Translational Medicine

Lakkis et al. Translational Medic 2012, S:1
DOI: 10.4172/2161-1025.S1-006

Research Article Open Access

Novel Biomarkers Detection and Identification by Microfluidic-Based MicroELISA

Montaha Lakkis[1]*, Junhai Kai[1], Nelson Santiago[1], Aniruddha Puntambekar[1],Victor Moore[1], Se Hwan Lee[1], David W Sehy[1], Ron Schultheis[1], Jungyoup Han[1] and Chong H Ahn[1,2]

[1]*Siloam Biosciences, Inc., Cincinnati, OH, USA*
[2]*School of Electronics and Computing Systems, University of Cincinnati, Cincinnati, Ohio, USA*

Abstract

Miniaturized, high throughput detection technologies including microfluidics systems represent powerful tools for biomarker discovery and analysis. Optimiser™ microplate technology combines microfluidics technology with standard SBS-configured 96-well microplate architecture and allows for the improvement of ELISA workflows. In this review, we present the "standard" and "high sensitivity" capabilities of the Optimiser™ ELISA in detecting human cytokine biomarker; IL-4, resulting in improving sensitivity by 1000-fold higher than the typical conventional ELISA. The Optimiser™ ELISA microplate employs standard ELISA equipment and protocols, selectively reduces sample volume 10-20 fold (Static Mode) and has the capability to amplify assay sensitivity by 1,000 fold (Repetitive Loading Flow-Through Mode) relative to conventional high sensitivity ELISA approaches. Optimiser™ allows sensitive and quantitative detection of biomarkers with demonstrated reproducibility, speed and linearity. In this study we demonstrate the utilization of Optmiser™ in the amplification of low-concentration markers. Optimiser™ microfluidic-based technology represents a revolutionary advancement in ELISA technology, and holds great promise for accurate, sensitive detection of novel biomarkers and its potential applications in clinical diagnosis.

Keywords: Optimiser™ Micro plate; Biomarker detection; Blow sample volume; Low abundant protein; Sandwich ELISA; High Sensitivity; Micro fluidics

Introduction

Microfluidic-based microplates for biomarker discovery & quantification: Biomarkers represent powerful tools for diseases detection, determining the efficacy and safety of novel therapeutic agents and monitoring disease recurrence.

Identification of key biomarkers at early stages of diseases that can be easily obtained by the least invasive methods is critical for early diagnosis of cancer diagnosis. Human body fluids, such as blood, present a promising resource for biomarkers. Routine detection of biomarkers is critical to bring substantial advances in molecular medicine and transform many areas of medicine from clinical trials to diagnosis and treatment of complex diseases [1,2].

Survival of cancer patients depends heavily on early detection and therefore, developing new technologies allows for a quick, sensitive and cost effective method is critical for cancer research [3]. For instance, early detection is an important factor in determining the survival of patients with hepatocellular carcinoma (HCC) [3,4]. However, early diagnosis of cancer is still difficult to achieve due to the lack of specific symptoms in early stages of the disease where detection of biomarkers is still unattainable due to low detection levels.

Recent advances in detection technologies hold promises to identify novel biomarkers reliably and accurately to predict outcomes during cancer treatment and management [4,5]. Additionally, the new technologies provide higher analytical capabilities, employing automated liquid handling systems for greater sensitivity, more robust and higher throughput sample analysis. A range of micro-scale and nano-scale approaches are becoming available for biomarker discovery and analysis. Miniaturized, low-cost microfluidic technologies hold great promise for accurate, sensitive and rapid detection of biomarkers. Optimiser™ Microplate technologies emerging as a suitable platform for biomarker discovery and validation by incorporating the power of microfluidics into a standard ELISA plate with dramatic improvement in reaction efficiency and signal sensitivity in comparison with the standard assay. The microfluidic channel at the base of each well serves as the "reaction chamber" thereby cutting down reaction volumes, reducing incubation times and simplifying assay operation by replacing the traditional labor-intensive wash with a simple "flush" step. The high surface area to volume ratio of the Optimiser™ allows for ultra-fast reaction kinetics translating to ~5-10 min incubation cycles per step in an assay sequence. Optimiser™ assays using similar protocols as conventional assays show comparable sensitivity with only 10 µL sample volume. Alternately, if the sample loading and incubation is repeated, the Optimiser™ allows for extraordinary gains in sensitivity using the same assay reagents. Optimiser™ uses the same equipment as conventional microplates including liquid handling systems for high-sensitivity mode. This greater sensitivity enables direct detection of low abundant proteins in body fluids. Another major advantage for this technology is the minimal sample and reagents requirement which allows the direct use of precious and perishable clinical samples while shortening the overall assay run to results time.

