User Manual

OptiMax™ Mouse IFN-gamma ELISA Kit

For the quantitative determination of mouse (MS) interferon-gamma in cell culture supernatants

Catalogue Numbers: OMA-M-IFNG-02; OMA-M-IFNG-10; OMA-M-IFNG-50

Manufactured by:

Siloam Biosciences, Inc.
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Cincinnati, Ohio 45240, USA

FOR RESEARCH USE ONLY

Not for use in clinical diagnostic procedures.

Read the User Manual in its entirety before using the OptiMax™ Mouse IFN-gamma ELISA Kit.

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Symbol indicates mandatory step required to ensure proper operation

Symbol indicates helpful tips to achieve optimal performance

Intended Use:

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

Introduction:

Mouse (Ms) interferon-gamma (IFN-γ), a cytokine, is one of the most important members of the interferon (IFN) family. It is a polypeptide of 155 amino acids having a molecular weight of 17.709 kDa. Mouse IFN-γ is the sole type II IFN, structurally unrelated to type I IFNs, and binds to different receptors and is encoded by a separate chromosomal locus (Schroder K et al., 2004). IFN-γ is primarily secreted by activated T cells and natural killer cells and can promote macrophage activation, mediate anti-viral and anti-bacterial immunity, enhance antigen presentation, orchestrate activation of the innate immune system, coordinate lymphocyte-endothelium interaction, regulate Th1/Th2 balance, and control cellular proliferation and apoptosis (Gattoni et al, 2006). Cytokines, most notably IL-12 and IL-18, control the production of IFN-γ during the course of the immune response. IFN-γ acts mainly through a receptor mediated signaling process (by IFNGR1/IFNGR2) and signals through the JAK-STAT pathway (Schroder K et al, 2004).

Siloam Biosciences' OptiMax™ Mouse Interferon-gamma ELISA Kit offers a rapid and sensitive chemifluorescent-based ELISA procedure for Ms IFN-γ that requires exceedingly small sample volumes. The speed, sensitivity, and small sample requirements are enabled by the unique microfluidic design of the Optimiser™ plate. The standard immunoassay reactions such as analyte capture and detection occur within a ~ 5 µL microfluidic reaction chamber. The unique microchannel geometry and small reaction volume favor rapid reaction kinetics. The Ms IFN-γ 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.

This OptiMax™ ELISA kit has been calibrated against the R&D Systems Quantikine® Mouse IFN-gamma ELISA kit. Data generated using the OptiMax™ Mouse IFN-γ kit should closely correlate with that generated using the R&D Systems Quantikine® Mouse IFN-gamma ELISA kit.

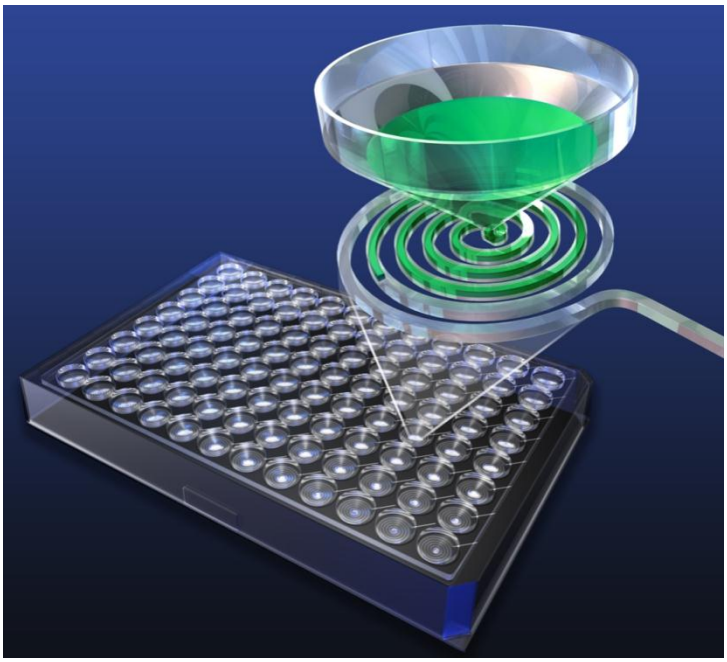


Figure 1. Optimiser™ microplate

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 microchannel 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.

Materials Provided:

OptiMax™ Ms IFN-γ ELISA kits provide the critical materials and reagents necessary for the measurement of Ms IFN-γ in tissue culture supernates (Tables 1 and 2). Table 1 identifies the kit contents, their function, and their required storage temperature. Table 2 restates the kit contents and indicates their individual product numbers and the amount of each component provided per kit. **Refer to the enclosed Certificate of Analysis (CofA) for expiration dating.**

Table 1. Materials Provided with the OptiMax™ ELISA Kit (Name, Function, and Storage Condition)

