

High throughput quantitation of cytokine biomarkers using LANCE *Ultra* TR-FRET assays.

Authors

Jen Carlstrom
Stephen Hurt
Roger Bosse
Revvity, Inc.

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Introduction

Injury to tissue can lead to inflammation, which is characterized by an accumulation of cytokines. The excessive production of pro-inflammatory cytokines can result in further tissue damage and has been implicated in many diseases, such as atherosclerosis and cancer. Anti-inflammatory drug development is therefore an important area of focus in the pharmaceutical industry. Discovery of these drugs through high throughput screening campaigns requires the ability to both robustly and reliably detect cytokine production in a variety of complex sample matrices.

Time-resolved fluorescence resonance energy transfer (TR-FRET) assays can be used to quantify the levels of cytokines in a sample. LANCE® *Ultra* TR-FRET homogeneous assays utilize the unique fluorescent properties of a europium-based chelate (Eu chelate) as the donor dye. Eu chelates have a high quantum yield, large Stokes' shift, a narrow-banded emission, and a long lifetime. These properties make Eu chelates ideal energy donors in TR-FRET assays, as they are less susceptible to interference and reduce background signal. *ULight*™ is a small, light-resistant acceptor dye that has red shifted emission and is therefore less sensitive to quenching by colored compounds. The power of LANCE *Ultra* TR-FRET assays comes from the combination of Revvity's Eu chelate donor dye and the *ULight*™ acceptor dye.



Using LANCE technology, Revvity has developed sensitive immunoassay kits to measure cytokines in a variety of samples (Figure 1). Here we demonstrate the ease and versatility of using LANCE *Ultra* TR-FRET assays to quantitate low levels of the pro-inflammatory cytokines TNF α , IL-6, and IL-1 β from PMA differentiated U937 cell supernatants and to detect measurable inhibition of cytokine secretion using the small molecule dexamethasone. In addition, we show the accuracy of miniaturization of the assay in both low volume 384- and 1536-well formats.

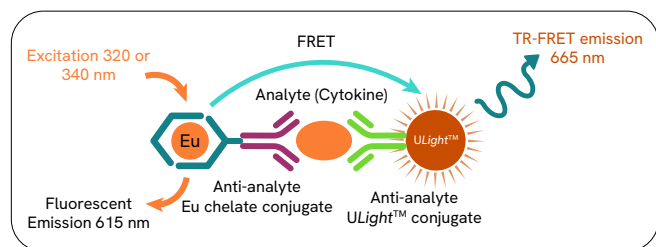


Figure 1: **LANCE *Ultra* TR-FRET assay.** The cytokine in the cell supernatant is captured by a Europium-labeled antibody and *ULight™* labeled antibody to form a complex. Excitation of the Eu chelate in the complex generates signal at 615 nm and after the energy transfer, signal is generated at 665 nm.

Materials and methods

Instrumentation

All LANCE *Ultra* TR-FRET measurements were performed on the Revvity EnVision Multimode Plate Reader Model 2104 equipped with dual detectors and a laser for FRET detection.

Cell culture

U937 cells (ATCC® cat# CRL1593.2) were differentiated for 48 hours with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) (Sigma cat# P1585-1MG), then changed into phenol red free RPMI (Sigma cat# R7509) media with 2% FBS and challenged for 21 hours with lipopolysaccharides (LPS; 10 μ g/mL) (Sigma cat# L4516-1MG) in 96-well CulturPlates (Revvity cat# 6005680).

Cell treatment

The U937 cells were differentiated using PMA and removed from the plate using cell dissociation buffer (Sigma cat# C5914-100ML). Then various concentrations of cells were plated in a 96-well CulturPlate and subjected to treatment with LPS to induce cytokine secretion. The supernatants from the cells (in phenol red-free RPMI) were collected and added directly to a 384-well OptiPlate for cytokine analysis.

For inhibition studies, U937 cells were differentiated with PMA for 48 hours and then treated simultaneously with LPS (10 μ g/mL) and increasing concentrations of dexamethasone (Sigma Cat# D2915-100MG) for 21 hours.

