Guidelines from PBMC isolation to cytokine assay optimisation.

This technical note describes how to purify PBMC from a buffy coat or whole blood sample using Ficoll gradient, and introduces the optimization steps needed for an appropriate PBMC cytokine quantification using HTRF assays.

Abstract

Revvity offers a comprehensive line of HTRF® cytokine assays to investigate the functional response of immune cells. These versatile, homogeneous assays are suitable for a variety of sample types, such as cell culture supernatants from immortalized cell lines or more physiological cell models such as Peripheral Blood Mononuclear Cells (PBMCs).

This technical note describes how to purify PBMC from a buffy coat or whole blood sample using Ficoll gradient, and introduces the optimization steps needed for an appropriate PBMC cytokine quantification using HTRF assays.

Materials and methods

Biological models and reagents

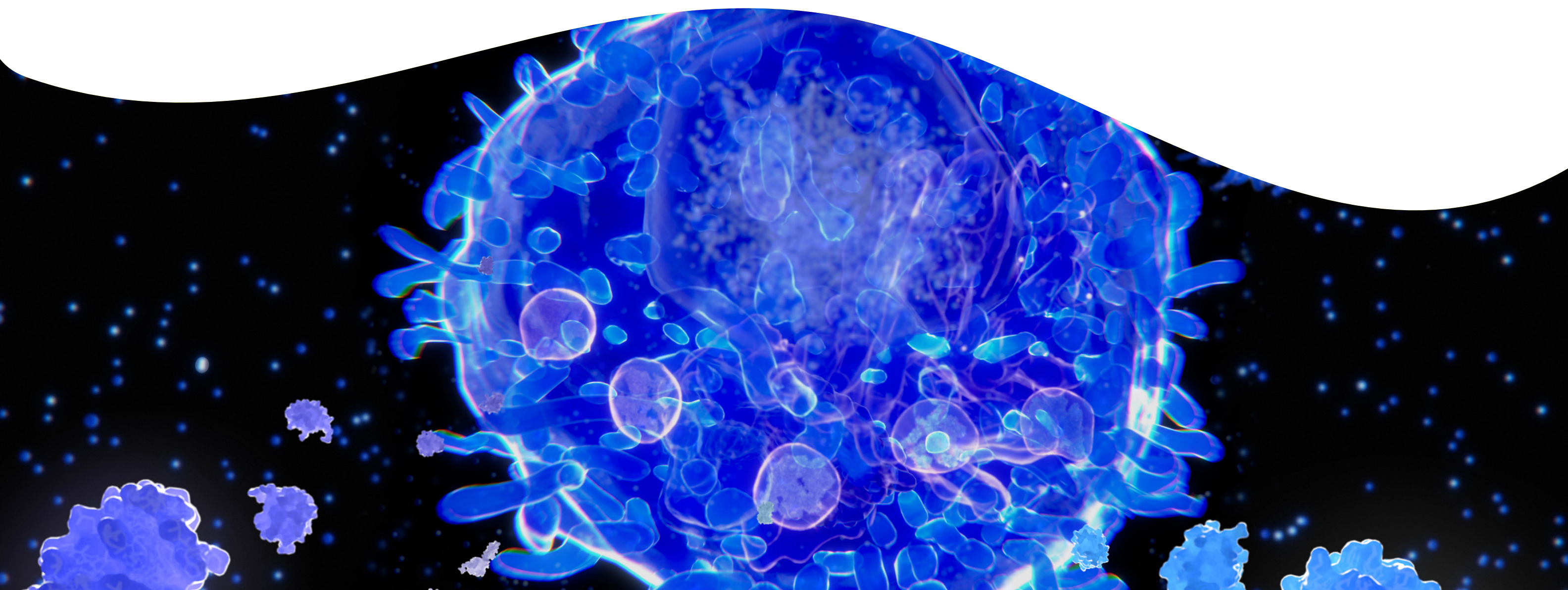
Buffy coat or whole blood samples were obtained from the French National Blood Service (EFS Pyrénées-Méditerranée)

HTRF cytokine-assays: Revvity, Human TNFα #62HTNFAPEG, Human IL1β #62HIL1BPEG, Human IL6 #62HIL06PEG, Human IL8 #62HIL08PEG

Agonists: LPS (Sigma #L4391)

PBMC extraction system: Ficoll gradient (Sigma #17-5442-02)

FACS antibodies: CD3-FITC (Milteny ref 130-109-460)
CD14-PerCP (Milteny ref 130-097-539)
CD19-PE (Milteny ref 130-091-247)
CD56-APC (Miltenyi ref 130-100-698)



HTRF Cytokine Assays

All HTRF cytokine assays are no-wash sandwich immunoassays where the fluorescent signal intensity is proportional to the analyte concentration in the sample. The detection is performed via analyte-specific antibodies coupled to donor and acceptor dyes.

