Human Cytokine Gene Expression Analysis Utilizing Novel STATE® and HPSA™ Fluorescent Assay Technology

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Introduction

Cytokines serve as key intercellular mediators for a variety of cytokine-responsive cell types in the maintenance and regulation of the immune system in a normal state, as well as in response to pathogenic states. Their role in pathogenesis, such as septic shock associated with intoxication of Gram-negative or mixed Gram-negative/positive lipopolysaccharide (LPS) antigen, often results in a cytokine storm or massive production of inflammatory cytokines by macrophages and T_H cells.[1] Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β) are two such cytokines secreted by monocyte-macrophages in this inflammatory response.[2,3] The therapeutic inhibition of these cytokine responses is critical in observed pathological patient outcomes.[4] The glucocorticoid hormone, dexamethasone, is a known inhibitor of TNF-α secretion in the human monocyte line,THP-1.[5] The bactericidal glycopeptide, vancomycin, is associated with the inhibition of TNF-α at the transcriptional level in human peripheral blood monocytes (PBMC).[6] Here, it is reported that dexamethasone and vancomycin can regulate the transcription of TNF-α and IL-1β in the human monocyte cell line, THP-1.

The elucidation of temporal cytokine gene expression profiles for multiple targets in response to stimulation with immunogenic biomolecules is paramount to full characterization of an induced cell system. However, common methods such as quantitative RT-PCR (Q-RT-PCR), Northern blots, RNase protection assays, and microarrays are often laborious and expensive, requiring extensive sample manipulation. Additionally, the data obtained may only be relative to a housekeeping gene, may be qualitative, and may display high variability among researchers even in the same lab.[7] The STATE (Simultaneous Transcriptome Analysis of Target Expression) and HPSA (High Performance Signal Amplification) assays provide rapid, absolute quantification of human cytokine response at the gene expression level. Target molecules are quantified using alkaline phosphatase conjugates and a novel, fluorogenic StarBright® Green substrate for highly sensitive detection without target amplification. The Human Cytokine STATE Panel offers quantification of seven targets and a housekeeping gene, while the HPSA Assay represents a single-target assay module. The use of the STATE Panel to develop a temporal cytokine expression profile for human monocytes in response to LPS is described. Pharmacological inhibition of the TNF-α and IL-1β cytokines in this LPS-induced THP-1 cell culture system was determined by analysis with target specific HPSA assays.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Cell Culture

The human monocyte line, THP-1, was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 medium (Product Code R 5886) supplemented with 10% fetal bovine serum (Product Code F 2442), 2 mM L-glutamine (Product Code G 7513), 0.1% β-mercaptoethanol (Product Code M 6250), 100 u/ml penicillin and 100 µg/ml streptomycin (Product Code P 4458). All cell culture was performed at 37 °C, 5% CO_2 in a humidified atmosphere.

Monocyte Stimulation for STATE Analysis

Monocytes were cultured with LPS from *Salmonella minnesota* (Product Code L 6261) at a concentration of 10 µg/ml in T-75cm^2 flasks in 15 ml of RMPI-1640 medium with supplements as previously described. The cultures were induced for up to 8 hours. Uninduced cell samples were harvested at time zero and induced cell samples were harvested at one, two, four, six, and eight hours by centrifugation at 300 *x g* for 5 minutes. Pellets were washed and resuspended with Dulbecco's Phosphate Buffered Saline (Product Code D 8662), collected by centrifugation as previously described and lysed with Lyse-N-Hyb Reagent provided in the kit to a final concentration of 2.0 x 10^6 cells/ml. Lysates were immediately stored at –80 °C for subsequent analysis.