LANCE assays

15 μ L of cell supernatants were transferred to 384-well OptiPlate plates (Revvity cat# 6007299) and 5 μ L of a mixture of Eu-antibody and *ULight™* antibody in *Ultra* Hiblock or LANCE assay buffer was added and incubated for one hour at room temperature. For 10- μ L reactions, 7.5 μ L of supernatants were transferred to 384-well ProxiPlate plates (Revvity cat# 6008289) and 2.5 μ L of a mixture of Eu-antibody and *ULight™* antibody in LANCE assay buffer was added and incubated for one hour at room temperature. For 5- μ L reactions, 3.75 μ L of supernatants were transferred to 1536-well OptiPlate (Revvity cat# 6004290) plates and 1.25 μ L of a mixture of Eu-antibody and *ULight™* antibody in LANCE assay buffer was added and incubated for one hour at room temperature. The LANCE *Ultra* TR-FRET kits used were IL-1 β (TRF1220C), TNF α (TRF1208C) and IL-6 (TRF1223C).

Data analysis

The data was analyzed using GraphPad Prism 5 Software. The standard curves were generated using nonlinear regression with a 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (minimize relative distances square). The signal as a function of cell number was plotted using a linear regression. The interpolation of cytokines for each cell number was determined using the interpolation of unknowns from standard curve function in GraphPad Prism 5 and then plotted against the number of cells per μ L. The Lower Detection Limit (LDL) is calculated by interpolating on the standard curve the value obtained from the addition of three times the standard deviation to the average of 12 blank samples.

LANCE *Ultra* TR-FRET signal in this application note was plotted as the ratio of the emission signal of the acceptor (665 nm) and the emission signal of the donor (615 nm).

Results

Quantifying recombinant proteins with LANCE Ultra TR-FRET assays

The LANCE Ultra cytokine detection kits contain recombinant proteins of known concentrations that can be used to correlate the LANCE signal with a quantifiable amount of protein detected in a sample. The LANCE signal output at each protein concentration can be measured as the emission signal of the acceptor dye at 665 nm or as the ratio of the emission signal of the donor (615 nm) and the acceptor (665 nm) dyes. Using the ratio can improve the variability in replicates, minimizing error in the data, and therefore leading to tighter results. In addition, using the ratio can also improve the sensitivity of the assay without affecting the EC_{50} . Figure 2 shows the standard curve for IL-1 β plotted as either the 665 nm measurement or the ratio of the 665 nm to 615 nm measurement. In this case, the sensitivity was improved four-fold (100 pg/mL down to 25 pg/mL) using the ratio.

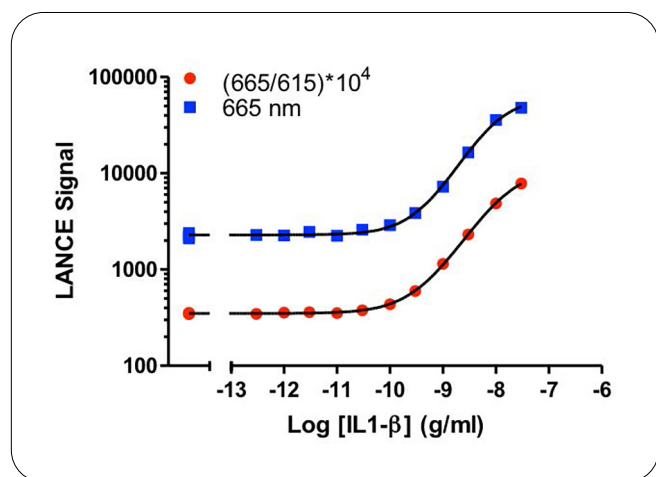


Figure 2: **Comparison of analysis methods.** Standard curves for IL-1 β detection using either the 665 nm measurement or the ratio of the 665 to 615 measurements.

Cytokine detection in supernatants from differentiated U937 cells

U937 is a pro-monocytic, human myeloid leukaemia cell line which can differentiate into mature monocytes or into macrophages upon PMA- treatment. After treatment, U937 suspension cells will adhere to the culture plates forming M0 type macrophages. The M0 cells are then polarized into the M1 macrophage phenotype by treatment with LPS. M1 type macrophages encourage inflammation and typical markers for M1-type macrophages are production of cytokines such as TNF α , IL-1 β , and IL-6.