Material	Function	Storage
Unopened Kit	Contains all provided materials	Store at 2 – 8 °C
Storage of Opened and Reconstituted Materials		
Material	Function	Storage / Handling
rMs IFN-γ standard	Construction of rMs IFN-γ standard curve	Refrigerated 2 - 8 °C (unopened). Reconstitute per directions in CofA. After reconstitution, aliquot and store at ≤ - 20 °C. Avoid repeated freeze-thaws.
Optimiser™ Holder	Holds Optimiser™ Plate and Optimiser™ Pad in proper alignment	Room temperature
Optimiser™ Plate	Contains microfluidic reaction chambers	
Optimiser™ Pad	Absorbs used reagent volume	
96-well v-bottom plate	For dilutions and reagent reservoir	
Standard Diluent	Diluent for lyophilized standard, standard curve, and samples	Refrigerated 2 – 8 °C
OptiBind™-G	Diluent for capture antibody	
OptiBlock™	Blocking solution and diluent for detection antibody and SAv-HRP	
OptiWash™	Washing solution	
OptiGlow™ - A	Components of chemifluorescent substrate	
OptiGlow™ - B		
OptiGlow™ - C		
Capture antibody	Captures Ms IFN-γ on solid phase	
Detection antibody	Binds captured Ms IFN-γ	
SAv-HRP	Binds detection antibody; interacts with substrate yielding chemifluorescence	

The reconstituted standard must be aliquoted and frozen on the day of reconstitution.

It is recommended that the package be opened and various components stored separately (as listed in Table 1) to conserve refrigerator shelf space.

All materials to be refrigerated are contained in a smaller box within the product package.

Table 2. Materials Provided with the OptiMax™ ELISA Kit[a]
(Quantity per assay kit per Product No.)

Material Provided	Volume/Unit	Product Number	Number of Units/Kit Type		
			Product No. OMA-M-IFNG-02 (2-plate kit)	Product No. OMA-M-IFNG-10 (10-plate kit)	Product No. OMA-M-IFNG-50 (50-plate kit)
Optimiser™ Holder	NA	OPH-02, or OPH-10, or OPH-50	1	1	1
Optimiser™ Plate	NA		2	10	50
Optimiser™ Pad	NA		4	20	100
96-well v-bottom plate	NA	OPT/FL-231	1	5	25
Standard Diluent	20 mL/vial	OM-059	1	2	10
OptiBind™-G	10 mL/vial	OM-052G	1	2	10
OptiBlock™	30 mL/vial	OM-055	1	2	10
OptiWash™	60 mL/vial	OM-054	1	2	10
OptiGlow™ - A	5 mL/vial	OM-056	1	2	10
OptiGlow™ - B	5 mL/vial	OM-057	1	2	10
OptiGlow™ - C	1 mL/vial	OM-058	1	2	10
Capture antibody	40 µL/vial	OM606102	1	2	10
Detection antibody	25 µL/vial	OM606202	1	2	10
rMs IFN-γ standard	Lyophilized	OM606302	2	10	50
SAv-HRP	25 µL/vial	OM0602	1	2	10

[a]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 OptiMax™ ELISA Kit:

1. Eppendorf or similar polypropylene tubes for centrifugation and dilutions
2. 0.22 µm filters (for sample filtration if required)
3. Kimwipes™ or other laboratory tissue paper
4. Reagent reservoirs (V-shape reservoir)
5. Pipet tips for delivering in the ranges of 1 -10, 10 - 100, and 100 - 1000 µL

Equipment Required:

1. Pipettor capable of accurately and precisely delivering 5 µL
2. Multichannel pipettor capable of accurately and precisely delivering 5 µL
3. Additional pipettors for delivery of liquids in the ranges of 1 -10, 10 - 100, and 100 - 1000 µL
4. Multichannel pipettor capable of delivering 30 µL
5. Vortex mixer
6. Microplate fluorescence reader and control software
7. Analytical software
8. Microcentrifuge capable of 13,000 rpm

Unique Considerations for Optimiser™ Microplate:

The operation sequence for immunoassays performed using Optimiser™ microplates is very similar to that for immunoassays performed using conventional microplates. By paying attention to a few key details listed here, users can ensure quality results and high success.

The Optimiser™ Plate and Assembly:

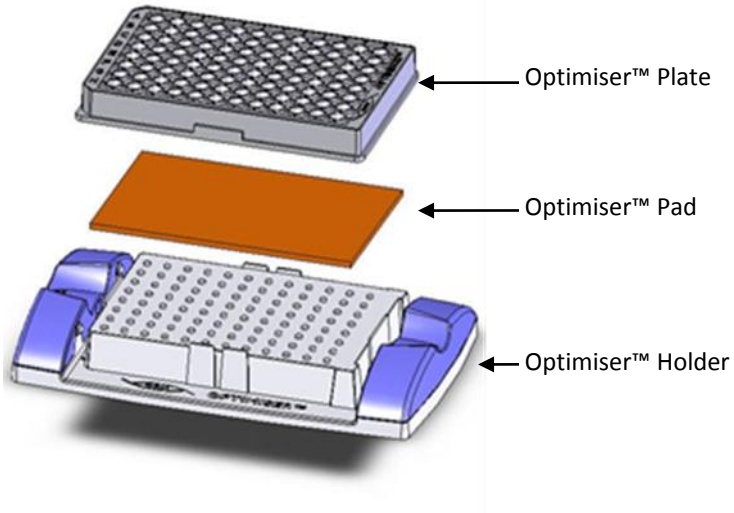


Figure 2. Optimiser™ microplate assembly
Position absorbent pad on holder, align the Optimiser™ microplate, and press down gently to click-lock the plate in holder.

