



NCL Method ITA-24

Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Human TNF- α 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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Please cite this protocol as:

Cedrone E, Potter TM, Neun BW, Dobrovolskaia MA, NCL Method ITA-24: Enzyme-Linked Immunosorbent Assay (ELISA) for detection of human TNF- α in culture supernatants. <https://ncl.cancer.gov/resources/assay-cascade-protocols>

1. Introduction

TNF- α is a proinflammatory cytokine produced by various types of cells in response to inflammatory stimulus, e.g. bacterial lipopolysaccharide LPS. This document describes experimental procedure for analysis of culture supernatants by ELISA to detect presence of TNF- α . 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 TNF- α . Cell culture supernatants are loaded onto the plate and TNF- α present in the supernatant is captured by the antibody. The excess sample is washed away and captured TNF- α 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 TNF- α presence in the test supernatant. The quantity of TNF- α is determined by comparing optical density of the test sample to that in the standard curve comprised of various concentrations of TNF- α 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 -80°C. When kept frozen supernatants can tolerate only three (3) freeze/thaw cycles; therefore, if repeat analysis in excess of 3 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 62.5 and 4000 pg/mL, respectively. The lower and upper limits of quantification are 62.5 and 4000 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 reagents and materials from alternate vendors can be substituted provided their performance in the assay is qualified and demonstrates acceptable

performance. 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 BupH Tris Buffered Saline Packs (Pierce/Thermo, 28376)
- 3.1.2 BupH Carbonate-Bicarbonate Buffer Packs (Pierce/Thermo, 28382)
- 3.1.3 Pooled human plasma, anti-coagulated with Li-heparin
- 3.1.4 Ultra TMB-ELISA Substrate (Pierce/Thermo, 34028)
- 3.1.5 Tween-20 (Sigma, P1379)
- 3.1.6 Bovine Serum Albumin (BSA) (Sigma, A9647)
- 3.1.7 NeutrAvidin Horseradish Peroxidase Conjugated 2 mg (Pierce, 31001)
- 3.1.8 Sulfuric acid
- 3.1.9 PBS (E Life Sciences, SH 30256.01)
- 3.1.10 Fetal bovine serum (GE Life Sciences, Hyclone, SH30070.03)
- 3.1.11 RPMI1640 (Invitrogen, 11875-119)
- 3.1.12 Pen/Strep solution (Invitrogen, 15140-148)
- 3.1.13 Human TNF- α monoclonal antibody, Mouse IgG1 500 μ g Clone# 28401 (R&D Systems, MAB610)
- 3.1.14 Human TNF- α Biotinylated Affinity Purified polyclonal antibody, Goat IgG 50 μ g (R&D Systems, BAF 210)
- 3.1.15 Recombinant Human TNF- α 20 μ g (R&D Systems, 210-TA-020)

3.2 Materials

- 3.2.1 Nunc Maxisorp flat bottom 96-well plate (eBioscience, 44-2404-21)
- 3.2.2 Sealing Tape for 96-well plates, pre-cut (Pierce/Thermo, 15036)
- 3.2.3 Microcentrifuge tubes, 1.5 mL
- 3.2.4 Reagent reservoirs

3.3 Equipment

- 3.3.1 Pipettes covering the range from 0.05 to 1 mL
- 3.3.2 Multichannel pipette (8 or 12-channels)
- 3.3.3 Microcentrifuge

- 3.3.4 Centrifuge capable of running at 2500xg, with a swinging basket set up for holding 5cc vacutainer tubes
- 3.3.5 Refrigerator, 2-8°C
- 3.3.6 Freezer, -80°C
- 3.3.7 Vortex
- 3.3.8 ELISA plate reader capable of operating at 450 nm

Table 1. Summary of reagents and their use per plate.

Reagent	Number of Plates
TNF- α monoclonal antibody (Clone 28401), Mouse IgG1	25
Human TNF- α Biotinylated Affinity Purified polyclonal antibody, Goat IgG	10
Recombinant Human TNF- α , carrier free	40
Neutravidin-HRP	2000

4. Preparation and Storage of Plasma for Preparation of Assay Diluent

Collect whole blood into heparinized tubes. Centrifuge for 10 minutes at 2500xg 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 pH 9.4. Filter through 0.2 μ m 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 μ L of Tween-20. Store 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 μ m 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 2-8°C and allow to equilibrate to room temperature. Incubate 30 minutes at 56°C in a water bath mixing every five minutes. Single use aliquots may be stored at 2-8°C for up to one month or at a nominal temperature of -20°C 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 μ g/mL streptomycin sulfate. Store at 2-8°C, protected from light for no longer than one month. Before use, warm in a 37°C water bath.

5.6 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.

5.6.1 Whole Blood Supernatants

Approach A: Thaw pooled Li-Heparin plasma, pulse-spin in a microcentrifuge or centrifuge 10 min at 2,500xg 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 2,500xg 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 frozen 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 2,500xg 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

5.7.1 **Stock:** Monoclonal Mouse IgG1 is supplied as a lyophilized powder. Prepare stock by reconstitution of provided material in sterile PBS at concentration 0.5 mg/mL, e.g. by adding 1 mL of sterile PBS to 0.5 mg of lyophilized powder. Prepare single use 40 µL aliquots and store at -70°C for up to 6 months.

5.7.2 Working Solution: On the day of assay thaw a 40 μ L aliquot at room temperature and add the entire amount to 10 mL of Coating Buffer (step 5.1) to yield the final concentration of 2 μ g/mL.

