Diaclone DIAplex

Human Th1 / Th2 / Inflammation

Instructions for use

For research use only

Fast Track Your Research…

コスモ・バイオ株式会社

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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 (IFN-γ, TNF-α, 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 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 :
IFN-γ, TNF-α, 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 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
IFN-γ	●		●		●
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. Each combination is supplied with a DIAplex accessory kit.

DIAplex Design design your own panel from the following:

☑		Cat no.	☑		Cat no.
☐	IFN-γ	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. Antibodies highly specific to the analytes to be detected are coupled to fluorescently labelled detection beads. Each bead is analogous to an individually coated well in an ELISA providing a capture surface for a specific analyte.

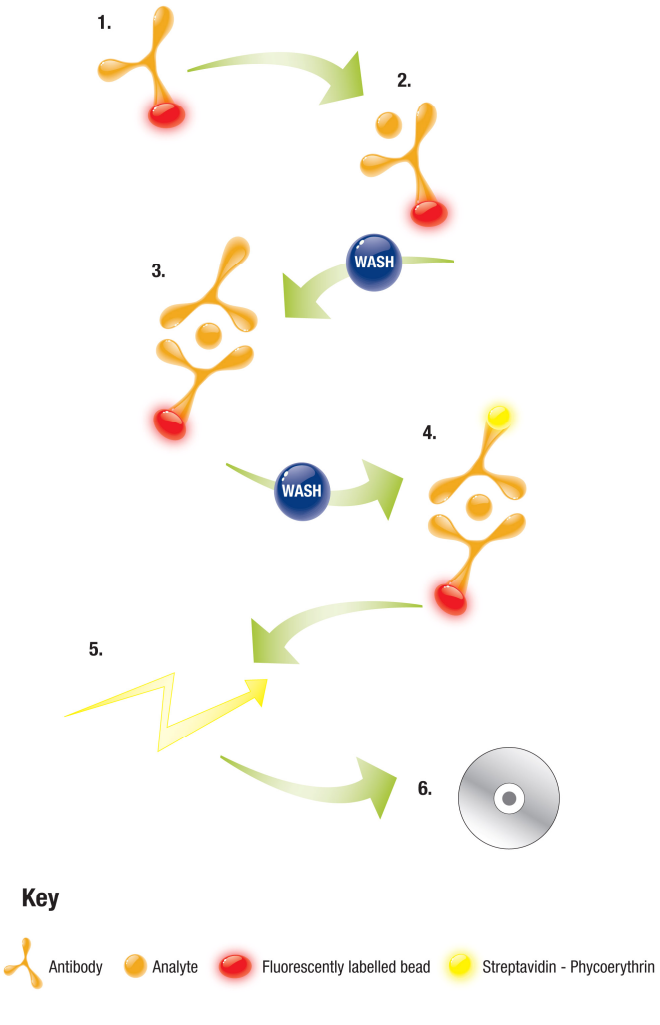
2. One (simplex) or more (multiplex) populations of the beads are combined in suspension with the sample or standard under test. Any analytes present in the sample will bind to the specific antibodies linked to the fluorescent beads.

3. A biotin-conjugated antibody mixture is then added which binds to any analytes captured by the first antibodies.

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

5. The unique size and fluroescent signature of each bead is differentiated by flow cytometry.

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



Sufficient reagents are supplied to be able to perform 96 tests for each of the chosen antibody specificities (including standard curves).

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 TNF-α	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 IFN-γ	L1 (5µm)	R1 (large)

Each detection set contains the following specific for each antibody specificity:

- 1 vial (300 µl) Fluorescent Beads coated with specific Antibody
- 2 vials lyophilized Standard
- 1 vial (150 µl each) Biotin-Conjugate

3.2. DIAplex accessory kit

Each 'DIAplex Design' or '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

4. Materials required but not provided

- A Flow Cytometer equipped with a laser (488 nm or 532 nm) capable of detecting and distinguishing fluorescence emissions at 575nm and far red (695-690 nm)
- 96 well V bottomed microtitre plates or appropriate tubes
- Centrifuge
- 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
- Beakers, flask's, measuring cylinders necessary for preparation of reagents
- Distilled water
- Vortex mixer
- Microplate shaker, tubes
- DIAplex Pro 1.0 Software - complimentary and can be ordered from Diaclone or downloaded from www.genprobe.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. The buffer should be filtered before use.

