

# Detection of cytokine release in THP-1 cells

## *Enzyme-linked immunosorbent assay (ELISA)*

AUTHORED BY:	DATE:
Cordula Hirsch	15.01.2014

REVIEWED BY:	DATE:
Harald Krug	09.04.2014

APPROVED BY:	DATE:

## DOCUMENT HISTORY

Effective Date	Date Revision Required	Supersedes
15.02.2014	DD/MM/YYYY	DD/MM/YYYY

Version	Approval Date	Description of the Change		Author / Changed by
1.0	DD/MM/YYYY	All	Initial Document	Cordula Hirsch

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>1/20</b>

## Table of Content

1	Introduction.....	3
2	Principle of the Method .....	3
3	Applicability and Limitations .....	3
4	Related Documents .....	3
5	Equipment and Reagents .....	4
5.1	Equipment .....	4
5.2	Reagents .....	4
5.3	Reagent Preparation .....	5
5.3.1	Complete cell culture medium .....	5
5.3.2	PMA stock solution.....	5
5.3.3	NaOH .....	5
5.3.4	Pluronic F-127.....	6
5.3.5	LPS .....	7
5.3.6	ELISA wash buffer .....	7
6	Procedure .....	7
6.1	General remarks.....	7
6.2	Flow chart 1 .....	8
6.3	Cell seeding.....	8
6.3.1	Cell culture.....	8
6.3.2	Cell seeding into 24-well plate .....	8
6.4	Cell treatment.....	9
6.4.1	Dilution of nanomaterials.....	9
6.4.2	Dilution of LPS (chemical positive control).....	12
6.4.3	Application of stimuli.....	12
6.5	Harvest of supernatant.....	13
6.6	ELISA performance as such.....	13
6.6.1	To get started .....	13
6.6.2	Flow chart 2 .....	14
6.6.3	TNF- $\alpha$ ELISA [eBioscience #88-7346].....	15
6.6.4	IL-8 ELISA [eBioscience #88-8086].....	16
6.7	Data evaluation .....	18
7	Quality Control, Quality Assurance, Acceptance Criteria.....	18

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>2/20</b>

8	Health and Safety Warnings, Cautions and Waste Treatment.....	19
9	Abbreviations .....	20
10	References.....	20

## 1 Introduction

In general inflammation describes a systemic and complex reaction of the body to harmful stimuli, as e.g. pathogens or irritants. This process involves (among others) the production of different cytokines by different cell types to allow for a coordinated defense reaction of the body. *In vitro* the release of cytokines from certain cell types can be studied using the enzyme-linked immunosorbent assay (ELISA) technique.

## 2 Principle of the Method

In the so called “sandwich” ELISA a first primary antibody is adsorbed to the surface of a high-affinity binding microwell plate. This antibody recognizes and binds the protein of interest in the cell culture supernatant. A second biotinylated antibody binding to the same protein of interest, but at a different epitope, serves as the detection antibody. It is visualized by horseradish peroxidase (HRP) linked to avidin and a subsequent enzymatic reaction using Tetramethylbenzidine (TMB) as the substrate. Absorbance of the resulting color is measured in an appropriate plate reader.

## 3 Applicability and Limitations

Cytokine expression and release is cell type dependent. Not all cell types release cytokines and not all cytokines are released by one cell type. Furthermore many commercial ELISA kits are available that are functional and can be used instead of the one described here. However, cell treatment conditions and sample titration will have to be optimized for each kit and cell type.

This SOP specifically addresses the measurement of Interleukin-8 (IL-8) and Tumor necrosis factor alpha (TNF- $\alpha$ ) in the supernatant of differentiated THP-1 cells. To be able to directly compare protein expression (by ELISA, described here) and gene regulation on the mRNA level (assessed by qRT-PCR, described in SOP “Detection of cytokine and Nrf2 signaling pathway component expression in THP-1 cells – qRT-PCR”) we harvest both biomolecules from the same sample. This necessitates optimized culture conditions as described in chapter 6 “Procedure”. The final measurement is done using the Ready-SET-Go!® ELISA kits from eBioscience.

Nanomaterial (NM) related considerations are addressed in the SOP: “NM interference in an ELISA”.

## 4 Related Documents

**Table 1:** Documents needed to proceed according to this SOP and additional NM-related interference control protocols.

Document ID	Document Title			
I_ELISA_interference	NM interference in an enzyme-linked immunosorbent assay (ELISA)			
Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>3/20</b>

cell culture_THP-1	<i>Culturing and differentiating THP-1 cells</i>
M_NM suspension_metal oxides	<i>Suspending and diluting Nanomaterials – Metal oxides and NM purchased as monodisperse suspensions</i>
M_NM suspension_carbon based	<i>Suspending and diluting Nanomaterials – Carbon based nanomaterials</i>

## 5 Equipment and Reagents

### 5.1 Equipment

- Absorbance reader for multi-well plates (to measure optical density (OD) at a wavelength of  $\lambda=650$  nm)
- Centrifuge (for cell pelleting; able to run 15 ml as well as 50 ml tubes at 200 x g)
- Conical tubes (15 ml and 50 ml; polypropylene or polystyrene; e.g. from Falcon)
- Flat bottom 24-well cell culture plates
- Flat bottom high-affinity binding 96-well plates (e.g. Corning Costar 9018 ELISA plate)
- Hemocytometer
- Laminar flow cabinet (biological hazard standard)
- Light microscope (for cell counting and cell observation)
- Microreaction tubes (1.5 ml; e.g. from Eppendorf)
- Multichannel pipette (with at least 8 positions; volume range per pipetting step at least from 50  $\mu$ l to 200  $\mu$ l)
- Vortex<sup>®</sup>

### 5.2 Reagents

For cell culturing and differentiation:

- Dimethyl sulfoxide (DMSO) [CAS number: 67-68-5]
- Fetal Calf Serum (FCS)
- L-glutamine
- Lipopolysaccharides (LPS) from *Salmonella enterica* [Sigma L7770; no CAS number]
- Neomycin<sup>1)</sup>
- Penicillin<sup>1)</sup>
- Phorbol 12-myristate 13-acetate (PMA) [CAS number: 16561-29-8]  
**Note: Carcinogenic! Handle with special care! Special waste removal** (see chapter 8)
- Phosphate buffered saline (PBS)
- Roswell Park Memorial Institute medium (RPMI-1640)
- Streptomycin<sup>1)</sup>
- Trypsin-EDTA (0.05%)

<sup>1)</sup> bought as a 100x concentrated mixture of Penicillin, Streptomycin and Neomycin (PSN) e.g. from Gibco.

