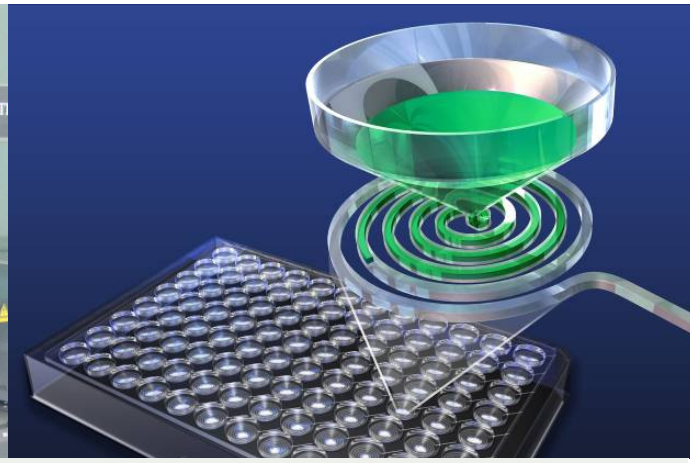

Automating ELISAs on Tecan's Freedom EVO® using Optimiser™ technology from Siloam Biosciences

Low volume, high sensitivity ELISAs using automation-compatible OptiMax™ plates

Introduction

One of the challenges in life sciences research today is to discover methods for running key assays more quickly, more reliably and using lower volumes of reagents and sample, but still with improved sensitivity. One area where this particularly holds true is for traditional enzyme-linked immunosorbent assays (ELISAs), whose application provides a useful measurement of antigens, including cytokines and a host of other biomarkers.

ELISAs are considered one of the most useful secondary or tertiary type assays in drug discovery, because they elucidate specific cellular pathways and associated mechanisms of action for target genes, proteins or small molecules. They are equally important to clinical biology laboratories, as they enable determination of biomarker concentrations in unknown biological samples.

There have been a number of attempts to replace the traditional plate-based ELISA with microfluidic-based technology, but in general these have all suffered from the need for specialized liquid handling systems. Until now, microfluidic technology has not been adapted to the SBS plate footprint, and could not make use of the plate-based liquid handling and detection instrumentation found in many life science laboratories.

Siloam Biosciences' Optimiser technology

Siloam Biosciences' OptiMax microplate (based on the Optimiser technology platform) offers rapid, sensitive and specific chemifluorescence-based ELISA procedures using exceedingly small sample volumes. The speed, sensitivity and small sample requirements are achieved as a result of the unique microfluidic design of the Optimiser technology. All reactions, including analyte capture and detection, occur within a ~5 µl microfluidic reaction chamber, which has a unique microchannel geometry and small reaction volume to favor rapid reaction kinetics. A typical assay requires only 5 µl of sample, and each reaction step is completed within 10 to 20 minutes. A typical Optimiser technology-based ELISA can be completed within two hours, including wash times, substrate incubations and read times. Exploiting these rapid reaction kinetics on a microscale, coupled with a microplate automation systems such as the Freedom EVO workstation, offers extremely high sensitivity or very fast assays. The OptiMax plate is SBS/ANSI-compliant and is compatible with Tecan fluorescence plate readers and the Freedom EVO workstation.

Principle of operation

The Optimiser technology-based ELISA workflow (Figure 1) mirrors standard ELISA steps, however the volumes used are significantly smaller and washing steps are reduced. Sample or reagent volumes as small as 1-10 µl are added to each well and drawn through the microchannel via capillary forces.

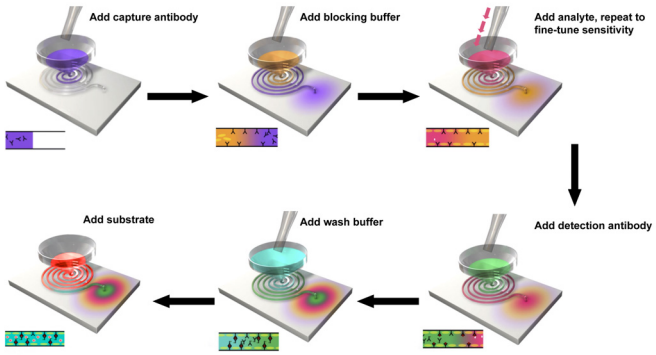

Figure 1. Optimiser technology-based assay workflow

The microfluidic reaction chamber – where binding occurs – holds 4.5 µl of liquid, and excess liquid is channeled to an absorbent pad under the microplate.