LANCE Ultra TR-FRET cytokine detection kits were used to quantify levels of TNF α , IL-1 β , and IL-6 cytokines secreted into cell supernatants from PMA and then LPS-treated U937 cells. First, the LANCE signal was measured as a function of increasing concentrations of recombinant cytokine protein. Figures 3A, 4A, and 5A show the standard curve of each cytokine using a recombinant protein diluted in RPMI media. From these standard curves, the lower detection limit of the assay in this media was determined. The lower detection limit (LDL) for the IL-1 β experiment was 19 pg/mL in RPMI media (Fig 3A). The lower detection limit (LDL) for IL-6 was 93 pg/mL (Figure 4A) and the lower detection limit (LDL) for TNF α was 30 pg/mL (5A).

As the number of cells per well increases, the amount of each cytokine released into the cell supernatant should also increase. Figures 3B, 4B, and 5B show the cell titration data for three different cytokines. In the absence of LPS, very little of each cytokine is detectable. However, when the cells are treated with 10 μ g/mL of LPS for 21 hours, there is a significant increase in cytokine secretion that increases linearly with cell number.

Finally, to quantify the amount of each cytokine released, the LANCE signal from Figures 3B, 4B, and 5B was interpolated onto the standard curves shown in Figures 3A, 4A, and 5A to obtain a value of each cytokine in g/mL. These data were then graphed as a function of the number of cells per μ L in the well. Figures 3C, 4C, and 5C, show the interpolated cell titration data using the standard curves. This data clearly shows that the LANCE Ultra cytokine detection kits can reliably detect and quantify the amount of cytokines within cell supernatants.

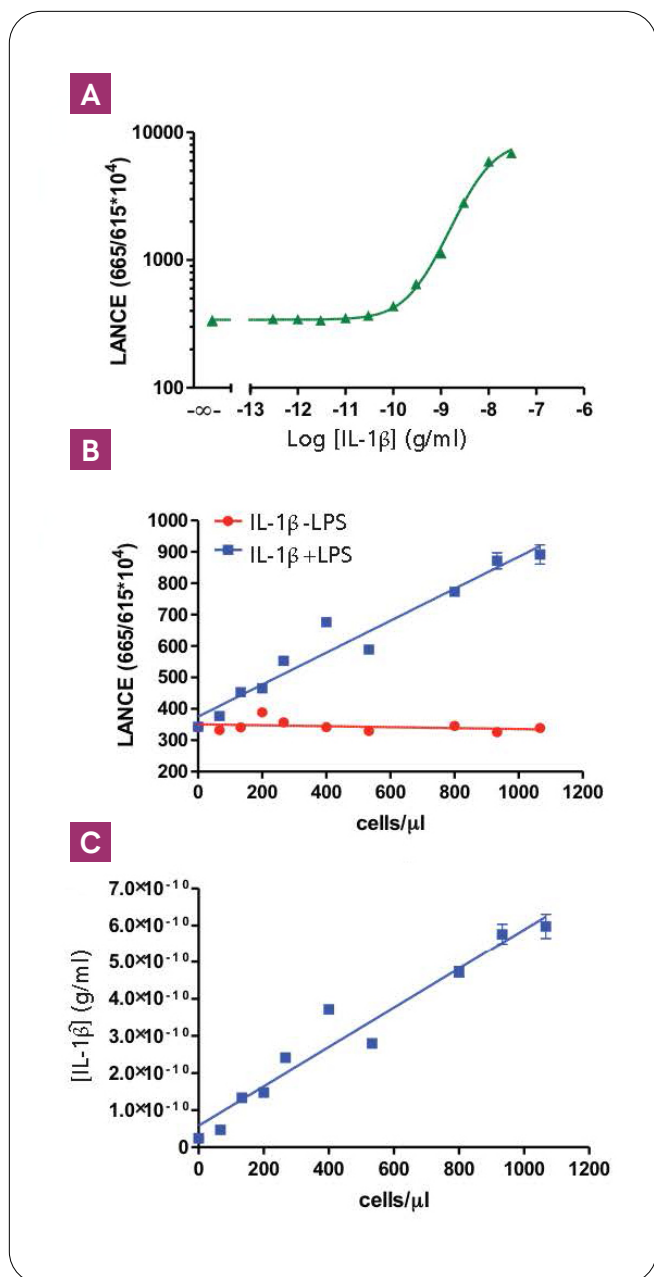


Figure 3: **Quantification of IL-1β secretion.** A) Standard curve for human recombinant IL-1β. B) Signal generated by cells differentiated for 48 hours with PMA and treated with LPS for 21 hours. 15 μL of each concentration were used in the assay. C) Interpolation of the amount of IL-1β secreted by U937 cells based on the standard curve data. Data points are means of triplicates.

