SpeedyPLEX Human Th1/Th2 Cytokine (6-plex) CBA Assay Panel 2 Datasheet

Please read it entirely before use

Cat.No.: KMF00004

Sample type: Serum, Plasma and Cell culture supernatant

Test number: 96 tests

Target name	Standard Curve Range (pg/mL)
IL-2	1.37-1000
IL-4	2.74-2000
IL-5	2.74-2000
IL-10	2.74-2000
TNF -alpha	2.74-2000
IFN-gamma	2.74-2000

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Table of content Page

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1. Background

Interleukin-2 (IL-2) is a four-helix bundle, type I cytokine that functions as a growth factor for a wide range of leukocytes. In the immune system, IL-2 is essential for immune homeostasis, normal T regulatory cell function, and self-tolerance. It regulates immune cell homeostasis and has been used to treat a range of disorders including cancer and autoimmune disease. IL-2 signals through heterodimerization of theIL-2R β and IL-2R γ receptor subunits.

Interleukin-4 (il-4), a member of the α -helical cytokine family, is produced by activated CD4+ T cells, basophils, and mast cells. It promotes the proliferation and differentiation of antigen presenting cells. This cytokine has been applied in the treatment of autoimmune disorder like multiple myeloma, cancer, psoriasis, and arthritis.

Interleukin-5 (IL-5), was originally discovered as a soluble T cell-derived factor, called T cell-replacing factor (TRF), that induced T cell-depleted activated B cells to secrete immunoglobulin. IL-5 is a key hematopoietic cytokine in eosinophil differentiation, maturation, recruitment and activation at sites of allergic inflammation. IL-5 also plays a role in the development, metabolism, and function of basophils. IL-5 exerts its biological activity through the IL-5 receptor (IL-5R), which is composed of at least two chains: an alpha chain that binds IL-5 with low affinity and a beta chain that does not bind IL-5, but together with the IL-5 alpha chain, constitutes the high affinity IL-5 receptor. The beta chain is common to the IL-3, IL-5 and GM-CSF receptors and has been shown to signal through the JAK/Stat pathway. IL-5 has long been associated with the cause of several allergic diseases including allergic rhinitis and asthma, wherein a large increase in the number of circulating, airway tissue, and induced sputum eosinophils have been observed.

Interleukin (IL)-10 is an anti-inflammatory cytokine, produced by T helper (Th) cells, macrophages, monocytes, and B cells, that plays a crucial role in preventing inflammatory and autoimmune pathologies. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL10 can block NF- κ B activity, and is involved in the regulation of the JAK-STAT signaling pathway. IL10, along with its receptors, describes an important role in pathogenesis of various diseases, including infectious, inflammatory, autoimmune diseases. IL10 mutations are associated with an increased susceptibility to HIV-1 infection and rheumatoid arthritis.

TNF, also known as TNF-alpha, or cachectin, is a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.

Interferon gamma (IFNG) is a soluble cytokine that is the only member of the type II class of interferons. It is secreted by Th1 cells, cytotoxic T cells and NK cells. The cytokine is associated with antiviral, immunoregulatory and anti-tumor properties and is a potent activator of macrophages. It plays a crucial role in pathogen clearance. Aberrant IFNG expression is associated with a number of autoinflammatory and autoimmune diseases. It has been identified in many studies as a biomarker for pleural tuberculosis (TB). Mutations in this gene are associated with aplastic anemia.

2. Principle

Immunoassay Principle: Prior to the assay, fluorescent dye, color-coded beads are conjugated to capture antibodies. During sample incubation, the capture antibodies and the corresponding biotinylated detection antibodies all bind to the target, thus assembling the sandwich complex. Then, streptavidin-PE is added, which is used for detecting the concentration of target proteins by flow cytometry (one unit of streptavidin-PE can bind to four units of the sandwich complex).

Fluorescence signal detection: In this part of the assay, the flow cytometer distinguishes the targets based on the fluorescence signals of APC/APC-Cy7, and detects the concentration of the target based on the fluorescence signal of PE.

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The beads contain two fluorescent dyes - APC and APC-Cy7, which are freely mixed in different proportions, resulting in different codes. The flow cytometer distinguishes different targets based on the proportion of fluorescent dyes in the coded beads. Each target is associated with only one type of coded bead (containing a specific proportion of fluorescent dyes). Meanwhile, the flow cytometer collects the level of PE fluorescence from different beads, and the target concentration in the sample can be determined by comparing the PE signal to a standard curve.

