NCL Method ITA-25

Enzyme-Linked Immunosorbent Assay (ELISA) for detection of human IFNγ 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.

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1. Introduction

IFNγ is type II interferon produced by lymphocytes (T-cells, NK cells, NKT cells) in response to viral infections and inflammatory stimuli, including but not limited to bacterial lipopolysaccharide LPS and CpG DNA. Factors mitogenic to T-cells (e.g. PHA-M) can also induce this interferon. IFNγ is essential in maintaining function of adaptive immunity through regulation of T cell function. It is also important for protection from intracellular bacteria (e.g. *M.tuberculosis*) and anti-tumor immunity. This document describes experimental procedure for analysis of culture supernatants by ELISA to detect presence of IFNγ. NCL protocol ITA-10 should be referred to for details of preparation of culture supernatants.

2. Principle

A 96 well plate is coated with capture antibody specific to IFNγ. Cell culture supernatants are loaded onto the plate and IFNγ present in the supernatant is captured by the antibody. The excess sample is washed away and captured IFNγ is detected with secondary antibody conjugated to biotin. Streptavidin-conjugated horse radish peroxidase is used to develop the plate and the absorbance is detected at 450 nm. Optical density of the test sample higher than that of the background control is indicative of IFNγ presence in the test supernatant. The quantity of IFNγ is determined by comparing optical density of the test sample to that in the standard curve comprised of various concentrations of IFNγ reference standard. The ELISA procedure described herein takes approximately 6 hours to complete. If ELISA cannot be conducted immediately after incubation of whole blood with nanoparticles is complete, the culture supernatants can be stored at room temperature for two (2) hours or frozen at -80C. When kept frozen supernatants can tolerate only two (2) freeze/thaw cycles, therefore if repeat analysis in excess of 2 times is desirable supernatants must be frozen in small aliquots to avoid repeated multiple freeze/thaw cycles. The lower and upper limits of detection are 31.3 and 2000 pg/mL, respectively. The lower and upper limits of quantification are 62.5 and 2000 pg/mL, respectively.

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 Nunc Maxisorp flat bottom 96 well plate eBioscience cat# 44-2404-21

3.2 BupH Tris Buffered Saline Packs Pierce cat# 28376

3.3 BupH Carbonate-Bicarbonate Buffer Packs Pierce cat# 28382

3.4 Sealing Tape for 96-Well plates, pre-cut Pierce cat# 15036

3.5 Pooled human plasma, anti-coagulated with Li-heparin

3.6 Ultra TMB-ELISA Substrate Pierce cat# 34028

3.7 Tween-20, Sigma, cat# P1379

3.8 Bovine Serum Albumin (BSA), cat# A9647-100G

3.9. Human IFNγ monoclonal antibody, Mouse IgG2a, 500 µg, Clone# K3.53, R&D Systems cat# MAB2852

3.10. Human IFNγ Biotinylated Affinity Purified polyclonal antibody, Goat IgG, 50 µg, R&D Systems cat# BAF 285

3.11. Recombinant Human IFNγ, 100 µg, R&D Systems cat# 285-IF-100

3.12. NeutrAvidin Horseradish Peroxidase Conjugated 2 mg Pierce cat# 31001

3.13. Sulfuric acid

3.14. PBS, GE Life Science, cat #SH 30256.01

3.15. Fetal bovine serum, GE Life Sciences, Hyclone, cat# SH30070.03

3.16. RPMI1640, Invitrogen, cat#11875-119

3.17. Pen/Strep solution, Invitrogen, cat#15140-148

3.18. Pipettes covering the range from 0.05 to 1 mL

3.19. Microcentrifuge tubes, 1.5 mL

3.20. Multichannel pipette (8 or 12-channels)

3.21. Microcentrifuge

3.22. Centrifuge capable of running at 2500 x g, with a swinging basket set up

for holding 5cc vacutainer tubes

3.23. Refrigerator, 2-8°C

3.24 Freezer, -80°C

3.25 Vortex

3.26 ELISA plate reader capable of operating at 450 nm

3.27 Reagent reservoirs

3.28 Summary of reagents and their use per plate

Reagent	**Number of plates**
IFNγ monoclonal antibody (Clone K3.53), Mouse IgG2a	25
Human IFNγ Biotinylated Affinity Purified polyclonal antibody, Goat IgG	50
Recombinant Human IFNγ, carrier free	10
Neutravidin-HRP	2000

Note: Information summarized above is provided for information purposes only and not to endorse any specific vendor. Equivalent reagents and materials from other vendors can be used provided their performance in the assay was qualified and demonstrated acceptable performance.

