NCL Method ITA-27

Multiplex Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Human Cytokines in Culture Supernatants

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

The method was developed in collaboration between Nanotechnology Characterization Lab and Quansys Biosciences.

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1. Introduction

Cytokines, chemokines and interferons (IFN) are soluble mediators of inflammation and a variety of responses that orchestrate both innate and adaptive immunity in health and disease [1-10]. Understanding the induction of these biomarkers in response to a drug product helps to establish the product's safety profile and shed light on the mechanism of action or mechanism of toxicity. This document describes experimental procedure for analysis of culture supernatants by multiplex ELISA to detect presence of type I IFNs (IFNα, IFNβ, IFNω), type II IFN (IFNγ), type III IFN (IFNλ), cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-21, IL-22, IL-23, IL-27, IP-10, MCP-1, MCP-2, MIP-1α, MIP-1β, RANTES, TNFα) in culture supernatants. NCL protocol ITA-10 should be referred to for details of preparation of culture supernatants. Protocols for single-plex ELISA (ITA-22, ITA-23, ITA-24 and ITA-25) can also be considered. The advantage of this protocol over single-plex ELISA is that it allows simultaneous analysis of a broad spectrum of markers.

2. Principle

A 96-well plate is coated with capture antibodies specific to the target cytokines and immobilized at particular location within each well. Cell culture supernatants are loaded onto the plate and cytokines present in the supernatant are captured by the antibodies. The excess sample is washed away, and captured cytokines are detected with secondary antibody conjugated to biotin. Streptavidin-conjugated horse radish peroxidase is then used to detect captured analytes. After additional wash, the conjugate remaining at each location is detected using chemiluminescent substrate. Images are taken using Quansys Image Pro reader and analyzed using Q-View Software. The intensity of the individual spot corresponding to a particular analyte is proportional to the level of the analyte in the test supernatant. The quantities of the individual cytokines are determined by comparing the intensity of spots corresponding to each of the cytokines in the test sample to that in the standard curve comprised of various concentrations of reference standards. The ELISA procedure described herein takes approximately 6 hours to complete. If ELISA cannot be conducted immediately

after incubation of whole blood/PBMC with nanoparticles is complete, the culture supernatants can be stored at -80°C.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

- 3.1.1 Custom Multiplex kit, (Quansys, 107749GR-Q-Plex™ Custom Chemi Kit)
- 3.1.2 Phosphate buffered saline (PBS), (GE Life Science, SH30256.01)
- 3.1.3 Cell culture grade water, GE Life Science, SH30529.02)
- 3.1.4 RPMI1640, (GE Life Sciences, Hyclone, SH30096.01)
- 3.1.5 Pen/Strep solution, (GE Life Sciences, Hyclone, SV30010)
- 3.1.6 Cytokine Calibration Standards (see section 10 for the example of Product Information Card showing individual cytokine's concentrations)

3.2 Materials

- 3.2.1 96-well, U-bottom plates
- 3.2.2 96-well, V-bottom polypropylene plates
- 3.2.3 Pipettes covering the range from 0.05 to 1 mL
- 3.2.4 Microcentrifuge tubes, 1.5 mL
- 3.2.5 Multichannel pipette (8 or 12-channels)
- 3.2.6 Reagent reservoirs

3.3 Equipment

- 3.3.1 Microcentrifuge
- 3.3.2 Refrigerator, 2-8°C
- 3.3.3 Freezer, -80°C

3.3.4 Vortex

3.3.5 Plate shaker

3.3.6 Plate washer

3.3.7 Multiplex ELISA plate reader capable of reading chemiluminescence

4. Preparation of Reagents and Controls

4.1 Calibrators

The kits come with 1 to 3 vials of lyophilized calibrators depending on the format being used. Each set of calibrators should be prepared with **sample diluent** according to the product information card included with the kit (see section 10). The calibrators will then be serially diluted, 3-fold, as described in the procedure below (see step 6.3).

4.2 Sample diluent (10 mL): Use as is.

4.3 Assay diluent (lyophilized): Reconstitute with 6 mL Cell Culture Grade water.

4.4 Detection reagent (lyophilized): Reconstitute with 6 mL Cell Culture Grade water.

4.5 Streptavidin-HRP (6 mL): Use as is.

4.6 Substrate A (3 mL) and Substrate B (3 mL): Mix both reagents together 15 minutes before use and store in the dark at room temperature.

