Diaclone DIAplex

Human Th1 / Th2 / Inflammation

Instructions for use

For research use only

Fast Track Your Research…

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1. Intended use

The DIAplex Human Th1 / Th2 / Inflammation Kit is a multiplexed fluorescent bead-based immunoassay for the quantification of multiple human cytokines (IFNγ, TNFα, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70 and IL-17A) in plasma, serum and culture supernatants by Flow Cytometry.

This kit has been configured for research use only and is not to be used in diagnostic or therapeutic procedures.

2. Introduction

2.1. Summary

Different populations of CD4+ T-Cells secrete differing patterns of cytokines that can lead to differing immune responses. The T Helper Type-1 (Th1) or Type-2 (Th2) cell cytokines can direct an antigenic response towards either a cell mediated or a humoral immune response respectively. The Th1 and Th2 cytokines cross regulate each other providing a mechanism for regulation of immune responses. A new subset of T helper cells that predominantly produce IL-17 (Th17 cells) are believed to be the major cell type involved in the induction of various pro-inflammatory cytokines.

DIAplex is a sensitive multiplex fluorescent bead-based immunoassay for the simultaneous quantification of multiple analytes from a single sample by Flow Cytometry. Utilising bead populations with distinct fluorescence intensities and unique antibody specificities DIAplex Human Th1 / Th2 / Inflammation kits can accurately measure multiple T helper cytokines in a single sample with a significantly reduced assay time and sample volume requirement compared to traditional ELISA techniques. Providing accurate cost effective and time saving cytokine quantification from a reduced sample volume.

Flexible by design

Any number of the following cytokines can be tested depending on your panel of choice:
IFNγ, TNFα, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70 and IL-17A.

DIAplex Complete provides multiplex assay sets which are preconfigured for the detection of a panel of analytes for investigation of a specific scientific area. An individual experiment can provide measurements of several analytes from a single sample. DIAplex Accessory Kit supplied free of charge.

DIAplex Complete ready configured detection panels:

Panel	Th1	Th2	Th1 / Th2	Inflammation	Th1 / Th2 / Inflammation
Cat No.	880.110.004	880.120.003	880.140.007	880.130.004	880.100.010
IFNγ	●		●		●
IL-2	●		●		●
IL-12p70	●		●		●
TNFα	●		●	●	●
IL-4		●	●		●
IL-6		●	●		●
IL-10		●	●		●
IL-8				●	●
IL-17a				●	●
IL-1β				●	●

DIAplex Design combines the benefits of multiplexing with the ability to design your own analyte detection panel. The assays have been designed for use individually (simplex) or combined to create any size flex system. The DIAplex Accessory Kit is not included with DIAplex design, it needs to be ordered individually.

DIAplex Design design your own panel from the following:

☑		Cat no.	☑		Cat no.
☐	IFNγ	880.000.001	☐	IL-4	880.020.001
☐	IL-2	880.010.001	☐	IL-17a	880.940.001
☐	IL-10	880.060.001	☐	IL-1β	880.006.001
☐	IL-8	880.050.001	☐	TNFα	880.090.001
☐	IL-6	880.030.001	☐	IL-12p70	880.070.001

2.2. Principle of the method

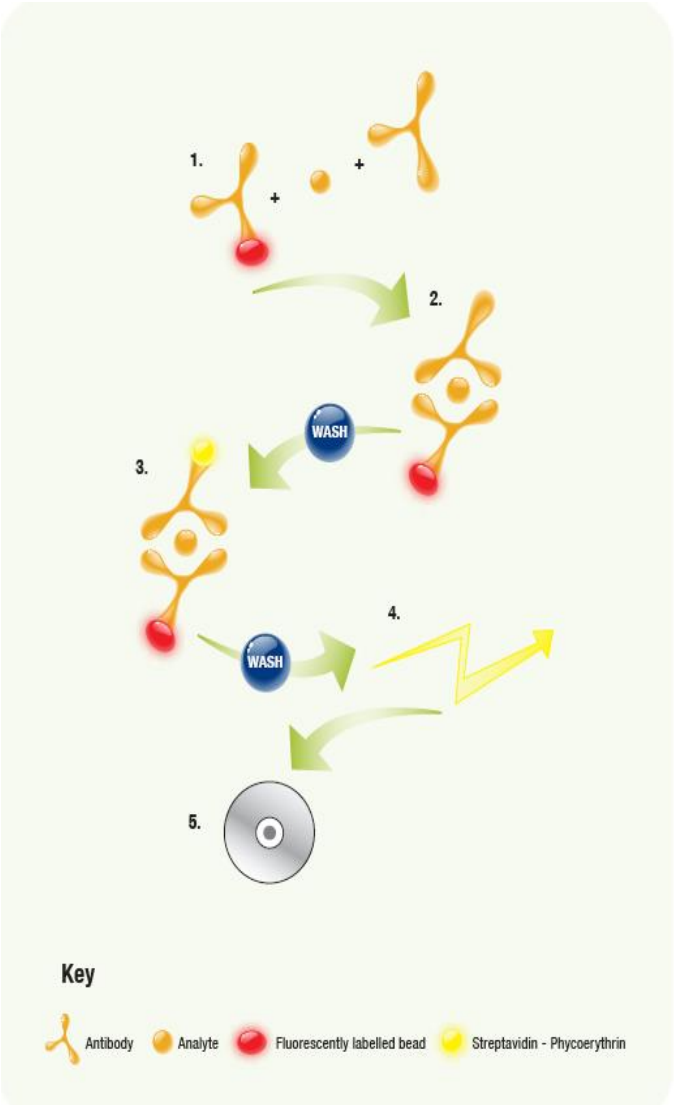
1 to 2. Antibodies highly specific to the analytes of interest are provided coupled to fluorescently labelled detection beads. One (simplex) or more (multiplex) populations of bead/antibody complexes are combined in suspension with the sample(s) or standard(s) under test. Any analytes present in the sample will bind to their specific bead/antibody complexes. A biotin-conjugated secondary antibody mixture is also added which binds specifically to any analytes captured by the primary antibodies.

3. Streptavidin-Phycoerythrin (PE) is added, which binds to the biotin conjugates and emits a yellow fluorescent signal.

4. The unique size and fluorescent signature of each bead is differentiated by flow cytometry.

5. Following sample data acquisition using a flow cytometer, analyte concentration in the test sample is calculated simply by using the DIAplex analysis software provided.

