NCL Method ITA-23

Enzyme-Linked Immunosorbent Assay (ELISA) for detection of human IL-1β 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

IL–1β is a pro-inflammatory chemokine produced by various types of cells in response to inflammatory stimulus, e.g., bacterial lipopolysaccharide (LPS). This document describes an experimental procedure for analysis of cell culture supernatants for the presence of IL–1β using an enzyme linked immunosorbent assay (ELISA). Refer to NCL protocol ITA-10 for details regarding culture supernatant preparation.

2. Principles

A 96 well plate is coated with capture antibody specific to IL-1β. Cell culture supernatants are then loaded onto the plate where IL-1β present in the supernatant is captured by the antibody. Excess sample is washed away, and captured IL-1β is detected with a secondary antibody conjugated to biotin. Streptavidin-conjugated horse radish peroxidase is used to develop the plate and the absorbance is detected at 450 nm. An optical density for the test sample which is higher than that of the background control is indicative of IL-1β in the supernatant. The quantity of IL-1β is determined by comparing the optical density of the test sample to that in a standard curve comprised of various concentrations of IL-1β reference standard.

The ELISA procedure takes approximately six hours. If the ELISA cannot be conducted immediately after culture supernatants are collected, the culture supernatants can be stored at room temperature for up to two hours or frozen at -80°C. Supernatants can tolerate three freeze/thaw cycles. Therefore, if repeat analysis in excess of three times is desirable, freeze supernatants in small aliquots to avoid repeated freeze/thaw cycles. The lower and upper limits of detection are 3.9 and 250 pg/mL, respectively. The lower and upper limits of quantification are 7.8 and 250 pg/mL, respectively.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted provided their performance in the assay is qualified and exhibits acceptable performance.

3.1 Reagents

1. Pooled human plasma, anti-coagulated with Li-heparin

2. Fetal bovine serum (GE Life Sciences, Hyclone, SH30070.03)
3. RPMI1640 (Invitrogen, 11875-119)
4. Pen/Strep solution (Invitrogen, 15140-148)
5. PBS (GE Life Sciences, SH 30256.01)
6. Bovine Serum Albumin (BSA) (Sigma, A9647-100G)
7. Tween-20 (Sigma, P1379)
8. BupH Tris Buffered Saline Packs (Pierce, 28376)
9. BupH Carbonate-Bicarbonate Buffer Packs (Pierce, 28382)
10. Ultra TMB-ELISA Substrate (Pierce, 34028)
11. NeutrAvidin Horseradish Peroxidase Conjugated, 2 mg (Pierce, 31001)
12. Sulfuric acid
13. Human IL-1β monoclonal antibody, Mouse IgG1, 500 μg, Clone# 2805 (R&D Systems, MAB601)
14. Human IL-1β biotinylated affinity purified polyclonal antibody, Goat IgG, 50 μg (R&D Systems, BAF 201)
15. Recombinant Human IL-1β, 5 μg (R&D Systems, 201-LB-005)

3.2 Materials

1. Nunc Maxisorp flat bottom 96 well plate (eBioscience, 44-2404-21)
2. Sealing Tape for 96-Well plates, pre-cut (Pierce, 15036)
3. Pipettes, 0.05 to 1 mL
4. Microcentrifuge tubes, 1.5 mL
5. Reagent reservoirs

3.3 Equipment

1. Multichannel pipette (8 or 12-channels)
2. Microcentrifuge
3. Centrifuge, 2500x*g*, with a swinging basket for 5cc vacutainer tubes
4. Refrigerator, 2-8°C
5. Freezer, -80°C
6. Vortex
7. ELISA plate reader, 450 nm

Table 1. Summary of reagents and their maximum number of uses.

Reagent	Number of Plates
IL-1β monoclonal antibody (Clone 28401), Mouse IgG1	50
Human IL-1β biotinylated affinity purified polyclonal antibody, Goat IgG	10
Recombinant Human IL-1β, carrier free	40
Neutravidin-HRP	2000

4. Preparation and Storage of Plasma for Assay Diluent

Collect whole blood in heparinized tubes. Centrifuge for 10 minutes at 2500x*g* at 2-8°C. Collect and pool plasma from at least three 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 pH 9.4. 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 (1X PBS + 1%BSA + 0.5% Tween)

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

5.4 Heat-Inactivated Fetal Bovine Serum

Thaw a bottle of FBS at room temperature, or overnight at 2-8ºC and allow to equilibrate to room temperature. Incubate 30 minutes at 56ºC in a water bath mixing

every five minutes. Single use aliquots may be stored at 2-8ºC for up to one month or at a nominal temperature of -20ºC indefinitely.

