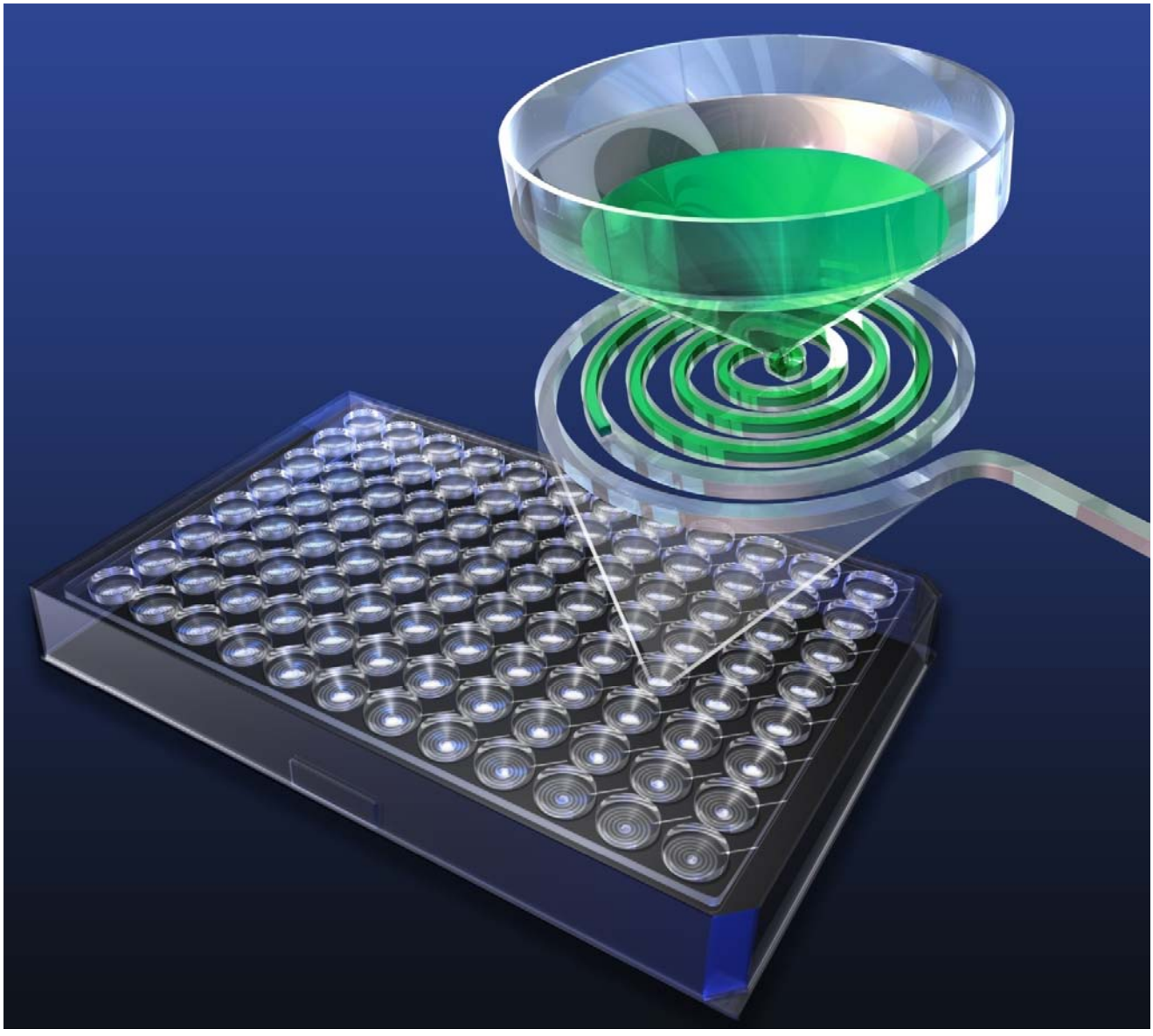
ASSAY TRANSFER GUIDE

For Transfer of Validated 96-well ELISA to Optimiser™

OPTIMISER™

THE NEXT GENERATION OF MICROPLATES



SILOAM biosciences

Better Immunoassays through Innovative Microfluidics

FOREWORD:

The Assay Transfer Guide is a detailed description of a procedure to be used for migrating a validated assay from conventional 96-well plates to Optimiser™ microplate. The Assay Transfer Guide 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. The Assay Transfer Guide 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.

The Assay Transfer Guide is intended for users who are already familiar with and have used the Optimiser™ microplate system.

First time users should use the OptiMax™ Evaluation Kit (OPV-IL6) which contains additional materials and information. The OPV-IL6 instruction manual also contains Section I that 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™. Section I also includes detailed instructions for an illustrative Hu IL-6 assay (all assay reagents and buffers are included with OPV-IL6). 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.

Assay Transfer Guide

For transfer of validated 96-well ELISA to Optimiser™

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Symbol indicates mandatory step required to ensure proper operation

Symbol indicates helpful tips to achieve optimal performance

INTRODUCTION:

Siloam Biosciences' Optimiser™ technology 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. The typical assay procedure utilizes a 5 µL sample and each reaction step is completed in 10 - 20 minutes. With wash time, substrate incubation time, and read time accounted for, a typical assay can be completed within approximately 2 hours.

