

> **IL-4 Singleplex MultiBead™ Kit**
Catalog # 985-006
Bead singleplex kit for use with serum, plasma, culture supernatants and urine

This singleplex kit must be used with the Extracellular MultiBead™ Buffer Pack 1 Catalog No., 987-001 that contains a detailed test procedure for measurement and calculation of results.

Important note: If you are running this kit in combination with other singleplex assays, use the instruction manual provided with the Extracellular MultiBead™ Buffer Pack 1.

Visit www.assaydesigns.com/multibead for additional information.

Introduction

Assay Designs' IL-4 Singleplex MultiBead Kit is a complete immunometric kit for the quantitative determination of Interleukin-4 (IL-4) in biological fluids. Please read the complete kit insert before performing this assay. The kit uses beads attached to a monoclonal antibody against IL-4 to bind the human IL-4 in the standards or sample. A recombinant human IL-4 standard is included in the kit. After a short incubation at room temperature the excess standard or sample is washed away and incubated with an (IgG)₂ antibody fragment that has phycoerythrin (PE) covalently attached to it. This antibody binds to the IL-4 captured on the bead. After a short incubation the excess antibody is washed away and the beads are analyzed on a flow cytometer. The fluorescent intensity of the bound PE is proportional to the concentration of IL-4 in either standards or samples. The relative fluorescent intensity is used to calculate the concentration of IL-4.

IL-4 is a cytokine produced by activated T cells, mast cells and basophils that functions in the activation of B-cells, regulates IgG class switching and increases MHC II expression. IL-4 induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4. It was initially identified as a B cell differentiation and stimulatory factor. IL-4 has been shown to have multiple biological effects on hematopoietic and non-hematopoietic cells, including B and T cells, monocytes, macrophages, mast cells, myeloid and erythroid progenitors, fibroblasts, and endothelial cells.

Materials Provided

- IL-4 Capture Bead**
100 µL, Catalog No., 80-1870 One 96-test vial at a 25X concentration containing antibody coupled beads (5.4 µm, intensity level 1). **Do not freeze.**
- Inflammation Human 8-plex Standard Cocktail 1**
2 each, Catalog No., 80-1860 2 vials containing 75 ng of recombinant standard. **Store at -20°C.**
- IL-4 Antibody Conjugate**
400 µL, Catalog No., 80-1879. One 96-test vial at a 25X concentration containing IL-4 antibody conjugated to PE. **Do not freeze.**

Materials Needed but Not Supplied

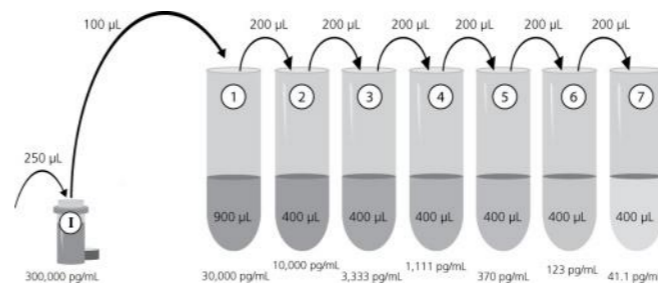
- Extracellular MultiBead™ Buffer Pack 1 (CN 987-001).
- A flow cytometer with a 488 nm and 635 nm laser capable of detecting and distinguishing fluorescence emissions at 576 nm and 670 nm.
- Multi-well plate vacuum manifold (e.g., Pall CN 5017).
- Disposable/round bottom polypropylene 12 x75 mm culture tubes (e.g., VWR CN 60818-281).
- Precision pipets for volumes between 5 µL and 1,000 µL.
- 5 mL and 10 mL pipets.
- Multichannel pipet, reservoir and disposable tips.
- A microplate shaker.
- Assay Designs Multibead Analysis Software (CN 28-0001, available for download at www.assaydesigns.com/multibead).