HTRF cytokine assays are homogeneous (no washing, no separation steps), with under 2h of bench time and requiring only 16µl of sample.

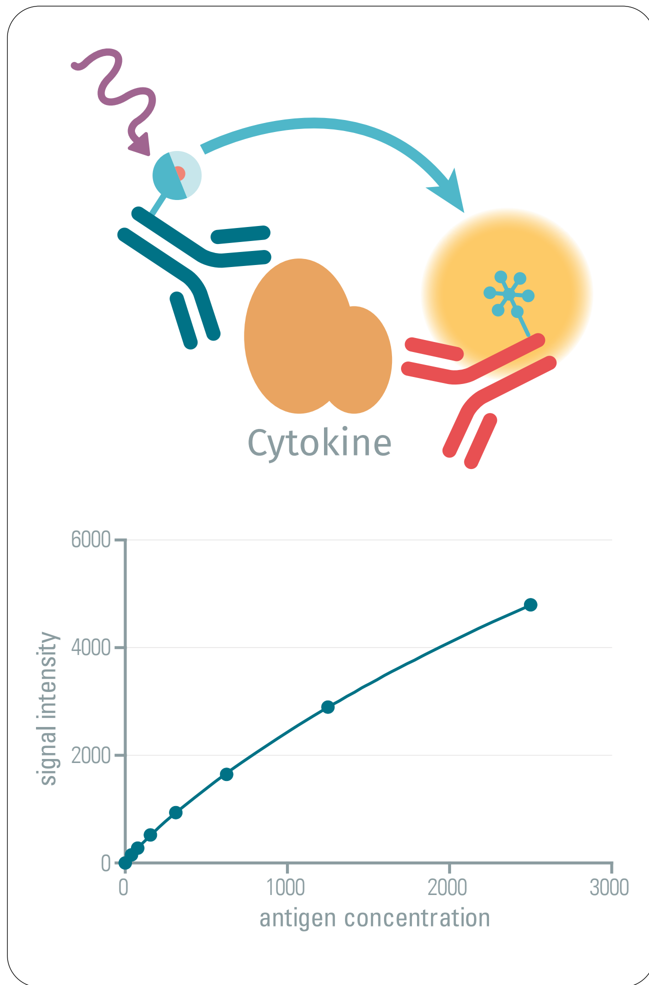


Figure 1: HTRF Cytokine assays

Flowchart

1. PBMC isolation using ficoll (Figure 2)

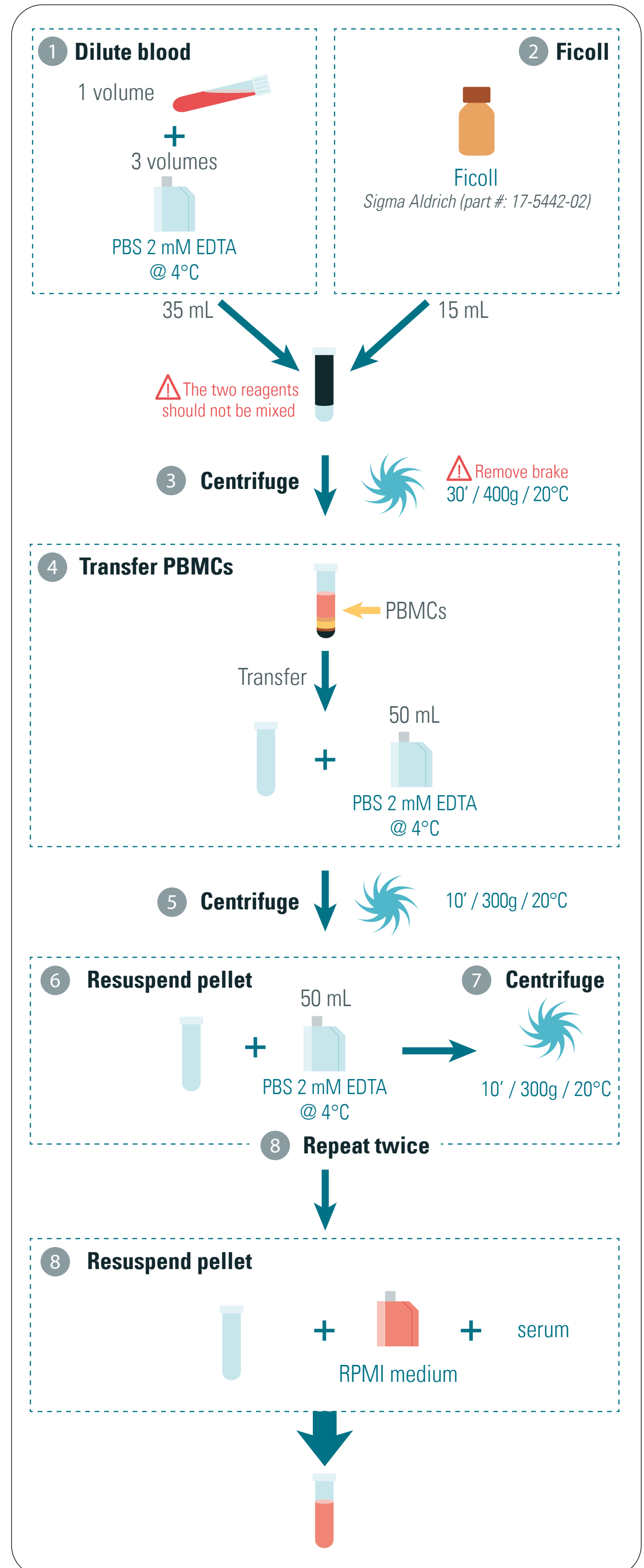


Figure 2

1. Dilute buffy coat or whole blood 3-fold with PBS and 2mM EDTA pre-cooled to 4°C. For example mix 8.750 mL of blood and 26.25 mL of cold PBS, which corresponds to 35 mL of PBS-diluted blood.
2. Dispense 15 mL of ficoll solution + 35 mL of the PBS-diluted blood (or 1:3.33 volume of Ficoll solution). Do not mix diluted blood and ficoll.
3. Centrifuge 35 minutes, 400g at 20°C. Remove the brake for this step.
4. PBMC will appear as an orange ring below the plasma. Slowly pipette the PBMC ring and transfer it into a different tube containing 50 mL of cooled PBS (4°C), 2mM EDTA.
5. Centrifuge 10 minutes at 300g, 20°C.
6. Discard the supernatant and resuspend the pellet with 50 mL 4°C PBS, 2mM EDTA.
7. Centrifuge 10 minutes at 300, 20°C.
8. Repeat steps 6 and 7.
9. Discard supernatant and resuspend the pellet (PBMCs) with an appropriate cell culture medium, usually FCS sup plemented RPMI culture medium.

