Quantification of cytokines on the SpectraMax® Paradigm® Multi-Mode Microplate Detection Platform using Alpha Technology

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Abstract

Inflammation is accompanied by increased endothelial chemokine production and adhesion molecule expression, which may result in an extensive neutrophil infiltration. As such, the search for novel anti-inflammatory substances able to downregulate these parameters, as well as the tissue damage, holds therapeutic promise. Here we describe how Alpha Technology, a bead-based, homogeneous assay for studying molecular interactions in a microplate format, has been used to detect anti-inflammatory metabolites from cyanobacteria in human endothelial cell-based *in vitro* assays. We show the quantification of cytokines (TNF-alpha) down to picogram levels with Molecular Devices' SpectraMax® Paradigm® Modular Multi-Mode Reader. Alpha Technology can be performed on the SpectraMax Paradigm Reader in various plate formats ranging from 96-well to 1536-well.

TNF-alpha Purification and Quantification

Pichia pastoris is a popular host for production of recombinant proteins. Due to its strong and inducible AOX (Alcohol Oxidase) promoter the system can produce up 35% of total cellular protein as recombinant protein. A further advantage of the system is the absence of endotoxins and therefore the lack of pro-inflammatory components which could potentially interfere with downstream human based cell culture assays. We have expressed the human cytokine TNF-alpha in the cytoplasm of *Pichia pastoris* and purified the protein with Immobilized Metal Affinity Chromatography (IMAC) and subsequent gel filtration. With the above described method we are able to purify 10-20 mg of biologically active protein per liter culture volume. In order to quantify the protein we have used the AlphaLISA® technology since this method is capable of analyzing protein samples down to the low picomolar level (see Figure 1).

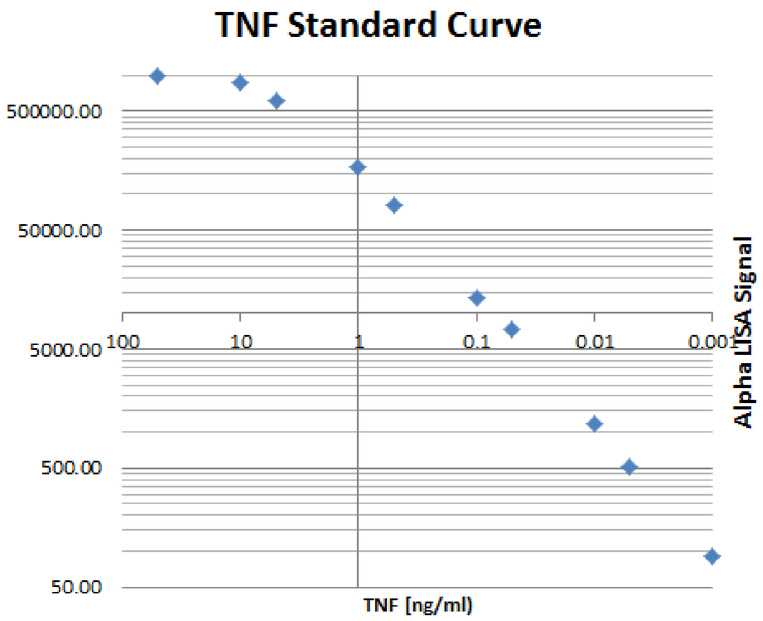


Figure 1: TNF-alpha quantification with AlphaLISA down to the low picomolar level. Samples were distributed in triplicate into a white, opaque 384-well plate and read on the SpectraMax® Paradigm® Multi-Mode Microplate Reader. We have shown an assay window of about 5 decades.

Detection of Pro-Inflammatory Factors

Human lung microvascular endothelial cells (HL-MVEC) were grown in 96-well plates (2x105 cells/mL) , allowed to adhere for 6 hours, and then incubated for 30 minutes with 10 ng/mL human recombinant TNF-alpha. Intercellular Adhesion Molecule 1 (ICAM-1) expression was up-regulated upon 18 hours of incubation with the pro-inflammatory cytokine, human Tumor Necrosis Factor (hTNF). Subsequently, the level of ICAM-1 expression was quantified by AlphaLISA® Detection Technology.

