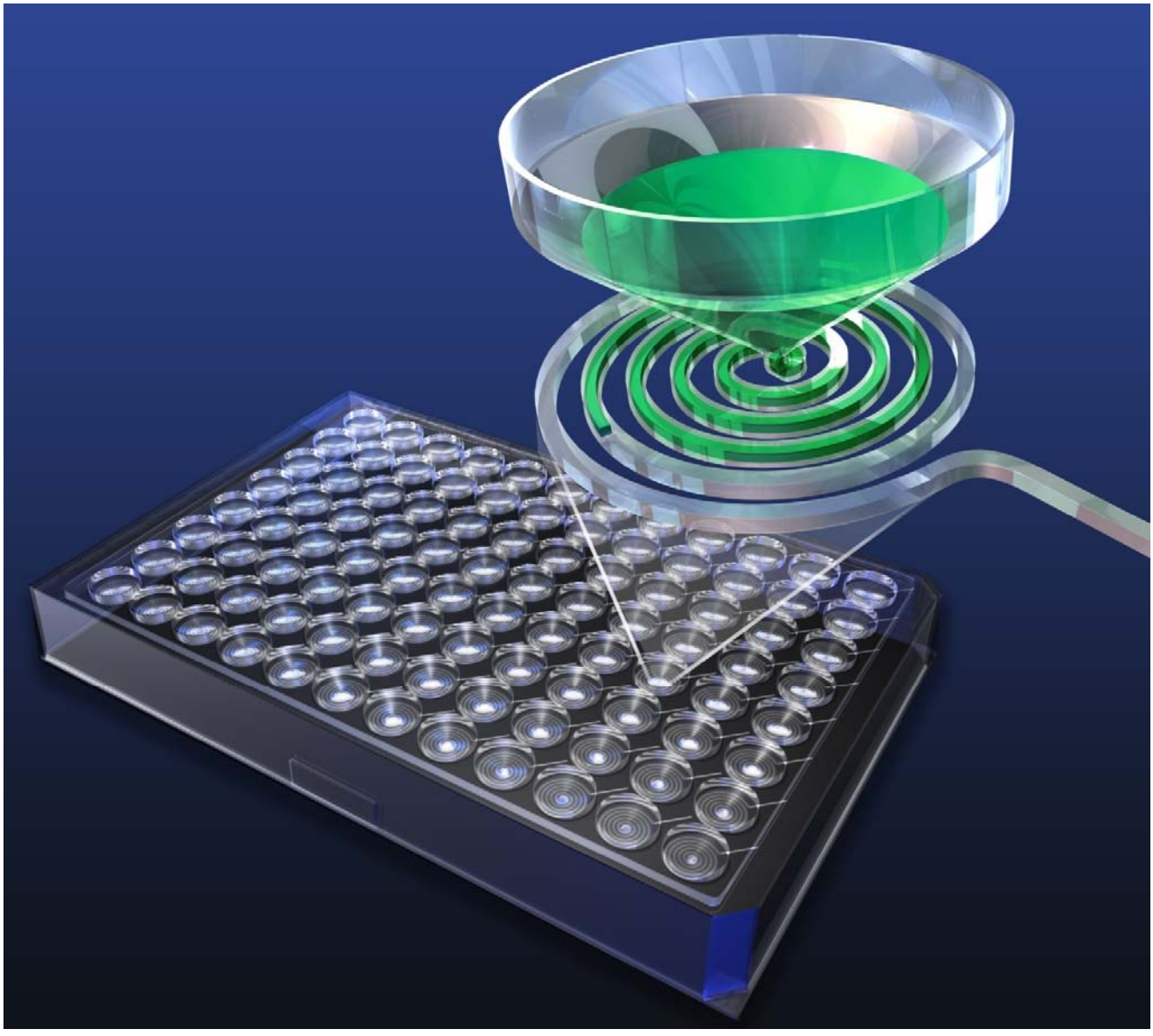
INSTRUCTION MANUAL

Optimiser™ Microplate Adoption Kit

OPTIMISER™

THE NEXT GENERATION OF MICROPLATES



SILOAM biosciences

Better Immunoassays through Innovative Microfluidics

INTENDED USE:

The Optimiser™ Microplate Adoption Kit (OPV-IL6WDR) and the associated Instruction Manual are specifically designed for first time user to provide a comprehensive overview to the Optimiser™ microplate system.

Section I serves as an Introduction to the Optimiser™ microplate system and guides the user through correct pipetting techniques with the Optimiser™. The pipetting technique instructions are accompanied by a Tutorial that will allow users to evaluate their pipetting proficiency on the Optimiser™ microplate.

Section I also includes detailed instructions for an illustrative IL-6 assay (all assay reagents and buffers included with OPV-IL6WDR). Completing the associated Tutorial will allow users to learn the assay operation sequence for Optimiser™ microplate assays. Successful completion of this assay will also help users understand the ***POWER OF MICROFLUIDICS*** to deliver high sensitivity ELISA results with only 5 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 Optimiser™ 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

Optimiser™ Microplate Adoption Kit

For adapting conventional ELISA plate assays to Optimiser™ Microplate System

Catalogue Numbers: OPV-IL6WDR

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 Optimiser™ Microplate Adoption Kit

Table of Content

Symbol indicates mandatory step required to ensure proper operation

Symbol indicates helpful tips to achieve optimal performance

SECTION I:

Using the Optimiser™ Microplate 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™ microplate. 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 50 - 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™ microplate platform.*

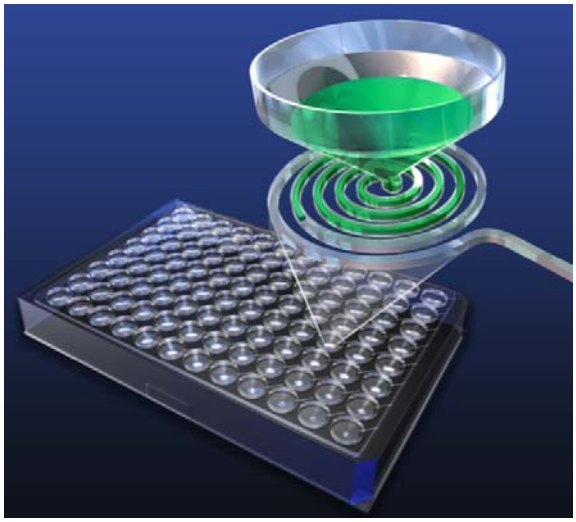


Figure 1. Optimiser™ microplate:
The Optimiser™ microplate is a revolutionary new microplate format. With an ANSI/SBS compliant 96-well layout, the Optimiser™ microplate integrates the **Power of Microfluidics** to allow for low volume, rapid, and uniquely high-sensitivity immunoassay protocols. Figure 1 shows the Optimiser™ microplate schematic with a magnified view of one "cell" of the Optimiser™ microplate. Each cell of the Optimiser™ 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 Optimiser™ 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.

Siloam Biosciences' Optimiser™ Microplate Adoption Kit is designed to provide first-time users with a comprehensive introduction to the methods of use and the capabilities of the Optimiser™ platform. Specifically:

- The Pipetting Instruction Section and Tutorial 1 are designed to guide users through the correct method for pipetting to the Optimiser™ microplate. Although very similar to the conventional 96-well ELISA plate, pipetting to the Optimiser™ requires careful attention to a few key details for reliable performance.
- Tutorial 2 is designed to allow users to complete a model IL-6 assay. Tutorial 2 illustrates that the workflow for Optimiser™ based assays is similar but much simplified when compared to conventional 96-well ELISA plates by eliminating the traditional wash step. Tutorial 2 also shows the capabilities of the Optimiser™ to deliver equivalent sensitivity to conventional 96-well ELISA plates while using only 5 µL sample volume.
- The Optimiser™ Assay Transfer Guide contains detailed instructions for users to transfer a working assay from the conventional 96-well ELISA plate to the revolutionary Optimiser™ platform. Following the step-by-step instructions, users can also successfully test their own assay on the Optimiser™ microplate.