Additionally necessary to dilute carbon based NM:

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>4/20</b>

- 10x concentrated RPMI-1640
- Sodium bicarbonate solution, 7.5% (NaHCO<sub>3</sub>) [CAS-number: 144-55-8]

ELISA kits:

- Human TNF alpha ELISA Ready-SET-Go!<sup>®</sup> [eBioscience # 88-7346]
- Human IL-8 ELISA Ready-SET-Go!<sup>®</sup> (2<sup>nd</sup> Generation) [eBioscience #88-8086]

For buffers and solvents not included in the ELISA kits:

- Pluronic F-127 [CAS number: 9003-11-6]
- Tween<sup>®</sup> 20 [CAS number: 9005-64-5]

For waste treatment:

- HCl (smoking) [CAS number: 7647-01-0]  
**Note: Corrosive and Irritating! Handle with special care!** (see chapter 8)
- NaOH [CAS number: 1310-73-2]  
**Note: Corrosive! Handle with special care!** (see chapter 8)

## 5.3 Reagent Preparation

### 5.3.1 Complete cell culture medium

Basic medium:

- RPMI-1640

supplemented with:

- 10% FCS
- 1x PSN, which results in final concentrations of:
  - 50 µg/ml Penicillin
  - 50 µg/ml Streptomycin
  - 100 µg/ml Neomycin
- 0.2 mg/ml L-glutamine

### 5.3.2 PMA stock solution

Prepare a 1 mM stock of PMA in DMSO. Therefore resuspend the 1 mg (standard packaging size) PMA powder in 1.62 ml DMSO. Aliquote and freeze at -20°C. Can be stored for years.

**Note: Carcinogenic! Handle with special care! Special waste removal.** (see chapter 8)

### 5.3.3 NaOH

Prepare a 5 M solution NaOH for PMA waste treatment.

- Dissolve 200 g NaOH pellets in 1 l ddH<sub>2</sub>O.

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>5/20</b>

**Note: Be careful, exothermic reaction, gets HOT. NaOH is corrosive, wear protective clothing (especially eye protection).**

### 5.3.4 Pluronic F-127

Stock:

- 160 ppm in ddH<sub>2</sub>O: 160 µg/ml (=16 mg/100 ml)

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>6/20</b>

### 5.3.5 LPS

Stock:

- 1 mg/ml in PBS: reconstitute the whole vial (1 mg) in 1 ml of sterile PBS
- Freeze this stock in 100 µl aliquots at -80°C.
- Can be stored for years.

Single use working stock:

- Dilute the 1 mg/ml stock solution to 100 µg/ml in PBS (1:10).
- Freeze this 100 µg/ml working stock as 50 µl **single use** aliquots at -80°C.
- Never re-freeze after thawing!

### 5.3.6 ELISA wash buffer

Prepare a 0.05% Tween-20® solution in PBS always freshly before usage. To perform an ELISA with one completely filled 96-well plate 1 l is needed. As Tween-20® is highly viscous, small volumes cannot be pipetted accurately. Weighing the liquid is thus the method of choice. With a density of 1.11 g/cm<sup>3</sup> you need:

- 0.56 g Tween-20®/1 l PBS

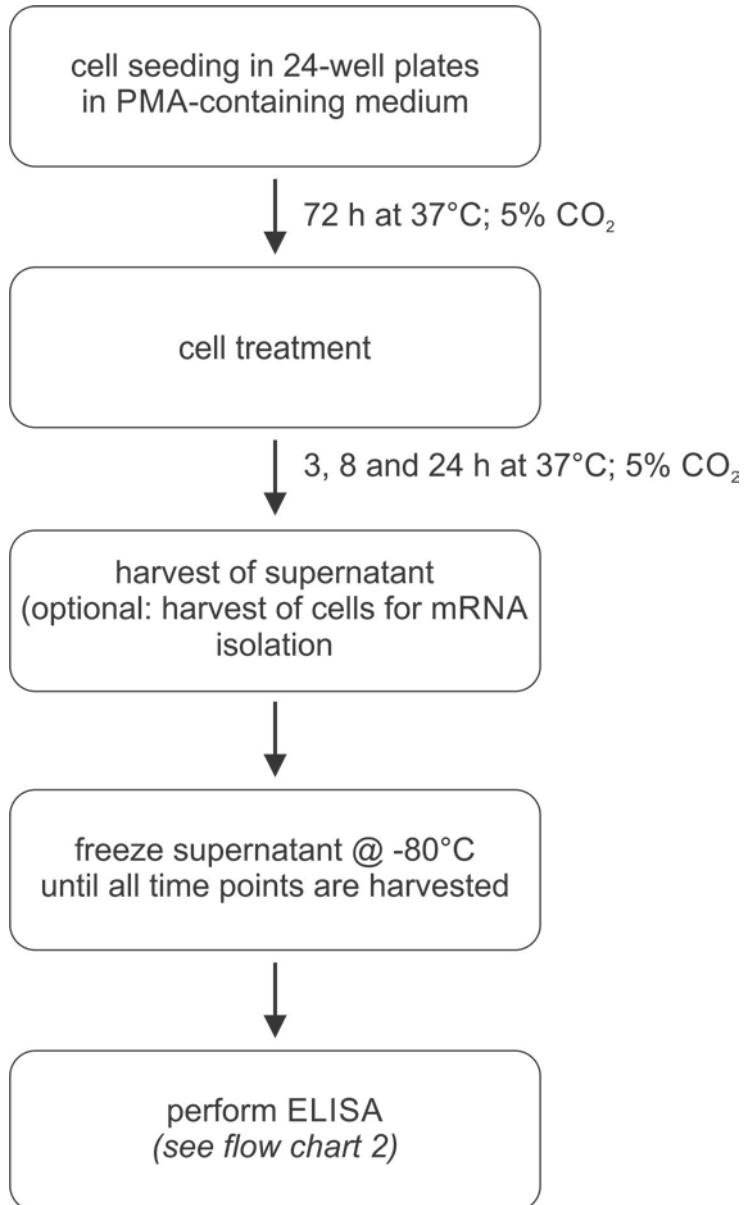
## 6 Procedure

### 6.1 General remarks

Well size and cell numbers are optimized to allow protein and mRNA measurements from the same sample. Supernatant (containing proteins) and cells (lysed to obtain mRNA) are harvested after 3, 8 and 24 hours of treatment. For technical reasons a separate 24-well plate for each time point is used.

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>7/20</b>

## 6.2 Flow chart 1



**Figure 1:** Brief outline of the workflow; from cell seeding and differentiation until harvest of supernatant (and cells for mRNA isolation; see SOP “Detection of cytokine and Nrf2 signaling pathway component expression in THP-1 cells”).

## 6.3 Cell seeding

### 6.3.1 Cell culture

THP-1 cells are grown in T75 cell culture flasks in a total volume of 20 ml of complete cell culture medium. They are kept at 37°C, 5% CO<sub>2</sub> in humidified air in an incubator (standard growth conditions according to SOP “Culturing and differentiating THP-1 cells”).

### 6.3.2 Cell seeding into 24-well plate

- Three days (72 h) prior to experimental start harvest and count cells as described in SOP “Culturing and differentiating THP-1 cells”.
- Seed  $2.5 \times 10^5$  cells in 500 µl complete cell culture medium containing 200 nM PMA per well into a 24-well cell culture plate.

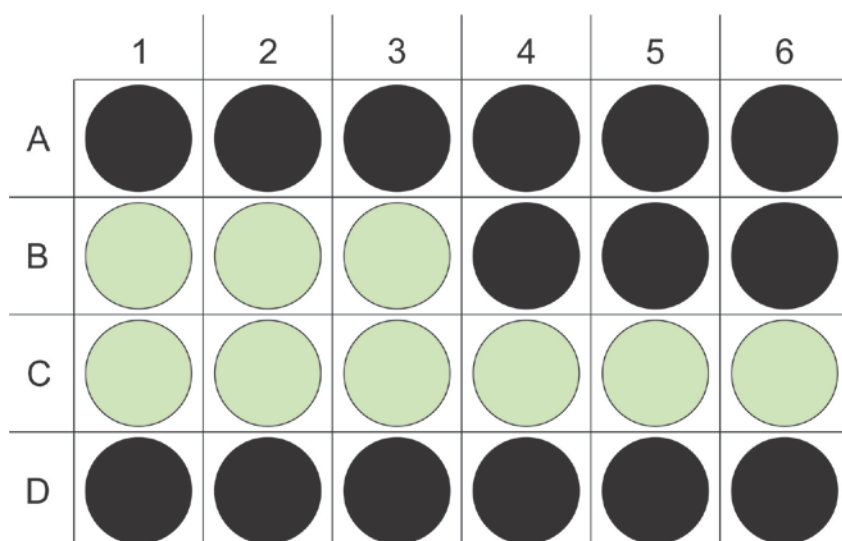
Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>8/20</b>



- For one 24-well plate (see Figure 2)  $2.5 \times 10^6$  cells are suspended in 5 ml complete PMA containing cell culture medium ( $5 \times 10^5$  cells/ml). To assess 3 time points, 3 plates are necessary ( $7.5 \times 10^6$  cells in 15 ml).

**Note:** PMA is diluted 1:5000 from the 1 mM stock (3  $\mu$ l/15 ml medium).

- Using a 1 ml micro-pipette 500  $\mu$ l of this cell suspension are distributed into each of the green wells (B1 to B3 and C1 to C6, figure 3).



**Figure 2: Cell seeding into a 24-well plate.**

Cells are seeded at a density of  $2.5 \times 10^5$  cells per well in 500  $\mu$ l complete cell culture medium (containing 200 nM PMA) into each of the green wells. Black wells receive 500  $\mu$ l complete cell culture medium each.

- Remaining wells (labeled in black in Figure 2) receive 500  $\mu$ l complete cell culture medium only.
- Differentiate cells for three days (72 hours) in a humidified incubator at standard growth conditions.

## 6.4 Cell treatment

### 6.4.1 Dilution of nanomaterials

For this SOP we distinguish two types of nanomaterials (NM) according to their solvent, suspension properties and highest concentrations used in the assay. See also respective related documents (3).

- (1) Metal oxide NM, Polystyrene beads and all NM delivered as monodisperse suspensions by the supplier: solvent either determined by the supplier or ddH<sub>2</sub>O; sub-diluted in ddH<sub>2</sub>O; highest concentration in assay 100  $\mu$ g/ml
- (2) Carbon based NM: suspended and sub-diluted in 160 ppm Pluronic F-127; highest concentration in assay 80  $\mu$ g/ml

Volumes given in the following dilution schemes are enough for three 24-well plates.