Bead Mixture
Once the bead mixture has been prepared it should be stored protected from light at 4°C for no longer than 2 hours before use.

Standards
Once reconstituted a standard vial and standard dilutions should be used and then discarded immediately.

Biotin-Conjugate
Once the biotin-conjugate mixture has been prepared it should be stored at 4°C for no longer than 2 hours before use.

Streptavidin- PE
The Steptavidin-PE should be prepared just before use, protected from light and discarded it after use.

Sample Storage
Samples should be analysed on the Flow Cytometer on the same day 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

Decide how many samples are to be tested and for what number of analytes.

We recommend that for each DIAplex assay the full set of standards 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	30
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

- Calculate the final volume of the Bead Mixture required:
  Final volume = 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
- Vortex each individual Bead vial.
- 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.
- 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).

Note: - Vortex “Bead Mix” before use
- Protect bead mixture from light

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 Mixure". **e.g. for a final required volume of 1000µl, add 50µl of each biotin conjugate.**
- Fill up to the final volume with Assay Buffer

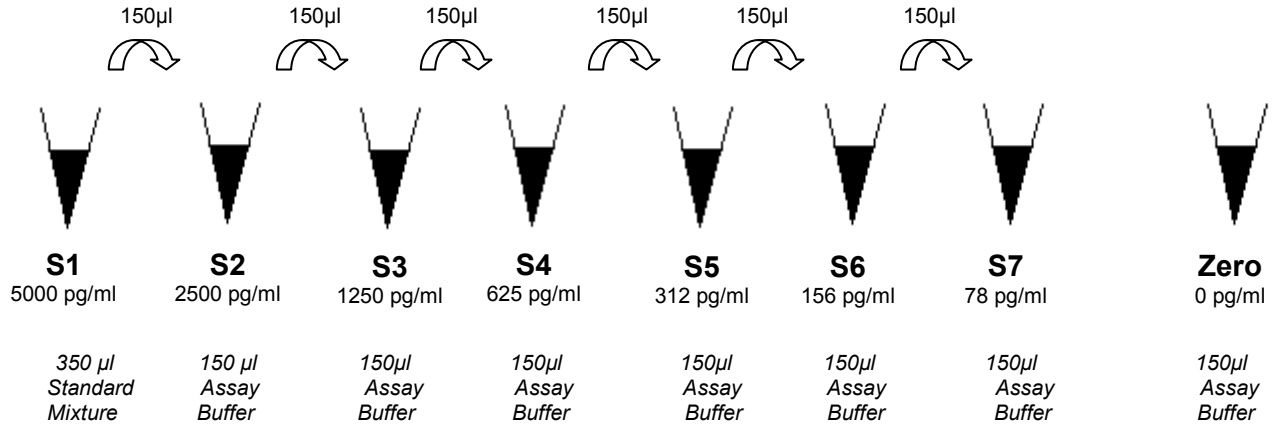
8.5. Standard Preparation

Reconstitute each of the required standard vials with the volume indicated on the vial. Standards must be prepared immediately prior to use.

- Resuspend each individual standard vial.
- Add 35µl of each required standard to a vial labelled standard 1 (S1)
- Fill up to a final volume of 350µl with Assay Buffer (If all ten standards were added no addition of assay buffer is required).
- S1 provides the top concentration of 5000 pg/ml of the analytes under test

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 the contents of tube S2 and transfer 150µl to tube S3 and so on down to tube S7.



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

8.6. Streptavidin-PE Preparation

Prepare Streptavidin-PE just before use (see section 9.0.)

- Calculate the final volume of the Streptavidin-PE required:
  Final volume required = total number of tests x 50µl
- Round 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
- Dilute the Streptavidin-PE concentrate supplied 1/1000 in Assay Buffer to achieve the required volume. **e.g. for 2500 µl of Streptavidin-PE dilute 2.5 µl into 2500 µl assay buffer**

9. Assay Method

In the case of microplate incubations create a plate map to identify the microwell position of all the required tests. Alternatively in the case of individual incubation tubes appropriately label the 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.).

Prepare all reagents as detailed in Section 8.0.

The Beads and all incubation steps should be performed protected from light.

It is important to perform assay additions in the order outlined below.

