AN0010: Application of Novel Optimiser™ Immunoassay Technology to the Rapid, Sensitive, and Specific Measurement of Human IL-6 in Tissue Culture Supernatants

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Introduction:

Siloam Biosciences' Hu IL-6 OptiMax™ ELISA Kit offers a rapid, sensitive, and specific chemifluorescent-based ELISA procedure for the measurement of Hu IL-6 using exceedingly small sample volumes. The speed, sensitivity, and small sample requirements are enabled by the unique microfluidic design of the Optimiser™ plate in which all reactions, including analyte capture and detection, occur within a ~5 µL microfluidic reaction chamber. The unique microchannel geometry and small reaction volume favor rapid reaction kinetics. A typical Hu IL-6 assay utilizes only a 5 µL sample and each reaction step is completed in 10 or 20 minutes. With wash time, substrate incubation, and read time accounted for, a typical OptiMax™ ELISA assay can be completed within two hours.

The Optimiser™ plate is SBS/ANSI-compliant and is compatible with all standard fluorescence plate readers, robotic sample processors, and other equipment and instruments used in conducting traditional 96-well microwell-based assays.

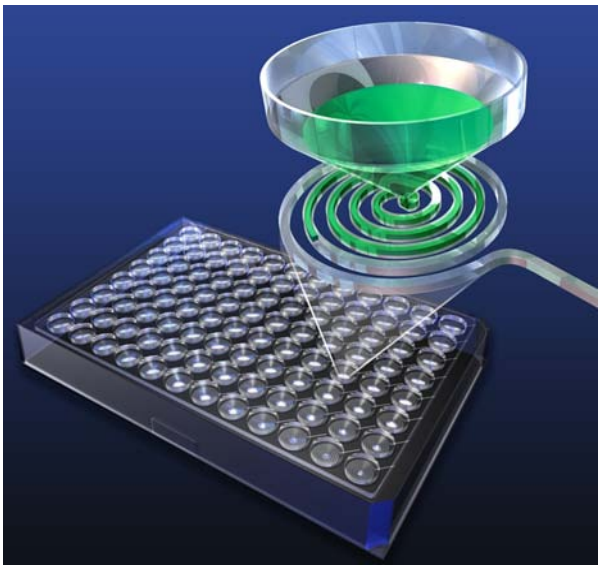

Figure 1: Optimiser™ microplate and magnified view of one "cell" of the Optimiser

Significance of IL-6:

IL-6 is a cytokine that functions in inflammation and in maturation of B cells. The protein is primarily produced at sites of acute and chronic inflammation where it is secreted into the serum and induces a transcriptional inflammatory response. This classical responsiveness to IL-6 is governed by a receptor complex consisting of two membrane-bound subunits, an 80-kDa cognate Alpha-chain (IL-6R Alpha) and a ubiquitously expressed 130-kDa Beta-chain receptor (gp130) which acts as the universal signal transducing element for all IL-6 family cytokines. Alternatively, IL-6 regulation of leukocyte trafficking relies upon signaling via its soluble IL-6R Alpha (termed IL-6 trans-signaling). Functioning of the gene is implicated in a wide variety of inflammation-associated disease states including susceptibility to diabetes mellitus and systemic juvenile rheumatoid arthritis.

The format for the Hu IL-6 ELISA, and most other ELISAs, has remained largely unchanged for the last few decades. Typically, conventional ELISAs are performed in a series of steps each of which requires about 100 µL of material and 1-2 hours incubation. These steps are separated by laborious wash steps. Optimiser™-based ELISAs follow a similar reaction sequence but with notable differences

- Optimiser™-based ELISAs use only 5 µL of sample or reagent per well [20 times less per well than a conventional ELISA]. This economizes the use of valuable samples (e.g. individual mouse sera) and reagents (chemiluminescent substrates).
- Most Optimiser™ incubations are 10 minutes in length [20 minutes for samples; 15 minutes for substrate] (Figure 2). The Optimiser™-based Hu IL-6 ELISA is completed in about 2 hours. In contrast, the conventional Hu IL-6 ELISA requires about 6.5 hours to complete [a savings of about 4.5 hours of labor].
- Optimiser™-based ELISAs use a unique "flush" step rather than the traditional, and laborious, "wash" step used in conventional ELISAs. To flush, the user simply dispenses wash buffer into the Optimiser™ well. The wash buffer "flushes" the used reagent/sample from the microchannel into an absorbent pad beneath the plate. The Optimiser™ "flush" is equally effective as the traditional washes. The flush step also simplifies assay automation.
- The Optimiser™ **is the only platform** capable of quantifying analytes from a 5 µL sample in true ELISA format and without the need for any special liquid handling systems.