Optimiser™ microfluidic-based immunoassays for accurate and sensitive detection of protein biomarkers: Enzyme-linked immune sorbent assays (ELISA) are a workhorse technology for the quantification of a broad range of analytes from complex matrices, such as serum and plasma and cell lysates. Assay specificity and sensitivity are enabled through the use of high affinity antibodies that are used to capture and detect analyte by two distinct epitopes, reducing the likelihood of cross-reactivity. The format of the sandwich ELISA is still considered the gold standard for reliable and efficient protein detection platform and is used extensively in both research and

***Corresponding author:** Montaha Lakkis, Siloam Biosciences, Inc., Cincinnati, USA, Email: mlakkis@siloambio.com

Received November 22, 2011; **Accepted** February 14, 2012; **Published** February 16, 2012

Citation: Lakkis M, Kai J, Santiago N, Puntambekar A, Moore V, et al. (2012) Novel Biomarkers Detection and Identification by Microfluidic-Based MicroELISA. Translational Medic S1:006. doi:10.4172/2161-1025.S1-006

clinical applications. However sandwich ELISA has not witnessed any significant improvement since its early onset. Current immunoassays typically measure proteins at concentrations around pictogram/mL to nanogram/mL[3]. But there is still the need to detect low abundant proteins, including some cytokines, which are expressed in serum at the femtogram level [6,7,8]. There are some high sensitive immunoassay technologies which have been developed recently [7,9,10,11] but most of them require special preparation or conjugation in reagents, or/and expensive equipment.

A novel Optimiser™ microplate combines the typical SBS footprint and layout of a 96-well microplate with a dedicated microfluidic channel connected to each well where assay reactions occurs (Figure 1). The conventional 96-well layout allows for the use of conventional peripheral microplate instrumentation such as automated pipetting stations and microplate readers; the microchannel dimensions (200 x 200 µm) promotes efficient binding reactions by a significant increase in surface area to volume ratio resulting in a superior binding efficiency for capture antibody binding compared to standard ELISA techniques. The Optimiser™ -based ELISA use the standard ELISA workflow but uses volumes significantly lower than those used in conventional ELISAs. Moreover, there is no need to use microplate washers. As each microchannel has a volume of only ~5 µL, the addition of excess wash buffer to the well flushes out the microchannel contents onto an absorbent pad beneath the microplate, simulating a wash process. The microchannel is arranged in a spiral pattern directly below the well of the microplate and with the final addition of substrate, defines the detection volume. As the area of the microchannel spiral is similar to a microplate well, a conventional fluorescence microplate reader can be used for detection.

In this study, we demonstrate assay performance using human IL-4 as an analyte and show the ability to tune assay sensitivity by repeating analyte loading/incubation cycles.

Material and Methods

Reagents and assay material

Purified monoclonal capture antibody (Clone 8D4-8), biotinylated monoclonal detection antibody (clone MP4-25D2) and recombinant human IL-4 protein (catalog # 14-8049) were purchased frome Bioscience (San Diego, CA). Ten percent fetal bovine serum in 1x RPMI cell culture medium (catalog# R1145, Sigma, St. Louis, MO) was used as diluent for IL-4 recombinant protein. Optimiser™ Plate (catalog# OPH-2) and OptiMax™ Reagent Pack (catalog# OMR-2 including OptiPrime™ priming buffer, OptiBind™ coating buffer, OptiWash™ wash buffer, OptiBlock™ blocking buffer) were manufactured internally at Siloam Biosciences (Cincinnati, OH). NUNC ELISA MaxiSorp® 96-well plates (Fisher Scientific, Santa Clara, CA) were used in comparison test.

