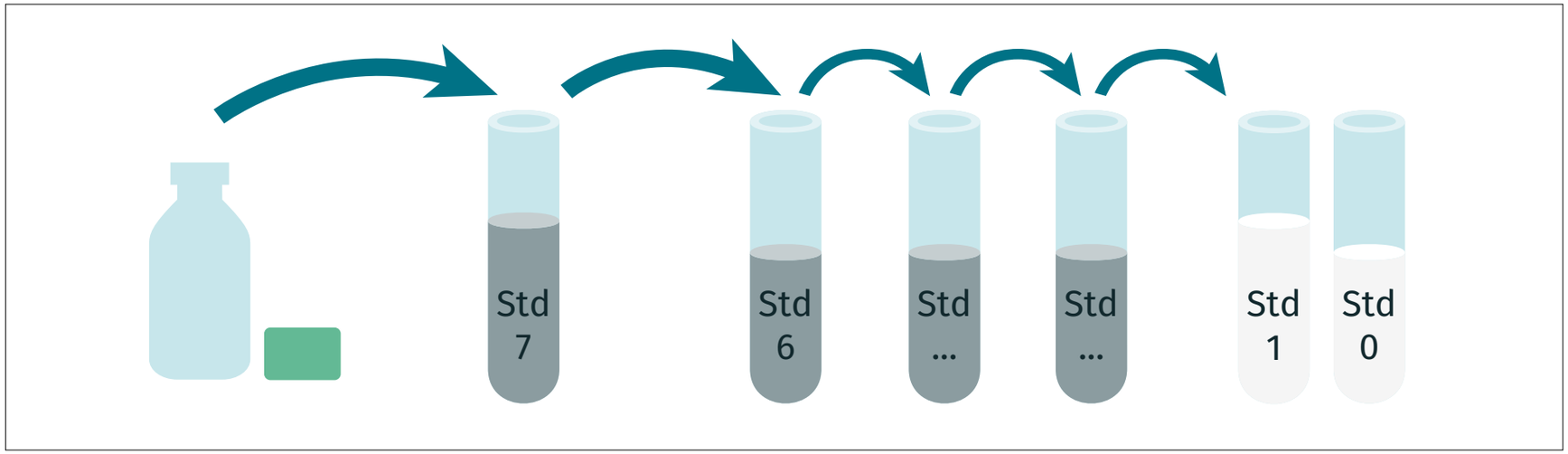
1. Reconstitute the standard vial with the volume indicated on the vial label using distilled water.

   *At this stage, the reconstituted standard solution can be aliquoted (10µL), frozen and stored at -60°C or below.*

2. Dilute the reconstituted standard stock solution 3-fold with your cell culture medium or with the diluent provided in the kit.
3. Prepare serial dilutions of the standard according to the cytokine package insert.

### Recommendations

- Each standard has been carefully positioned to enable optimal determination of low cytokine concentrations, thus any change to the dilution procedure may lead to improper curve fitting and result inconsistency.
- Cytokine may be intrinsically unstable, therefore check the stability of the frozen aliquot over time.
- Cytokine may be intrinsically sticky, therefore check the stability of the frozen aliquot over time.