Assay step		Detail
1.	addition	Add 50µl of either **Assay Buffer** (used as Zero Standard) or **Standard S1 to S7** or **Samples** under test into 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 and Incubate on the microplate shaker (500rpm) at room temperature (18° to 25°C) for **2 hours**
5.	wash	a) Centrifuge for 1 min at 1200g at room temperature (18° to 25°C) b) Carefully discard the supernatant c) Vortex tubes a few seconds to detach caps d) Add 100µl of **Washing Buffer** to all tubes including the blank tubes e) Repeat steps 5a), 5b) & 5c)
6.	prep	Prepare Streptavidin-PE just before use (see section 8.6.)
7.	addition	Add 50µl of **Streptavidin-PE** to all tubes
8.	incubation	Protect tubes from light with aluminium foil and Incubate on the microplate 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 tubes
11.	wash	Repeat **wash steps** 5a), 5b) and 5C)
12.	addition	Add 50µl of **PBS** to all tubes
13.	transfer	Transfer into appropriately labelled acquisition tubes for a flow cytometer
14.	addition	Add 200µl of **PBS** to all tubes
Samples are now ready for analysis on the Flow Cytometer – keep samples protected from light		

Before analysing samples on the Flow Cytometer adjust the Cytometer set up
(refer to Cytometer Setup in Section 10).

10. Cytometer Set Up & Data Acquisition

10.1. Instrument set up

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

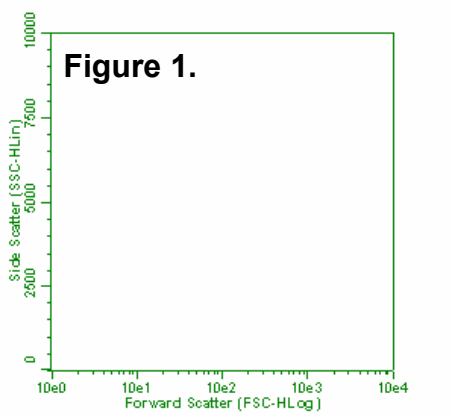
These tubes are used for the following
- Adjusting FSC & SSC parameters
- Creating regions for the two bead populations (R1 & R2)
- Defining the number of events counted
- Ensuring bead population of S1 is visible on the screen

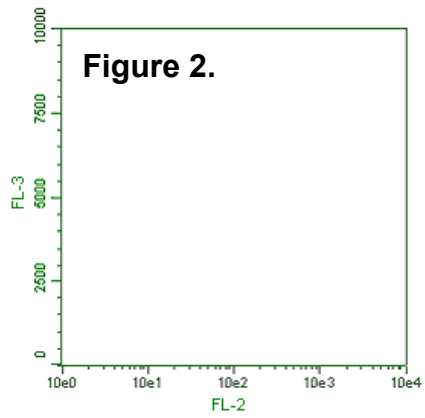
With every new experiment before starting sample acquisition adjust Cytometer parameters in SETUP mode.

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

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

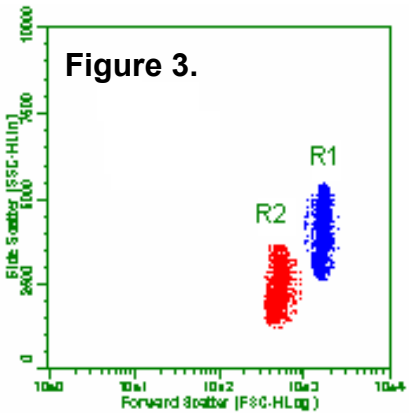
	X-axis (Red)	Y-Axis (Yellow)
BD Facscalibur	FL-3	FL-2
Guava Technologies	RED-Hlog	YLW-Hlog
Beckman Coulter	FL-4	FL-2





- 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 as shown in 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.)

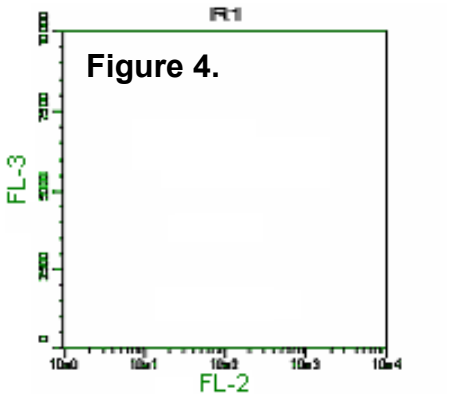


Figure 4.