Successfull performance of DIAplex has been reported using FC500, Navios and Gallios from Beckman Coulter; FacsCalibur, FacsCanto II and LSR II from BD, Guava Easy Cyte Plus from Millipore, and MACSQuant Analyser from Miltenyi Biotec.



Sufficient reagents are supplied to perform a total of 96 tests for each of the chosen antibody specificities (including standard curves) which can be split into a maximum of two experiments.

3. Reagents provided

3.1. DIAplex detection sets

The exact DIAplex detection sets supplied will depend on the 'DIAplex Design' or 'DIAplex Complete' set ordered but will comprise one or more of the following antibody specificities:

Antibody specificity	Bead Population	Bead Size / Region
h IL-12	L10 (4µm)	R2 (small)
h IL-6	L9 (5µm)	R1 (large)
h TNFα	L8 (4µm)	R2 (small)
h IL-8	L7 (5µm)	R1 (large)
h IL-1β	L6 (4µm)	R2 (small)
h IL-10	L5 (5µm)	R1 (large)
h IL-17A	L4 (4µm)	R2 (small)
h IL-2	L3 (5µm)	R1 (large)
h IL-4	L2 (4µm)	R2 (small)
h IFNγ	L1 (5µm)	R1 (large)

Note: The beads are internally dyed with different intensities of fluorescent dye. L1 to L10 correspond to these differing levels of red-fluorescence, L1 is the lower and L10 the higher.

Each detection set contains the following, per antibody specificity:

- 1 vial (300 µl) Fluorescent Beads coated with specific primary Antibody
- 2 vials lyophilized Standard
- 1 vial (150 µl) Biotin-Conjugate (specific secondary Antibody conjugated to biotin)

3.2. DIAplex accessory kit

Each 'DIAplex Complete' set is supplied with an accessory kit containing the following:

- 1 bottle Assay Buffer 10X (5 ml)
- 1 bottle Wash Buffer 10X (5 ml)
- 1 bottle PBS 10X (5 ml)
- 1 vial Streptavidin-PE
- 1 bottle Serum Sample Buffer (3 ml)

4. Materials required but not provided

- A Flow Cytometer equipped with a Argon or He-Ne laser (488 nm or 532 nm), and capable of detecting and distinguishing fluorescence emissions at 575nm and at 695-690 nm (far red).
- Filter vacuum 96 well plate or 96 well V bottomed microtitre plates or appropriate tubes
- Adhesive Film for microtitre plates
- Manifold vacuum system or Centrifuge (and if required an adaptator to centrifuge plates)
- Sample acquisition tubes for a Flow Cytometer
- Aluminium foil
- 5 ml and 10 ml graduated pipettes
- 100 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 40 µl to 200 µl adjustable single channel micropipettes with disposable tips
- 5 µl to 40 µl adjustable single channel micropipettes with disposable tips
- 1 µl to 10 µl adjustable single channel micropipettes with disposable tips
- Beakers, flask's, measuring cylinders necessary for preparation of reagents
- Distilled water
- Vortex mixer
- Microplate shaker
- Appropriate shaker for incubations using tubes
- DIAplex Pro 1.0 Software - complimentary and can be ordered from Diaclone or downloaded from www.diaclone.com

5. Storage Instructions

Store all components of this kit between 2°C and 8°C. The expiry date of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by previous handling.

Diluted Assay Buffer, Wash Buffer and PBS
Once diluted the buffers can be stored in a sealed container for up to 1 month at 4°C. These buffers should be filtered before use.

Serum Sample Buffer
Ready to use. Store at + 4°C.

Bead Mixture
Once the bead mixture has been prepared it can be stored protected from light at 4°C but must be used within 2 hours.

Biotin-Conjugate
Once the biotin-conjugate mixture has been prepared it can be stored protected from light at 4°C but must be used within 2 hours.

Standards
Once reconstituted, standard vials and standard dilutions should be used immediately, any remaining solution should be discarded, do not freeze or re-use reconstituted standard vials or standard dilutions.

Streptavidin- PE
The Streptavidin-PE should be prepared immediately before use, protected from light throughout and any remaining solution discarded after use.

Sample Storage
Samples must be analysed by Flow Cytometry within 24 hours of preparation.

6. Specimen collection, processing & storage

Cell culture supernatant, Serum and Plasma have been validated for use with this assay. Other biological samples may also be suitable for use with the assay kit.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Serum: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

- Reagents are intended for in vitro use and are not for use in diagnostic or therapeutic procedures.
- All chemicals in this kit should be considered as potentially hazardous. We therefore recommend that this product is handled only by persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- Reagents containing preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times.
- Respect incubation times described in the assay procedure.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.

8. Reagent Preparation

8.1. Assay Design

Calculate the appropriate number of tests for your desired protocol.

We recommend that for each DIAplex assay a full set of standards (7) and a zero are run in duplicate, with one additional 5000 pg/mL standard and one additional zero test prepared for Flow cytometer set up.

For example, for the analysis of 10 samples (in duplicate) the following number of tests is required:

Condition	Repetition	No. of tests
8 standards (includes zero)	X 2	16
Zero for FC set up	x 1	1
5000 pg/mL for FC set up	x 1	1
10 Samples	x 2	20
Total Number of Tests		**38**

Note: Bring the required DIAplex detection sets and all the DIAplex accessory reagents to room temperature before use

8.2. Buffer Preparation

- Mix by inversion the contents of the bottle labelled Assay Buffer (10X). Add contents (5.0 ml) **Assay Buffer (10X)** to 45 ml distilled water and mix gently to avoid foaming. Store at 2° to 8°C and filter before use (0.2μm).

- Mix by inversion the contents of the bottle labelled Wash Buffer (10X). Add contents (5.0 ml) **Wash Buffer (10X)** to 45 ml distilled water and mix gently to avoid foaming. Store at 2° to 8°C and filter before use (0.2μm).

- Mix by inversion the contents of the bottle labelled PBS (10X). Add contents (5.0 ml) **PBS (10X)** to 45 ml distilled water and mix. Store at 2° to 8°C and filter before use (0.2μm).

8.3. Bead Mixture Preparation

a) Calculate the final volume of the Bead Mixture required: **Final volume = total number of tests x 25µl**
b) Round up the volume required for pipetting reservoir:
e.g. for 38 tests: final volume = 38 x 25 µl = 950 µl, round up to final volume = 1000µl

c) Vortex each individual Bead vial.
d) Pipette 1/10 of the final volume required of each bead suspension into a vial labelled “Bead Mix”
e.g. for a final required volume of 1000µl, add 100µl of each vial of beads.
e) Fill up to the final volume with Assay Buffer if needed (If all ten analytes are to be detected no addition of assay buffer is required).