**Samples** → **Standard curve** → **Detection**



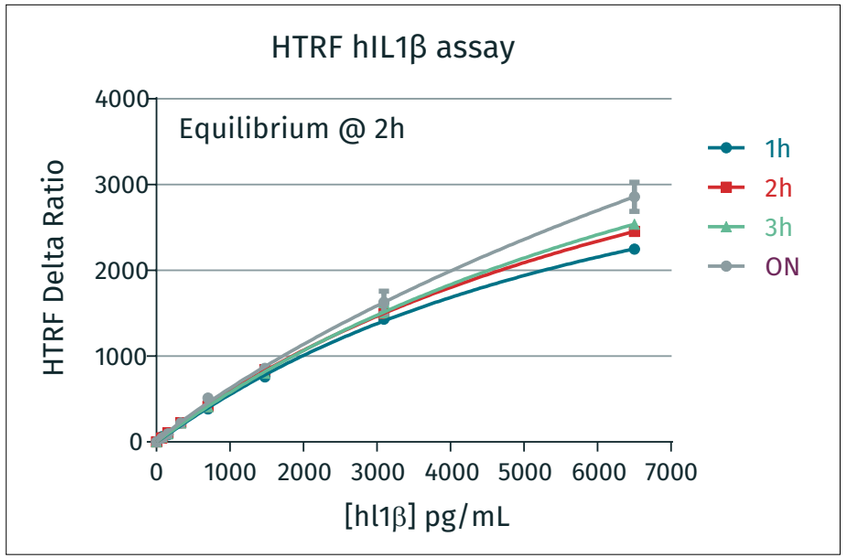
## TABLE OF CONTENTS

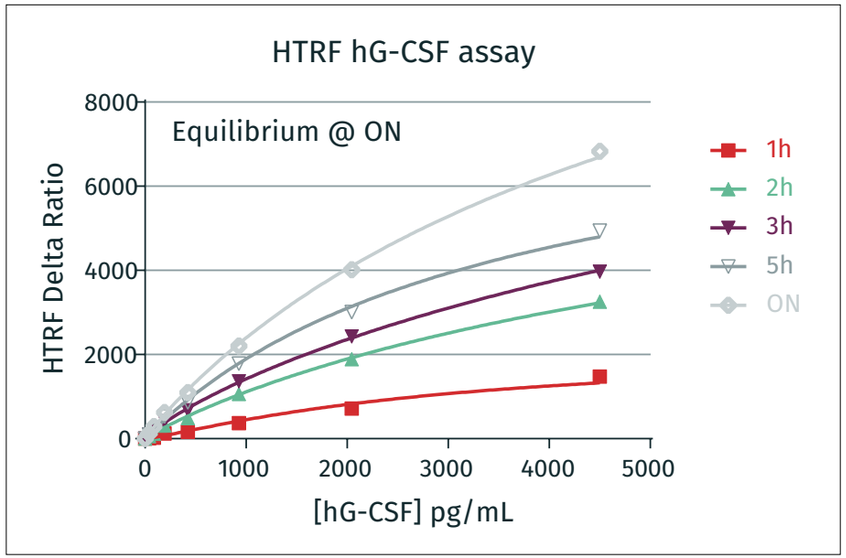
# ASSAY OPTIMIZATION
# Detection

## Detection kinetic

The HTRF signal reaches an equilibrium after a defined incubation time with HTRF detection reagents. This time is determined for each HTRF cytokine assay.

In the example below, the hIL1β assay reaches its equilibrium phase after 2h of incubation, whereas the HTRF signal of an hG-CSF assay increases up to ON incubation. However at 2h, the HTRF signal is usually high enough to assess whether the experiment delivers the expected results.




