INSTRUCTION MANUAL

OptiMax™ Microplate Adoption Kit
For Automated Pipetting Systems

OPTIMISER™

THE NEXT GENERATION OF MICROPLATES

SILOAM biosciences

Better Immunoassays through Innovative Microfluidics

INTENDED USE:

The OptiMax™ Microplate Adoption Kit for Automated Pipetting System and the associated Instruction Manual are specifically designed for first time user to provide a comprehensive overview to the OptiMax™ microplate system.

Section I serves as an Introduction to the OptiMax™ microplate system and guides the user through correct setting for using automatic pipetting system with the OptiMax™. The instructions are accompanied by a Tutorial that will allow users to evaluate their automated pipetting system with OptiMax™.

Section I also includes detailed instructions for an illustrative IL-6 assay (all assay reagents and buffers included with OMP-AK). Completing the associated Tutorial will allow users to learn the assay operation sequence and programming for OptiMax™ microplate assays. Successful completion of this assay will also help users understand the ***POWER OF MICROFLUIDICS*** to deliver high sensitivity ELISA results with only5 µL sample volume and a ~ 2 hour assay protocol.

Section II is a detailed method description to be used for migrating a validated assay from conventional 96-well plates to OptiMax™ microplate. Section II is presented as a series of 3 experiments, where each experiment sequence is described in complete detail including reagent preparation steps, assay plate layouts, assay procedures, calculations and data analysis methods. Section II also describes an illustrative IL-6 assay and the results from the IL-6 assay experiments are used to illustrate the data analysis methods used in the assay transfer guide.

Instruction Manual

OptiMax™ Microplate Adoption Kit

For adapting conventional ELISA plate assays to OptiMax™ Automation Plates using Automated Pipetting Systems

Catalogue Numbers: OMP-AK

Manufactured by:

Siloam Biosciences, Inc.
413 Northland Blvd.
Cincinnati, Ohio 45240
USA

FOR RESEARCH USE ONLY

Not for use in clinical diagnostic procedures.

Read the Instruction Manual in its entirety before using the OptiMax™ Microplate Adoption Kit

OptiMax™ microplates are warranted to perform in conformance with published product specifications in effect at the time of sale as set forth in product documentation and/or package inserts. Products are supplied for Research Use Only. The use of this product for any clinical diagnostic applications is expressly prohibited. The warranty provided herein is valid only when used by properly trained individuals and is limited to six months from the date of shipment and does not extend to anyone other than the original purchaser. No other warranties express or implied, is granted, including without limitation, implied warranties of merchantability, fitness for any particular purpose, or non-infringement. Buyers' exclusive remedy for non-conforming product during the warranty period is limited to replacement of or refund for the non-conforming product.

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SECTION I:

Using the OptiMax™ Microplate System With Automated Pipetting System

INTRODUCTION

Siloam Biosciences' Optimiser™ technology platform offers a rapid and sensitive chemifluorescent-based ELISA procedure that uses very small sample volumes. The speed, sensitivity, and small sample requirements are enabled by the unique microfluidic design of the Optimiser™ & OptiMax™ microplates. Standard immunoassay reactions such as analyte capture and detection occur within a ~ 5 µL microfluidic reaction chamber. The unique microchannel geometry and small reaction volumes favor rapid reaction kinetics. Typical Optimiser™ assay procedures utilize 5 µL sample volumes and each reaction step is completed within 5 - 10 minutes. Most standard Optimiser™ technology-based ELISAs are completed within approximately 2 hours.

Please refer to the Optimiser™ Technology page on Siloam's website (www.siloambio.com) *for more details regarding the principles behind the Optimiser™ &OptiMax™ microplate platform.*

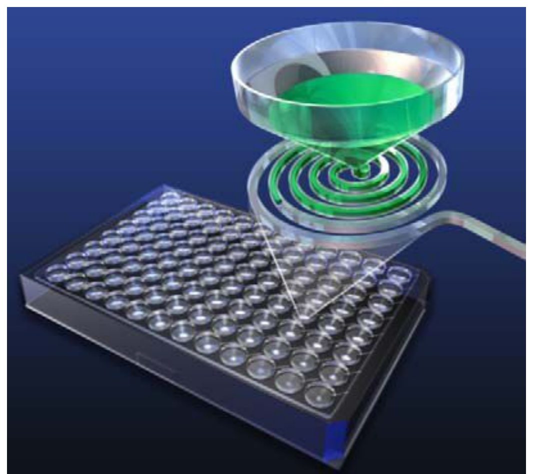


Figure 1.OptiMax™ microplate:
The OptiMax™ microplate is a revolutionary new microplate format. With an ANSI/SBS compliant 96-well layout, the OptiMax™ integrates the **Power of Microfluidics** to allow for low volume, rapid, and uniquely high-sensitivity immunoassay protocols. Figure 1 shows the OptiMax™ microplate schematic with a magnified view of one "cell" of the OptiMax™ microplate. Each cell of the OptiMax™ microplate has a loading well (only used to add reagents) and a microfluidic reaction chamber. Reagents/samples are added to the well and transported via capillary action to an absorbent pad (not shown). The unique design of the OptiMax™ microplate allows the well to be drained but each liquid is trapped in the channel by capillary forces. As the next liquid volume is added, the capillary barrier is broken and the liquid within the microchannel is drawn out by the absorbent pad and replaced by the new reagent. All assay reactions occur within the microfluidic reaction chamber.

The automation-compatible OptiMax™ microplate is built on the same principles as the Optimiser™ microplate. The OptiMax™ plate integrates the holder and absorbance pads within the plate body. The resulting footprint is identical to the standard ANSI/SBS 96-well layout and fits on the corresponding hardware of all robotic liquid handling systems. The built-in pads provide adequate absorbent capacity for all anticipated ELISA procedures and do not need to be removed prior to plate reading. The opaque sealing layer of the OptiMax™ plate prevents interference by liquids absorbed within the pad and allows the plate to be read (intact) **without disassembly**.

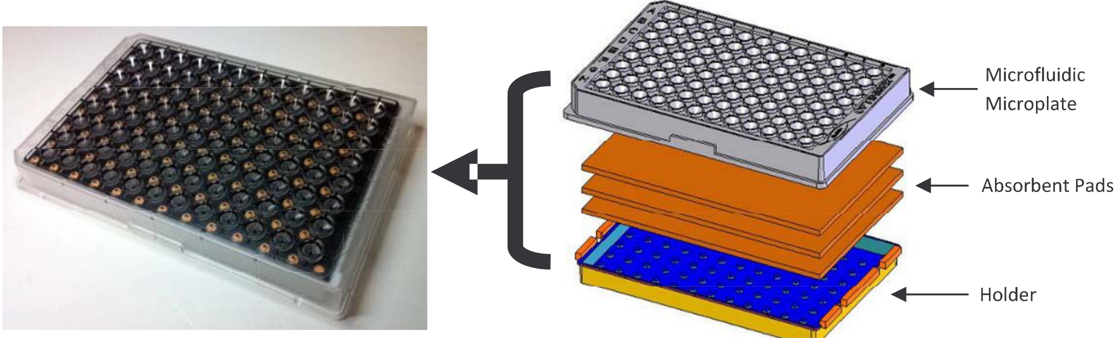


Figure 2.Assembled OptiMax™ microplate (left) and illustration of plate components (right).

NOTE:
Optimiser™ and OptiMax™ microplates are virtually identical in performance.
Optimiser™ microplates are limited to manual use (external holder must be manually detached prior to reading).
OptiMax™ plates are fully automation-compatible with no need for disassembly.

MATERIALS PROVIDED AND REQUIRED:

Materials Provided:

OptiMax™ Microplate Adoption Kit for Automated Pipetting System provides the critical materials and reagents necessary for the Tutorial described in this manual and for the user to develop and optimize an ELISA assay on the OptiMax™ plate. Table 1 identifies the kit contents, their function, and their required storage temperature. **It is recommended that the package be opened and various components stored separately (as listed in Table 1).**

Table 1. Materials Provided with the OptiMax™ Microplate Adoption Kit*

Material	Quantity	Function	Storage / Handling (before and after opening)
OptiMax™ Microplate	5	Contains microfluidic reaction chambers One for reader setup and liquid testing; One for IL-6 demo assay; Three for assay development	Room temperature
96-well polypropylene v-bottom plate	5	For dilutions and reagent reservoir	
OptiBind™, A-L	1 vial each (4 mL)	Coating buffer panel for screening to determine optimal coating buffer for capture antibody	Refrigerated (2 – 8°C)
OptiBind™-H	1 vial (10 mL)	Coating buffer for model IL-6 assay	
OptiBlock™	1 vial (30 mL)	Blocking buffer and diluent for detection antibody and SAv-HRP	
Protein Free Blocking buffer (free sample)**	1 vial (7 mL)	Wash buffer for assays whose capture antibody requires OptiBind™ Buffer A-E as coating buffer.	
OptiWash™	1 vial (60 mL)	Wash buffer	
OptiGlow™ - A	1 vial (5 mL)	Components of chemifluorescent substrate	
OptiGlow™ - B	1 vial (5 mL)		
OptiGlow™ - C	1 vial (1 mL)		
Red dye solution	1 vial (7 mL)	Dyed blocking buffer solution for pipetting exercise	
Green dye solution	1 vial (7 mL)	Dyed wash buffer solution for pipetting exercise	
IL-6 standard	1 vial	Lyophilized recombinant IL-6 protein for model assay standard curve	Refrigerated (2 – 8 °C) **After reconstitution, standard must be aliquoted and stored at ≤ -20 °C. Avoid repeated freeze-thaw cycles for standard.**
IL-6 Capture Antibody	1 vial	Captures IL-6 on solid-phase	
IL-6 Detection Antibody	1 vial	Detects captured IL-6, biotin-conjugated	
SAv-HRP	1 vial	Binds biotin on detection antibody; activates substrate to yield chemifluorescent signal.	