When assaying serum or plasma samples, follow these additional steps:
f) Spin the beads mixture 5 min at 2000 g.
g) Remove the majority of the supernatant leaving 50µl in the tube.
e.g. for a volume of 1000µl, carefully remove 950 µl
h) Add with the same volume of Serum Sample Buffer and resuspend the beads.
e.g. if 950 µl was removed in step g) replace with 950 µl of Serum Sample Buffer.

Note: Vortex “Bead Mix” before use and always protect bead mixture from light and use within 2 hours of preparation.

8.4. Biotin-Conjugate Antibody Mixture Preparation

- Calculate the final volume of the Biotin-Conjugate Mixture required:
  Final volume required = total number of tests x 25µl
- Round up the volume required for pipetting reservoir:
  e.g. for 38 tests: final volume = 38 x 25 µl = 950 µl, round up to final volume = 1000µl

- Pipette 1/20 of the final volume of each Biotin-Conjugate into a vial labelled “Biotin-Conjugate Mixture”.
  e.g. for a final required volume of 1000µl, add 50µl of each biotin conjugate.
- Fill up to the final volume with Assay Buffer.

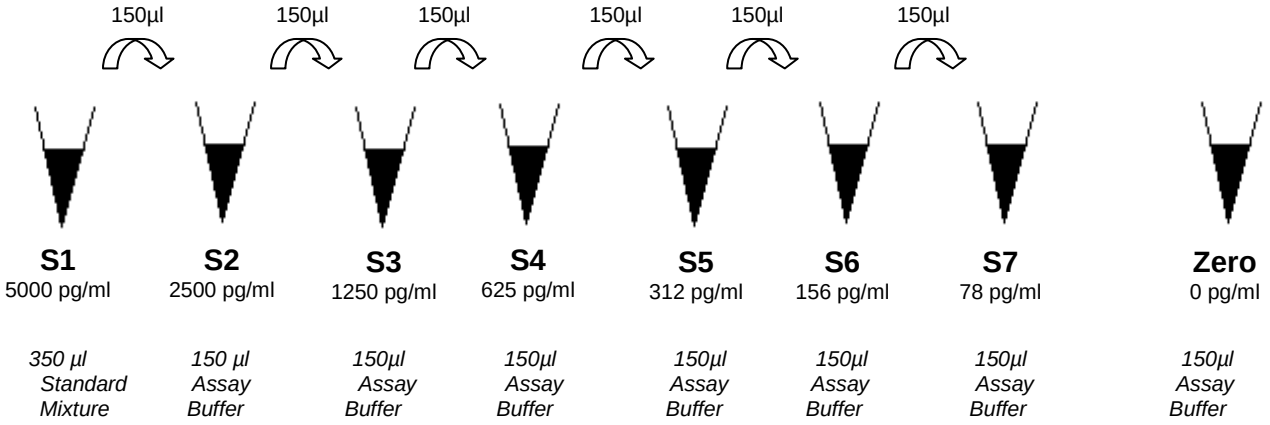
8.5. Standard Preparation

Reconstitute each of the required standard vials with the volume indicated on the vial. Standards must be prepared immediately prior to use. **DO NOT VORTEX** standard vials.

- Resuspend each individual standard vial with Assay Buffer.
- Add 35µl of each required standard into a vial labelled standard 1 (S1) this represents a 1/10 dilution of each reconstituted standard.
- Fill up to a final volume of 350µl with Assay Buffer if required (If all ten standards are used no additional assay buffer is required).
- This vial (S1) provides the top concentration standard of 5000 pg/ml of each analyte tested.

Perform 1:1 serial dilutions to achieve standard concentrations of 5000 to 78 pg/ml:

- Add 150µl Assay Buffer to 7 tubes labelled S2 to S7 and Zero
- Transfer 150µl of S1 to tube S2, mix gently by pipetting. Then transfer 150µl of S2 to tube S3 and so on down to tube S7, providing a standard curve ranging from 5000pg/ml to 78pg/ml.



S1:	35µl of each reconstituted standard, fill up to the final volume of 350µl with Assay Buffer if necessary
S2:S7:	1:1 serial dilutons into assay buffer from S1
Zero:	Assay buffer only

8.6. Streptavidin-PE Preparation

Streptavidin-PE must be prepared immediately before use (see section 9).

- Calculate the final volume of the Streptavidin-PE required: **Final volume required = total number of tests x 50µl or x 100µl when assayed with Serum Sample Buffer**
- Fill up the volume required for pipetting reservoir:
  e.g. for 38 tests: final volume = 38 x 50 µl = 1900 µl, round up to final volume = 2500µl
  or 38 x 100 µl = 3800 µl, round up to final volume = 4000µl when assayed with Serum Sample Buffer

- Dilute the concentrated Streptavidin-PE in Assay Buffer per ratio indicated on the vial.
  e.g. PE to dilute at 1/1000 : Add 2.5 µl into 2500 µl Assay Buffer or 4µl into 4000µl

9. Assay Method

9.1. Using tubes

Prepare all reagents as detailed in Section 8.

We recommend you appropriately label a required number of tubes. Be sure to include the required number of Standards and Zeros, ensuring additional Zero and S1 tubes for Analysis set up are included (see section 8.1.).

It is important to perform assay additions in the order outlined below and all incubation steps must be performed protected from light.

Note: The bead mixture and the Biotinylated Antibody mixture once prepared can be mixed in the same tube and then 50 µl of this solution added to all tubes.