**Samples** → **Standard curve** → **Detection**



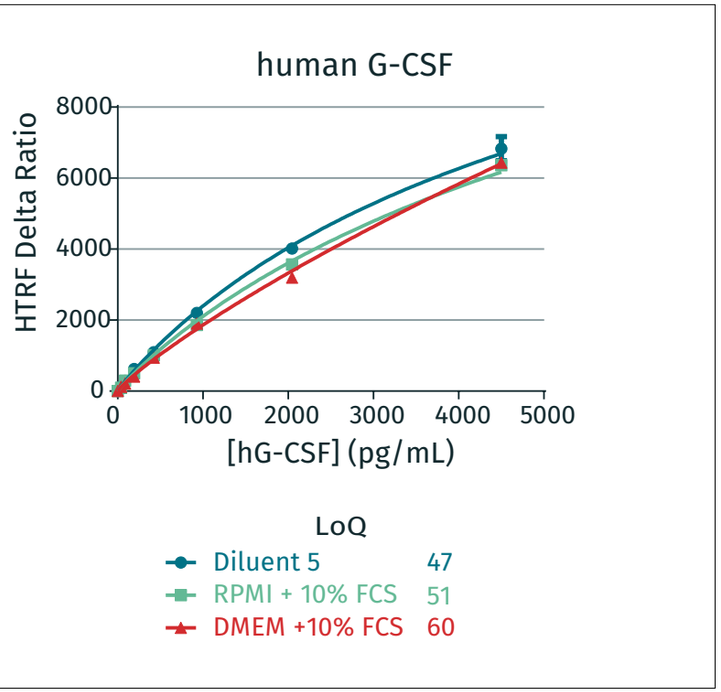
## TABLE OF CONTENTS

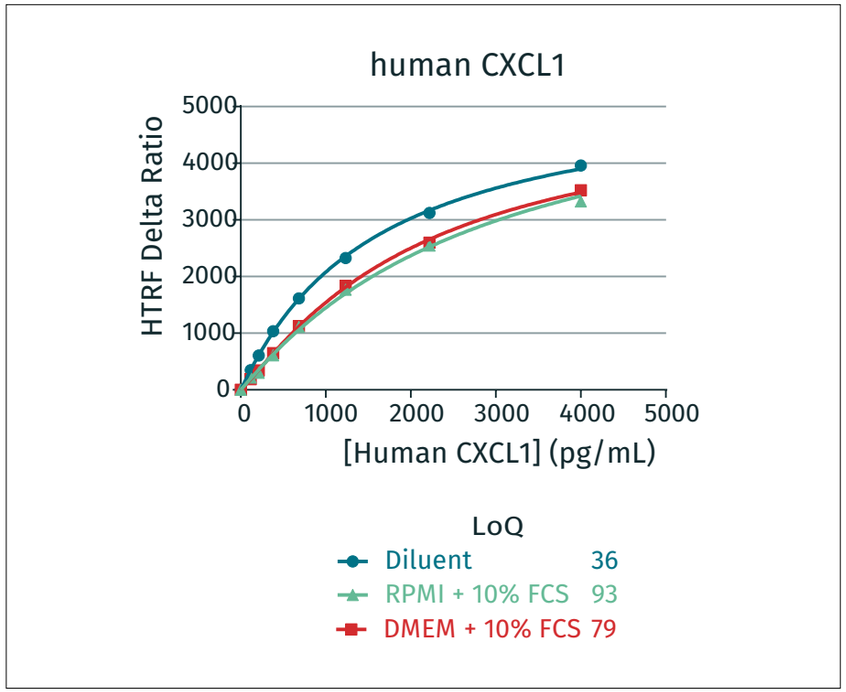
# ASSAY OPTIMIZATION
# Detection

## Cell culture medium

Depending on the composition of the cell culture medium, the limit of quantification may vary, as illustrated on human G-CSF and human CXCL1.

Therefore dilution of the standards in the cell culture medium used for samples is recommended.