***Material Safety Data Sheets (MSDS) are available on the Siloam Biosciences' web site. (http://www.siloambio.com)**

**** Protein Free (PBS) Blocking buffer; Thermo Scientific (Catalog#37584, 37572) – provided at no cost.**

AUTOMATIC PIPETTING SYSTEM SETUP

System Requirements:

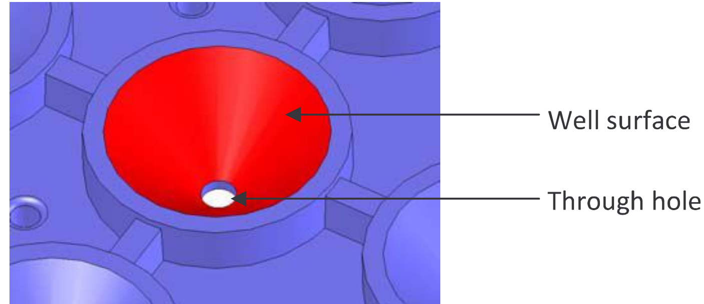
- The system must be capable of precisely dispensing 5µL and 30 µL volumes.
- The system must have carrier(s) capable of holding SBS standard 96-well plates.
- The system should be equipped with 8 (or 12) channel pipettor and/or 96-channel pipettor head
- OptiMax™ microplate is compatible with both disposable and fixed tips. ***Do not use low retention tips.***
- Minimize dust in the working environment.

Reagent and Sample Preparation:

- Solutions must be free of visible insoluble material (*e.g.,* particulates, precipitate).
- Centrifugation for 10 minutes at 13,000 *x g* is necessary during sample preparation.

Position of Tips While Dispensing:

While dispensing the liquid into the OptiMax™ microplate, the pipettor tips **<u>must contact the well surface</u>**. Solutions must be dispensed onto the surface of the conical well (shown as the red area in figure below):



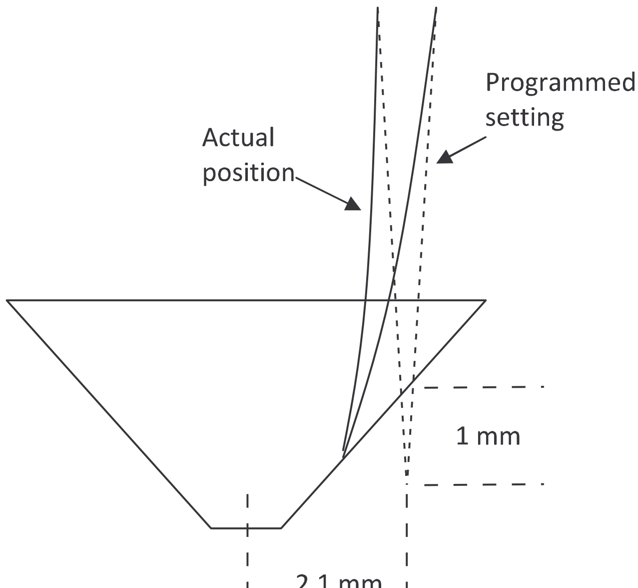
Once the liquid touches the well surface, it will flow into the microchannel beneath the loading well via the through hole. The basic structure of an OptiMax™ well is shown above. *Please see Appendix 1 at Page 29 for detailed plate drawing*.

Siloam recommends positioning pipettor tips relative to the loading well as illustrated below:

With the programmed setting illustrated on right, the tip will bend slightly as it comes in contact with the well surface. The resulting tip-to-well contact will ensure proper delivery of solutions. This setting has been used successfully with various automated fluid handling systems. Please see comprehensive list on page6.

IMPORTANT:

- **DO NOT POSITION THE TIP INSIDE THE THROUGH HOLE!**
- **TIP MUST CONTACT WELL SURFACE DURING DISPENSING!**



Liquid Class Configurations:

The following recommendations for precisely dispensing small liquid volumes into the OptiMax™ microplate can ensure correct dispensing operation with requisite precision and accuracy, but without introducing problematic air bubbles.

1) **Dispensing rate**: Use low dispensing rate (20-50 µl/sec) to dispense small liquid volumes into the OptiMax™ microplate.
2) **Do not "blow out" the liquid in dispensing step**.
3) **Aspirate extra liquid**: Aspirate the volume that you intend to dispense (*e.g.,* 5 µL) plus an additional 2-5 µL of extra solution to avoid dispensing air into the well and creating a bubble.
4) **For aspiration**: Small quantities of assay reagents will be used in most of steps. Siloam suggests using reservoirs with v-shaped bottoms for most reagents and always aspirating from near the bottom of the v-shaped reservoir.
5) **Dispensing sample volume less than 5 µL:** Sample volumes can be reduced to as low as 2 µL for OptiMax™-based assay procedures without compromising assay sensitivity. Please consult with Siloam technical support for appropriate settings for precisely dispensing less than 5 µL solutions onto the OptiMax™ microplate well surfaces.

Siloam Biosciences has tested OptiMax™ microplates on the following automated fluidic handling systems. Siloam Biosciences can provide more detailed settings and programs for the automated fluidic handling systems listed below. Please contact Siloam Biosciences' Technical Support Department for more information.

Table 1.Automated Fluidic Handling Systems Verified with OptiMax™ Microplates

Manufacturer	Model	System configuration	Tip
BioTek*	Precision	8 channel pipettor	Disposable tip
Tecan*	EVO100	8 channel pipettor, RoMa Arm, and M200 reader	Disposable & Fixed
Hamilton	MicroLab Star	12 channel pipettor and 96-channel head	Disposable
Beckman Coulter	BioMek FXP	8 channel pipettor and 96-channel head	Disposable

* contact Siloam Biosciences' Technical Support Department for program scripts to run the assay methods in this guide.

FLUORESCENCE PLATE READER SETUP

OptiMax™ microplate-based assays are compatible with standard fluorescence plate readers and multi-mode plate readers with fluorescence read capability. Below is the general guidance for setting up the readers. *The "Technical Support" section on Siloam's website offers detailed guidance on the set up of several major brands of instruments as illustrative examples.*

<u>**Step 1**: Selecting the wavelength for excitation and emission light:</u>

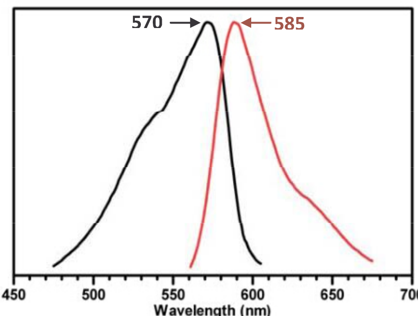


Figure 3. Normalized absorption (left) and emission (right) spectra of OptiGlow™ chemifluorescent substrate.

Assays performed on the OptiMax™ platform use the OptiGlow™ substrate system which can be detected using the appropriate excitation and emission settings (Figure 3). Quantitation does not require filters that precisely match the excitation/emission maxima. However, a non-overlapping filter set with a band-pass that includes the excitation/emission spectra is required. Wavelengths can be set at 530-575 nm for excitation and at 585-630 nm for emission. Below are examples for different types of readers:

- **Filter-based readers**: Install 528/20 nm (or similar) band-pass filter for excitation and 590/35 nm (or similar) band-pass filter for emission.
- **Monochromator-based readers**: Set excitation wavelength at 544 nm and emission wavelength at 590 nm.
- **Readers with pre-configured optical set:** Select the wavelength setting for Rhodamine or Cy3.

<u>**Step 2**: Selecting the plate type:</u>
The OptiMax™ microplate fits the 96-well SBS standard in all specifications. Please use "96-well standard" or similar selection when setting the plate type.

<u>**Step 3**: Selecting the probe direction:</u>
Use "top reading" for probe direction.

<u>**Step 4**: Selecting the sensitivity/gain:</u>
When defining reading parameters for fluorescence analysis, setting the photomultiplier tube (PMT) sensitivity (referred to as "gain" in some types of fluorescence readers) is important for obtaining useful measurements. A manual sensitivity/gain setting is recommended for reading OptiMax™ microplates. The procedure is described below:

Fluorescence Solutions Preparation: Serial Dilution of Activated OptiGlow™ Substrate

1) In well A1 of a supplied 96-well v-bottom plate, add 50 µL of OptiGlow™-A, 50 µL of OptiGlow™-B, **5 µ**L of OptiGlow™-C, and 1 µL of supplied SAv-HRP stock solution, mix well, and wait for 2 minutes. The substrate will be fully developed to a red fluorescence dye solution, and stable for hours.
2) Prepare 1:2 serial diluted solutions with OptiWash™ to prepare 15 fluorescence solutions with 1 zero point (blank):
   a) Load 50 µL of <u>OptiWash™</u> to well B1-H1, and A2-H2. Do not use other buffers.
   b) Transfer 50 µL solution from well A1 to well B1 and mix well.
   c) <u>Change the tip</u>, repeat same procedure till well H1, then continue to well B2 and repeat till well G2, leaving well H2 as zero point (blank).