Equipment

Detection of the fluorescent signal from the Optimiser™ plate was performed with an FLx800™ Fluorescence Microplate Reader (BioTek® Instruments, Winooski, VT), with 528/20 nm excitation filter and 590/35 nm emission filter, with sensitivity set at 45. Detection of colorimetric signal from the NUNC plate was performed with a ChroMate® Absorbance Microplate Reader (Awareness Technology, Palm City, FL).

The Precision™ Microplate Pipetting System (BioTek® Instruments, Winooski, VT) was used for multiple loading of analyte (IL-4) for experiments tuning assay sensitivity and for reagent addition (Figure 2). The instrument can operate up to 3 Optimiser™ ELISA plates in one run. A customized plate holder has been used for Optimiser™ ELISA plate in this study.

Optimiser™ plate operation

Figure 3 demonstrates the principle of Optimiser™ operation.

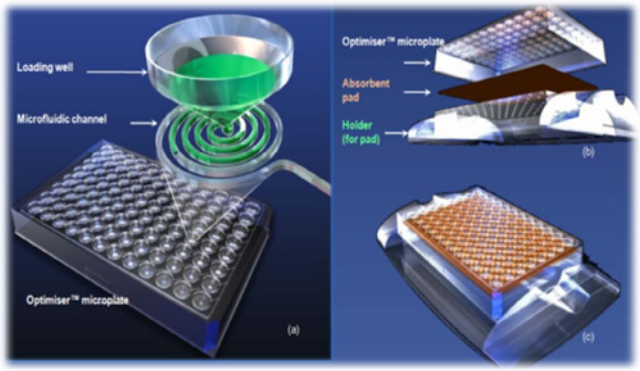

Figure 1: Optimiser™ microplate illustration: (a) Optimiser™ microplate with magnified view of one cell; (b) exploded and (c) assembled view of Optimiser™ microplate with absorbent pad and holder.

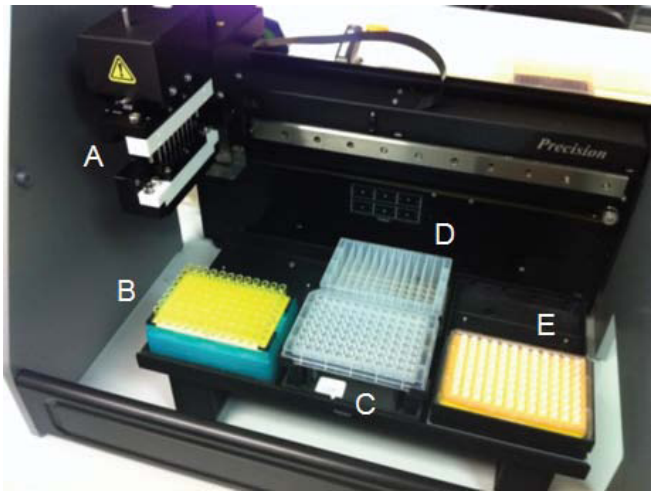

Figure 2: Precision Microplate Pipetting System layout for Optimiser™ automation. A: 8-channel pipetting head; B: Tip box; C: Sample (i.e. IL-4) reservoir; D: Reagent reservoir; E: Optimiser™ plate.

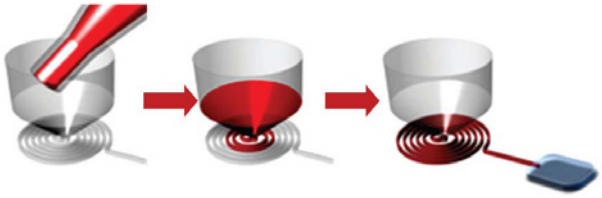

Figure 3: Principle of Optimiser™ operation – initial well loading with reagent serves to load microchannel. Once a well is empty and microchannel filled, capillary forces retain fluid in the microchannel for an incubation step. Addition of subsequent reagents relieves capillary forces causing flushing of initial reagent into an absorbent pad beneath the plate (shown on the side in the figure for clarity). Once the well is again empty, capillary forces induce another incubation step.

