pbl assay science

VeriPlex™ Mouse Cytokine 9-Plex ELISA Kit

Catalog No. 52500

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 12-16 and read the manual of the Q-View™ imager and software available for download at www.quansysbio.com.

TABLE OF CONTENTS

52500 Rev. 00

A. 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 and at least one Interferon Beta (IFN-β) gene. Other Type I IFNs in the mouse include Limitin and Epsilon.[1] IFN-α and IFN-β 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 oligodeoxynucleotides and lipopolysaccharides (LPS). Upon binding to their cellular receptor chains IFNAR1 and IFNAR2, Type I IFNs signal through the Jak-Stat pathway to further elicit a host of antiviral actions including production of protein kinase A and 2'5' Oligoadenylate Synthetase (OAS).[2] Type I IFNs are used therapeutically to treat viral infections, cancers and autoimmune 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 IFN consists of Interferon Gamma (IFN-γ). IFN-γ is produced by a host of immune cell lymphocytes, CD4+ T cells, NK cells, and such antigen presenting cells (APCs) as macrophages, monocytes, and dendritic cells.[5] IFN-γ uses receptor chains IFNGR1 and IFNGR2. IFN-γ, a homodimer, binds two IFNGR1 subunits, thereby generating binding sites for two IFNGR2 chains, a process that subsequently triggers intracellular 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, antiviral action, activation of microbicidal functions in immune cells, and leukocyte trafficking.[5]

The VeriPlex™ Mouse Cytokine 9-Plex ELISA has been developed to simultaneously detect IFN-α, IFN-β, IFN-γ, and other key pro-inflammatory cytokines released upstream and downstream of interferon signaling, including TNF-α, IL-1α, IL-1β, IL-6, IL-10, and RANTES. 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. 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, mouse serum, mouse plasma, and buffers.

MATERIALS PROVIDED

- Pre-coated microtiter plate
- Plate sealers
- Wash Solution Concentrate
- Mouse Cytokine 9-Plex Antigen Standard
- Mouse Cytokine 9-Plex Detection Mix
- HRP Concentrate
- Mouse Cytokine 9-Plex Sample Diluent
- Substrate A
- Substrate B+

ADDITIONAL MATERIALS REQUIRED *(NOT PROVIDED)*

- Variable volume microtiter pipettes
- Adjustable multichannel pipette (50-300 µl)
- Reagent reservoirs
- Wash bottle or plate washing system
- Distilled or deionized water
- Serological pipettes (1, 5, 10 or 25 ml)
- Disposable pipette tips (polypropylene)
- Plate shaker
- Q-View™ Imager or Q-View™ Imager LS
- Q-View™ software

Specifications: This kit quantitates Mouse Interferon Alpha (IFN-α), Mouse Interferon Beta (IFN-β), Mouse Interferon Gamma (IFN-γ), Mouse Interleukin-1a (IL-1a), Mouse Interleukin-1b (IL-1b), Mouse Interleukin-6 (IL-6), Mouse Interleukin-10 (IL-10), Mouse RANTES, and Mouse Tumor Necrosis Factor-Alpha (TNF-α) in sera, plasma and tissue culture media by sandwich enyzme 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 20 min

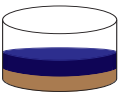
Specificity: No cross-reactivity with human IFN alpha 2a, human IFN beta 1a, human IFN gamma, rat IFN alpha, rat IFN beta, or human IFN lambda 1, 2, or 3. Refer to Cross Reactivity on p. 21 for details.

Storage Conditions/Comments: For retention of full 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), 22-25°C, before use. We have not fully evaluated the long term stability of reconstituted materials in liquid or frozen form.

For laboratory research use only. Not for use in diagnostic or therapeutic procedures.

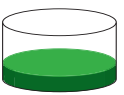
B. ASSAY PROCEDURE - QUICK REFERENCE

Total Time: 3 hr, 20 min



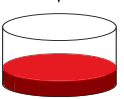
Add **50 µl** Standard, Test Sample, or Blank
(For serum, plasma, and media samples, dilute 1:1)*

Incubate **2 hrs** (shake at 500 rpm)
Aspirate and Wash **3x**



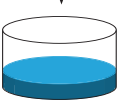
Add **50 µl** Detection Mix

Incubate **1 hr** (shake at 500 rpm)
Aspirate and Wash **3x**



Add **50 µl** HRP Concentrate

Incubate **20 min** in the dark (shake at 500 rpm)
Aspirate and Wash **6x**



Add **50 µl** mix of Substrate A and Substrate B+
Image plate within 15 min

Note: ALL incubations are at room temperature (22-25°C)

* Refer to pg. 9 Test Sample Preparation

C. PREPARATION OF REAGENTS

Supplied Mouse Cytokine 9-Plex Antigen Standard, Mouse Cytokine 9-Plex Detection Mix, and HRP Concentrate should be kept on wet ice (4ºC).