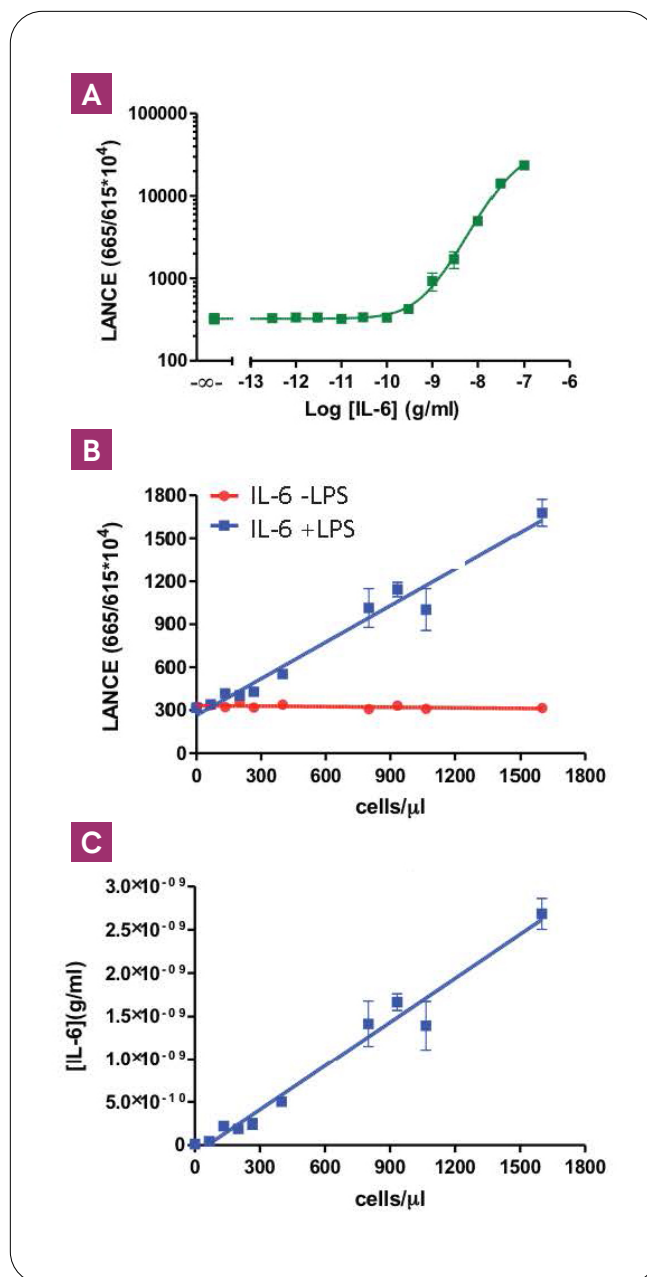


Figure 4: **Quantification of IL-6 secretion.** A) Standard curve for human recombinant IL-6. B) Signal generated by cells differentiated for 48 hours with PMA and treated with LPS for 21 hours. 15 μL of each concentration were used in the assay. C) Interpolation of the amount of IL-6 secreted by U937 cells based on the standard curve data. Data points are means of triplicates.