Assay step		Detail
Prepare Bead Mixture, Biotin-Conjugate Mixture, and the standards (see section 8.3., 8.4. and 8.5.)		
1.	addition	Add 50µl of **Assay Buffer** (used as Zero Standard), **Standards, Samples and Flow Cytometer set-up solutions** to appropriate tubes.
2.	addition	Add 25µl of **Bead Mixture** to all tubes
3.	addition	Add 25µl of **Biotin-Conjugate Mixture** to all tubes
4.	incubation	Protect tubes from light with aluminium foil Incubate on an appropriate shaker (500rpm) at room temperature (18° to 25°C) for **2 hours when cells supernatants are assayed** Or Incubate on an appropriate shaker (500rpm) at room temperature (18° to 25°C) for **3 hours when serum samples are assayed**
5.	wash	a) Centrifuge for 1 min at 1200g at room temperature (18° to 25°C) b) Carefully discard the supernatant c) Add 200µl of **Washing Buffer** to all tubes. d) Repeat once steps a and b
6.	preparation	Prepare Streptavidin-PE just before use (see section 8.6.)
7.	addition	Add **50µl of Streptavidin-PE** to all tubes when cell supernatants samples are assayed Or Add **100µl of Streptavidin-PE** to all tubes when serum samples are assayed
8.	incubation	Protect tubes from light with aluminium foil and Incubate on an appropriate shaker (500rpm) at room temperature (18° to 25°C) for **1 hour**
9.	wash	Repeat wash step 5.
10.	addition	Add 200µl of **PBS** to all tubes
11.	wash	Repeat wash steps 5 a and b only
12.	addition	Add 50µl of **PBS** to all tubes
13.	transfer	Transfer into appropriately labelled clean Flow Cytometer acquisition tubes
14.	addition	Add 200µl of **PBS** to all tubes
Samples are now ready for analysis using a suitable Flow Cytometer, always keep samples protected from light prior to analysis. The tubes **must be analysed within 24 hours** following the completion of step 14.		

Before analysis the Flow Cytometer requires appropriate calibration, please refer to Section 10.1 (Instrument set-up) of this instruction manual.

9.2. Using a plate (filter plate or V-bottomed)

Prepare all reagents as detailed in Section 8.

We recommend the creation of a plate map to identify the position of all tests including the required number of Standards and Zeros, ensuring additional Zero and S1 wells for Flow Cytometer set up are included (see section 8.1).

It is important to perform assay additions in the order outlined below and all incubation steps must be performed protected from light.

Note: The bead mixture and the Biotinylated Antibody mixture once prepared can be mixed in the same tube and then 50 µl of this solution added to all wells.

Assay step		Detail
Prepare Bead Mixture, Biotin-Conjugate Mixture, and the standards (see section 8.3., 8.4. and 8.5.)		
1.	addition	Add 50µl of **Assay Buffer** (used as Zero Standard), **Standards, Samples and Flow Cytometer set-up solutions** to appropriate wells.
2.	addition	Add 25µl of **Bead Mixture** to all wells
3.	addition	Add 25µl of **Biotin-Conjugate Mixture** to all wells
4.	incubation	Protect tubes from light with aluminium foil Incubate on an appropriate shaker (500rpm) at room temperature (18° to 25°C) for **2 hours when cells supernatants are assayed** Or Incubate on an appropriate shaker (500rpm) at room temperature (18° to 25°C) for **3 hours when serum samples are assayed**
5.	wash	a) Centrifuge for 1 min at 1200g at room temperature (18° to 25°C) and carefully discard the supernatant OR b) Apsirate the supernantant with vaccum manifold system c) Add 100µl of **Washing Buffer** to all wells including the blank d) Repeat once steps a or b
6.	preparation	Prepare Streptavidin-PE just before use (see section 8.6.)
7.	addition	Add **50µl of Streptavidin-PE** to all tubes when cell supernatants samples are assayed Or Add **100µl of Streptavidin-PE** to all tubes when serum samples are assayed
8.	incubation	Protect the plate from light with aluminium foil and Incubate on a plate shaker (500rpm) at room temperature (18° to 25°C) for **1 hour**
9.	wash	Repeat wash step 5
10.	addition	Add 100µl of **PBS** to all wells
11.	wash	Repeat wash steps 5 a and b only
12.	addition	Add 200µl of **PBS** to all wells
Samples are now ready for analysis using a suitable Flow Cytometer, always keep samples protected from light prior to analysis. The plate **must be analysed within 24 hours** following the completion of step 12.		

Before analysis the Flow Cytometer requires appropriate calibration, please refer to Section 10.1 (Instrument set-up) of this instruction manual.

10. Cytometer Set Up & Data Acquisition

10.1. Instrument set up

Ensure additional Zero and S1 tubes/wells are available to enable Cytometer setup (See Section 8.1).

These tubes/wells are used for the following

- Adjusting FSC & SSC parameters
- Creating regions for the two bead populations (R1 & R2)
- Defining the number of events counted
- Ensuring bead population of S1 is visible on the screen

With every new experiment before starting sample acquisition adjust Cytometer parameters in SETUP mode.

- Perform instrument start up following the manufacturers instructions
- Perform a flow check as per the manufacturers instructions
- Create a new protocol
- Create a Dot Plot window with FSC (Forward Scatter) on X-axis and SSC (Side Scatter) on Y-axis. Set FCS to Log mode and SSC to Linear mode (Figure 1).
- Create a second and a third Dot Plot windows with FL-2 (Yellow) on X-axis and FL-3 (Red) on Y-axis. See examples shown below.

	Y-axis (Red)	**X**-Axis (Yellow)
BD Facscalibur	FL-3	FL-2
BD Facscanto II	PerCP-Cy5.5 or APC	PE
Guava Technologies	RED-Hlog	YLW-Hlog
Beckman Coulter FC500	FL-4	FL-2

Note: FL Channel number for the specific wavelength range will vary depend on the instrument used

Successfull performance of DIAplex has been reported using FC500, Navios and Gallios from Beckman Coulter; FacsCalibur, FacsCanto II and LSR II from BD, Guava Easy Cyte Plus from Millipore and MACSQuant Analyser from Miltenyi Biotec.

- Set FL-2 and FL-3 to Log mode (Figure 2.)

Figure 1.

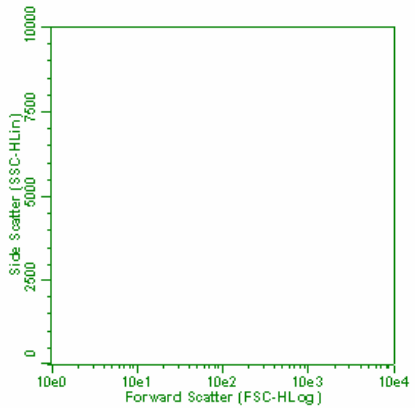
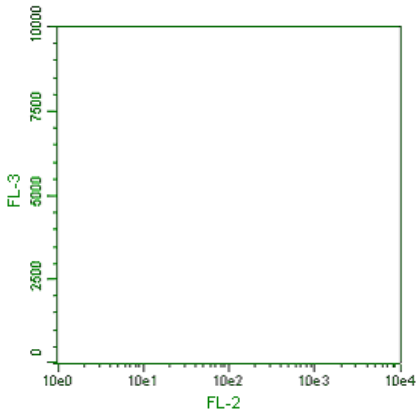


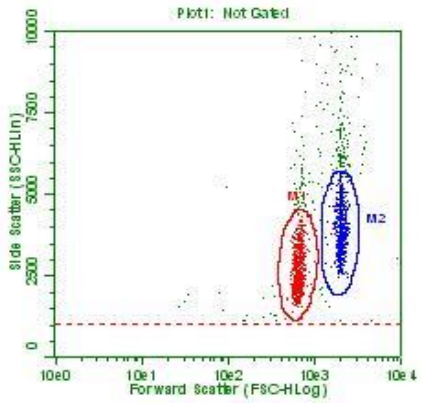
Figure 2.