Both the microplate and holder have standard markings (A-H rows, 1-12 columns) to aid in alignment. The microplate can be mounted on the holder in one orientation only.

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

Pipetting for Optimiser™-Based Assays:

Avoiding Bubbles While Pipetting:

1. Bubbles will compromise the performance of Optimiser™-based assays by interfering with the flow of liquid within the microchannels.
2. In particular, the Standard Diluent and OptiBlock™ reagents may form bubbles readily if incorrectly pipetted.
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 pipettor to the second stop (refer to illustration below).
    b. Immerse the pipet tip in the liquid (to be pipetted) to a depth of about 2 mm and slowly 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 Optimiser™ loading well by gently and steadily pressing the pipettor's operating button to the first stop. Briefly hold the button in this position.
    e. With the button in this position, move the tip from the receiving well to the source of the liquid to be pipetted, immerse the tip in the liquid, and aspirate.

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™.

If bubbles are accidentally dispensed/ created, they can be easily disrupted using a clean 26 gauge needle or similar clean, sharp-tipped object.

Figure 3.

Reverse Pipetting Procedure

	Pipetting Step			
Ready Position	1	2	3	4
First Stop	↓	↑	↓	↑
Second Stop	↓	↑		

Accurate and Precise Delivery of 5 µL Volumes: Optimiser™ assays require the accurate and precise delivery of 5 µL volumes. The following guidance is offered to users.

1. Use multichannel and single channel pipettors for which the upper limit of their operating range is ≤ 10 µL.
2. Use pipet tips appropriate for 5 µL pipetting.
3. To aspirate liquid, hold the pipettor nearly vertical and immerse the pipet tip in the liquid to a depth of approximately 2 mm. Withdraw the operating button slowly and steadily. Wait ~ 1 second. Withdraw the tip from the liquid.
4. To dispense liquid, hold the pipettor nearly vertical. With the pipet tips touching the surface of the Optimiser™ well, depress the operating button slowly and steadily until the liquid is dispensed.
5. **Note:** The pipet 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).

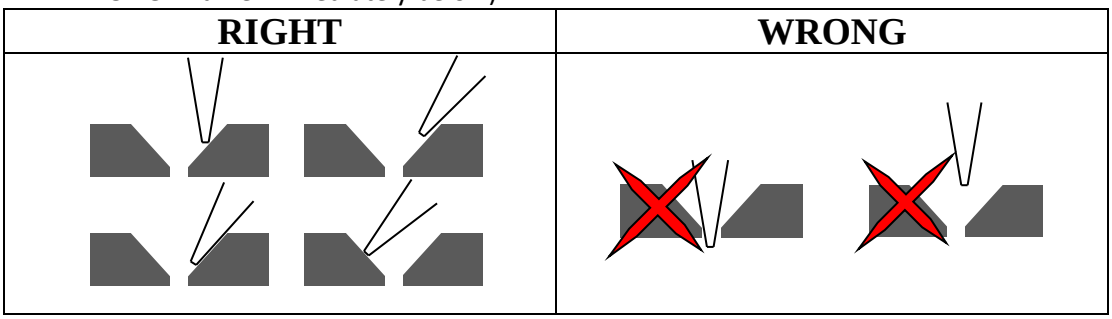


Figure 4. Pipet tip positioning for dispensing in the Optimiser™

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 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 (i.e minor variations in the time required for all liquid to drain from wells) do not affect assay results.

Reader Settings:

OptiMax™ ELISA procedures are compatible with standard fluorescence plate readers and multi-mode microplate readers with fluorescence reading capability. The "Technical Support" section on Siloam's website offers detailed guidance on set up of the BioTek FLx800™ instrument and general guidance for other readers. Siloam Biosciences has verified the compatibility of OptiMax™ ELISA assays using OptiGlow™ chemifluorescence substrate in combination with BioTek Instruments' FLx800™ Fluorescence Microplate Reader. Siloam Biosciences uses the following wavelengths and corresponding products.

Table 3. Required Filters for BioTek FLx800™ Fluorescence Reader

Function	BioTek Part Number	Wavelength
Excitation	7082247	528/20 nm (or similar)
Emission	7082224	590/35 nm (or similar)

The use of an automatic multi-channel pipette simplifies operation and minimizes potential for bubbles.

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

Small flow rate variations (minor variations in time required for liquid to drain from wells) do not affect assay performance. The incubation step smoothes out any flow variation differences.

The Optimiser™ has an ANSI/SBS compliant layout. Z-axis adjustment is not required for reading the Optimiser™ plate. Use the same setting used for a conventional 96-well microplate.

For the FLx800™ instrument and the filters listed above, a **sensitivity setting of 45** is recommended for the reader. For more detailed information and technical support for BioTek instruments or Gen5™ software, please contact BioTek Instruments at 1-888-451-5171.

