Amplifying Immunoassay Sensitivity with the Optimiser™ Microplate Technology

Repetitive Sample Loading of the Optimiser Microplate Allows for Amplification of Assay Sensitivity from Picogram/mL Levels to Femtogram/mL Levels

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A combination of microfluidics technology with standard SBS-configured 96-well microplate architecture allows for the improvement of ELISA workflows, conservation of samples and reagents, improved reaction kinetics, and the ability to tune the sensitivity of the assay by multiple analyte loading. Multiple analyte loading was facilitated by the use of an automated pipetting station.

Key Words:

ELISA

High Sensitivity

fg/mL

Cytokines

Biomarkers

Optimiser™

Microfluidics

Introduction

Enzyme-linked immunosorbent 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 chance of cross-reactivity. Possible confounding factors such as Rh factor, HAMA, soluble receptors, and protein binding factors are eliminated by careful selection of matching antibody pairs which are not affected by soluble receptor in the binding site, and the use of proprietary diluents to match matrix effects. The technology has origins in the 70's and received a boost in popularity through the development of monoclonal antibodies in the mid '70's and the advent of microplate-based instrumentation in the early '80's. The format of the sandwich ELISA has not changed significantly from those times and is used extensively in both research and clinical applications.

A novel microplate (Optimiser™) has been recently developed that combines the typical SBS footprint and layout of a 96-well microplate with a dedicated microfluidic channel connected to each well where binding events occur (see 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 large increase in surface area to volume ratio of capture antibodies compared to standard ELISA techniques where capture antibodies are immobilized on the microplate well bottom. The ELISA assay workflow is similar using the Optimiser except addition volumes are significantly less and there is no

need to use microplate washers. As each microchannel has a volume of only 4.5 μL, the addition of excess wash buffer (10 μL) 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.
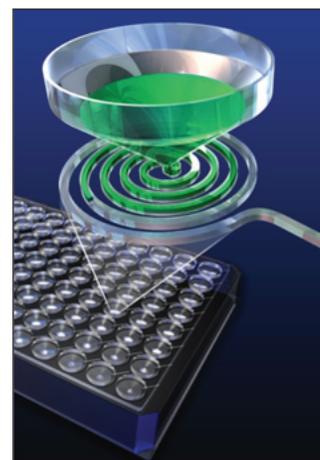

Figure 1. Overview of the Optimiser Microplate illustrating 96-well density on an SBS footprint and an exploded few of a microchannel associated with one of the wells. Microchannels are loaded passively through capillary action associated with well loading. The repeat addition of fluid to the well expels fluid from the microchannel into an absorbent pad. The detection volume is defined by the microchannel spiral and can be read using conventional microplate readers.

In this application note we will demonstrate assay performance using a number of cytokines as analyte and demonstrate the ability to tune assay sensitivity by repeating analyte loading/incubation cycles.

Materials and Methods

Materials

OptiMax human IL-4 Assay Kit (OMA-H-IL4-2) including the Optimiser plate (OPH-2) (or OptiMax Plate (OMP-2)) and OptiMax Buffer Reagent (OMR-2) were all provided by Siloam Biosciences (Cincinnati, OH). Pipette tips used were from BioTek Instruments (200 µL tip, cat# 98214).