IL-8 is a potent chemoattractant for neutrophils and is secreted by activated (TNF-alpha or LPS stimulated) endothelial cells. Quantification of secreted IL-8 from endothelial cells can therefore provide valuable information about the pro-inflammatory status of vascular endothelial cells in vitro. To assess the level of secreted IL-8 we analyzed the cell culture supernatant of stimulated HL-MVEC cells using AlphaLISA.

Figure 2: Up-regulation of ICAM-1 upon TNF–alpha stimulation for 18 hours in HL-MVEC cells, measured using AlphaLISA. Detection was performed with the SpectraMax® Paradigm® Multi-Mode Microplate Reader.

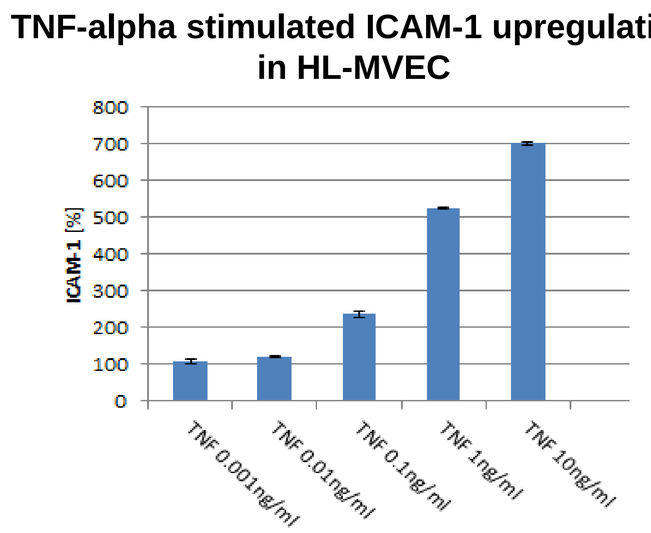
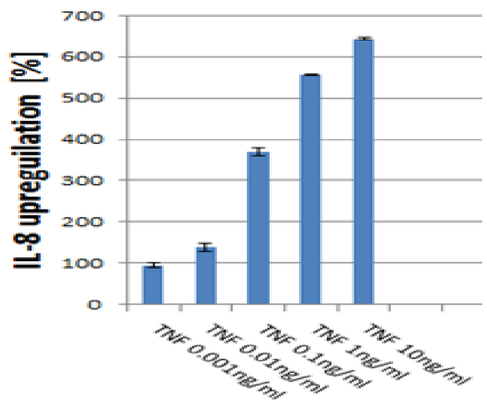


Figure 3: Up-regulation of secreted IL-8 upon TNF-alpha stimulation for 18 hours on HL-MVEC cells.



An Endothelial Cell-Based Assay for Identification of Anti-Inflammatory Compounds from Cyanobacteria

In view of the remarkable diversity of their metabolites, cyanobacteria have been shown to contain therapeutically promising compounds, some of which are currently being tested for the treatment of inflammatory diseases (1,2,3). We have used the methods described above to screen for anti-inflammatory compounds from cyanobacteria. For this purpose we pre-treated HL-MVEC cells with different fractions isolated from *Nostoc* sp. and subsequently stimulated with 0.4 ng of human TNF-alpha. 18 hours later, ICAM-1 up-regulation was detected with AlphaLISA technology, and cell culture supernatants were further analyzed for IL-8 secretion. We found that fraction 8721,6 (circled in figure below) showed a significant reduction in ICAM-1 production. This fraction also showed a clear reduction in IL-8 secretion (data not shown). Fractions were tested for cytotoxicity prior to AlphaLISA and shown to have negligible effect on cell health.