Please refer to the Optimiser™ Technology page on Siloam's website for more details on the principles behind the Optimiser™ microplate platform.

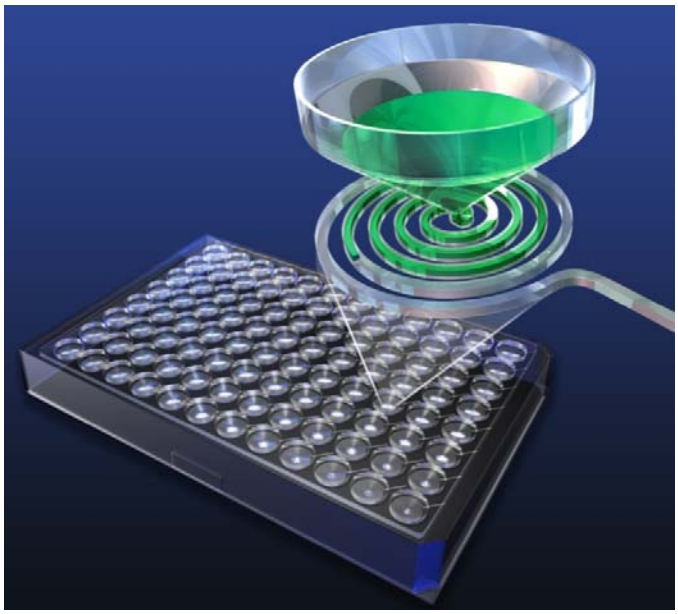


Figure 1. Optimiser™ microplate:

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

MATERIALS REQUIRED:

Optimiser™ Microplate System and OptiMax™ Reagents are required for developing and optimizing an ELISA assay on the Optimiser™ platform. **Please read the Assay Transfer Guide in its entirety before ordering the required supplies.**

Related Products:

Catalog #	*Product Description*
OPH-02 [1]	• **2-Plate Optimiser™ pack with holder** – 1 Optimiser™ holder, 2 Optimiser™ microplates and 4 Optimiser™ Pad
OMR-02X [2]	• **2-Plate OptiMax™ Reagent Buffers** – Selected OptiBind™, OptiWash™, and OptiBlock™ buffers, and OptiGlow™ detection substrate (3-component; OptiGlow™-A, OptiGlow™-B, and OptiGlow™-C).
OMR-TEST	• **OptiMax™ Test Buffers to Select Optimal Antibody Coating Buffer (Experiment 1)** – 1 set (12 vials) of OptiBind™ A-L, OptiWash™, OptiBlock™, and OptiGlow™ detection substrate (3-component; OptiGlow™-A, OptiGlow™-B, and OptiGlow™-C). Designed for screening up to 10 different assays.
OMR-HRP	• **OptiMax™ Streptavidin-HRP for use with Optimiser™ Microplates** –The concentration and activity have been characterized and optimized for use with the Optimiser™ microplate system. For assays on 50 Optimiser ™ microplates.
OPV-IL6	• **OptiMax™ Evaluation Kit** – Allow users to practice pipetting to Optimiser™, run a trial sandwich immunoassay, and transfer ONE validated conventional 96-well ELISA protocol to Optimiser™

[1] *Also available in 10-plate and 50-plate sizes*
[2] *OMR buffer packs are available with one of 12 different coat buffers. For instance, OMR-02 with coat buffer A is item OMR-02A. Please refer to the Guide for procedure to select the optimal coat buffer. OMR buffer packs are also available in 10-plate and 50-plate sizes.*

Materials Required for Testing but Not Supplied by Siloam:

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. 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:

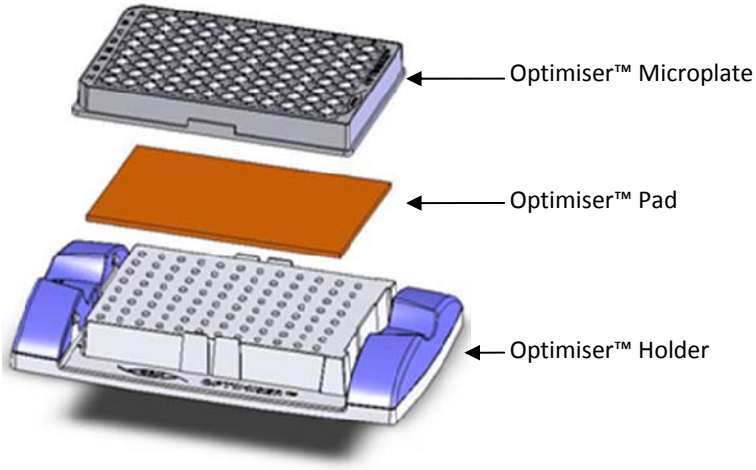


Figure 2. Optimiser™ microplate assembly
Position absorbent pad on holder, align the Optimiser™ microplate and press down gently to click-lock the plate in holder

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:

First time users are strongly encouraged to use the Optimiser Starter™ Kit (Cat# OPS-IL6) or OptiMax™ Evaluation Kit (Cat# OPV-IL6), which includes a tutorial for hands-on training for pipetting on Optimiser™.