Instrumentation

The Precision™ Microplate Pipetting System (BioTek Instruments, Winooski, VT) was used for multiple loading of analyte (IL-4) for experiments tuning assay sensitivity and reagent addition (see Figure 2). The instrument can operate up to 3 Optimiser™ ELISA plates. A customized plate holder has been used for Optimiser™ ELISA plate in this study, although an integrated Optimiser plate with integrated absorbent pad will be available in the near future from Siloam Biosciences, which does not need the customized plate holder. The off-the-shelf OptiMax™ plate can handle up to 30 repeat loads of sample. For the data shown with 100 loads Siloam Biosciences can provide a customized jig to allow for sample loading >30 repetitions.
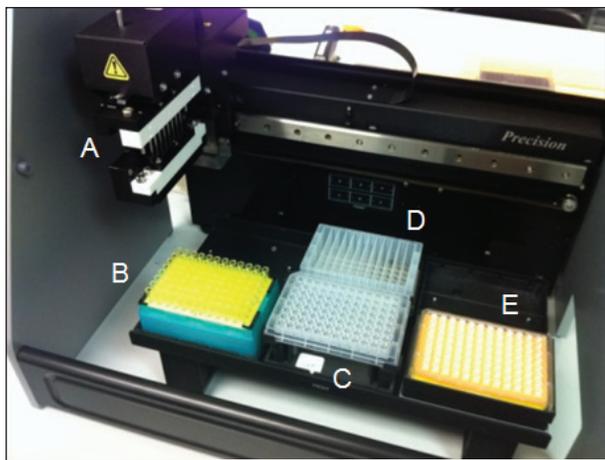

Figure 2. Precision Microplate Pipetting System layout for Optimiser automation. A: 8-channel pipetting head; B: Tip box; C: Sample (i.e. IL-4) reservoir; D: Reagent reservoir; E: Optimiser plate.

Detection of the fluorescent signal from the Optimiser plate was performed with an FLx800™ Fluorescence Microplate Reader. The following spectral filters were used for detection.

Function	BioTek Part Number	Wavelength
Excitation	7082247	528/20 nm
Emission	7082224	590/35 nm

Optimiser Operation

Figure 3 demonstrates the principle of Optimiser operation. A slight excess of first reagent (5 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.
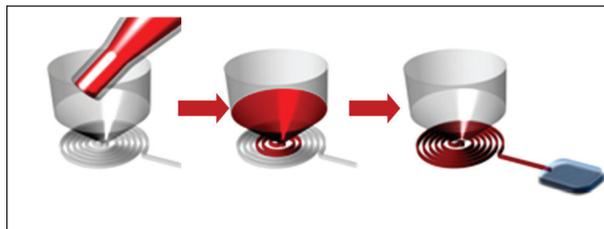

Figure 3. Principle of Optimiser operation – initial well loading with reagent serves to load microchannel. Once a well is empty and microchannel filled, capillary forces retain fluid in the microchannel for an incubation step. Addition of subsequent reagents relieves capillary forces causing flushing 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.

Optimiser Workflow

The Optimiser workflow mirrors the steps involved in conducting a typical ELISA. The main difference is the volumes of addition are much smaller. Each step here consists of the addition of 10 µL of reagent and either 10 µL of analyte (IL-4) for a single addition or repetitive additions of 5 µL for tuning sensitivity. Apart from step 3, all steps were the same for all experiments where 2 µg/mL of capture antibody (clone 8D4-8), blocking buffer, sample, 2 µg/mL of biotinylated detection antibody (clone MP4-25D2), wash buffer, and then Streptavidin-HRP, followed by 2 aliquots (30 µL each) of wash buffer and finally substrate were added in 10 µL aliquots.
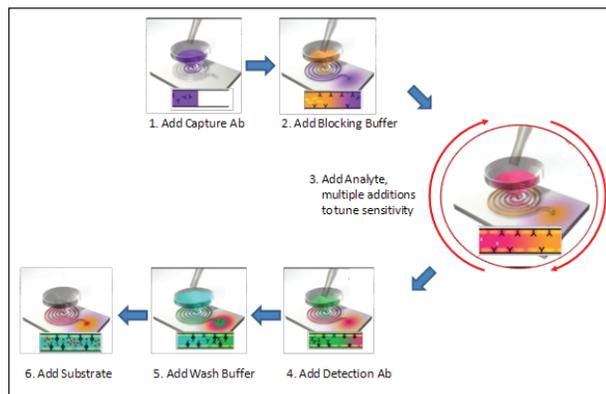

Figure 4. Optimiser workflow – the steps of the workflow mirror typical ELISA workflows. The figure above is an illustrative representation of key steps and the exact assay protocol is described in the text.