**Samples** → **Standard curve** → **Detection**



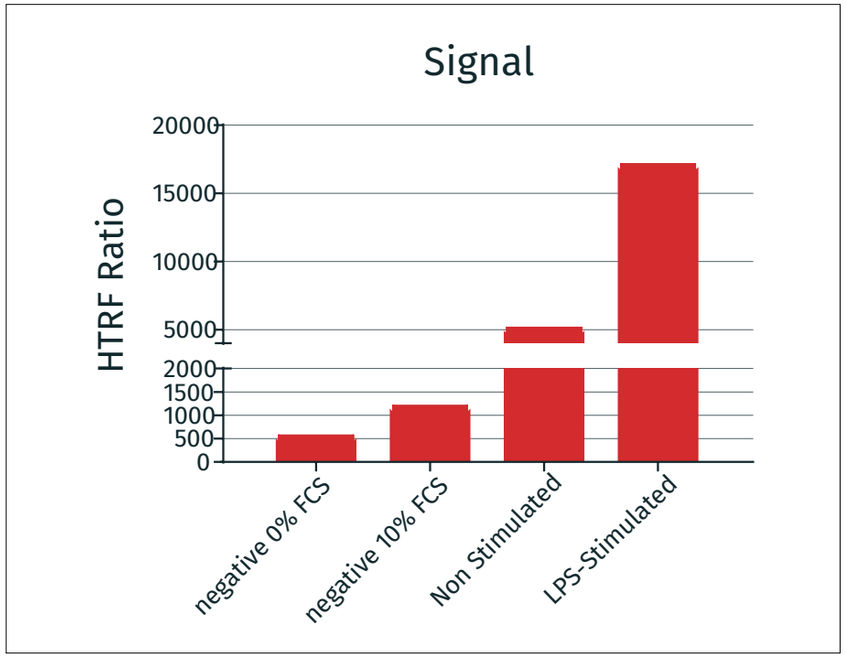
## TABLE OF CONTENTS

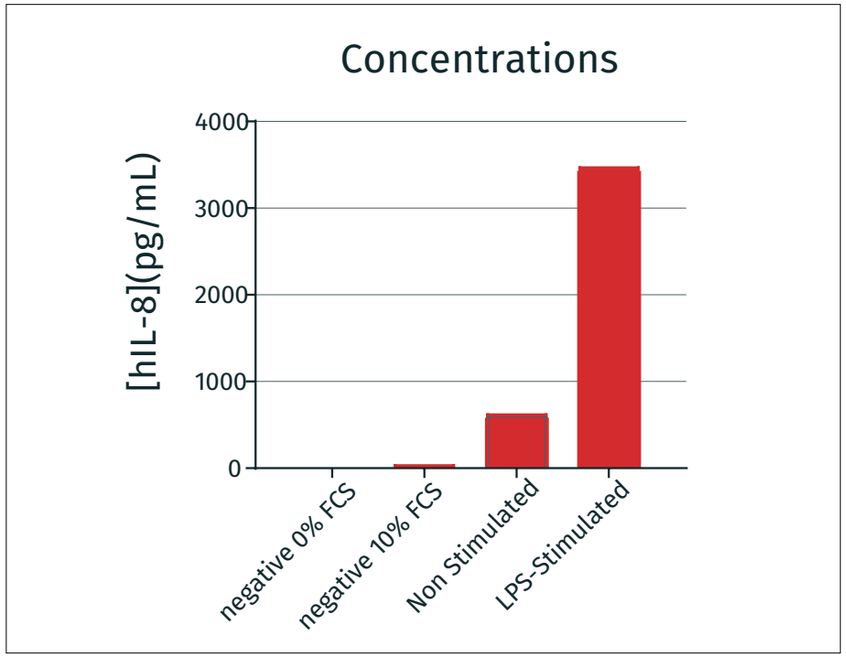
# ASSAY OPTIMIZATION
# Detection

## FCS effect

In addition, a small but detectable amount of certain cytokines like IL8 or TGFβ may be present in the FCS used in cell culture medium.

As shown here on IL8, a significant HTRF signal is detected in the presence of 10% FCS, compared to 0% FCS. It corresponds to 30pg/mL of IL8 present in the FCS used for this experiment, which is negligeable compared to the Non Stimulated condition. Therefore particular attention must be paid to cell culture medium composition.