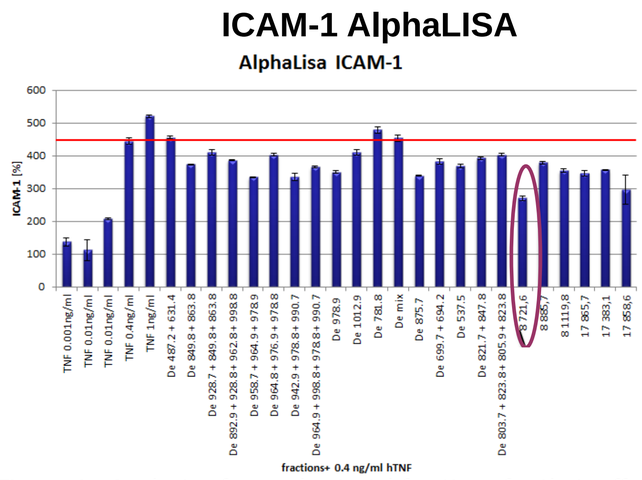


Figure 4. Analysis of cyanobacterial fractions for their effect on ICAM-1 up-regulation in response to TNF-alpha stimulation.

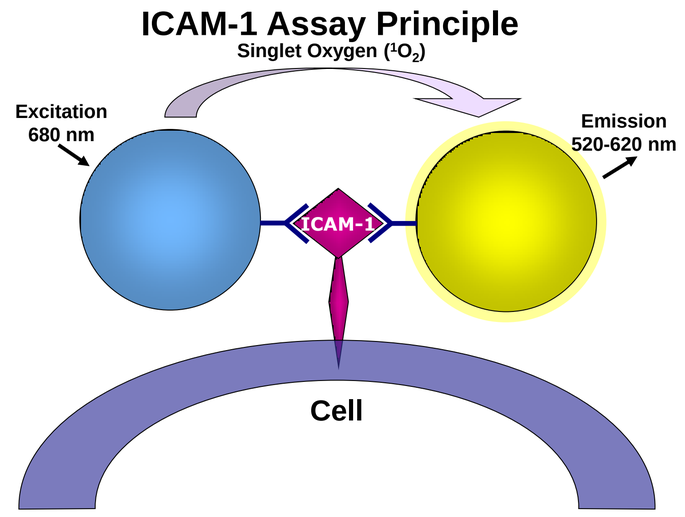


Figure 5. ICAM-1 on the cell surface is bound by antibodies located on the surface of donor and acceptor beads, bringing the beads into close proximity to each other and enabling excitation of the acceptor bead by singlet oxygen. Emission at the indicated wavelengths is detected as a readout of the amount of ICAM-1 present on the cell surface.

SpectraMax® Paradigm® Multi-Mode Microplate Reader

Advantages of SpectraMax® Paradigm® System

- Flexible design for future expansion
- User upgradable in < 2 minutes
- High speed & sensitivity

Benefits for Alpha Technology

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- Easy upgrade to AlphaScreen capability
  - AlphaScreen cartridge can be added to any Paradigm system, any time.
- Pre-configured protocols
  - Get your results faster.
- Better data, less crosstalk
  - Microplate optimization
  - Interlaced reading to reduce crosstalk

Summary

We report here the use of AlphaLISA Technology for establishment of a pathologically relevant, endothelial cell-based model using the SpectraMax® Paradigm® Multi-Mode Microplate Reader.

Furthermore, we have shown that this model is capable of screening and identification of anti-inflammatory secondary metabolites from cyanobacteria.

References

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Romay, C.; Armesto, J.; Remirez, D.; Gonzalez, R.; Ledon, N.; Garcia, I. *Inflamm. Res.* **1998**, *47*(1), 36.[31] Prinsep, M. R.; Thomson, R. A.; West, M. L.; Wylie, B. L. *J. Nat. Prod.* **1996**, *59*(8), 786.

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