5.5 Complete RPMI-1640 Medium

The complete RPMI medium should contain the following reagents: 10% FBS (heat inactivated); 2 mM L-glutamine; 100 U/mL penicillin; and 100 μg/mL streptomycin sulfate. Store at 2-8ºC, protected from light for no longer than one month. Before use, warm in a 37ºC water bath.

5.6 Assay Diluent

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

5.6.1 Whole Blood Supernatants

Approach A: Thaw pooled Li-Heparin plasma, pulse-spin in a microcentrifuge or centrifuge 10 min at 2,500x*g* to remove fibrinogen fibers and any other aggregated material, and dilute this plasma in complete RPMI to a final concentration of 20%. For example, add 10 mL plasma into 40 mL complete RPMI media. The plasma does not have to match the donors used in a given experiment. A large pool of plasma from various donors can be prepared in advance, aliquoted, and stored at -20°C. Use this diluent fresh and 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 and discard any leftover amount after experiment is complete.

Note: If autologous plasma was frozen, pulse-spin in a microcentrifuge or centrifuge 10 min at 2,500x*g* to remove fibrinogen fibers and any other aggregated material.

Approach C: Utilize the unused portion of blood diluted for the experiment (see NCL ITA-10) to prepare untreated blood supernatants from each individual donor and pool these supernatants to make a pooled assay diluent representing all donors used in the given experiment. Add 1 mL of complete RPMI per each

4 mL of pooled supernatant to match the matrix of the study samples by both composition and concentration. Use fresh and discard any leftover amount after experiment is complete.

Note: If the experiment cannot be completed within the same day, the diluent can be froze at -20°C. If this diluent is stored frozen and thawed prior to use in the assay, pulse-spin in a microcentrifuge or centrifuge 10 min at 2,500x*g* to remove fibrinogen fibers and any other aggregated material.

5.6.2 PBMC Supernatants

For PBMC supernatants use complete RPMI as the assay diluent.

5.6.3 Supernatants From Other Cultures

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 the assay diluent.

5.7 Coating Antibody

1. Stock: Anti human IL-1β monoclonal mouse IgG1 is supplied as a lyophilized powder. Prepare stock by reconstitution of provided material in sterile PBS at a concentration of 0.5 mg/mL, e.g. by adding 1 mL of sterile PBS to 0.5 mg of lyophilized powder. Prepare single use 20 µL aliquots and store at -70ºC for up to 6 months.
2. Working Solution: On the day of assay thaw a 20 µL aliquot at room temperature and add the entire amount to 10 mL of Coating Buffer (step 5.1) to yield a final concentration of 1 µg/mL.

5.8 Recombinant Human IL-1β Stock

Recombinant Human IL-1β Standard is supplied as a lyophilized powder. Reconstitute lyophilized material in sterile PBS containing 0.1% BSA to a final concentration of 25 µg/mL, e.g., by adding 200 µL sterile PBS containing 0.1% BSA to 5 µg of lyophilized protein. Prepare single use 5 µL aliquots and store at -70ºC for up to 6 months. This is the stock solution to be used for calibration standards and quality controls.

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

5.9 Secondary Antibody

1. Stock: Human IL-1β Polyclonal Goat IgG-Biotin Conjugate is supplied as a lyophilized powder. Prepare stock by reconstitution of provided material in sterile PBS at a concentration of 0.2 mg/mL, e.g. by adding 250 µL of sterile PBS to 50 µg of lyophilized antibody. Prepare single use 25 µL aliquots and store at -70ºC for up to 6 months.
2. Working Solution: On the day of assay thaw a 25 µL aliquot at room temperature and add the entire amount to 10 mL of Blocking Buffer (step 5.3) to yield a final concentration of 0.5 µg/mL.

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

5.10 NeutrAvidin Horseradish Peroxidase Conjugate

1. Stock: NeutrAvidin Horseradish Peroxidase Conjugate is supplied as a 2 mg lyophilized powder. Reconstitute with 0.4 mL 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 uL aliquots. Avoid repeated freezing and thawing.