2. Cytokine secretion from PBMC (Figure 3)

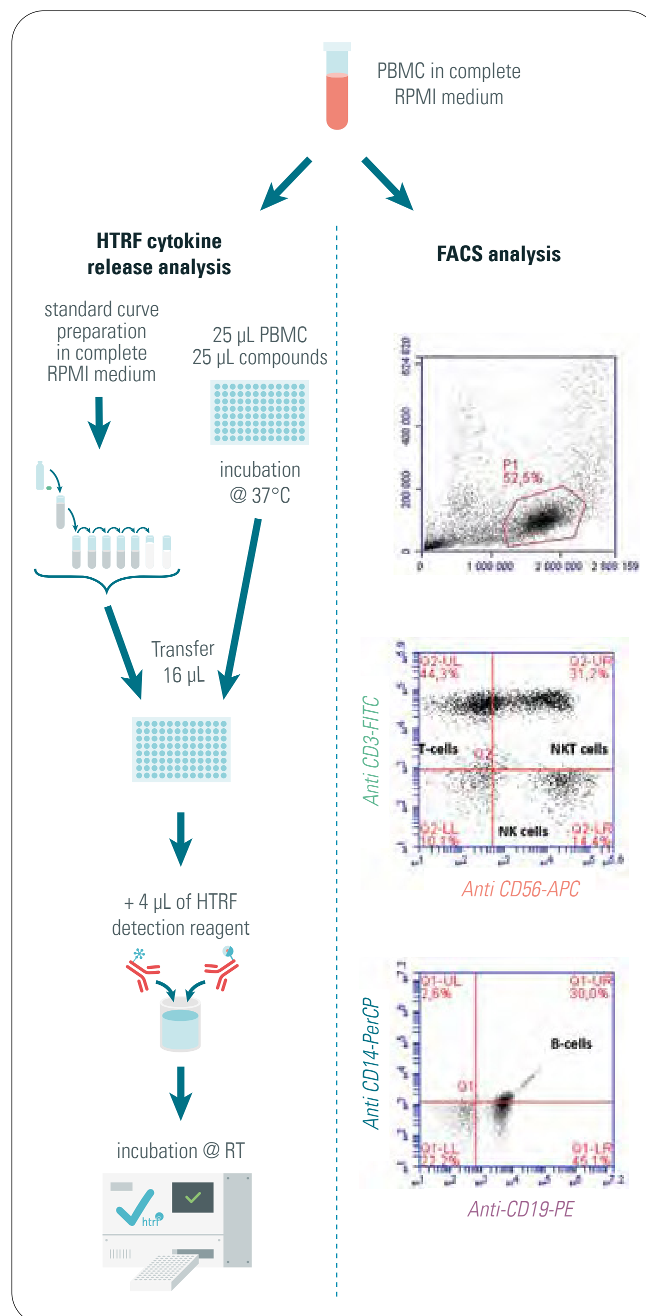


Figure 3

3. Supernatant preparation

When working with an unknown concentration of cytokine, we recommend testing dilutions of your supernatants (for example 1:3 and 1:9) to ensure that you are within the dynamic range of the HTRF cytokine assay. Dilutions of supernatant can be made in your culture medium or in the diluent provided in the kit.

IMPORTANT: Make sure you use the same medium (culture or diluent) for supernatant dilutions and standard curve preparation.

4. HTRF detection

- Transfer 16 µL of PBMC supernatants or 16 µL of standards into a detection plate, either a Cisbio 96 well low volume white microplate or a 384 well small volume white microplate. You can find a list of compatible HTRF detec tion plates here: http://www.cisbio.com/htrf-microplaterecommendations
- Dispense 4 µL of pre-mixed HTRF reagents. Include a neg ative control by replacing the supernatant by 16 µL of the corresponding buffer (culture medium or diluent).
- Proceed to incubation according to the kit's protocol. Record the HTRF signal on an HTRF-compatible reader You can find the list of compatible readers and their setup here: http://www.cisbio.com/htrf-compatible-readers.

For more information about the HTRF cytokine protocol you can watch our step by step video: http://www.cisbio.com/cytokine-assay-protocol.

5. FACS analysis

The PBMC isolation process was assessed by flow cytometry. T cells and B cells populations were quantified via anti-CD3/anti-CD56 and anti-CD1/anti-CD19 respectively.

Data handling

- The HTRF Ratio was calculated for each well of standard or samples independently, using the following formula:

$$\text{HTRF Ratio} = \frac{\text{(signal 665 nm)}}{\text{(signal 620 nm)}} \times 10^4$$

- The standard curve was obtained by data reduction using the four parameter logistic (4PL $1/y^2$ weighted) curve fit. Learn more about HTRF data reduction and curve fitting at http://www.cisbio.com/4PL-regression.
- Means and Standard Deviations (SD) are represented on the graphs.

Optimization steps

Cell density

Define the optimal cell density for each cytokine assay release. In this example, PBMC samples were dispensed at different cell densities ranging from 50,000 to 400,000 cells per well of a 96 well microplate. The PBMCs were stimulated with LPS at 0.2 µg/mL for 3 hours. As illustrated hereafter by their secretion level, the quantification of TNFα will require more PBMC than IL8.

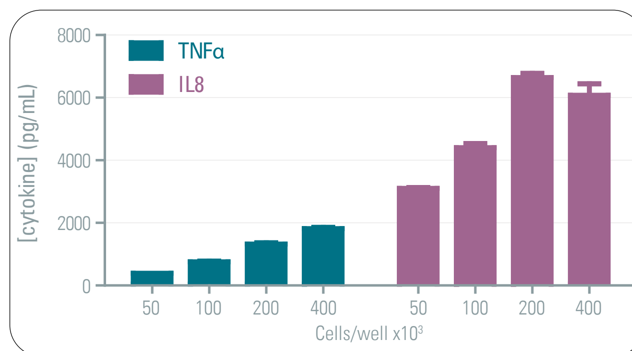


Figure 4: Quantification of TNFα and IL8 release at various PBMC densities after LPS

Kinetic of treatment

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

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 3h or overnight (ON). As illustrated here by the secretion level, an ON stimulation enables a stronger IL1β and IL6 release than 3h.