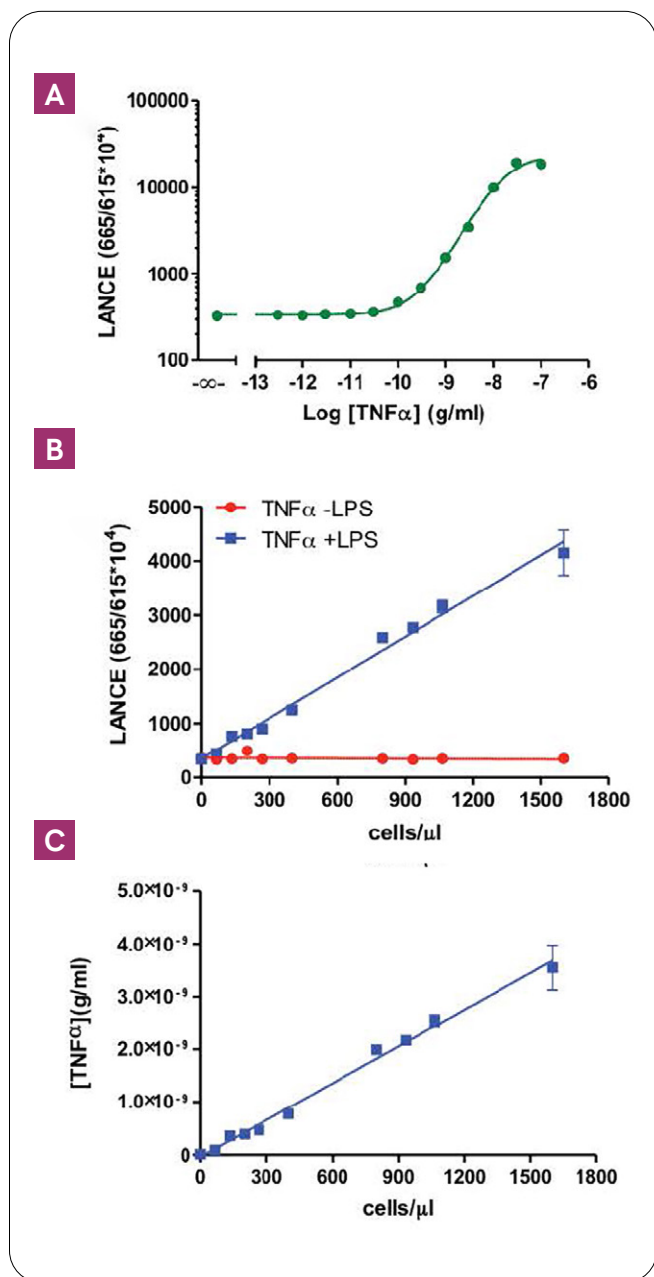


Figure 5: **Quantification of TNFα secretion.** A) Standard curve for human recombinant TNFα. B) Signal generated by cells differentiated for 48 hours with PMA and treated with LPS for 21 hours. 15 μL of each concentration were used in the assay. C) Interpolation of the amount of TNFα secreted by U937 cells based on the standard curve data. Data points are means of triplicates.

Cytokine secretion inhibition by dexamethasone

NF-κB controls transcriptional activity of the promoters of the pro-inflammatory cytokines IL-1β, TNFα and IL-6. Glucocorticoids inhibit NF-κB activity and down-regulate the transcription of cytokines. The effect of the glucocorticoid dexamethasone on secretion of IL-1β, TNFα, and IL-6 was determined using LANCE Ultra kits. Under these conditions, the IC₅₀ for dexamethasone inhibition of secretion of these cytokines were determined. Figure 6 shows the IC₅₀ for TNFα secretion is 5 nM, for IL-1β is 20 nM, and for IL-6 is 2.5 nM.