Quantification of Human Cytokine mRNA with STATE Panel

Lysates were assayed for IFN-γ, IL-1β, IL-2, IL-6, IL-8, RANTES, TNF-α and β-actin expression using the Human Cytokine STATE Panel (Product Code ST 1000) according to the protocol supplied by Sigma (Figure 1). Kit components include a 96-well black capture plate, blocking solution, wash buffer, Lyse-N-Hyb Reagent, target-specific detection probe, *in vitro* transcript standard, *in vitro* transcript positive and negative control, alkaline-phosphatase labeled streptavidin, StarBright Green fluorogenic substrate, and stop solution. Briefly, 100 µl of lysate was captured on a covalently linked probe immobilized in each well of the 96-well plate and simultaneously hybridized with 10 µl of biotinylated detection probe. All samples were analyzed in duplicate. Standard curves for absolute

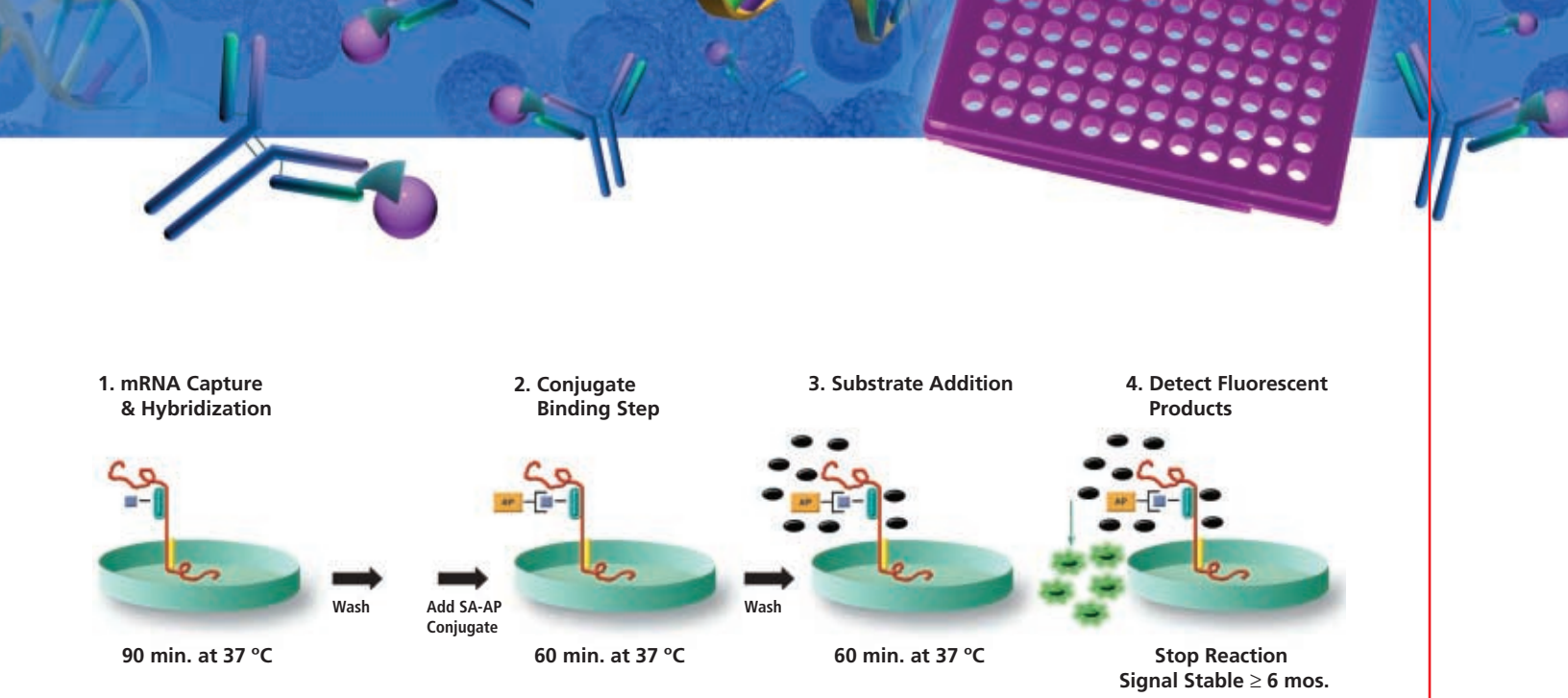

Figure 1. Overview of STATE Panel and HPSA Assay Procedure.