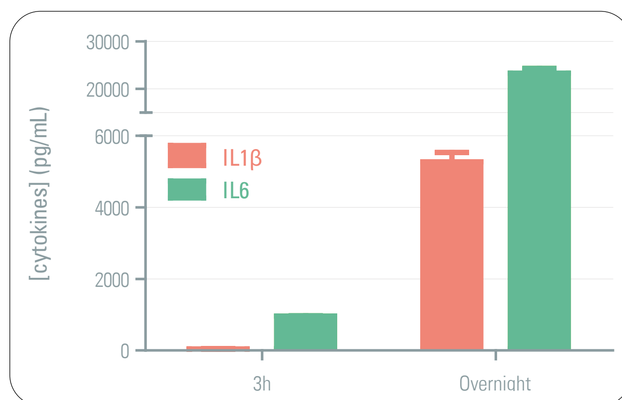


Figure 5: Quantification of human IL1β and IL6 release after 3h and ON of LPS stimulation

Concentration of compound

Define the optimal concentration of compound to obtain comfortable biological response (activation or inhibition) without compound toxicity effect.

In the example below, PBMCs were dispensed at 100,000 cells per well into a 96 well microplate, then stimulated with different LPS concentrations overnight. As illustrated here by the secretion level, whereas increasing the LPS concentration up to 2 µg/mL improves the secretion level of IL1β, LPS used at 0.02 µg/mL is optimal for TNFα and IL6.

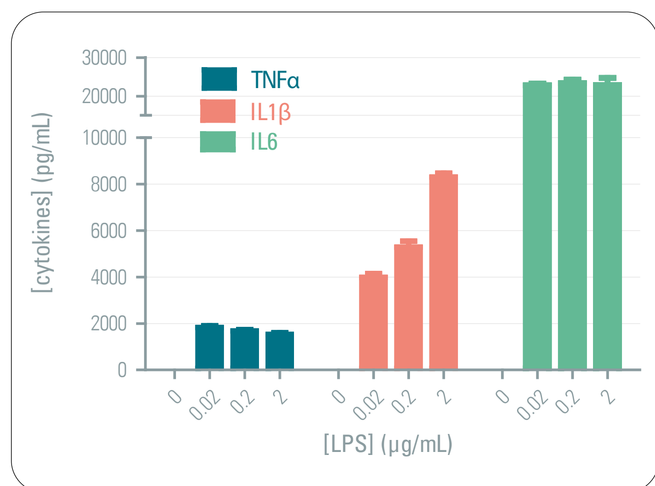


Figure 6: Quantification of human TNFα, IL1β and IL6 release after PBMC stimulation using various LPS concentrations

Optimized experimental conditions enable multiparametric approach

Once cell density, compound concentration and treatment duration have been properly defined for each cytokine, HTRF measurement of a panel of cytokines from the same sample represents an attractive approach. The analysis of multiple cytokines from a single sample not only enables you to save precious biological materials but it also gives access to a more global biological landscape.

In the example below, 200,000 PBMCs were treated with LPS at 0.02 µg/mL for 3 hours.

The supernatant was collected and according to the first tests performed:

- Supernatant for IL8 detection was diluted ¼ in RPMI + 10% FCS
- Supernatant for IL6 was diluted ½ in RPMI + 10% FCS
- Neat supernatants for TNFα and IL1β detection were not diluted

As mentioned before, the standard curve was also prepared in RPMI + 10% FCS to avoid any matrix effect.

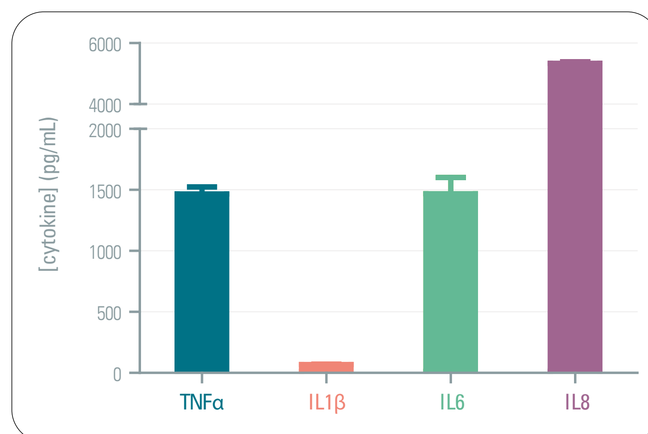
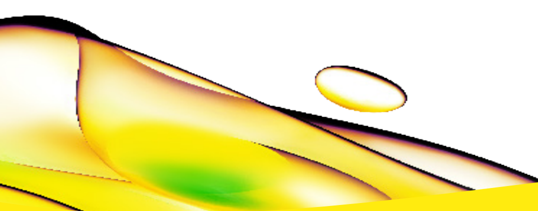


Figure 7: Quantification of IL1β, IL6, IL8 and TNFα release after PBMC stimulation with LPS



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