Reagent Preparation

- IL-4 Capture Bead**
Beads are supplied as a 25X concentrated stock. To make the working stock, mix one part concentrate with 24 parts assay buffer. For example, 50 assays would require 1250 µL of working stock (25 uL/assay). Vortex the 25X stock vigorously and add 50 µL to 1200 µL of Extracellular Assay Buffer 1 to prepare the working stock. The bead working stock should be stored at 4°C and vortexed before use.
- IL-4 Antibody Conjugate**
Conjugate is supplied at a 25X concentration. To make the working stock, mix one part concentrate with 24 parts assay buffer. For example, 50 assays would require 5000 µL of working stock (100 µL/assay). Vortex the 25X stock add 200 µL to 4800 µL of Extracellular Assay Buffer 1 to prepare the working stock. The working stock should be stored at 4°C and used within one hour.

Inflammation Human 8-plex Standard Cocktail 1

Reconstitute the lyophilized Standard Cocktail 1 vial with 250 µL of Extracellular Assay Buffer 1 and gently vortex. Wait 5 minutes and vortex again. Label seven tubes #1 through #7. Pipet 900 µL of Extracellular Assay Buffer 1 in tube #1 and 400 µL of Extracellular Assay Buffer 1 into tubes #2-#7. Add 100 µL of the reconstituted standard to tube #1. Tube #1 now contains 30,000 pg/mL of IL-4. Add 200 uL of tube #1 into tube #2 and vortex thoroughly. Continue for tubes #2 through #7.



Diluted standards should be used within one hour of preparation. The reconstituted standard (300,000 pg/mL) is stable at 4°C for one hour and may be aliquoted and stored at -70°C in polypropylene tubes for three months.

Filter Plate Assay Procedure

The kit has been optimized for use in 96-well filter plates. Alternatively the assay may be performed in microtubes or 96-well filter or V-bottom plates. The performance of the reagents has not been validated in these alternate formats.

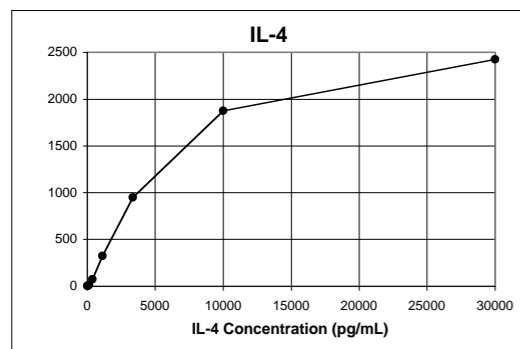
Refer to the Assay Layout Sheet to determine the number of wells to be used. **Place plate sealer over unused wells to facilitate efficient vacuum filtration.**

- Pipet 100 µL of Extracellular Assay Buffer 1 to the bottom of each well to be used. Aspirate with vacuum manifold. Tap the plate firmly on a lint free paper towel to remove any buffer at bottom of plate. **Important!** Correct pressure settings of the vacuum manifold are critical to ensure proper washing and recovery of the beads. Detailed instructions may be found in the Extracellular 1 Buffer Pack Instruction Manual.
- Pipet 25 µL of the diluted (1X) beads to the bottoms of the appropriate wells.
- Pipet 50 µL of Standards #1 through #7 or sample to the bottoms of the appropriate wells. **Important!** Include an extra replicate of Standard #1 to be used for Instrument Setup.
- Pipet 50 µL of Extracellular Assay Buffer 1 to the bottoms of the S0 (0 pg/mL standard) wells.
- Pipet 25 µL of Extracellular Assay Buffer 1 to the bottoms of each well to be used.