   Note: One 5 µL aliquot is enough to prepare 50 mL of working solution sufficient to process five ELISA plates. To maximize the use of the conjugate, consider accumulating samples for five plates. Processing one or two plates at a time 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.
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.

5.11 Stop Solution (2 N Sulfuric Acid)

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

6. Preparation of Calibration Standards and Quality Controls for ELISA

6.1 Human IL-1β Calibration Standards

Calibration standards are prepared by dilution of recombinant IL-1β stock (step 5.8) in the Assay Diluent. See Table 2 for dilutions. Int A and Int B are intermediate solutions used only to prepare the calibration curve samples.

Table 2. Calibration Standards

Sample	Nominal Concentration pg/mL	Preparation Procedure
Int A	25,000	5 µL of stock + 4995 µL of Assay Diluent
Int B	2,500	100 µL of Int A + 900 µL of Assay Diluent
Std 1	250	100 µL of Int B + 900 µL of Assay Diluent
Std 2	125	250 µL of Std 1 + 250 µL of Assay Diluent
Std 3	62.5	250 µL of Std 2 + 250 µL of Assay Diluent
Std 4	31.3	250 µL of Std 3 + 250 µL of Assay Diluent
Std 5	15.6	250 µL of Std 4 + 250 µL of Assay Diluent
Std 6	7.8	250 µL of Std 5 + 250 µL of Assay Diluent
Std 7	3.9	250 µL of Std 6 + 250 µL of Assay Diluent

6.2 Quality Controls

Quality control samples are prepared by dilution of recombinant IL-1β stock (step 5.8) in the Assay Diluent. See Table 3 for dilutions. Int A and Int B are intermediate solutions 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 (Table 2), the latter set of intermediate solutions should not be used to prepare QC in order to

avoid duplication of any possible errors. Prepare Int A and Int B for QC separately from that used to make calibration standards.

Table 3. Quality Controls

Sample	Nominal Concentration pg/mL	Preparation Procedure
Int A	25,000	5 µL of stock + 4995 µL of Assay Diluent
Int B	2,500	100 µL of Int A + 900 µL of Assay Diluent
QC 1	100	150 µL of Int B + 3600 µL of Assay Diluent
QC 2	50	500 µL of QC 1 + 500 µL of Assay Diluent
QC 3	12.5	200 µL of QC 2 + 600 µL of Assay Diluent

6.3 Inhibition/Enhancement Controls (IEC)

Approach A: Use culture supernatants from the positive control sample and spike with the test nanoparticle at four concentrations (refer to 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 IL-1β in the positive control supernatant will be 1.3 times lower. Compare the IL-1β 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%, the test-nanoparticle does not interfere with the ELISA.

Approach B: Use cell free controls and spike them with IL-1β standard. For example, add 10 µL of Int B from step 6.2 to 490 µL of cell-free supernatant from ITA-10. Compare this IEC to QC2. If the difference in test results is within 25%, the test-nanoparticle does not interfere with the ELISA.

7. Experimental Procedure

1. Refer to the Appendix (Section 10) for an ELISA plate template to determine the number of plates needed. Coat the plate with capture antibody by adding 100 µL of working

solution (step 5.7.2) to each well. Cover the plate with a plate sealer and incubate overnight at 4°C.

2. Aspirate coating solution and dry the plate by tapping on a paper towel. Add 100 µL of blocking buffer (step 5.3) per well and incubate for 1 hr at room temperature.
3. During this incubation time, prepare calibration standards, quality controls, and IEC as directed in steps 6.1, 6.2, and 6.3, respectively.
4. Aspirate blocking buffer and dry the plate by tapping on a paper towel. 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 1 hr at room temperature. All standards, controls, and samples should be analyzed in duplicate.

   Note: If samples were stored frozen, pulse-spin in a microcentrifuge to remove fibrinogen fibers and any other aggregated material. If it takes longer than 5-10 min to load all samples onto the plate, prepare an intermediate plate and use multichannel pipettor to transfer the diluted samples from the intermediate plate onto ELISA plate.
5. Carefully remove the adhesive plate cover. Wash the plate six times with wash buffer (step 5.2). When using an 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.
6. Add 100 µL of Secondary Antibody working solution (step 5.9.2) per well, cover the plate with a plate sealer, and incubate for 1 hr at room temperature.
7. Carefully remove the adhesive plate cover. Wash the plate six times with wash buffer (step 5.2). When using an 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.
8. Add 100 µL of NeutrAvidin HRP working solution (step 5.10.2) to each well, cover the plate with plate sealer, and incubate for 1 hr at room temperature.
9. Carefully remove the adhesive plate cover. Wash the plate six times with wash buffer (step 5.2). When using an 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.