Principle of Method:

The OptiMax™ Mouse IFN-γ ELISA procedure is a chemifluorescent immunoassay in which traditional ELISA reactions take place within the unique Optimiser™ plate architecture. Briefly, anti-IFN-γ capture antibody is immobilized on the internal surfaces of the plate's microchannels. Following a flush step (which is equivalent to a wash step in conventional plates), any unreacted sites on the microchannel surface are blocked with a blocking solution. Recombinant (r) Ms IFN-γ standard, control, and samples are diluted in Standard Diluent and dispensed to the Optimiser™ wells. Ms IFN-γ present in standards, controls, and samples will be specifically captured on the microchannel surface by the immobilized capture antibody. Following another flush, a biotin-labeled anti-Ms IFN-γ detection antibody is added to the wells. The biotin-labeled antibody will bind Ms IFN-γ that has been captured and immobilized on the microchannel surface thus "sandwiching" the Ms IFN-γ between the capture and detection antibodies. Following another flush, horseradish peroxidase-labeled streptavidin (SAv-HRP) is added to the Optimiser™ wells. The SAv of SAv-HRP binds specifically to the biotin moiety of the biotin-labeled antibody if it is present in the [capture antibody + Ms IFN-γ + detection antibody] complexes formed and immobilized on the microchannel surface. Following two additional flushes, 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 chemifluorescent 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 Ms IFN-γ in standards, controls, and samples and will be quantifiable when the plate is read using a microplate fluorescence reader.

Reagent Preparation:

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

1. OptiBind™: OptiBind™ is provided in a ready-to-use form. No further preparation is required. **Do not substitute other coating buffers for OptiBind™**.
2. Capture Antibody: The procedure requires 5 µL of capture antibody working solution for each assay well to be used.
    a. Prepare the capture antibody working solution by diluting the capture antibody stock 1:62.5 in OptiBind™ in a clean polypropylene tube according to the following table:

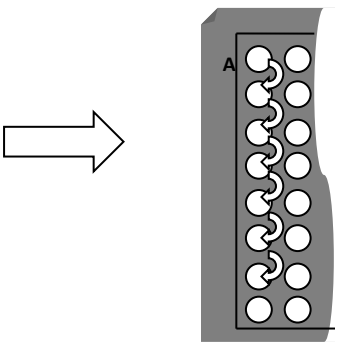
Number of Wells to be Used	Vol. of Capture Antibody Stock	Vol. of OptiBind™	Final Preparation
48 Wells (1/2 plate) (6 columns)	8 µL	0.5 mL	Dispense 55 µL of the working solution into each well of a single column in the polypropylene 96-well v-bottom plate.
96 Wells (full plate) (12 columns)	16 µL	1 mL	Dispense 120 µL of the working solution into each well of a single column in the polypropylene 96-well v-bottom plate, or transfer the entire volume of working solution into a v-shape reagent reservoir.

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

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

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 detection antibody and SAv-HRP.
4. Standard Diluent: Standard Diluent (SD) is used to reconstitute the lyophilized rMs IFN-γ standard and for the preparation of rMs IFN-γ standards 1 – 7. SD is also the diluent for Ms IFN-γ controls and for samples where sample dilution is required. SD is dispensed to the blank wells during the sample incubation step. It is provided ready to use.
5. Recombinant (r) Ms IFN-γ standard:
    a. Stock Solution: The rMs IFN-γ standard is provided in lyophilized form.
        i. Reconstitute the lyophilized standard by adding 420 µL of Standard Diluent.
        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. Refer to the enclosed Certificate of Analysis (CofA) for the concentration of the reconstituted standard.
        v. Use freshly prepared material on the day of reconstitution, **or**
        vi. Prepare single-use aliquots by dispensing reconstituted standard to appropriately-sized polypropylene vials and store frozen at ≤ -20 °C.
        vii. Use single-use aliquots one time only on the day of thawing. Avoid repeated freeze-thaws.
    b. Working Solution: The concentration of the reconstituted rMs IFN-γ standard is specified in the CofA enclosed with each assay kit. Prepare a 210 pg/mL standard (Standard 1) by diluting the rMs IFN-γ standard appropriately in Standard Diluent. Refer to CofA for dilution instruction.
        i. Vortex the 210 pg/mL standard briefly to mix.
    c. Standard Curve: Prepare the remaining rMs IFN-γ standards by performing six serial two-fold dilutions in Standard Diluent beginning with the 210 pg/mL standard as follows:
        i. Dispense 200 µL of Standard 1 (210 pg/mL) to well A1 of the 96-well polypropylene v-bottom plate.
        ii. Dispense 100 µL Standard Diluent to each of the seven wells of the same column immediately below the 210 pg/mL-containing well (wells B1 – H1).

	1
A	200 µL Std 1
B	100 µL SD
C	100 µL SD
D	100 µL SD
E	100 µL SD
F	100 µL SD
G	100 µL SD
H	100 µL SD



	1
A	210
B	105
C	53
D	26
E	13
F	6.6
G	3.3
H	0

        iii. Transfer 100 µL of the 210 pg/mL standard from well A1 to well B1 immediately below it.

The Certificate of Analysis includes instructions for the reconstitution of the lyophilized standard and for preparation of Standard 1.

To ensure accurate preparation of the standard, pipet at least 10 µl of the stock standard using an appropriate pipettor.

The standard curve preparation described here is an illustrative example using the first column of a v-bottom plate. For subsequent use, the 2nd or additional columns of the v-bottom plate may be used.