**Note:** "Mixing" in the context of diluting NMs means, the solvent containing tube is put on a continuously shaking Vortex® and the previous sub-dilution (or stock suspension, respectively) is put

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		9/20

dropwise into the shaking solvent. The resulting suspension stays on the Vortex® for additional 3 seconds before proceeding with the next sub-dilution.

### (1) Metal oxide NM:

Prepare serial sub-dilutions of the stock suspension (1 mg/ml) in ddH<sub>2</sub>O:

- Label six microreaction tubes (1.5 ml total volume) with 1 to 6 (relates to steps 1-6 below).
  - Add 1 ml NM stock suspension to tube no. 1.
  - Add 350 µl ddH<sub>2</sub>O to tubes no. 2, 4, 5 and 6.
  - Add 390 µl ddH<sub>2</sub>O to tube 3.
1. 1 ml NM stock suspension in ddH<sub>2</sub>O → 1 mg/ml (1)
  2. 350 µl of 1 mg/ml stock suspension (1) are mixed with 350 µl of ddH<sub>2</sub>O → 500 µg/ml (2)
  3. 260 µl of 500 µg/ml (2) are mixed with 390 µl ddH<sub>2</sub>O → 200 µg/ml (3)
  4. 350 µl of 250 µg/ml (3) are mixed with 350 µl ddH<sub>2</sub>O → 100 µg/ml (4)
  5. 350 µl of 100 µg/ml (4) are mixed with 350 µl ddH<sub>2</sub>O → 50 µg/ml (5)
  6. 390 µl ddH<sub>2</sub>O → solvent control (6)

Preparation of final dilutions:

- Label six conical tubes (15 ml total volume) as follows:
  1. 100 µg/ml
  2. 50 µg/ml
  3. 20 µg/ml
  4. 10 µg/ml
  5. 5 µg/ml
  6. Solvent control
- Add 1.8 ml complete cell culture medium to each tube.
- Mix on the Vortex® with 200 µl of the respective NM sub-dilutions or the solvent (ddH<sub>2</sub>O):
  1. 200 µl of the stock suspension (1 mg/ml) are mixed with 1.8 ml medium → 100 µg/ml (1)
  2. 200 µl of the 500 µg/ml sub-dilution are mixed with 1.8 ml medium → 50 µg/ml (2)
  3. 200 µl of the 200 µg/ml sub-dilution are mixed with 1.8 ml medium → 20 µg/ml (3)
  4. 200 µl of the 100 µg/ml sub-dilution are mixed with 1.8 ml medium → 10 µg/ml (4)
  5. 200 µl of the 50 µg/ml sub-dilution are mixed with 1.8 ml medium → 5 µg/ml (5)
  6. 200 µl of ddH<sub>2</sub>O (solvent) are mixed with 1.8 ml medium → solvent control (6)

### (2) Carbon based NM:

Prepare serial sub-dilutions of the stock suspension (500 µg/ml) in 160 ppm Pluronic F-127:

- Label six microreaction tubes (1.5 ml total volume) with 1 to 6 (relates to steps 1-6 below).
- Add 1 ml NM stock suspension to tube no. 1.
- Add 500 µl 160 ppm Pluronic F-127 to tubes 2 to 6.

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		10/20

1. 1 ml NM stock suspension in 160 ppm Pluronic → 500 µg/ml (1)
2. 500 µl of 500 µg/ml stock suspension (1) are mixed with 500 µl of Pluronic F-127 → 250 µg/ml (2)
3. 500 µl of 250 µg/ml (2) are mixed with 500 µl Pluronic F-127 → 125 µg/ml (3)
4. 500 µl of 125 µg/ml (3) are mixed with 500 µl Pluronic F-127 → 62.5 µg/ml (4)
5. 500 µl of 62.5 µg/ml (4) are mixed with 500 µl Pluronic F-127 → 31.25 µg/ml (5)
6. 500 µl 160 ppm Pluronic F-127 → solvent control (6)

Preparation of final dilutions:

- Prepare the appropriate dilution of a 10x concentrated medium stock as follows. This mixture (A) is used in all following steps for the preparation of the final NM concentrations. Mixing NM sub-dilutions with (A) will result in 1x concentrated medium containing the correct concentrations of all supplements and the respective NM concentrations.

Reagent	Volume
10x RPMI	1.7 ml
100x PSN	170 µl
100x L-Glutamine	170 µl
7.5% NaHCO <sub>3</sub>	450 µl
100% FCS	1.7 ml
ddH <sub>2</sub> O	10 ml

- Label six conical tubes (15 ml total volume) as follows:
  1. 80 µg/ml
  2. 40 µg/ml
  3. 20 µg/ml
  4. 10 µg/ml
  5. 5 µg/ml
  6. Solvent control
- Add 2.1 ml (A) to each tube.
- Mix on the Vortex<sup>®</sup> with 400 µl of the respective NM sub-dilutions or the solvent (160 ppm Pluronic F-127):
  1. 400 µl of the stock suspension (500 µg/ml) are mixed with 2.1 ml medium → 80 µg/ml (1)
  2. 400 µl of the 250 µg/ml sub-dilution are mixed with 2.1 ml medium → 40 µg/ml (2)
  3. 400 µl of the 125 µg/ml sub-dilution are mixed with 2.1 ml medium → 20 µg/ml (3)
  4. 400 µl of the 62.5 µg/ml sub-dilution are mixed with 2.1 ml medium → 10 µg/ml (4)
  5. 400 µl of the 31.25 µg/ml sub-dilution are mixed with 2.1 ml medium → 5 µg/ml (5)
  6. 400 µl of 160 ppm Pluronic F-127 (solvent) are mixed with 2.1 ml medium → solvent control (6)

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		11/20

### 6.4.2 Dilution of LPS (chemical positive control)

Prepare a 10 µg/ml sub-dilution of the LPS working stock (100 µg/ml) in PBS:

- mix 27 µl of PBS with 3 µl of the working stock.