MATERIALS PROVIDED AND REQUIRED:

Materials Provided:

Optimiser™ Microplate Adoption Kit provides the critical materials and reagents necessary for the Tutorials described in this manual and for the user to develop and optimize an ELISA assay on the Optimiser™ microplate. 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 Optimiser™ Microplate Adoption Kit*

Material	Quantity	Function	Storage / Handling, (before and after opening)
Optimiser™ Holder	1	Holds Optimiser™ Microplate and Optimiser™ Pad in proper alignment	Room temperature
Optimiser™ Microplate	10	Contains microfluidic reaction chambers Six Optimiser™ microplates for protocols described in manual; Four spare Optimiser™ microplates	
Optimiser™ Pad	20	Absorbs used reagent volume, single use	
96-well polypropylene v-bottom plate	3	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	
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	Binds captured IL-6, biotin conjugated	
SAv-HRP	1 vial	Binds detection antibody, interacts with substrate to yield chemifluorescence signal. 1:150 diluted with OptiBlock™ to make working solution	

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

Materials Required for Testing but Not Supplied With Optimiser™ Microplate Adoption Kit:

1. Eppendorf or similar tubes for centrifugation and dilutions
2. Kimwipes™ or other laboratory tissue paper
3. Reagent reservoirs (V-shape reservoir)
4. Pipette tips for delivering in the ranges of 1 -10, 10 -100, and 100 – 1000 µL

Equipments Required:

1. Pipette capable of accurately and precisely delivering liquids in the ranges of 1 -10, 10 -100, and 100 – 1000 µL
2. Multichannel pipette capable of accurately and precisely delivering 5 µL
3. Multichannel pipette capable of delivery of 30 µL
4. Vortex mixer
5. Fluorescence plate reader and control software
6. Analytical software
7. Microcentrifuge
8. Timer

UNIQUE CONSIDERATIONS FOR OPTIMISER™ MICROPLATE

Optimiser™ Microplate and Assembly:

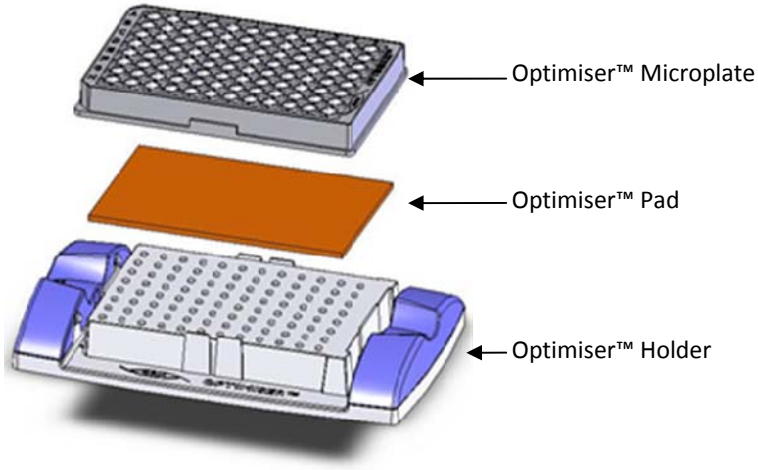


Figure 2. Optimiser™ microplate assembly
Position absorbent pad on holder, align the Optimiser™ microplate and press down gently to click-lock the plate in holder

The pad must be oriented correctly with the smooth surface (tape side) facing the holder and absorbent surface touching the microplate

Optimiser™ Microplate Pipetting Instruction:

Tutorial 1 provides hands-on training for first time users to practice pipetting with Optimiser™ microplate. Please read the entire Pipetting Instruction section before attempting Tutorial 1.

Avoiding Bubbles While Pipetting:

1. Bubbles will compromise the performance of assays on Optimiser™ microplate by interfering with the flow of liquid within the microchannels.
2. OptiBlock™ reagent may form bubbles readily with standard pipetting techniques.
3. To avoid complications due to bubbles, Siloam Biosciences recommends the use of the "Reverse Pipetting" technique during all pipetting steps.
    a. To aspirate liquid, press the operating button of the pipette to the second stop (refer to illustration below).
    b. Immerse the pipette tip in the liquid to a depth of about 2 mm and steadily release the operating button completely.
    c. Withdraw the tip from the liquid, touching it against the edge of the reservoir to remove excess liquid.
    d. Dispense the liquid into the loading well of Optimiser™ microplate by gently and steadily pressing the pipette's operating button to the first stop. Briefly hold the operating button in this position.
    e. With the button in this position, move the tip from the loading well to the reagent reservoir, immerse the tip in the liquid and aspirate.

	Pipetting step			
Ready position	1	2	3	4
First stop	↓	↑	↓	↑
Second Stop	↓	↑		

Figure 3. Reverse Pipetting procedure

THE USE OF PROPER PIPETTING TECHNIQUE IS CRITICAL TO AVOID AIR-BUBBLES.

THE USE OF PROPER PIPETTING TECHNIQUE IS CRITICAL TO AVOID AIR-BUBBLES.
Air bubbles will occlude the microfluidic channel and stop the flow of the Optimiser™.

Accurate and Precise Delivery of 5 µL Volumes:

Assays on Optimiser™ microplate require the accurate and precise delivery of 5 µL volumes. The following guidance is offered to users.

1. Use pipette for which the upper limit of their operating range is ≤10 µL.
2. Use pipette tips appropriate for 5 µL pipetting.
3. To aspirate liquid, hold the pipette near vertical and immerse the pipette tip in the liquid to a depth of approximately 2 mm in the liquid. Withdraw the operating button steadily. Wait ~ 1 second. Withdraw the tip from the liquid.
4. To dispense liquid, hold the pipette nearly vertical. With the pipette tips **touching the surface of the Optimiser™ well**, depress the operating button steadily until the liquid is dispensed.
5. **Note:** The pipette tip must make contact with the well surface for proper dispensing (see "RIGHT" frame below). Do not pipet directly into the hole at the bottom of the well (see "WRONG" frame).

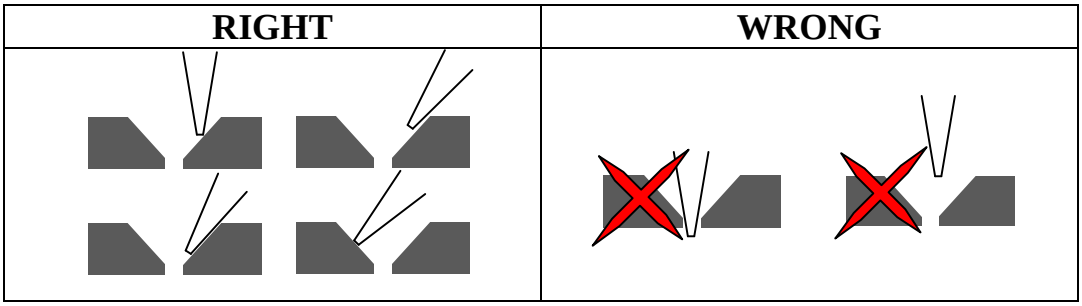


Figure 4. Pipette tip positioning for dispensing in the Optimiser™ microplate

Additional Technical Considerations:

1. The Optimiser™ system has been qualified with aqueous liquids only. Do not use solvent-containing samples.
2. The buffer reagents provided with the assay kit have been developed and validated for the Optimiser™ microplate. **Do not substitute alternate buffers or reagents.**
3. The presence of particulates in liquids dispensed to Optimiser™ wells may block liquid flow through the microchannels.
    a. Centrifuge serum samples and serum-containing tissue culture supernates for 10 minutes at 13,000 rpm prior to testing.
4. Small flow rate variations (time to empty well) do not affect assay results.

Using Electronic Multi-channel Pipette:

An electronic multi-channel pipette is ideally suited for use with Optimiser™ microplates since (a) it *eliminates* possibility of injecting bubbles and (b) can be used for convenient repetitive loads with single aspiration step for rapid reagent transfers.

General setup for using an electronic multi-channel pipette:

- Select pipette capable of delivery 5 µL & 30 µL (e.g., with volume range of 5-120 µL).
- Choose "Reverse Pipetting" in function setting.
- Use "Multiple Dispensing" mode to transfer the solution into the Optimiser™ microplate. *For example, to transfer capture antibody solution in to a full Optimiser™ microplate, set the program for 12 times dispensing, 5 µL per dispensing. Then the pipette will automatically aspirate 60 µL of solution and dispense 5 µL volumes 12 times. Users will not need to move pipette back and forth to transfer solution.*

Multichannel pipette must be used for transferring solution into the Optimiser™ plate.

If the pipette tip is pushed inside the through-hole, the tip may cause the sealing tape at the base of the Optimiser™ to de-laminate and lead to flow failure

If the pipette tip does not touch the surface of well, the solution may stick on the pipette tip end and not dispensed into the well OR may lead to air-bubbles.

Small variations in flow rates (time to empty well) do not affect assay performance. The incubation step smoothes out any flow variation differences.