- Adjust the parameters of FSC and SSC to obtain two distinct populations of beads in the **Zero set up tube**
- Create regions R1 for the large bead population and R2 for the small bead population. Both bead populations should be visible in these regions (refer to Figure 3.).

We recommend to pause and restart acquisition frequently until obtaining results similar to those shown in figure 3 with two clear and distinct size populations.

Figure 3.



- Select gate R1 for the large bead population in the first FL-2/FL-3 Dot plot (Figure 4.)
- Select gate R2 for the small bead population in the first FL-2/FL-3 Dot plot (Figure 5.)

Figure 4.

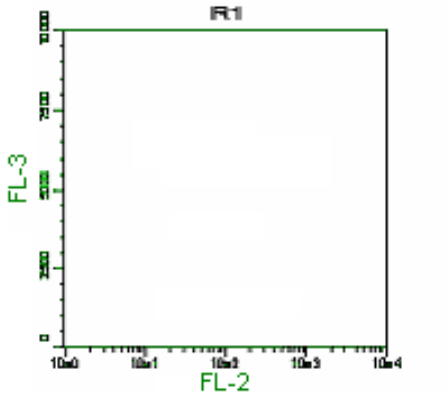
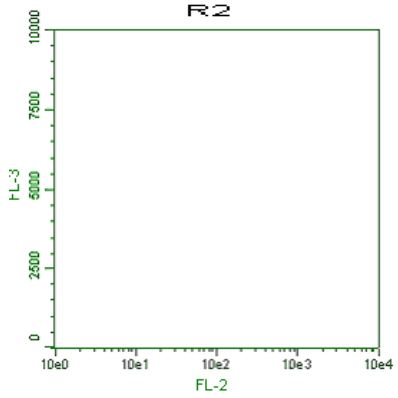


Figure 5.



- Create areas for each bead population M1 – M5 (Figure 6. & Figure 7.)

Figure 6.

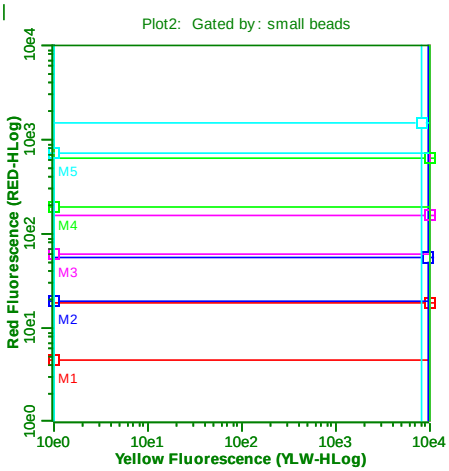
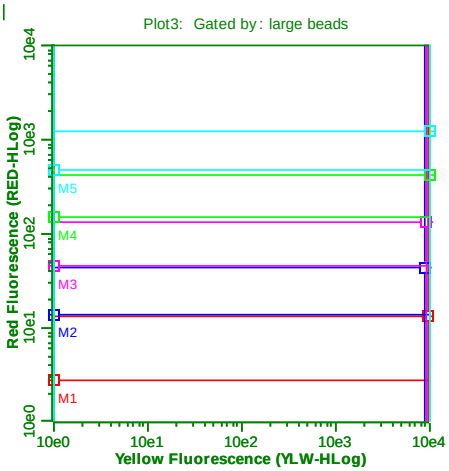


Figure 7.



Before starting the acquisition of standards and samples, adjust the settings using the blank and S1 set up tubes/wells :

- Using the blank tube, adjust the voltage of FL-2 to move the bead populations to the left side of the plot and adjust the voltage of far red emissions to generate clearly separated bead populations.

Figure 8

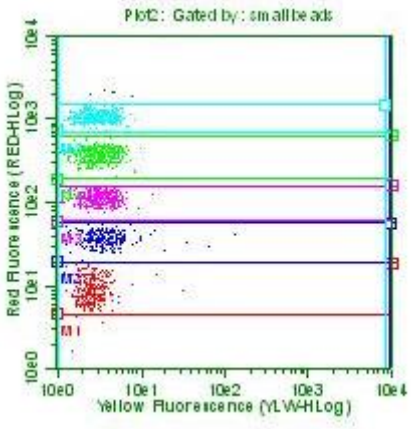
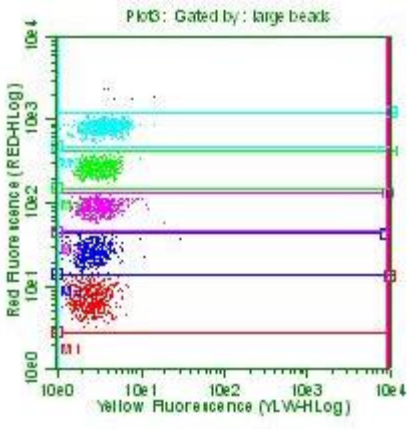


Figure 9



- Using the S1 Set up tube, adjust the voltage of FL-2 until the Bead population with the highest PE (FL-2) signal is on the right hand axis (see Figure 8. & Figure 9.). It is important to place the bead populations towards the right side of the acquisition plot to optimise the distribution of each standard concentrations across the FL-2 scale.