Prepare the final concentrations in complete cell culture medium as follows:

- 1 µg/ml: 2 ml medium + 20 µl working stock (100 µg/ml)
- 0.1 µg/ml: 2 ml medium + 20 µl sub-dilution (10 µg/ml)

Apply NM as well as LPS as described below.

### 6.4.3 Application of stimuli

**Note:** All NM dilutions have to be vortexed directly before application to the cells.

Three days (72 hours) after cell seeding!

- Remove PMA-containing medium. **Toxic! Discard this supernatant separately! See Chapter 8 “Waste treatment”.**
- Wash cells twice with 1 ml of pre-warmed PBS per well.
- Add 500 µl per well of complete cell culture medium containing the corresponding LPS and NM concentrations according to the pipetting scheme shown in Figure 3.
- Culture cells for appropriate time points (3 h, 8 h, 24 h) under standard growth conditions.

	1	2	3	4	5	6
A	●	●	●	●	●	●
LPS (µg/ml) B	0	0.1	1	●	●	●
NM conc. <sup>1)</sup> (µg/ml) C	0	5	10	20	50	100
D	●	●	●	●	●	●

**Figure 3: Application of stimuli.** NMs as well as LPS are applied in 500 µl complete cell culture medium per well after two washing steps in PBS.

<sup>1)</sup> NM concentrations given here refer to metal oxide NMs. Carbon based NM concentrations are detailed in the text.

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		12/20

## 6.5 Harvest of supernatant

- After appropriate time points (3 h, 8 h, 24 h) transfer the supernatant of each well (500 µl) to a separate 1.5 ml microreaction tube. (At this point remaining cells can be harvested for RNA isolation. Therefore remaining cells are lysed in 350 µl RLT buffer per well. See SOP “Detection of cytokine and Nrf2 signaling pathway component expression in THP-1 cells – qRT-PCR”).
- Spin down for 5 minutes at 200 x g.
- Take 400 µl of the supernatant and transfer to a new 1.5 ml microreaction tube.
- Freeze at -80°C until all time points are harvested and for long time storage. (Supernatants can be stored for at least one year at -80°C.)

## 6.6 ELISA performance as such

All volumes given are for one 96-well plate where all samples are performed in duplicates.

### 6.6.1 To get started

- Prepare appropriate amount of 1x assay diluent from the 5x stock:  
10 ml 5x assay diluent + 40 ml ddH<sub>2</sub>O
- Prepare appropriate amount of wash buffer:  
0.56 g Tween-20® / 1 l PBS
- Prepare appropriate amount of 1x coating buffer from the 10x stock:  
1.5 ml 10x coating buffer + 13.5 ml ddH<sub>2</sub>O