3. Required Materials

3.1 A flow cytometer equipped with two lasers (488 nm or 532 nm for PE fluorescent and 635 nm for APC and APC-Cy7).

Flow cytometer	Reporter channel	Bead channels
Beckman (CytoFlexs)	PE	APC/APC-Cy7
Challenbio (Fongcyte)	PE	APC/APC-Cy7
Agilent (Novocyte)	PE	APC/APC-Cy7

3.2 Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.

3.3 Hand-held microplate magnet or platewasher with a magnetic platform for 96-Well Plate flat bottom microplate, or centrifugal machine for 96-Well Plate V-bottom microplate.

3.4 Absorbent paper towels.

3.5 Glass or plastic tubes to prepare standard and sample dilutions.

3.6 Beakers and graduated cylinders.

3.7 Log-log or semi-log graph paper or computer and software for Data analysis. A four-parameter logistic (4-PL) regression analysis is recommended. Proteintech data analysis website:https://www.ptgcn.com/products/elisa-kits/

3.8 Microplate thermostatic shaker.

3.9 Deionized or distilled water.

4. Kit Components and Storage

Microplate - 96-Well Plate (Flat bottom)[1]	1 plate	**Unopened Kit:** **Store all reagents at 2-8°C for 12 months** **Please use a new standard for each assay.**
Microplate - 96-Well Plate (V-bottom)[2]	1 plate	
Setup Beads Cocktail - 500 µL/vial*	1 vial	
PE Control Beads - 500 µL/vial*	1 vial	
Protein Standard Cocktail; lyophilized	2 bottles	
Capture Beads Cocktail (50x) - 120 µL/vial*	1 vial	
Biotinylated Detection Antibody Cocktail (50x) - 120 µL/vial*	1 vial	
Sample Diluent PT 3-ef -30 mL/bottle	1 bottle	
Detection Diluent - 30 mL/bottle	1 bottle	
Additional Diluent ATK00004 - 6 mL/bottle; Only for human serum and plasma samples.	1 bottle	
Wash Buffer Concentrate (20x) - 30 mL/bottle	1 bottle	
Streptavidin-PE (50x)-120 µL/vial*	1 vial	
Plate Cover Seals	4 pieces	

1: Microplate - 96-Well Plate(Flat bottom) match with Hand-held microplate magnet or platewasher with a magnetic platform in the wash step

2: Microplate - 96-Well Plate (V-bottom) match with centrifugal machine in the wash step

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* Centrifugation immediately before use

5. Safety Notes

5.1 Do not use the kit after the expiration date.

5.2 The kit components are designed to be used as a collective unit. Do not mix components from different lots or kits.

5.3 Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.

5.4 This product is for research use only and not for use in human or animal therapeutics or diagnostics.

5.5 If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate sample diluent and repeat the assay.

5.6 Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

5.7 Protect the beads and Streptavidin-PE (1x) from light at all times

5.8 Quantitative results or protein levels for the same sample or recombinant protein run in ELISA and CBA assays may differ.

6. Sample Collection and Storage

6.1 Serum: Allow blood samples to clot for 30 min, followed by centrifugation for 15 min at 1000 x g . Clear serum can be assayed immediately or aliquoted and stored at - 20 ℃. Avoid repeated freeze-thaw cycles.

6.2 Plasma: Use EDTA or heparin as an anticoagulant for plasma collection. Centrifuge for 15 min at 1000 x g within 30 min of collection. The plasma can be assayed immediately or aliquoted and stored at - 20 ℃. Avoid repeated freeze-thaw cycles.

6.3 Cell Culture Supernatant: Remove particulates by centrifugation for 5 min at 500 x g and assay immediately or aliquot and store samples at ≤ - 20 ℃. Avoid repeated freeze-thaw cycles.

7. Reagent Preparation

7.1 Wash Buffer (1x): If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate (20x) to 570 mL deionized or distilled water to prepare 1x Wash Buffer.

7.2 Antibody Cocktail (1x): Capture beads cocktail and biotinylated detection antibody cocktail are provided as a 50x concentrate in separate tubes. Prior to use, the two components must be mixed together and diluted in detection diluent to form the 1x Antibody Cocktail.

7.2.1 Vortex to resuspend capture beads cocktail and biotinylated detection cocktail before removing the cap.

7.2.2 Mix and dilute the capture beads cocktail (50x) and biotinylated detection antibody cocktail (50x) with the detection diluent to create the Antibody Cocktail (1x).