4 Preparation and storage of plasma to be used for preparation of Assay Diluent.

Collect whole blood into heparinized tubes. Centrifuge for 10 minutes at 2500 x g and 2 – 8 °C; collect and pool plasma from at least 3 donors. Plasma must be prepared within 30-60 min after blood collection and either used immediately or aliquoted and stored at -20°C. Avoid repeated freeze-thaw cycles.

5. Preparation of reagents and controls

5.1. Coating Buffer (BupH Carbonate-Bicarbonate)

Dissolve one pack of BupH Carbonate-Bicarbonate in 500 mL distilled water and mix well. This produces 0.2 M carbonate-bicarbonate buffer with pH9.4 Filter through 0.2 μm filter and store at room temperature for up to one month.

5.2. Wash Buffer (1X TBS+0.05% Tween)

Dissolve one pack of BupH Tris Buffered Saline Pack in 500 mL distilled water mix well and add 250 μL of Tween-20. Store at at room temperature for one month.

5.3. Blocking Buffer (1XPBS+1%BSA+0.5% Tween)

Weigh 5g BSA and dissolve in 500 mL of 1 x PBS; then add 2.5 mL Tween-20 and mix well. Filter through 0.2 μm low protein binding filter and store at 4C for up to one month.

5.4. Complete RPMI

The complete RPMI medium should contain the following reagents:

10% FBS (heat inactivated)

2 mM L-glutamine

100U/mL penicillin

100 μg/mL streptomycin

Store at 2-8 °C protected from light for up to 1 month.

5.5. Assay Diluent

IMPORTANT: Composition of the assay diluent should mimic the sample matrix, i.e. it should be identical to the culture medium used to prepare study supernatants.

FOR WHOLE BLOOD SUPERNATANTS use the following as assay diluent:

Approach A: thaw pooled Li-Heparin plasma, palse-spin in a microcentrifuge or 10 min at 2,500 x g to remove fibrinogen fibers or any other aggregated material, and dilute this plasma in complete RPMI to a final concentration of 20 %, for example by adding 10 mL plasma into 40mL complete RPMI media. The plasma does not have to match the donors used in a given

experiment; large pool of plasma from various donors can be prepared in advance, aliquoted and stored at -20 °C. Use fresh, discard any leftover amount after experiment is complete.
Approach B: autologous plasma collected from the same donor as used in the culture experiment (see NCL ITA-10) can be used. The limitation of this approach is that a separate standard curve and quality control set should be prepared for each donor. Use fresh, discard any leftover amount after experiment is complete.
Note: if autologous plasma was frozen, palse-spin in a microcentrifuge or 10 min at 2,500 x g to remove fibrinogen fibers or any other aggregated material
Approach C: utilize unused portion of the blood diluted for the experiment (see NCL ITA-10) to prepare untreated blood supernatants from each individual donor and pool these supernatants to make pooled assay diluent representing all donors used in the given experiment. Then add 1mL of complete RPMI per each 4 mL of the pooled supernatant to match the matrix of study samples by both composition and concentration. Use fresh, discard any leftover amount after experiment is complete.
Note: If the experiment cannot be completed within the same day, it is OK to freeze this diluent at -20 °C. If this diluent is stored frozen and thawed prior to use in the assay palse-spin in a microcentrifuge or 10 min at 2,500 x g to remove fibrinogen fibers or any other aggregated material

FOR PBMC SUPERNATANTS use complete RPMI as the assay diluent

FOR SUPERNATANTS FROM OTHER CULTURES use complete culture medium specific to the analyzed cell line, e.g. if cells are grown in DMEM supplemented with 20 % FBS, then DMEM supplemented with 20 % FBS should be used as assay diluent.