	1	2
A	no dilution	1/256
B	1/2	1/512
C	1/4	1/1024
D	1/8	1/2048
E	1/16	1/4096
F	1/32	1/8192
G	1/64	1/16384
H	1/128	Zero (OptiWash only)

Transfer to OptiMax™ plate and read:

Transfer **4µL solution** of each well in the V-bottom plate to the corresponding well on column 1 and 2 of OptiMax™ microplate. Wait for all the wells to empty. Read the OptiMax™ microplate with fluorescence reader.

In order to read OptiMax™ microplate, adjust your reader setting as listed in page 6 to enable the following requirements:

- For the capacity to run a wide dynamic range assay (*e.g.,*729 fold), the reader should have:
  - Detectable dose response from well A1 to D2 (*e.g.,* 19114 vs. 37 RFU) AND
  - Clearly distinguish well D2 to well H2. (*e.g.,* 37 vs. 10 RFU)
- For the capacity to run a more-limited dynamic range assay (64 fold), the reader should have:
  - Detectable dose response from well C1 to D2 (e.g., 7864 vs. 37 RFU) AND
  - Clearly distinguish well D2 to well H2. (*e.g.* 37 vs. 10 RFU)

When using same reader setting to read IL-6 demo assay on the OptiMax™ plate, the top signal should be close to value of well A1 and the background signal should be close to value of well B2-A2. **Record the value of RFU_{max} (from A1), which will be used as reference in assay transfer procedure.**

Note: please transform your reading from RFU (relative fluorescence unit) to percentage of RFU_{Max} (from well A1) for comparing your data to the typical data provided below.

The following results show data on a BioTek FLx800 Fluorescence Plate Reader, with excitation filter at 529/20, emission filter at 590/35, and sensitivity at 45.

Well #	Dilution from 5x fully developed substrate	RFU	Percentage of Max
A1	1	19114	100.00%
B1	2	13082	68.44%
C1	4	7865	41.15%
D1	8	4438	23.22%
E1	16	2379	12.45%
F1	32	1240	6.49%
G1	64	630	3.30%
H1	128	327	1.71%
A2	256	188	0.98%
B2	512	106	0.55%
C2	1024	62	0.32%
D2	2048	37	0.19%
E2	4096	25	0.13%
F2	8192	17	0.09%
G2	16384	15	0.08%
H2	OptiWash (blank)	10	0.05%

Desired range for reader. Detectable dose response should extend ~4-8x dilutions less than expected assay background

Typical assay background

Clear difference in signal intensity between well D2 and H2

TUTORIAL 1: PIPETTING TO THE OPTIMAX™ MICROPLATE USING AUTOMATED PIPETTING SYSTEMS:

Materials Required for Pipetting Tutorial and Supplied with OptiMax™ Microplate Adoption Kit:

1. Same OptiMax™ plate used for reader setup (column 1 and 2 have been used)
2. One 96-well v-bottom plate
3. OptiWash™ buffer
4. Green dye solution
1. Red dye solution

Other Materials/Equipment Required:

1. Single channel pipette with tip capable of delivering 100 µL
2. Automatic pipetting system

Pipetting Procedure with Dye Solution:

1. Transfer 100µL of green dye solution into each well of Column 1 on 96-well v-bottom reservoir. Transfer 100 µL of red dye solution into Column 2. Transfer 100 µL of OptiWash™ solution into column 3.
2. Place 96-well v-bottom reservoir and an OptiMax™ plate in the automatic pipetting system. Program the system to transfer solutions in triplicate into Column 4-6 of the OptiMax plate as illustrated below.

#1: 5 µl Green dye; wait for 10 min.

↓

#2: 5 µl Red dye; wait for 5 min.

↓

#3: 5 µl Red dye; wait for 10 min.

↓

#4: 5 µl Red dye; wait for **20** min.

↓

#5: 30 µl OptiWash™; wait for 10 min.

OBSERVATIONS AND CONCLUSIONS

- Except in step #2, all wells should be empty within time limits listed (left). There may be a little residual volume left in step #2, but all wells will drain completely in step #3. If a well is not empty after 10 minutes, most likely a bubble will be evident near the microchannel interface with the well. *This bubble was likely injected due to* ***incorrect pipetting setting****s.* Please refer to the pipetting guidelines to correct the pipetting settings, and try again. Verify that pipettor tips are positioned correctly to contact well during dispensing.
- Observe the wells as they drain out. Note the variation in time to empty each well. ***Bear in mind that, as long as each well drains out in 10 minutes, this variation has NO EFFECT ON ASSAY PERFORMANCE.***
  - ➢ Most assay protocols on OptiMax™ recommend a 10 minute incubation interval The 20 minute incubation step with red dye shows that ALL incubations can be extended up to 20 minutes. This may be useful for processing multiple OptiMax™ microplates in parallel.
  - ➢ **Incubation steps should be at least 5 minutes and no more than 30 minutes.**

3. **CHECK THAT ALL WELLS DRAIN WITHIN 10 MINUTES FOR EACH DISPENSING STEP.**

4. **If any wells take longer than 10 minutes the most likely cause is incorrect setting in pipetting causing a visually-evident micro-bubble. Please refer to the Automatic Pipetting System Setup and repeat the pipetting test. Verify that pipettor tips are positioned correctly to contact well during dispensing.**

TUTORIAL 2: IL-6 DEMONSTRATION ASSAY ON THE OPTIMAX™ MICROPLATE USING AUTOMATED PIPETTING SYSTEM:

The OptiMax™ Microplate Adoption Kit also contains necessary reagents of an IL-6 sandwich ELISA to demonstrate the capabilities of OptiMax™-based assays when used with automatic pipetting system. The representation of the expected data produced from ***OptiMax™ Microplate Adoption Kit is not intended for use as a commercial assay kit***; this control ELISA is provided for training purposes only.

Materials Required for Demonstration Assay and Supplied with OptiMax™ Microplate Adoption kit:

1. One new OptiMax™ microplate(#2)
2. One new 96-well v-bottom plate
3. OptiBind™-H buffer
4. OptiBlock™ buffer
5. OptiWash™ buffer
6. OptiGlow™ substrate kit, contains component A, B and C
7. IL-6 capture antibody
8. Lyophilized IL-6 standard
9. IL-6 detection antibody, biotinylated
10. Streptavidin–HRP

Very small volumes of assay reagents are required and provided for OptiMax™ Microplate-based assays.
NOTE: ***A quick-spin (mini-centrifuge) of each reagent tube is CRUCIAL to recover all material in Items 7-10.***

Materials Required for Assay but Not Supplied

1. Eppendorf or similar tubes for centrifugation and dilutions

Equipment Required:

1. Automatic pipetting system.
2. Single channel Pipettes capable of accurately and precisely delivering liquids in the ranges of 1 -10, 10 -100, and 100 – 1000 µL for reagent preparation.
3. Vortex mixer
4. Microplate fluorescence reader and control software
5. Analytical software

Assay Layout:

The plate layout of IL-6 standard concentration is shown in below. Each concentration will be run in triplicate; three columns of one OptiMax™ microplate will be used.

Table 2. Plate layout of IL-6 concentrations (pg/mL) for demonstration assay

	1	**2**	**3**
A	3000	3000	3000
B	1000	1000	1000
C	333.3	333.3	333.3
D	111.1	111.1	111.1
E	37.0	37.0	37.0
F	12.3	12.3	12.3
G	4.1	4.1	4.1
H	0	0	0

Reagent Preparation:

All reagents, **except OptiGlow™ substrate**, shall be prepared and transferred into the 96-well v-bottom plate (reservoir) for the assay procedure. OptiGlow™ substrate will be loaded into the reservoir at the end of procedure (see Assay Procedure). Below is the reagent layout in the reservoir.

Table 2.Reagents preparation in96-well v-bottom reservoir

Column#	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8-12**
Reagent	Capture antibody 60 µL/well	OptiBlock™ 80 µL/well	IL-6 standards 80 µL/well	Detection antibody 60 µL/well	SAv-HRP 60 µL/well	OptiWash™ 300 µL/well	Substrate 60 µL **(Load at the end)**	Not used

Each reagent will be prepared and transferred into the 96-well v-bottom reservoir for easy aspiration; an extra volume of 40 µL is provided for each reagent.

Bring all reagents to room temperature before use and prepare all necessary dilutions before beginning the test procedure.

1. Capture Antibody: The procedure requires 5 µL of capture antibody working solution for each OptiMax™ Microplate assay well to be used.
   a. Prepare a **1:62.5 dilution** of the capture antibody stock solution in OptiBind™-H buffer in a clean, polypropylene tube, such as an Eppendorf tube. (Add 8 µl of capture antibody stock solution to 0.5 mL of OptiBind™ buffer.)
   b. Dispense 60 µL of the working solution into each well of column 1 in the polypropylene 96-well v-bottom plate.

2. OptiBlock™: OptiBlock™ buffer is provided in ready-to-use form and is used to block the surfaces of the OptiMax™ microplate's microfluidic reaction chambers following their incubation with the capture antibody solution. OptiBlock™ is also used as the diluent for the standard, biotinylated detection antibody and SAv-HRP in this experiment.