Example for **Antibody Cocktail (1x)**:

Total volume (µL)	Capture beads Cocktail (50x) (µL)	Biotinylated Detection Antibody Cocktail (50x) (µL)	Detection Diluent (µL)
1000	20	20	960
2500	50	50	2,400
5000	100	100	4,800

Mix the components gently but thoroughly. The total volume of Antibody Cocktail (1x) should be made according to the number of wells to be used. An additional 100 µL or 200 µL should be made to account for dead volume.

7.3 Sample Dilution: Different samples should be diluted with corresponding Sample Diluent.

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7.3.1 Samples may require further dilution if the readout values are higher than the highest standard. Therefore, it is recommended to conduct a preliminary experiment to determine the dilution factor of the sample.

7.3.2 Variations in sample collection, processing and storage may affect the results of the measurement. Especially for cell culture supernatant samples, the sample values can be affected by various factors such as culture conditions, stimulation concentration, stimulation method, and cell batch.

7.3.3 Recommended Dilution for different sample types: 1:2 is recommended for human serum, plasma, 1:2 to 1: 512 is recommended for cell culture supernatant.

Note: The recommended dilution factor is for reference only. The actual dilution factor should be determined based on the sample being tested.

7.4 Streptavidin-PE (1x): Streptavidin-PE (50x) need to be diluted before use.

Example for **Streptavidin-PE (1x)** :

Total volume (µL)	Streptavidin-PE (50x) (µL)	Detection Diluent (µL)
1000	20	980
2500	50	2,450
5000	100	4,900

Mix gently but thoroughly. Adjust the total volume of **Streptavidin-PE** (1x) according to the number of wells being tested. When calculating the total volume, an additional 100 µL or 200 µL should be made to account for dead volume.

7.5 Standard Serial Dilution:

1. Label the tubes in the 7-Tube Strip: Std2, Std3, Std4, Std5, Std6, Std7, Std8.
2. Add 2000 µL Sample diluent into the lyophilized protein standard cocktail to reconstitute. This is the Std1.
3. Add 600 µL Sample Diluent into Std2–Std8 tubes.
4. Transfer 300 µL of reconstituted standard from Std1 tube into Std2 tube using new pipette tip.
5. Mix by pipetting up and down 10 times.
6. Repeat steps 4–5 for tubes Std2–Std7, changing pipette tips between dilution steps.
7. Std8 serve as a background and only contain sample diluent.

Note: Please use a new standard for each assay.

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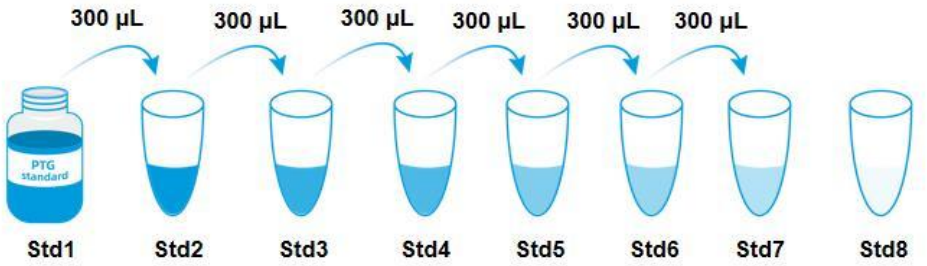

	Std1	Std2	Std3	Std4	Std5	Std6	Std7	Std8
# µL of Sample Diluent PT 3-ef	2000	600	600	600	600	600	600	600
Add # µL of Standard diluted in the previous step	—	300	300	300	300	300	300	-
IL-2	1000	333.33	111.11	37.04	12.35	4.12	1.37	0
IL-4	2000	666.67	222.22	74.07	24.69	8.23	2.74	0
IL-5	2000	666.67	222.22	74.07	24.69	8.23	2.74	0
IL-10	2000	666.67	222.22	74.07	24.69	8.23	2.74	0
TNF - alpha	2000	666.67	222.22	74.07	24.69	8.23	2.74	0
IFN-gamma	2000	666.67	222.22	74.07	24.69	8.23	2.74	0

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8. Assay Procedure Summary

Note: 1) Protect the beads and Streptavidin-PE (1x) from light at all times

2) Make sure that all beads are kept in the wells of the plate throughout all the steps, especially in the Washing step.