The sequence of reagent/sample additions is demonstrated in Figure 1. For each successive addition, the capillary barrier is broken at the microchannel inlet, and all previous reagents are flushed onto the absorbent pad. Flushing excess liquid effectively removes unbound materials and also prevents cross-contamination of reagents within the microchannel. By making multiple additions of the sample containing the analyte, sensitivity can be 'tuned' according to the number of repeat sample loads (for example, 20 repeat loads increases sensitivity 20-fold), with demonstrated sensitivity gains of up to 1,000-fold over the same assay reagents in a conventional 96-well microplate.

The flush action in the OptiMax microplates is far more suited to automated liquid handling than a conventional wash step, since it only requires a dispensing step. In fact, the entire OptiMax plate-based assay workflow only requires the sequential pipetting of samples and reagents to the loading well.

Automating the process further enhances ELISA efficiency and throughput, and the Freedom EVO workstation's parallel processing capabilities allow operation of multiple OptiMax microplates. Automation also offers distinct performance benefits, including:

- improved pipetting precision at very low volumes;
- precise control of dispensing times, allowing shorter incubation cycles;
- precise operation of the repeat load process, dramatically improving sensitivity;
- increased productivity allowing the user to attend to other tasks while the instrument is running;
- fully integrated readout, using a Tecan microplate reader such as the Infinite® 200 PRO.

Materials and methods

Equipment

An overview of the equipment required for the automation of Siloam's OptiMax plates is given in Table 1. The Freedom EVO workstation is equipped with an 8-channel Liquid Handling (LiHa) Arm with fixed or disposable tips. A Robotic Manipulator (RoMa) Arm transports the plates to an integrated Infinite M200 PRO multimode reader with fluorescence option.

Tecan equipment	• Freedom EVO 100 • 8-channel Liquid Handling (LiHa) Arm with fixed or disposable tips • Low volume option, including 500 µl syringes, low volume tubing, and low volume washable and disposable tips Trough carrier with 100 ml (regular) or 25 ml (maximum retention) troughs • Tube racks • Microplate carriers Infinite M200 PRO plate reader • Freedom EVOware™ Standard and Magellan™ software
Siloam Biosciences materials	• OptiMax microplates • OptiMax buffers[1] • Polypropylene V-bottom plate • Streptavidin-HRP Coat buffer test panel[1] • OptiMax mouse IL-2 assay kit[2] • OptiMax mouse IFN-γ assay kit[2] • OptiMax mouse IL-17A assay kit[2] • OptiMax human IL-4 assay kit[2]

Table 1. Overview of equipment

1. *OptiMax buffers contain specific OptiBind™ coating buffer suitable for a given assay, test panel contains one each of twelve coating buffers used in screening tests*
2. *For this study, OptiMax automation-compatible microplates were substituted for the Optimiser manual use microplates included in the OptiMax assay kits in all experiments.*

The data shown in this application note has been created with a Liquid Handling Arm with low volume option, using both low volume washable and disposable tips. This option includes 500 µl syringes and low volume tubing, which ensures precise free dispensing of volumes between 0.5 and 500 µl.

For higher throughputs or to ensure accurate pipetting times even with very short incubation times, a MultiChannel Arm™ (MCA) 96 can be used.

Automated workflow

Automation enhances efficiency and increases the throughput of OptiMax assays. A typical workstation layout is shown in Figure 2. The Freedom EVO workstation automatically loads analytes and reagents into the microfluidic microplate, and enables multiple OptiMax microplates to be analyzed in a single batch. Precise, reproducible scheduling of the dispensing steps ensures robust assay results every time.

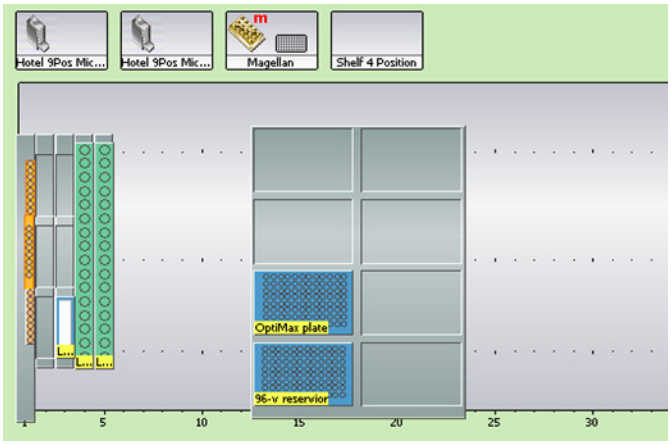

Figure 2. Typical worktable layout for OptiMax ELISAs

All reagents, including capture antibody, blocking buffer, detection antibody, streptavidin-HRP and protein standards, are preloaded in a 96-well V-bottom reservoir plate. Wash buffer is placed in a 100 ml trough, and samples are loaded in tubes.