Materials and Methods:

The OptiMax™ Hu IL-6 ELISA kit provides all of the necessary materials for testing including the Optimiser™ plate, holder, and pad, and the recombinant Hu IL-6 standard, the capture and biotinylated detection antibodies, HRP-labeled Streptavidin, substrate solution, and the necessary buffers for plate coating, plate blocking, plate washing, and for diluting the assay reagents.

The assay protocol on the right (Figure 2) illustrates the typical sequence for an Optimiser™-based ELISA such as the Hu IL-6 assay addressed in this application note. The protocol requires about 2 hours to complete including substrate development time and reading. [A conventional Hu IL-6 ELISA using a traditional 96-well microplate requires about 6.5 hours to complete. Users of Optimiser™-based ELISAs can realize a ~ **4.5 hour time and labor savings** compared to users of conventional ELISAs.]

The mean signal of triplicate blank wells is calculated after which the mean blank signal is subtracted from the standards, controls, and samples. A standard curve is created by reducing the data using computer software capable of generating a five parameter logistic curve fit. The Hu IL-6 concentrations of samples and controls are interpolated from the standard curve. [Alternatively, plot the standard curve on log-log graph paper with Hu IL-6 concentration on the x-axis and background-adjusted signal on the y-axis. A best-fit curve is drawn through the points corresponding to the background-adjusted signal for each standard concentration.]

Unique Considerations in Conducting OptiMax™ ELISA Assays:

The OptiMax™ Hu IL-6 ELISA procedure requires accurate and precise pipetting of 5 and 10 µL volumes. Specific guidance for the accurate and precise pipetting of 5 and 10 µL volumes is included in the User Manual provided with each kit.

OptiMax™ incubations are 10, 15, or 20 minutes in length. To ensure optimal assay performance, all assay reagents must be transferred to the Optimiser™ plate within about one minute. To facilitate rapid, yet accurate and precise transfer, Siloam Biosciences provides a standard polypropylene 96-well v-bottom plate with each kit in which the standards, controls, samples, blanks, and assay reagents can be prepared prior to their transfer to the Optimiser™ plate using a multichannel pipettor capable of the accurate and precise delivery of 5 and 10 µL volumes. The buffers and reagents provided with the OptiMax™ Hu IL-6 ELISA kit are specially formulated for compatibility with the microfluidic design of the plate.

OptiMax™ ELISA

Place Optimiser™ Plate and Pad on Holder
↓
Dispense 5 µL capture Ab
↓ 10 min RT
Flush with 5 µL OptiWash™
↓ 10 min RT
Dispense 5 µL OptiBlock™
↓ 10 min RT
Dispense 5 µL standard, sample, blank
↓ 20 min RT
Flush with 5 µL OptiWash™
↓ 10 min RT
Dispense 5 µL Detection Ab
↓ 10 min RT
Flush with 5 µL OptiWash™
↓ 10 min RT
Dispense 5 µL SAv-HRP
↓ 10 min RT
Flush 2x with 30 µL OptiWash™
↓ 2 x 10 min RT
Dispense 10 µL OptiGlow™
↓ 15 min RT
Read Optimiser™ plate

Figure 2. Typical protocol for Optimiser™-based ELISA

Validation Summary:

Siloam Biosciences has validated the OptiMax™ Hu IL-6 ELISA kit. Data acquisition and analysis utilized Gen5™ software, Excel, and Prism. A summary of the validation results follows. The rHu IL-6 standard curve ranges from **2.3 to 150 pg/mL**. Concentration (x-axis) and signal (y-axis) are plotted on Log scales. A typical standard curve is presented in Figure 3.

Validation samples were prepared by spiking rHu IL-6 into RPMI medium supplemented with 10% fetal bovine serum. Each sample was tested in 24 replicates in each of six independently performed assays. Both Intra and inter assay precision were determined by calculating the mean concentration, standard deviation, and percent coefficient of variation for each of the samples. The recovery of the OptiMax™ Hu IL-6 ELISA assay was determined by comparing the concentration determined using the OptiMax™ ELISA kit with the known Hu IL-6 concentration of the validation samples.