An electronic multi-channel pipette can allow for loading all reagents with a single aspiration step – Ideally suited for processing multiple Optimiser™ microplates in parallel

Frequently Asked Questions: Pipetting

Almost all pipetting protocols specify users NOT to touch the well surface during pipetting. Why does the Optimiser™ microplate user guide suggest the exact opposite?

In conventional 96-well ELISA plates, if the pipette tip touches the bottom surface of the well, it may physically disrupt some of the bound bio-molecules. In the Optimiser™ microplate all the assay reactions occur *within the microchannel*. Hence, touching the pipette tip on the loading well of the Optimiser™ has absolutely no effect on the assay performance.

For most dispensing steps in Optimiser™ based assays, users are dispensing only 5 µl volumes. If the pipette tip does NOT touch the well surface, the dispensed well volume may "bead" and stick to the end of the tip. The well geometry of the Optimiser™ is engineered to ensure smooth filling of well/microchannel provided the liquid is dispensed steadily and directly on the well surface.

See the Optimiser™ Technology page on Siloam's website for instructional videos on pipetting techniques.

Why must all materials be transferred to the Optimiser™ microplate within one minute at each step in the assay procedure?

Optimiser™ incubation steps are from 10 to 20 minutes in length. Longer time to transfer material will cause time difference between each well in incubation, which may affect the assay accuracy.

I don't have a multichannel pipette – can I try the kit with a single channel pipette?

A multichannel pipette is essential to ensure that all dispense steps can be comfortably completed in 1 minute or less. With a single channel pipette it is very difficult to complete pipetting to even 3 columns in 1 minute.

How critical is the accuracy of 5 µl dispensing volume?

The Optimiser™ microplate is designed such that the 5 µl volume represents a slight excess compared to the microchannel internal volume. Provided that the dispense volume is greater than 4.5 µl, slight (even up to 10%) dispense volume variations will not affect assay results.

Why has the recommended operating volume been changed to 5 µL? I remember seeing 10 µL as recommended volume in earlier version of the FAQ.

1) Minimizing the volume helps with improving the precision. When using the 10 µl protocol, there is higher variation in the "time to empty" for different wells on each plate. This is related to the flow rate of the microchannel and larger volume show more net effect on flow duration (and variation of the duration).
2) The new 5 µl protocol also reduces the incidences of "slow" or "stopped" flow. With proper pipetting technique and by use of the new protocol, our lab tests show that flow failure rate (well does not empty after 10 minutes) is now less than ~ 0.2%.
3) We have verified through extensive assay tests that change from 10 µl to 5 µl does not affect the assay sensitivity. This is partly owing to improvements made to the OptiMax™ buffer formulations.

FLUORESCENCE PLATE READER SETUP

Optimiser™ 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.*

Step 1: Selecting the wavelength for excitation and emission light:

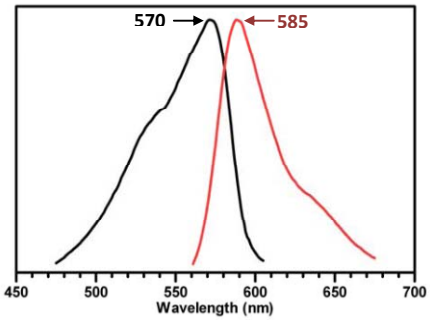


Figure 5.Normalized absorption (left) and emission (right) spectra of OptiGlow™ chemifluorescent substrate.

Assays performed on the Optimiser™ platform use the OptiGlow™ substrate system which can be detected using the appropriate excitation and emission settings (Figure 5). 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.

Step 2: Selecting the plate type:

The Optimiser™ microplate fits the 96-well SBS standard in all specifications. Please use "96-well standard" or similar selection when setting the plate type.

Step 3: Selecting the probe direction:

Use "top reading" for probe direction.

Step 4: Selecting the sensitivity/gain:

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 Optimiser™ 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 µL 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 OptiWash™ 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) Change the tip, 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 Optimiser™ microplate and read:

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

In order to read Optimiser™ microplate, adjust your reader setting as listed in page 8 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.,* 39747 vs. 95 RFU) AND
  - Clearly distinguish well D2 to well H2. (*e.g.,* 95 vs. 22 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.*, 17735 vs. 95 RFU) AND
  - Clearly distinguish well D2 to well H2. (*e.g.,* 95 vs. 22 RFU)

When using same reader setting to read IL-6 demo assay on the Optimiser™ microplate, 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.

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

Well #	Dilution from 5x fully developed substrate	RFU	Percentage of Max
A1	1	39747	100.00%
B1	2	28662	72.11%
C1	4	17735	44.62%
D1	8	9943	25.01%
E1	16	5225	13.15%
F1	32	2776	6.98%
G1	64	1443	3.63%
H1	128	771	1.94%
A2	256	458	1.15%
B2	512	266	0.67%
C2	1024	162	0.41%
D2	2048	95	0.24%
E2	4096	62	0.15%
F2	8192	41	0.10%
G2	16384	32	0.08%
H2	OptiWash (blank)	22	0.06%

TUTORIAL 1: PIPETTING TO THE OPTIMISER™ MICROPLATE:

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

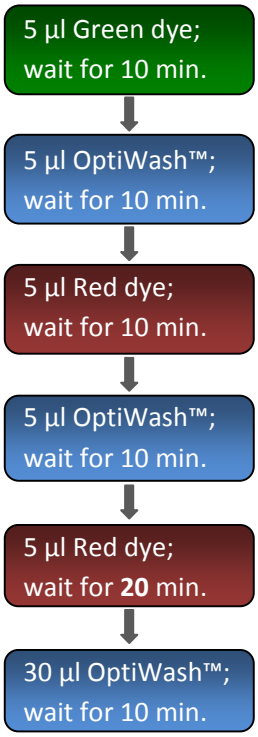
1. One Optimiser™ holder
2. Same Optimiser™ microplate (#1) used for reader setup, 3 (or more) unused columns will be used for pipetting
3. One new Optimiser™ microplate (#2), **all 12 columns** will be used for pipetting
4. One Optimiser™ pad, single use
5. OptiWash™ buffer
6. Green dye solution
6. Red dye solution

Other Materials/Equipments Required:

1. Kimwipes™ or other laboratory tissue paper
2. Reagent reservoirs (V-shape reservoir)
3. Single channel pipette capable of delivering in the ranges of 100 – 1000 µL
4. Pipette tips for delivering in the ranges of 100 – 1000 µL
5. Multichannel pipette capable of accurately and precisely delivering 5 µL
6. Multichannel pipette capable of delivery of 30 µL

Procedure with Manual Multi-channel Pipette:

1. Assemble the Optimiser™ Microplate, Optimiser™ Pad, and Optimiser™ Microplate Holder as described on Page 5.
2. Transfer 1.5 mL of green dye solution into a V-shape reagent reservoir. Transfer 1.5 mL of red dye solution into another V-shape reagent reservoir. Transfer 3 mL of OptiWash™ solution into a V-shape reagent reservoir.
3. To aspirate liquid, hold the multi-channel pipette nearly vertical and immerse the pipette tip in the liquid to a depth of approximately 2 mm in the liquid. Withdraw the operating button steadily. Wait ~ 1 second. Withdraw the tip from the liquid.
4. Dispense into columns 2-4 (3-columns) for Optimiser™ microplate (#1) per the sequence illustrated below.
    a. To dispense liquid, hold the multi-channel pipette nearly vertical. With **ALL** the pipette tips **touching the surface of the Optimiser™ wells**, depress the operating button steadily until the liquid is dispensed. **DO NOT** position pipette tips into the hole at the bottom of the surface.



OBSERVATIONS AND CONCLUSIONS

- In each step, all wells should be empty within 10 minutes. If a well is not empty after 10 minutes, please inspect under low-power microscope and most likely a bubble will be evident near microchannel interface with well. *This bubble was accidentally injected due to incorrect pipetting technique.* Please refer to the pipetting guidelines and try again.
- Note that as the dye reagent is changed in the well; even a 5 µL volume will "clear" the previous reagent in the microchannel. This demonstrates the efficiency of the "flushing" action instead of the traditional wash step.
- 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 Optimiser™ microplate 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 Optimiser™ microplates in parallel.
    - **Incubation steps should be at least 5 minutes and no more than 30 minutes. Use at least 20 minutes incubation for sample/standard.**

5. Assemble a new Opitmiser™ microplate (#2) and pad on the holder.
6. Repeat the dispensing protocol shown above again in **ALL 12 Columns of Optimiser™ microplate #2**. Time the dispensing cycles and check that all dispensing steps are completed within 1 minute.