quantification of the targets were generated in triplicate using *in vitro* transcripts for each target at concentrations of 500, 125, and 31 attomoles/well or 1000, 250, and 62 attomoles/well for IL-8. Hybridization was carried out at 37 °C at 500 rpm for 90 minutes. The plate was washed three times with 200 µl/well wash buffer. The biotinylated target complex was probed with 100 µl/well alkaline phosphatase-labeled streptavidin for 60 minutes at 37 °C. The plate was washed three times with 200 µl/well wash buffer. Bound alkaline phosphatase was detected using 100 µl/well of the StarBright Green fluorogenic substrate for 60 minutes at 37 °C. The reaction was stopped with the addition of 100 µl/well stop buffer. Fluorescent product was immediately detected at excitation 440 nm and emission 510 nm using the Wallac Victor 2™ (Perkin Elmer Life Sciences, Boston, MA) plate reader. Microsoft Excel and SigmaPlot were used for statistical analysis.

Pharmacological Inhibition of LPS-Induced IL-1β and TNF-α Gene Expression

For determination of dexamethasone regulation of IL-1β and TNF-α gene expression, THP-1 cultures were pretreated with dexamethasone (Product Code D 4902) at concentrations of 100 µM, 10 µM, and 1 µM for 30 minutes in T-25cm^2 flasks in 5 ml of RMPI-1640 medium supplemented as described above. Monocytes were then stimulated with 10 µg/ml LPS. Uninduced samples were harvested at time zero and three hours. Induced cell samples were harvested at 45, 90, 135, and 180 minutes. Dexamethasone-treated cell samples were harvested at 45, 90, 135, and 180 minutes. All cells were harvested following the method described under "Monocyte Stimulation for STATE Analysis" with the exception that lysates were collected at a concentration of 1.0 x 10^6 cells/ml. Determinations for uninduced dexamethasone-treated samples were included as a negative control.

For determination of vancomycin regulation of IL-1β and TNF-α gene expression, THP-1 cultures were simultaneously treated with vancomycin (Product Code V 1130) at concentrations of 1000 µg/ml, 100 µg/ml, and 10 µg/ml and LPS at 10 µg/ml in T-25cm^2 flasks in 5 ml of RMPI-1640 medium supplemented as described above. Samples were harvested at time points of 45, 90, 135, and 180 minutes as described for dexamethasone-treated samples. Determinations for uninduced vancomycin-treated samples were included as a negative control.

Quantification of IL-1β, TNF-α and GAPDH mRNA with HPSA Assays

Lysates were assayed for IL-1β, TNF-α and GAPDH expression using the HPSA Gene Expression Kits (Product Codes HP1000, HP4000 and HK1000) according to the protocol supplied by Sigma and as outlined under "Quantification of Human Cytokine mRNA with STATE Panel." Specifically, standard curves were generated in duplicate for quantification of 500, 250, 125, 62, and 31 attomoles/well for IL-1β and TNF-α or 1000, 500, 250, 125, and 62 attomoles/well for GAPDH. All samples were analyzed in duplicate.

Results and Discussion

The Human Cytokine STATE Panel was used to simultaneously identify transcription levels of multiple cytokines following stimulation of THP-1 human monocytes with 10 µg/ml LPS for up to 8 hours. Gene expression was determined for the cytokines IL-1β, IFN-γ, IL-2, IL-6, RANTES, TNF-α, IL-8 and housekeeping gene, β-actin (Figure 2). Absolute quantification of the eight mRNA targets was determined using the standard curves generated for each target using *in vitro* transcripts, all of which were linear with an R-squared value of 0.98 or greater (Figure 3). The uninduced lysates had high levels of endogenous IL-8 expression, at 160 attomoles per 1.0 x 10^6 cells. Significant baseline expression of IFN-γ, IL-2, TNF-α, and IL-1β was observed with 43, 13, 7.6, and 4.0 attomoles per 1.0 x 10^6 cells detected, respectively. IL-8 expression was elevated throughout the entire eight-hour induction, with peak expression of 690 attomoles at eight hours. Expression of IFN-γ was slightly elevated one hour post-induction at 53 attomoles and declined thereafter. IL-2 expression was above baseline, ranging from 17-24 attomoles at all timepoints, with the excep-