A slight excess of first reagent (10 µL), relative to the volume of the microchannel, is loaded in the well. Capillary forces transport the reagent to the microchannel and slightly further to the absorbent pad. The reagent will drain from the well but flow stops due to capillary forces when the well is empty but still filling the channel. This step serves as an incubation step. The next reagent addition to the well breaks the capillary barrier at the inlet of the microchannel and flow resumes until reagent 2 is drawn from the well completely and flow will stop again.

IL-4 Optimiser™ ELISA assay

The Optimiser™ assay mirrors the steps involved in conducting a typical sandwich ELISA. The entire assay is performed in room temperature. Briefly, after assembling the Optimiser™ Plate (with absorbent pad and holder) and priming with 10 µL of priming buffer, 10 µL OptiBind™ coating solution containing 2 µg/mL IL-4 capture antibody was dispensed into wells of Optimiser™ plate and incubated for 10 minutes. Then 10µL Opti Block™ blocking buffer was dispensed into wells and incubated for 10 minutes. Subsequently, for a single-loading assay, 10 µL recombinant human IL-4 was dispensed into the wells and incubated for 10 minutes. For multiple-loading assay, 5 µL recombinant human IL-4 was dispensed into the wells and incubated for 5 minutes and then repeated for multiple cycles. Then 10 µL Opti Wash™ wash buffer was dispensed into the wells and soaked for 10 minutes to "flush" the microchannel. After "flushing", 10 µL OptiBlock™ blocking solution containing 2 µg/mL IL-4 detection antibody was added and incubated for 10 minutes. Following a flush with 10 µL wash buffer the wells were loaded with 10 µL HRP-conjugated streptavidin diluted in blocking buffer solution for 10 minutes. Following two "flushes" with 30 µL wash buffer each, the plates were loaded with 10 µL of OptiGlow™ chemifluorescence substrate working solution into each well. The captured horseradish peroxidase on the microchannel surface reacts with the substrate solution and yields a fluorescent signal when excited at the appropriate wavelength. After 5 minutes, the plates were detached from the holder and the residues on the bottom of plate were wiped off with Kim wipe. The fluorescence signal was read with the Flx800™ fluorescence reader at 15 minutes after addition of substrate solution.

A conventional sandwich IL-4 ELISA assay with NUNC MaxiSorp® plate was performed as comparison. Briefly, 100 µL PBS solution containing 2 µg/mL IL-4 capture antibody was added to 96-well plate and incubated overnight at 4°C. The plates were then washed and blocked with Starting Block® blocking buffer (Pierce, Rockford, IL) for 2 hours. Subsequently, 100 µL solution containing recombinant IL-4 was added to the wells and the plate incubated at room temperature for 2 hours. After washing, 100 µL blocking buffer solution containing 2 µg/mL IL-4 detection antibody was added and incubated at room temperature for 1 hour. Subsequent to washing, the wells were incubated with 100 µL HRP-conjugated streptavidin at room temperature for 30 minutes. Following multiple washes, the wells were loaded with 100 µL of TMB buffer and incubated for 15 minutes at room temperature. The optical absorbance (OD 450 nm, corrected with OD 630) of each well was measured.

Manual pipetting was used for Optimiser™ assay with single-loading antigen and conventional ELISA assay. An automatic pipetting system was used in the Optimiser™ assay with repetitive sample loading.

For all assays, the mean background signal from the blank well were calculated and subtracted from the mean signal of individual standards. A standard curve was generated by plotting the standard concentration (x-axis) vs the background-adjusted signal (y-axis). The Limit of Detection (LOD) was determined by performing 20 replicates of assay diluent (blank) and calculating the mean signal + 2 standard deviations (SD) of the 20 values.

Results and Discussion

Comparisons to standard ELISA

When a single load of 10 µL sample containing analyte is added to the Optimiser™ plate, analytical performance with respect to limits of detection and sensitivity, defined as the slope of a linear calibration curve, are similar to standard ELISAs using 100 µL of sample containing analyte (Figure 5). This level of analytical performance is suitable for a wide range of applications quantifying analyte in the pg/mL range from numerous samples, such as serum and plasma. The advantage of using Optimser™ in this instance is a significant reduction in reagent and sample consumption which reduces costs and preserves precious samples. The Optimiser™ total assay time for this case is only ~ 1.5 hours which also represents a significant time saving and increase in work throughput.