5.6. Coating Antibody

5.6.1. Stock: Anti human IFNγ monoclonal mouse IgG2a is supplied as a lyophilized powder. Prepare stock by reconstitution of provided material in sterile PBS at concentration 1 mg/mL, e.g. by adding 0.5mL of sterile PBS to 0.5mg of lyophilized powder. Prepare single use 20 µL aliquots and store at -70 ºC for up to 6 months.

5.6.2. *Working Solution*: On the day of assay thaw 20 µL aliquot at room temperature and add the entire amount to 10mL of **Coating Buffer** from step 4.1 to yield the final concentration of 2 µg/mL.

5.7. Recombinant Human IFNγ Stock

Recombinant Human IFNγ Standard is supplied as lyophilized powder. Reconstitute lyophilized material in sterile PBS containing 0.1 % BSA to a final concentration of 200 µg/mL e.g. by adding 500 µL sterile PBS containing 0.1 % BSA to 100µg of lyophilized protein. Prepare single use 5 µL aliquots and store at -70 ºC for up to 6 months. This is stock solution to be used to prepare calibration standards and quality controls

Note: If protein from a source other than that tested in validation is used, the final dilution of this protein can be adjusted to provide more optimal assay performance (i.e. minimum background, high signal-to-noise ratio).

5.8. Secondary Antibody

5.8.1. Stock: Human IFNγ Polyclonal Goat IgG-Biotin Conjugate is supplied as lyophilized powder. Reconstitute the powder to a final concentration of 0.2 mg/mL in sterile PBS, e.g. by adding 250 µL of sterile PBS to 50 µg of lyophilized antibody. Aliquot 25 µL and store at -70 ºC for up to 6 months.

5.8.2. Working Solution: On the day of experiment thaw aliquot at room temperature and add the entire amount to 10 mL of **Blocking Buffer** from step 4.3 to a final concentration of 0.5 µg/mL.

Note: If antibody from a source other than that tested in validation is used, the final dilution of this antibody can be adjusted to provide more optimal assay performance (i.e. minimum background, high signal-to-noise ratio).

5.9. NeutrAvidin Horseradish Peroxidase Conjugate

5.9.1. Stock: NeutrAvidin Horseradish Peroxidase Conjugate is supplied as 2 mg lyophilized powder. Reconstitute with 0.4mL distilled water and further dilute to 2 mL by adding 1.6 mL sterile PBS to achieve a stock concentration of 1 mg/mL. For long

term storage freeze reconstituted product in single use 5 µL aliquot. Avoid repeated freezing and thawing.

Note: One 5µL aliquot is enough to prepare 50 mL of working solution sufficient to process 5 ELISA plates. To maximize the use of the conjugate one may want to accumulate samples for 5 plates; alternatively processing 1-2 plates at a time is OK but will result in loss of the conjugate as storage of lower than 5 µL aliquots or repeated freeze/thaw of the same aliquot is suboptimal and not recommended.

5.9.2. Working solution: On the day of experiment thaw one aliquot of the stock NeutrAvidin and dilute in **Blocking Buffer** to a final concentration of 0.1 µg/mL, e.g. by adding 1 µL of the stock into 10 mL Assay diluent.

5.10. Stop Solution (2N sulfuric acid)

Slowly add 27.7 mL H_2SO_4 into 200 mL of dH2O water, mix the solution thoroughly, let it cool and bring the solution to 500 mL with dH_2O using a 1000 mL graduated cylinder. Mix well and store in a bottle at room temperature.

6. Preparation of Calibration Standard and Quality Controls for ELISA.

6.1. Preparation of Human IFNγ Calibration Standards

Calibration standard are prepared by dilution of recombinant IFNγ stock prepared in step 5.7 in the **Assay Diluent**. Follow the table below for directions. Int A and Int B are intermediate solutions used only to prepare calibration curve.