Avoiding Bubbles While Pipetting:

1. Bubbles will compromise the performance of assays on Optimiser™ 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.
Air bubbles will occlude the microfluidic channel and stop the flow of the Optimiser™.

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

Accurate and Precise Delivery of 5 µL Volumes:

Assays on Optimiser™ 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 immediately below).

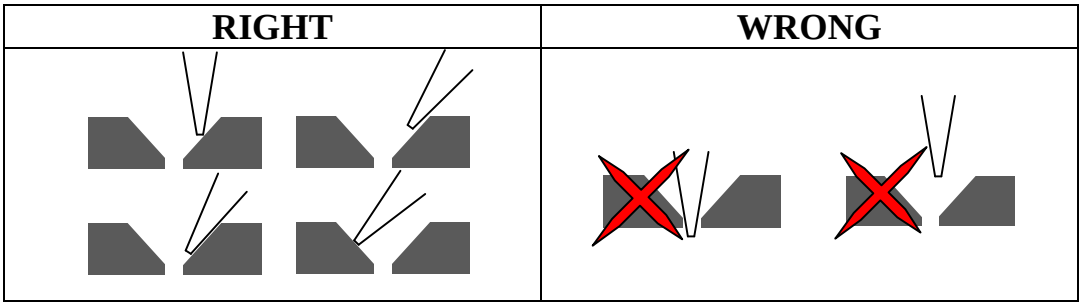


Figure 4. Pipette tip positioning for dispensing in the Optimiser™

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 by Siloam 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

READER SETUP:

Optimiser™ based assays are compatible with standard fluorescence plate readers and multi-mode plate readers with fluorescence reading capability. Below is the general guidance for setting up the readers. For further assistance, please contact Siloam's technical support.

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

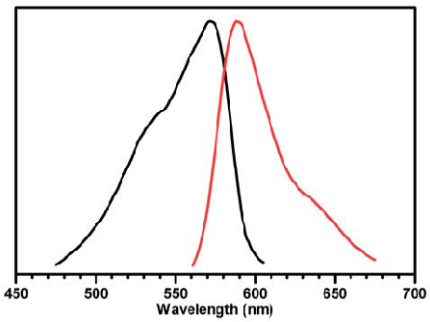


Figure 5. Normalized absorption (left) and emission (right) spectra of OptiGlow™ chemifluorescent substrate.

Assays on Optimiser™ uses OptiGlow™ substrate, 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 bandpass that includes the excitation/emission spectra is required. Wavelengths at 530-575 nm for excitation and 585-630 nm for emission can be used for detection. Below are examples for different types of readers:

- **Filter-based readers**: install 528/20 nm (or similar) filter for excitation, and 590/35 nm (or similar) filter for emission
- **Monochromator-based readers**: in wavelength setting, set excitation at 528/20 nm, and emission at 590/35 nm
- **Readers with pre-configured optical set:** select the wavelength setting for Rhodamine or Cy3.

Step 2: Selecting the plate type:

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

Step 3: Selecting the probe direction:

Please use "top reading" for probe direction.

Step 4: Selecting the sensitivity/gain:

When defining reading parameters for fluorescence analysis, setting the PMT sensitivity (or "gain" in some types of fluorescence reader) is important for obtaining useful measurements. A manual sensitivity/gain setting is recommended for reading Optimiser™ microplates. The procedure is as described below:

1) In a clean plastic tube, add 50 µL of OptiGlow™-A, 50 µL of OptiGlow™-B, 1 µL of OptiGlow™-C, and 1 µL of SAv-HRP stock solution provided by Siloam, mix well, and wait for 2 minutes. The substrate will be fully developed and stable for hours.
2) Load 4 µL of mixture into one well of Optimiser™ microplate and wait until the well is empty (do not use pad/holder)
3) Read that well in reader with various gain setting.
4) Select the gain which gives the RFU reading closest to 11,000[1].
5) Use the same gain setting, read one blank well of Optimiser™, the readout should be less than 50.
6) Save or record this gain setting.
7) This defines the max reading (**RFU_{max}**) that Optimiser™ based assays can reach with this reader gain/sensitivity setting.

The gain setting will be valid for all Optimiser™ based assays. Repeat Step 4 if a) changing the reader or b) changing the optical unit such as light bulb, filters, etc.

The "Technical Support" section on Siloam's website offers detailed guidance on set up of the BioTek FLx800™ instrument as an illustrative example.

[1]The RFU_{max} value of 11,000 is selected for readers with linear operating range up to ~ 100,000 RFU. Setting RFU_{max} = 11,000 will allow users to: (a) compare results with Siloam data and allow for effective trouble-shooting if needed and (b) use the wide dynamic range protocols as described in Appendix 1 without changing the gain settings.