Wash Solution: The Wash Solution Concentrate may contain crystals. Place the bottle in a warm water bath and gently mix until completely dissolved. Prepare a 1:20 working wash solution (e.g. Add 50 ml of Wash Solution Concentrate to 950 ml of distilled or deionized water and mix thoroughly). Diluted Wash Solution can be stored at RT (22-25ºC) when not in use.

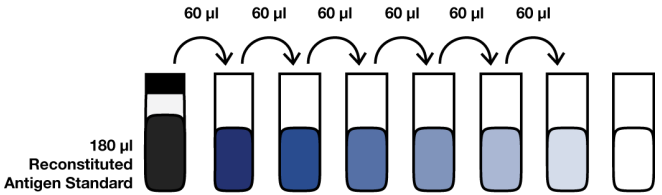
Standard: Reconstitute the supplied Mouse Cytokine 9-Plex Antigen Standard by adding the volume of Mouse Cytokine 9-Plex Sample Diluent indicated in the lot specific Certificate of Analysis. Mix gently until the Antigen Mix is completely dissolved and store on wet ice until use. Do not vortex. Do not introduce bubbles.

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

Detection Mix: Ready for use. Store on ice until use in Step 2 of the Assay Procedure.

Substrate Mix: Prepare during Step 2 incubation with Detection Mix. Prepare by mixing equal volumes of Substrate A and Substrate B+. Use full contents of Substrate A and Substrate B+. Store at RT (22-25ºC), in the dark, until use in Step 4. Do not put on ice or store at 4ºC. The mix is advised to be prepared at least 10 minutes prior to use and can be stored at RT for up to 5 days.

Fig. 1: 7-Point Standard Curve Prepared in Sample Diluent



Sample Dilution Label	S1	S2	S3	S4	S5	S6	S7	Blank
Sample Diluent (µl)	0	120	120	120	120	120	120	120

D. ASSAY PROCEDURE

All incubations should be performed in a closed chamber at RT (22-25°C), keeping the plate away from drafts and other temperature fluctuations. Use a plate shaker at 500 rpm where indicated. Use plate sealers to cover the plates as directed. During all wash steps, remove contents of plate by inverting and shaking over a sink and blotting the plate on lint-free absorbent paper; tap the plate. Wash each well with a minimum of 300 µl of diluted Wash Solution for each wash step. See Preparation of Reagents for details on dilution of concentrated solutions.

1. **Standards and Test Samples:** We recommend running the standard, blanks and test samples in duplicate. All serum, plasma, and media samples require a 1:1 dilution in the supplied Sample Diluent. Mix all samples thoroughly. Refer to the model plate setup in Figure 2. **The highest point of the standard curve (S1) must be in wells A1 and A2.**

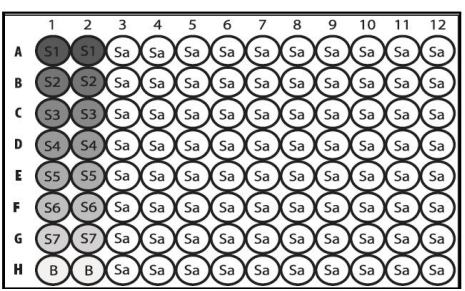
Adding Standards, Blanks and Test Samples:
- Add 50 µl of Standard to wells designated as Standard
- Add 50 µl of Blanks (Sample Diluent or sample matrix) to wells designated as Blanks
- Add 50 µl Test Samples to wells designated as Test Samples

Cover with plate sealer and shake plate at 500 rpm at RT (22-25ºC) for 2 hours.

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

2. **Mouse Cytokine 9-Plex Detection Mix:** Add 50 µl of Detection Mix to each well. Cover with plate sealer and shake plate at 500 rpm at RT (22-25ºC) for 1 hour.