**Samples** → **Standard curve** → **Detection**

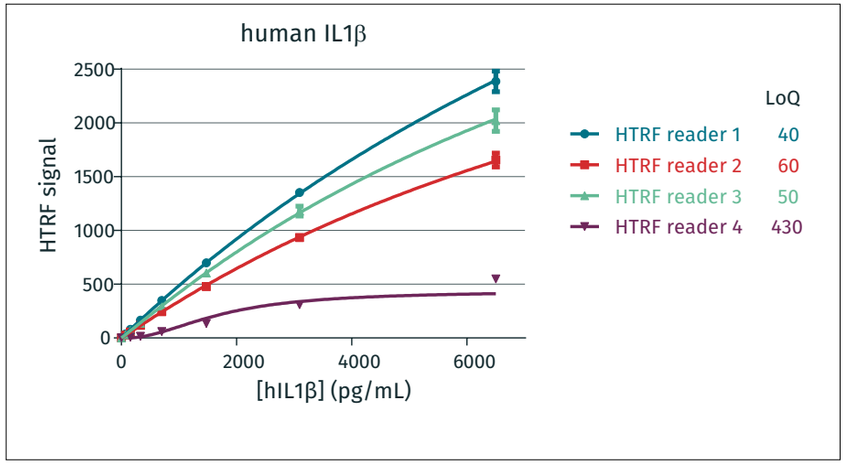


## TABLE OF CONTENTS

# ASSAY OPTIMIZATION
# Detection

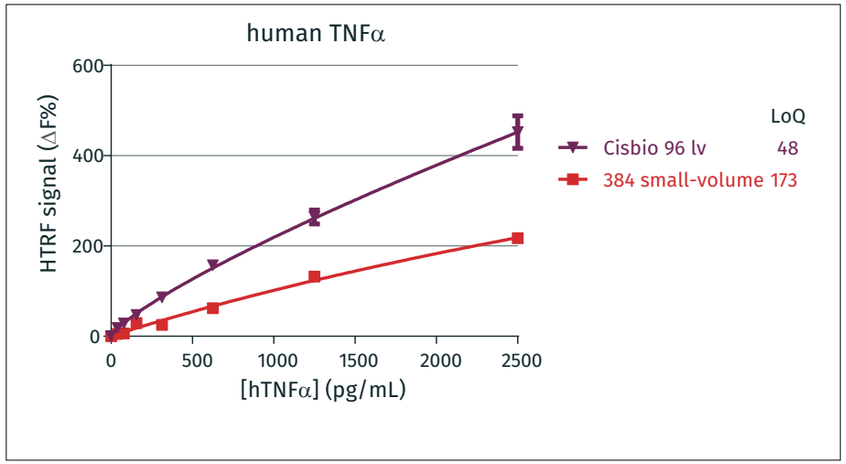
## Reader performance

Depending on the sensitivity of your HTRF compatible reader, the limit of quantification also may vary, as illustrated on human IL1β.




## Detection plate

Depending on the reader, alternative detection formats may sometimes improve data quality, as illustrated on human TNFα where the LoQ is significantly improved by the use of a Revvity 96 lv well plate compared to 384 small-volume plates.




**Samples** → **Standard curve** → **Detection**

DETECTION

# Choosing the Right Plate Reader

Choosing the suitable microplate reader ensures you'll get an optimal readout. A multimodal reader gives your research the ideal flexibility and expands the field of possibilities by measuring a wide range of technologies.

Revvity offers multi mode plate readers that are certified for use with HTRF technology. Certification involves rigorous testing of plate reader with HTRF technology to ensure optimal readout. HTRF assays are also compatible with multimode readers from other providers as well.


**EnVision® Nexus™**

Provides lightning speed and superior sensitivity across all established detection technologies with advanced options for ultimate performance. It is the next generation of high-throughput screening, ideal for your most demanding assays. .


**VICTOR® Nivo™**

Packs all the latest major detection technologies into the industry's smallest benchtop footprint. The perfect microplate reader for everyday biochemical and cell-based assays.



## TABLE OF CONTENTS

# DATA REDUCTION
# Standard curve fitting

The standards of HTRF cytokine assays have been carefully positioned to maximize both

- assay sensitivity at lowest cytokine concentrations.
- assay dynamic at higher cytokine concentrations.

The recommended mathematical model for fitting is four parameter logistic 4PL with $1/y^2$ weight. This model enables better accuracy at lowest analyte concentrations, as indicated in green in the table below where the recovery percentage corresponds to the difference between the expected and the measured concentration.

However 4PL without $1/y^2$ weight is also possible, especially if cytokines are not in a low concentration range.

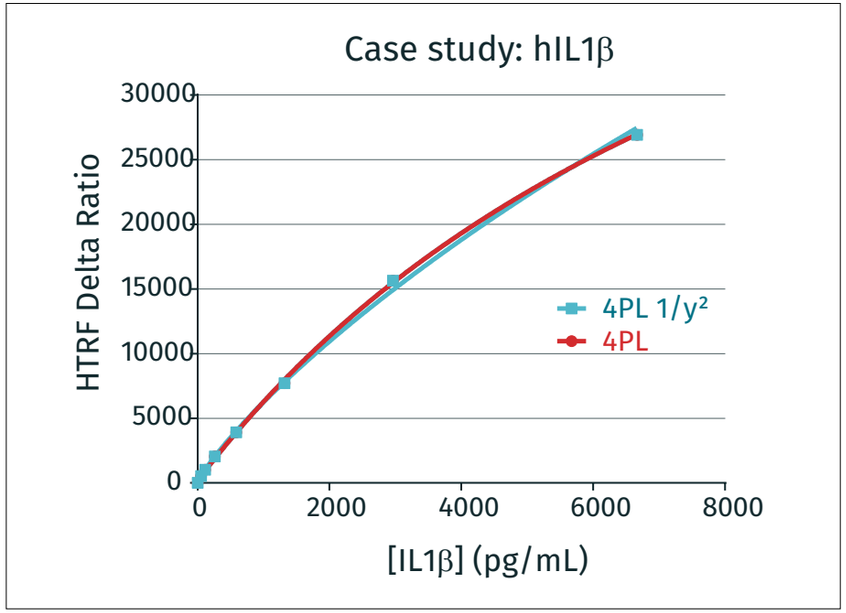
| Expected concentrations | Measured concentrations | | | | | |
|---|---|---|---|---|---|---|
| | 4PL 1/y² | | | 4PL | | |
| | Mean | CV | % Recovery | Mean | CV | % Recovery |
| 22.8 | 23 | 5% | 101% | 30.7 | 20% | 135% |
| 34.25 | 36.3 | 12% | 106% | 48.1 | 25% | 140% |
| 77 | 75.1 | 8% | 98% | 95.5 | 9% | 124% |
| 173.4 | 166.5 | 1% | 96% | 191 | 9% | 110% |
| 390.2 | 379.2 | 4% | 97% | 403.8 | 7% | 103% |
| 877.9 | 874.1 | 3% | 100% | 869.8 | 3% | 99% |
| 1975.3 | 2013.4 | 0% | 102% | 1953 | 3% | 99% |
| 4444.4 | 4650.6 | 0% | 105% | 4567.2 | 1% | 103% |