Samples, reagents and wash buffer are pipetted sequentially into the OptiMax plate according to the individual assay protocol, and the plate incubated at room temperature for the specified duration for each assay step.

Full integration of the Infinite M200 PRO allows precise scheduling of the fluorescence readout of the OptiMax microplates, enhancing the reproducibility and robustness of the assay results.

Sample saving

Procedure

In order to investigate the influence of reduced sample volumes on assay sensitivity, a series of different concentrations of spiked mouse IL-2 serum (diluted 1:2 in assay diluent) were measured with load volumes ranging from 1 to 5 µl (load volumes were twice sample volume). Each assay was run in triplicate, and the total run time was 58 minutes for each assay.

The effect of the sample volume on the analytical sensitivity of the assay was determined, and the precision of the 1 µl load volume (0.5 µl serum sample) verified by replicate analysis (n=12) of spiked serum samples at seven different concentrations.

Results

The analytical sensitivity of the 1 µl load volume (0.5 µl serum sample) assay method is ~75 % compared to the 5 µl load volume assay method (Figure 3).

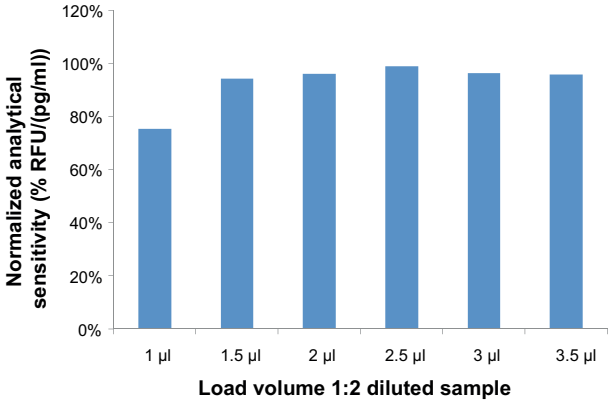

Figure 3. Analytical sensitivity by load volume. Load volume = 2 x sample volume in this experiment

Results in Figure 3 and Figure 4 show that load volumes in excess of 1 µl show similar sensitivity.

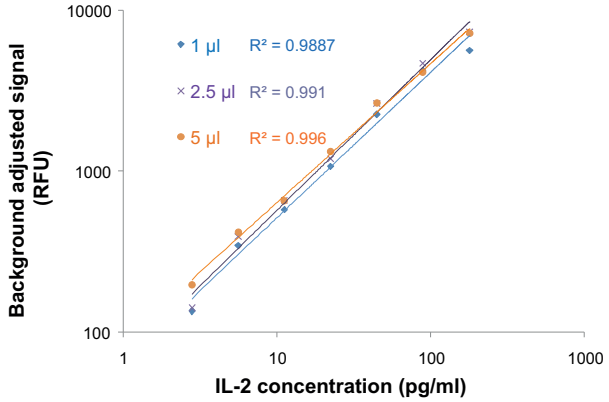

Figure 4. IL-2 assay in spiked mouse serum with varying sample volumes

The raw signal precision of the 1 µl load volume assay method ranged from 7 to 12 % at different concentrations and is comparable to the 5 µl method.

A critical requirement for running ELISAs with ultra-low volumes (down to 1 µl) is the use of the highly accurate and precise liquid handling of the Freedom EVO workstation, ensuring free dispensing of aqueous liquids down to 0.5 µl. Similarly, the signal readout requires a state-of-the-art microplate reader. The Infinite M200 PRO is designed to read 96- and 384-well plates and, in fluorescence mode, the excitation is focused within a 3 mm diameter spot in the center of each well. This represents a 1.5 µl volume within the spiral microfluidic reaction chamber in the OptiMax microplate and, consequently, even 1 µl load volumes produce a strong signal.

Multi-analyte detection

Procedure

The ultra low volume handling capability of the Freedom EVO 100 is ideal for Optimiser technology-based multi-analyte assays using extremely small sample volumes. A fully automated multi-analyte assay for the simultaneous detection of products of mouse activated Th1 (IFN-g), Th2 (IL-4, IL-10) and Th17 (IL-17A, CCL20, G-CSF) cell effector molecules was demonstrated, with a total assay time of just 95 minutes. An eight point standard curve (in triplicate) was generated for each analyte, and 16 independently spiked cell culture samples were measured. Commonly available antibody pairs and antigens were used for all assays, without any special modifications.