Figure 2: Example of a Typical Plate Setup



B: Blank
S1-S7: Standard Curve
Sa: Sample

Prepare the Substrate Mix as instructed on pg. 8 under Preparation of Reagents.

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

3. **HRP Concentrate:** Add 50 µl of supplied HRP Concentrate to each well. Cover with plate sealer and shake, in the dark, at 500 rpm at RT (22-25ºC) for 20 minutes.

After 20 minutes, empty the contents of the plate and wash the wells six times with at least 300 µl of 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 15 minutes of adding the Substrate Mix. Refer to pg. 12-16 for detailed instructions on imaging the plate.

Note: It is recommended to use this product as a screening tool. It is not uncommon to obtain a 5-point standard curve for certain analytes as signal curves may saturate at the top end of the standard curve. (4-point curves may also occur sparingly.)

For best results, it is recommended to run samples in pre-screened serum from the strain being tested. Differential recovery dependent on mouse strain is expected. Refer to Additional Studies on p. 22 for details.

E. IMAGING PROCEDURE

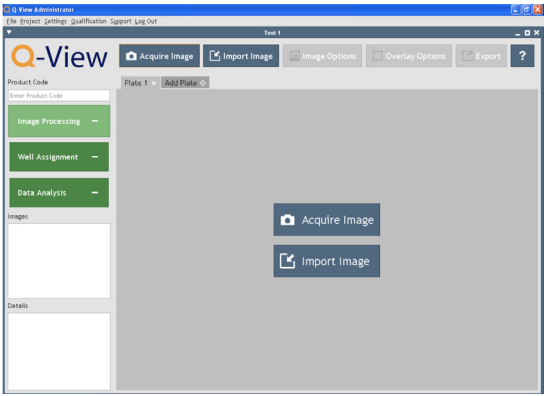
1. Quansys Q-View™ Imager—Acquiring an Image

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 is available for free. The download is available at http://www.quansysbio.com/q-view-software. The user manual for the software can be found under Manuals in the Support section.

A. Select New Project if starting a new project and save under a new name. 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. The connection status can be confirmed under Settings → Administration → Manage Imagers. If needed, click on Refresh.
C. Optional. Uncheck the box "Discard sub-images after stacking is complete" under Settings → Preferences in order to see images of different exposure times after the imaging process is 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 Settings → Administration → Manage Imagers. Ensure that there is no plate in the plate housing slot in the imager and that the housing door is closed. Select Calibrate.
E. Optional. If the imager has not been focused previously, place a Quansys focusing plate in the imager (do not close

plate housing door) and adjust the focus (under Settings → Administration → Manage Imagers) until the image is clear. Remove focusing plate. Close the Manage Imagers section.

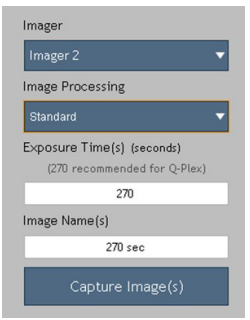


Q-View™ Version 3.1

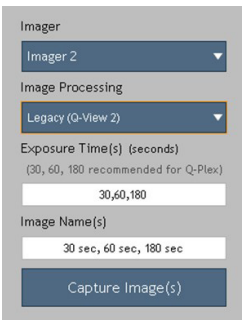
F. Select Acquire Image.
G. Ensure that the Q-View™ Imager is recognized. If not, follow step B. and click on Refresh.
H. Select Image Processing method: Legacy (similar to previous versions of Q-View™) or Standard (new feature in Q-View™).
I. Enter Exposure Time(s). Recommended exposure times are 30, 60 and 180 seconds for the Legacy setting or 270 seconds for the Standard setting. When using Legacy, each exposure will have a different image. The software will also display a stacked image.
J. Place the plate to be read in the plate housing slot, close the plate housing door and select Capture Image(s). The imaging should begin. Once acquired, the image will appear in the Q-View™ Software main screen.

K. If the image is needed for use in other programs, save the acquired image(s) by clicking Export. Export the image(s) as TIFF file(s).

Exposure Times: These times can be modified to meet your specific assay, but 30, 60 and 180 second exposure times (Legacy) or 270 second exposure time (Standard) are recommended for most assays. Once set, select Capture Image to start the reading.