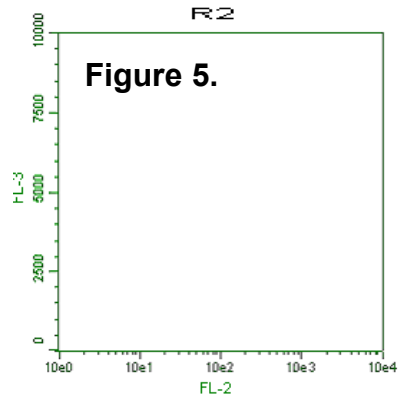


Figure 5.

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

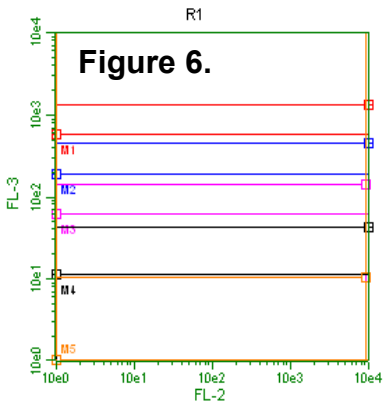


Figure 6.

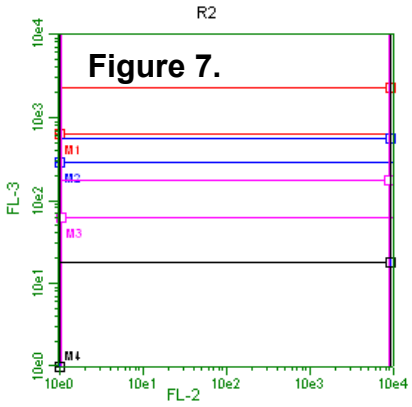


Figure 7.

- Before starting the acquisition of standard and samples, adjust setting using the **S1 set up tube**
- Adjust the parameters of FL-2 until obtaining the Bead population with the highest PE (FL-2) signal on the right hand axis (Figure 8. & Figure 9.). It is important to place the bead populations at the very right margin of the acquisition plot to opimise the distribution of standard concentrations across the FL-2 scale.
- 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.

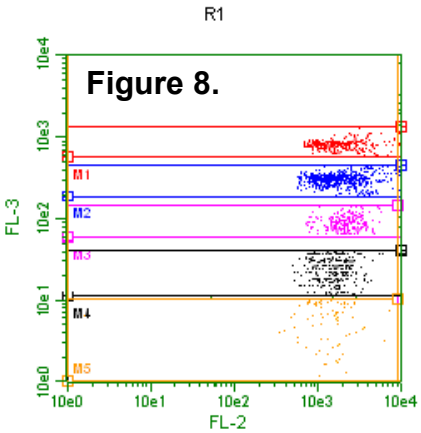


Figure 8.

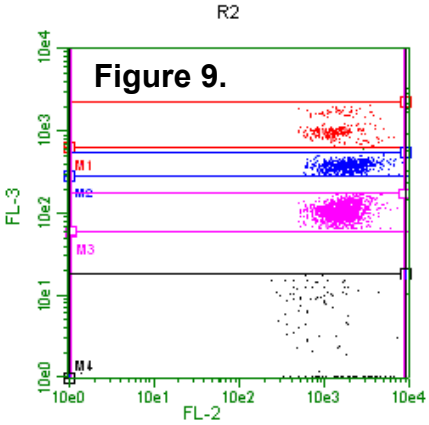


Figure 9.

Example final setup settings are given below.

Note: the setup sequence described in Section 10.1 should be performed for each new experiment.

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	651	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	12.8	% FL-2

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 : 21.5%

After all settings and compensation has been optimised switch to acquisition mode and start measuring Standards and Samples.

10.2. Acquisition

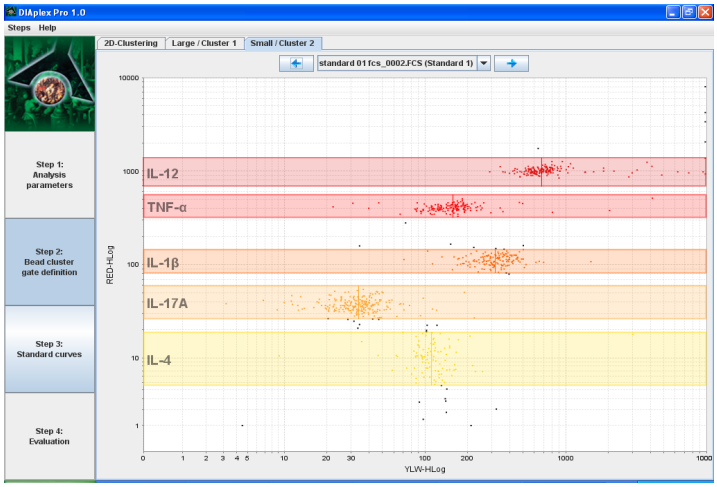
We highly recommend adjusting the setting with an additional Standard 1 and blank tubes before starting the acquisition of standard and samples.

