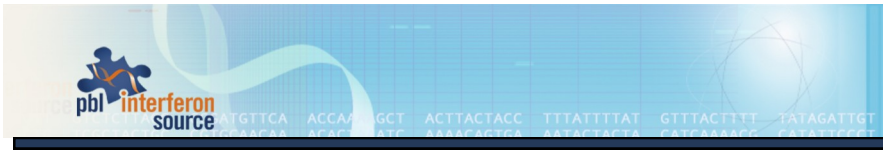
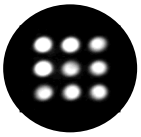




VeriPlex™ Human Interferon 9-plex ELISA Kit

Product # 51500-1

Store **all** components at 2-8°C

We recommend reading the protocol in its entirety prior to use. First time users must pay particular attention to pages 12-24 and read the manual of the Q-View™ imager and software available for download at www.quansysbio.com.

Table of Contents

Part	Title	Page
A	PRODUCT INFORMATION	3
B	PREPARATION OF REAGENTS	7
C	ASSAY PROCEDURE	8
D	ASSAY PROCEDURE-QUICK REFERENCE	11
E	IMAGING PROCEDURE	
1	-Q-View™ Imager-Acquiring	12
2	-Q-View™ Software-Importing an Image	14
3	-Alpha Innotech HD2 Camera	15
4	-Alpha Innotech FC2 Camera	16
5	-Bio-Rad VERSADOC 4000 Camera	18
6	-Bio-Rad CHEMIDOC XRS Camera	20
7	-FUJIFILM LAS-3000 Camera	22
F	PRODUCT PERFORMANCE CHARACTERIZATION	
1	Matrix Studies	25
2	Cross-Reactivity Studies	32
3	Additional	35
G	REFERENCES	36

A. PRODUCT INFORMATION

Specifications: This kit quantitates Human Interferon-Alpha (IFN-α), Human Interferon-Beta (IFN-β), Human Interferon-Gamma (IFN-γ), Human Interferon-Omega (IFN-ω), Human Interferon Lambda (IFN-λ1,2, and 3), Human Tumor Necrosis Factor-alpha (TNF-α), Human Interleukin-6 (IL-6), Human Interferon-Gamma inducible protein (IP-10), and Human Interleukin 1-alpha (IL-1α) in sera, plasma and tissue culture media by sandwich enzyme linked immunosorbent assay (ELISA) using the Q-Plex™ Multiplex technology.

Detection Ranges: Refer to the supplied lot specific Certificate of Analysis

Speed: Incubation time, 3 hr 15 min

Specificity: The IFN-λ antigen in the Standard is an equal mix of IFN-λ1 and λ2. The IFN-λ antibody pair in the product detects IFN-λ1,λ2, and λ3, although IFN-λ2 and 3 are detected less effectively.
Rhesus monkey IFN-α, Cynomolgus monkey IFN-α, and Cynomolgus monkey IFN-β are detected by the product. Mouse IFN-β, Mouse IFN-γ, and Mouse IFN–λ3 do not cross-react with the product. 10 ug/ml of Mouse IFN-α demonstrated 0.004 % cross-reactivity. Refer to pages 32-34 for details.

Storage Conditions/Comments: For retention of activity, all reagents should be kept at 2-8°C in the dark when not in use. Diluents and buffer reagents should be warmed to room temperature (RT) before use. We have not fully evaluated the long term stability of reconstituted materials in liquid or frozen form.

MATERIALS PROVIDED

- Human IFN Multiplex 96-Well Microtiter plate
- Plate Sealers
- Wash Solution Concentrate
- Human IFN Multiplex Antigen Standard
- Sample Diluent
- Assay Diluent
- Human IFN Multiplex Detection Mix
- HRP Concentrate
- Substrate A
- Substrate B
- Diluent Additive II
- Diluent Additive III

ADDITIONAL MATERIALS REQUIRED (*NOT PROVIDED)*

- Variable volume micro-titer pipettes
- Adjustable multi-channel pipette (50-300µl)
- Reagent reservoirs
- Wash bottle or plate washing system
- Distilled or deionized water
- Serological pipettes (1, 5, 10 or 25ml)
- Disposable pipette tips (polypropylene)
- Plate shaker
- One of the following cameras/imagers:
    - Q-View™ Imager (recommended)
    - Alpha Innotech HD2 and FC2 Camera
    - Bio-Rad VERSADOC 4000 Camera
    - Bio-Rad CHEMIDOC XRS Camera
    - Fujifilm LAS 3000 Camera
    - KODAK 4000MM Camera
- Q-View™ software Version 2.0