3) This experiment offers two washing methods, just choose one washing method based on the equipment available.

Microplate - 96-Well Plate **(Flat bottom)** match with Hand-held microplate magnet or platewasher with a magnetic platform in the wash step;

Microplate - 96-Well Plate **(V-bottom)** match with centrifugal machine in the wash step.

8.1 Preset the layout of the microplate, including control group, standard group, and sample group.

8.2 Add 50 µL standard or sample to appropriate wells.

For serum or plasma, add 25 µL of Additional Diluent to the appropriate wells (No need incubation and wash);

For cell culture supernatant, no need to add Additional Diluent, directly follow the next step.

8.3 Vortex antibody cocktail solution (1x) (refer to Reagent Preparation 7.2) to resuspend the beads and add 50 µL solution each well. Seal plate with cover seal and incubate at 37 °C on a microplate thermostatic shaker set at 800 rpm for 1 h (**Note**: The magnetic beads are iron-containing plastic spheres. They will settle at the bottom of the tube in normal conditions. Therefore, before each addition, please resuspend the solution to ensure they are evenly dispersed in the liquid).

8.4 Wash (Choose one washing method based on the equipment available)

Method 1: When use the flat bottom plate,follow the washing steps below：

1) Place the plate on the hand-held microplate magnet for 2 min to allow the beads to firmly adhere to the bottom of the plate. Then discard the liquid from wells by aspirating or decanting while retaining the magnetic beads inside the wells.

Note: Ensure the plate and the microplate magnet are not separated during this step.

2) Take out the plate from microplate magnet, add 1x Wash Buffer 100 µL per well. Seal plate with cover seal and resuspend beads on a microplate thermostatic shaker set at 800 rpm for 1 min.

3) Place the plate on the hand-held microplate magnet for 2 min to allow the beads to firmly adhere to the bottom of the plate. Then discard the liquid from wells by aspirating or decanting while retaining the magnetic beads inside the wells.Note: Ensure the plate and the microplate magnet are not separated during this step. Take out the plate from microplate magnet

Method 2: When use the V- bottom plate,follow the washing steps below：

1) Centrifuge the plate at 200 x g for 5 minutes. Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting.

2) Add 1x Wash Buffer 100 µL per well. Seal plate with cover seal and resuspend beads on a microplate thermostatic shaker set at 800 rpm for 1 min.

3) Centrifuge the plate at 200 x g for 5 minutes. Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting.

8.5 Add 50 µL **Streptavidin-PE (1x)** (refer to Reagent Preparation 7.4) solution to each well. Incubate at 37 °C on a microplate thermostatic shaker set at 800 rpm for 15 min.

8.6 Repeat Step 8.4.

8.7 Resuspend the wells with 120 µL 1x Wash buffer.

8.8 Read samples on the flow cytometer and collect data from the software.

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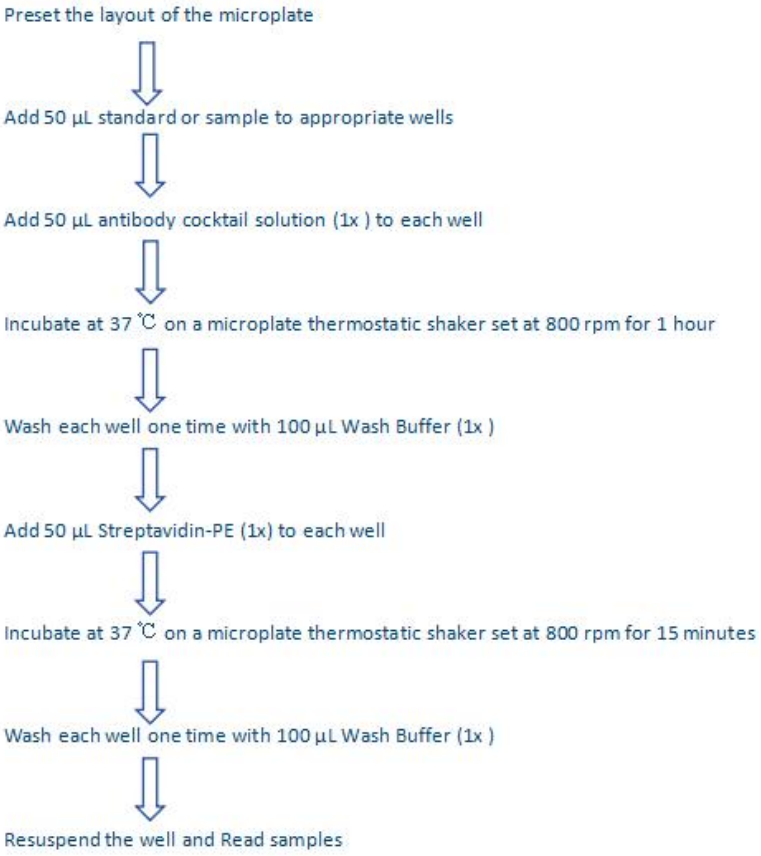
Procedure summary