## Note that

The HTRF Delta Ratio represents a specific signal and is calculated as follows:

*HTRF Delta Ratio = HTRF Ratio (Standard 1-7) – HTRF Ratio Standard 0*

For optimal fitting, the value 0 of the standard 0 must be integrated in the standard curve.






## TABLE OF CONTENTS

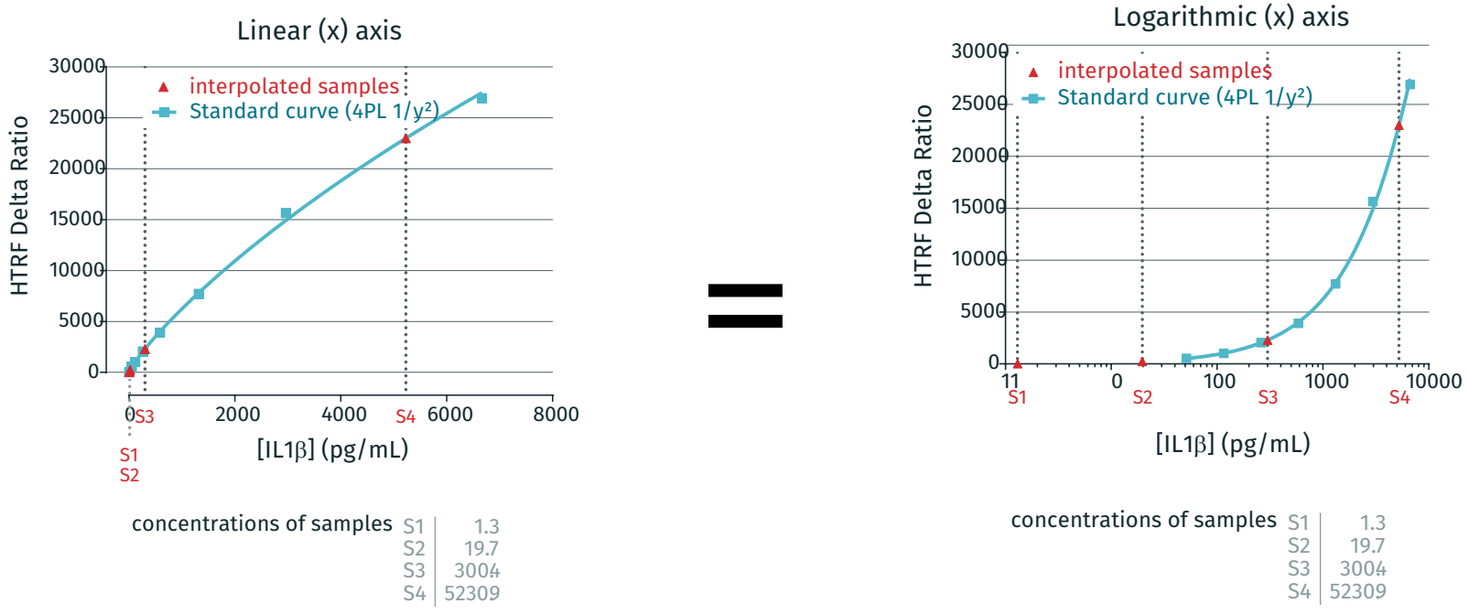
## DATA REDUCTION

# Standard curve representation

The standard curve of HTRF cytokine assays is usually represented with a linear (x) axis.