INTRODUCTION

Interferons (IFNs) are a group of cytokines which exhibit pleiotropic activities that play major roles in both innate and adaptive immunity. There are three types of interferons, namely type I, II, and III. Type I IFNs consist of multiple Interferon-Alpha (IFN-α) genes, at least one Interferon-Beta (IFN-β) gene, and one Interferon-Omega (IFN-ω) gene in most vertebrates.[1] IFN-α, IFN-β, and IFN-ω are released by a host of mammalian cells on exposure to viruses or double-stranded RNAs,[2] and on triggering of Toll-like receptors (TLR3/4/7/8/9) by CPG DNAs and lipopolysaccharide (LPS). Upon binding to their cellular receptor chains IFN-α-Rc1 and IFN-α-Rc2, type I interferons signal through the Jak-Stat pathway to further elicit a host of anti-viral actions including production of protein kinase A and 2'5' Oligoadenylate Sythetase (OAS).[2] Type I interferons are used therapeutically to treat viral infections, cancers, and auto-immune disorders. IFN-α is used therapeutically to treat hepatitis B and hepatitis C infections. Additionally, IFN-α is known to have significant biological activity in inhibition of proliferation of multiple cancers.[3] IFN-β is used therapeutically to treat multiple sclerosis.[4] Type II Interferon consists of Interferon-gamma (IFN-γ) IFN-γ is produced by a host of immune cells-lymphocytes, CD4+ T cells, NK cells, and such antigen presenting cells (APCs) as macrophages, monocytes, and dendritic cells. [5] IFN-γ uses receptor chains IFN-γ-R1 and IFN-γ-R2. IFN -γ, a homodimer, binds two IFN-γ-R1 sub-units, thereby generating binding sites for two IFN-γ-R2 chains, a process that subsequently triggers intra-cellular signaling and activation of Jak1, Jak2, and Stat1 that in turn induce genes with the γ activation sequence in the promoter. [2, 6] IFN-γ plays a role in several immunomodulatory functions including up-regulation of pathogen recognition, anti-viral action, activation of microbicidal functions in immune cells, and leukocyte trafficking.[5] The newly characterized type III interferons consist of Interferon-lambda1 (IFN-λ1 or IL-29), Interferon-lambda2 (IFN-λ2

or IL-28A), and Interferon-lambda3 (IFN-λ3 or IL-28B). The members of Type III family share close homology to one another with IFN-λ2 and IFN-λ3 sharing 96 % amino acid identity and with IFN-λ1 sharing 81% homology with IFN-λ2 and IFN-λ3. [7] Type III interferons are functionally similar to type I interferons and are known to have similar downstream effects i.e. type III interferons promote the phosphorylation on STAT1 and STAT2, induce the ISRE3 complex, elevate OAS and type I IFN inducible Myxovirus resistance protein A (MxA) expression and exhibit anti-viral activity *in vitro* [10]; however, type III interferons and their heterodimeric receptor subunits-CRF2-12(IFN-λR1)[9] and CRF2-4 (IL-10R2)[9] are known to be more prominent in cells of the epithelial tissues.[8]

IFN-λ1 is known to modulate the development of Th1/Th2 cells.[10] IFN-λ2 has been shown to inhibit the replication of hepatitis B and hepatitis C virus in murine hepatocyte cell lines,[11] and IFN-λ1 is being explored as a potential therapeutic for hepatitis C. [12]

The *VeriPlex™* Human Interferon 9-plex ELISA has been developed to simultaneously detect IFN-α, IFN-β, IFN-γ, IFN-ω, IFN-λ1/2/3, and other key pro-inflammatory cytokines released upstream and downstream of interferon signaling, including TNF-α, IP-10, IL-1α, and IL-6. This assay has been developed using the Q-Plex™ array spotting technology, in which capture antibodies to the different analytes are spotted in a single well in a 3x3 array. The functional format of the assay is as that of a sandwich ELISA with a chemiluminiscent output. The assay is compatible with multiple matrices including tissue culture media, human serum, human plasma, and buffers.

B. PREPARATION OF REAGENTS

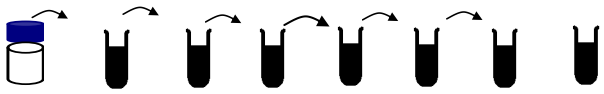
Supplied Human IFN Multiplex Antigen Standard, Human IFN Multiplex

Detection Mix, and HRP Concentrate should be kept on wet ice.

Wash Solution: Prepare a 1:20 working wash solution. Add 50ml of the Wash Solution Concentrate to 950ml of distilled or deionized water, mix thoroughly. The working Wash Solution can be stored at (2-25°C) when not in use.

Standard: Reconstitute the supplied Human IFN Multiplex Antigen Standard by adding the volume of Sample Diluent indicated in the lot specific Certificate of Analysis. Mix gently until the Antigen Mix is completely reconstituted and store on wet ice until use. Do not vortex. Do not introduce bubbles.

Standard Curve Preparation:
Label 7 polypropylene tubes as S2-S7. Prepare a 3 fold dilution series using the reconstituted Antigen Standard and Sample Diluent as per figure 1 below. Mix thoroughly between each dilution by pipeting 5X. The high point (S1) in the series is the reconstituted Antigen Standard.