tion of two hours, when expression diminished below baseline. RANTES expression was induced at four hours and increased to 14 attomoles at eight hours. IL-6 expression was not detected at any point following LPS-stimulation, up to eight hours. Furthermore, IL-1β expression peaked at 48 attomoles at four hours post-stimulation, while TNF-α expression peaked at two hours, with 13 attomoles detected.

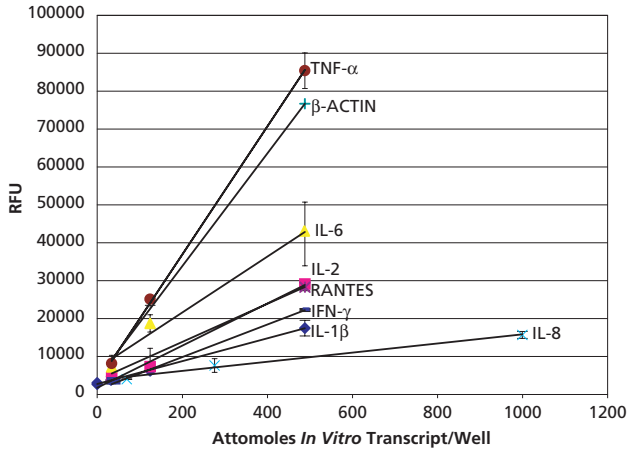

Figure 2. STATE assay standard curves generated from in vitro transcripts. *The mean signal and standard deviation for each standard assayed in triplicate are reported.*

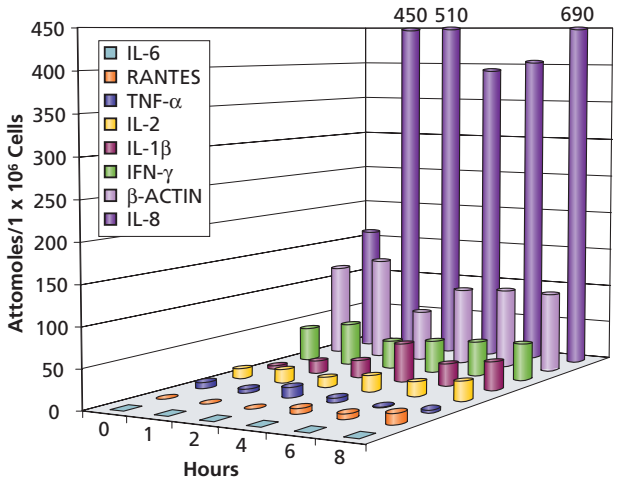

Figure 3. Temporal expression profile of cytokine mRNA detected following LPS-stimulation of THP-1 cells. *Expression levels of several human cytokines and the housekeeping gene β-actin were determined for up to eight hours following treatment with 10 μg/ml LPS using the STATE assay. IL-6 expression was not detected under these experimental conditions.*

To further investigate the LPS-induced early expression of IL-1β and peak TNF-α expression in a biological context and in the presence of potential inhibitors, the HPSA single-target assay modules were utilized. IL-1β, TNF-α and the housekeeping gene, GAPDH, were assayed at 45, 90, 135, and 180 minutes in the presence of dexamethasone at concentrations of 1, 10, and 100 μM and vancomycin at concentrations of 10, 100, and 1,000 μg/ml, as well as LPS-stimulated alone (Figure 4). The time course data obtained suggests that GAPDH expression increases with LPS-stimulation. At three hours the GAPDH expression in the uninduced samples is at 56 attomoles/1 x 10^6 cells, whereas the induced samples expressed at 100 attomoles/ 1 x 10^6 cells (Data not shown). The expression of IL-1β was highest at the three-hour time point, with 17 attomoles expressed, compared with a baseline level determination of 5.4 attomoles. TNF-α expression was highest between 135 and 180 minutes with greater than 15 attomoles expressed compared to baseline levels of 13 attomoles.