Sensitivity improvement with the Optimiser™ using repetitive sample loading for amplification

Cytokine secretion tends to produce high local concentrations of cytokines that can induce both autocrine and paracrine signaling–yet the concentration of cytokine in the cell supernatant in a microplate well is typically several orders of magnitude lower requiring exquisite sensitivity [11]. In some applications and biological model systems (*in vitro, fresh ex vivo*, serum), there is a critical need for higher sensitivity. An example would be monitoring cytokine secretion from peripheral blood mononuclear cells where sample volume is limited, cell number is limited or the response is antigen-specific, rather than polyclonal

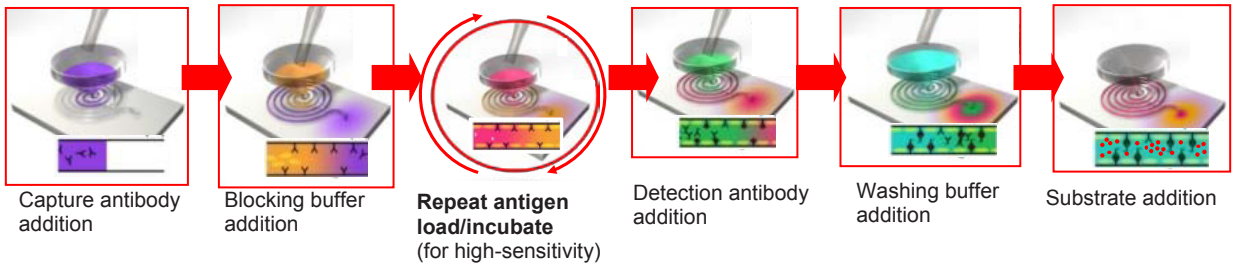

Figure 4: Assay procedure with Optimiser™ microplate. The "repeat loading" sequence for high sensitivity mode was completed on a BioTek® Precision automatic pipetting system.

or mitogen-driven. In many cases, ELISA kits provide insufficient sensitivity for these applications. As the Optimiser™ technology uses a flow-through principle where subsequent reagent/analyte additions flush microchannel contents on to the absorbent pad, multiple analyte additions can be used with dilute samples to increase sensitivity. This ability is demonstrated in Figure 6 where IL-4 is the analyte and standard amounts are spiked into cell culture media to construct calibration curves. In this log-log plot, the standard workflow described in Figure 4 is used, but the difference between the four sets of data is the number of analyte loadings, 1 x 10 µL, 20 x 5 µL, 100 x 5 µL and 260 x 5 µL.

Analytical sensitivity can be represented as the slope of a linear calibration curve as this defines the change in detection signal induced by a change in analyte concentration. Figure 7 quantifies the sensitivity gains attributable to multiple loadings of analyte using the Optimiser™ plate. It is apparent that an increase approximately proportional to the number of loading times in sensitivity is gained for each successive experiment with multiple loading.

Similar data was obtained using human IL-6, mouse IL-2, mouse IFN-g and mouse IL-17A as shown in Figure 8.

Left shifting of the data sets with increasing analyte loading illustrates the gain in sensitivity. A trade-off to multiple analyte loading is the overall time to complete the analysis. Each loading requires 5 minute incubation between additions. Total assay time for a single loading of 10 µL is ~1.5 hour, repeatedly loading 5 µL samples 20 times (total sample volume is 100 µL) requires a total assay time of ~3 hours, repeatedly loading 5 µL samples 100 times (total sample volume is 500 µL) requires a total assay time ~10 hours, and repeatedly loading 5 µL sample 260 times (total sample volume is 1.3 mL) requires a total assay time ~24 hours. The 5 µl x 20 load case uses the same sample volume as a conventional ELISA, but yields ~ 15x improvement in sensitivity. The 5 µL x100 load case represents the typical time required for a conventional ELISA but with an extraordinary 125x gain in sensitivity. The 5 µL x 260 load case represents the typical time required for a conventional "high-sensitivity" ELISA but with an attogram/mL or ~ 1,000x higher sensitivity, as determined by the related fluorescence units (RFU). This data clearly shows the ability of Optimiser™ to "tune" assay sensitivity simply by changing the sample volume used in the exact same assay.