3. Recombinant IL-6 Standard:
   a. Stock Solution: The IL-6 standard is provided in lyophilized form.
      i. Reconstitute the lyophilized standard by adding 420 µL OptiBlock™ blocking buffer.
      ii. Mix by gentle swirling until all of the lyophilized material has dissolved.
      iii. Vortex gently to ensure thorough mixing of the reconstituted standard.
      iv. Use freshly prepared material on the day of reconstitution
   b. Working Solution: The concentration of the reconstituted IL-6 standard is 4ng/mL. Prepare a 3000 pg/mL standard (Standard 1) by mixing 120µL IL-6 standard appropriately with 40 µL OptiBlock™ blocking buffer. Vortex the 3000 pg/mL standard briefly to mix.
   c. Standard Curve: Prepare the remaining IL-6 standards by performing six serial three-fold dilutions in OptiBlock™ beginning with the 3000 pg/mL standard as follows:
      i. Dispense 120 µL of Standard 1 (3000 pg/mL) to well A3 of the 96-well polypropylene v-bottom plate.
      ii. Dispense 80 µL OptiBlock™ to each of the seven wells of the same column immediately below the 3000 pg/mL-containing well (wells B3 – H3).
      iii. Transfer 40 µL of the 3000 pg/mL standard from well A3 to well B3 immediately below it. Mix the contents of well B3 gently. Then transfer 40 µL from well B3 to well C3, **change tips**, and titrate.
      iv. Continue serial dilutions **while changing tips after each 40 µL transfer** and before mixing until the 4.1pg/mL standard has been created in the seventh well (well G3) of the column.
      v. Do not transfer IL-6 solution to the eighth well (H3). It contains OptiBlock™ only and will provide material for the blank wells.

	3
A	120 µL Std 1
B	80 µL Blocking
C	80 µL Blocking
D	80 µL Blocking
E	80 µL Blocking
F	80 µL Blocking
G	80 µL Blocking
H	80 µL Blocking

	3
A	3000
B	1000
C	333.3
D	111.1
E	37.0
F	12.3
G	4.1
H	0

4. Detection Antibody: The procedure requires 5 µL of the detection antibody working solution for each assay well to be used.
   a. Prepare a **1:25 dilution** of the detection antibody stock in OptiBlock™ in a clean plastic tube (add 20 µL of detection antibody stock solution to 480 µL of OptiBlock™).
   b. Dispense 60 µL of the working solution into each well of column 4 in the 96-well v-bottom plate.
5. SAv-HRP: The procedure requires 5 µL of the SAv-HRP working solution for each assay well to be used.
   a. Prepare a **1:150 dilution** of the stock in OptiBlock™ in a clean plastic tube (Add 4 µL of SAv-HRP stock solution to 0.6 mL of OptiBlock™).
   b. Dispense 60 µL of the working solution into each well of column 5in the 96-well v-bottom plate.
6. OptiWash™: OptiWash™ is provided in ready-to-use form. No further preparation is required. The procedure requires 60 µL of OptiWash™ for each assay well to be used. Dispense 300µL OptiWash™ buffer into each well of column 6 in the 96-well v-bottom plate.
7. Substrate solution: The procedure requires 5µL of the working substrate solution for each assay well to be used.
   a. Prepare the working substrate solution no more than 30 minutes before the anticipated time for reading the completed assay.
   b. To create the substrate working solution, combine OptiGlow™-A, OptiGlow™-B, and OptiGlow™-C in a ratio of **50:50:5** parts respectively in a clean plastic tube and vortex gently to mix (add 250 µl of OptiGlow™-A, 250 µl of OptiGlow™-B, and 25 µl of OptiGlow™-C).
   ****OptiGlow™-C is a solid at 4C. It can be thoroughly thawed at RT or 37C to enable you to pipette correctly and for it to function effectively. Warm the reagent in a 37° C incubator/oven/heater or by warming the vial gently in your hands.***
   c. Dispense 60 µL of the working solution into each well of column 7 in the 96-well v-bottom plate.

DO NOT SUBSTITUTE OTHER BUFFERS OR REAGENTS FOR THOSE PROVIDED WITH THE KIT. OptiMax™ buffers are specially formulated to work with the OptiMax™ microplate and substitute buffers or reagents may lead to poor and unpredictable assay performance.

Procedure:

Prepare reagents in 96-well v-bottom reservoir as described in Page 12 and perform the assay. Please program the automatic pipetting system to transfer reagents as illustrated below. A timer may be used to remind the user to prepare the substrate final working solution.

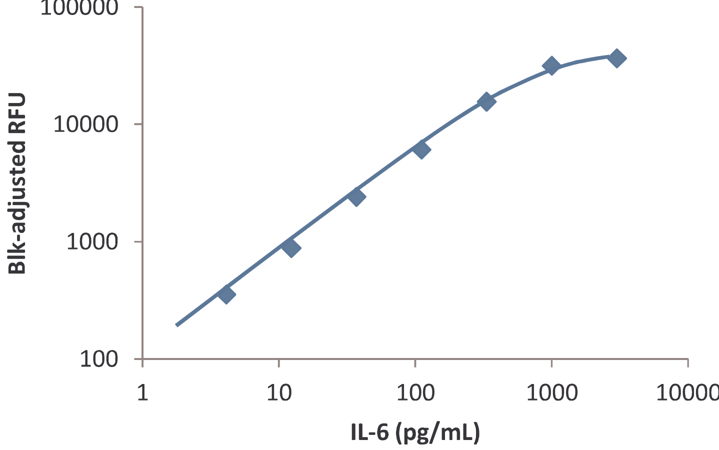
Program	Solution to transfer into OptiMax	Volume (µL) transferred to each well of OptiMax	Incubation/waiting time (min)
Program start →	Capture antibody	5	10
1 hour and 25 minutes (Capture antibody through OptiWash)	OptiBlock*	5	5
	OptiBlock	5	10
	Standards	5	10
	OptiWash	5	5
	Detection antibody	5	10
	OptiWash	5	5
	SAv-HRP	5	10
	OptiWash	30	10
	OptiWash	30	10
Program pause →	Prepare and transfer the OptiGlow™ working solution into the 96-well v-bottom plate*		
Program resume →	Substrate	5	15
	Read with fluorescence reader		

Calculations:

1. Calculate the mean background signal from the blank wells (wells containing OptiBlock™ only at the sample incubation step).
2. Subtract the mean background signal from the signal of individual standard.
3. Create a standard curve by plotting the standard concentration (x-axis) vs. the background-adjusted signal in relative fluorescence units (RFU) (y-axis). **A five parameter logistic curve fit with appropriate software is recommended.**

Typical Data:

The IL-6 standard curve ranges from **4.1 to 3000 pg/mL**. Concentration (x-axis) and signal (y-axis) are plotted on Log scales. A typical standard curve is presented below. Note again that tripling dilutions are used for a wider dynamic range than is typically run in such assays.



IL-6 (pg/ml)	Average	Blank-Subtracted
3000	18333	18254
1000	15861	15782
333.3	7882	7803
111.1	3127	3048
37.0	1286	1207
12.3	521	442
4.1	257	178
0	79	0

Figure 4. IL-6 Standard Curve with Tabulated Data

SECTION II:

Assay Transfer Guide

PROCEDURE FOR MIGRATING A VALIDATED ASSAY FROM CONVENTIONAL 96-WELL MICROPLATE TO OPTIMAX™ MICROPLATE USING AUTOMATED PIPETTING SYSTEMS

SANDWICH ELISA ASSAY TRANSFER GUIDE USING AUTOMATED PIPETTING AND OPTIMAX™ MICROPLATES:

The OptiMax™ Microplate ELISA procedure is a chemifluorescent immunoassay procedure in which traditional ELISA reactions take place within the unique OptiMax™ microplate architecture. Briefly, capture antibody is immobilized on the internal surfaces of the plate's microchannels. Following a wash step, any unreacted sites on the microchannel surface are blocked with a blocking solution. Standards and samples are dispensed to the OptiMax™ wells. Antigen present in samples and standards will be specifically captured on the microchannel surface by the immobilized capture antibody. Following another wash, a biotin-labeled detection antibody is added to the wells. The biotin-labeled antibody will bind antigen that has been captured and immobilized on the microchannel surface thus "sandwiching" the antigen between the capture and detection antibodies. Following another wash, horseradish peroxidase-labeled streptavidin (SAv-HRP) is added to the OptiMax™ microplate wells. The streptavidin of SAv-HRP binds specifically to the biotin moiety of the biotin-labeled antibody if it is present in the [capture antibody+antigen+detection antibody] complexes formed and immobilized on the microchannel surface. Following two additional washes, a chemifluorescent substrate is added to the wells. If horseradish peroxidase has been captured on the microchannel surface during the sequence of reactions cited above, the enzyme will activate the substrate solution and will yield a fluorescent signal when excited at the appropriate wavelength. Within the linear portion of the curve, the light signal emitted will be directly proportional to the concentration of antigen in standards and samples, and will be quantifiable when the plate is read using a fluorescence plate reader.

In order to achieve the best assay performance with OptiMax™ microplate platform, optimization studies must be conducted for each assay of interest in order to identify ideal reagent use and best assay performance. A well-characterized and robust assay on 96-well platform is a mandatory prerequisite for the Assay Transfer Process. The assay transfer is a 3-step process with step 1 being data collection for conventional assay. Second, run one experiment to screen 12 types of supplied OptiBind™ coating buffers to determine the best coating buffer to use for this assay. Third, run a checkerboard titration experiment to determine the concentrations of capture antibody and detection antibody which yield the best signal:noise ratio. Finally, run an assay with wide range of target protein concentrations using the selected optimal coating buffer and antibody concentrations to determine the measurable/dynamic range of the assay.