Note that the fitted standard curve may also be drawn with a logarithmic (x) axis from the same data set.

As shown below, the logarithmic representation does not modify further data processing and consequently, the determination of sample concentrations.




## TABLE OF CONTENTS

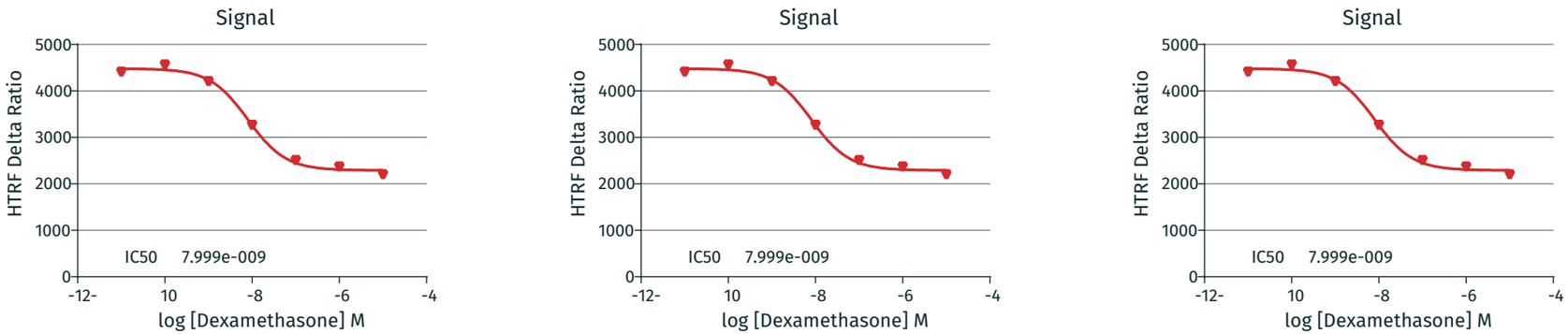
# DATA REDUCTION
# Representation of results

Results may be represented either in HTRF signal or in concentrations interpolated on the corresponding standard curve.

When absolute quantification is not required, e.g. comparing cytokine concentrations between two or more biological conditions, result analysis can be simply and rapidly performed on the HTRF signal.