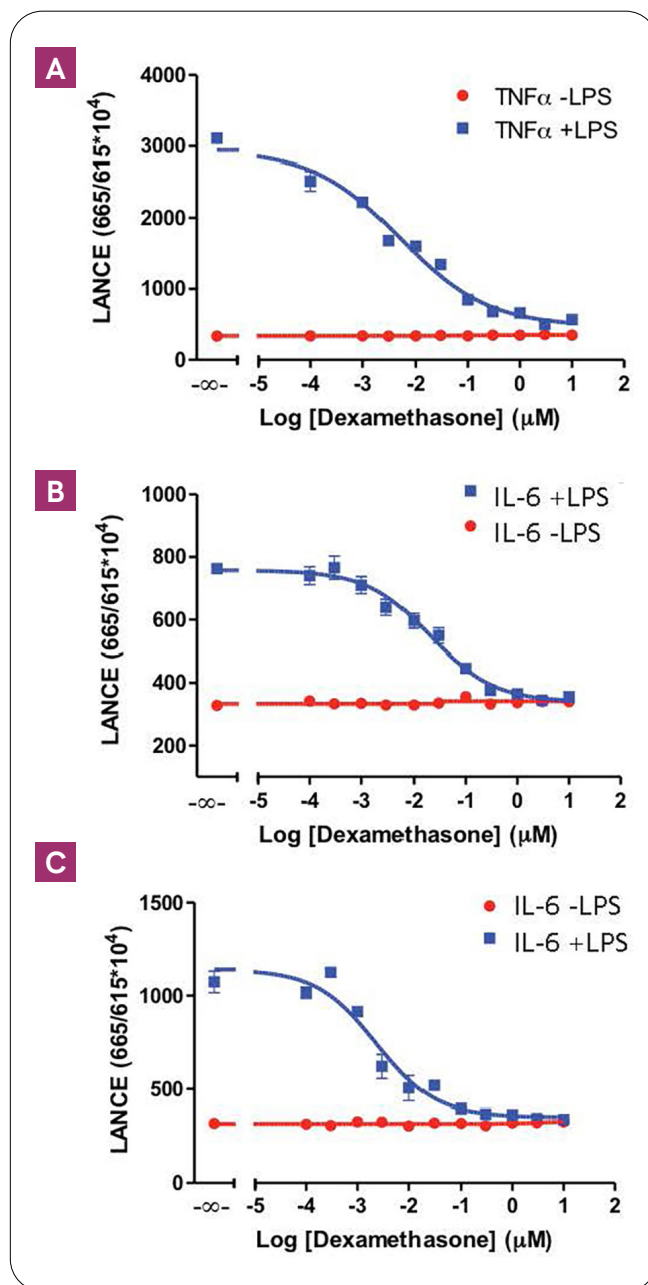


Figure 6: **Secretion inhibition by dexamethasone.** U937 cells were differentiated with PMA for 48 hours and then 800 cells/μL were plated and treated simultaneously with LPS (10 μg/mL) and increasing concentrations of dexamethasone for 21 hours.

Comparison of fresh and frozen samples

It is not often convenient to run an assay the same day that the stimulated cell supernatants are ready to be collected. Therefore, to verify that the LANCE Ultra TR-FRET cytokine kits can reliably measure cytokines in both fresh and frozen samples, the supernatants from the stimulated cells were collected and then the volume split between two plates for testing. Fresh samples were measured right after stimulation treatment and the duplicate plate was carefully sealed and frozen for five days at $-80\text{ }^{\circ}\text{C}$ in 96-well U-bottom StorPlates (Revvity cat# 6008190). The plate was set to thaw at room temperature and then $15\text{ }\mu\text{L}$ of each supernatant was transferred to a 384-well OptiPlate for the assay. As shown in Figure 7, the LANCE Ultra signal is constant between supernatants that were measured freshly after stimulation and those that were frozen at $-80\text{ }^{\circ}\text{C}$.

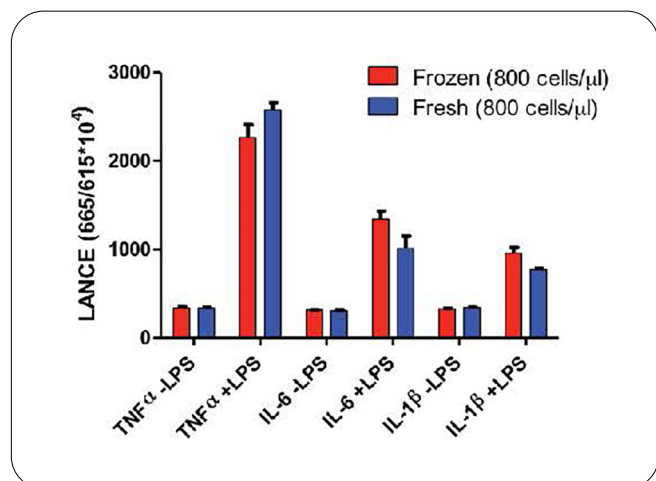


Figure 7: **Comparison of fresh and frozen supernatants.** Fresh and frozen cellular samples (800 cells/ μL) were compared in LANCE TNF α , IL-1 β , and IL-6 cytokine assays.

LANCE Ultra TR-FRET TNF α intraplate variability

High throughput screening of inhibitors of cytokine production requires robust assays with minimal variability in the measurements. One method for determining the robustness of an assay is to measure the intra-plate variability. Figure 8 shows 20 identical samples measuring TNF α secretion from cell supernatants (800 cells/ μL) treated with or without $10\text{ }\mu\text{g/mL}$ LPS. The calculated Z' from the data shown in Figure 8 for this assay is 0.76, indicating suitability for high throughput screening of compounds.