SANDWICH ELISA ASSAY TRANSFER GUIDE:

The OptiMax™ ELISA procedure is a chemifluorescent immunoassay 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 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.

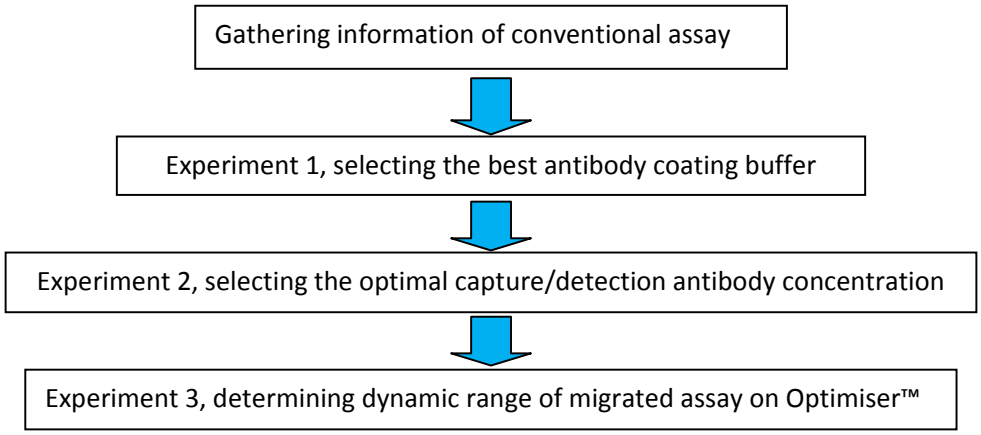


Figure 6. Schematic assay optimization procedure with Optimiser™ microplate.

Requirements for Conventional Assay to be Transferred to Optimiser™

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

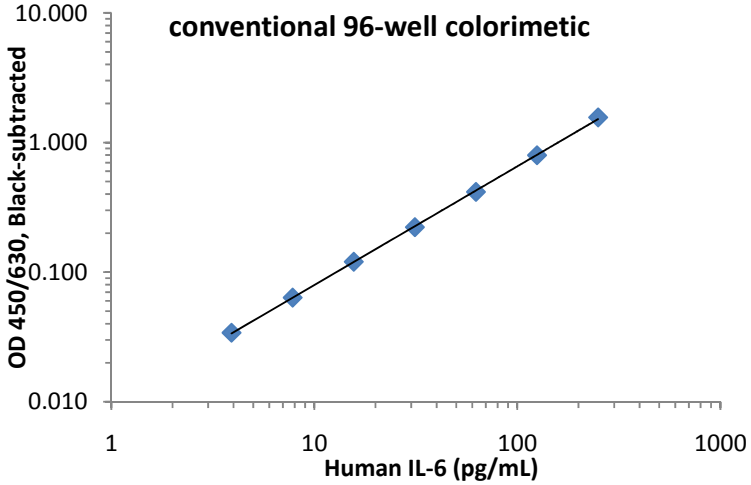
- **Reasonable background (zero) reading**: for an colorimetric assay, the OD450/630 absorbance reading of background shall be less than 0.15
- **Reasonable dose response** with various concentrations of standard: for an absorbance assay, the OD450/630 absorbance reading of highest concentration in detectable range shall be higher than 1.5

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 RIGHT BEFORE THE ASSAY MIGRATION PROCESS TO ENSURE MATERIAL QUALITY. DO NOT USE MANUFACTURER SPECS WITHOUT A CONFIRMATORY EXPERIMENT.

***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	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 7. 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 each concentrations for Capture and Detection antibody.

Details for illustrative IL-6 assay:

- Assay metrics
  - Background OD = 0.025 (less than 0.15)
  - Max signal OD = 1.58 (more than 1.5)
- 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 swamped by high HRP concentrations. The use of a biotinylated detection antibody is recommended with SAv-HRP provide by Siloam (Cat# OMR-HRP) to obtain the best response.

- **For biotinylated detection antibody**: Use appropriate formulized SAv-HRP provided by Siloam (Cat# OMR-HRP). Please prepare the working solution at 1:150 dilution with OptiBlock™.

It is strongly recommended that the SAv-HRP provided by Siloam (Cat# OMR-HRP) be used for all assays on Optimiser™. 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 (anti-mouse, anti-goat, etc.)**: use secondary antibody at 0.1-0.2 µg/mL concentration.
- **For using HRP directly conjugated detection antibody**: Characterization studies at Siloam with multiple direct labeled antibodies confirm a high background issue in most assays, which is mostly caused by high concentration of free HRP molecule in the solution. The use of direct HRP conjugated detection antibody in Optimiser™ based assay except **is not recommended** (unless the conjugated antibody is purified thoroughly to remove all free HRP). Please contact Siloam technical support for further assistance.

Unit Conversions — Comparing Absorbance and Fluorescence Assay Readings

In order to compare the assay performance between Optimiser™ and conventional plate, conversion between fluorescence readout (RFU) and absorbance readout (OD) is required. Please follow the guidance below:

Experiment

In a plastic tube, add 50 µL of OptiGlow™-A, 50 µL of OptiGlow™-B, 1 µL of OptiGlow™ C, and 1 µL of SAv-HRP **stock** solution provided by Siloam, mix well, wait for 2 minutes.