Sample/reagent prep in the v-bottom plate is highly recommended to allow easy transfer of materials to the Optimiser™ using a multi-channel pipettor.

iv. Change tips. Mix the contents of well B1 by gently aspirating and dispensing the liquid 8 - 10 times while avoiding the creation of significant bubbles in the well.
v. Transfer 100 µL from well B1 to well C1, change tips, and gently mix.
vi. Continue serial dilutions while changing tips after each 100 µL transfer and before mixing until the 3.3 pg/mL standard has been created in the seventh well (well G1) of the column.
vii. Do not transfer rMs IFN-γ to the eighth well (H1). It contains Standard Diluent only and will provide material for the blank wells.

6. Samples: Prepare samples for testing by diluting samples, if required, in Standard Diluent.
   a. Sample concentrations should be derived by interpolation from within the standard curve range. Dilute samples if necessary so that sample signal falls within the range of the standard curve.
   b. Dispense 60 µL of each diluted sample into a single well of the **v-bottom plate** in columns (illustrated below).

	1	2	….	12
A	Std 1	Sample 1		
B	Std 2	Sample 2		
C	Std 3	Sample 3		
D	Std 4	Sample 4		
E	Std 5	Sample 5		
F	Std 6	Sample 6		
G	Std 7	Sample 7		
H	Blank	Sample 8		

7. Detection Antibody: The procedure requires 5 µL of the working detection antibody solution for each assay well to be used.
   a. Prepare a 1:125 dilution of the detection antibody stock in OptiBlock™ in a clean polypropylene tube according to the directions in the following table.

Number of Wells	Vol. of Detection Antibody Stock	Volume of OptiBlock™	Final Preparation
48 Wells (1/2 plate) (6 columns)	4 µL	0.5 mL	Dispense 55 µL of the working solution into each well of a single column in the polypropylene 96-well v-bottom plate.
96 Wells (full plate) (12 columns)	8 µL	1 mL	Dispense 120 µL of the working solution into each well of a single column in the polypropylene 96-well v-bottom plate, or transfer the entire volume of working solution into a v-shape reagent reservoir.

8. SAv-HRP: The procedure requires 5 µL of the working SAv-HRP solution for each assay well to be used.
   a. The SAv-HRP provided with the kit is a stock solution. The stock SAv-HRP must be diluted with OptiBlock™ on the day of use to create a working solution.
   b. Calculate the amount of SAv-HRP working solution required for the assay to be performed (5 µL per well + sufficient excess).

√

The Certificate of Analysis includes instructions for the preparation of the SAv- HRP working solution.

c. Prior to beginning the assay, dilute the SAv-HRP stock solution with OptiBlock™ according to the directions in the CofA to create the appropriate volume of SAv-HRP working solution.

9. Substrate solution: The procedure requires **10 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 50:50:1 parts respectively according to the following table and vortex gently to mix.

OptiGlow™ - C must be thoroughly thawed to function effectively. Warm the reagent in a 37 ^{o}C incubator or water bath or by rotating the vial gently between one's hands.

Number of Wells	Volume OptiGlow™A	Volume OptiGlow™B	Volume OptiGlow™C	Final Preparation
48 Wells (1/2 plate)	0.45 mL	0.45 mL	9 µL	Dispense 100 µL of the working solution into each well of a single column in the polypropylene 96-well v-bottom plate.
96 Wells (full plate)	0.9 mL	0.9 mL	18 µL	Dispense 200 µL of the working solution into each well of a single column in the polypropylene 96-well v-bottom plate, or transfer the entire volume of working solution into a v-shape reagent reservoir.

10. 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.
    a. Dispense OptiWash™ buffer to a v-shaped reagent reservoir according to the following table:

Number of Wells	Volume of OptiWash™	Final Preparation
48 (1/2 plate) (6 columns)	5 mL	Transfer 5 mL of OptiWash™ into a v-shaped reagent reservoir.
96 (full plate) (12 columns)	10 mL	Transfer 10 mL of OptiWash™ into a v-shaped reagent reservoir.

Figure 5. **Schematic Procedure**

Assemble the Optimiser™ plate and the Optimiser™ pad in the Optimiser™ plate holder.

↓

Dispense 5 µL of capture antibody to the required number of wells in the Optimiser™ plate.

Incubate 10 minutes at room temperature (RT).

↓

Dispense 5 µL of OptiWash™ to the wells. Wait 10 minutes to proceed to the next step.

↓

Dispense 5 µL of OptiBlock™ to the wells. Incubate 10 minutes at RT.

↓

Dispense 5 µL of standard, control, sample, and blank to the wells. Incubate **20 minutes** at RT.

↓

Dispense 5 µL of OptiWash™ to the wells. Wait 10 minutes to proceed to the next step.

↓

Dispense 5 µL of detection antibody to the wells. Incubate 10 minutes at RT.

↓

Dispense 5 µL of OptiWash™ to the wells. Wait 10 minutes to proceed to the next step.

↓

Dispense 5 µL of SAv-HRP to the wells. Incubate 10 minutes at RT.

↓

Dispense 30 µL of OptiWash™ to the wells. Wait 10 minutes to proceed to the next step.

↓

Again, dispense 30 µL OptiWash™ to the wells. Wait 10 minutes to proceed to the next step.