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

8. **If any wells take longer than 10 minutes the most likely cause is an error in pipetting causing a visually evident or micro-bubble. Please refer to the pipetting instructions and repeat steps 6 and 7. User MUST complete dispensing protocol on to entire Optimiser™ microplate with all wells on that microplate draining in 10 minutes (for all steps in protocol).**

Optimiser™ microplate is a powerful tool for ELISAs and requires correct pipetting procedures to ensure repeatable results.
USERS MUST BE ABLE TO COMPLETE THE DISPENSING PROTOCOLS FOR A COMPLETE Optimiser™ MICROPLATE WITH ALL WELLS DRAINING IN LESS THAN 10 MINUTES (FOR ALL STEPS OF THE PROTOCOL).

THIS SIMPLE STEP IS CRITICAL TO ENSURE USERS CAN ACHIEVE EXCELLENT ASSAY RESULTS ON OPTIMISER™ MICROPLATE.

Two additional Optimiser™ microplates are included with the package to allow users to further practice and perfect pipetting to the Optimiser™ microplate. Excess volumes of the red/green dye and OptiWash™ are also included.

(OPTIONAL) Procedure with Electronic Multi-channel Pipette (5-100 µL volume):

1. Repeat the protocol described for Manual multi-channel pipette with an Electronic multi-channel pipette.
2. Choose "Reverse Pipetting" in function settings for Electronic pipette.
3. Choose "Multiple Dispensing" mode, and program for 12 dispense cycles with 5 µl dispense volume per cycle (for dispensing to all 12 columns).

OBSERVATIONS:

- All observations and conclusions listed for previous protocol.
- Note the difference in time required to load 12 columns with an electronic multi-channel pipette.

If one well drains in (say) 1 minute and another in (say) 8 minutes, how is it possible that they provide comparable results?

Although it may seem that difference of minutes may have an impact on the assay precision, Siloam has demonstrated with multiple assays that ***well optimized assays on Optimiser™ microplate easily achieve CV < 6-10%.*** The minimal effect of flow rate on precision is a combination of multiple factors:

1. On the micro-scale reaction kinetics are vastly different compared to the macro-scale kinetics of conventional 96-well ELISA plate. In microfluidic channels, most surface binding reactions are saturated in ~ 5 minutes. Optimiser™ characterization data shows that up to ~ 75% of peak adsorption is completed in only **10 seconds** and assay binding reactions saturate in ~ 5 minute. This is a result of two factors:
    a. The diffusion distances in the microchannel are extremely small (the channel has a cross-section of only 200 µm x 200 µm) hence diffusion is no longer a limiting factor
    b. The surface area of the microchannel is ~ 1.5 times the surface area at the base of a conventional 96-well ELISA plate. The volume contained in the microchannel is ~ 5 µl leading to ~ 50x higher surface area to volume ratio which allows for extremely efficient binding reactions.

2. Even for the well that drains in 8 minute, the initial section of the microchannel (towards the center) is filled up in ~ 2-3 minutes. Optimiser™ characterization data shows that the first few loops of the microchannel contribute ~ 95% of the optical signal hence even if the last 1-2 loops take significantly longer to fill, their contribution to the signal is almost negligible. Consequently, variations in signal from the last loops have little impact on overall (assay) signal variation.
3. For most reaction steps in the assay sequence (except for sample/standard loading step) the biomolecules are present in vast abundance and the binding reactions are completed extremely quickly. To ensure good precision, it **IS** recommended that the sample/standard incubation should be 20 min.
4. Finally, the incubation interval (when there is no liquid left in the well) "smooths out" the effect of flow rate variances.

How does the variance (CV) of Optimiser™ microplates compare to conventional plates?

In most assays (conventional plates), raw signal variance for triplicates is <10% which is also true for Optimiser™ microplates. Please see Siloam's website for a Technical Note detailing variance studies on the Optimiser™ platform. For **first-time users**, it is common to see variances at ~ 15% and even up to 20%. In almost all cases, this is related to pipetting techniques and as any other platform "practice makes perfect" -- most users see noticeable improvement after running a few Optimiser™ plates. The most common pipetting related issues that are resolved with careful attention to details and practice include:

1. Tips do not touch well surface leading to incomplete dispensing. This can lead to very high signal (e.g. if block buffer is not properly dispensed) or very low signals (e.g. if SAv-HRP is not properly dispensed).
2. Inconsistent load times as users learn pipetting procedures. With significant differences between loading intervals across the plate there is higher variance – with practice users can establish a steady "rhythm" with improved precision.
3. Failure to change tips in reagent preparations and/or between dispensing steps for assay sequence.
4. Use of inappropriate pipette tips and/or pipettes

Most users after completing ~ 10-12 Optimiser™ based assays can achieve (a) background signal <2-3% of peak signal (see Page 15) and (b) variance <10% for all points on standard curve. These can be used as metrics to determine user proficiency on the Optimiser™ system.

TUTORIAL 2: IL-6 DEMONSTRATION ASSAY ON THE OPTIMISER™ MICROPLATE:

The Optimiser™ Microplate Adoption Kit also contains necessary reagents of an IL-6 sandwich ELISA Assay to demonstrate the capabilities of Optimiser™ based assays under controlled conditions by a user. The representation of the expected data produced from ***Optimiser™ Microplate Adoption Kit is not intended to be used as a commercial assay kit***; this control ELISA is provided for training purposes only.

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

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

Materials Required for Assay But Not Supplied with Optimiser™ Microplate Adoption Kit:

1. Eppendorf or similar tubes for centrifugation and dilutions
2. Kimwipes™ or other laboratory tissue paper
3. Reagent reservoirs (V-shape reservoir)
4. Pipette tips for delivering in the ranges of 1 -10, 10 -100, and 100 – 1000 µL

Equipment Required:

1. Pipettes capable of accurately and precisely delivering liquids in the ranges of 1 -10, 10 -100, and 100 – 1000 µL
2. Multichannel pipette capable of accurately and precisely delivering 5 µL
3. Multichannel pipette capable of delivery of 30 µL
4. Vortex mixer
5. Microplate fluorescence reader and control software
6. 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 Optimiser™ microplate will be used.

Table 2. Plate layout of IL-6 concentration (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

Very small volumes of assay reagents are required and provided for Optimiser™ based assays.

A quick-spin (mini-centrifuge) is **CRUCIAL** to recover all material in Items 9-12.

Use a spin **each time** the assay reagents are to be used.

Reagent Preparation:

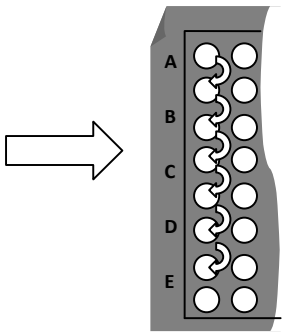
The incubation times for Optimiser™ microplate are only 10-20 minutes. Preparing all the reagents, samples, standards in advance will allow for proper timing (especially for first time users).

Always prepare extra volume of solution for easy transferring. Siloam suggest to prepare 30 µL extra volume each well in 96-well v-bottom plate. This volume can be reduced with careful pipetting if sample is very limited or precious.

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

1. OptiBind™: OptiBind™-H is provided in a ready-to-use form. No further preparation is required. **Do not substitute other coating buffers for OptiBind™-H**.
2. Capture Antibody: The procedure requires 5 µL of capture antibody working solution for each assay well to be used.
    a. Prepare a **1:62.5 dilution** of the capture antibody stock in OptiBind™-H buffer in a clean, polypropylene tube, such as Eppendorf tube. (Add 8 µl of capture antibody stock solution to 0.492 mL of OptiBind™-H buffer).
    b. Dispense 60 µL of the working solution into each well of a single column in the polypropylene 96-well v-bottom plate.
3. OptiBlock™: OptiBlock™ buffer is provided in ready-to-use form and is used to block the surfaces of the Optimiser™ microplate's microfluidic reaction chambers following their incubation with the capture antibody solution. OptiBlock™ is also used as the diluent for the standard, detection antibody and SAv-HRP in this experiment.
4. 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 4 ng/mL. Prepare a 3000 pg/mL standard (Standard 1) by mixing 120 µL 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 A1 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 B1 – H1).
        iii. Transfer 40 µL of the 3000 pg/mL standard from well A1 to well B1 immediately below it. Mix the contents of well B1 gently. Then transfer 40 µL from well B1 to well C1, **change tips**, and titrate.
        iv. Continue serial dilutions **while changing tips after each 40 µL transfer** and before mixing until the 4.1 pg/mL standard has been created in the seventh well (well G1) of the column.
        v. Do not transfer IL-6 solution to the eighth well (H1). It contains OptiBlock™ only and will provide material for the blank wells.