	IL-2	IL-4	IL-6	IL-10	IL-17A	IL23	IFN-γ	TNF-α	G-CSF	CCL20
Reagent manufacturer	Siloam	Life Technologies™	Life Technologies™	R&D Systems®	Siloam	R&D Systems®	Siloam	R&D Systems®	R&D Systems®	R&D Systems®
Reagent cat #	OMR-M-IL-2	CMC0043	CMC0063	DY417	OMR-M-IL-17A	DY1887	OMR-M-IFNγ	DY410	DY414	DY760
Limit of quantification	2.8 pg/ml	3.9 pg/ml	23.4 pg/ml	31.25 pg/ml	2.8 pg/ml	39 pg/ml	3.3 pg/ml	31 pg/ml	31 pg/ml	31 pg/ml
Correlation factor (slope of curve)	0.95	1.00	0.87	1.15	0.93	0.9	0.97	1	1.02	0.99
Correlation coefficient (r^2 value)	0.98	0.96	0.98	0.99	1	0.99	0.99	0.99	0.98	1

Table 2. Results summary for the 10 analyte panel

Results

Figures 5 and 6 show representative examples of the correlation curves for IL-2 and IFN-γ assays. Table 2 summarizes the results for the 10 analyte panel, where the complete panel is detected from a 15 µl sample volume.

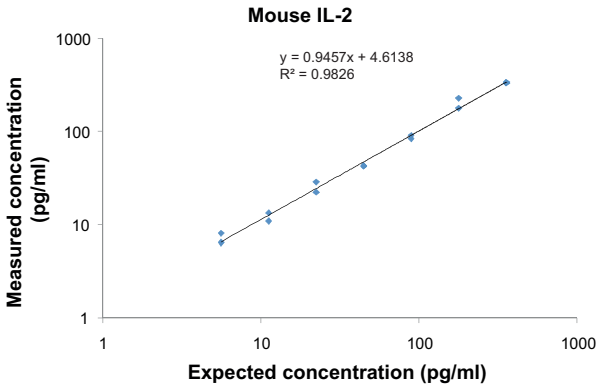

Figure 5. Correlation curve for mouse IL-2 assay as part of a 10 analyte panel

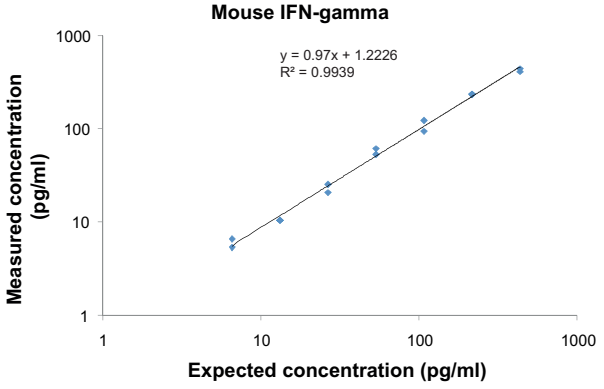

Figure 6. Correlation curve for mouse IFN-γ assay as part of a 10 analyte panel

These results demonstrate that the Optimiser technology using OptiMax automation plates is a powerful tool for monitoring a variety of analytes using minimal sample volumes, while maintaining excellent sensitivity.

Sensitivity 'tuning'

Procedure

The Optimiser technology-based assay protocol closely matches the standard ELISA workflow shown previously (Figure 1). By repeating the sample addition step multiple times, the sensitivity of the assay can be 'tuned' to the desired level. In addition to the number of repeat loads, the incubation time for the sample can be adjusted. Incubation intervals of 5 minutes or longer typically achieve near saturation of antigen binding.

Shorter incubation times are less efficient in antigen capture, but the loss in efficiency can be compensated for by repeated sample loads, maintaining the same overall assay time. To demonstrate the influence of repeated sample loading on assay sensitivity, a model IL-4 assay was performed with a constant total assay duration of approximately one hour, and varying numbers of repeat loads of sample, adjusting incubation times for each case (Table 3). Automation of the procedure on the Freedom EVO allows precise scheduling and volume control without any operator intervention.

Solution loaded	Volume/well (µl)	Incubation time (min)
Capture antibody (4 µg/ml)	4	5
Blocking buffer	5	5
IL-4 standards	2	20 min, (4 x 5 min), (10 x 2 min)
AP-detection antibody (2 µg/ml)	3	5
Washing buffer	5	3
Sav-HRP (0.07 µg/ml)	3	10
Washing buffer	30	5
Washing buffer	30	5
OptiGlow™ substrate	2	13

(AP – alkaline phosphatase)

Table 3. Assay protocol for IL-4 assay

Results

Figure 7 shows the assay results for the various repeat load cases, where a curve shift to the left indicates higher sensitivity with repeat loads.