Recon. Antigen STD.(S1)	Blank	S2	S3	S4	S5	S6	S7
ml		0.12 ml	0.12 ml	0.12 ml	0.12 ml	0.12 ml	0.12 ml	0.12

Volume of Sample Diluent

Figure 1: Standard curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
B	S2	S2	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
C	S3	S3	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
D	S4	S4	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
E	S5	S5	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
F	S6	S6	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
G	S7	S7	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa
H	BK	BK	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa	Sa

B K= Blank;

S1-S7 = Standard Curve;

Sa = Test Samples

Figure 2: Model Plate Setup

Step 1B: Adding Standards, Blanks, and Test Samples

Add 50µl of Standards to wells designated as Standard

Add 50µl of Blanks (Sample Diluent) to wells designated as Blanks

Add 50µl Test samples to wells designated as Test Samples

After 2 hours, empty the contents of the plate and wash the wells 3 times with 300 µl of the working Wash Solution (refer to Preparation of Reagents) per well.

2. **Detection Antibody:** Add 50µl of reconstituted Detection Mix (refer to Preparation of Reagents) to each well. Cover with Plate Sealer and shake plate at RT (22- 25°C) for 1 hour.

   During the incubation, prepare a Substrate Mix by mixing equal volumes of Substrate A and Substrate B. Use full contents of Substrate A and Substrate B. Store the mix at RT (22-25°C), in the dark, until use in Step 4. The mix must be prepared at least 10 minutes prior to use in Step 4.

After 1 hour, empty the contents of the plate and wash the wells 3 times with 300 µl working Wash Solution per well.

3. **Streptavidin-HRP**: Add 50µl of supplied Streptavidin-HRP to each well. Cover with plate sealer and shake, in the dark, at RT (22-25°C) for 15 minutes.

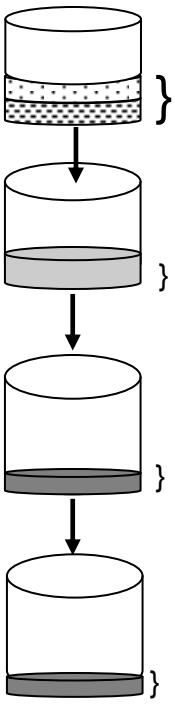
After 15 minutes, empty the contents of the plate and wash the wells 6 times with 300 µl working Wash Solution per well.

4. **Substrate Mix and Imaging:** Add 50 µl of the prepared Substrate Mix to each well.

Image the plate within 20 minutes of adding the Substrate Mix. Refer to page 12 onwards for detailed instructions on imaging the plate using different imagers.

D. ASSAY PROCEDURE—QUICK REFERENCE

Total time: 3 hours and 15 minutes



1) Add 50µl Assay Diluent
2) Add 50µl Test Sample, Standard, or Blank

Incubate 2 hours with shaking (medium to fast)
Aspirate and wash 3 x

Add 50 µl reconstituted Detection Antibody

Incubate 1 hour with shaking (medium to fast)
Aspirate and wash 3 x

Add 50 µl Streptavidin-HRP with shaking (medium to fast)

Incubate 15 minutes in the dark
Aspirate and wash 6 x

Add 50µl Mix of Substrate A and Substrate B
Image plate within 20 minutes

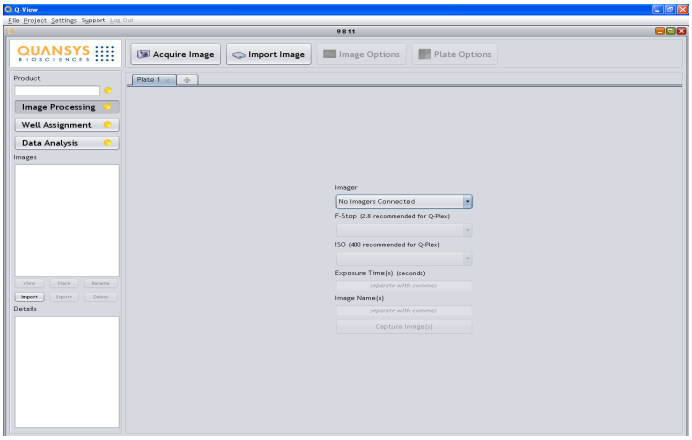
E. IMAGING PROCEDURE

1. Quansys Q-View™ Imager-Acquiring an Image

These are basic instructions for using the Q-View™ Imager and Software to image your plate only. A comprehensive software manual for use of Q-View™ software is available.

A full version of the Quansys Q-View™ Software Version 2.07 is available for free. The download is available at: http://www.quansysbio.com/pbl-q-view-software.
The user manual for the software can be found under Support → Help.

A. Select New Project if starting a new project. Otherwise, select Open Project to browse and select a previous project. The new image will not overwrite prior images in the project.
B. Ensure that the Q-View™ Imager is connected to the computer.
C. Optional. Uncheck the box "Discard sub-images after stacking is complete" under Preferences under Settings to see images of different exposure times after the imaging process in complete. Otherwise, a stacked image will be displayed.
D. Optional. It is recommended to periodically calibrate the Q-View™ Imager. To calibrate the imager, select Manage Imagers under Administration under Settings. Ensure that there is no plate in the plate housing slot in the imager. Select Calibrate.
E. Optional. If the imager has not been focused previously, place the mouse pad in the imager (do not close the plate housing door) and adjust the focus (under Manage Imagers under Administration under Settings) until the letters on the mouse pad are in focus. Remove the mouse pad. Close the Manage Imagers section.