	1
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



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

5. 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 a single column in the polypropylene 96-well v-bottom plate.
6. 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 detection antibody 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 a single column in the polypropylene 96-well v-bottom plate.
7. Substrate solution: The procedure requires **5 µL** 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 in 4°C. It can be thoroughly thawed at RT or 37°C to enable you to pipette correctly and for it to function effectively. Warm the reagent in a 37°C incubator/oven/heater or by holding the vial gently in your hands.
   c. Dispense 60 µL of the working solution into each well of a single column in the polypropylene 96-well v-bottom plate.
8. OptiWash™: OptiWash™ is provided in ready-to-use form. No further preparation is required. The procedure requires 75 µL of OptiWash™ for each assay well to be used. Dispense 4 mL OptiWash™ buffer to a v-shaped reagent reservoir and use for all wash steps in the assay.

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

ENSURE THAT TIP CHANGES AS RECOMMENDED FOR STANDARD PREPARATION ARE FOLLOWED. Continued use of same tip may lead to errors in dilution and consequent assay signals.

Procedure:

1. Assemble the Optimiser™ Microplate, Optimiser™ Pad, and Optimiser™ Microplate Holder as described on Page 5.
2. **Hint:** Optimiser™ incubation steps are from 10 to 20 minutes in length. To achieve optimal assay performance, all materials must be transferred to the Optimiser™ microplate within one minute at each step. To accomplish this, first place the materials to be transferred in the enclosed 96-well polypropylene v-bottom plate or v-shape reagent reservoir (*instructed in Reagent Preparation, page 14*). Then transfer the materials to the Optimiser™ wells using a **multi-channel pipette**.
3. Dispense 5 µL capture antibody working solution to the required number of wells in the Optimiser™ microplate. Incubate 10 minutes at room temperature (RT).
4. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
5. Dispense 5 µL OptiBlock™ to the capture antibody-coated wells. Incubate 10 minutes at RT.
6. Dispense 5 µL of the standard and blank to the required number of replicate wells of the plate. Incubate **20 minutes at RT**.
7. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
8. Dispense 5 µL detection antibody working solution to each well. Incubate 10 minutes at RT.
9. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
10. Dispense 5 µL SAv-HRP to each well. Incubate 10 minutes at RT.
11. Dispense 30 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
12. Again dispense 30 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
13. Dispense 5 µL OptiGlow™ working solution to each well. Incubate for 15 minutes at RT.
    a. **Caution:** Observe the wells during the incubation. When the substrate has completely drained from all wells, remove the plate and pad from the holder. Discard the pad. Wipe the bottom of the plate with a Kimwipe™ to remove any liquid on the bottom surface of the plate. Step 13a will be completed within the 15 minute substrate incubation time.
14. Place the plate in the reading chamber of a fluorescence plate reader. Promptly at the conclusion of the 15 minute incubation, read the plate.

If all the assay reagent preparation steps and protocol are followed correctly, the "wells" (microfluidic channels) corresponding to top 2 (up to top 3) standards clearly appear pink (owing to developed substrate). If the pink color is not evident even for topmost standard, one or more reagent preparation steps or assay steps was not performed correctly.

It is common to see slight differences in the time required for different wells to empty. This difference has no impact on assay performance.

To facilitate work flow, incubations designated as 10 minutes may be extended to 20 minutes with no impact on method performance.

Optimiser™ "washes" are performed by simply dispensing OptiWash™ to the wells.

Wipe the plate bottom thoroughly. Any liquid residue on the bottom surface will cause false positive signal.

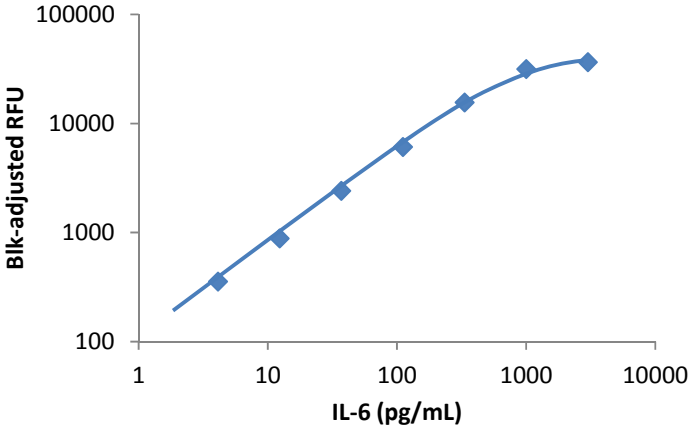
In rare cases (<0.2%), a well may not empty in 10 min. If so, blot the reagent from the well with a tissue**. Do not include data from this well in calculations.**

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	36665	36506
1000	31722	31564
333.3	15764	15606
111.1	6254	6096
37.0	2572	2414
12.3	1041	883
4.1	514	356
0	158	

Figure 6. IL-6 Standard Curve with Tabulated Data

Note: The IL-6 assay provided with the adoption kit is only designed for first-time users to verify their methods to run Optimiser™ based assays. The LoQ of this assay (lot-to-lot variations) is typically between 4.1 pg/mL – 37.0 pg/mL.

Assay Background

SIGNIFICANCE OF ASSAY BACKGROUND

The reader setup in this example sets a value of ~ 11,000 RFU as the high value for the 50:50:1 "saturated" substrate signal. *Regardless of the substrate ratio used*, the background RFU readings (blank signal) should not exceed ~ 350 RFU (~3% of max value in reader setup).

- Background signals higher than 3% of RFU_{max} (established during reader setup) indicate that one or more steps of the assay was performed incorrectly and users should repeat the assay.
- Background signals higher than ~ 6% of RFU_{max} (established during reader setup); corresponding to ~ 700 RFU in current example, indicate a failure and the assay must be repeated.

CAUSES:

- High backgrounds are most commonly a result of pipetting errors and can be resolved with careful attention to the procedure and additional practice.
- Another common cause for high background is use of alternate SAv-HRP or direct HRP labeled detection antibodies. Optimiser™ based assays are exquisitely sensitive to HRP concentration and the SAv-HRP provided by Siloam has been carefully optimized to achieve best performance. Use of alternate buffers (especially blocking buffers) can also lead to high background signals.

Please consult with Siloam's Tech Support team before substituting any of the buffers provided with the kits or as part of the OptiMax™ assay buffer reagent sets.

TUTORIAL 2: TARGETED OUTCOME

First time users can run a complete assay on the Optimiser™ microplate and confirm that they can generate similar data as listed in the User Manual. This Tutorial is intended to serve two purposes: (a) to familiarize users with the assay operation sequence on the Optimiser™ microplate and ensure performance matches with Siloam's data and (b) to provide users an introduction to the capabilities of the Optimiser™ microplate to deliver high-sensitivity assay data even when using only 5 µl sample volumes and a 2 hour assay protocol.

SECTION II:

Assay Transfer Guide

PROCEDURE FOR MIGRATING A VALIDATED ASSAY FROM CONVENTIONAL 96-WELL MICROPLATE TO OPTIMISER™ MICROPLATE

SANDWICH ELISA ASSAY TRANSFER GUIDE:

The Optimier™ microplate ELISA procedure is a chemifluorescent immunoassay procedure in which traditional ELISA reactions take place within the unique Optimiser™ 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 Optimiser™ 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 Optimiser™ 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 react with 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 best assay performance with Optimiser™ platform, serial optimization tests for each type of assay need to be performed before measuring the real sample. A well-characterized and robust assay on 96-well platform is a mandatory pre-requisite 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 concentration of capture antibody and detection antibody which gives the best signal/noise ratio. Finally, run an assay with wide range of target protein concentration with the selected optimal coating buffer and antibodies concentrations to determine the measurable/dynamic range of the assay.