↓

Load 4 µL of mixture into one well of Optimiser™ plate and wait until the well is empty (do not use pad/holder)

↓

Read in fluorescence plate reader, record the readout: RFU_{max}

Conversion Calculation

Convert RFU to OD:

$$OD = \frac{RFU}{RFU_{max}} \times 3.5$$

Conversion equation is identical for all assays. If the reader and/or optical unit of reader are changed, RFU_{max} may change.

Conversion Examples

For example, using reader set up instructions (Page 8) RFU_{max}=11000.

RFU reading	Equivalent OD
9425	3.00
5000	1.59
3150	1.00
200	0.06

Note that OD_{max} is set to 3.5 as this is the maximum OD value read by most readers

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

What detection modes can use with the Optimiser™?

- The current version of Optimiser™ 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™.

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 20 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™ plates in parallel.
- All incubation steps must be at least 5 minutes (at least 20 minute for sample). Incubation steps should not exceed 20 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™?

- 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 Note (under the Technical Support Tab) on Siloam's website (authored by BioTek) that describes the use of a Precision automation station to increase assay sensitivity more than 100x! Please discuss your application with Siloam 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™ 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. It requires one assay experiment and uses one full Optimiser™ microplate.

Optimiser™ Microplate System (Cat# OPH-2), OptiMax™ Test Buffer (Cat# OMR-TEST), and OptiMax™ SAv-HRP (Cat# OMR-HRP) are required to complete Experiment 1. Alternately, OptiMax™ Evaluation Kit (Cat# OPV-IL6) contains all required components for Experiment 1.

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™ COAT BUFFER IS MANDATORY FOR OPTIMISER™ ASSAYS - DO NOT USE ANY OTHER COAT 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 7). 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 by Siloam. 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 3.
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 10*). 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 **10 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)

Selecting the Best Coating Buffer:

Select the OptiBind type which gives the maximum signal. This particular OptiBind™ coat buffer should be used for all further experiments of this assay.

- 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 8 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 (based on RFU_{max} ~ 11,000; *see Unit conventions, page 8*). Data acquisition and analysis utilized Gen5™ software and Excel.

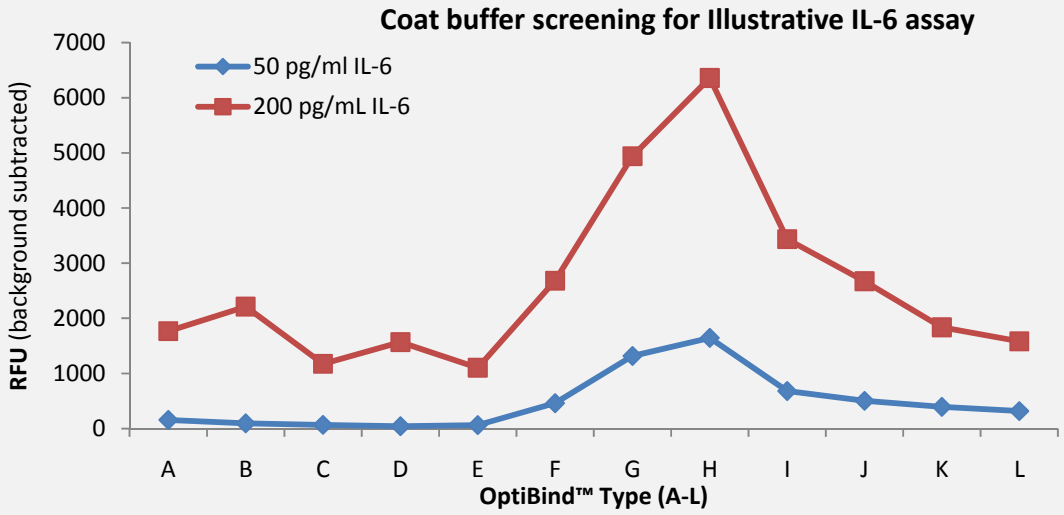


Figure 8. Results for coat buffer screening test for Illustrative IL-6 assay.

For the data shown in Figure 8, both curves for screening test result are valid and either curve can be used to select the best OptiBind™ formulation. Both curves also show 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/detection Antibody Concentration:

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.

Optimiser™ Microplate System (Cat# OPH-2), OptiMax™ Reagent Buffers (Cat# OMR-02X) with the optimal OptiBind from Experiment 1, and OptiMax™ SAv-HRP (Cat# OMR-HRP) are required to complete Experiment 2. Alternately, OptiMax™ Evaluation Kit (Cat# OPV-IL6) contains all required components for Experiment 2.