F. Select Acquire Image.
G. Ensure that the Q-View™ Imager is recognized. If not, click on Refresh.
H. Type the following settings in the ISO, and F-Stop fields:
ISO: 400, F-Stop: 2.8
I. Recommended exposure times are 30, 60, and 180 seconds. Each exposure will have a different image. The software will also display a stacked image.
J. Enter the names of the image (s). For example, Expt1 30 sec, Expt 1, 60 sec, and Expt 1 180 sec.
K. Place the plate in the plate housing slot, close the plate housing door, and select Capture Image. The imaging should begin. Once acquired, the image will appear in the Q-View™ Software main screen.
L. Save the acquired image (s) by clicking Image Analysis → Export Image. Export the image (s) as TIFF file(s).

2. Q-View™ Software-Importing an Image File

Q-View™ Software can process images in the following formats: CR2 (raw image files from Canon cameras), TIFF, JPEG, PNG and BMP. Users should take images using supported imaging systems (See Page 4).
To acquire an image by importing an image file, select Import Image. Browse and select the image.
When prompted, select the imager used to acquire the image. If you imager is not listed, select Other. Also select Other, if the image was acquired using the Q-View Imager to override repeated vignette correction.
The time to upload the image will vary depending on the image file type and size. Once imported, the image will appear in the Q-View Software main screen.

Images acquired using the following imagers can be imported into the Q-View™ software for analysis. Refer to page 14 for details on importing images to Q-View™ software.

3. Acquiring an image using the Alpha Innotech **HD2** Camera and Software Setup

A. Open the camera door.
B. Set the adjustable tray on the lowest level.
C. Place the place the mouse pad in the center of the tray for focusing.
D. Open the aperture on the camera all the way to the lowest value (~.95).
E. Open the software on the computer.
F. Click the acquire button.
G. Close the door on the camera slightly so some light can get in and the letters on the mouse pad can be seen on the computer screen.
H. Adjust the focus on the actual camera lens until the letters on the mouse pad are in focus.
I. Remove the mouse pad.
J. Place the plate in the center of the tray and make sure it is in the center of the photo path (on the computer screen).
K. Close all doors on the camera and ensure there are no light leaks.
L. Ensure all cabinet lights are off and that the filter wheel is set to position "1".

M. Set the software settings on the computer as follows:
  i. No lights on.
  ii. Resolution to “Normal/Ultra”.
  iii. Select only “noise reduction”
  iv. Set the exposure time to 3 min for the first time.
N. Click on Acquire Image.
O. Once the image is acquired, save it and look at the pixel intensity of the high points on the standard curve. On average, most of the high points on the curve should be in the 45,000 – 55,000 pixel intensity range, and on the second spot they should be in the 20,000 – 40,000 pixel intensity range.
  i. If there are spots where the PI (pixel intensity) on the high point of the curve is at 65,000 and the second point is 60,000 or higher, then acquire another image of the plate for a shorter period of time.
  ii. If the spots on the high point of the curve in general fall below 40,000 PI, then reacquire the image for a longer period of time.

4. Acquiring an image using the Alpha Innotech FC2 Camera and Software Setup

A. Open the camera door.
B. Set the adjustable tray on the top shelf.
C. Place the mouse pad in the center of the tray for focusing.
D. Open the aperture on the camera all the way to the lowest value (~1.8).
E. Open the software on the computer.
F. Click the Acquire button.

G. Close the door on the camera slightly so some light can get in and the letters on the mouse pad can be seen on the computer screen.
H. Adjust the focus on the actual camera lens until the letters on the mouse pad are in focus.
I. Remove the mouse pad.
J. Place the plate in the center of the tray and make sure it is in the center of the photo path (on the computer screen).
K. Close all doors on the camera and ensure there are no light leaks.
L. Ensure all cabinet lights are turned off and that the filter wheel is set to "1".
M. Set the software settings on the computer.
  i. No lights on.
  ii. Resolution to "Normal/Ultra".
  iii. Select only "noise reduction",
  iv. Set the exposure time to 6 min for the first time.
N. Click on Acquire Image.
O. Once the image is acquired, save it and look at the pixel intensity of the high points on the standard curve. On average, most of the high points on the curve should be in the 45,000 – 50,000 pixel intensity range, and on the second spot they should be in the 20,000 – 40,000 pixel intensity range.
  i. If there are spots where the PI (pixel intensity) on the high point of the curve is at 65,000 and the second point is 60,000 or higher, then acquire another image of the plate for a shorter period of time.
  ii. If the spots on the high point of the curve in general fall below 40,000 PI, then reacquire the image for a longer period of time.