Level	Nominal Conc. pg/mL	Preparation Procedure
Int A	200,000	5 µL of stock +4995 µL of Assay Diluent
Int B	20,000	100 µL of Int A +900 µL of Assay Diluent
Std 1	2000	100 µL of Int B +900 µL of Assay Diluent
Std 2	1000	250 µL of Std 1+250 µL of Assay Diluent
Std 3	500	250 µL of Std 2+250 µL of Assay Diluent

Std 4	250	250 µL of Std 3+250 µL of Assay Diluent
Std 5	125	250 µL of Std 4+250 µL of Assay Diluent
Std 6	62.5	250 µL of Std 5+250 µL of Assay Diluent
Std 7	31.3	250 µL of Std 6+250 µL of Assay Diluent

6.2. Preparation of Quality Controls

Quality control samples are prepared by dilution of recombinant IFNγ stock prepared in step 5.7 in the **Assay Diluent** and follow the table below for directions. Int A and Int B are intermediate solutions A and B used to prepare quality controls only. Although Int A and Int B have the same nominal concentrations as Int A and Int B used to prepare calibration standards, the latter set of intermediate solutions should not be used to prepare QC in order to avoid duplicating error. Prepare Int A and Int B for QC separately from that used to make calibration standards.

Level	Nominal Conc. pg/mL	Preparation Procedure
Int A	200,000	5 µL of stock +4995 µL of Assay Diluent
Int B	20,000	100 µL of Int A +900 µL of Assay Diluent
QC1	800	150 µL of Int B + 3600 µL of Assay Diluent
QC2	400	500 µL of QC1 +500 µL of Assay Diluent
QC3	200	300 µL of QC2 +300 µL of Assay Diluent

6.3. Preparation of Inhibition/Enhancement Controls (IECs)

Two approaches can be used to prepare IECs.

Approach A: Use culture supernatant from the positive control sample and spike it with the test nanoparticle at 4 concentrations (refer to the NCL ITA-10). For example, add 100 µL of nanoparticle working dilution into 400 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 IFNγ in the positive control supernatant

will be 1.3 times lower. Compare the IFNγ level in the positive control supernatant with that in IEC x 1.3 to account for the dilution factor. If the difference in test results is within 25 %, test-nanoparticle does not interfere with ELISA

Approach B: Use cell free controls and spike them with IFNγ standard. For example, add 5 µL of Int B from step 6.2 to 245 µL of cell-free supernatant from ITA-10. Compare this IEC to QC2. If the difference in test results is within 25 %, test-nanoparticle does not interfere with ELISA

7. Experimental Procedure

7.1. Refer to Section 9 of this protocol for the ELISA plate template to determine the number of plates to be used. Coat the plate with capture antibody by adding100 µL of working solution from step 5.6.2. to each well; cover plate with a plate sealer and incubate overnight at 4°C.

7.2. Aspirate coating solution and dry the plate by tapping on a paper towel. Add 100 µL of blocking buffer per well and incubate for one (1) hour at room temperature

7.3. During incubation time in step 7.2 prepare calibration standards and quality controls and as directed in section 6.1 and 6.2 respectively.

7.4. Aspirate blocking buffer, pet-dry the plate and add 100 µL of standards, test samples, quality controls and inhibition/enhancement controls to appropriate wells. Carefully cover the plate with an adhesive plate cover. Ensure that all edges and strips are sealed tightly. Incubate for one (1) hour at room temperature. **All standards, controls, and samples are analyzed in duplicate.**

Notes:

- *if samples were stored frozen, palse-spin in a microcentrifuge to remove fibrinogen fibers or any other aggregated material*
- *if it takes you longer than 5-10 minutes to load all samples on the plate, prepare an intermediate plate and use multichannel pipettor to transfer the diluted samples from the intermediate plate onto ELISA plate.*

7.5. Carefully remove the adhesive plate cover. Wash the plate 6 times with wash buffer. When using automatic plate washer, turn plates after first wash cycle (i.e. after first 3

washes). After final wash, tap the plate on absorbent paper to remove traces of wash buffer from wells

7.6. Add 100 µL per well of Secondary Antibody working solution from step 5.8.2, cover the plate with plate sealer and incubate for one (1) hour at room temperature.

7.7. Wash the plate 6 times with wash buffer. When using automatic plate washer turn plates after first wash cycle (i.e. after first 3 washes). After final wash, tap plate thoroughly on absorbent paper to remove traces of wash buffer from the wells.