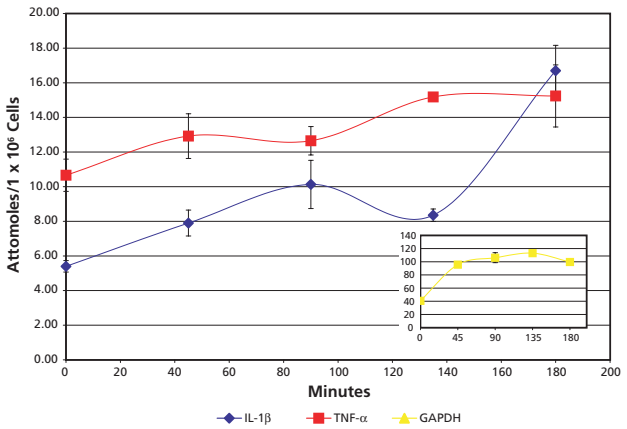

Figure 4. Expression of IL-1β and TNF-α in LPS-stimulated THP-1 cells. *THP-1 human monocytes were stimulated with 10 μg/ml LPS and demonstrate increased expression levels of IL-1β and TNF-α up to 3 hours, at 45-minute intervals. Levels of the housekeeping gene GAPDH are also reported.*

Examination of the effects of dexamethasone and vancomycin at the early time-point of 45 minutes post-LPS-induction and pharmacological treatment demonstrated significant inhibition of IL-1β expression in a dose-dependent manner for both dexamethasone and vancomycin (Figures 5A, 5B). However, the significant inhibition of IL-1β at the 100 μM dexamethasone concentration may be related to a potential toxic effect of the hormone, as GAPDH levels also significantly decreased (Figures 5E, 5F). Vancomycin was shown to significantly down-regulate IL-1β expression at a concentration of 100 μg/ml. Although dexamethasone and vancomycin have been reported to inhibit TNF-α expression in response to LPS stimulation in similar cell culture systems, this was not observed at 45 minutes (Figures 5C, 5D) and may occur at later time points.

The HPSA and STATE fluorescent assays provide a highly sensitive and reproducible method for absolute quantification of human cytokine gene expression for a variety of applications, including temporal and pharmacological analysis. The utilization of lysates allows for rapid and high-throughput sample collection for subsequent analysis, and the multiwell format is highly amenable to automated platforms. The use of these assays for analysis of LPS-induced cytokine expression in a THP-1 human monocyte cell culture system demonstrates the utility of

the multi-target STATE Panel in the simultaneous determination of a cytokine expression profile for eight targets with a wide dynamic range. Furthermore, the single-target HPSA assays provide a flexible format for more extensive analysis of specific cytokine targets.