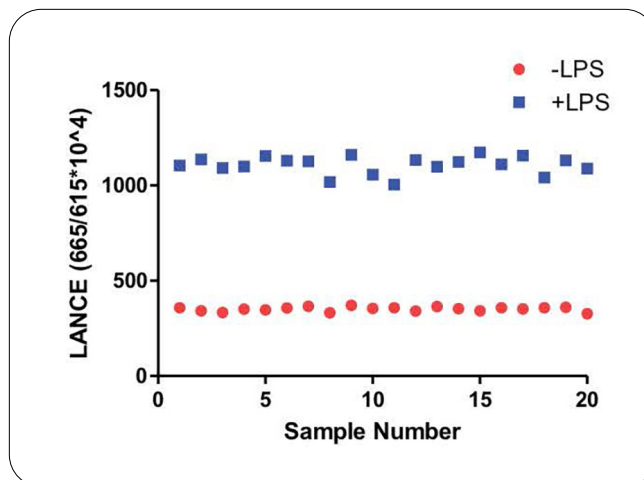


Figure 8: **Intra-plate variability.** TNF α secretion by PMA-differentiated U937 cells was determined by comparing LANCE signal of cells treated or not with LPS for 21 hours.

Assay miniaturization

Successful assay miniaturization requires that an assay maintain the same sensitivity while also providing an acceptable assay window (S/B ratio) as the recommended assay format. Here, we miniaturized the LANCE Ultra TR-FRET TNF α assay from a $20\text{-}\mu\text{L}$ reaction in a 384-well OptiPlate to a $10\text{-}\mu\text{L}$ low-volume reaction in a 384-well ProxiPlate and a $5\text{-}\mu\text{L}$ reaction in a 1,536-well OptiPlate format.

Figure 9A shows a comparison of the TNF α standard curves in all three assay formats. Both high- and low-volume 384-well plate formats show comparable assay sensitivity and assay window (S/B=75), while a slightly lower, but still exceptional assay window (S/B=35) was obtained in the 1536-well format. Assay sensitivity was not affected by miniaturization to a $10\text{-}\mu\text{L}$ reaction, however, the 1536-well assay format has higher LDL (190 pg/ml) compared to those obtained in 384-well plate format. The assay window and sensitivity are still within acceptable ranges for the assay in the 1536-well plate format. As shown in Figure 9B and 9C, the quantification of TNF α in supernatants from differentiated U937 cells is comparable between the two assay formats. In general, LANCE Ultra TR-FRET assays are highly amenable to miniaturization due to the brightness of the Eu chelate compared to other fluorescent based detection assays.

