Project:

Determination of cytokine concentrations

Methodology for multiplex analysis by Luminex technology (Bioplex 200)

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1.0	22.02.2016	All	Initial Document	Neill Liptrott
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Table of Content

1 Introduction

Cytokines, chemokines, and growth factors are a diverse group of cell signaling proteins expressed and secreted by virtually all cell types, including cells of endothelial, epithelial, and immune origin. These proteins interact with specific receptors on target cells to mediate important physiological responses such as growth, immunity, inflammation, and hematopoiesis. Dysregulation of expression is associated with pathological conditions ranging from cancer and diabetes to infection and autoimmune disease.

Bio-Plex Pro™ assays enable researchers to quantify multiple protein biomarkers in a single well of a 96-well plate in 3–4 hours. These robust immunoassays require as little as 12.5 µl serum or plasma or 50 µl cell culture supernatant or other biological fluid. The use of magnetic (MagPlex) beads allows researchers to automate wash steps on a Bio-Plex Pro (or similar) wash station. Magnetic separation offers greater convenience and reproducibility compared to vacuum filtration.

2 Principle of the Method

Whole blood or PBMC from healthy volunteers are incubated with test materials and appropriate controls as described in EUNCL-ITA-010. The Bio-Plex® multiplex system is built upon the three core elements of xMAP technology:

Fluorescently dyed microspheres (also called beads), each with a distinct color code or spectral address to permit discrimination of individual tests within a multiplex suspension. This allows simultaneous detection of up to 500 different types of molecules in a single well of the 96-well microplate on the Bio-Plex® 3D system, up to 100 different types of molecules on the Bio-Plex® 200 system, and up to 50 different types of molecules on the Bio-Plex® MAGPIXTM system

On the Bio-Plex 200 and Bio-Plex 3D systems, a dedicated flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads. In the Bio-Plex MAGPIX, the entire sample load volume is injected into a chamber where the beads are imaged using LED and CCD technology

A high-speed digital signal processor that efficiently manages the fluorescence data.

Bio-Plex Pro™ assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA. Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator or reporter.

Data from the reactions are acquired using a Bio-Plex system or similar Luminex-based reader. When a multiplex assay suspension is drawn into the Bio-Plex 200 reader, for example, a red (635 nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus assay identification. At the same time, a green (532 nm) laser excites PE to generate a reporter signal, which is detected by a photomultiplier tube (PMT). A high-speed digital processor manages data output, and Bio-Plex ManagerTM software presents data as median fluorescence intensity (MFI) as well as

concentration (pg/ml). The concentration of analyte bound to each bead is proportional to the MFI of reporter signal.

Using Bio-Plex Data ProTM software, data from multiple instrument runs can be combined into a single project for easy data management, quick visualization of results, and simple statistical analysis.

3 Applicability and Limitations (Scope)

This assay is intended for the detection of secreted analyte from immune cells treated with test materials and is intended as a guide fort he determination of possible cytokine storm. The particular cell type that is responding to the test material is not directly defined and follow up experiments using isolated immune cell subsets should be conducted.

4 Related Documents

Table 1:

Document ID	Document Title
EUNCL_ITA_010	*Preparation of blood and PBMC for cytokine secretion*

5 Equipment and Reagents

5.1 Equipment

5.1.1 Pipettes covering a range of 0.05 to 10 mL

5.1.2 96-well round bottom plates

5.1.3 Polypropylene tubes, 50 and 15 mL

5.1.4 Microcentrifuge tubes

5.1.5 Centrifuge

5.1.6 Refrigerator, 2-8 ºC

5.1.7 Freezer, -20 ºC

5.1.8 Biohazard safety cabinet approved for level II handling of biological material

5.1.9. Vortex

5.1.10 Bioplex 200 running Bioplex Manager software 6.0 or above

5.1.11 Automated plate washer capable of handling magnetic beads.

5.2 Reagents

Bioplex reagents for the detection of secreted analytes by Luminex technology. The reagents used for this assay are specific for the analytes to be analysed and should be chosen depending on the intended analytical outcome.