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>13/20</b>

## 6.6.2 Flow chart 2

general work flow

specific features

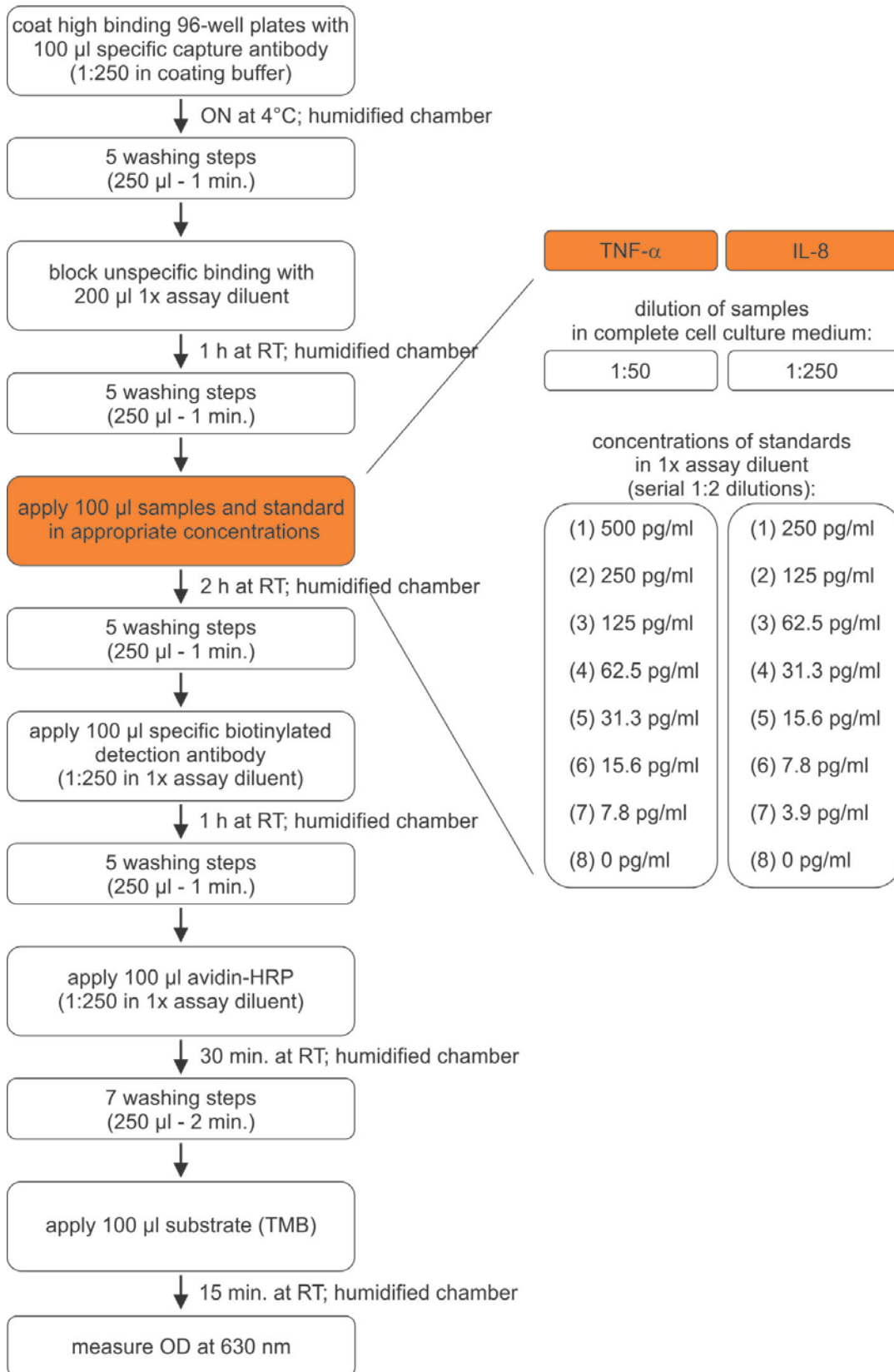


Figure 4: Brief outline of the ELISA workflow.

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		14/20

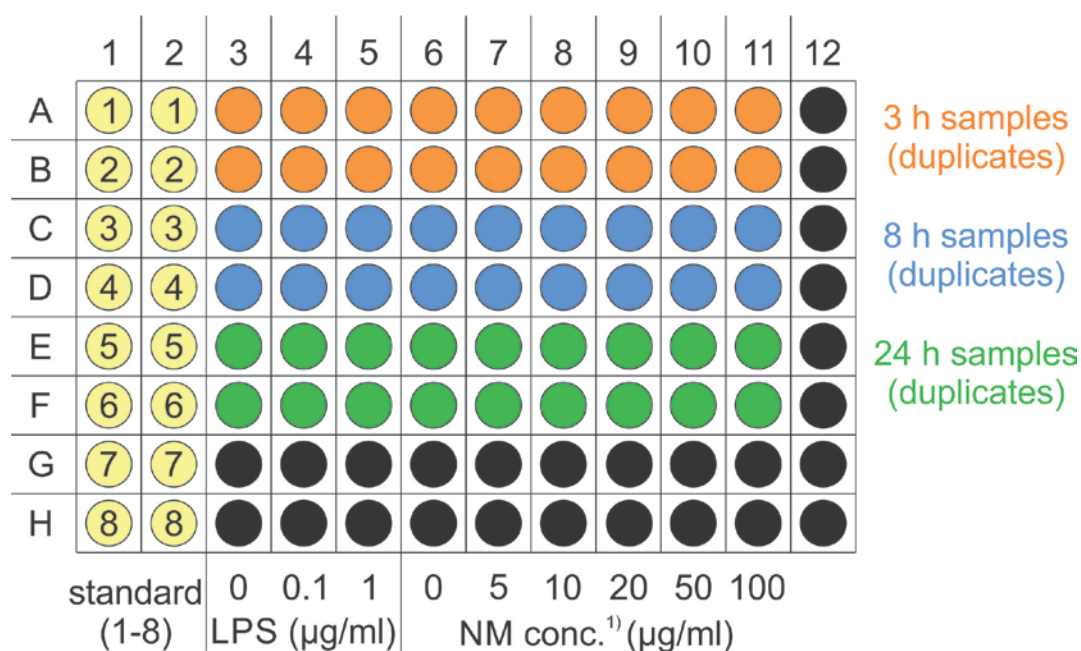
### 6.6.3 TNF- $\alpha$ ELISA [eBioscience #88-7346]

- Standard curve: Prepare serial 1:2 dilutions of the recombinant TNF- $\alpha$  protein (contained as single use aliquots in the ELISA kit) in 1x assay diluent. **Stock concentration: 1  $\mu$ g/ml.**
  - Label eight microreaction tubes (1.5 ml total volume) with 1 to 8 (relates to steps 1-8 below).
  - Add 1000  $\mu$ l of 1x assay diluent to tube no. 1
  - Add 300  $\mu$ l to tubes 2 to 8.
  1. 0.5  $\mu$ l of the stock suspension (1  $\mu$ g/ml) are mixed with 1000  $\mu$ l of 1x assay diluent  $\rightarrow$  500 pg/ml (1)
  2. 300  $\mu$ l of 500 pg/ml (1) are mixed with 300  $\mu$ l 1x assay diluent  $\rightarrow$  250 pg/ml (2)
  3. 300  $\mu$ l of 250 pg/ml (2) are mixed with 300  $\mu$ l 1x assay diluent  $\rightarrow$  125 pg/ml (3)
  4. 300  $\mu$ l of 125 pg/ml (3) are mixed with 300  $\mu$ l 1x assay diluent  $\rightarrow$  62.5 pg/ml (4)
  5. 300  $\mu$ l of 62.5 pg/ml (4) are mixed with 300  $\mu$ l 1x assay diluent  $\rightarrow$  31.3 pg/ml (5)
  6. 300  $\mu$ l of 31.3 pg/ml (5) are mixed with 300  $\mu$ l 1x assay diluent  $\rightarrow$  15.6 pg/ml (6)
  7. 300  $\mu$ l of 15.6 pg/ml (6) are mixed with 300  $\mu$ l 1x assay diluent  $\rightarrow$  7.8 pg/ml (7)
  8. 300  $\mu$ l 1x assay diluent  $\rightarrow$  solvent control (8)

Keep all dilutions on ice (4°C) till needed.