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9. Instrument Set and Data Analysis

Note：

1) **In this experiment, we use Agilent instrument (Version:Novocyte/Software:Novoexpress) as an example to illustrate the settings steps. When using a different instrument, the same general setup can be followed.**

2) **If the instrument software can simultaneously perform the functions of data reading and analysis, it is recommended to follow the instrument operation procedures for setting up and reading the data.**

For Novocyte flow cytometers, the PE channel is used for reporting sample concentration and the APC /APC-Cy7channel is used for beads classification. In general, there is no need for compensation between these channels if the machine is set up properly following the setup procedure described below. This procedure is required in the following situations:

• Running the SpeedyPLEX Human Th1/Th2 Cytokine (6-plex) CBA Assay Panel 2 for the first time.

• Running kits of the same Catalog Number but from different lots.

9.1. Start up the Instrument

Complete the startup and quality control procedure according to the instrument manual. Ensure the instrument is in good condition before proceeding to the next steps. Confirm that the excitation channels of the PE, APC and APC-Cy7 are in an enabled state.

9.2 Creating a New Template for Data Acquisition:

If a template is not available, create a new template following the instructions below. **If a template has been created, please skip this setup and proceed directly to Section 9.5**, Pre-defined gates and settings that allow the user to skip the setup procedure and perform sample acquisition directly.

9.3 Set up Density Plots

1) From the Novoexpress software home screen, click File >> New>> New Blank Experiment, then a blank experiment window is produced. Select the test well from plate or tube and right-click the mouse and choose “new”. Then the sample wells information (eg: A1) will be presented in the lower right window.

2) From the toolbar, click the dot plot sign and a dot plot will appear with FSC-H (forward scatter) for X-axis and SSC-H (side scatter) for Y-axis (Figure 1). FSC-H/SSC-H dot plot is used for "Total beads" gate to separate target beads from debris. Set the FSC-H and SSC-H to linear mode. To change the mode, click on the coordinate axis, then right click the mouse and select property.

3) Create APC and APC-Cy7 dot plot from the "Total beads" parent gate (Figure 2), beads filled with different ratio APC and APC-Cy7 content will be dispersed in respective positions. The plots should all be in log mode. To change the mode, click on the density plot, right click the mouse and select property.

4) Create PE and Count histogram from the "Total beads" parent gate (Figure 3).

5) Set the collect volume of each well ≥ 70 μL. It is recommended that the number of beads for each target ≥ 200 beads/well to ensure the accuracy of the data.

9.4 Set up PMT Voltages and Gates

1) Vortex the vial of Setup Bead Cocktail for 30 seconds to resuspend the beads.

2) Set the flow cytometer flow rate to low. Run the Setup Bead Cocktail 120 μL. Adjust the gains in the Parameters (the upper left of the window) for FSC-H and SSC-H, so that all bead populations are visible (Figure 1). From the toolbar, click the Elliptical Gate (Other graphics gate can also be used) and use the mouse to drag the populations to the middle of the dot plot. **Pause and restart acquisition frequently during the setup procedure to refresh the bead populations after adjusting settings.**

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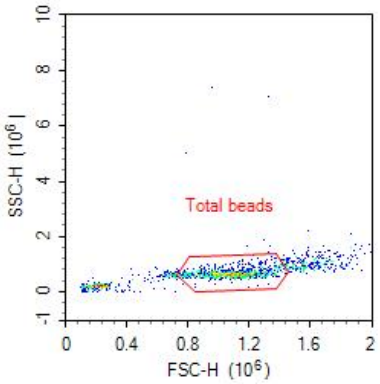

Figure 1

3) Meanwhile, adjust the gains in the Parameters (the upper left of the window) for APC and APC-Cy7 channel to make sure all beads are evenly distributed and well separated throughout a prominent area. If the APC and APC-Cy7 fluorescence signal exceeds the maximum collection range of the graph, reduce the gains in the Parameters (the upper left of the window) for APC and APC-Cy7 channel. Adjust the PMT gain of the flow cytometer. From the toolbar, click the Elliptical Gate (Other graphics gate can also be used) and use the mouse to drag the populations to the middle of the dot plot. Gate each cluster of beads and name the corresponding target (Figure 2).