Figure 10

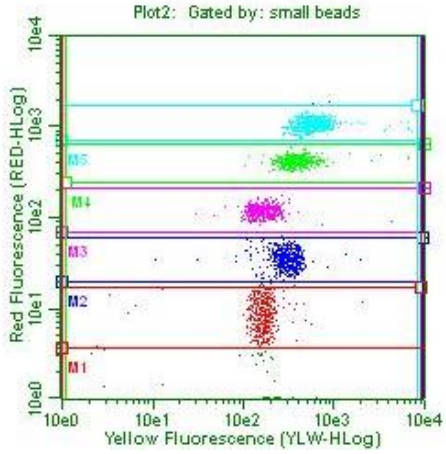
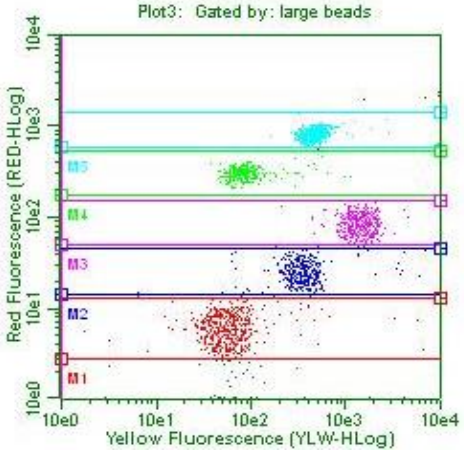
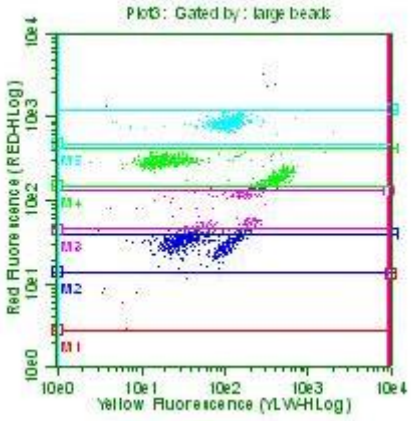


Figure 11



- Adjust (increase if tailing up or decrease if tailing down) the compensation setting for FL-3 - %FL-2 if the bead populations are not in a horizontal position (see below).

Figure 12



- Following the above adjustments please re-check using the extra blank tube/well that the blank bead populations have not moved too from Y-axis. Some movement will occur due to the FL-2 voltage increase, however this movement must allow the blank population to remain as close to Y-axis as possible and must be within the first decade (log scale), following set-up of the S1 populations between the third and forth decade.

10.2. Example final setup settings

Please note these settings should be used as a guidance and the setup sequence described in Section 10.1 should be performed for each new experiment, and settings optimised in each individual laboratory.

BD Facscalibur

Detector	Voltage	Amp Gain	Mode
FSC	E00	5.4	LIN
SSC	320	2.00	LIN
FL-1	601	1.00	LIN
FL-2	650	1.00	LOG
FL-3	611	1.00	LOG

Compensation		
FL-1	0	% FL-2
FL-2	0	% FL-1
FL-2	0.2	% FL-3
FL-3	13.0	% FL-2

No Threshold

BD Facscanto II

Detector	Voltage	Mode
FSC	77	LOG
SSC	346	LIN
PE	401	LOG
PerCP-Cy5.5	527	LOG

Compensation		
PE	0.0	% PerCP-Cy5.5
PerCP-Cy5.5	19.8	% PE

Threshold : 500

Guava Technologies Easy Cyte Plus

Gain and PMT Voltage	
FSC	X32 min-max scale 136%
SSC	380V
FL-1	586V
FL-2	495V
FL-3	706V
NIR	514V

Compensation
All to 0% except RED-% Yello : 20%

10.3. Acquisition

DO NOT USE your in-house set-up, section 10.1 must be followed for optimal results.

- Switch from SETUP Mode to ACQUISITION Mode
- Ensure previously optimised (section10.1) settings are retrieved
- Define/set the number of events so that the recommended 300 events per analyte are measured. (And only collect events within the previously identified R1 and R2 regions)

  Note: it is not necessary to acquire more events a range between 200-400 events per analyte is advised otherwise hugh data files will be generated which will significantly inhibit data analysis.

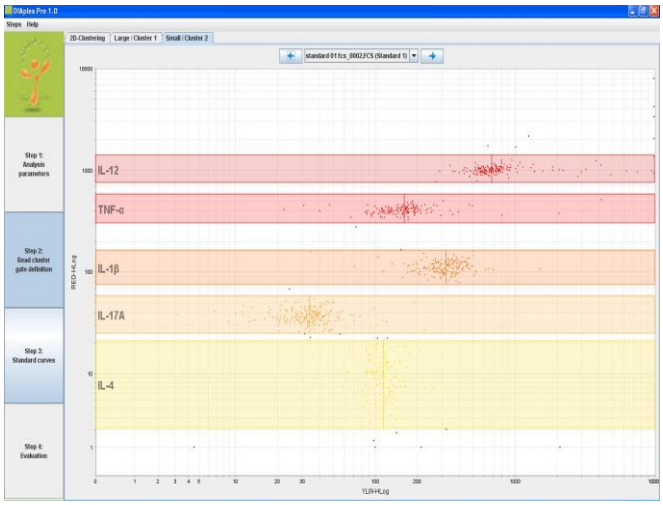
- Begin analysing the test samples in the following recommended order:
  1. Negative control tube (zero)
  2. Standards (S1 – S7)
  3. Samples (unknowns)

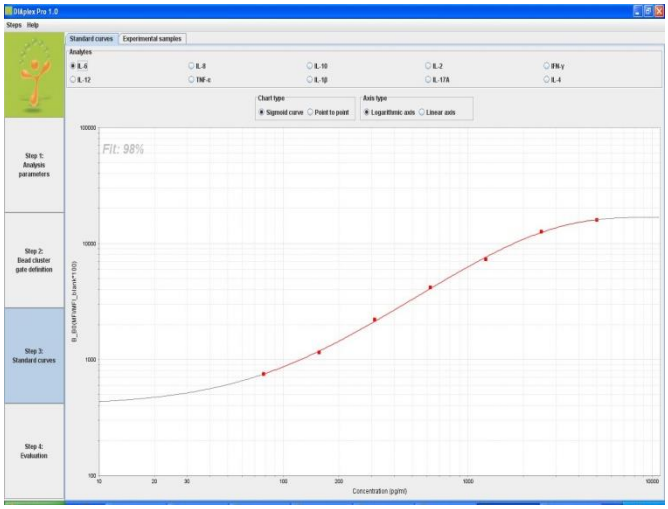
When saving/naming acquisition files to simplify auto file loading in the DIAplex Pro 1.0 Software we advise to save all measured data with the same file name and consecutive numbering (e.g. Sample.001, Sample.002, Sample.003, …).