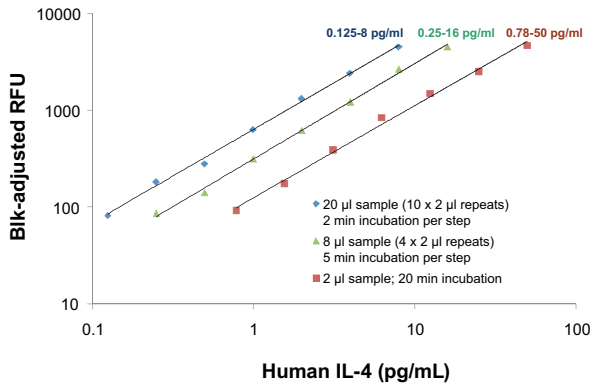

Figure 7. IL-4 assay sensitivity 'tuning' with repeat sample load protocols

Sample volume (µl)	Incubation time (min)	Measurable range (pg/ml)
2	20	0.78 – 50
8 (4 x 2 µl)	20 (4 x 5 min)	0.25 – 16
20 (10 x 2 µl)	20 (10 x 2 min)	0.125 – 8

Table 4. IL-4 assay sensitivity as a function of the number of sample repeat loads

As shown in Table 4, four repeat loads result in an approximately three-fold sensitivity gain, and loading 10 times shows an approximately six-fold sensitivity gain at constant assay time.

In addition, previous results show that the sensitivity gain can be proportional to the number of repeat loads if the incubation times allow saturation of the antigen binding to be reached. This means that, under optimized conditions, a 10-fold increase in sensitivity can be achieved by 10 repeat sample loads.

Maximum throughput

Procedure

All of the features described above – low volume handling, reduced incubation times and automated microplate processing – can be combined to optimize ELISAs for significantly increased throughputs. In this experiment, a β-hCG assay (typically used as a benchmark to compare diagnostic assay platforms) has been performed using the assay protocol shown in Table 5.

The β-hCG assay with a triplicate standard curve and 24 duplicate samples (spiked male human serum) can be completed in just 30 min, using the Optimiser technology-based assay on the Freedom EVO 100 workstation. In a separate experiment, sample replicates at high, medium and low concentrations (n=14 for each) were assessed for precision and recovery using the same assay protocol.

Solution loaded	Volume/well (µl)	Incubation time (min)
Capture antibody (16 µg/ml)	4	4
Blocking buffer	5	4
Standards, sample	3	4
AP -detection antibody (16 µg/ml)	3	4
Washing buffer	5	3
Sav-HRP (0.07 µg/ml)	3	10
Washing buffer	20	4
Washing buffer	20	5
OptiGlow substrate	2	5

(AP – alkaline phosphatase)

Table 5. Assay protocol for β-hCG assay

Results

Figure 8 shows the correlation results for the β-hCG sample analysis. The replicate study results established precision (CV) ranging from 11.0-12.7 % and recovery values ranging from 92-101 % at the various concentrations. This shows that, by carefully balancing the number of repeat sample loads and incubation times for each step, each ELISA can be optimized for both sensitivity and maximum throughput.

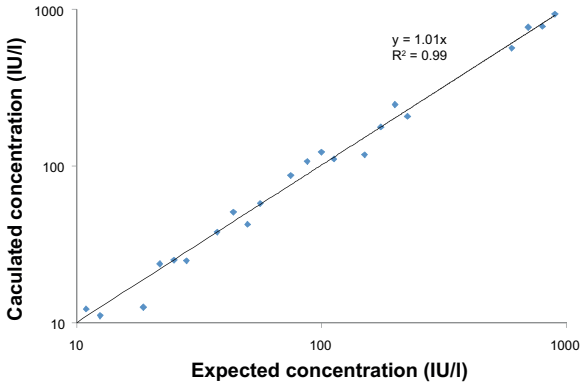

Figure 8. β-hCG sample analysis correlation results

Conclusion

The combination of Tecan's Freedom EVO workstation and Siloam Biosciences' Optimiser technology offers distinct performance benefits, including improved pipetting precision at very low volumes, precise control of dispensing times – allowing for short incubation cycles – and operation of the repeat load process, dramatically improving sensitivity. Fully integrated readout of the chemifluorescence signal is achieved with a Tecan Infinite M200 PRO reader. The increased productivity allows users to attend to other tasks while the instrument is in operation.

Acknowledgements

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