↓

Dispense **10 µL** OptiGlow™ working solution to the wells. Incubate **15 minutes** at RT.

↓

Determine the fluorescence of the wells using a microplate reader.

Procedure:

1. Assemble the Optimiser™ Plate, Optimiser™ Pad, and Optimiser™ Plate Holder.
    a. Place the Optimiser™ Plate Holder on the laboratory bench with the Optimiser™ logo facing the user.
    b. Note that the top and bottom surfaces of the absorbent pad differ from one-another. The top side has an absorbent surface whereas a thin plastic film covers the bottom side of the pad.
    c. Place the Optimiser™ Pad on the Optimiser™ Plate Holder with the bottom side of the pad facing down on the Optimiser™ Holder surface.
    d. With the absorbent side of the pad facing up, place the Optimiser™ plate on top of the pad.
    e. Carefully align the plate holder, pad, and plate and push the plate down firmly using thumbs and index fingers on the 4 plate corners until the plate snaps in place on the holder.

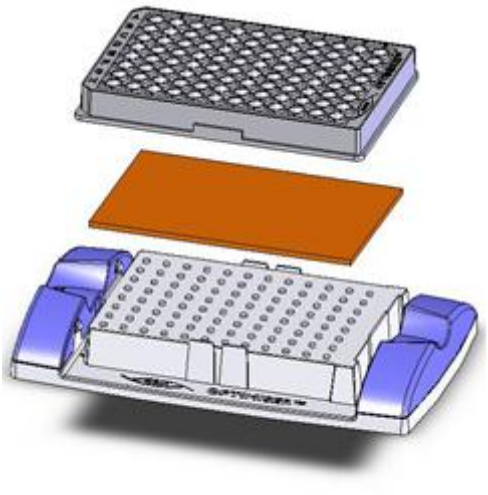


Figure 6. Proper Alignment of the Optimiser™ Holder, Optimiser™ Pad, and Optimiser™ Plate.

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™ plate 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. Then transfer the materials to the Optimiser™ wells using a **multi-channel pipettor capable of accurate and precise delivery of 5 and 10 µL volumes**. See Figure 7.
3. Dispense 5 µL capture antibody working solution to the required number of wells in the Optimiser™ plate. 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 rMs IFN-γ standards, controls, samples, 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 must be completed within the 15 minute substrate incubation time.

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.

In rare cases (<1%), 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.**

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.

14. Place the plate in the reading chamber of a fluorescence microplate reader. Promptly at the conclusion of the 15 minute incubation, read the plate.

Figure 7. **Illustrative example for ½ plate assay**

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Samp 1	Samp 9									
B	Std 2	Samp 2	Samp 10									
C	Std 3	Samp 3	Samp 11									
D	Std 4	Samp 4	Samp 12									
E	Std 5	Samp 5	Samp 13									
F	Std 6	Samp 6	Samp 14									
G	Std 7	Samp 7	Samp 15									
H	Blank	Samp 8	Samp 16									

↑ Polypropylene v-bottom plate containing diluted standards, samples, and blank.

← 5 µL of standard, sample, and blank are transferred from individual wells of polypropylene v-bottom plate to duplicate cells of Optimiser™.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1; 210 pg/ml		Sample #1		Sample #9							
B	Std 2; 105 pg/mL		Sample #2		Sample #10							
C	Std 3; 53 pg/mL		Sample #3		Sample #11							
D	Std 4; 26 pg/mL		Sample #4		Sample #12		Shaded cells not used in this assay					
E	Std 5; 13 pg/mL		Sample #5		Sample #13							
F	Std 6; 6.6 pg/mL		Sample # 6		Sample #14							
G	Std 7; 3.3 pg/mL		Sample #7		Sample #15							
H	Blank (0 pg/mL)		Sample #8		Sample #16							

↑ Optimiser™ plate to which standards, samples, and blank will be dispensed

√

Unused wells in a previously used plate can be used in a subsequent assay. Simply replace the used absorbent pad. The Optimiser™ design prevents movement of liquids between wells.

Calculations:

1. Calculate the mean background signal from the blank wells (wells containing Standard Diluent only at the sample incubation step).
2. Subtract the mean background signal from the signal of individual standard, sample, and control wells.
3. Create a standard curve by plotting the standard concentration (x-axis) vs the background-adjusted signal (y-axis). Draw a best fit curve through the points of the graph. A five parameter logistic curve fit with appropriate software is recommended.
4. Interpolate the Ms IFN-γ concentration of individual sample and control wells from the standard curve using the appropriate sample dilution factor as required.

5. **Note:** Sample concentrations should be derived by interpolation from within the standard curve range. Dilute samples if necessary so that sample signal falls within the range of the standard curve.
6. Calculate the mean concentration of each sample.

Typical Data:

Siloam Biosciences has validated the OptiMax™ Ms IFN-γ ELISA kit. Data acquisition and analysis utilized Gen5™ software, Excel, and Graphpad Prism®. A summary of the validation results follows.

Standard Curve:

The rMs IFN-γ standard curve ranges from **3.3 to 210 pg/mL**. Concentration (x-axis) and signal (y-axis) are plotted on Log scales. A typical standard curve is presented below.