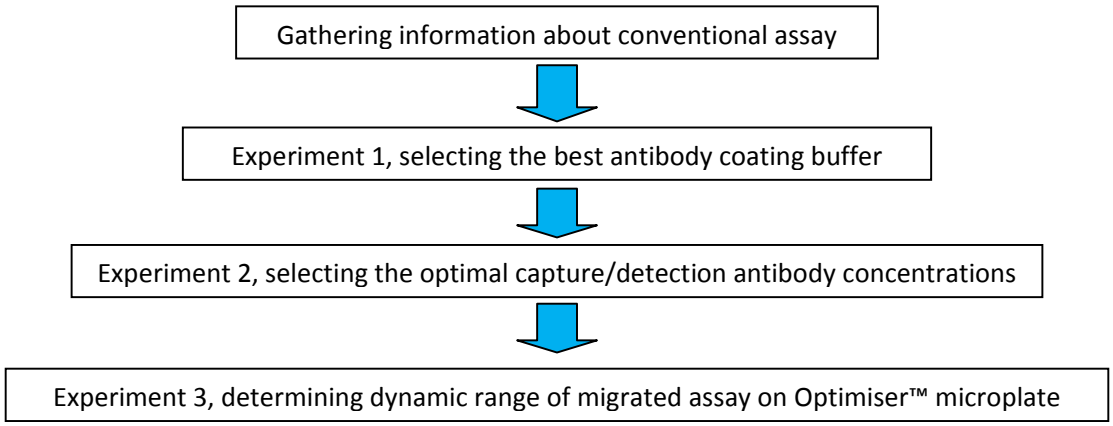


Figure 7. Schematic assay optimization procedure for Optimiser™ microplate.

Requirements for Conventional Assay to be Transferred to Optimiser™ Microplate

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

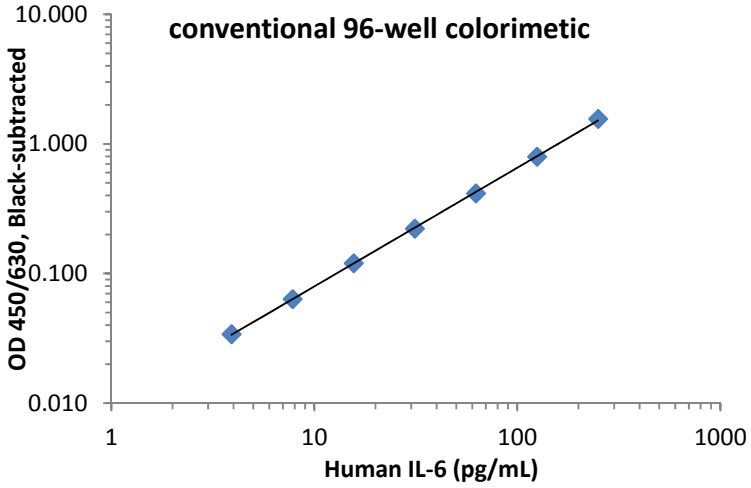
- **Reasonable background (zero) reading**: for a colorimetric assay, the OD450/630 absorbance reading of background shall be less than 0.15 (*e.g.,* after a standardized colorimetric substrate development time, such as 15 minutes).
- **Reasonable dose response** with various concentrations of standard: 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 8. 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 = 0.025 (less than 0.15); (*e.g*., after a standardized colorimetric substrate development time, such as 15 minutes).
  - Max signal OD = 1.58 (more than 1.5); (*e.g*., after a standardized colorimetric substrate development time, such as 15 minutes).

- Conventional colorimetric assay; Known information:
    - Concentration of capture antibody = 2 µg/mL
    - 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 Assays on Optimiser™:

Optimiser™ 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 HRP conjugate to ensure that the sensitive response from Optimiser™ 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 Optimiser™ microplate. The concentration and activity have been characterized and optimized for use with the Optimiser™ microplate system. Use of alternate SAv-HRP may lead to low signals or very high backgrounds.

- **For using HRP conjugated secondary antibody (*e.g.,* anti-mouse IgG, anti-goat IgG, etc.)**: Reagent must be titrated extensively to determine optimal working conditions.
- **For using HRP directly conjugated detection antibody**: Reagent must be titrated extensively to determine optimal working conditions.

Frequently Asked Questions: Optimiser™ based ELISA

Can I use Optimiser™ microplates for all the assay types that I run on a normal 96-well plate?
The current version of Optimiser™ microplate has been validated for ELISA applications, such as direct, indirect, sandwich and competitive immunoassays. For other applications, please discuss your application with Siloam's tech support.

What detection modes can be used with the Optimiser™ microplate?

- The current version of Optimiser™ microplate is well suited for fluorescence/chemifluorescence mode detection.
- We will have a version suitable for chemiluminescence mode available in the near future.
- Absorbance mode does NOT work with the Optimiser™ microplate.

I am not sure if the 10 minute incubation in room temperature will work – I would prefer to incubate for at least 30 minutes or in 37 °C. Is that OK?
Actually – NO. We strongly recommend that incubation times should not exceed 20 minutes. Incubating beyond 30 minutes or at 37 °C, will cause evaporative losses.

How critical is the 10 minute incubation window?

- Most binding reactions on Optimiser™ microplates saturate in ~ 5 minutes. Users can actually use even 5 minute incubation steps (except for sample which should be at least 20 minutes). The Application note section (Technical Support Tab) of Siloam's website has an article that describes this in greater detail.
- We recommend that you start with at least 10 minute incubation cycles/step – but you can certainly use longer (up to 20 minutes) incubation steps. This may be useful when you are processing multiple Optimiser™ microplates in parallel.
- All incubation steps must be at least 5 minutes (at least 20 minute for sample). Incubation steps should not exceed 30 minutes.

Can I use cell lysate supernatants or other biological fluids such as serum or urine?

- The flow does work in some circumstances even with particulates in the solution. However, we have seen that the flow is not very repeatable. For these fluids, we recommend using supernatant after centrifuging at 13,000 g for 10 minutes or pass through 0.2 µm filter.

How can I improve sensitivity of my assay using Optimiser™ microplate?

- In most cases, using the assay optimization protocol described in the "Assay Transfer Guide" you should be able to achieve slightly better sensitivity.
- A **guaranteed** method to significantly increase sensitivity is the use of repeat load process for sample/standard steps. Please see the Application Notes (under the Support Tab) on Siloam's website (authored by Tecan/BioTek) that describe the use of automation stations to increase assay sensitivity more than 100x! Please discuss your application with Siloam's tech support and we can offer more accurate guidance.

Experiment 1 – Selecting the Best Antibody Coating Buffer:

In Optimiser™ 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 plate, the capture antibody adsorption in Optimiser™ microplate 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 Optimiser™ microplate.

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

OptiBind™ COATING BUFFER IS MANDATORY FOR OPTIMISER™ ASSAYS - DO NOT USE ANY OTHER COATIONG BUFFER.

Reagent Preparation:

The incubation times for Optimiser™ are only 10-20 minutes. Preparing all the reagents, samples, standards in advance will allow for proper timing (especially for first time users).

Always prepare extra volume of solution for easy transferring. Prepare ~30 µL extra volume in each well of 96-well v-bottom plate. The extra volume may be reduced with careful pipetting if sample is very limited or precious.

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

1. OptiBind™: Twelve types of OptiBind™ are provided in ready-to-use form. No further preparation is required. **Do not substitute other coating buffers for OptiBind™**.
2. Capture Antibody: Use same concentration as conventional assay, prepare the capture antibody working solution by diluting the capture antibody stock in 12 types of OptiBind™ to make **100 µL** final working solution. Dispense the capture antibody working solutions in different OptiBind™ coat buffers into a single row in the 96-well v-bottom plate (one per well).
   2 µg/mL capture antibody concentration is used in the example assay (Page 20). 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.
3. OptiBlock™: For blocking step, prepare **1 mL** of OptiBlock™ in a v-shape reservoir.
4. High Concentration Protein Standard: Prepare **1 mL** protein standard with concentration of 80% of top standard, diluted in OptiBlock™ in a v-shape reservoir.
   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 **1 mL** protein standard with concentration of 20% of top standard, diluted in OptiBlock™ in a v-shape reservoir.
   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): Prepare **1 mL** of OptiBlock™ in a v-shape reservoir.
7. Detection Antibody: Use same concentration as conventional assay, prepare the detection antibody working solution by diluting the detection antibody stock in OptiBlock™ to make **1mL** final working solution in a v-shape reservoir.
   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 **8 µL** of SAv-HRP stock solution to **1.2 mL** of OptiBlock™ (**1:150 dilution**) in a v-shape reservoir (mix well).
9. Substrate solution: The procedure requires 10 µL of the working substrate solution for each assay well to be used. Prepare the substrate working solution in a v-shape reagent reservoir by mixing **0.9 mL** of OptiGlow™-A, **0.9 mL** of OptiGlow™-B, and **18 µL** of OptiGlow™-C.
10. OptiWash™: OptiWash™ is provided in ready-to-use form. No further preparation is required. The procedure requires total of 75 µL of OptiWash™ for each assay well to be used. Prepare **10 mL** of OptiWash™ into a v-shaped reagent reservoir and use it for all wash steps in the assay.