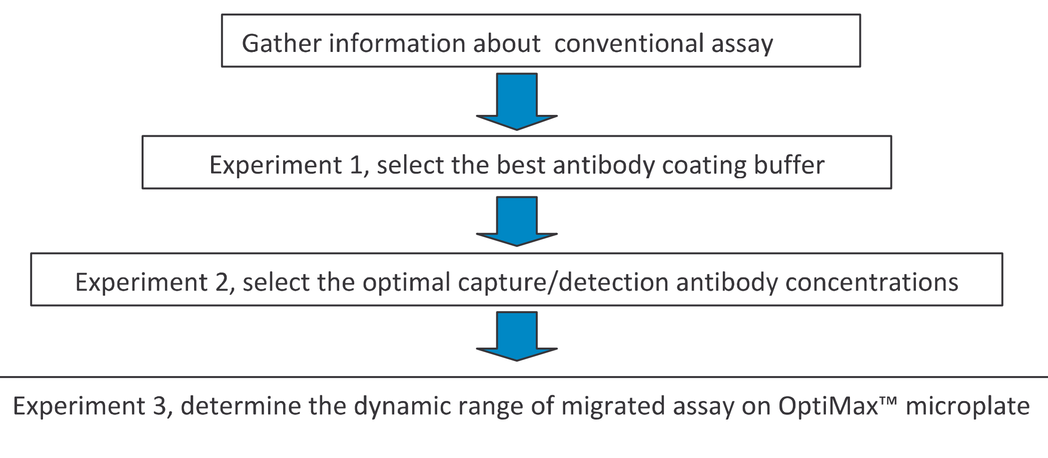


Figure 5. Schematic assay transfer procedure for OptiMax™ microplate.

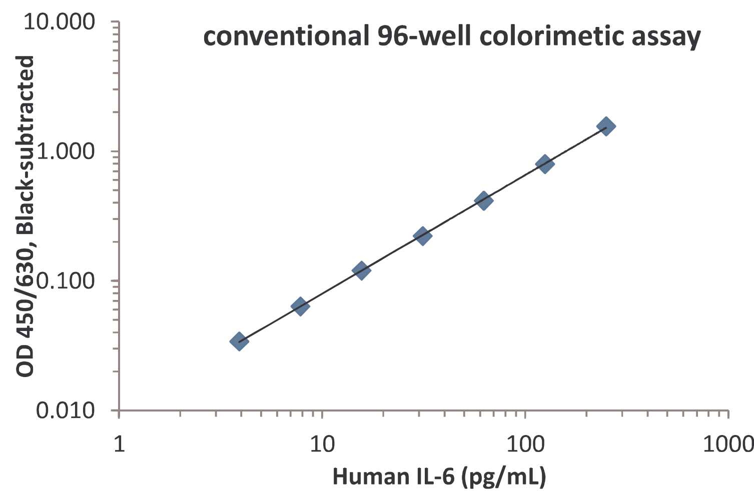
Requirements for Conventional Assay to be Transferred to OptiMax™ Microplate

For transfer to OptiMax™ microplate, a robust conventional (96-well plate) assay is expected with the following **minimum performance metrics:**

- **Reasonable background (zero) reading**: for a colorimetric assay, the OD450/630 absorbance reading of background shall be less than 0.15 (*e.g.,* after a standardized colorimetric substrate development time, such as15 minutes).
- **Reasonable dose response** with various concentrations of standard: for an absorbance assay, the OD450/630 absorbance reading of highest concentration in detectable range shall be higher than 1.5(*e.g.*, after a standardized colorimetric substrate development time, such as 15 minutes).In addition to satisfying the minimum performance metrics, the following information is required for the assay transfer process:
- **Known concentrations or dilution ratio** for capture antibody and detection antibody working solution. The capture and detection antibody solutions must be in a format such that at least 4x concentration solutions (as compared to working concentration for conventional 96-well plate) can be prepared.
- **HRP conjugate:** the detection antibody must NOT be directly labeled with HRP; a biotin labeled detection antibody is most preferred (*see next page for details*)
- **Known dynamic range for assay** is used as a starting point for the assay transfer process.

IT IS STRONGLY RECEOMMENDED THAT ALL ASSAY MATERIALS USED SHOULD BE TESTED IN COLORIMETRIC FORMAT RIGHT BEFORE THE ASSAY MIGRATION PROCESS TO ENSURE MATERIAL QUALITY. DO NOT USE ELISA REAGENT MANUFACTURER'S SPECIFICATIONS WITHOUT A CONFIRMATORY EXPERIMENT VERIFYING PERFORMANCE IN COLORIMETRIC MODE.

As an example*, a working IL-6 colorimetric assay with conventional 96-well plate is shown below. The assay transfer guide uses this assay as an example to illustrate the transfer process.*



IL-6 (pg/mL)	Average	OD450 (Blank Subtracted)
250	1.586	1.561
125	0.823	0.798
63	0.441	0.416
31	0.247	0.222
16	0.145	0.120
8	0.089	0.064
4	0.059	0.034
0	0.025	

Figure 6. Standard curve of IL-6 assay run in conventional 96-well plate, using TMB substrate and colorimetric detection of absorbance at 450 nm and corrected with 630 nm.2 µg/mL concentration for Capture and Detection antibody.

Details for illustrative IL-6 assay:

- Assay metrics
  - Background OD = <u>0.025</u> (less than 0.15); (e.g., after a standardized colorimetric substrate development time, such as 15 minutes).
  - Max signal OD = <u>1.58</u> (more than 1.5); (e.g., after a standardized colorimetric substrate development time, such as 15 minutes).
- <u>Conventional colorimetric assay; Known information:</u>
  - Concentration of capture antibody = <u>2 µg/mL</u>

- Concentration of detection antibody = 2 µg/mL
- Conjugate for detection antibody: biotin conjugated
- Assay dynamic range = 4 pg/mL – 250 pg/mL

HRP Concentrations in OptiMax™ Microplate Assays:

OptiMax™ microplates are an exquisitely-sensitive platform for high-sensitivity ELISA with minimal sample/reagent volume requirements. It is **CRITICALLY IMPORTANT** to follow the guidelines for the HRP conjugate to ensure that the sensitive response from the OptiMax™ microplate is not overwhelmed by erroneously-high HRP concentrations. The use of a biotinylated detection antibody is recommended with the well-characterized and validated SAv-HRP provided by Siloam Biosciences (Cat# OMR-HRP) to obtain the best response.

- **For biotinylated detection antibody**: a vial of appropriate SAv-HRP is included in the kit. Please prepare the working solution at 1:150 dilution with OptiBlock™ buffer.

It is strongly recommended that the SAv-HRP provided by Siloam (Cat# OMR-HRP) be used for all assays on OptiMax™. The concentration and activity have been characterized and optimized for use with the OptiMax™ microplate system. Use of alternate SAv-HRP may lead to low signals or very high backgrounds.

- **For using HRP conjugated secondary antibodies (e.g., anti-mouse IgG, anti-goat IgG, etc.)**: Reagent must be titrated extensively to determine optimal working conditions.
- **For using an HRP labeled detection antibody**: Characterization studies at Siloam with multiple directly-labeled antibodies demonstrate that a high background may occur. ,
- ***[PLEASE DELETE THIS SPECULATION/EDITORIALIZING ABOUT THE POTENTIAL CAUSES OF THE HIGH BACKGROUND.]***

Materials Required for Demonstration Assay and Supplied with OptiMax™ Microplate Adoption Kit:

1. One OptiMax™ microplate(#3)
2. One new 96-well v-bottom plate
3. OptiBind™ buffer set, A-L
4. OptiBlock™ buffer
5. OptiWash™ buffer
6. OptiGlow™ substrate kit, contains component A, B and C
7. Streptavidin–HRP

Materials Required for Assay but Not Supplied

1. Eppendorf or similar tubes for centrifugation and dilutions
2. **Wash buffer reservoir in automation system for holding OptiWash™**

Equipment Required:

1. Automatic Pipetting System
2. Single channel Pipettes capable of accurately and precisely delivering liquids in the ranges of 1 -10, 10 -100, and 100 – 1000 µL
3. Vortex mixer
4. Microplate fluorescence reader and control software
5. Analytical software

Experiment 1 —Selecting the Best Capture Antibody Coating Buffer for OptiMax™ Microplates Using Automated Pipetting Systems:

In the OptiMax™ microplate, all assay reactions occur in the microfluidic microchannel. The high surface area to volume ratio and short diffusion distances of the microchannels allow rapid protein adsorption onto the surface. Unlike the assay in conventional plates, the capture antibody adsorption in OptiMax™ microplates is dominated by the reaction rate of protein adsorption, which is strongly affected by the coating buffer. The first step of assay development is to screen twelve types of OptiBind™ coating buffer provided in the kit. It requires one assay experiment and uses one full OptiMax™ microplate.

The assay sensitivity can vary as much as 10x depending on the coating buffer used for capture antibody coating. This additional assay optimization step is critical for OptiMax™ microplates to achieve best performance.

<u>OptiBind™ COATING BUFFER IS MANDATORY FOR OPTIMAX™ ASSAYS - DO NOT USE ANY OTHER COATING BUFFER.</u>

Assay Layout:

Row #		OptiBind™ Type	1	2	3	4	5	6	OptiBind™ Type	7	8	9	10	11	12
A		A	High Standard		Low Standard		Zero		F	High Standard		Low Standard		Zero	
B		B							G						
C		C							H						
D		D							I						
E		E							J						
F									K						
G									L						
H															

Reagent Preparation:

All reagents, **except OptiGlow™ substrate**, shall be prepared and transferred into the 96-well v-bottom plate for the assay procedure. OptiGlow™ substrate will be loaded into the reservoir at the end of procedure (see Assay Procedure). A wash buffer reservoir is required for OptiWash™ buffer. Below is the reagent layout in the 96-well v-bottom plate. Bring all reagents to room temperature before use and prepare all necessary dilutions before beginning the test procedure.