An example oft he contents of the reagents supplied is shown below;

Table 1. Contents of Bio-Plex Pro cytokine, chemokine, and growth factor assays.*

Component	1 x 96-Well Format	10 x 96-Well Format
Standard diluent*	10 ml	100 ml
Sample diluent*	40 ml	80 ml
Assay buffer	50 ml	500 ml
Wash buffer	200 ml	1.5 L
Detection antibody diluent	5 ml	50 ml
Streptavidin-PE (100x)	1 tube	1 tube
Filter plate and/or flat bottom plate (96-well)	1 plate	10 plates
Sealing tape	1 pack of 4	10 packs of 4
Assay Quick Guide	1 booklet	1 booklet
Standard	1 vial	10 vials
Human and Mouse Cytokine (Group I and II)		
Coupled magnetic beads (10x)	1 tube	1 tube
Detection antibodies (10x)	1 tube	1 tube
Mouse Cytokine (Group III) and Rat Cytokine (Group I)		
Coupled magnetic beads (20x)	1 tube	1 tube
Detection antibodies (20x)	1 tube	1 tube

* Bio-Plex Pro high dilution reagent kit, 1 x 96-well, contains 70 ml serum-based diluent in lieu of standard diluent and sample diluent.

5.3 Instrument Preparation

Start up and calibrate the Bio-Plex® 100/200 or similar system with Bio-Plex ManagerTM software prior to setting up the assay. The calibration kit should be run daily or before each use of the instrument to standardize the fluorescent signal. To prepare either a Bio-Plex 3D or Bio-Plex® MAGPIXTM reader, consult its respective user manual.

The validation kit should be run monthly to ensure performance of fluidics and optics systems. Refer to either the software manual or online Help for directions on how to conduct validation.

Start Up System (Bio-Plex 100, 200, or Similar)

1. Empty the waste bottle and fill the sheath fluid bottle before starting if high throughput fluidics (HTF) are not present. This will prevent fluidic system backup and potential data loss.

2. Turn on the reader, XY platform, and HTF (if included). Allow the system to warm up for 30 min (if not already done).

3. Select Start up and follow the instructions. If the system is idle for 4 hr without acquiring data, the lasers will automatically turn off. To reset the 4-hr countdown, select Warm up and wait for the lasers/ optics to reach operational temperature.

Calibrate System

1. Select Calibrate and confirm that the default values for CAL1 and CAL2 are the same as the values printed on the bottle of Bio-Plex calibration beads. Use the Bio-Plex system low RP1 target value even if assays will be run at high RP1.

2. Select OK and follow the software prompts for step-by-step instructions for CAL1 and CAL2 calibration.

Note: In Bio-Plex Manager version 6.1 and higher, startup, warm up, and calibration can be performed together by selecting the "Start up and calibrate" icon.

PMT setting for standard curves

Refer to the latest assay guide for the reagents of choice to ensure up-to-date information is used for the analysis and is appropriate for the Luminex system used.

5.4 Prepare wash method

Bio-Plex ProTM assays are compatible with both magnetic separation and vacuum filtration methods. However, for best results, it is recommended to perform the assays in a flat bottom plate with magnetic separation.

Setting up the Bio-Plex Pro or Bio-Plex Pro II Wash Station The wash station does not require calibration; however, it should be primed before use.

1. Install the appropriate plate carrier on the wash station.
2. Use the prime procedure to prime channel 1 with wash buffer.

Setting Up the Bio-Plex Handheld Magnetic Washer

Place an empty flat bottom plate on the magnetic washer by sliding it under the retaining clips. Push the clips inward to secure the plate. Make sure the plate is held securely. If needed, the clips can be adjusted for height and tension.

6 Procedure

6.1 General remarks

It is essential to reconstitute and dilute standards exactly as described in this section. Incorrect preparation may lead to low signal, high background, or inconsistent measurements from plate to plate

- The peel-off label provided with the standards lists the concentration of the most concentrated dilution point, S1. Enter this information into Bio-Plex ManagerTM software as instructed in Section 8
- For users who wish to mix assays from different panels, such as diabetes assays with group I cytokines, guidance is provided here for mixing 2 different lyophilized standards. Bead regions were chosen to avoid overlap whenever possible. However, performance of multiplexes containing assays from different groups have not been extensively validated. Therefore, users must confirm that the assay performance is still fit for purpose
- Bring all assay components and samples to room temperature before use.
- Use calibrated pipettes and pipet carefully, avoiding bubbles. Use new pipet tips for every volume transfer.
- Assay incubations are carried out in the dark on a shaker at 850rpm +/- 50 rpm. Cover the plate with sealing tape and protect from light with aluminium foil.

Selecting a Diluent for Standards

Refer to Table 2 for recommended diluents based on different sample types.

As a general rule, reconstitute and dilute standards in a diluent similar to the final sample type or sample matrix.