5. Acquiring an image using the Bio-Rad VERSADOC 4000 Camera and Software Setup

A. File setup

a. Open the software on the computer.

b. Click on “File”, then select “Versadoc”.

c. Make sure that only “Channel 1” is enabled.

d. Click on the “Select” button, then select “Custom” and then “Create”.

e. When the new window opens, name this custom setup as “Quansys 1X1 binning”, and change the settings to:

i. Filter: None

ii. Illumination: None

iii. Gain: 1X

iv. Binning: 1X1

f. Click on “OK”. (Now that this setting is saved you can use it again for future exposures. Instead of selecting “Create”, select “Quansys 1X1 binning”.)

B. Camera Setup and Focus

a. Open the imager door.

b. Place a box or stand in the cabinet below the camera to increase the imaging height between 4 and 6 inches.

c. Place the “imaging target sheet” or the mouse pad in the cabinet on top of the box or stand.

d. Open the aperture on the camera all the way to the lowest value.

e. Leave the door slightly open to let in light while focusing.

f. Click “Focus” in the software, and turn the focus on the camera until the imaging targets on the screen on the computer are in focus or the letters on the mouse pad are in focus. Click “Stop” when in focus.

g. Replace the “imaging target sheet”/mouse pad with the plate, and ensure the plate is centered in the imaging screen and is straight.

h. Close the cabinet door.

C. Image Acquisition

a.On “Step III – Set exposure time” on the software, change the exposure time to 30 seconds and select Acquire.

b. When the exposure is complete, convert the image into negative (black background with white spots).

i. Click Image, and new menu appears.

ii. Select Transform, and then check the box that says “Invert Display”.

iii. Click OK.

c. Save the image.

D. Image Optimization

a. Once the image is acquired, look at the pixel intensity of the high points on the second dilution in the standard curve, and make sure they are not saturated. On average, most of the high points on the curve should be in the 45,000 – 55,000 Pixel Intensity range, and on the second spot they should be in the 20,000 – 40,000 pixel Intensity range.

i. If there are spots where the PI (pixel intensity) on the high point of the curve is at 65,000 and the second point is 60,000 or higher, then re-expose image of the plate for a shorter period of time (1 minute).

ii. If the spots on the high point of the curve in general fall below 40,000 PI, then re-expose the image for a longer period of time (i.e. 3 minutes).

E. File Conversion

a. After acquiring the images, you need to convert them to TIFF files.

b. Click on “File” then “Export to Tiff image”.

c. Select “Export raw data”, click on “Export”, then click on “Save”.

6. Acquiring an image using the Bio-Rad CHEMIDOC XRS Camera and Software Setup

A. File setup

a. Open the software on the computer.

b. Click on “File”, then select “ChemiDoc XRS”.

c. Under “Step I – Select Application” press the “Select” button, then select “Custom” and then “Create”.

d. When the new window opens, name this custom setup as “Quansys 1X1 binning”. Under “Illumination” select“ none”, and under “gain &binning” select “2X, 1X1” and click “OK”. (Now that this setting is saved you can use it again for future exposures. Instead of selecting “Create”, select “Quansys 1X1 binning”.)

B. Camera Setup and Focus

a. On the software select “Live Focus”.

b. On the cabinet press the plus button to open the aperture or iris all the way (the lowest number).

c. Open the camera's drawer, place the plate in the middle of the drawer, then close the drawer.

d. Press the "Epi White" button on the camera cabinet.

e Press the zoom buttons on the cabinet until the plate fills most of the screen on the computer.

f. Replace the plate with the "imaging target sheet" or the mouse pad and close the door.

g. Press the focus buttons on the cabinet until the targets or letters on the mouse pad are in focus on the computer screen.

h. Replace the imaging target sheet/mouse pad with the plate, close the drawer, and make sure the plate is in the center of the imaging screen and straight.

i. Turn off the "Epi White" button on the cabinet, and select "freeze" in the software.

C. Image Acquisition

a.On "Step III – Acquire Image" on the software, change the exposure time to 30 seconds and select Manual Expose.

b. When the exposure is complete, convert the image into negative (black background with white spots).

i. Click Image, and new menu appears.

ii. Select Transform, and then check the box that says "Invert Display".

iii. Click OK.

c. Save the image.

D. Image Optimization

a. Once the image is acquired, look at the pixel intensity of the high points on the standard curve. On average, most of the high points on

the curve should be in the 45,000 – 55,000 Pixel Intensity range, and on the second spot they should be in the 20,000 – 40,000 pixel Intensity range.

i. If there are spots where the PI (pixel intensity) on the high point of the curve is at 65,000 and the second point is 60,000 or higher, then re-expose image of the plate for a shorter period of time (i.e. 1 minute).

ii. If the spots on the high point of the curve in general fall below 40,000 PI, then re-expose the image for a longer period of time (i.e. 3 minutes).

E. File Conversion

a. After acquiring the images, you need to convert them to TIFF files.

b. Click on "File" then "Export to Tiff image".

c. Select "Export raw data", click on "Export", then click on "Save".