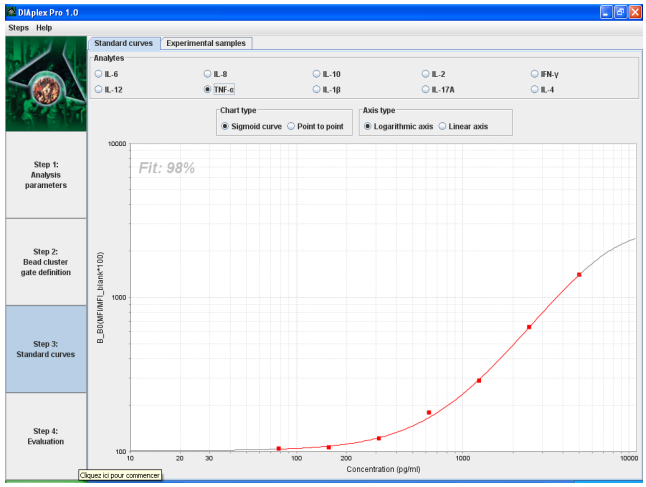
- Switch from SETUP Mode to ACQUISITION Mode
- Ensure previously optimised settings are retrieved
- Define the number of events so that approximately 300 events per analyte are measured e.g. if all 10 analytes are being measured count 1500 events of gated population R1 (300 events x 5 analytes)
- Set number of events to be collected to ‘all events’
- Begin analysing the test samples in the following order:
    1. Negative control tube (zero)
    2. Standards (S1 – S7)
    3. Samples (unknowns)

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.genprobe.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.

Concentration pg/ml	Fluorescent Intensity				
	h IL-6	h IL-1β	h TNF-α	h IL-8	hIL-17A
0	3.2	2.9	2.3	3.6	6.3
78	96.5	10.5	6.8	43.9	22.5
156	156.8	18.6	11.9	92.2	30.2
312	257.1	49.6	31.0	161.8	60.4
625	445.0	132.7	71.0	294.3	95.6
1250	679.3	282.6	133.4	410.5	164
2500	956.0	542.5	273.8	530.4	287.8
5000	1235.4	850.5	482.6	664.2	385.4

Concentration pg/ml	Fluorescent Intensity				
	hIL-2	hIL-10	hIL-4	hIL-12p70	hIFNγ
0	2.0	3.0	3.0	3.9	2.4
78	4.2	66.7	34.0	12.2	56.7
156	9.8	106.5	64.9	21	73
312	25.2	191.1	111.4	41.8	133.4
625	73.6	368.5	191.1	82.8	252.5
1250	179.4	685.4	278.8	139.5	372.0
2500	388.9	1444.4	385.4	264.2	525.7
5000	667.1	1810.6	437.1	399.5	604.3

13. Limitations

The statistically determined Limit of Detection (LOD) of 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 for the following parameters: Sensitivity, Specificity, Reproducibility, Linearity and Spike recovery.

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 30 Zero's in one session) + 2 times the standard deviation of the fluorescence results.

Sensitivity (LOD)	IFN-γ	0.8 pg/mL	IL-4	4.3 pg/mL
	IL-2	12.4 pg/mL	IL-17a	8.7 pg/mL
	IL-10	1.7 pg/mL	IL-1β	3.5 pg/mL
	IL-8	1.3 pg/mL	TNFα	9.8 pg/mL
	IL-6	1.4 pg/mL	IL-12p70	3.4 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 2 differing recombinant protein spiked pooled human serum samples. Mean CV = **5.8%**