Sample Type	Diluent for Standards	Add BSA
Serum and plasma	Standard diluent	None
Culture media, with serum	Culture media	None
Culture media, serum-free	Culture media	To 0.5% final
Lavage, sputum, other fluids	Bio-Plex® sample diluent	To 0.5% final*
Lysate	Bio-Plex sample diluent	To 0.5% final*

*At least 0.5% final w/v BSA is recommended to stabilize analytes and reduce absorption to labware.

Table 2. Summary of recommended diluents for standards

6.2 Reconstitute a single vial of standards

This procedure prepares enough material to run each dilution in duplicate. 1. Gently tap the vial containing the lyophilized standard.

6.2.1 Add 500 µl of the appropriate diluent (see Table 5). Do not use assay buffer to reconstitute the standards.

6.2.2 Gently vortex the reconstituted standard for 5 sec then incubate on ice for 30 min. Be consistent with the incubation time in every assay to ensure best results.

6.2.4 During the incubation period, prepare the samples as instructed in section 6.4.

6.3 Prepare Standard Dilution Series from a single antigen vial

The following procedure produces an eight-point standard curve with a fourfold dilution between each point. Pipet carefully using calibrated pipets and use new pipet tips for every volume transfer.

6.3.1 Label nine 1.5 ml polypropylene tubes S1 through S8 and Blank.

6.3.2 Add the specified volume of standard diluent to each tube.

6.3.3 Vortex the reconstituted standards gently for 5 sec before removing any volume. Add 128 µl into the S1 tube containing 72 µl of standard diluent. Vortex at medium speed for 5 sec, then use a new pipet tip to transfer 50 µl from S1 tube to S2 tube. Vortex.

6.3.4 Continue with 1:4 (fourfold) serial dilutions from tube S2 to S8. Use reconstituted and diluted standards immediately. Do not freeze for future use.

6.4 Sample preparation

General guidelines for preparing different sample types are provided here. For more information, contact Bio-Rad Technical Support.

Once thawed, keep samples on ice. Prepare dilutions just prior to the start of the assay and equilibrate to room temperature before use

Do not freeze diluted samples

6.4.1 Collect supernatants and centrifuge at 1,000 x g for 15 min at 4°C. For cell lines cultured in serum-free culture media, collect samples and add BSA as a carrier protein to a final concentration of at least 0.5% to stabilize protein analytes and to prevent adsorption to labware.

6.4.2 Transfer to a clean polypropylene tube. If cellular debris or precipitates are present, centrifuge again at 10,000 x g for 10 min at 4°C.

6.4.3 It is recommended to test undiluted samples first. If levels are anticipated to be high, samples can be further diluted in culture medium. Rarely would samples need to be diluted greater than 1:10.

6.4.4 Assay immediately or store samples in single-use aliquots at –70°C. Avoid repeated freeze-thaw cycles.

6.5 Prepare coupled beads

Instructions are provided for diluting the coupled beads to a 1x concentration.

6.5.1 Calculate the volume of coupled beads and assay buffer needed using the most up-to-date guides provided by the manufacturer.

6.5.2 Add the required volume of Bio-Plex assay buffer to a 15 ml polypropylene tube.

6.5.3 Vortex the stock coupled beads at medium speed for 30 sec. Carefully open the cap and pipet any liquid trapped in the cap back into the tube. This is important to ensure maximum bead recovery. Do not centrifuge the vial; doing so will cause the beads to pellet.

6.5.4 Dilute coupled beads to 1x by pipetting the required volume into the 15 ml tube. Vortex.

Each well of the assay plate requires either 2.5 µl (20x stock) or 5.0 µl (10x stock) adjusted to a final volume of 50 µl in assay buffer.

6.5.5 Protect the beads from light with aluminum foil. Equilibrate to room temperature prior to use.

Note: To minimize volume loss, use a 200–300 µl capacity pipet to remove beads from the stock tube. If necessary, perform the volume transfer in two steps. Do not use a 1,000 µl capacity pipet and/or wide bore pipet tip.

6.6 Running the assay

6.6.1 Add Coupled Beads, Standards, and Samples

6.6.2 Cover unused wells with sealing tape.

6.6.3 Vortex the diluted (1x) coupled beads for 30 sec at medium speed. Pour the diluted coupled beads into a reagent reservoir and transfer 50 µl to each well of the assay plate.

6.6.4 Wash the plate two times with 100 µl Bio-Plex® wash buffer using the wash method of choice.

6.6.5 Gently vortex the diluted standards, blanks, samples, and controls (if applicable) for 5 sec. Transfer 50 µl to each well of the assay plate, changing the pipet tip after every volume transfer.

6.6.6 Cover plate with a new sheet of sealing tape and protect from light with aluminum foil. Incubate on shaker at 850 ± 50 rpm at room temperature (RT). See Table 3 for incubation time.