- Prepare a 1:250 dilution of the TNF- $\alpha$  capture antibody in 1x coating buffer.  
10 ml 1x coating buffer + 40  $\mu$ l TNF- $\alpha$  capture antibody
- Coat high affinity binding 96-well plate with 100  $\mu$ l/well of this TNF- $\alpha$  capture antibody dilution. Incubate the plate in a humidified chamber overnight (ON) at 4°C.
- **Washing** (performed this way throughout the whole procedure):  
Aspirate all wells (using a vacuum pump equipped with an 8-channel adapter) and wash 5 times for at least 1 min. with 250  $\mu$ l/well wash buffer. After the last washing step (after aspiration of wash buffer) blot plate on absorbent paper to remove any residual buffer.
- Block wells with 200  $\mu$ l/well 1x assay diluent. Incubate in a humidified chamber for 1 h at RT.
- Perform 5 washing steps as describe above: 250  $\mu$ l/well washing buffer, 1 min. each.
- Dilute samples 1:50 in complete cell culture medium:  
6  $\mu$ l sample + 294  $\mu$ l complete cell culture medium
- Apply 100  $\mu$ l of standard and sample dilutions per well according to pipetting scheme in Figure 5 and incubate in a humidified chamber for 2 h at RT.

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		15/20



**Figure 5: Distribution of standard and sample dilutions.** Yellow: duplicates of the 8 dilutions of the recombinant standard protein. 100 µl/well are distributed into wells A1 to H8. 100 µl/well of the sample dilutions are distributed into wells A3 to F11 (orange, blue and green). Black: empty wells.

<sup>1)</sup> NM concentrations given here refer to metal oxide NMs. Carbon based NM concentrations are detailed in the text.

- Perform 5 washing steps as describe above: 250 µl/well washing buffer, 1 min. each.
- Prepare a 1:250 dilution of the TNF-α detection antibody in 1x assay diluent.  
10 ml 1x assay diluent + 40 µl TNF-α detection antibody
- Apply 100 µl/well of this TNF-α detection antibody dilution. Incubate in a humidified chamber for 1 h at RT.
- Perform 5 washing steps as describe above: 250 µl/well washing buffer, 1 min. each.
- Prepare a 1:250 dilution of Avidin-HRP in 1x assay diluent.  
10 ml 1x assay diluent + 40 µl Avidin-HRP
- Apply 100 µl/well of this Avidin-HRP dilution. Incubate in a humidified chamber for 30 min. at RT.
- Perform 7 washing steps as describe above: 250 µl/well washing buffer, 2 min. each.
- Apply 100 µl/well Substrate Solution (TMB) and incubate in a humidified chamber for 15 min. at RT.
- Read plate at 650 nm.

#### 6.6.4 IL-8 ELISA [eBioscience #88-8086]

- Standard curve: Prepare serial 1:2 dilutions of the recombinant IL-8 protein (contained as single use aliquots in the ELISA kit) in 1x assay diluent. **Stock concentration: 1 µg/ml.**
  - Label eight microreaction tubes (1.5 ml total volume) with 1 to 8 (relates to steps 1-8 below).
  - Add 1000 µl of 1x assay diluent to tube no. 1.
  - Add 300 µl to tubes 2 to 8.

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		16/20



1. 0.25 µl of the IL-8 stock solution (1 µg/ml) are mixed with 1000 µl of 1x assay diluent → 250 pg/ml (1)
2. 300 µl of 250 pg/ml (1) are mixed with 300 µl 1x assay diluent → 125 pg/ml (2)
3. 300 µl of 125 pg/ml (2) are mixed with 300 µl 1x assay diluent → 62.5 pg/ml (3)
4. 300 µl of 62.5 pg/ml (3) are mixed with 300 µl 1x assay diluent → 31.3 pg/ml (4)
5. 300 µl of 31.3 pg/ml (4) are mixed with 300 µl 1x assay diluent → 15.6 pg/ml (5)
6. 300 µl of 15.6 pg/ml (5) are mixed with 300 µl 1x assay diluent → 7.8 pg/ml (6)
7. 300 µl of 7.8 pg/ml (6) are mixed with 300 µl 1x assay diluent → 3.9 pg/ml (7)
8. 300 µl 1x assay diluent → solvent control (8)

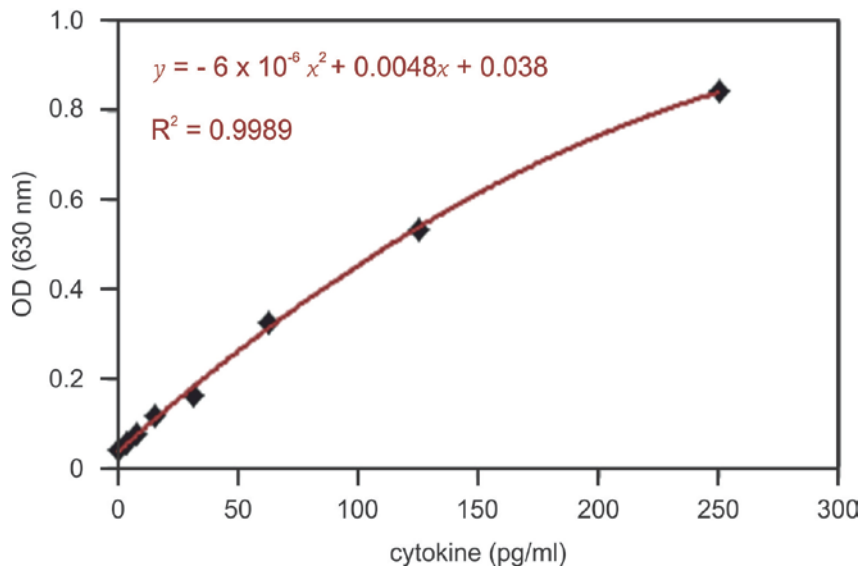
Keep all dilutions on ice (4°C) till needed.