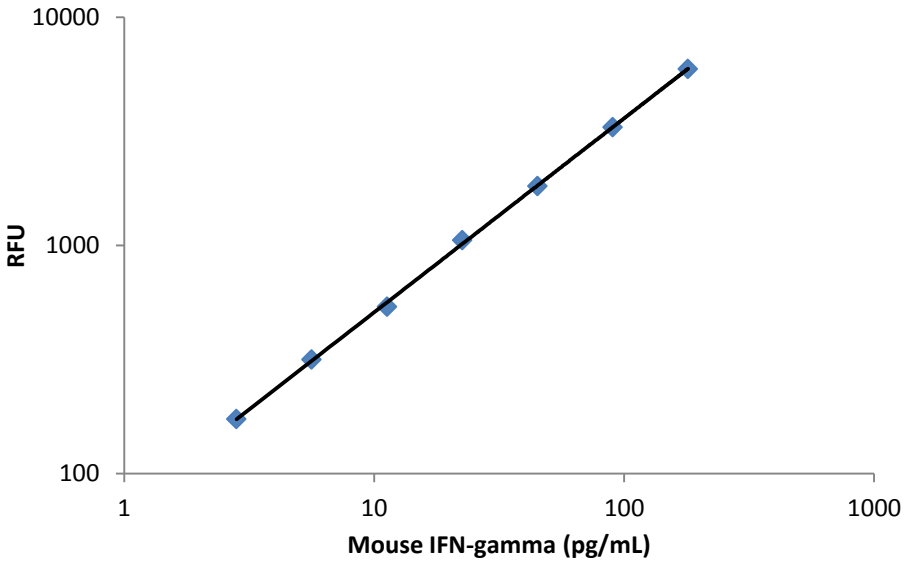


Figure 8. rMs IFN-γ Standard Curve with Tabulated Data

mouse IFN-γ (pg/mL)	RFU 1	RFU 2	Mean	Blank- subtracted
210	7366	7720	7543	7291
105	4187	4523	4355	4103
53	2232	2492	2362	2110
26	1536	1515	1525.5	1273
13	871	892	881.5	629
6.6	516	589	552.5	300
3.3	401	421	411	159
0	221	284	252.5	

Table 4. Tabulated RFU Data for Ms IFN-γ Standard Curve

This OptiMax™ ELISA kit has been calibrated against the R&D Systems Quantikine® Mouse IFN-gamma ELISA kit. Data generated using the OptiMax™ Mouse IFN-γ kit should closely correlate with that generated using the R&D Systems Quantikine® Mouse IFN-gamma ELISA kit.

Precision and Recovery:

Validation samples were prepared by spiking rMs IFN-γ into RPMI medium supplemented with 10% fetal bovine serum. Each sample was tested in 24 replicates in each of four independently performed assays. Both Intra and inter assay

precision were determined by calculating the mean concentration, standard deviation (SD), and percent coefficient of variation (%CV) for each of the samples.

The recovery of the OptiMax™ Moude IFN-γ ELISA assay was determined by comparing the concentration determined using the OptiMax™ ELISA kit with the known Ms IFN-γ concentration of the validation samples as follows:

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

Table 5. Intra-assay and Inter-assay Precision of OptiMax™ Mouse IFN-γ ELISA

	Intra-assay precision				**Inter-assay precision**		
Sample (pg/mL)	**158**	**78.8**	**39.4**		**158**	**78.8**	**39.4**
Mean of calculated concentration (pg/ml)	153	77.4	36.6		156.0	75.8	35.2
Standard deviation	11.30	6.80	3.41		6.2	5.0	1.2
CV (%)	7.4%	8.8%	9.3%		4.0%	6.7%	3.3%

The percent recovery ranged from 92% to 97% (mean = 95%).

Limit of Detection:

The Limit of Detection (LOD) [minimum detectable dose (MDD)] was determined by performing 20 replicates of Standard Diluent (blank) alone and calculating the mean signal + 2 standard deviations of the 20 values. The LOD is defined as the Ms IFN-γ concentration corresponding to the mean assay blank + 2 SD. The LOD was determined to be **< 0.8 pg/ml**.

Detection of Native Protein:

Six million mouse splenocytes were cultured in 3 mL of RPMI-1640 medium with the following supplements: 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. The splenocytes were stimulated with 10 µg/ml of Concanavalin-A, incubated at 37 °C, 5% CO_2 for 2 days. The cell culture supernatant was assayed for endogenous mouse IFN-γ using the OptiMax™ Mouse IFN-γ ELISA and the R&D Systems Quantikine® Mouse IFN-gamma ELISA with comparable results.

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 6.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.

Technical Assistance: If you require assistance, please contact Siloam Biosciences, Inc. Technical Support at 513-429-2976 or techsupport@siloambio.com.

References:

1. Interferon-gamma: an overview of signals, mechanisms and functions. Schroder K, Hertzog PJ, Ravasi T, Hume DA. J Leukoc Biol. 2004 Feb; 75 (2): 163-89. Epub 2003 Oct 2. Review. PMID: 14525967
2. Interferon-gamma: biologic functions and HCV therapy (type I/II) (1 of 2 parts). Gattoni A, Parlato A, Vangieri B, Bresciani M, Derna R. Clin Ter. 2006 Jul-Aug; 157(4): 377-86. Review. Retraction in: Clin Ter. 2008 May-Jun; 159 (3): 207. PMID: 17051976

APPENDIX 1

Alternative OptiMax™ ELISA Procedures

A 90 Minute OptiMax™ ELISA:

The standard OptiMax™ ELISA procedure, as described on page 12 of this User 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).