4.7 Wash buffer, 20X (50 mL): Mix with 950 mL of distilled water for a 1X solution.

5. Preparation of Inhibition/Enhancement Controls (IECs)

Two approaches can be used to prepare IECs:

5.1 <u>Approach A</u>

Use culture supernatant from the positive control sample and spike it with the test nanoparticle at 4 concentrations (refer to NCL ITA-10). For example, add 6 µL of 10X nanoparticle working solution into 60 µL of positive control supernatant. The final concentration of nanoparticle in this sample will mimic that in the supernatants from nanoparticle treated cells. The concentration of the analyte in the IEC supernatant will be 1.1X lower. Compare the analyte

level in the positive control supernatant with that in IEC x 1.1 to account for the dilution factor. If the difference in test results is within 25%, test-nanoparticle does not interfere with ELISA.

5.2 Approach B

Use cell free controls and spike them with calibration standard. For example, add 6 µL of calibrator 1 from step 6.3 to 60 µL of cell-free supernatant from ITA-10. This IEC should be approximately comparable to calibrator 3 (step 6.3.3). If the difference in test results is within 25%, test-nanoparticle does not interfere with ELISA.

6. Experimental Procedure for 4-plex Interferon Multiplex

6.1 Prepare assay diluent, sample diluent and wash buffer as described in Section 4. Store all buffers at room temperature and use on the same day as preparation.

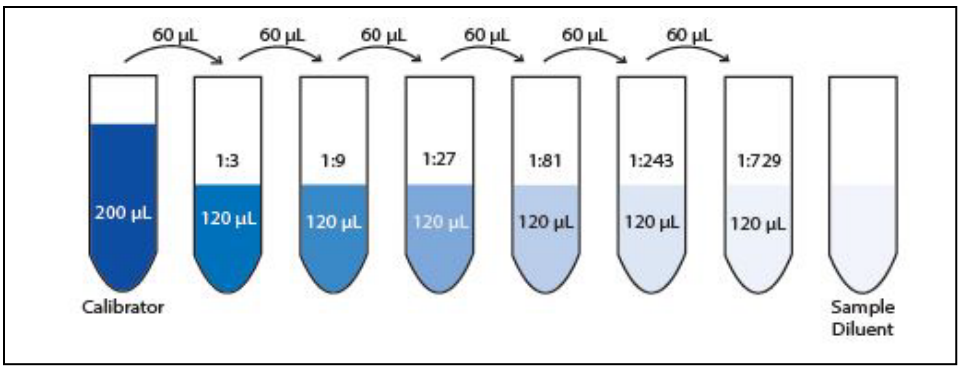
6.2 Reconstitute calibrator(s) with sample diluent according to the included information card (see Section 10 for an example information card).

6.3 Prepare calibration standards in microtubes or a 96-well tray as follows:

6.3.1 Add 200 µL of the stock calibrator to the first tube/well labeled Standard or Calibrator 1.

6.3.2 Add 120 µL of the **sample diluent** to an additional 7 tubes/wells labeled sequentially as Standard or Calibrator 2 through 8.

6.3.3 Perform a serial, 3-fold dilution of the first standard by transferring 60 µL of the Standard 1 to the Standard 2 tube/well, mix by repeated up-and-down pipetting, continue transferring 60 µL serially into the next tubes/wells until Standard 7 is reached. Standard 8 is sample diluent only (see image below).



6.4 Thaw frozen culture supernatants at room temperature or use freshly collected supernatants.

6.5 Dilute culture supernatants 2-fold with **sample diluent** by mixing 60 µL of the culture supernatants with 60 µL of the **sample diluent**.

6.6 Add 50 µL of the **assay diluent** to each well on the multiplex ELISA plate.

6.7 After adding the **assay diluent**, load 50 µL of the standards (step 6.3) and culture supernatants (step 6.5) to the 96-well multiplex plate, seal and incubate at room temperature on a shaker set at approximately 500 rpm for 2 hours.

6.8 Wash the plate 3 times with the wash buffer prepared in step 6.1 (section 4.7). Use 300 µL of the wash buffer per well. Tap the plate on paper towel to remove excess buffer and immediately proceed to the next step.

6.9 Add 50 µL per well of previously prepared detection reagent (section 4.4), seal and incubate the plate at room temperature on a shaker set to approximately 500 rpm for 1 hour.

6.10 Repeat step 6.8.

6.11 Add 50 µL per well of the ready-to-use Streptavidin-HRP conjugate provided with the kit, seal and incubate the plate at room temperature on a shaker set to approximately 500 rpm for 15 minutes.