Table3. Reagent preparation in96-well v-bottom reservoir for coating buffer selection, using automated pipetting

	1	2	3	4	5	6	7	8	9	10
	Capture antibody diluted in various Optibind									
A	Optibind A	Optibind F	Protein free blocking buffer 100 µL/well	OptiBlock 150 µL/well	High standard 60 µL/well	Low standard 60 µL/well	Zero 60 µL/well	Detection antibody 100 µL/well	SAv-HRP 100 µL/well	Substrate 100 µL **(Load at the end)**
B	Optibind B	Optibind G								
C	Optibind C	Optibind H								
D	Optibind D	Optibind I								
E	Optibind E	Optibind J								
F	/	Optibind K	/							
G		Optibind L								

Each reagent in this protocol contains 40 µl extra volume in each well. This extra volume can be reduced with precisely-configured automatic system.

1. <u>Capture antibody in various OptiBind™ coating buffer:</u> Use same capture antibody concentration as was found to be optimal for corresponding conventional assay, prepare the capture antibody working solution by diluting the capture antibody stock in 12 types of OptiBind™ buffer to make **100 µL** final working solution, and transfer into the 96-well v-bottom plate as illustrated in **Table 3.**
   2 µg/mL capture antibody concentration is used in the example assay (Page 16). Hence, 100 µL (each) of 2 µg/mL capture antibody working solution in each of OptiBind™-A, OptiBind™-B, OptiBind™-C….. OptiBind™-L would be prepared for this step.

2. Protein free blocking buffer: Load 100 µL into well A3 to E3. This Protein-Free Blocking Buffer is for use in assays requiring OptiBind™ Buffers A-E only. [For assays requiring dilution of capture antibody in OptiBind™ F-L, use OptiBlock™ buffer for blocking/washing.]
3. OptiBlock™: Load 150 µL into well A4 to G4.
4. High Concentration Protein Standard: Prepare more than 500 µL of protein standard in OptiBlock™ with concentration of 80% of top standard, load60 µL into well A5 to G5. *250 pg/mL of IL-6 is the top standard in the example assay. Hence, 200 pg/mL IL-6 standard would be prepared for this step.*
5. Low Concentration Protein Standard: Prepare more than 500 µL of protein standard in OptiBlock™ with concentration of 20% of top standard, load 100 µL into well A6 to G6
250 pg/mL of IL-6 is the top standard in the example assay. Hence, 50 pg/mL IL-6 standard would be prepared for this step.
6. Blank (zero): Load100 µL of OptiBlock™ into well A7 to G7.
7. Detection Antibody: Using the same concentration as was determined to be optimal for conventional plate assay, prepare the detection antibody working solution by diluting the detection antibody stock in OptiBlock™ buffer to make **1mL** final working solution and transfer 100 µL into well A8 to G8. *2 µg/mL detection antibody concentration is used in the example assay. Hence, 1 mL of 2 µg/mL detection antibody working solution in OptiBlock™ would be prepared for this step.*
8. SAv-HRP: Use SAv-HRP stock solution provided in the kit. Prepare the SAv-HRP working solution by adding **6 µL** of SAv-HRP stock solution to **900µL** of OptiBlock™ (**1:150 dilution**) and transfer 100 µL into well A9 to G9.
9. Substrate solution: Prepare the substrate working solution in by mixing **0.5 mL** of OptiGlow™-A, **0.5 mL** of OptiGlow™-B, and **10µL** of OptiGlow™-C. **Prepare the working substrate solution no more than 30 minutes before the anticipated time for reading the completed assay**. Transfer 100 µL of working solution into well A10 to G10.
10. OptiWash™: OptiWash™ is provided in ready-to-use form. No further preparation is required. The procedure requires a total of 70 µL of OptiWash™ buffer for each assay well to be used. Prepare **10 mL** of OptiWash™ into a buffer reservoir and use it for all wash steps in the assay.

Procedure

Program the automatic pipetting system to transfer reagents as illustrated below. A timer may be used to remind the user when to prepare the substrate final working solution needed for Step 11.

Step #	Aspiration			Dispensing		Incubation/waiting time (min)
	Solution to transfer	Well # of reservoir	Aspirated Volume (µL)	Well # of OptiMax	Dispensed Volume (µL)	
1	Capture antibody	A1-E1	30	A1-E6	5	10
		A2-G2	30	A7-G12	5	
2	Protein free blocking	A3-E3	30	A1-E6	5	5
	OptiBlock	A4-G4	30	A7-G12	5	
3	OptiBlock	A4-G4	60	A1-G12	5	10
4	Standards	A5-G5	20	A1-G2, A7-G8	5	10
		A6-G6	20	A3-G4, A9-G10	5	
		A7-G7	20	A5-G6, A11-G12	5	
5	OptiWash	/	60	A1-G12	5	5
6	Detection antibody	A8-G8	60	A1-G12	5	10
7	OptiWash	/	60	A1-G12	5	5
8	SAv-HRP	A9-G9	60	A1-G12	5	10
9	OptiWash	/	360	A1-G12	30	10
10	OptiWash	/	360	A1-G12	30	10
Prepare and transfer the OptiGlow™ working solution into the 96-well v-bottom plate						
11	Substrate	A10-G10	60	A1-G12	5	15
Read with fluorescence reader						

Calculations:

1. Calculate the mean background signal from the blank wells for assay with each type of OptiBind™ coating buffer (for example, average signal from well G1 and H1 for background signal of assay with OptiBind™-A coating buffer).
2. Calculate the mean sample signal from the high concentration protein standard wells for assay with each type of OptiBind™ coating buffer (for example, average signal from well A1, B1, C1 for high signal of assay with OptiBind™-A coating buffer).
3. Calculate the mean sample signal from the low concentration protein standard wells for assay with each type of OptiBind™ coating buffer (for example, average signal from well D1, E1, F1 for low signal of assay with OptiBind™-A coating buffer).
4. Create screening curves by plotting the OptiBind™ coating buffer types (x-axis) vs. the background-adjusted signal (y-axis).
5. Choose the type of OptiBind™ coating buffer which gives highest signal (after subtracting background). **See Figure 9.**

Selecting the Best Coating Buffer:

Select the OptiBind™ buffer type which yields the maximum signal. This particular OptiBind™ coating buffer should be used for all further experiments of this assay (*i.e.,* using this particular capture antibody clone). .

- Use the HIGH concentration curve ONLY if the peak RFU value is less than 90% of RFU_{max}.
- Use the LOW concentration curve ONLY if the peak RFU value is more than 10% of RFU_{max}.
- If both curves are valid per criteria listed above; usually the same OptiBind™ formulation will show best results.
- If there is discrepancy in choice of OptiBind™ formulation from LOW and HIGH concentration curves, use the HIGH concentration curve to make the selection.

Example Data:

Figure 9 shows screening test results for the illustrative IL-6 assay. Protein standard concentrations of 200 pg/mL (80% of max) and 50 pg/mL (20% of max) were used. 2 µg/mL of capture antibody and 2 µg/mL of detection antibody were used for this assay. Data read using Biotek FLx800 fluorescence plate reader with excitation filter at 528/20nm and emission filter at 590/35, sensitivity at 45.Data acquisition and analysis utilized Gen5™ software and Excel.

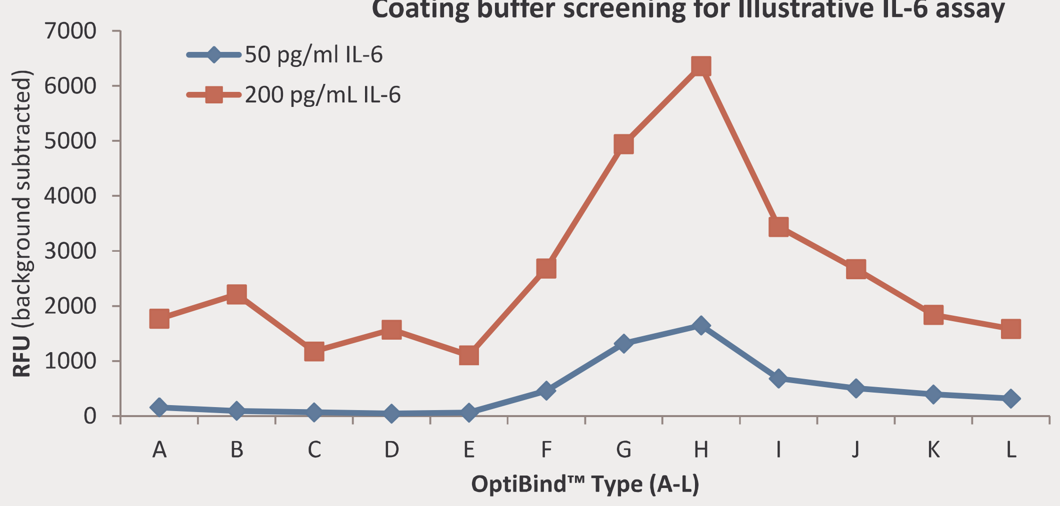


Figure 9. Results for coating buffer screening test for Illustrative IL-6 assay.