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 by Siloam. 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 3.
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 14*). 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 **10 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 following steps to determine the optimal combination of capture/detection antibody concentrations:

1) For most assays tested at Siloam to-date, using the **same** concentration of capture and detection antibody results in approximately the same dynamic (operating) range for the assay on Optimiser™
2) Select a higher capture (or detection) antibody ONLY when the data shows at least 1.5x higher signal than lower concentration combination. If the signal increase is less than 50% the net gain in assay sensitivity will not be significant. *See example data below*.
3) DO NOT select antibody concentrations which give background reading higher than 4% of RFU_{max}. *See example data below*.
4) Higher concentrations slightly increase the consumption (hence cost); although even with 4 times higher concentrations, Optimiser™ assays still use 5 times less antibody than conventional plate. Select the higher concentrations only if your assay requires higher sensitivity.

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 OD readings

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	0.861		0.107		0.744		0.111		0.525		0.114	
	B												
4 µg/mL	C	0.788		0.095		0.667		0.074		0.550		0.085	
	D												
2 µg/mL	E	0.746		0.076		0.660		0.092		0.491		0.093	
	F												

- All background readings are less than 4% of RFU_{max} (RFU reading = 440 for RFU_{max} = 11,000).
- 8 µg/mL capture; 2 µg/mL detection antibody concentrations selected (signal 50% higher than 2 µg/mL capture; 2 µg/mL detection).
- Increasing capture antibody concentration has most significant effect on signal. Using 8 µg/mL capture; 8 µg/mL detection shows only ~15% signal increase. This will not have a significant effect on assay sensitivity.

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

		Capture Antibody											
		4 times as conventional assay				**2 times as conventional assay**				**Same as conventional assay**			
Detection antibody		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
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 results from Experiment 1 and Experiment 2 are used for the final Experiment to determine the dynamic range of the assay. This experiment will run a standard curve of the assay with wide range of concentrations which covers the expected dynamic range. **Appropriate standard diluents must be used for this experiment. For example, use cell culture medium as standard diluent for measuring cell culture supernatant**. 3 rows in one Optimiser™ microplate will be used.

Optimiser™ Microplate System (Cat# OPH-2), OptiMax™ Reagent Buffers (Cat# OMR-02X) with the optimal OptiBind from Experiment 1, and OptiMax™ SAv-HRP (Cat# OMR-HRP) are required to complete Experiment 3. Alternately, OptiMax™ Evaluation Kit (Cat# OPV-IL6) contains all required components for Experiment 3.

Reagent Preparation:

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

1. OptiBind™: Use the optimal OptiBind™ coat buffer selected from experiment 1.
2. Capture Antibody: Use optimal concentration of capture antibody determined in experiment 2. Prepare the capture antibody working solution by diluting the capture antibody stock in *selected* OptiBind™ to make **300 µL** final working solution. Use 3 wells in one column on the polypropylene 96-well v-bottom plate, load **90 µL** of working solution into each well.
3. OptiBlock™: OptiBlock™ is provided in ready-to-use form and is used as blocking buffer. OptiBlock™ is also used as a diluent for the detection antibody and SAv-HRP. For blocking step, prepare **1 mL** of OptiBlock™ in a v-shape reservoir.
4. Standard diluent: Appropriate standard diluent shall be used for this experiment. As an example, use cell culture medium as standard diluent for measuring cell culture supernatant
5. Protein Standards: Prepare **100 µL** of protein standard at defined top concentration with standard diluent in a plastic tube. Using one row of 96-well v-bottom plate, load 45 µL of standard diluent in wells 2-12. Load 90 µL of top standard into the first well, transfer 45 µL of top standard to well 2, change tip and mix with the standard diluent to create a 1:2 dilution. Continue this process till well 11; **do not** transfer diluted standard to well 12; leave well 12 as blank.

   T**op concentration**: Based on the converted OD reading from experiment 2 (and assuming the standard curve is linearly proportional to antigen concentration), calculate the concentration for which the signal will be higher than OD 3.5.

   As an example, for the IL-6 assay, expected top concentration = (50 pg/mL x 3.5)/0.746 = 234 pg/mL. For easy calculation and dilution, 250 pg/mL would be used as top concentration for this experiment.
6. Detection Antibody: Use optimal concentration of detection antibody determined in experiment 2. Prepare the detection antibody working solution by diluting the detection antibody stock in OptiBlock™ to make **300 µL** final working solution. Use 3 wells in one column in the polypropylene 96-well v-bottom plate, load **90 µL** of working solution into each well.
7. SAv-HRP: Use SAv-HRP stock solution provided by Siloam. 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 **0.4 mL** of OptiGlow™-A, **0.4** mL of OptiGlow™-B and **8 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 **4 mL** of OptiWash™ into a v-shaped reagent reservoir and use it for all wash steps in the assay.

Assay Layout:

Protein standards concentration

	1	2	3	4	5	6	7	8	9	10	11	12
A	Top concentration*	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	0
B												
C												

T**op concentration**: Based on the RFU reading from experiment 2 (and assuming the standard curve is linearly proportional to antigen concentration), calculate the concentration for which the signal will be higher than RFU_{max}.