Assay	Incubation Time
Bio-Plex Pro human cytokine (group I and II)	30 min
Bio-Plex Pro mouse cytokine (group I and II)	30 min
Bio-Plex Pro mouse cytokine (group III)	1 hr
Bio-Plex Pro rat cytokine (group I)	1 hr

Note: Be consistent with this incubation time for optimal assay performance and reproducibility.

Table 3. Sample Incubation Times

6.7 Prepare and add Detection Antibodies

6.7.1 While the samples are incubating, calculate the volume of detection antibodies and detection antibody diluent needed according tot he manufacturers up-to-date instructions. Detection antibodies should be prepared 10 min before use.

6.7.2 Add the required volume of Bio-Plex detection antibody diluent to a 15 ml polypropylene tube.

6.7.3 Vortex the stock detection antibodies for 15–20 sec at medium speed, then perform a 30 sec spin to collect the entire volume at the bottom of the tube.

6.7.4 Dilute detection antibodies to 1x by pipetting the required volume into the 15 ml tube.

Each well of the assay requires either 1.25 µl (20x stock) or 2.5 µl (10x stock) adjusted to a final volume of 25 µl in detection antibody diluent.

6.7.5 After incubating the beads, samples, standards, and blank, slowly remove and discard the sealing tape.

6.7.6 Wash the plate three times with 100 µl wash buffer.

6.7.7 Vortex the diluted (1x) detection antibodies gently for 5 sec. Pour into a reagent reservoir and transfer 25 µl to each well using a multichannel pipet.

6.7.8 Cover plate with a new sheet of sealing tape and protect from light with aluminum foil. Incubate on shaker at 850 ± 50 rpm for 30 min at room temperature.

6.8 Prepare and add Streptavidin-PE (SA-PE)

6.8.1 While the detection antibodies are incubating, calculate the volume of SA-PE (100x) and assay buffer needed according tot he manufacturers up-to-date instructions. Streptavidin-PE should be prepared 10 min before use.

6.8.2 Add the required volume of assay buffer to a 15 ml polypropylene tube.

6.8.3 Vortex the 100x SA-PE for 5 sec at medium speed.

6.8.4 Perform a 30 sec spin to collect the entire volume at the bottom of the tube.

6.8.5 Dilute SA-PE to 1x by pipetting the required volume into the 15 ml tube. Vortex and protect from light until ready to use.

6.8.6 Each well of the assay requires 0.5 µl (100x stock) adjusted to a final volume of 50 µl in assay buffer.

6.8.7 After the detection antibody incubation, slowly remove and discard the sealing tape.

6.8.8 Wash the plate three times with 100 µl wash buffer.

6.8.9 Vortex the diluted (1x) SA-PE at medium speed for 5 sec. Pour into a reagent reservoir and transfer 50 µl to each well using a multichannel pipet.

6.8.10 Cover plate with a new sheet of sealing tape and protect from light with aluminum foil. Incubate on shaker at 850 ± 50 rpm for 10 min at room temperature.

6.8.11 After the streptavidin-PE incubation step, slowly remove and discard the sealing tape.

6.8.12 Wash the plate three times with 100 µl wash buffer.

6.8.13 To resuspend beads forplate reading, add 125µl of assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake at room temperature at 850 ± 50 rpm for 30 sec, and slowly remove the sealing tape. Ensure that the plate cover has been removed before placing the plate on the reader.

6.8.14 Remove the sealing tape and read the plate using the settings below. Refer to the kit instructions provided by the manufacturer fort he most up-to-date instructions.

6.9 Read the plate

Bio-Plex ManagerTM software is recommended for all Bio-Plex ProTM assay data acquisition and analysis. Instructions for Luminex xPONENT software are also included. For instructions using other xMAP system software packages, contact Bio-Rad Technical Support or your regional Bio-Rad field applications specialist.

- Prepare Protocol in Bio-Plex Manager Software v 6.0 and Higher
- The protocol should be prepared in advance so that the plate is read as soon as the experiment is complete.
- A protocol file specifies the analytes used in the reading, the plate wells to be read, sample information, the values of standards and controls, and instrument settings.
- Bio-Plex Manager software contains protocols for most Bio-Plex® assays. Choose from available protocols or create a new protocol. To create a new protocol, select File, then New from the main menu. Locate and follow the steps under Protocol Settings.