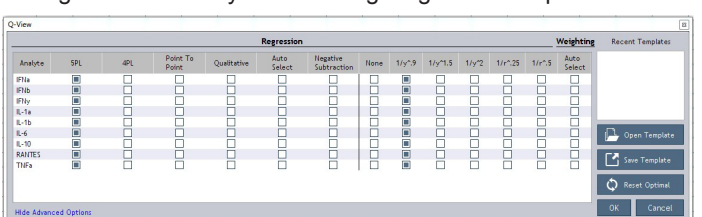


Standard Image Acquisition Option

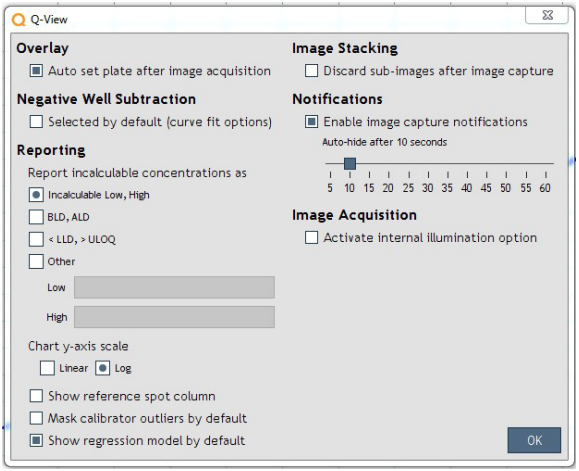


Legacy Image Acquisition Option

Once the run has completed, click on “Data Analysis” and then “Curve Fit Options” to access the regression and weighting options for analysis. We recommend selecting “5PL” parameter fit for regression and “1/y^0.9” for weighting to obtain optimal results.



*Please note that when the software is updated, 'Mask calibrator outliers by default' is automatically pre-selected. We recommend de-selecting automatic outlier masking before analyzing your data. This option can be found in Settings → Preferences.



→

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

Q-View™ Software can open images in the following formats: TIFF, CR2 (raw image files from Canon cameras), JPEG, PNG and BMP. However, it should be noted that lossy or low bit depth images (JPEG, PNG and BMP) are insufficient to use for analysis and therefore should be imported for the purpose of display only. Users should take images using supported imaging systems.

To acquire an image by importing an image file, select Import Image. Browse and select the desired image.

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.

F. PRODUCT PERFORMANCE CHARACTERIZATION

1. Matrix Studies

i. Levels of analytes in pooled lots of normal mouse serum and plasma

The endogenous levels of analytes in the samples were extrapolated from a Standard Curve prepared in Sample Diluent.

	Pooled Lots of Mouse Serum (n=14)		Pooled Lots of Mouse Plasma (n=7)	
Analyte	**Avg (pg/ml)**	**Range (pg/ml)**	**Avg (pg/ml)**	**Range (pg/ml)**
IFNa	<LLOD	<LLOD-5.0	4.5	<LLOD-16.6
IFNb	<LLOD	N/A	<LLOD	N/A
IFNg	5.5	<LLOD-10.5	8.6	3.36-18.8
IL-1a	<LLOD	N/A	<LLOD	N/A
IL-1b	<LLOD	N/A	<LLOD	N/A
IL-6	17	<LLOD-52.07	8.5	<LLOD-15.6
IL-10	<LLOD	N/A	<LLOD	N/A
RANTES	20.5	17.7-23.8	39.1	31.2-54.1
TNFa	1.8	<LLOD-2.6	3.3	2.0-5.0

ii. Spike Recovery

Low, medium, and high spikes were prepared using the kit standard in normal mouse serum and Sample Diluent from the product. Medium spikes were also prepared using the kit standard in normal mouse plasma with different anti-coagulants. The concentrations of spikes were extrapolated from a Standard Curve prepared in Sample Diluent. The recoveries were calculated after subtracting measured levels of endogenous analytes (in cases of normal mouse serum and plasma) and background (in case of Sample Diluent).