Procedure:

1. Assemble the Optimiser™ Microplate, Optimiser™ Pad, and Optimiser™ Microplate Holder as described on Page 3.
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 18*). 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 **10 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.**

Determine the Dynamic Range:

Use the following steps to determine dynamic (or operating) range of the assay:

1) The top measurable concentration should give signal between 60%-90% of RFU_{max}.
2) For best linearity and easy operation, a 7-point, two-step dilution (with one zero) is most commonly used as standard curve. Using the selected top measurable concentration, determine the lowest concentration (6 columns to the right of the top measurable concentration).
   Please check Appendix 1 to see a procedure for extending dynamic range of assays on Optimiser™.

Example Data:

As an example, a dynamic range test has been performed for IL-6 assay. Top concentration of IL-6 is 250 pg/mL. 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:

IL-6 (pg/mL)	250	125	62.5	31.3	15.6	7.8	3.9	2.0	1.0	0.5	0.2	0
Mean of RFU	9764	6396	3539	2116	1263	764	471	358	316	230	217	176
Converted OD	3.10	2.03	1.12	0.67	0.40	0.24	0.15	0.11	0.10	0.07	0.06	0.05

The (lowest) top measurable concentration of this IL-6 assay is 125 pg/mL. Hence, the lowest measurable concentration is at least 2 pg/mL.

Using 4x higher concentration of the capture antibody allows Optimiser™ based IL-6 assay to achieve higher sensitivity while still using less reagents than a conventional plate. The sample volume for the Optimiser™ assay is only 5 µL (compared to 100 µL on the conventional plate) and the total assay time for Optimiser™ based assay is only ~2 hours (compared to ~8 hours for conventional plate).

OTHER ASSAY FORMATS ON THE OPTIMISER™:

Indirect Immunoassay: Siloam has developed protocols for indirect ELISA on Optimiser™. These will be added to the Assay Transfer Guide in future revisions. Please contact Siloam's technical support for assistance.

Competitive Immunoassay: Siloam has also developed protocols for competitive ELISA on Optimiser™. These will be added to the Assay Transfer Guide in future revisions. Please contact Siloam's technical support for assistance.

TROUBLESHOOTING:

The Optimiser™ technology and OptiMax™ ELISA kits have 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 due to the unique features of the Optimiser™ technology as well as problems that can be encountered with immunoassays in general.

Table 3.Trouble Shooting Guidelines

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™ and the tape side (smooth) is facing down to touch holder. • Ensure the topside of the pad is touching the bottom of Optimiser™ plate 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 pipet tips when handling different buffers/reagents.
Poor precision	Pipetting error (technique or equipment)	• Follow recommendations for pipetting small volumes.
Curve is nonlinear.	Pipetting	• Follow guidelines for in-plate serial two-fold dilutions.
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: ALTERNATIVE ASSAY PROCEDURES ON OPTIMISER™

Rapid Assay on Optimiser™:

The standard Optimiser™ assay procedure, as described on page 11 of this Instruction Manual, requires approximately 2 hours (125 minutes) to complete. Most incubation steps are 10 minutes in length with the exceptions of sample incubation (20 minutes) and substrate incubation (15 minutes).

Siloam Biosciences has developed an alternative method that can be completed in 90 minutes. The sample incubation time (20 minutes), final two washes (10 minutes) and substrate incubation time (15 minutes) are unchanged. However, the remaining incubation times can be reduced from 10 minutes to 5 minutes. The plot in Figure 9 shows the adsorption kinetics on the Optimiser™ showing that in ~ 5 minutes, ~ 92% of peak adsorption (or binding) is completed. More importantly, from 5 – 30 min (next time point) the adsorption only changes from ~ 92% to ~ 96%.

In doing so, the total assay time is reduced from 125 minutes to 90 minutes with no change in method performance. Siloam strongly recommends that only users proficient in the use of the Optimiser™ microplate system attempt the rapid test protocol. It is especially important to ensure that pipetting for each step is completed within ~ 30 seconds. It is also critically important to maintain consistency in pipetting and incubation intervals when using the accelerated protocol.

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

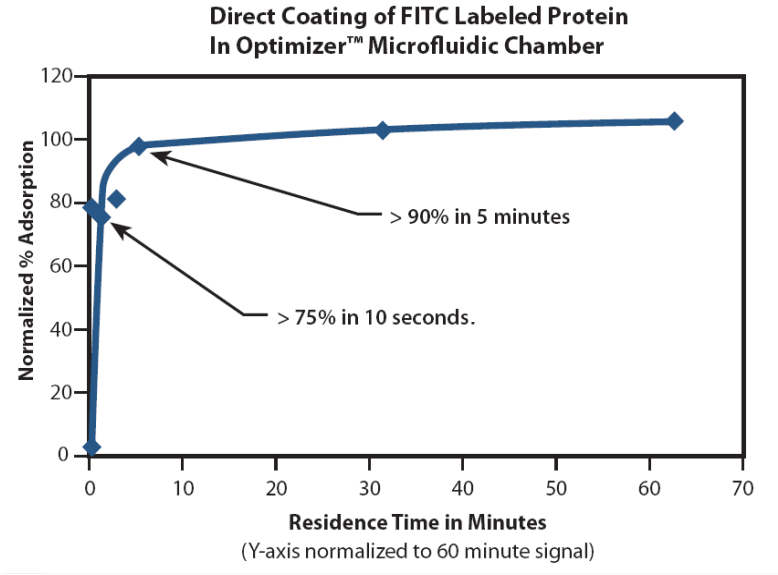


Figure 9. Adsorption characteristics of capture antibody on the Optimiser™ microchannel surface.