Assay Layout:

OptiBind™ Type	A	B	C	D	E	F	G	H	I	J	K	L
	1	2	3	4	5	6	7	8	9	10	11	12
A		High concentration protein standard, concentration at 80% of top standard used in conventional assay										
B												
C												
D		Low concentration protein standard, concentration at 20% of top standard used in conventional assay										
E												
F												
G		Zero (blank)										
H												

Procedure:

1. Assemble the Optimiser™ Microplate, Optimiser™ Pad, and Optimiser™ Microplate Holder as described on Page 5.
2. **Hint:** Optimiser™ incubation steps are from 10 to 20 minutes in length. To achieve optimal assay performance, all materials must be transferred to the Optimiser™ microplate within one minute at each step. To accomplish this, first place the materials to be transferred in the enclosed 96-well polypropylene v-bottom plate or v-shape reagent reservoir (as *instructed in Reagent Preparation, page 23*). Then transfer the materials to the Optimiser™ wells using a **10 µL multi-channel pipette**.
3. Dispense 5 µL capture antibody solution to the required number of wells in the Optimiser™ microplate. Incubate 10 minutes at room temperature (RT).
4. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
5. Dispense 5 µL OptiBlock™ to the capture antibody-coated wells. Incubate 10 minutes at RT.
6. Dispense 5 µL of the standard and blank to the required number of replicate wells of the plate. Incubate 20 minutes at RT.
7. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
8. Dispense 5 µL detection antibody working solution to each well. Incubate 10 minutes at RT.
9. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
10. Dispense 5 µL SAv-HRP to each well. Incubate 10 minutes at RT.
11. Dispense 30 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
12. Again dispense 30 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
13. Dispense 5 µL OptiGlow™ working solution to each well. Incubate for 15 minutes at RT.
    a. **Caution:** Observe the wells during the incubation. When the substrate has completely drained from all wells, remove the plate and pad from the holder. Discard the pad. Wipe the bottom of the plate with a Kimwipe™ to remove any liquid on the bottom surface of the plate. Step 13a will be completed within the 15 minute substrate incubation time.
14. Place the plate in the reading chamber of a fluorescence plate reader. Promptly at the conclusion of the 15 minute incubation, read the plate.

It is common to see slight differences in the time required for different wells to empty. This difference has no impact on assay performance.

To facilitate work flow, incubations designated as 10 minutes may be extended to 20 minutes with no impact on method performance.

Optimiser™ "wash" is performed by simply **dispensing** OptiWash™ to the wells.

Caution: In rare cases, a well may not empty in 10 min. If so, blot the reagent from the well with a tissue. **Do not analyze signal from this well.**

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.

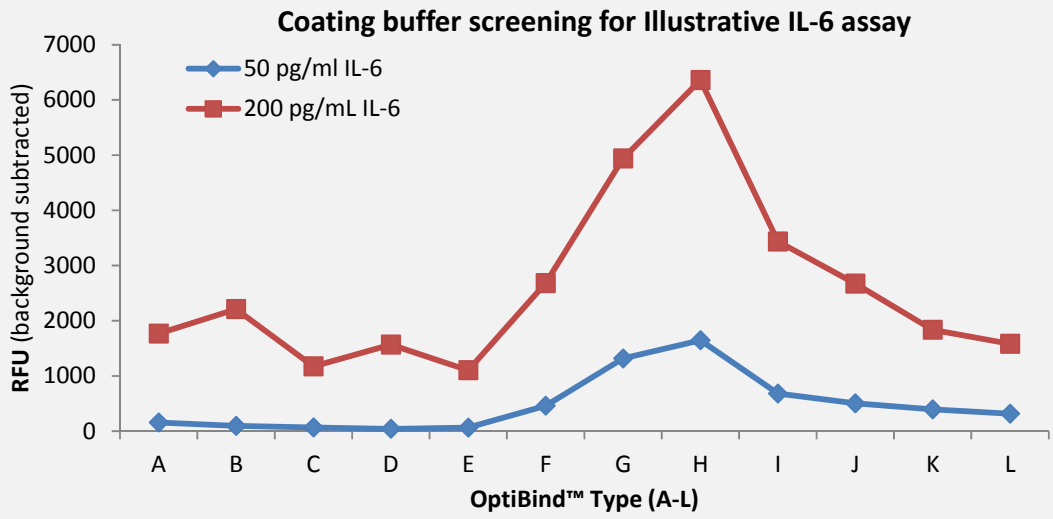


Figure 9. Results for coat buffer screening test for Illustrative IL-6 assay.

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 coat buffer for this assay.

Data logging and Calculation Worksheets
Experiment 1 — Selecting the Correct Antibody Coating Buffer

Assay Target: _____

Capture antibody information: _____, and working concentration: _____µg/mL

Detection antibody information: _____, and working concentration: _____µg/mL

Protein standard information: _____, and top concentration: _____

High protein standard, 80% of top standard concentration: _____

Low protein standard, 20% of top standard concentration: _____

RFU_{max}: _____

Test results

OptiBind™ type		A	B	C	D	E	F	G	H	I	J	K	L
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
High standard	**A**												
	B												
	C												
	Mean												
Low standard	**D**												
	E												
	F												
	Mean												
Blank	**G**												
	H												
	Mean												

OptiBind™ type _____ gives highest signal, if that signal of high standard between 10% and 90% of RFU_{max}

With this type of OptiBind™, protein standard at concentration _____ give signal between 10% and 50% of RFU_{max}. It will be used for experiment 2.

Tested By: _____ Date: _____

Experiment 2 –Selecting the Optimal Capture and Detection Antibody Concentrations:

The larger surface area and very high surface area to volume ratio in the microfluidic channel of Optimiser™ microplate allows more capture antibody to be adsorbed onto the surface, which may improve assay sensitivity. 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 Optimiser™ microplate will be used for this experiment.
Based on the result from Experiment 1, concentration of protein standard which gives maximum signal between 10% to 50% of RFU_{max} will be used for this experiment.

Reagent Preparation:

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

1. OptiBind™: Use the OptiBind™ coat buffer selected from experiment 1.
2. 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 assay. Prepare the capture antibody working solution by diluting the capture antibody stock in *selected* OptiBind™ to make **250 µL** final working solution. Use a single row in the polypropylene 96-well v-bottom plate, load **60 µL** of working solution to each well. Load wells 1-4 with 4x concentration capture antibody solution, wells 5-9 with 2x concentration, and wells 9-12 with "same as conventional" concentration.
3. OptiBlock™: OptiBlock™ is provided in ready-to-use form and is used to block the surfaces of the Optimiser™'s microfluidic reaction chambers following their incubation with the capture antibody solution. OptiBlock™ is also used as the diluent for the protein standard, detection antibody and SAv-HRP. For blocking step, prepare **1 mL** of OptiBlock™ in a v-shape reservoir.
4. Protein Standard: Prepare **1 mL** of protein standard with OptiBlock™ in a v-shape reservoir. Select a concentration which gives the max signal between 10% to 50% of RFU_{max}.
   From results for Illustrative IL-6 assay; a concentration of 50 pg/mL would be used for this experiment.
5. Blank (zero): Prepare **1 mL** of OptiBlock™ in a v-shape reservoir.
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. Use a single column in the polypropylene 96-well v-bottom plate, load **90 µL** of working solution in to each well. Load wells A and B with 4x concentration detection antibody solution, wells C and D with 2x concentration, wells E and F with "same as conventional" concentration.
7. SAv-HRP: Use SAv-HRP stock solution provided in the kit. Prepare the SAv-HRP working solution by adding **8 µL** of SAv-HRP stock solution to **1.2 mL** of OptiBlock™ (1:150 dilution) in a v-shape reservoir.
8. Substrate solution: The procedure requires **10 µL** of the working substrate solution for each assay well to be used. Prepare the substrate working solution in a v-shape reagent reservoir by mixing **0.9 mL** of OptiGlow™-A, **0.9 mL** of OptiGlow™-B and **18 µL** of OptiGlow™-C.
9. OptiWash™: OptiWash™ is provided in ready-to-use form. No further preparation is required. The procedure requires total of 75 µL of OptiWash™ for each assay well to be used. Prepare **10 mL** of OptiWash™ into a v-shaped reagent reservoir and use it for all wash steps in the assay.