Percent Recovery= (determined concentration ÷ actual concentration) x 100

The percent recovery ranged from 91 to 108% (mean = 98%).

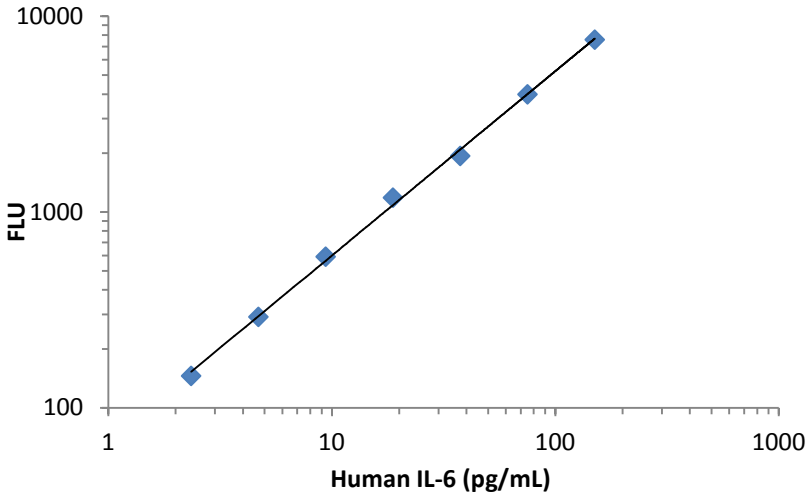

Figure 3. Hu IL-6 Standard curve generated using the OptiMax™ Hu IL-6 ELISA kit

IL-6 (pg/mL)	Average FLU	Blank-Subtracted
150	7782	7600
75	4177	3995
37.5	2118	1936
18.8	1367	1185
9.4	774	592
4.7	474	292
2.3	328	146
0	182	NA

Table 1. Data Summary for Hu IL-6 assay

Table 2. Intra-assay and inter-assay precision

	Intra-assay precision			Inter-assay precision		
Sample	1	2	3	1	2	3
Mean (pg/ml)	103.9	51.4	26.9	113.8	54.6	26.9
Standard deviation	6.5	4.3	1.8	5.4	3.7	2.3
% CV	6.2%	8.4%	6.5%	4.7%	6.8%	8.4%

The Limit of Detection (LOD) [minimum detectable dose (MDD)] was determined by performing 20 replicates of assay diluent (blank) and calculating the mean signal + 2 standard deviations (SD) of the 20 values. The LOD is defined as the Hu Il-6 concentration corresponding to the mean assay blank + 2 SD. **The LOD was determined to be < 0.59 pg/mL**.

Comparison with Conventional ELISA Using Traditional 96-well Microplates

The assay reagents provided with the OptiMax™ Hu IL-6 ELISA kit (OMA-H-IL6) were incorporated in a conventional 96-well microplate-based Hu IL-6 ELISA. The Optimiser™-based and conventional Hu IL-6 ELISAs were performed concurrently for comparison (Figure 4 and Table 3).

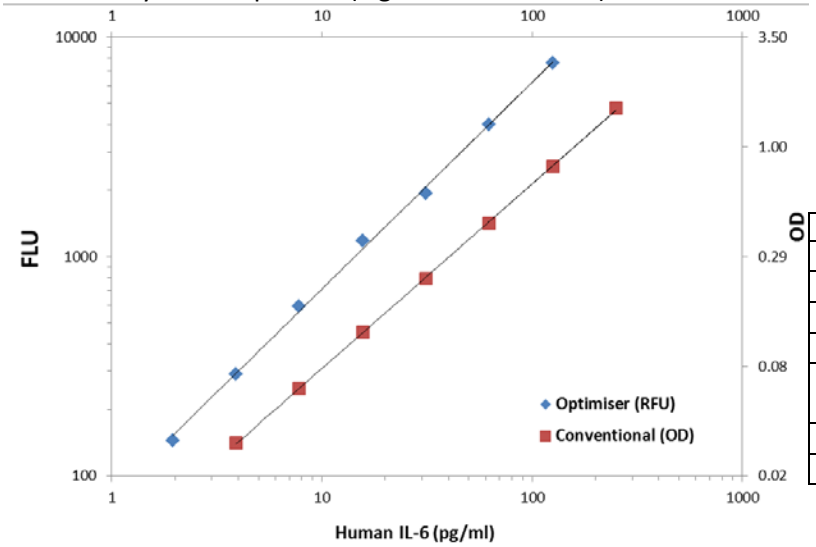

Figure 4. Comparison of conventional and Optimiser™-based Hu IL-6 ELISA assays.