Note: Changes in voltage will shift the overall position of the beads, but their relative positions to each other will remain unchanged.

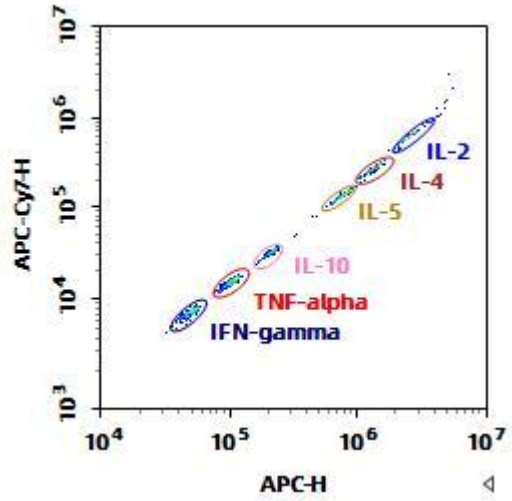

Figure 2

4) Vortex the vial of PE control Beads for 30 seconds to resuspend the beads. Run the PE control Beads 120 µL.

5) Adjust the gains in the Parameters (the upper left of the window) for PE channel so that the MFI (PE channel) is around(1.5-5)×10^5 for Total beads (Figure 3).

Note: PE channel voltage is critical to obtain the best standard curve. If the voltage is set too low, the low end of the standard curve may plateau, resulting in low sensitivity of the assay. If the voltage is set too high, the high end of the standard curve may plateau.

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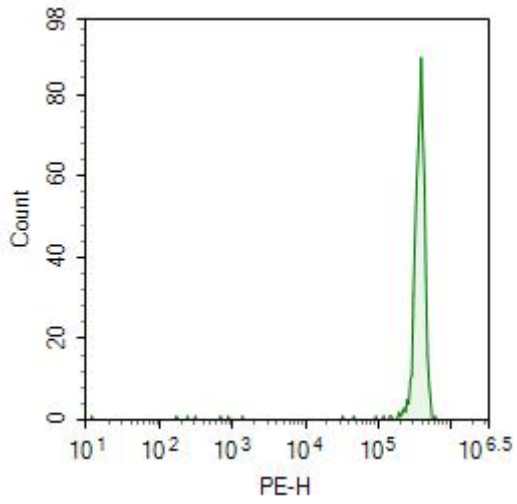

Figure3

6) Save the template and rename the template then proceed to the next step of the setup.

9.5 Sample Acquisition use Saved Template

To use an existing template for sample acquisition, follow the instructions below.

1) From the Novoexpress Software home screen, go to click File >> New>> New Blank Experiment.

2) Select all sample wells from plate or tube and right-click the mouse and select new. Then all the sample well information (eg: A1,A2,A3) will be presented in the lower right window.

3) Choose any sample wells (eg: A1), right-click the mouse and select import >> import template >>, Select the corresponding template >> OK, **Note: This template is only applicable to the particular one sample wells (eg: A1).**

4) Drag the sample wells to Specimen, from the popup window, choose paste template to all samples. At this point, the template was applied to all the samples.

5) From the lower left of the windows, click run plate >> Select well(s) to run >> Run >> Name your test experiment.

6) The flow cytometer is now ready for sample acquisition.

9.6 Export Data and Analyze:

1) After all test wells are collected, from the toolbar, click Statistical table >> Create New, then a table will pop up. We can select the parameters we need (Including the beads number and MFI of each gate) and export the table.