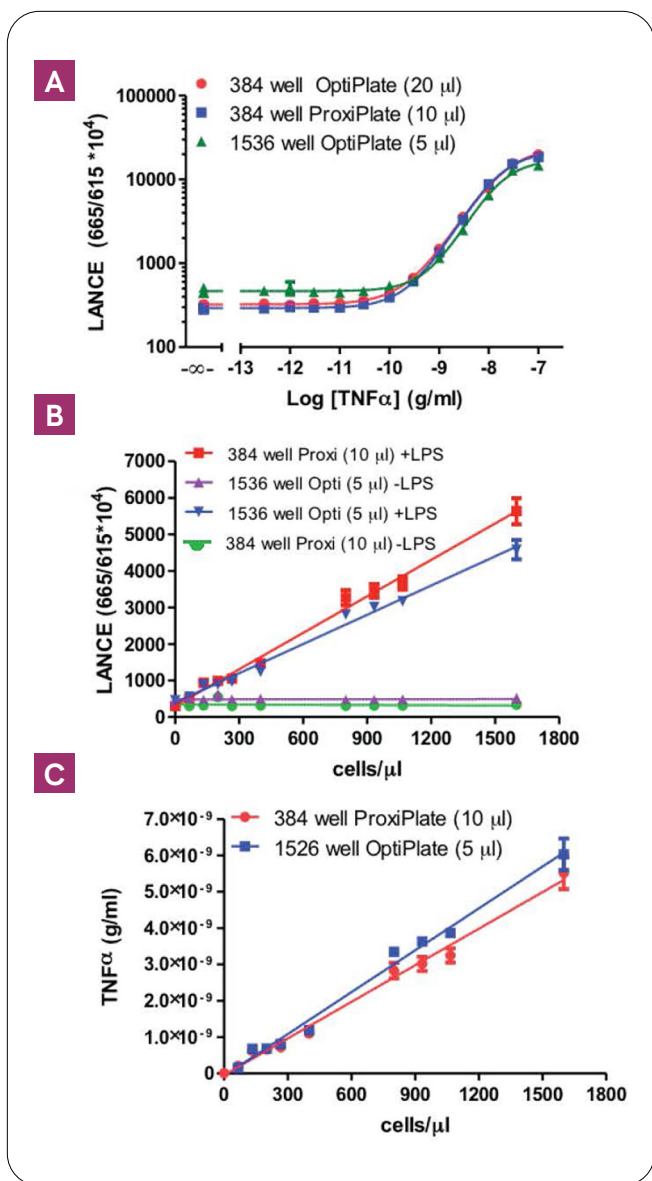


Figure 9: **TNF α miniaturization assays.** A) Standard curve for human recombinant TNF α measured in either a 20- μ L reaction in a 384-well OptiPlate, a 10- μ L reaction in a 384-well ProxiPlate, or a 5- μ L reaction in a 1536-well OptiPlate. B) Signal generated by cells differentiated for 48 hours with PMA and treated with LPS for 21 hours. 7.5 μ L (384-well ProxiPlate) or 3.75 μ L (1536-well OptiPlate) of each concentration were used in the assay. C) Interpolation of the amount of TNF α secreted by U937 cells based on the standard curve data. Data points are means of triplicates.

Dexamethasone inhibition of TNF α secretion was also tested in the miniaturized assay formats. As shown in Figure 10, the IC₅₀ obtained from dexamethasone inhibition (3.1 nM for 10- μ L/384-well format, 4.6 nM for 5- μ L/1536-well format) are similar to those obtained in the 20- μ L format (5 nM). These data show that the assays are highly amenable to miniaturization and well-suited for automation.

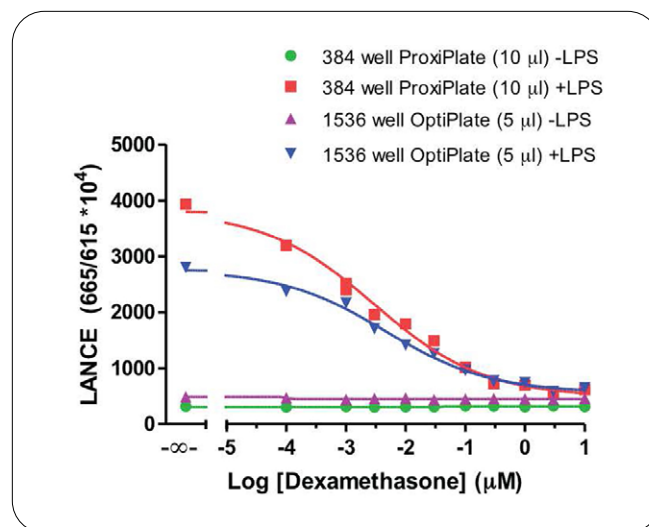


Figure 10: **Dexamethasone Titration.** U937 cells were differentiated with PMA for 48 hours and then 800 cells/ μ L were plated and treated simultaneously with LPS (10 μ g/mL) and increasing concentrations of dexamethasone for 21 hours.

Conclusion

LANCE Ultra TR-FRET kits for the detection of cytokine biomarkers successfully detect multiple cytokines secreted by stimulated U937 cells in RPMI media, with high sensitivity compared values reported with other quantification technologies. Inhibition of IL-1 β , TNF α , and IL-6 secretion by a known inhibitor of inflammatory cytokines (dexamethasone) in a dose dependent manner was demonstrated. Supernatants can be frozen and assayed at a later date without losing signal. In addition, Z'-factor determination illustrates the robust performance and HTS suitability of LANCE Ultra TR-FRET assay formats. Finally, assays are easy to set up and can be reliably miniaturized in a 10- μ L (384-well ProxiPlate) reaction or a 5- μ L (1536-well OptiPlate) reaction.

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