7.8. Add 100 µL of NeutrAvidin HRP working solution from step 5.9.2. to each well, cover the plate with plate sealer and incubate for one (1) hour at room temperature.

7.9. Wash the plate 6 times with wash buffer. When using automatic plate washer turn plates after first wash cycle (i.e., after first 3 washes). After final wash, tap plate thoroughly on absorbent paper to remove traces of wash buffer from the wells.

7.10. Add 100 µL of TMB substrate per well, cover the plate with plate sealer and incubate plate for 20-30 minutes at room temperature. **Protect from light.**

Note: You can pre-read plate at 650 nm at about 15-20 min of incubation to decide whether to stop or continue incubation up to 30 min. Criteria for decision to stop and/or continue the incubation is acceptance of calibration standards and QC (see section 7) and steepness of the standard curve (it is better to avoid high concentration standards reaching maximum OD). OD units seen 650 nm will be lower after addition of stop solution and analysis at 450 nm.

7.11. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

7.12. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

8. Acceptance Criteria

8.1 % CV and PDFT for each calibration standard and quality control should be within 20 %. The exception is Cal 7, for which 25 % is acceptable.

8.2 Run (each plate) is accepted if 2/3 of all QC levels and at least one of each level have demonstrated acceptable performance (rule 4-6-20). If not, entire run should be repeated.

8.3 % 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.

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

8.5 Within the acceptable run if two of three replicates of unknown sample fail to meet acceptance criterion described in 8.3 this unknown sample should be re-analyzed.

9. Example of ELISA Plate Template

B0	Std1 2000pg/ml	Std2 1000pg/ml	Std3 500pg/ml	Std4 250pg/ml	Std5 125pg/ml	Std6 62.5pg/ml	Std7 31.3pg/ml	QC1 800 pg/ml	QC2 400g/ml	QC3 200pg/ml	TS1
B0	Std1 2000pg/ml	Std2 1000pg/ml	Std3 500pg/ml	Std4 250pg/ml	Std5 125pg/ml	Std6 62.5pg/ml	Std7 31.3pg/ml	QC1 800 pg/ml	QC2 400g/ml	QC3 200pg/ml	TS1
TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4	TS1 CF
TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4	TS1 CF
TS1 CF	TS1 CF	TS2 CF	TS2 CF	TS2 CF	TS3 CF	TS3 CF	TS3 CF	TS4 CF	TS4 CF	TS4 CF	IEC1
TS1 CF	TS1 CF	TS2 CF	TS2 CF	TS2 CF	TS3 CF	TS3 CF	TS3 CF	TS4 CF	TS4 CF	TS4 CF	IEC1
IEC2	IEC3	IEC4	NC	NC	PC	PC	VC	VC	QC1 800 pg/ml	QC2 400g/ml	QC3 200pg/ml
IEC2	IEC3	IEC4	NC	NC	PC	PC	VC	VC	QC1 800 pg/ml	QC2 400g/ml	QC3 200pg/ml

Std = standard; TS = test sample; QC = quality control; B0 = blank (assay diluent); NC- negative control supernatant; PC – positive control supernatant; VC- vehicle control supernatant; TS1, TS2, TS3 and TS 4 – supernatant from nanoparticle test sample at concentration 1,2,3 and 4, respectively; IEC1, IEC2, IEC3 and IEC4 – inhibition enhancement controls for nanoparticles at test concentrations 1,2,3, and 4, respectively; CF – cell free

10. Abbreviations

Std	standard
QC	quality control
B0	blank (assay diluent)
NC	negative control supernatant
PC	positive control supernatant
TS	test sample supernatant
IEC	inhibition enhancement control
CF	cell free
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute
VC	vehicle control supernatant
PBMC	peripheral blood mononuclear cells
FT	freeze/thaw
IFN	interferon
LPS	lipopolysaccharide
PHA-M	phytohemagglutinin-M
CpG DNA	deoxyribonucleic acid oligonucleotide containing CpG motif
NK	natural killer
NKT	natural killer T cells