- Seal the plate and incubate for one hour with shaking* at room temperature.
- Aspirate plate on vacuum manifold and wash by adding 200 µL of wash buffer to every well. Place plate sealer over unused wells to facilitate efficient vacuum filtration. Repeat 2 more times for a total of 3 washes. After the final wash, remove the plate from the vacuum manifold and tap the plate firmly on a lint free paper towel to remove any residual buffer from the bottom of plate.
- Pipet 100 µL of the diluted (1X) IL-4 Antibody Conjugate into every well. Seal the plate and incubate for one hour with shaking at room temperature. During the incubation prepare cytometer for sample acquisition.
- Aspirate plate on vacuum manifold and wash by adding 200 µL of wash buffer to every well. Place plate sealer over unused wells to facilitate efficient vacuum filtration. Repeat 2 more times for a total of 3 washes. After the final wash remove the plate from the vacuum manifold and tap the plate firmly on a lint free paper towel to remove any residual buffer from the bottom of plate.
- Pipet 250 µL of wash buffer into each well. Transfer 250 µL of beads to 12x75 mm tubes by vigorously pipetting up and down 3X with the pipet tip near the membrane surface.
- If beads cannot be analyzed immediately they may be stored at 4°C protected from light for up to 18 hours.
- For flow cytometer setup and multiplex instruction refer to Extracellular Buffer Pack Instruction Manual available for download at www.assaydesigns.com/multibead.
* Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120 -700 rpm.

Assay Performance

Typical Data



The results shown below are for illustration only and should not be used to interpret results from another assay.

Sensitivity

The sensitivity, or limit of detection, of this assay is 19.9 pg/mL. It was determined by interpolation at 2 standard deviations above the mean signal at background, using data from 10 standard curves. The upper limit of detection was determined to be the point at which non-linear values were observed. For this assay the upper limit of detection is 10,000 pg/mL*.

* Performance testing indicated non-linearity of recovered values above this level. Any value above this level may be unreliable and may require further dilutions of the sample.

Dilutional Linearity

Samples were serially diluting into Extracellular Assay Buffer 1. Non-conditioned Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) was spiked with recombinant cytokines and synthetic eicosanoids and diluted in the assay buffer. The assay buffer was spiked to the same concentration and used as a control to determine linearity of the culture medium. Pools of human serum, plasma, and urine were also diluted in Extracellular Assay Buffer 1 to produce values within the dynamic range of the assay. The values represent the percent of the lowest dilution tested within the dynamic range of the assay.

Dilution Factor	Assay Buffer	Serum	Plasma	Urine	Medium
Neat	109	111	117	38	64
1:2	118	134	108	91	96
1:4	117	139	113	81	94
1:8	111	124	115	115	82
1:16	100	100	100	122	93

Based on the linearity data, the minimum required dilutions to eliminate matrix interference in the assay are listed below. Due to differences in samples, further dilution may be required.

A minimum 1:2 dilution in Extracellular Assay Buffer 1 is required for most serum and plasma samples. Most culture supernates without serum supplementation require a minimum 1:2 dilution in Extracellular Assay Buffer 1. No dilution is required for most supernates supplemented with 10% fetal bovine serum (FBS). A minimum 1:4 dilution in Extracellular Assay Buffer 1 is required for most urine samples.

Specificity

The reagents have been validated with human cytokines and the cross-reactivity with other species such as monkey, rat and mouse has not been tested. The antibodies used in the Inflammation (human) 8-plex MultiBead Panel have been screened for reactivity with their target molecules. Six immunometric bead sets were multiplexed in the presence of the Cytokine Antibody Cocktail. Individual cytokines (10,000 pg/mL) were then added to show that the cytokine did not bind to the other 5 antibody pairs. The 6-plex background PE fluorescence levels are shown in duplicate. The data demonstrates that the antibody pairs chosen are specific and do not cross-react with other cytokines.