11. Data Analysis

For analysis of data please refer to the DIAplex Pro Software instructions

DIAplex Pro Software and instructions - complimentary and can be ordered from Diaclone or downloaded from www.diaclone.com





12. Typical Data

Representative standard curves for each analyte are shown below. These curves should not be used to derive test results. A standard curve must be run for each group of samples assayed. Note this 'Typical Data' was gathered using the plate method described above and the results acquired using a Guava easy cyte plus flow cytometer.

Concentration pg/ml	Fluorescent Intensity				
	IL-12p70	TNFα	IL-1β	IL-17A	IL-4
0	4.27	3.73	6.66	4.35	2.95
78	39.79	17.80	9.71	15.74	8.66
156	66.11	34.79	12.46	28.83	13.34
312	118.10	67.92	18.52	48.26	20.90
625	214.93	140.12	40.50	95.28	35.70
1250	323.42	241.45	95.69	161.93	50.93
2500	433.51	472.45	260.83	278.82	75.13
5000	510.62	675.09	604.68	387.19	93.95

Concentration pg/ml	Fluorescent Intensity				
	IL-6	IL-8	IL-10	IL-2	IFNγ
0	3.66	4.25	4.27	3.16	3.11
78	59.35	7.90	23.03	4.75	6.04
156	116.14	11.41	42.10	6.79	11.68
312	195.44	21.10	76.70	11.54	26.00
625	349.24	39.77	150.24	24.70	65.52
1250	568.89	71.21	276.49	55.35	155.44
2500	906.12	110.03	502.54	116.33	372.64
5000	1380.81	149.20	777.01	214.10	685.51

13. Limitations

The statistically determined Limit of Detection (LOD) for each analyte using the DIAplex Human Th1 / Th2 / Inflammation kit has been found to be comparable with conventional Diaclone ELISA for the analytes under test. The actual LOD of an analyte in a given experiment may vary slightly due to the complexity and kinetics of multiple-analyte analysis (See section 14.1).

Quantitative results or protein levels for the same sample run in the DIAplex Human Th1 / Th2 / Inflammation kit and in Diaclone ELISA assays may differ. Possible differences in quantification can be investigated by performing spike recover experiments (See Section 14.5).

A standard curve must be run for each group of samples assayed as exact conditions may vary from assay to assay.

14. Performance Characteristics

The DIAplex Human Th1 / Th2 / Inflammation kit has been rigorously tested in house and routinely generates the typical values shown here. However, please note that each laboratory using this product should establish its own performance characteristics, and these may vary from those presented in the manual.

14.1. Sensitivity

The theoretical limit of detection (LOD) of each analyte was statistically determined by extrapolation of the mean fluorescent of the Zero (average fluorescence of a minimum of 36 Zero's in one session) + 2 times the standard deviation of the fluorescence results.

Sensitivity (LOD)	IFNγ	1.5 pg/mL	IL-4	13.6 pg/mL
	IL-2	3.9 pg/mL	IL-17a	1.7 pg/mL
	IL-10	1.1 pg/mL	IL-1β	2.6 pg/mL
	IL-8	10.6 pg/mL	TNFα	3.6 pg/mL
	IL-6	0.8 pg/mL	IL-12p70	1.6 pg/mL

14.2. Specificity

Testing of each analyte individually, found no cross reactivity with the other analytes in the DIAplex range.

14.3. Reproducibility

Intra-assay

The within assay reproducibility was evaluated by measuring analyte concentration in 6 replicates of a recombinant protein spiked pool human serum and one in Assay buffer. Mean CV = **5.4%**

The data below shows the mean intra-assay coefficient of variation for each analyte.

Sample 1 Serum	mean (pg/ml)	SD (pg/ml)	CV (%)	Sample 2 Buffer	mean (pg/ml)	SD (pg/ml)	CV (%)
IL-1β	1046.9	26.6	2.6	**IL-1β**	1622.2	34.1	2.2
IL-2	784.9	42.1	5.3	**IL-2**	893.1	46.0	5.1
IL-4	693.1	35.9	5.1	**IL-4**	870.4	53.7	6.2
IL-6	1547.8	97.9	6.3	**IL-6**	949.0	43.6	4.5
IL-8	648.6	53.1	8.2	**IL-8**	968.1	53.7	5.5
IL-10	923.5	73.1	8.1	**IL-10**	1262.7	33.2	2.9
IL-12	486.2	29.8	5.8	**IL-12**	775.8	28.7	3.8
IL-17A	485.0	41.6	8.7	**IL-17A**	1439.9	78.1	5.3
IFNγ	1214.3	72.2	6.3	**IFNγ**	1398.8	39.4	2.9
TNFα	298.7	25.8	8.6	**TNFα**	1477.9	59.6	3.9
						Overall Mean CV	**5.4%**

Inter-assay

The assay to assay reproducibility was evaluated in 3 independent experiments by measuring analyte concentration in 6 replicates of 2 differing samples. Mean CV = **6.0%**

The data below shows the mean inter-assay coefficient of variation for each analyte.

Sample 1	Expt 1	Expt 2	Expt 3	Mean	CV Mean (%)
IL-1β	1042.5	1020.4	1084.9	1049.3	3.8
IL-2	792.0	795.3	810.3	799.2	5.1
IL-4	658.6	624.4	640.3	641.1	4.9
IL-6	1484.8	1436.3	1463.7	1461.6	5.0
IL-8	663.2	661.0	660.7	661.6	8.0
IL-10	980.2	985.3	1000.5	988.7	7.0
IL-12	397.8	403.7	498.0	429.4	11.8
IL-17A	431.1	416.4	383.1	411.8	8.8
IFNγ	1252.4	1394.5	1456.8	1367.9	8.4
TNFα	294.5	284.4	281.5	286.8	7.5
				Overall Mean CV	**7.0 %**

Sample 2	Expt 1	Expt 2	Expt 3	Mean	CV Mean (%)
IL-1β	1704.1	1604.3	1713.7	1674.0	3.8
IL-2	909.8	916.7	917.8	914.8	5.5
IL-4	850.4	867.7	898.4	872.2	5.7
IL-6	958.6	946.0	959.8	954.8	4.7
IL-8	1023.4	985.4	1021.1	1010.0	5.8
IL-10	935.0	953.8	948.9	945.9	3.9
IL-12	1149.1	1088.2	1139.2	1125.5	4.7
IL-17A	1455.5	1500.9	1469.3	1475.2	5.0
IFNγ	1500.7	1658.5	1505.3	1554.8	5.7
TNFα	1596.4	1649.2	1539.6	1595.1	4.6
				Overall Mean CV	**4.9 %**

14.4. Linearity

Cell culture supernatants were spiked with two levels of analyte and serially diluted in assay buffer to assess measurement linearity.