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

    Note: You can pre-read plate at 650 nm after about 15-20 min of incubation to decide whether to stop or continue incubation up to 30 min. The criteria for deciding whether to stop or continue the incubation is acceptance of the calibration standards and QC (see Section 8) and steepness of the standard curve. It is better to avoid high concentration standards reaching maximum OD. The OD units seen at 650 nm will be lower after addition of the stop solution and analysis at 450 nm.
11. Add 50 µL of Stop Solution (step 5.11) 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.
12. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

8. Acceptance Criteria

1. The %CV and PDFT for each calibration standard and quality control should be within 20%. The exception is Std 7, for which 25% is acceptable.
2. The %CV for each test sample including supernatants from cell/blood cultures treated with positive control, negative control and nanoparticle samples should be within 20%. At least one replicate of positive and negative control should be acceptable for the run to be accepted.
3. If both replicates of positive control or negative control fail to meet acceptance criterion described in step 2, the run should be repeated.
4. Within the acceptable run, if two of three replicates of the unknown sample fail to meet acceptance criterion described in step 2, this unknown sample should be re-analyzed.
5. An elevation of IL-1β level in the test sample ≥2-fold above that observed in the baseline sample is considered a positive response.
6. Nanoparticles do not interfere with the ELISA if the difference in test results between the IEC and QC or PC (refer to approaches A and B in step 6.3) is within 25%.

9. Abbreviations

BSA	bovine serum albumin
CV	coefficient of variation
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
HRP	horseradish peroxidase
IEC	inhibition enhancement control
IL	interleukin
IFN	interferon
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	positive control
PDFT	percent difference from theoretical
QC	quality control
Std	standard
TBS	Tris buffered saline
TNF	tumor necrosis factor

10. Appendix

Example ELISA Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	B0	Std 1 250 pg/mL	Std 2 125 pg/mL	Std 3 62.5 pg/mL	Std 4 31.3 pg/mL	Std 5 15.6 pg/mL	Std 6 7.8 pg/mL	Std 7 3.9 pg/mL	QC 1 100 pg/mL	QC 2 50 pg/mL	QC 3 12.5 pg/mL	TS 1
B	B0	Std 1 250 pg/mL	Std 2 125 pg/mL	Std 3 62.5 pg/mL	Std 4 31.3 pg/mL	Std 5 15.6 pg/mL	Std 6 7.8 pg/mL	Std 7 3.9 pg/mL	QC 1 100 pg/mL	QC 2 50 pg/mL	QC 3 12.5 pg/mL	TS 1
C	TS 1	TS 1	TS 2	TS 2	TS 2	TS 3	TS 3	TS 3	TS 4	TS 4	TS 4	TS 1 CF
D	TS 1	TS 1	TS 2	TS 2	TS 2	TS 3	TS 3	TS 3	TS 4	TS 4	TS 4	TS 1 CF
E	TS 1 CF	TS 1 CF	TS 2 CF	TS 2 CF	TS 2 CF	TS 3 CF	TS 3 CF	TS 3 CF	TS 4 CF	TS 4 CF	TS 4 CF	IEC 1
F	TS 1 CF	TS 1 CF	TS 2 CF	TS 2 CF	TS 2 CF	TS 3 CF	TS 3 CF	TS 3 CF	TS 4 CF	TS 4 CF	TS 4 CF	IEC 1
G	IEC 2	IEC 3	IEC 4	NC	NC	PC	PC	VC	VC	QC 1 10 pg/mL	QC 2 50 pg/mL	QC 3 12.5 pg/mL
H	IEC 2	IEC 3	IEC 4	NC	NC	PC	PC	VC	VC	QC 1 10 pg/mL	QC 2 50 pg/mL	QC 3 12.5 pg/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; TS 1, TS 2, TS 3 and TS 4 = supernatant from nanoparticle test sample at four different concentrations; IEC 1, IEC 2, IEC 3 and IEC4 = inhibition enhancement controls for nanoparticles at the four test concentrations