5.8 Recombinant Human TNF- α Stock

Recombinant Human TNF- α Standard is supplied as lyophilized powder. Reconstitute lyophilized material in sterile PBS containing 0.1 % BSA to a final concentration of 100 μ g/mL e.g. by adding 200 μ L sterile PBS containing 0.1 % BSA to 20 μ g of lyophilized protein. Prepare single use 5 μ L aliquots and store at -70°C 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 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

5.9.1 Stock: Human TNF- α 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 μ L of sterile PBS to 50 μ g of lyophilized antibody. Aliquot 25 μ L and store at -70 °C for up to 6 months.

5.9.2 Working Solution: On the day of experiment thaw an aliquot at room temperature and add the entire amount to 10 mL of **B**locking Buffer (step 5.3) to a final concentration of 0.5 μ g/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

5.10.1 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 μ L aliquots. Avoid repeated freezing and thawing.

Note: One 5 μ L 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 μ L aliquots or repeated freeze/thaw of the same aliquot is suboptimal and not recommended.

5.10.2 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 μ g/mL, e.g. by adding 1 μ L of the stock into 10 mL Assay Diluent.

5.11 Stop Solution (2 N Sulfuric Acid)

Slowly add 27.7 mL H₂SO₄ into 200 mL of dH₂O water, mix the solution thoroughly, allow to cool, and bring the solution to 500 mL with dH₂O using a 1000 mL graduated cylinder. Mix well and store at room temperature.

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

6.1 Preparation of Human TNF- α Calibration Standards

Calibration standard are prepared by dilution of recombinant TNF- α 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 calibration curve samples.

Table 2. Calibration Standards

Sample	Nominal Concentration pg/mL	Preparation Procedure
Int A	100,000	5 μ L of stock + 4995 μ L of Assay Diluent
Int B	10,000	100 μ L of Int A + 900 μ L of Assay Diluent
Std 1	4000	200 μ L of Int B + 300 μ L of Assay Diluent

Std 2	2000	250 µL of Std 1 + 250 µL of Assay Diluent
Std 3	1000	250 µL of Std 2 + 250 µL of Assay Diluent
Std 4	500	250 µL of Std 3 + 250 µL of Assay Diluent
Std 5	250	250 µL of Std 4 + 250 µL of Assay Diluent
Std 6	125	250 µL of Std 5 + 250 µL of Assay Diluent

6.2 Quality Controls

Quality control samples are prepared by dilution of recombinant TNF- α 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, the latter set of intermediate solutions should not be used to prepare QC in order to avoid duplication of any possible error. 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	100,000	5 µL of stock + 4995 µL of Assay Diluent
Int B	10,000	100 µL of Int A + 900 µL of Assay Diluent
QC1	1500	600 µL of Int B + 3399 µL of Assay Diluent
QC2	750	500 µL of QC1 + 500 µL of Assay Diluent
QC3	375	500 µL of QC2 + 500 µL of Assay Diluent

6.3 Inhibition/Enhancement Controls (IEC)

Approach A: Use culture supernatants from the positive control sample and spike it 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 TNF α in the positive control supernatant will be 1.3 times lower. Compare the TNF α 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 TNF α standard. For example, add 50 μ L of Int B from step 6.2 to 615 μ L 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 an ELISA plate template to determine the number of plates to be used. Coat the plate with capture antibody by adding 100 μ L of working solution from step 5.7.2. 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 μ L of blocking buffer per well and incubate for 1 hr at room temperature.
- 7.3 During this incubation time, prepare calibration standards, quality controls and inhibition/enhancement controls as directed in section 6.1, 6.2 and 6.3 respectively.
- 7.4 Aspirate blocking buffer and dry the plate by tapping on a paper towel. Add 100 μ L 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.

- 7.5 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.
- 7.6 Add 100 μ L 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.
- 7.7 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.
- 7.8 Add 100 μ L 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.
- 7.9 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.
- 7.10 Add 100 μ L 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.
- 7.11 Add 50 μ L 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.
- 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 The %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 The run (each plate) is acceptable if 2/3 of all QC levels and at least one of each level have demonstrated acceptable performance (rule 4-6-20). If not, the entire run should be repeated.
- 8.3 The %CV for each test sample including supernatants from whole 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.
- 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 the unknown sample fail to meet acceptance criterion described in 8.3, 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	B0	Std 1 4000 pg/mL	Std 2 2000 pg/mL	Std 3 1000 pg/mL	Std 4 500 pg/mL	Std 5 250 pg/mL	Std 6 125 pg/mL	Std 7 62.5 pg/mL	QC 1 1500 pg/mL	QC 2 750 pg/mL	QC 3 375 pg/mL	TS 1
B	B0	Std 1 4000 pg/mL	Std 2 2000 pg/mL	Std 3 1000 pg/mL	Std 4 500 pg/mL	Std 5 250 pg/mL	Std 6 125 pg/mL	Std 7 62.5 pg/mL	QC 1 1500 pg/mL	QC 2 750 pg/mL	QC 3 375 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 1500 pg/mL	QC 2 750 pg/mL	QC 3 375 pg/mL
H	IEC 2	IEC 3	IEC 4	NC	NC	PC	PC	VC	VC	QC 1 1500 pg/mL	QC 2 750 pg/mL	QC 3 375 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; CF = cell free; 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

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.

10. Abbreviations

B0	blank (assay diluent)
CF	cell free
FT	freeze/thaw
IEC	inhibition enhancement control
NC	negative control supernatant
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	positive control supernatant
QC	quality control
Std	standard
TNF	tumor necrosis factor
TS	test sample supernatant
VC	vehicle control supernatant