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

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 illustrates the adsorption kinetics of 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 the performance of the method. 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.

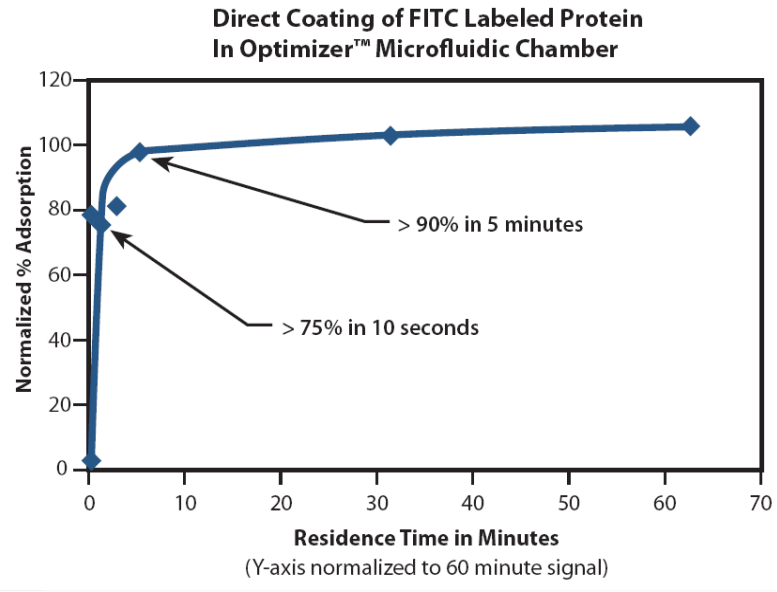


Figure 9. Adsorption characteristics of capture antibody on the Optimiser™ microchannel surface.

APPENDIX 1 (Continued)

An Ultrasensitive OptiMax™ ELISA Procedure:

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 the use of a robotic 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 in place of 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 robotic 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 approximately 20-fold.

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

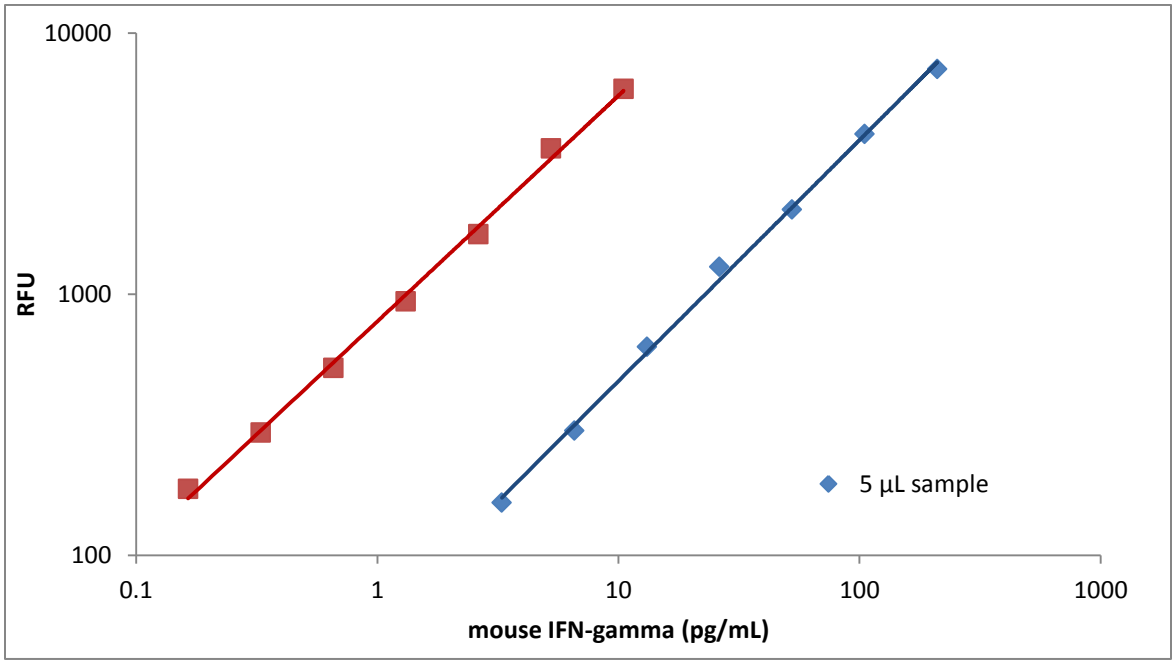


Figure 10. Ultra-sensitive assay using repeat sample loading technique with the OptiMax™ Mouse IFN-γ ELISA kit with a robotic sample processor.

Contact Siloam Biosciences for additional details.

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

- Material Safety Data Sheets (MSDS)
- 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™

All assay reagents for the OptiMax™ are provided by IMGENEX under agreement.
QuantaRed substrate is supplied by Thermo Fisher Scientific Inc.

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