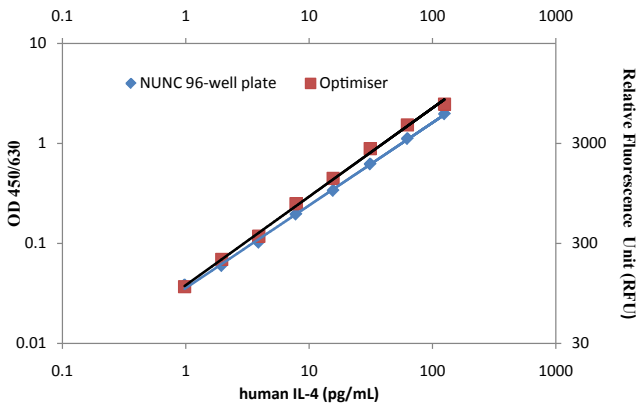

Figure 5: Calibration curves for IL-4 quantification using a standard 96-well microplate (NUNC – 100 µL sample load, total assay time ~ 12 hours) and the Optimiser™ plate (10 µL sample load, total assay time ~ 1.5 hours).

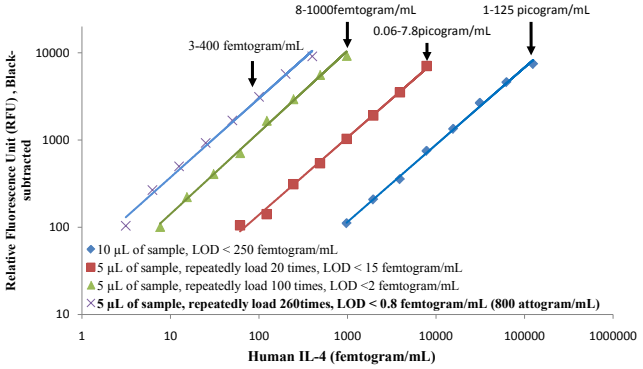

Figure 6: Human IL-4 assay in Optimiser™ ELISAmicroplate using repetitive sample loading for amplification of signal sensitivity. The various ranges of signal detection with the different loading conditions are indicated in pg and fg levels.

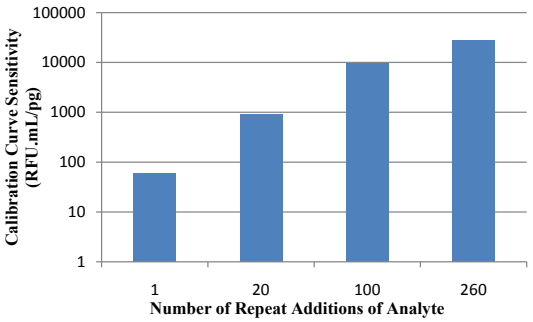

Figure 7: Sensitivity of human IL-4 assay for 10 µL x1, 5 µL x20,5 µL x100 and 5 µL x260 analyte.

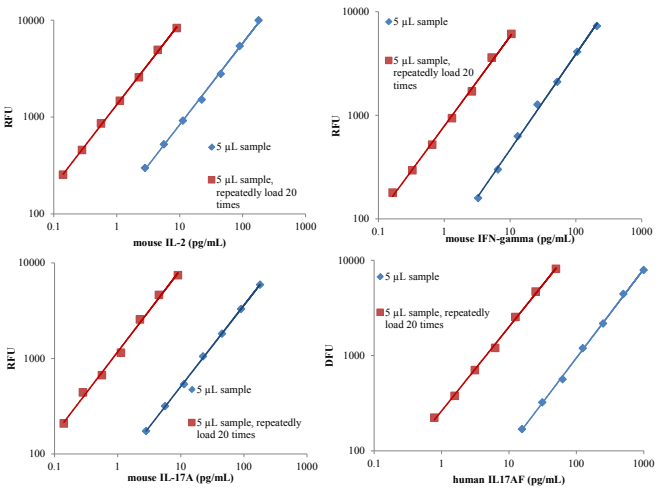

Figure 8: Assay with Optimiser™ for Human IL-6, mouse IL-2, mouse IFN-γ, and mouse IL-17A using repetitive sample loading for amplification of sensitivity.