**HTRF signal from samples** → **Interpolation on standard curve** → **Sample concentrations**

Example 1: hTNFα

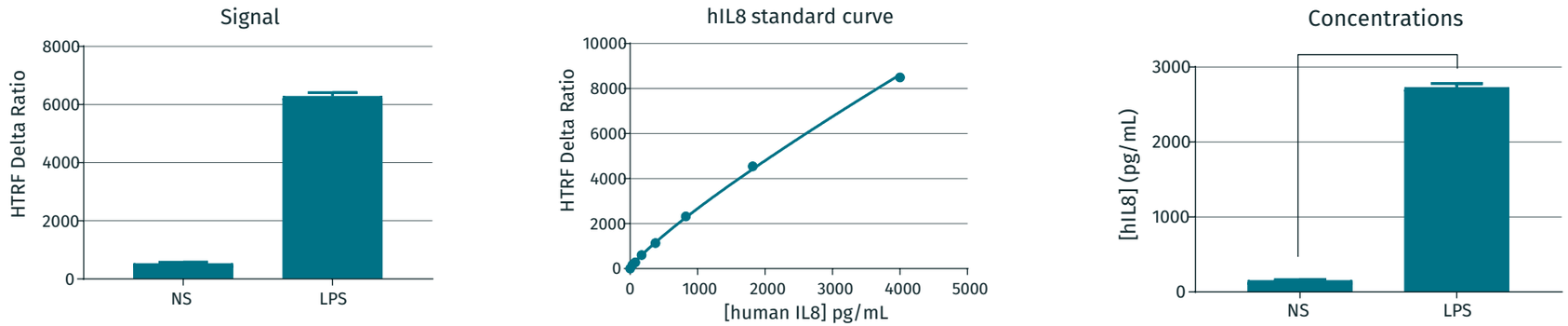



## TABLE OF CONTENTS

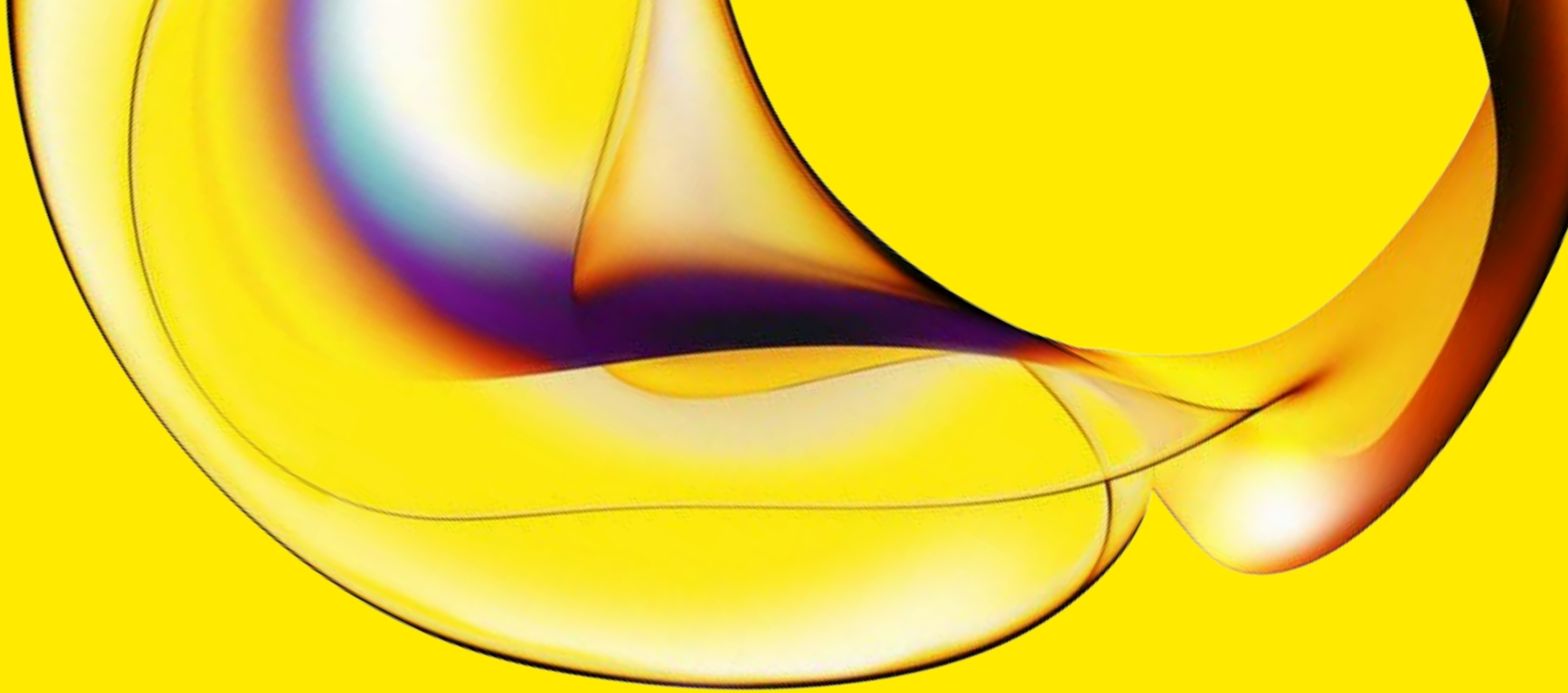
# DATA REDUCTION
# Representation of results

**HTRF signal from samples** → **Interpolation on standard curve** → **Sample concentrations**

Example 2: hIL8




## TABLE OF CONTENTS



**www.revvity.com**

**Revvity, Inc.**
940 Winter Street, Waltham, MA 02451 USA
(800) 762-4000 | www.revvity.com

**For a complete listing of our global offices, visit www.revvity.com**


654272 (544540)