a. High Spike % Recovery

Analyte	Sample Diluent	Serum
IFNa	76.4	106.9
IFNb	102.0	95.8
IFNg	77.0	118.2
IL-1a	66.5	106.9
IL-1b	84.0	105.8
IL-6	81.5	113.3
IL-10	68.0	114.5
RANTES	61.7	110.6
TNFa	90.0	103.7

b. Medium Spike % Recovery

Analyte	Sample Diluent	Serum
IFNa	91.6	89.2
IFNb	99.4	88.2
IFNg	84.2	84.1
IL-1a	91.1	81.7
IL-1b	94.2	89.5
IL-6	93.5	84.6
IL-10	93.2	86.7
RANTES	77.0	88.5
TNFa	107.2	100.9

c. Low Spike % Recovery

Analyte	Sample Diluent	Serum
IFNa	79.3	73.7
IFNb	81.9	73.9
IFNg	77.7	68.7
IL-1a	91.0	70.1
IL-1b	85.1	76.1
IL-6	90.7	62.8
IL-10	80.5	74.5
RANTES	77.7	69.6
TNFa	96.6	89.8

d. Medium Spike % Recovery in Mouse Plasma with Various Anti-Coagulants

Analyte	Na-Citrate	Na Heparin	K Oxalate/ NaF	CPD	CTAD	K-2 EDTA	K-3 EDTA
IFNa	82.4	76.7	67.8	78.5	77.6	82.2	73.2
IFNb	70.6	72.7	66.2	56.2	61.5	66.3	80.6
IFNg	68.4	69.4	60.3	69.1	70.4	73.9	47.6
IL-1a	46.8	45.5	46.1	42.9	45.4	44.2	47.9
IL-1b	70.1	74.5	70.7	67.1	69.4	70.6	66.8
IL-6	76.9	79.3	77.6	79.7	88.3	79.5	74.9
IL-10	48.1	37.9	47.2	57.1	47.6	43.6	50.8
RANTES	58.1	59.2	52.6	80.8	67.6	75.7	70.0
TNFa	76.8	68.4	72.0	79.7	83.5	74.9	74.0

Citrate-Phosphate-Dextrose (CPD); Citrate-Theophylline-Adenosine-Dipyridamole (CTAD); Dipotassium/Tripotassium Ethylenediaminetetraacetic Acid (K-2/K-3 EDTA)

iii. Intra-assay and Inter-assay % CV

Low, medium, and high spikes were prepared using the kit standard in normal mouse serum, normal mouse plasma with different anti-coagulants, and Sample Diluent from the product. Sample Diluent spikes were assayed undiluted in triplicate; mouse serum and plasma spikes were assayed with a 1:1 dilution in Sample Diluent in triplicate. The concentrations of spikes were extrapolated from a Standard Curve prepared in Sample Diluent. The intra-assay % CV represented on the following page is the average of the intra-assay % CVs calculated from within each run. The second table represents inter-assay % CV. In the case of normal mouse serum, this is the average of the inter-assay % CVs calculated for each normal mouse serum lot.

Intra-Assay % CVs:

	Sample Diluent			Mouse Serum		
Analyte	**Low Spike**	**Medium Spike**	**High Spike**	**Low Spike**	**Medium Spike**	**High Spike**
IFNa	8.7	3.9	3.4	9.6	6.6	4.1
IFNb	7.9	3.8	9.6	6.5	4.9	13.7
IFNg	8.6	4.8	5.2	13.0	8.5	5.8
IL-1a	4.8	3.8	5.8	6.3	4.6	6.4
IL-1b	5.6	3.7	3.0	9.3	2.2	7.7
IL-6	14.4	6.4	6.1	5.2	5.2	4.4
IL-10	8.5	3.0	4.6	15.0	3.7	12.6
RANTES	8.3	5.2	7.2	12.4	10.4	8.5
TNFa	6.4	5.7	3.7	13.4	5.5	4.5

Inter-Assay % CVs:

	Sample Diluent			Mouse Serum		
Analyte	**Low Spike**	**Medium Spike**	**High Spike**	**Low Spike**	**Medium Spike**	**High Spike**
IFNa	6.8	5.4	5.7	11.9	4.6	10.8
IFNb	14.8	6.1	*	10.7	0.2	6.0
IFNg	2.4	4.4	5.8	8.9	10.6	12.9
IL-1a	2.0	2.9	3.9	5.8	2.9	12.5
IL-1b	19.6	3.9	1.8	13.9	0.8	8.1
IL-6	8.0	6.5	4.6	15.4	5.8	6.4
IL-10	11.8	4.8	*	24.3	5.1	5.3
RANTES	7.6	10.3	8.0	16.5	0.2	14.2
TNFa	6.8	5.6	2.7	12.0	4.3	7.2

* No data on this spike set due to signal above quantitative limits on one plate.