7. Acquiring an image using the Fujifilm LAS-3000 Camera and Software Setup

A. File setup

a. Open the software on the computer.

b. Under "Exposure Type" select "Precision" in the drop down menu.

c. Under "Exposure Time" set the imager to take a 30-second image by selecting "Manual" then entering "30" in the first box and selecting "sec" in the second box.

d. Under "Sensitivity" select "Standard" in the drop down menu.

e. Ensure the box next to "Image Acquire &Digitize" is checked.

f. Click on the “Method/Tray Position” button. In the window that appears, select “Chemiluminescence”, and under tray position select “2”. Then select “OK” to close the window.

B. Camera Setup and Focus

a. Open the camera box and make sure the tray is in position “2”.

b. Place the mouse pad on the tray or other imaging target sheet and close the door.

c. On the software select “Focus” and a new window appears. In the “Adjust” area, click up or down on the arrows until the letters on the mouse pad are in focus.

d. Remove the mouse pad from the imager and place the plate in the center of the tray. Look on the computer screen to make sure the plate is centered and straight in the imaging screen. Close the imager door when the plate is centered.

e. Select the “Return” button on the software to close the focusing window.

C. Image Acquisition

a. When the plate is ready to image, press the “Start” button.

b. After the plate has imaged invert the image to black with white spots by clicking on “View” then selecting “Positive Gray” in the drop down menu.

c. Save the image by pressing the “Save” button. In the new window select “16 bit linear tiff” in the “Save as type” drop down menu. Then type a name for the file and select “Save”.

d. Press the “Complete” button to allow the imager to take another image.

e. Take multiple images at different exposure times to ensure you get the best reading possible. Example exposure times are 20 seconds, 45 seconds, 60 seconds, 90 seconds, and 120 seconds.

D. Image Optimization

a. Once the image is acquired, look at the pixel intensity of the high points on the standard curve. On average, most of the high points on the curve should be in the 45,000 – 55,000 Pixel Intensity range, and on the second spot they should be in the 20,000 – 40,000 pixel Intensity range.

i. If there are spots where the PI (pixel intensity) on the high point of the curve is at 65,000 and the second point is 60,000 or higher, then re expose image of the plate for a shorter period of time (i.e. 20 seconds).

ii. If the spots on the high point of the curve in general fall below 40,000 PI, then re-expose the image for a longer period of time (i.e. 2 minutes).

F. PRODUCT PERFORMANCE CHARACTERIZATION

1. Matrix studies:

i. Levels of analytes in Normal human serum and Normal human plasma

Normal human serum from 20 individual donors and Normal human plasma with different anti-coagulants (Na-EDTA, Na-Citrate, and Na-Heparin) from 13 other individual donors were tested in the assay. The levels of analytes in the samples were extrapolated from a Standard Curve prepared in Sample Diluent.

Serum and plasma from all donors had detectable levels of IP-10. The average concentration of IP-10 in serum was 55.6 pg/ml, while in plasma was 86.9 pg/ml.

Serum from one donor had 16.0 pg/ml of IFN-α, serum from a second donor had 6.4 pg/ml of IL-1 α and 40 pg/ml of IL-6, serum from a third donor had 9.1 pg/ml of IL-1 α, 1935.2 pg/ml of IL-6, and 27.0 pg/ml of TNF-α, and serum from a fourth donor had 43.4 pg/ml of IL-6.
Serum from remaining 16 donors had undetectable levels of analytes other than IP-10.

Plasma from all 13 donors had undetectable levels of analytes other than IP-10.

	Normal human serum (20 donors)		Normal human plasma (13 donors)	
Analyte	Average (pg/ml)	Range (pg/ml)	Average (pg/ml)	Range (pg/ml)
IFN-α	<LLOD	<LLOD to 16.0	<LLOD	N/A
IFN-β	<LLOD	N/A	<LLOD	N/A
IFN-γ	<LLOD	N/A	<LLOD	N/A
IFN-λ 1/2	<LLOD	N/A	<LLOD	N/A
IFN-ω	<LLOD	N/A	<LLOD	N/A
IL-1α	<LLOD	<LLOD to 9.1	<LLOD	N/A
IL-6	102.7 *	<LLOD to 1935.2	<LLOD	N/A
IP-10	55.6	9.14 to 130.3	86.9	21.3 to 182.2
TNF-α	<LLOD	<LLOD to 27.0	<LLOD	N/A

* Average value of IL-6 in normal human serum was high due to presence of 1935.2 pg/ml of IL-6 in serum from a particular donor. Levels in serum from 17 donors were either close to LLOD or < LLOD.

N/A-Not applicable because levels in serum or in plasma from all donors were <LLOD.

ii. Spike Recovery

Low, medium, and high spikes were prepared using the Multiplex Antigen Standard in Normal human serum from a single donor, Normal human plasma with different anti-coagulants, TCM-DMEM+10% FBS, and Sample Diluent. The concentration of spikes were extrapolated from a Standard Curve prepared in Sample Diluent. The recoveries in Normal human serum and in Normal human plasmas were calculated after subtracting measured levels of endogenous analytes, in the matrices, from the recovered values.