6.12 Repeat step 6.8 two times and immediately proceed to the next step.

6.13 Add 50 µL per well of the previously prepared ChemiLum substrate (section 4.6).

6.14 Read the plate using the Quansys ImagePro reader.

7. Experimental Procedure for 14-plex and 15-plex multiplex

The procedure for both the 14-plex and 15-plex is the same as the IFN 4-plex, with the exception that in **the 15-plex there is no assay buffer** as in step 6.6.

8. Acceptance Criteria

8.1 The % CV and PDFT for each calibration standard and quality control should be within 25%.

8.2 The % CV for each test sample, including supernatants from whole blood cultures treated with positive control, negative control and nanoparticle samples, should be within 25%. At least one replicate of positive and negative control should be acceptable for run to be accepted.

8.3 If both replicates of positive control or negative control fail to meet acceptance criterion described in 8.2 the run should be repeated.

8.4 Within the acceptable run, if two of three replicates of unknown sample fail to meet acceptance criterion described in 8.2, this unknown sample should be re-analyzed.

9. Example of ELISA Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1 Neat	Std2 1:3	Std3 1:9	Std4 1:27	Std5 1:81	Std6 1:243	Std7 1:729	B0	IEC1	IEC2	IEC3	IEC4
B	Std1 Neat	Std2 1:3	Std3 1:9	Std4 1:27	Std5 1:81	Std6 1:243	Std7 1:729	B0	IEC1	IEC2	IEC3	IEC4
C	Unt	Unt	Unt	NC	NC	NC	PC	PC	PC	VC	VC	VC
D	Unt	Unt	Unt	NC	NC	NC	PC	PC	PC	VC	VC	VC
E	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
F	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
G	TS1 CF	TS1 CF	TS2 CF	TS2 CF	TS3 CF	TS3 CF	TS4 CF	TS4 CF				
H	TS1 CF	TS1 CF	TS2 CF	TS2 CF	TS3 CF	TS3 CF	TS4 CF	TS4 CF				

Rows G & H do not contain cells.

Std: standard; B0 = blank (assay diluent); Unt: Untreated supernatant; NC: negative control supernatant; PC: positive control supernatant; VC: vehicle control supernatant; TS1-4: test sample supernatants at concentrations 1-4; IEC1-4: inhibition enhancement controls for TS1-4 concentrations; CF: cell free

10. Example of Product Information Card

Q-Plex™ ARRAY

Human Custom Product Card

Software Product Code: **HCUM151006NH**

Calibrator Lot Number: HCUM151006, HA1M150909, HA2M150611

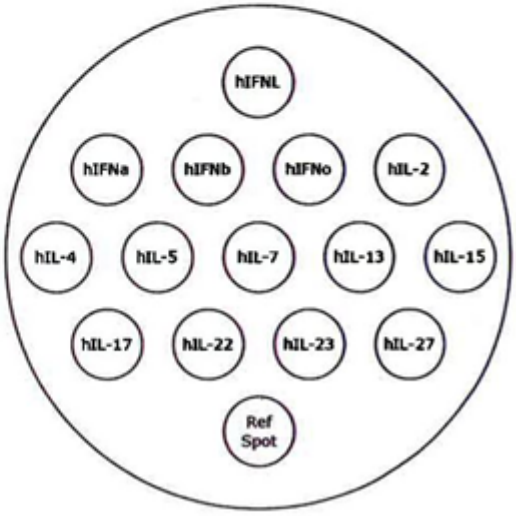
Reconstitution Volume (Vial 1): 700uL

Reconstitution Volume (Vial 2): 550uL of Vial 1

Reconstitution Volume (Vial 3): 500uL of Vial 2

101896GR

Analyte	Calibrator Conc.	Unit
hIFNL	11000	pg/ml
hIFNa	2500	pg/ml
hIFNb	16000	pg/ml
hIFNo	6200	pg/ml
hIL-2	4400	pg/ml
hIL-4	2200	pg/ml
hIL-5	3100	pg/ml
hIL-7	1050	pg/ml
hIL-13	3600	pg/ml
hIL-15	3100	pg/ml
hIL-17	4000	pg/ml
hIL-22	53000	pg/ml
hIL-23	32000	pg/ml
hIL-27	38000	pg/ml
Ref Spot	N/A	N/A



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12. Abbreviations

B0	blank (assay diluent)
CF	cell free
IEC	inhibition enhancement control
IFN	interferon
LLD	lower limit of detection
LPS	lipopolysaccharide
NC	negative control supernatant
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	positive control supernatant
PHA-M	phytohemagglutinin-M
Std	standard
TS	test sample supernatant
ULD	upper limit of detection
VC	vehicle control supernatant