For the data shown in Figure 9, both curves for screening test result are valid and either curve can be used to select the best OptiBind™ buffer formulation. Both curves also demonstrate that OptiBind™-H is the best coating buffer for this assay capture antibody.

Experiment 2 —Selecting the Optimal Capture and Detection Antibody Concentrations Using Automated Pipetting:

This experiment uses a checkerboard titration pattern with 3 concentrations of capture antibody and 3 concentrations of detection antibody. Six rows (72 wells) in an OptiMax™ microplate will be used for this experiment.
Based on the result from Experiment 1, the concentration of protein standard which yields a maximum signal between 10% to 50% of RFU_{max} will be used for this experiment.

Assay Layout:

Detection antibody concentration		Capture antibody concentration											
		4 times as conventional assay				2 times as conventional assay				Same as conventional assay			
		1	2	3	4	5	6	7	8	9	10	11	12
4 times as conventional assay	A	Protein standard		Blank		Protein standard		Blank		Protein standard		Blank	
	B												
2 times as conventional assay	C												
	D												
Same as conventional assay	E												
	F												

Reagent Preparation:

All reagents, **except OptiGlow™ substrate**, shall be prepared and transferred into the 96-well v-bottom plate (reservoir) for the assay procedure. OptiGlow™ substrate will be loaded into the reservoir at the end of procedure (see Assay Procedure). A wash buffer reservoir shall be used for OptiWash buffer. Below is the reagent layout in the 96-well v-bottom plate. Bring all reagents to room temperature before use and prepare all necessary dilutions before beginning the test procedure

Table 4.Reagent preparation in96-well v-bottom reservoir for selecting antibody concentration

	1	2	3	4	5	6	7	9	9	10
A	4x concentrate capture antibody 60 µL/well	2x concentrated capture antibody 60 µL/well	1x concentrated capture antibody 60 µL/well	Protein free blocking buffer or OptiBlock 100 µL/well	OptiBlock 100 µL/well	Protein standard 60 µL/well	Blank (zero) 60 µL/well	4x concentrated detection antibody 100 µL/well	SAv-HRP 100 µL/well	Substrate 100 µL **(Load at the end)**
B										
C								2x		
D										
E								1x		
F										

1. Capture Antibody: Three concentrations of capture antibody working solution will be tested: a) same, b) 2 times and c) 4 times as that used in conventional plate assay. Prepare the capture antibody working solution by diluting the capture antibody stock in *selected* OptiBind™ buffer to make >500 µL final working solution. Load wells A1-F1with 4x concentration capture antibody solution, wells A2-F2 with 2x concentration, and wells A3-F3with1x concentration.

2. Protein free blocking buffer (if use OptiBind™ A-E for dilution of capture antibody): Load 100 µL into well A4 to F. [**Replace with OptiBlock™ buffer** if using OptiBind™ F-L for dilution of capture antibody.]

3. OptiBlock™: Load 100 µL into well A5 to F5

4. Protein Standard: Based on the result from conventional assay and Experiment 1, select a concentration expected in the mid-low range of dynamic range. Load 60 µL into well A6-F6. *From results for Illustrative IL-6 assay; a concentration of 50 pg/mL would be used for this experiment.*

5. Blank (zero): Load 60 µL of OptiBlock into well A7-F7.

6. Detection Antibody: Three concentrations of detection antibody working solution will be tested: a) same, b) 2 times and c) 4 times as that used in conventional assay. Prepare the detection antibody working solution by diluting the detection antibody stock in OptiBlock™ to make >200 µL final working solution. Load 100 µL of 4x concentrated detection antibody solution into wellA8 and B8, 2x concentrated detection antibody solution into well C8 and D8, 1x concentrated detection antibody solution into well E8 and F8.
7. SAv-HRP: Prepare the SAv-HRP working solution by adding **6 µL** of SAv-HRP stock solution to **900µL** of OptiBlock™ (1:150 dilution) in a v-shaped reservoir. Load 100 µL into wells A9-F9
8. Substrate solution: Prepare the substrate working solution in by mixing **0.5 mL** of OptiGlow™-A, **0.5 mL** of OptiGlow™-B, and **10 µL** of OptiGlow™-C. **Prepare the working substrate solution no more than 30 minutes before the anticipated time for reading the completed assay**. Transfer 100 µL of working solution into wells A10 to G10.
9. OptiWash™: OptiWash™ is provided in ready-to-use form. No further preparation is required. The procedure requires total of 70 µL of OptiWash™ for each assay well to be used. Prepare **10 mL** of OptiWash™ into a buffer reservoir and use it for all wash steps in the assay.

Procedure:

Program the automatic pipetting system to transfer reagents as illustrated below. A timer may be used to remind the user to prepare substrate working solution before Step #11.

Step #	Aspiration			Dispensing		incubation/waiting time (min)
	Solution to transfer	Well # of reservoir	Aspirated Volume (µL)	Well # of OptiMax	Dispensed Volume (µL)	
1	Capture antibody	A1-F1	20	A1-F4	5	10
		A2-F2	20	A5-F8	5	
		A3-F3	20	A9-F12	5	
2	Protein free blocking (or OptiBlock)	A4-F4	60	A1-F12	5	5
3	OptiBlock	A5-F5	60	A1-F12	5	10
4	Protein standard	A6-F6	30	A1-F2, A5-F6, A9-F10	5	10
	Blank (zero)	A7-F7	30	A3-F4, A7-F8, A11-F12	5	
5	OptiWash	/	60	A1-F12	5	5
6	Detection antibody	A8-F8	60	A1-F12	5	10
7	OptiWash	/	60	A1-F12	5	5
8	SAv-HRP	A9-F9	60	A1-F12	5	10
9	OptiWash	/	360	A1-F12	30	10
10	OptiWash	/	360	A1-F12	30	10
Prepare and transfer the OptiGlow™ working solution into the 96-well v-bottom plate						
11	Substrate	A10-F10	60	A1-F12	5	15
Read with fluorescence reader						

Selecting the Optimal Antibody Concentrations:

Use the signal:noise (S/N) ratio to determine the optimal combination of capture and detection antibody concentrations:

Example Data:

As an example, an antibody optimization test has been performed for IL-6 assay. Protein standard concentration is 50 pg/mL. Three concentrations of capture antibody (2, 4, 8 µg/mL) and three concentrations of detection antibody (2, 4, 8 µg/mL) have been used for this assay. Use Biotek FLx800 fluorescence plate reader with excitation filter at 528/20nm and emission filter at 590/35, sensitivity at 45. Data acquisition and analysis utilized Gen5™ software and Excel. Results are shown as below:

Mean value of results

Detection antibody concentration		Capture antibody concentration											
		8 µg/mL				4 µg/mL				2 µg/mL			
		1	2	3	4	5	6	7	8	9	10	11	12
8 µg/mL	A	2707		337		2339		348		1650		359	
	B												
4 µg/mL	C	2476		297		2097		233		1730		266	
	D												
2 µg/mL	E	2344		238		2073		289		1543		292	
	F												

Converted to S/N ratio

Detection antibody concentration		Capture antibody concentration											
		8 µg/mL				4 µg/mL				2 µg/mL			
		1	2	3	4	5	6	7	8	9	10	11	12
8 µg/mL	A	8.03				6.72				4.60			
	B												
4 µg/mL	C	8.34				9.00				6.50			
	D												
2 µg/mL	E	9.85				7.17				5.28			
	F												

- 8 µg/mL capture antibody concentration and 2 µg/mL detection antibody concentrations are selected for the highest S/N ratio.

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Experiment 3 — Determine Assay Measurable Range:

The coating buffer selected from Experiment 1 and antibody concentrations selected from Experiment 2 will be used for the final Experiment to determine the dynamic range of the assay. This experiment will run a standard curve of the assay with a wide range of concentrations which covers the expected dynamic range. Most OptiMax™ microplate-based assays are expected to have a dynamic range of ~730 fold (1:3 dilution, 7 concentrations). **Appropriate standard diluents must be used for this experiment. For example, use cell culture medium as standard diluent for measuring cell culture supernatant**; it is important to match the matrix of your intended sample.

ADVANCED ASSAY PROTOCOL ON THE OPTIMAX™:

Unique Ultra-High Sensitivity Repeat-Loading Protocol: As the OptiMax™ microplate uses a flow-through principle where subsequent reagent/analyte additions flush microchannel contents onto the absorbent pad, multiple analyte additions can be used to increase sensitivity. Please see detail description in Appendix II, and example assays in Application Notes on Siloam's website.

Ultra-Fast Sandwich ELISA Protocol: The total assay time for a standard OptiMax™ based assay is less than 2 hours, which already represents a significant time saving and increase in throughput. However, based on kinetics study, even 5 min of incubation time will offer a stable assay response. Furthermore, using automation systems it is possible to control the dispense times precisely, and incubation times can be further reduced to less than 5 min for OptiMax™ based assays. This is less ''efficient'' in terms of capture efficiency but allows for tremendous time savings. The entire assay can take less than 30 minutes. Please contact Siloam's technical support for assistance.

Ultra-Low Sample Volume (2 µL) ELISA Protocol: The typical assay protocol requires 5 µL on OptiMax in each sample addition. It is possible to further reduce the sample consumption down to 2µL without loss in sensitivity. Please see application note in Siloam's web site or contact Siloam's technical support for assistance.

OTHER ASSAY FORMATS ON THE OPTIMAX™:

Indirect Immunoassay: Siloam has developed protocols for indirect ELISA on OptiMax™. Please contact Siloam's technical support for assistance.