Well	Target 1 Count	Target 2 Count	Target 3 Count	Target ……N Count	Target 1 Median PE-H	Target 2 Median PE-H	Target 3 Median PE-H	Target……N Median PE-H
A1								
A2								
A3								
A4								
A5								
A6								
A7								
A8								
A9								
A10								
A11								
A12								
……								

2) Use the VBA analysis tool listed on the Proteintech website to process the raw data. The link is as follows:

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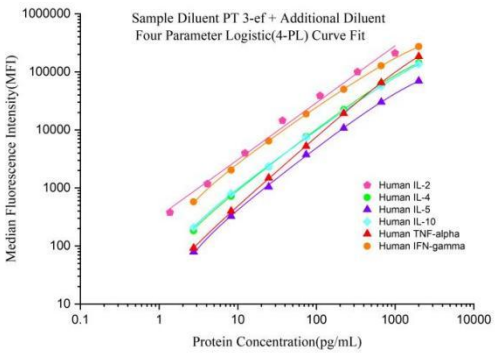
https://www.ptglab.com/media/bzzdgyfz/vba-en-version.xlsm

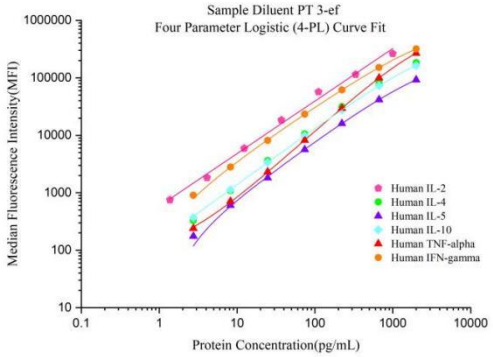
3) Calculate the average of MFI for each standard and sample and subtract the average of the zero standard MFI. Construct a standard curve by plotting the mean MFI for each standard on the y-axis against the concentration on the x-axis, use four-parameter logistic curve-fit (4-PL) analysis to do this. If the samples were diluted, the concentration readout from the standard curve must be multiplied by the dilution factor used.

10. Validation Data

10.1 Standard curve

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.





10.2 Precision

Intra-assay Precision (Precision within an assay) Three samples of known concentration were tested in one assay with 8 replicates to assess intra-assay precision.

Inter-assay Precision (Precision between assays) Three samples of known concentration were tested in four separate assays with 4 replicates to assess inter-assay precision.

Analyte	Sample	Intra-assay Precision (n=8)			Inter-assay Precision (n=16)		
		Mean (pg/mL)	SD	CV (%)	Mean (pg/mL)	SD	CV (%)
IL-2	Sample 1	334.4	4.1	1.2	334.6	4.4	1.3
	Sample 2	35.9	1.0	2.9	35.6	0.3	0.9
	Sample 3	2.4	0.1	2.1	2.5	0.1	2.8
IL-4	Sample 1	700.6	6.6	0.9	690.3	6.2	0.9
	Sample 2	69.7	5.6	8.1	67.3	1.4	2.1
	Sample 3	4.6	0.2	5.3	5.1	0.4	7.0
IL-5	Sample 1	685.5	8.1	1.2	680.1	9.3	1.4
	Sample 2	63.5	1.2	1.8	65.3	1.8	2.8
	Sample 3	4.4	0.2	3.6	4.9	0.4	7.6
IL-10	Sample 1	700.5	8.3	1.2	685.1	15.1	2.2
	Sample 2	66.1	1.0	1.5	66.0	1.4	2.1
	Sample 3	4.6	0.2	4.4	4.9	0.2	4.9
TNF -alpha	Sample 1	722.0	5.5	0.8	707.2	12.2	1.7
	Sample 2	56.3	0.5	0.8	58.3	1.5	2.7
	Sample 3	4.2	0.2	5.0	5.0	0.5	10.6

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Analyte	Sample	Intra-assay Precision (n=8)			Inter-assay Precision (n=16)		
		Mean (pg/mL)	SD	CV (%)	Mean (pg/mL)	SD	CV (%)
IFN-gamma	Sample 1	683.3	5.0	0.7	675.3	9.1	1.3
	Sample 2	70.8	0.9	1.3	69.4	1.2	1.7
	Sample 3	4.7	0.1	1.8	4.8	0.1	3.1

10.3 Recovery

The serum and cell culture supernatant samples were prediluted two-fold with sample diluent, then the recovery of analyte spiked to three different levels throughout the range of the assay were evaluated.

Analyte	Serum		Cell culture supernatant	
	Mean (%)	Range (%)	Mean (%)	Range (%)
IL-2	103	72-126	105	95-119
IL-4	96	70-116	95	81-113
IL-5	106	91-119	85	72-99
IL-10	102	89-112	98	74-120
TNF -alpha	97	84-108	114	99-130
IFN-gamma	108	91-127	100	83-118

10.4 Sample values

Human serum - Twelve samples were evaluated for detectable levels of IL-2, IL-4, IL-5, IL-10, TNF-alpha and IFN-gamma in this assay, the concentrations measured are shown below.