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

Ultra-sensitive Assay on Optimiser™:

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 ultralow sensitivity is required. The additional sample applications can be performed manually for a limited number of repeat sample loads but Siloam strongly recommends use of a laboratory sample processor for the ultra-high sensitive protocol.

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

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 a laboratory sample processor.

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.

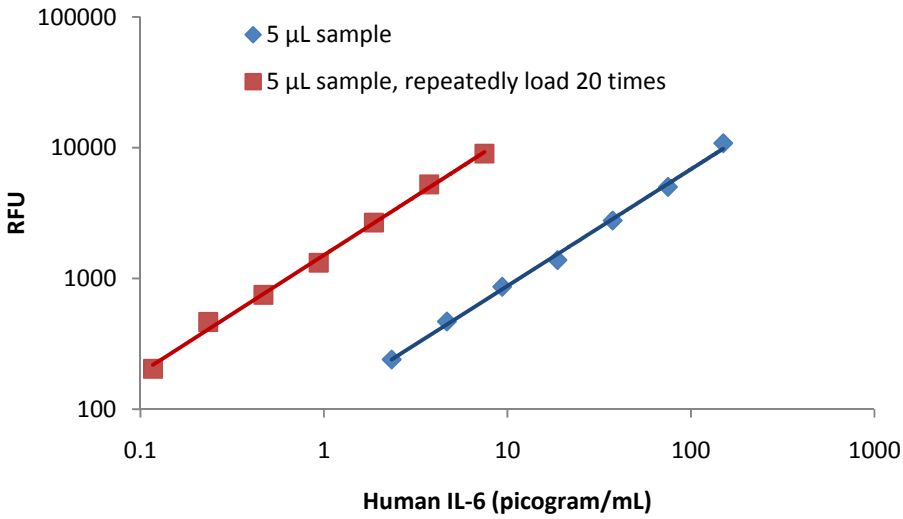


Figure 10. Ultra-sensitive assay using repeat sample loading technique with the OptiMax™ Human IL-6 ELISA kit with an automated pipetting station.

Assay with Wider Measurable Range on Optimiser™:

In standard assay procedure, the measurable range is typically ~ 64 to 100 fold. Generally, a 7-point double dilution with one zero is commonly used as standard curve, which give 64 fold between highest and lowest measurable concentration.

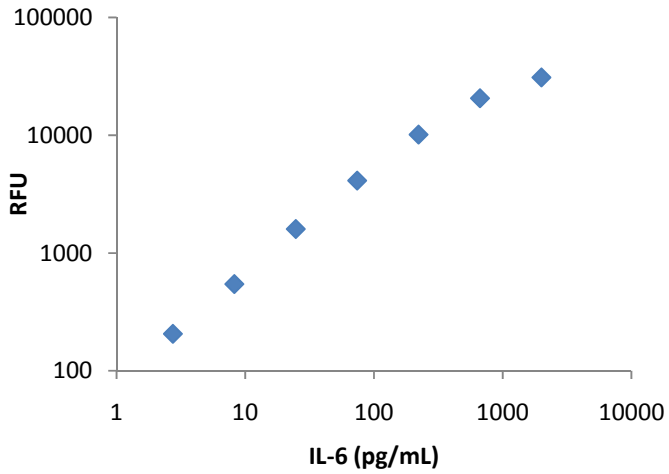
Siloam Biosciences has developed an alternative method that can provide **10 times** wider measurable range with same assay protocol described in page 11, except following changes:

1) Change the mixing ratio of OptiGlow™ substrate:
    a. To create the substrate working solution, combine OptiGlow™-A, OptiGlow™-B, and OptiGlow™-C in a ratio of **50:50:5** parts respectively according to the following table and vortex gently to mix.
2) Keep similar lowest concentration, use 7-point **serial three-fold dilutions** with one zero when making the standard. Below is an example for IL-6 assay:

	IL-6 with two-fold dilution (pg/mL)	IL-6 with three-fold dilution (pg/mL)
A	150	2000
B	75	667
C	38	222
D	19	74
E	9.4	25
F	4.7	8.2
G	2.3	2.7
H	0	0

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

The data in the figure below illustrates the wider measurable range obtained using the standard Optimiser™ assay procedure with changes described above.



IL-6 (pg/mL)	Mean	Blank-subtracted
2000	31149	30977
667	20794	20622
222	10308	10136
74	4292	4120
25	1772	1600
8.2	718	546
2.7	378	206
0	172	

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

NOTE:

NOTE:

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™ 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™

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QuantaRed™ substrate is supplied by Thermo Fisher Scientific Inc.

Better Immunoassays Through Innovative Microfluidics

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