Analyte	MultiBead Kit					
	IL-8	IL-1 β	IL-4	TNF α	IFN γ	IL-6
IL-8	4069	5.9	3.7	12.2	13.6	3.3
IL-1 β	4.0	1981	3.0	12.2	15.1	3.6
IL-4	4.5	5.5	1241	12.2	14.6	3.6
TNF α	5.0	5.5	3.5	1716	14.1	3.6
IFN γ	4.5	5.7	3.3	12.6	1949	3.6
IL-6	4.5	9.5	3.5	11.8	14.1	2996
Background	4.3	5.9	3.6	10.9	14.6	3.2
Background	4.1	5.9	2.9	12.2	13.6	3.2

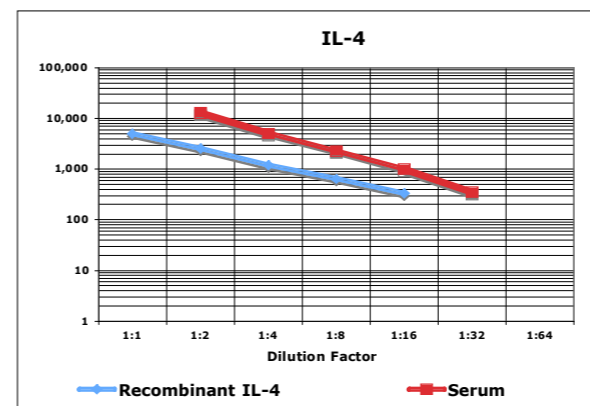
Spike and Recovery

After diluting each sample matrix to its minimum required dilution, recombinant analyte was spiked at high, medium and low concentrations. The recovery of each standard in spiked samples was compared to the recovery of identical spikes in the assay buffer. The mean of percent recovery at the three concentrations are indicated below for each matrix.

Sample Matrix (# of samples)	Minimum Required Dilution	Spike Concentration (pg/mL)	Recovery of Spike (Range)
Medium (n=1)	1:2	3333	124%
		370	94%
		50	38%
Plasma (n=5)	1:2	3333	103%
		370	129%
		50	46%
Serum (n=5)	1:2	3333	119%
		370	120%
		50	81%

Parallelism

Parallelism experiments were carried out to determine if the recombinant standards accurately determine analyte concentrations in biological matrices. To assess parallelism, the concentration of various analytes in serum samples were determined from a standard curve using four parameter logistic curve fitting. The observed concentration was plotted against the dilution factor. Parallelism of the curves demonstrates that the antibody binding characteristics are similar enough to allow the accurate determination of analyte levels in diluted samples.



Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing IL-4 in a single assay.

pg/mL	%CV
7735.7	8.2
1284.1	6.7
348.8	5.1

Inter-assay precision was determined by measuring buffer controls of varying IL-4 concentrations in multiple assays over several days.

pg/mL	%CV
7466.7	19.1
1417.9	6.5
333.7	12.3

Tips & Troubleshooting

- ✓ If buffers other than those recommended are used in the assay, the end-user must determine the appropriate dilution and validation requirements.
- ✓ Beads must be vortexed well prior to use.
- ✓ Pipet the reagents to the sides of the wells to avoid possible contamination.
- ✓ Use a fresh pipet tips for each sample, standard, and reagent.
- ✓ Insufficient washing or residual wash buffer in the wells may cause variation in assay results.
- ✓ All standards, controls, and samples should be assayed in duplicate.
- ✓ Do not invert the plate when tapping residual buffer off the bottom of the plate during wash procedure.
- ✓ Seal unused wells during wash procedure to maintain vacuum efficiency.
- ✓ Correct pressure settings of the vacuum manifold are critical to ensure proper washing and recovery of the beads. Detailed instructions may be found in the Extracellular 1 Buffer Pack Instruction Manual.
- ✓ Seal the plate tightly with provided plate sealer to avoid leakage during incubations.
- ✓ Flow cytometer setup may vary depending on which instrument is used.
- ✓ If a well does not aspirate during filtration pipet vigorously up and down to remove the clog taking care not to puncture the membrane.

Limited Warranty

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

Assay Designs must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if Assay Designs is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy to the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.