a. High Spike

Analyte	Sample Diluent	DMEM+10 %FBS	Normal human serum (donor A)	Normal human plasma with Na-EDTA (donor B)	Normal human plasma with Na-EDTA (donor C)	Normal human plasma with Na-Heparin (donor C)	Normal human plasma with Na-Citrate (donor D)
IFN-α	82.8%	105.3%	91.6%	38.0%	42.7%	114.4%	86.5%
IFN-β	79.6%	77.8%	125.8%	87.2%	110.0%	108.1%	102.7%
IFN-γ	113.8%	88.0%	98.8%	95.5%	92.5%	100.7%	92.7%
IFN-λ 1/2	96.5%	95.5%	94.0%	89.9%	94.0%	101.3%	83.3%
IFN-ω	82.9%	77.3%	82.5%	76.8%	104.8%	104.2%	86.7%
IL-1α *	92.4%	85.0%	76.9%	0.1%	79.7%	71.6%	63.2%
IL-6	81.8%	75.6%	69.1%	50.0%	77.1%	79.1%	68.4%
IP-10	87.2%	77.7%	83.8%	73.5%	64.6%	83.6%	75.0%
TNF-α	91.4%	72.6%	81.3%	79.2%	87.0%	88.5%	60.6%

b. Medium Spike

Analyte	Sample Diluent	DMEM+10 %FBS	Normal human serum (donor A)	Normal human plasma with Na-EDTA (donor B)	Normal human plasma with Na-EDTA (donor C)	Normal human plasma with Na-Heparin (donor C)	Normal human plasma with Na-Citrate (donor D)
IFN-α	100.0%	121.8%	107.1%	33.0%	43.0%	106.5%	73.3%
IFN-β	82.6%	97.9%	138.4%	103.3%	98.7%	100.2%	98.1%
IFN-γ	125.5%	97.5%	110.6%	104.9%	89.4%	94.4%	77.3%
IFN-λ 1/2	114.9%	118.0%	115.5%	104.2%	84.2%	87.7%	72.6%
IFN-ω	97.2%	87.5%	101.1%	95.7%	105.8%	109.6%	101.4%
IL-1α *	102.3%	89.9%	80.6%	0.0%	59.5%	55.6%	47.9%
IL-6	98.2%	84.5%	79.1%	57.2%	75.8%	71.7%	61.3%
IP-10	97.7%	81.0%	96.1%	77.4%	63.4%	75.6%	50.8%
TNF-α	99.3%	77.5%	86.2%	77.8%	85.1%	88.2%	57.1%

c. Low Spike

Analyte	Sample Diluent	DMEM+10 %FBS	Normal human serum (donor A)	Normal human plasma with Na-EDTA (donor B)	Normal human plasma with Na-EDTA (donor C)	Normal human plasma with Na-Heparin (donor C)	Normal human plasma with Na-Citrate (donor D)
IFN-α	91.5%	124.2%	109.0%	23.9%	35.5%	91.2%	81.0%
IFN-β	69.8%	94.2%	161.8%	111.3%	95.2%	87.4%	98.3%
IFN-γ	133.9%	85.1%	113.3%	108.4%	44.7%	72.7%	63.1%
IFN-λ 1/2	93.2%	94.2%	75.5%	91.1%	47.4%	56.1%	54.4%
IFN-ω	90.8%	82.1%	106.0%	89.2%	62.1%	78.7%	79.5%
IL-1α *	102.2%	73.2%	64.1%	0.0%	25.3%	20.6%	9.0%
IL-6	95.4%	82.0%	78.4%	67.3%	65.0%	54.2%	49.9%
IP-10 ++	117.2%	67.8%	113.9%	79.6%	-16.5%	-9.4%	-70.8%
TNF-α	102.1%	69.4%	86.0%	75.6%	66.9%	60.9%	41.9%

* The recovery of high, medium, and low IL-1α spikes in normal human plasma with Na-EDTA from donor B was particularly poor.

++ Poor recoveries of low IP-10 spikes in plasma are due to presence of ~110 pg/ml of apparent endogenous IP-10 in the plasma lots.

iii. Intra-assay and Inter-assay %CV

a. Intra-assay %CV

Analyte	Sample Diluent	Normal human serum (donor A)	DMEM+10 %FBS	Normal human plasma with Na-EDTA (donor B)
IFN-α	4.3%	3.91	4.6%	7.0%
IFN-β	4.3%	8.2%	3.8%	8.0%
IFN-γ	6.3%	3.8%	6.5%	6.5%
IFN-λ 1/2	7.0%	7.7%	11.4%	9.2%
IFN-ω	4.6%	7.0%	5.5%	6.5%
IL-1α	4.9%	6.3%	5.6%	15.7%
IL-6	7.2%	5.1%	7.4%	2.1%
IP-10	3.4%	8.5%	3.0%	3.3%
TNF-α	4.4%	4.3%	4.6%	3.9%

b. Inter-assay %CV

Analyte	Sample Diluent	Normal human serum (donor A)	DMEM+10% FBS	Normal human plasma with Na-EDTA (donor B)
IFN-α	8.6%	3.8%	7.6%	15.5%
IFN-β	9.8%	13.9%	12.1%	12.6%
IFN-γ	8.9%	8.9%	14.3%	15.6%
IFN-λ 1/2	14.1%	12.4%	20.2%	23.9%
IFN-ω	6.2%	7.3%	11.0%	8.0%
IL-1α	9.2%	9.8%	14.9%	ND
IL-6	12.8%	19.0%	19.8%	25.1%
IP-10	8.4%	10.0%	13.9%	21.5%
TNF-α	13.2%	12.4%	12.9%	12.7%