- Prepare a 1:250 dilution of the IL-8 capture antibody in 1x coating buffer.  
10 ml 1x coating buffer + 40 µl IL-8 capture antibody
- Coat high affinity binding 96-well plate with 100 µl/well of this IL-8 capture antibody dilution. Incubate the plate in a humidified chamber ON at 4°C.
- **Washing** (performed this way throughout the whole procedure):  
Aspirate all wells (using a vacuum pump equipped with an 8-channel adapter) and wash 5 times for at least 1 min. with 250 µl/well wash buffer. After the last washing step (after aspiration of wash buffer) blot plate on absorbent paper to remove any residual buffer.
- Block wells with 200 µl/well 1x assay diluent. Incubate in a humidified chamber for 1 h at RT.
- Perform 5 washing steps as describe above: 250 µl/well washing buffer, 1 min. each.
- Dilute samples 1:250 in complete cell culture medium:  
1.5 µl sample + 373.5 µl complete cell culture medium
- Apply 100 µl of standard and sample dilutions per well according to pipetting scheme in Figure 5 and incubate in a humidified chamber for 2 h at RT.
- Perform 5 washing steps as describe above: 250 µl/well washing buffer, 1 min. each.
- Prepare a 1:250 dilution of the IL-8 detection antibody in 1x assay diluent.  
10 ml 1x assay diluent + 40 µl IL-8 detection antibody
- Apply 100 µl/well of this TNF-α detection antibody dilution. Incubate in a humidified chamber for 1 h at RT.
- Perform 5 washing steps as describe above: 250 µl/well washing buffer, 1 min. each.
- Prepare a 1:250 dilution of Avidin-HRP in 1x assay diluent.  
10 ml 1x assay diluent + 40 µl Avidin-HRP
- Apply 100 µl/well of this Avidin-HRP dilution. Incubate in a humidified chamber for 30 min. at RT.
- Perform 7 washing steps as describe above: 250 µl/well washing buffer, 2 min. each.
- Apply 100 µl/well Substrate Solution (TMB) and incubate in a humidified chamber for 15 min. at RT.
- Read plate at 650 nm.

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		17/20

## 6.7 Data evaluation

The mean is calculated from the OD-values of the standard curve duplicates. These mean values are plotted against their corresponding concentrations (see Figure 6).



**Figure 6:** Example of standard curve measurement and polynomic curve fitting. Resulting quadratic equation (1) and correlation coefficient ( $R^2$ ) are given.

Polynomic curve fitting with two unknowns results in quadratic equation (1):

$$y = ax^2 + bx + c \quad (1)$$

Solving the equation for x results in equation (2):

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad (2)$$

Using equation (2) the cytokine content (in pg/ml) can be calculated from sample OD values (OD values equal y). In the example shown in Figure 6 the following values can be attributed to the variables:

$$a = -6 \times 10^{-6}$$

$$b = 0.0048$$

$$c = 0.038$$

**Note:** This is only an example! Measurements have to be performed and values calculated with every ELISA performance and for every cytokine.

## 7 Quality Control, Quality Assurance, Acceptance Criteria

Highest concentrations of the recombinant standard proteins (500 pg/ml for TNF- $\alpha$  and 250 pg/ml for IL-8) should result in OD (650 nm) values of at least 0.8. Values lower than 0.8 indicate improper

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		18/20

binding of antibodies to the plate or unfolding of the recombinant standard proteins. In both cases the detection limit of the whole assay will be lowered.

The correlation coefficient  $R^2$  (as depicted in Figure 6) is a measure for the strength of the relationship of two variables. A  $R^2$  of 1 would be the perfect correlation (all values exactly on the curve). A  $R^2$  of 0 would be no correlation at all (random distribution of the measured values). To assure accurate ELISA performance  $R^2$  should be above a value of 0.8.

To assure proper cell performance LPS treatment should result in considerable TNF- $\alpha$  as well as IL-8 induction. As a rough estimate check the following fold changes compared to the respective untreated control:

	3 h	8 h	24 h
TNF- $\alpha$	3-fold	6-fold	3-fold
IL-8	3-fold	6-fold	8-fold

At least these fold changes have to be reached and at least for two of the three time points in each experiment.

## 8 Health and Safety Warnings, Cautions and Waste Treatment

Cell seeding has to be carried out under sterile conditions in a laminar flow cabinet (biological hazard standard). For this only sterile equipment must be used and operators should wear laboratory coat and gloves (according to laboratory internal standards). Special care has to be taken during PMA handling (carcinogenic potential of the substance!).

**PMA waste treatment:** use a separate exhaust extraction system with a collecting flask containing already 20 ml 5 M NaOH to neutralize PMA. The resulting non-toxic solution is very alkaline and has to be neutralized using HCl before final disposal in the sink.

NaOH is **corrosive**. It causes severe burns. Wear especially eye/face protection. Dissolution of NaOH is an exothermic reaction, the solution will get fairly hot – be careful! It is strongly recommended to wear eye protection when handling 5 M NaOH.

HCl is **corrosive and irritant**. It is very hazardous in case of skin contact, of eye contact and of ingestion. It is slightly hazardous in case of inhalation. Therefore avoid inhalation as well as contact with skin and eyes and avoid exposure in general.

Discard all materials used to handle cells (including remaining cells themselves) according to the appropriate procedure for special biological waste (i.e. by autoclaving).

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		19/20

## 9 Abbreviations

ddH <sub>2</sub> O	double-distilled water
DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
g	constant of gravitation
HRP	horseradish peroxidase
IL-8	interleukine 8
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
OD	optical density
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
ppm	parts per million
PSN	Penicillin, Streptomycin, Neomycin
qRT-PCR	quantitative real-time polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
TMB	Tetramethylbenzidine
TNF- $\alpha$	tumor necrosis factor alpha

## 10 References

Document Type	Document ID	Version	Status	Page
SOP	I_ELISA_THP-1	1.0		<b>20/20</b>