Analyte	Dilution	Serum (pg/ml)	Plasma (pg/ml)	Culture medium
IL-1β	**Neat**	1129,4	1083,6	1445,6
	1 :2	491,4	509,1	695,9
	1 :4	122,1	137,0	338,6
	1 :8	10,0	23.0	113,4
	R^2	0.998	0.997	0.999
IL-2	**Neat**	1505,6	1782,3	1390,2
	1 :2	801,9	864,8	737,0
	1 :4	370,1	403,9	409,1
	1 :8	184,3	201,0	204,7
	R^2	0.998	1.000	0.999
IL-4	**Neat**	641,6	224,8	1387,0
	1 :2	388,1	150,5	687,0
	1 :4	239,6	86,2	380,7
	1 :8	114,9	45,0	186,5
	R^2	0.986	0.970	0.999
IL-6	**Neat**	1621,7	1965,1	1683,3
	1 :2	796,9	875,0	803,2
	1 :4	406,6	404,4	422,6
	1 :8	213,6	201,4	228,0
	R^2	1.000	0.998	0.999
IL-8	**Neat**	1311,7	1647,3	1780,6
	1 :2	742,0	865,3	752,7
	1 :4	426,2	441,0	431,8
	1 :8	226,5	231,5	212,8
	R^2	0.997	1.000	0.991
IL-10	**Neat**	1039,2	1085,4	1719,3
	1 :2	554,8	504,2	772,9
	1 :4	311,1	258,3	385,8
	1 :8	161,2	150,8	189,9
	R^2	0.999	0.997	0.998
IL-12	**Neat**	370,1	459,2	1116,1
	1 :2	247,6	319,6	533,9
	1 :4	151,8	202,5	277,8
	1 :8	83,6	130,4	138,4
	R^2	0.974	0.975	0.999
IL-17A	**Neat**	466,1	956,2	1783,2
	1 :2	322,1	488,8	845,7
	1 :4	225,9	235,4	397,9
	1 :8	157,9	160,9	200,3
	R^2	0.986	0.998	0.999
IFNγ	**Neat**	1328,9	1700,0	3372,2
	1 :2	868,3	830,1	1493,1
	1 :4	521,1	471,5	761,3
	1 :8	290,9	279,4	360,7
	R^2	0.980	0.998	0.997
TNFα	**Neat**	692,7	719,6	1630,5
	1 :2	438,9	399,7	756,9
	1 :4	265,5	212,7	352,8
	1 :8	174,4	114,4	163,8
	R^2	0.992	0.998	0.999

14.5. Spike recovery

Individual analyte protein was spiked into Serum and Plasma within the range of assay quantification. The average % analyte recovery was measured.

Analyte	Serum		Plasma		Culture Medium	
	Average recovery (%)	Range (%)	Average recovery (%)	Range (%)	Average recovery (%)	Range (%)
IL-1β	54	54-54	54	53-55	93	93-93
IL-2	67	67-68	77	77-78	59	58-61
IL-4	37	37-37	10	10-11	84	83-85
IL-6	74	74-75	90	89-91	79	77-80
IL-8	60	60-60	62	62-63	86	86-86
IL-10	52	51-52	47	46-47	90	89-91
IL-12	29	29-30	30	30-31	84	84-85
IL-17A	24	23-25	37	36-38	107	106-108
IFNγ	32	32-32	41	41-42	81	74-87
TNFα	28	27-30	30	28-31	85	85-86

Same experiments was done with Serum Sample Buffer

Analyte	Serum		Plasma		Culture Medium	
	Average recovery (%)	Range (%)	Average recovery (%)	Range (%)	Average recovery (%)	Range (%)
IL-1β	68	61-71	76	71-79	96	80-107
IL-2	76	70-81	96	87-102	78	61-89
IL-4	47	36-56	20	15-24	78	59-89
IL-6	86	78-96	108	98-122	90	75-98
IL-8	73	63-86	95	91-99	91	76-102
IL-10	92	65-109	111	86-149	115	80-154
IL-12	51	44-56	84	74-90	87	59-106
IL-17A	33	19-47	75	63-89	100	67-119
IFNγ	63	55-69	86	79-96	86	61-102
TNFα	69	55-84	69	62-75	76	55-85

15. Troubleshooting / Technical Tips

Problem	Potential solution	
Variation between duplicate tests	**Mixing**	Vortex each sample tube before FACS measurement
Low bead number in samples	**Mixing**	Ensure bead mixture is vortexed fully prior to adding to the standards or sample tubes Ensure solutions are pipetted accurately into the bottom of tubes to avoid loss on the tubes sides Do not wash or re-suspend beads in volumes higher than those recommended
No detection of protein in sample	**Dilution**	Sample may be too dilute; try various dilutions
Sample fluorescence is greater than the highest standard	**Dilution**	Samples may require further dilution prior to testing
Poor standard curve	**Assay procedure**	Ensure procedure was followed correctly paying particular attention to preparation of reagents, incubation times, thorough mixing. Use a new vial of standard
Overlap of bead populations	**Setup optimization**	Ensure the Flow Cytometer setup has been optimized
Less bead populations than expected or unequal population distribution	**Mixing**	Ensure equal volume of beads were added to each assay tube Ensure thorough mixing of beads before addition
Poor assay sensitivity / Poor standard curve	**Setup optimization** **Light Protection**	It is important to place the bead populations of S1 at the very right margin of the acquisition plot to optimize the distribution of standard concentrations across the FL-2 scale. All steps after the addition of the Streptavidin-PE should be protected from light to avoid loss of fluorescence intensity
No events shown on Flow Cytometer screen	**Setup optimization**	Prime the instrument with sufficient fluid to remove any air bubbles
Additional bead populations found	**Set-up optimization**	Doublets and triplets found (two or more beads passing the laser at the same time) these should be excluded from both gate A and B on the Flow Cytometer.
Scattered bead populations	**Washing step**	Bead populations appear scattered and not focused. This can occur from poor mixing of tubes/wells following addition of Streptavidin-PE. Add Streptavidin-PE on the bottom of the well or tube and mix thouroughly
Only a bead population appears on the screen, instead of two.	**Set-up optimization**	Ensure the threshold is not to high, the beads are 4 and 5 µm.

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