Metric	Conventional	Optimiser™
Dynamic range	3.9 – 250 pg/mL	2.3 - 150 pg/mL
LOD	< 1 pg/mL	< 0.59 pg/mL
Reagent/step	100 µL	5 µL
Sample requirement	100 µL	5 µL
Incubation/step	1-2 Hours	10 min (reagents) 20 min (sample)
Total assay time	6.5 Hours	2 Hours
Precision	< 10%	< 10 %

Table 3. Comparison of conventional and Optimiser™-based HU IL-6 ELISA's.

The assay protocol for the conventional ELISA using the traditional 96-well microplate was briefly: 100 µL of 2 µg/mL capture antibody added and incubated overnight, 3x wash, 100 µL block buffer added and incubated for 1.5 hour, 3x wash, 100 µL of sample (or control, standard) added and incubated for 2 hours, 3x wash, 100 µL biotinylated detection antibody added and incubated for 1.5 hours, 3x wash, 100 µL SAv-HRP added and incubated for 0.5 hours, 3x wash, 100 µL of colorimetric substrate (TMB) added and incubated for 15 minutes and plate was read. The Optimiser™-based ELISA was performed as described in Figure 2. **The left shift of the Optimiser™ standard curve (Figure 4) clearly shows the higher sensitivity of the Optimiser™-based ELISA while using significantly smaller volumes of valuable sample, standard, and reagents than the conventional ELISA and completing the assay in significantly less time (and labor) than the conventional ELISA.**

Summary:

Siloam Biosciences has successfully applied its novel Optimiser™ technology to the rapid, accurate, and precise quantitation of Hu IL-6 in tissues culture supernatants. The Optimiser™ procedure utilizes a 5 µL sample volume; or one-twentieth of the volume required for most commercial ELISA kits**, AND** the Optimiser™-based method achieves better sensitivity. The procedure was completed in approximately two hours; or less than one-half the time required for most commercially available ELISA procedures. The accuracy and precision of the method met or exceeded the performance of other commercial kits.

- Siloam has also validated the Hu IL-6 assay using a **90 minute assay protocol** where each reagent incubation time is only 5 minutes. An example "90 minute assay" is described in a separate application note available in the "Technical Support → Application Notes" section of Siloam's website. The operating range of the 90 minute assay protocol is the same as the one reported here and the other assay metrics are comparable.
- Furthermore, the sensitivity of the assay can be readily "tuned" by repeating the sample addition step. Sensitivity of **femtogram/mL** is readily achievable by repeated sample loading. An example of this is described in a separate application note available in the "Technical Support → Application Notes" section of Siloam's website. Repeated sample addition (followed by 5 min incubation for each repeat) allows for a "pre-concentration" effect by allowing the surface bound capture antibodies to capture more analyte from the sample. Note that the total assay time increases slightly with each repeated sample addition step. Siloam recommends that up to 3 (no more than 5) sample addition repeats can be completed manually. If sensitivity gains beyond that achieved by 3-5 repeat loads are required, the use of a robotic sample processor is strongly recommended.

OptiMax™ kits are available from Siloam Biosciences, Inc. and its distributors.

Item	Catalogue Number	Comment
Optimiser™ Evaluation Pack	OPV-IL6	Contains training material and directions for migrating a conventional ELISA to the Optimiser™ assay system
OptiMax™ Human IL-6 ELISA kit	OMA-H-IL6-02	2-plate kit
Optimiser™ microplates	OPH-02	Optimiser™ 2-pack[a]
OptiMax™ buffers	OMR-02**#**[b]	2-plate reagent pack[a]
Test reagent pack	OMR-**TEST**	To determine optimal coating buffer for a capture antibody

[a] Also available in 10 plate and 50 plate sizes.
[b] OMR buffer packs are available with one of 12 different coat buffers. For instance, OMR-02 with coat buffer A is item OMR-02A

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SILOAM biosciences

Better Immunoassays Through Innovative Microfluidics

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Capture antibody, detection antibody, rHu IL-6 standard, and SAv-HRP provided by IMGENEX Corp under agreement. QuantaRed™ substrate is supplied by Thermo Fisher Scientific Inc.