Analyte	Range (pg/mL)	No.of Detectable	Mean (pg/mL)	% of Detectable
IL-2	ND-0.21	1	0.02	10%
IL-4	ND	0	ND	0%
IL-5	ND-11.41	6	2.13	60%
IL-10	1.83-12.00	10	8.51	100%
TNF-alpha	1.66-5.93	10	3.76	100%
IFN-gamma	0.19-3.49	10	0.9	100%

ND*=Non-detectable

Cell culture supernatant - Human peripheral blood mononuclear cells (PBMC) (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 10% fetal bovine serum, 5μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 μg/mL PHA for 1 day. Aliquots of the Cell culture supernatants were removed and assayed for levels of IL-2, IL-4, IL-5, IL-10, TNF -alpha and IFN -gamma.

	IL-2 (pg/mL)	IL-4 (pg/mL)	IL-5 (pg/mL)	IL-10 (pg/mL)	TNF-alpha (pg/mL)	IFN-gamma (pg/mL)
Unstimulated (1 day)	11.97	ND	4.58	93.44	13.10	9.21
Stimulated (1 day)	12,140.19	274.62	323.14	15,566.40	7,270.43	29,671.91

ND*=Non-detectable

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10.5 Sensitivity

The minimum detectable dose of the analyte was defined as the concentration corresponding to the mean Median Fluorescence Intensity MFI (PE channel) of 20 zero- standard replicates plus two standard deviations.

Analyte	Sensitivity (pg/mL)
IL-2	0.03
IL-4	0.08
IL-5	0.36
IL-10	0.21
TNF-alpha	0.35
IFN-gamma	0.02

10.6 Linearity

Serum was spiked with high concentrations of analyte and diluted with the appropriate sample diluent to produce samples with values within the dynamic range of the assay.

	Human Serum	IL-2	IL-4	IL-5	IL-10	TNF-alpha	IFN-gamma
1:2	Average % of Expected	118	90	97	95	74	115
	Range (%)	118-119	87-94	95-99	88-101	73-75	113-116
1:6	Average % of Expected	120	103	107	106	78	124
	Range (%)	120-121	100-105	107-107	103-109	76-109	124-124
1:18	Average % of Expected	120	117	108	110	94	125
	Range (%)	118-123	116-118	105-111	110-110	89-99	122-127
1:54	Average % of Expected	111	110	104	112	89	120
	Range (%)	104-119	104-115	103-105	112-113	84-94	116-123

Cell culture supernatants were diluted with the appropriate **Sample Diluent** to produce samples with values within the dynamic range of the assay.(Cell culture supernatant were initially diluted 1:16 for IL-2, 1:8 for IL-10, 1:2 for TNF-alpha and 1:32 for IFN-gamma).

	Cell culture supernatant	IL-2	IL-4	IL-5	IL-10	TNF-alpha	IFN-gamma
1:2	Average% of Expected	100	100	100	100	100	100
	Range (%)	-	-	-	-	-	-
1:4	Average% of Expected	105	108	105	98	109	107
	Range (%)	104-107	108-108	99-112	94-102	106-113	103-110
1:8	Average% of Expected	116	109	104	98	106	111
	Range (%)	115-117	107-111	96-112	98-99	101-111	104-118
1:16	Average% of Expected	123	112	106	98	99	109
	Range (%)	120-125	109-114	92-120	98-98	94-104	105-114

10.7 Specificity

The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference.

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IL-2	IL-4	IL-5	IL-10	TNF-alpha	IFN-gamma
Human IL-1α	Human GM-CSF	Human IL-3	Human IL-10 RB	Human TNF-β	Human IFN-γ R1
Human IL-1β	Human gp130	Human IL-5 Ra	Human IL-10 RA	Rat TNF-α	Human IFN-β
Human IL-2Ra	Human IL-4 R	Mouse IL-5	Rat IL-10	Mouse TNF-α	Mouse IFN-γ
Human IL-2 gamma chain	Mouse IL-4		Mouse IL-10	Human TNF RI	Rat IFN-γ
Human IL-4			Human IL-22	Human TNF RII	
Human IL-7				Human TNFSF13	
Human IL-9					
Human IL-15					
Human IL-21					
Mouse IL-2					
Rat IL-2					

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