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	Protein standard		Blank		Protein standard		Blank		Protein standard		Blank	
	D												
Same as conventional assay	E	Protein standard		Blank		Protein standard		Blank		Protein standard		Blank	
	F												

Procedure:

1. Assemble the Optimiser™ Microplate, Optimiser™ Pad, and Optimiser™ Microplate Holder as described on Page 5.
2. **Hint:** Optimiser™ incubation steps are from 10 to 20 minutes in length. To achieve optimal assay performance, all materials must be transferred to the Optimiser™ microplate within one minute at each step. To accomplish this, first place the materials to be transferred in the enclosed 96-well polypropylene v-bottom plate or v-shape reagent reservoir (as *instructed in Reagent Preparation, page 27*). Then transfer the materials to the Optimiser™ wells using a **10 µL multi-channel pipette**.
3. Dispense 5 µL capture antibody solution to the required number of wells in the Optimiser™ microplate. Incubate 10 minutes at room temperature (RT).
4. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
5. Dispense 5 µL OptiBlock™ to the capture antibody-coated wells. Incubate 10 minutes at RT.
6. Dispense 5 µL of the standard and blank to the required number of replicate wells of the plate. Incubate 20 minutes at RT.
7. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
8. Dispense 5 µL detection antibody working solution to each well. Incubate 10 minutes at RT.
9. Dispense 5 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
10. Dispense 5 µL SAv-HRP to each well. Incubate 10 minutes at RT.
11. Dispense 30 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
12. Again dispense 30 µL OptiWash™ to each well. Wait 10 minutes to proceed to the next step.
13. Dispense 5 µL OptiGlow™ working solution to each well. Incubate for 15 minutes at RT.
    a. **Caution:** Observe the wells during the incubation. When the substrate has completely drained from all wells, remove the plate and pad from the holder. Discard the pad. Wipe the bottom of the plate with a Kimwipe™ to remove any liquid on the bottom surface of the plate. Step 13a will be completed within the 15 minute substrate incubation time.
14. Place the plate in the reading chamber of a fluorescence plate reader. Promptly at the conclusion of the 15 minute incubation, read the plate.

It is common to see slight differences in the time required for different wells to empty. This difference has no impact on assay performance.

To facilitate work flow, incubations designated as 10 minutes may be extended to 20 minutes with no impact on method performance.

Optimiser™ "wash" is performed by simply **dispensing** OptiWash™ to the wells.

Caution: In rare cases, a well may not empty in 10 min. If so, blot the reagent from the well with a tissue. **Do not analyze signal from this well.**

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.

Data logging and Calculation Worksheets
Experiment 2 — Selecting the Optimal Capture/detection Antibody Concentrations

Assay Target: _____

Capture antibody information: _____,

and working concentration: 1x= _____µg/mL, 2x= _____µg/mL, 4x=_____µg/mL

Detection antibody information: _____,

and working concentration: 1x= _____µg/mL, 2x= _____µg/mL, 4x=_____µg/mL

Protein standard information: _____,

and concentration will be used (from experiment 1): _____

Test results

		Capture Antibody											
Detection antibody		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1x	**A**												
	B												
2x	**C**												
	D												
4x	**E**												
	F												

Calculated Mean

Detection antibody		**Capture Antibody**								
		4 times as conventional assay			**2 times as conventional assay**			**Same as conventional assay**		
		Standard	**Zero**	**S/N**	**Standard**	**Zero**	**S/N**	**Standard**	**Zero**	**S/N**
4 times as conventional assay	**A**									
	B									
2 times as conventional assay	**C**									
	D									
Same as conventional assay	**E**									
	F									

Optimal capture antibody concentration: _____µg/mL

Optimal detection antibody concentration: _____µg/mL

With optimal antibody concentrations, the top concentration of protein standard for experiment 3 is: _____

Tested By: _____ Date: _____

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 Optimiser™ 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 OPTIMISER™ MICROPLATE:

Unique Ultra-High Sensitivity Repeat-Loading Protocol:

As the Optimiser™ 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 I, and example assays in Application Notes on Siloam's website.

Ultra-Fast Sandwich ELISA Protocol:

The total assay time for a standard Optimiser™ 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 Optimiser™ 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 Optimiser™ microplate 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 OPTIMISER™ MICROPLATE:

Indirect Immunoassay:

Siloam has developed protocols for indirect ELISA on Optimiser™ microplate. Please contact Siloam's technical support for assistance.

Competitive Enzyme Immunoassay (EIA):

Siloam has also developed protocols for competitive ELISA on Optimiser™ microplate. Please contact Siloam's technical support for assistance.

TROUBLESHOOTING:

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

Problem	Possible Cause	Solution
Liquid does not drain from the Optimiser™ well or does not drain within 10 minutes.	A bubble is in the well.	• Disrupt the bubble with a clean 26 gauge needle. • 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.
	Plate has lost contact with the absorbent pad or is positioned incorrectly.	• Ensure that the absorbent side (rough) of the pad is in contact with Optimiser™ micropate and the tape side (smooth) is facing down to touch holder. • Ensure the topside of the pad is touching the bottom of Optimiser™ microplate by pushing down firmly on the 4 corners of the plate. • Ensure the plate and pad are securely aligned in the holder.
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.
	Substrate working solution has degraded.	• Prepare substrate no more than 30 minutes before plate is read.
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 pipette tips when handling different buffers/reagents.
Poor precision	Pipetting errors, use of alternate assay buffers or SAv-HRP	• Follow recommendations for pipetting small volumes (Page 6). Variance <10% and background < 3% of RFU_{max} (established during reader setup) are expected. • Do not substitute provided assay buffers or SAv-HRP.
Signal of lower standard(s) are < 0 following background subtraction.	Degraded standard	• Use standard on the day of its reconstitution, or • Thaw single use aliquots fresh on each test day. • Avoid repeated freeze-thaws.
	Degraded capture antibody	• Use within specified expiration period. • Store according to recommended storage temperature.

APPENDIX 1: ULTRA-HIGH SENSITIVITY ASSAYS ON OPTIMISER™ MICROPLATES

Because of the unique features of the Optimiser™ plate 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.***

PLEASE CONTACT TECHNICAL SUPPORT FOR ASSISTANCE WITH THIS PROTOCOL. The description provided here should not be used a formal protocol.

The additional sample applications can be performed manually for a limited number of repeat sample loads but Siloam strongly recommends use of an automated fluidic handler (automated pipetting station) for the ultra-high sensitive protocol.

The data in the figure below illustrates the sensitivity and dynamic range obtained using the standard Optimiser™ 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 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 methods 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.

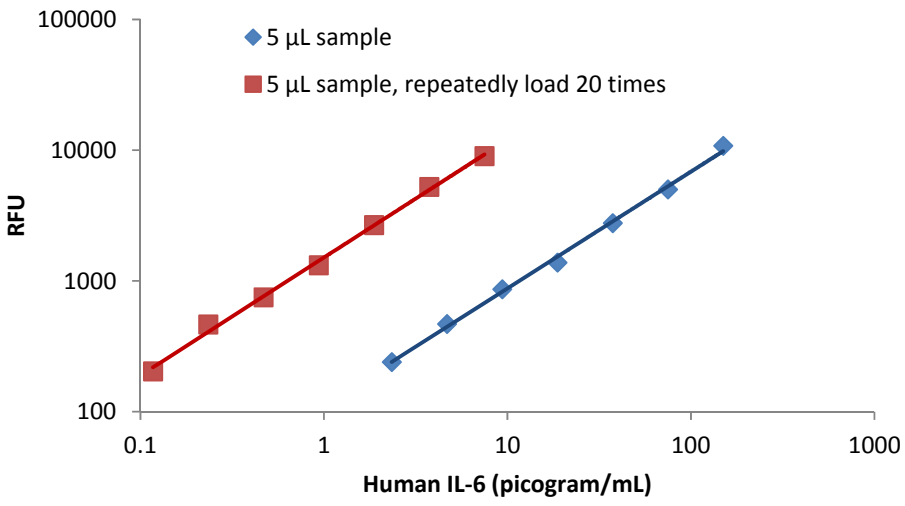


Figure 10. Ultra-high sensitive human IL-6 sandwich ELISA using repeat sample-loading technique with Optimser™ microplate in conjunction with an automated pipetting station.

Technical Assistance: If you require assistance, please contact Siloam Biosciences, Inc. Technical Support at 513-429-2976 or techsupport@siloambio.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
- Optimiser™ Microplate User's Guide
- Reader Settings
- Quick Reference Guide
- 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™

Better Immunoassays Through Innovative Microfluidics

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