2. Cross-Reactivity Studies

i. IFN-λ subtypes:

Independant curves of recombinant human IFN-λ1, recombinant human IFN-λ2, and recombinant human IFN-λ3 were prepared in Sample Diluent. A separate standard curve was prepared using the human IFN Multiplex Antigen Standard supplied in the product. The % recovery of those points with pixel intensitites withtin the range of pixel intensities of IFN-λ1/2 in the multiplex standard were averaged to estimate the % Reactivity for each subtype.

Subtypes	% Reactivity
IFN-λ1	167.4%
IFN-λ2	39.5%
IFN-λ3	41.7%

ii. Human IFN-α subtypes, Rhesus Monkey IFN-α, Cynomolgus monkey IFN-α (ILe16), and Cynomolgus monkey IFN-β

Independent curves of recombinant analytes listed in the table below were prepared in Sample Diluent. A separate standard curve was prepared using the Human IFN Multiplex Antigen Standard supplied in the product. The % recovery of those points on the curves of the test analytes with pixel intensities within the range of pixel intensities of IFN-α A 2a in the curve prepared using the Multiplex Antigen Standard were averaged to estimate the % Reactivity for each analyte.

Catalog Number	Analyte	% Reactivity
11105-1	Human IFN-α 2 (α2b)	66.7%
11100-1	Human IFN-α A (α2a)	55.7%
11175-1	Human IFN-α 1 {α D (Ala 114)}	30.6%
11165-1	Human IFN-α K (α6)	20.0%
11120-1	Human IFN-α C (α10)	15.6%
11160-1	Human IFN-α J1 (α7)	14.2%
11180-1	Human IFN-α 4b (α4)	10.7%
11135-1	Human IFN-α G (α5)	3.7%
11145-1	Human IFN-α H2 (α14)	0.3%
11130-1	Human IFN-α F (α21)	0.1%
11190-1	Human IFN-α WA (α16)	0.1%
11150-1	Human IFN-α I (α17)	0.1%
11115-1	Human IFN-α B2 (α8)	0.0%
14110-1	Rhesus Monkey IFN-α	10.6%
16100-1	Cynomolgus Monkey IFN-α A Ile16	6.0%

2.3 ng/ml of Cynomolgus IFN-β was measured in tissue culture supernatant of a mammalian cell line expressing Cynomolgus IFN-β. No other antigen was detected in the supernatant.

iii. Mouse IFN-α A, Mouse IFN-β,Mouse IFN-γ, and Mouse IFN-λ3

Independent curves, starting at 10 μg/ml, of recombinant analytes listed below were prepared in Sample Diluent. A separate standard curve was prepared using the Human IFN Multiplex Antigen Standard supplied in the product. The % recovery of those points with pixel intensities within the range of pixel intensities of corresponding human analytes in the curve prepared using the Human IFN Multiplex Antigen Standard were averaged to estimate the % cross reactivity for each analyte.

Catalog Number	Analyte	% Cross-reactivity
12100-1	Murine IFN-α A	0.004%
12400-1	Murine IFN-β	0.0%
12500-1	Murine IFN-γ	0.0%
12820-1	Murine IFN-λ3	0.0%

3. Additional Studies

Serum samples from 27 Rheumatoid Arthritis (RA) patients' were tested. Only 1 sample gave false positive. The spike recovery was acceptable.

20 human plasma samples with constituents known to interfere in immuno-assays were tested. No sample gave false positive.

Please note that detection of analytes in serum and plasma from patients on certain therapeutics can be affected due to the presence of antibodies against analytes from the multiplex in such samples.

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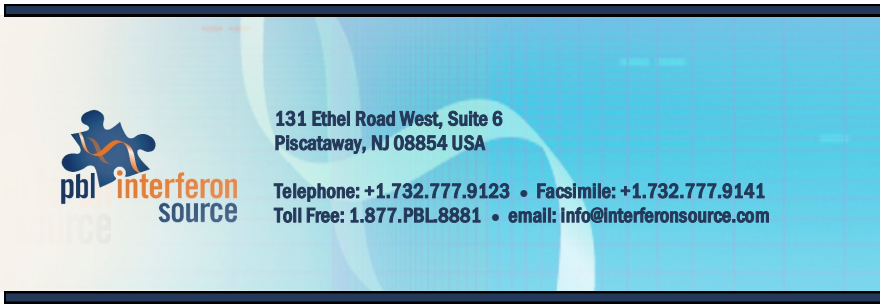
pbl interferon source
131 Ethel Road West, Suite 6
Piscataway, NJ 08854 USA
Telephone: +1.732.777.9123 • Facsimile: +1.732.777.9141
Toll Free: 1.877.PBL.8881 • email: info@interferonsource.com