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

Sample 1	mean (pg/ml)	SD (pg/ml)	CV (%)	Sample 2	mean (pg/ml)	SD (pg/ml)	CV (%)
IL-1β	958.0	36.7	3.8	**IL-1β**	1995.8	61.4	3.1
IL-2	952.1	52.2	5.5	**IL-2**	1862.0	114.2	6.1
IL-4	501.7	28.6	5.7	**IL-4**	3610.4	243.0	6.7
IL-6	971.1	54.8	5.6	**IL-6**	1689.9	73.7	4.4
IL-8	817.5	27.6	3.4	**IL-8**	1970.3	89.4	4.5
IL-10	1003.9	37.9	3.8	**IL-10**	2243.7	58.5	2.6
IL-12	1016.3	92.7	9.1	**IL-12**	2353.3	194.8	8.3
IL-17A	1011.1	80.1	7.9	**IL-17A**	2354.2	166.1	7.1
IFNγ	1293.0	109.3	8.5	**IFNγ**	1318.3	61.2	4.6
TNFα	821.7	62.2	7.6	**TNFα**	2220.5	168.2	7.6
						Overall Mean CV	**5.8%**

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 = **5.7%**

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	847.9	885	847.5	860.1	2.5
IL-2	952.1	978.6	918	949.6	3.2
IL-4	544.3	549.1	501.7	531.7	4.9
IL-6	887.6	872.6	850.6	870.2	2.1
IL-8	820.3	842.0	817.5	826.6	1.6
IL-10	531.4	483.5	479.2	498.0	5.8
IL-12	1291.1	1403.8	1179.2	1291.4	8.7
IL-17A	778.8	793.4	767.9	780.0	1.6
IFNγ	884.7	795.5	836.2	838.8	5.3
TNFα	776.4	802.9	821.7	800.3	2.8
				Overall Mean CV	**3.9 %**

Sample 2	Expt 1	Expt 2	Expt 3	Mean	CV Mean (%)
IL-1β	1985.5	1995.8	1857.3	1946.2	4.0
IL-2	1703.9	1862	1963	1843.0	7.1
IL-4	2971.4	3610.4	3805.4	3462.4	12.6
IL-6	1823.0	1895.9	1689.9	1802.9	5.8
IL-8	1813.4	1970.3	1885.0	1889.6	4.2
IL-10	827.5	849.0	1106.5	927.7	16.7
IL-12	2353.3	2331.3	2137.6	2274.1	5.2
IL-17A	2354.0	2527.6	2125.6	2335.7	8.6
IFNγ	3361.4	3091.9	3196.7	3216.7	4.2
TNFα	1852.6	1648.3	1630.8	1710.6	7.2
				Overall Mean CV	**7.6 %**

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	Sample 1 (pg/ml)	Sample 2 (pg/ml)
IL-2	**Neat**	2625	1436
	1 :2	892	389
	1 :4	439	332
	1 :8	212	159
	R^2	0.977	0.927
IL-10	**Neat**	3463	2556
	1 :2	2269	1240
	1 :4	1237	878
	1 :8	540	365
	R^2	0.968	0.987
IL-8	**Neat**	1819	823
	1 :2	704	358
	1 :4	352	262
	1 :8	171	134
	R^2	0.988	0.980
IL-6	**Neat**	2848	1792
	1 :2	1586	819
	1 :4	852	557
	1 :8	385	257
	R^2	0.995	0.989
IFNγ	**Neat**	635	493
	1 :2	368	183
	1 :4	223	159
	1 :8	103	61
	R^2	0.991	0.953
IL-17A	**Neat**	2250	1478
	1 :2	1194	575
	1 :4	562	408
	1 :8	279	186
	R^2	0.998	0.975
IL-1β	**Neat**	5721	2849
	1 :2	2193	1525
	1 :4	1199	615
	1 :8	575	360
	R^2	0.999	0.985
TNFα	**Neat**	4648	2882
	1 :2	2352	1166
	1 :4	1186	746
	1 :8	508	295
	R^2	0.999	0.985
IL-12	**Neat**	724	513
	1 :2	447	257
	1 :4	259	184
	1 :8	122	83
	R^2	0.983	0.987
IL-4	**Neat**	480	304
	1 :2	279	136
	1 :4	152	101
	1 :8	63	38
	R^2	0.989	0.980

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	
	Average recovery (%)	Range (%)	Average recovery (%)	Range (%)
IL-1β	100	93-108	66	45-85
IL-2	65	49-80	123	83-164
IL-4	67	61-73	108	95-121
IL-6	122	109-136	83	74-92
IL-8	78	78-79	65	53-77
IL-10	121	119-123	112	110-115
IL-12	112	106-118	81	43-119
IL-17A	102	96-108	111	104-117
IFNγ	117	112-121	62	59-65
TNFα	123	91-155	112	75-150

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.

16. Bibliography

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