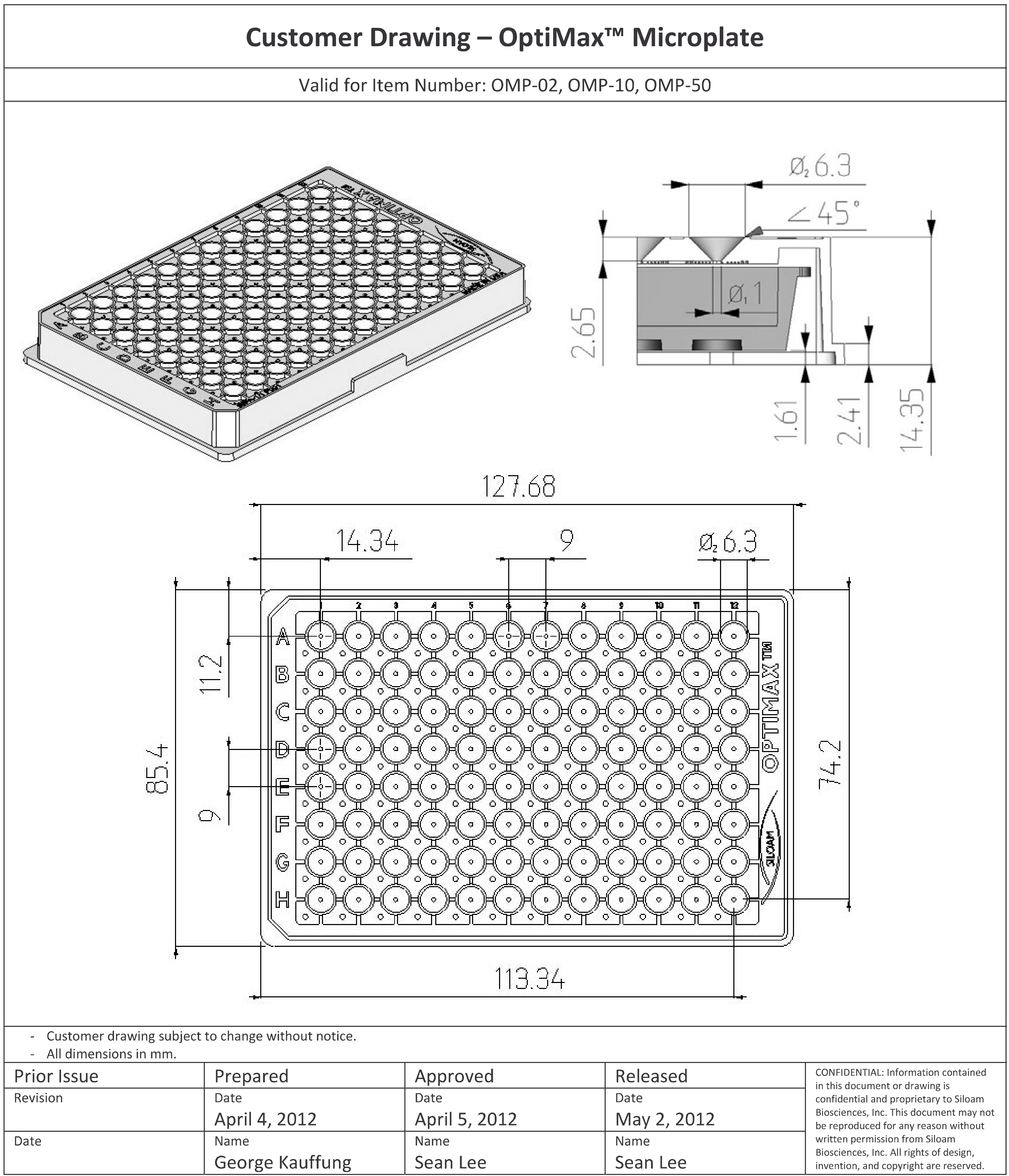
Competitive Enzyme Immunoassay (EIA): Siloam has also developed protocols for competitive ELISA on OptiMax™. Please contact Siloam's technical support for assistance.

TROUBLESHOOTING:

The OptiMax™ microplate has been designed and manufactured to ensure problem-free sample analysis. However, Siloam Biosciences has prepared the following guidance for trouble-shooting problems that might be encountered.

Problem	Possible Cause	Solution
Liquid does not drain from the OptiMax™ well or does not drain within 10 minutes.	A bubble is in the well.	• Follow recommended pipetting guidelines. • Prepare excess reagent to avoid aspirating air. • Do not use detergents.
	Sample contains particulates.	• Centrifuge sample for 10 min at 13,000 RPM, or • Filter the sample using a 0.2 µm filter.
No signal or unexpectedly low signal	Standard has degraded.	• Use standard on the day of its reconstitution, or • Thaw single use aliquots fresh on each test day. • Avoid repeated freeze-thaws.
	Incorrect reader filters	• Confirm filters meet requirements for substrate.
	Antibodies or SAv-HRP are degraded.	• Use within specified expiration period. • Store according to recommended storage temperature.
	Substrate was prepared incorrectly.	• Thaw OptiGlow™ - C thoroughly before preparing substrate working solution.
Unexpectedly high signal	Incorrect reader filters with overlapped wavelength bandwidth	• Confirm filters meet requirements for substrate.
	Reagent contamination	• Avoid cross contamination in reagents. Always change the pipet tips when handling different buffers/reagents.
	Substrate working solution has degraded.	• Prepare substrate no more than 30 minutes before plate is read.
Poor precision	Pipetting errors, use of alternate assay buffers or SAv-HRP	• Follow recommendations for pipetting setup (Page 5). • Do not substitute provided assay buffers or Sav-HRP.

APPENDIX 1: CUSTOMER DRAWING OF OPTIMAX™ MICROPLATE



Customer Drawing – OptiMax™ Microplate

Valid for Item Number: OMP-02, OMP-10, OMP-50

- Customer drawing subject to change without notice.
- All dimensions in mm.

Prior Issue	Prepared	Approved	Released	CONFIDENTIAL: Information contained in this document or drawing is confidential and proprietary to Siloam Biosciences, Inc. This document may not be reproduced for any reason without written permission from Siloam Biosciences, Inc. All rights of design, invention, and copyright are reserved.
Revision	Date April 4, 2012	Date April 5, 2012	Date May 2, 2012	
Date	Name George Kauffung	Name Sean Lee	Name Sean Lee	

APPENDIX 2: ULTRA-HIGH SENSITIVITY ASSAYS ON OPTIMAX™ MICROPLATES USING AUTOMATED PIPETTING

Because of the unique features of the OptiMax™ microplate and OptiMax™ ELISA procedures, users can apply sample to individual microfluidic reaction chambers multiple times. The result is a significant improvement in assay sensitivity ***when ultra-high sensitivity is required***.

The data in the figure below illustrates the sensitivity and dynamic range obtained using the standard OptiMax™ ELISA procedure (a single 5 µL sample addition) and the improvement in sensitivity that is gained by performing 20 consecutive 5 µL sample applications to individual reaction chambers using an automated fluidic handler (automated pipetting station).

Each additional sample incubation is 5 minutes in length. Thus with 95 additional minutes of assay time, the total assay time is approximately 3 hours with a corresponding increase in assay sensitivity of 20-fold.

The repeat sample loading method is a reliable and simple method to “tune” the sensitivity of the assay to the desired range simply by adjusting the number of sample addition (and incubation steps).

Contact Siloam Biosciences for additional details and specific guidance on running this alternate protocol.

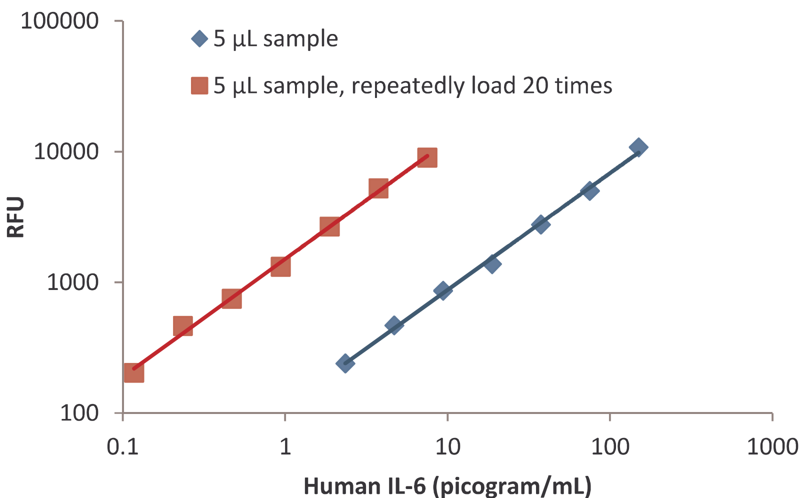


Figure 8.Ultra-high sensitive human IL-6 sandwich ELISA using repeat sample-loading technique with the OptiMax™ in conjunction with an automated pipetting station.

Technical Assistance: If you require assistance, please contact Interchim Technical Support at interbiotech@interchim.com .

Additional technical assistance is available under the Technical Support tab on the Siloam Biosciences web site (http://siloambio.com/).

- Using Optimiser™ Immunoassay Microplate Video
- OptiMax™ Microplate User's Guide
- Reader Settings
- Frequently Asked Questions
- Application Notes

Two additional videos appear under the Technology tab of the web site.

- Optimiser™ Principles of Operation
- Running an Assay with Optimiser™

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DOC ID: OPTI-2-MS-0076-A0

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