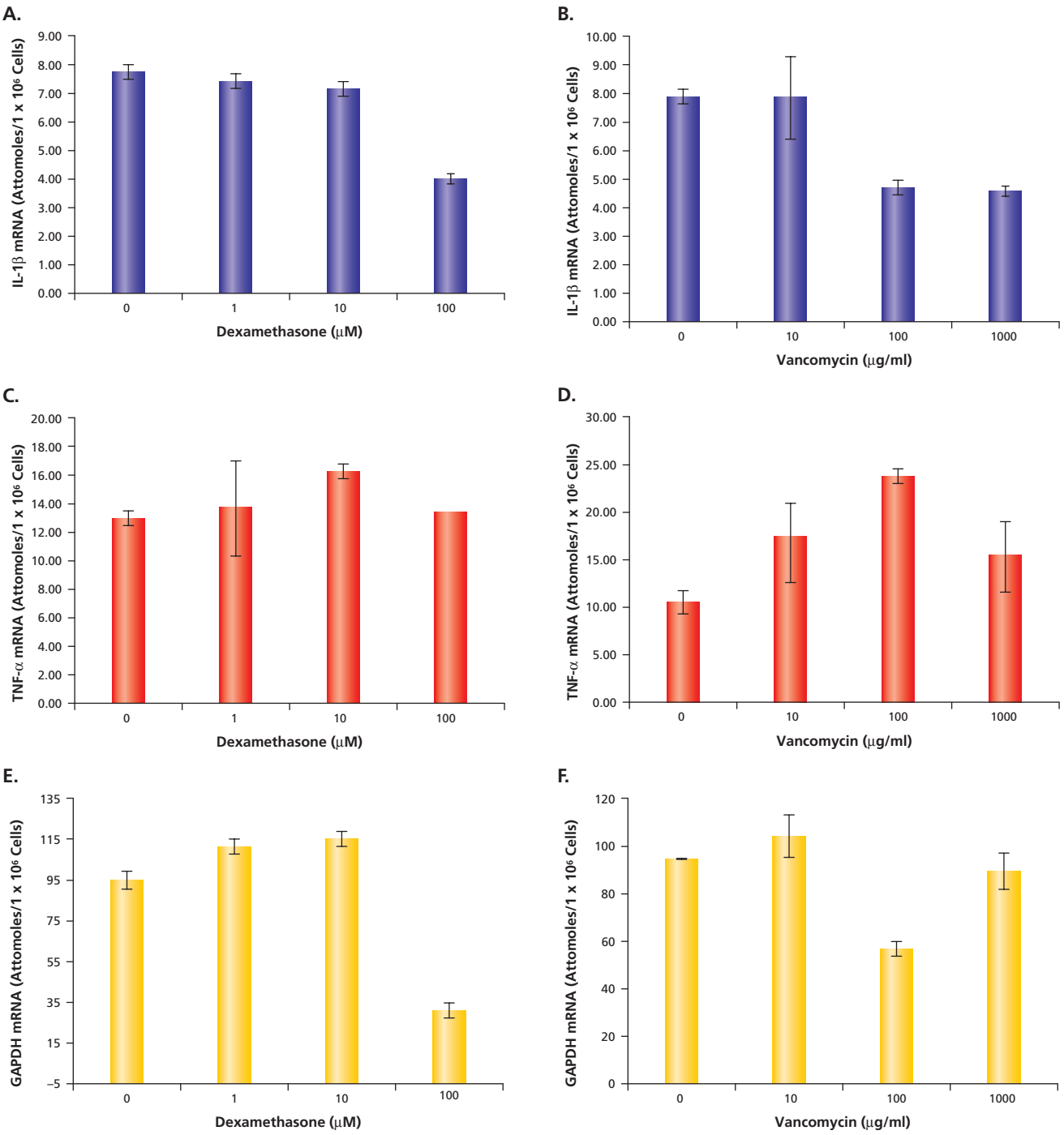

Figure 5. Inhibition of IL-1β expression in LPS-induced THP-1 cells. *The effects of various concentrations of dexamethasone and vancomycin were tested at 45 minutes post LPS stimulation. Dexamethasone* ***(A)*** *and vancomycin* ***(B)*** *were assayed at concentrations indicated and the effect on IL-1β expression was determined. Dexamethasone* ***(C)*** *and vancomycin* ***(D)*** *at same concentrations and effect on TNF-α expression were determined. Effects of the potential immunomodulators dexamethasone* ***(E)*** *and vancomycin* ***(F)*** *on expression of the housekeeping gene GAPDH were determined.*

Acknowledgements

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Ordering Information

Product	Description	Unit
ST1000	Human Cytokine Panel Gene Expression Kit	1 kit
HP1000	IL-1β Human Gene Expression Kit	1 kit
HP4000	TNF-α Human Gene Expression Kit	1 kit
HK1000	GAPDH Human Gene Expression Kit	1 kit

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