2. Cross Reactivity Studies

Independent curves, starting at 10 µg/ml, of recombinant analytes listed in the table below were prepared in Sample Diluent. A separate standard curve was prepared using the Mouse 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 corresponding mouse analytes in the curve prepared using the Multiplex Antigen Standard were averaged to estimate the % Reactivity for each analyte.

Catalog No.	Analyte	% Reactivity
11101-2	Human IFN-α 2 (α2a)	0%
11410-2	Human IFN Beta 1a	0%
11500-1	Human IFN Gamma	0%
13100-1	Rat IFN Alpha	0%
13400-1	Rat IFN Beta	0%
11725-1	Human IFN Lambda 1	0%
11720-1	Human IFN Lambda 2	0%
11730-1	Human IFN Lambda 3	0%

3. Additional Studies

Average % Recovery in Media and Different Strains of Serum

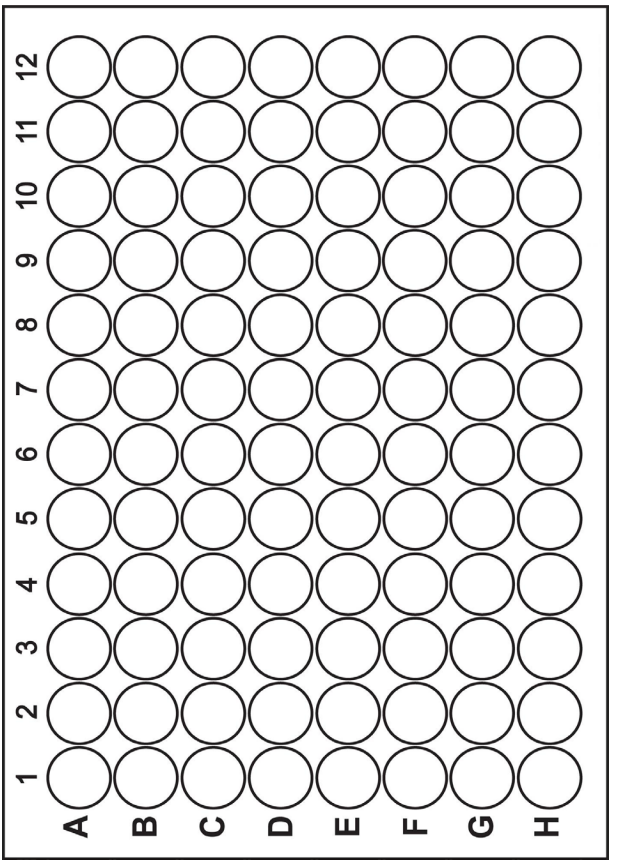
Analyte	RPMI + 10% FBS	Pooled CD-1 Sera	Pooled BALB/c Sera	Pooled C57 BL/6 Sera
IFNa	64.0	90.8	78.4	76.3
IFNb	149.8	115.7	115.8	99.0
IFNg	89.0	81.6	76.0	66.7
IL-1a	81.4	66.1	45.6	37.0
IL-1b	92.8	86.2	73.4	91.6
IL-6	119.7	93.1	80.4	72.5
IL-10	79.7	54.6	50.3	51.5
RANTES	76.0	84.8	77.9	64.9
TNFa	97.3	88.7	82.6	64.9

G. REFERENCES

1. Pestka, S., *et al*. (2005) “Evolution of the class 2 cytokines and receptors, and discovery of new friends and relatives”; in *Pharmacolo*gy & Therapeutics. 106(3): 299-346.
2. Pestka, S., *et al*. (2004) “Interferons, Interferon-like cytokines, and their receptors”; in *Immunological Reviews*. 202: 8-32.
3. Dorr, RT. (1993) “Interferon Alpha in malignant and viral diseases: a review”; in *Drugs*. 45(2).
4. Zivadinov, R., *et al*. (2008) “Interferon-treatment for relapsing multiple sclerosis”; in *Expert Opinion in Biological Therapeutics*. 8(9): 1435-37.
5. Hume, DA., *et al*. (2004) “Interferon-γ: an overview of signals, mechanism, and functions”; in *Journal of Leukocyte Biology*. 75: 163-189.

PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.



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