6.10 Flow chart

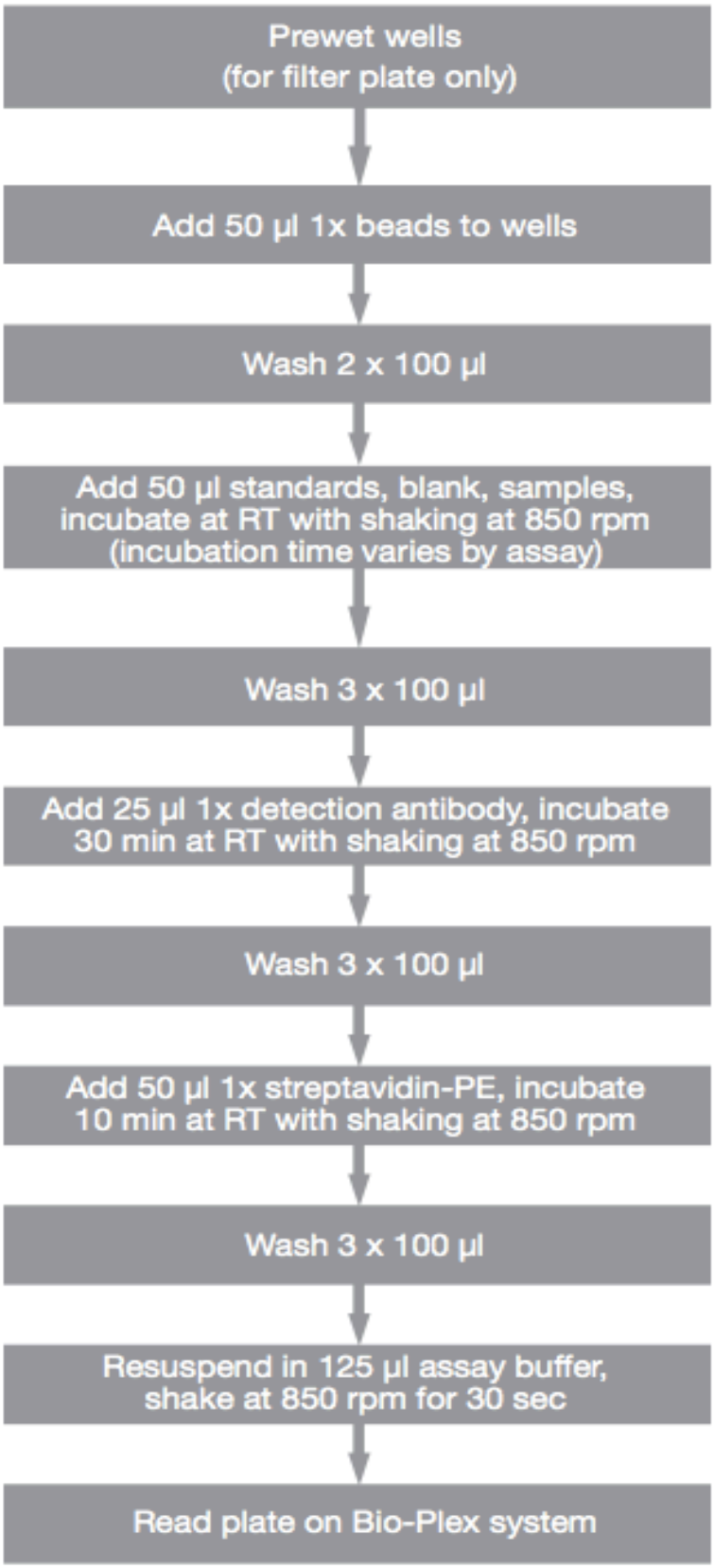

Figure 1: Brief outline of the workflow.

7 Quality Control, Quality Assurance, Acceptance Criteria

7.1 %CV and PDFT for each calibration standard and quality control should be within 20%.

7.2 % CV for each test sample including supernatants from whole blood cultures treated with positive control, negative control and nanoparticle sample should be within 20 %. At least one replicate of positive and negative control should be acceptable for run to be accepted.

7.2 If both replicates of positive control or negative control fail to meet acceptance criterion described in 7.2 the run should be repeated.

7.4 Within the acceptable run if two of three replicates of unknown sample fail to meet acceptance criterion described in 7.2 this unknown sample should be re-analyzed.

8 Health and Safety Warnings, Cautions and Waste Treatment

Universal precautions must be used when handling human peripheral blood.

- No sharps are to be used in the preparation of cell isolates of plasma from human healthy volunteer blood.
- All liquid waste must be discarded into 1% Virkon solution and left overnight (18 hours) in a class II BSC with the lid loose to allow for venting.