Results and Discussion

Comparisons to Standard ELISA

When single loads of sample containing analyte (10 µL) are 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 (see 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 is 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/ throughput increase.
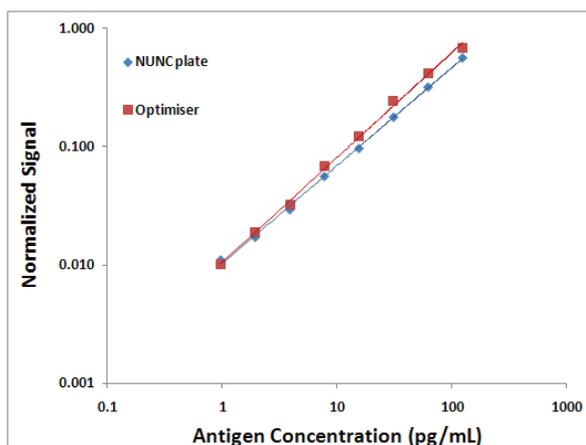

Figure 5. Calibration curves for IL-4 quantification using a standard 96-well microplate (NUNC – 100 µL sample load) and the Optimiser plate (10 µL sample load). Assay signals were normalized to achieve similar scaling.

Sensitivity Tuning with the Optimiser

In some applications, there is a need for higher sensitivity to the assay. An example would be monitoring cytokine secretion from peripheral blood mononuclear cells. 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. 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 unto 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 three sets of data is the number of analyte loadings, 1x 10 µL, 20x 5 µL and 100x 5 µL.

Left shifting of the data sets with increasing analyte loading illustrates the gain in sensitivity. A trade-off to multiple analyte loading is time to complete the analysis: each loading requires a 5 minute incubation between additions. Total assay time for a single loading of 10 µL is ~1.5 hour, repeated loading of 5 µL sample 20 times (total sample volume is 100 µL) requires a total assay time of ~3 hours, and repeatedly loading 5 µL sample 100 times (total sample volume is 500 µL) requires a total assay time ~10 hours.
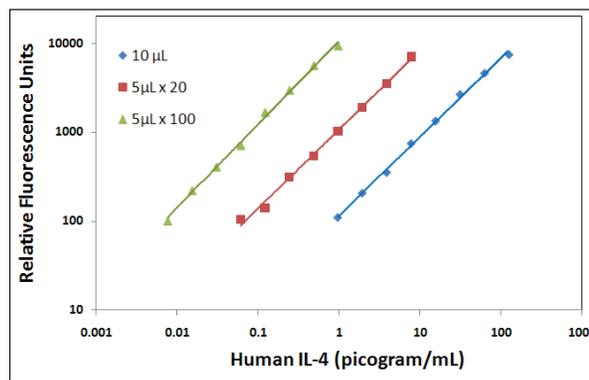

Figure 6. Human IL-4 assay in Optimiser ELISA microplate.

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 approximate 10-fold increase 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 (in-house data not shown).
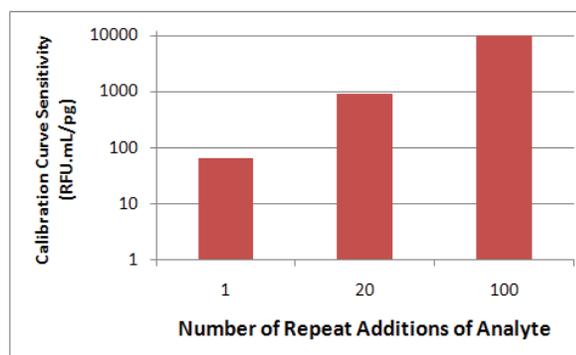

Figure 7. Sensitivity for 1x 10 µL, 20x 5 µL and 100x 5 µL.

Conclusions

We have demonstrated the ability of the Optimiser microplate to compare favorably to standard ELISA methods with respect to conserving costly reagents and precious samples. The flow-through nature of the technology also allows for tuning of sensitivity to fg/mL levels that would be beneficial for cytokine secretion studies. Our next efforts will be to demonstrate this capability in assays monitoring TH2 cytokine secretion from peripheral blood mononuclear cells.