Conclusion

The Optimiser™ platform is a simple and inexpensive microfluidic ELISA technology that offers high sensitivity and scalability. It combines the power of microfluidics technology with the standard SBS-configured 96-well microplate architecture. Together, Optimiser™ -based ELISAs result in a remarkable improvement in ELISA workflows, significant improvement in reaction kinetics, with

a remarkable conservation of precious samples and reagents. And with the repeat loading application, it offers investigators the ability to tune the sensitivity of their assays by multiple analyte loading.

In this review, we have demonstrated the ability of the Optimiser™ microplate to compare favorably to conventional ELISA methods with respect to conserving costly reagents and precious samples, yielding comparable sensitivity while using 10-20 fold less experimental sample. The flow-through nature of the technology also allows for tuning of sensitivity for 1000-10,000 fold gains in sensitivity that are beneficial for cytokine secretion studies of PBMCs in culture, where analyte levels are lower than those conventional methods can detect (*e.g.*, lowly-expressed cytokines (Th2 family, IL-12 family) from low starting cell number, and when monitoring antigen-specific responses, where responding cells are very rare). Similarly, the Optimiser™ Repetitive Sample Loading Method enables measurement of currently undetectable levels of serum cytokines in various conditions, without *in vivo* stabilization. The rationale of the innovative In Vivo Cytokine Capture Assay (IVCCA)[9] is that systemic cytokine levels (*e.g.*, typically measured by assessment of serum samples) are typically many orders of magnitude below the LOD of any ELISA, because cytokines act primarily locally (at the cell: cell synapse), and because of receptor-mediated uptake (and turn-over by the kidneys), they are degraded so rapidly they can't be measured without *in vivo* stabilization. By increasing the sensitivity level another 10,000 fold, the Optimiser™ enables a unique detection approach for lowly-expressed analytes which are currently impossible to detect by any other method. Unlike recently-developed high sensitivity immunoassay technologies, the Optimiser™ microplate uses comparable reagents as conventional 96-well plate ELISAs, a simple operation protocol, and an inexpensive standard fluorescence microplate reader. Optimiser™ presents a powerful tool for researchers to detect proteins in ultra-low concentrations with minimum effort and cost and has the potential to identify therapeutic markers and profile drug responses. Continued refinement of techniques and methods to determine the abundance and status of proteins holds great promise for the future study of diseases and the development of reliable therapies and eventually the translation of basic discoveries into daily clinical practice.

References

1. Hanash S (2003) Disease proteomics. Nature 422: 226-232.
2. Fung ET, Wright GL Jr, Dalmasso EA (2000) Proteomic strategies for biomarker identification: progress and challenges. Curr Opin Mol Ther 2: 643-650.
3. Gonzalez SA, Keeffe EB (2011) Diagnosis of hepatocellular carcinoma: role of tumor markers and liver biopsy. Clin Liver Dis 15: 297-306.
4. Srinivas PR, Kramer BS, Srivastava S (2001) Trends in biomarker research for cancer detection. Lancet Oncol 2: 698-704.
5. Patton W (2002) Detection technologies in proteome analysis. Journal of Chromatography B 771: 3-31.
6. Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8: 871-874.
7. Marko-Varga GA, Nilsson J, Laurell T (2004) New directions of miniaturization within the biomarker research area. Electrophoresis 25: 3479–3491.
8. Power CA, Grand CL, Ismail N, Peters NC, Yurkowski DP, et al. (1999) Avalid ELISPOT assay for enumeration of ex vivo, antigen-specific, IFNgamma-producing T cells. J Immunol Methods 227: 99-107.
9. Nam JM, Thaxton CS, Mirkin CA (2003) Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. Science 301: 1884-1886.
10. Rissin DM, Kan CW, Campbell TG, Howes SC, Fournier DR, et al. (2010) Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat Biotechnol 28: 595–599.
11. Finkelman FD, Morris SC (1999) Development of an assay to measure in vivo cytokine production in the mouse. Int Immunol 11: 1811-1818.

This article was originally published in a special issue, **Discovering Novel Biomarkers** handled by Editor(s). Dr. K. StephenSuh, Hackensack University Medical Center, USA; Dr. Takemi Tanaka, Thomas Jefferson University, USA; Dr. Valli De Re, Centro di Riferimento Oncologico, Italy