Project: VIGO

EMPA
Materials Science & Technology

Detection of cytokine release in A549 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:

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Table of Content

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) in the supernatant of A549 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 expression in A549 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 kit 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)*
cell culture_A549	*Culturing A549 cells*
M_NM suspension_metal oxides	*Suspending and diluting Nanomaterials – Metal oxides and NM purchased as monodisperse suspensions*
M_NM suspension_ carbon based	*Suspending and diluting Nanomaterials – Carbon based nanomaterials*

5 Equipment and Reagents

5.1 Equipment

- Absorbance reader for multi-well plates (to measure optical density (OD) at a wavelength of λ=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 µl to 200 µl)
- Vortex®

5.2 Reagents

For cell culturing:

- Bovine serum albumin (BSA) *[CAS number: 9048-46-8]*
- Fetal Calf Serum (FCS)
- L-glutamine
- Neomycin[1)]
- Penicillin[1)]
- Phosphate buffered saline (PBS)
- Recombinant tumor necrosis factor alpha (TNF-α) *[CAS number: 94948-59-1]*
- Roswell Park Memorial Institute medium (RPMI-1640)
- Streptomycin[1)]
- Trypsin-EDTA (0.05%)

[1)] bought as a 100x concentrated mixture of Penicillin, Streptomycin and Neomycin (PSN) e.g. from Gibco.

Additionally necessary to dilute carbon based NM:

- 10x concentrated RPMI-1640
- Sodium bicarbonate solution, 7.5% ($NaHCO_3$) *[CAS-number: 144-55-8]*

ELISA kit:

- Human IL-8 ELISA Ready-SET-Go!® (2nd Generation) *[eBioscience #88-8086]*

For buffers and solvents not included in the ELISA kits:

- Pluronic F-127 *[CAS number: 9003-11-6]*
- Tween® 20 *[CAS number: 9005-64-5]*

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 Pluronic F-127

Stock:

- 160 ppm in ddH_2O: 160 µg/ml (=16 mg/100 ml)

5.3.3 Recombinant TNF-α

Solvent:

- 0.1% BSA in PBS: 0.1 g BSA/100 ml PBS

Stock:

- 100 µg/ml in 0.1% BSA in PBS: reconstitute the whole vial (50 µg) in 500 µl of sterile PBS containing 0.1% BSA
- Freeze this stock in single use aliquots at -20°C.
- Never re-freeze after thawing!
- Can be stored for years.

5.3.4 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^3 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.

6.2 Flow chart 1

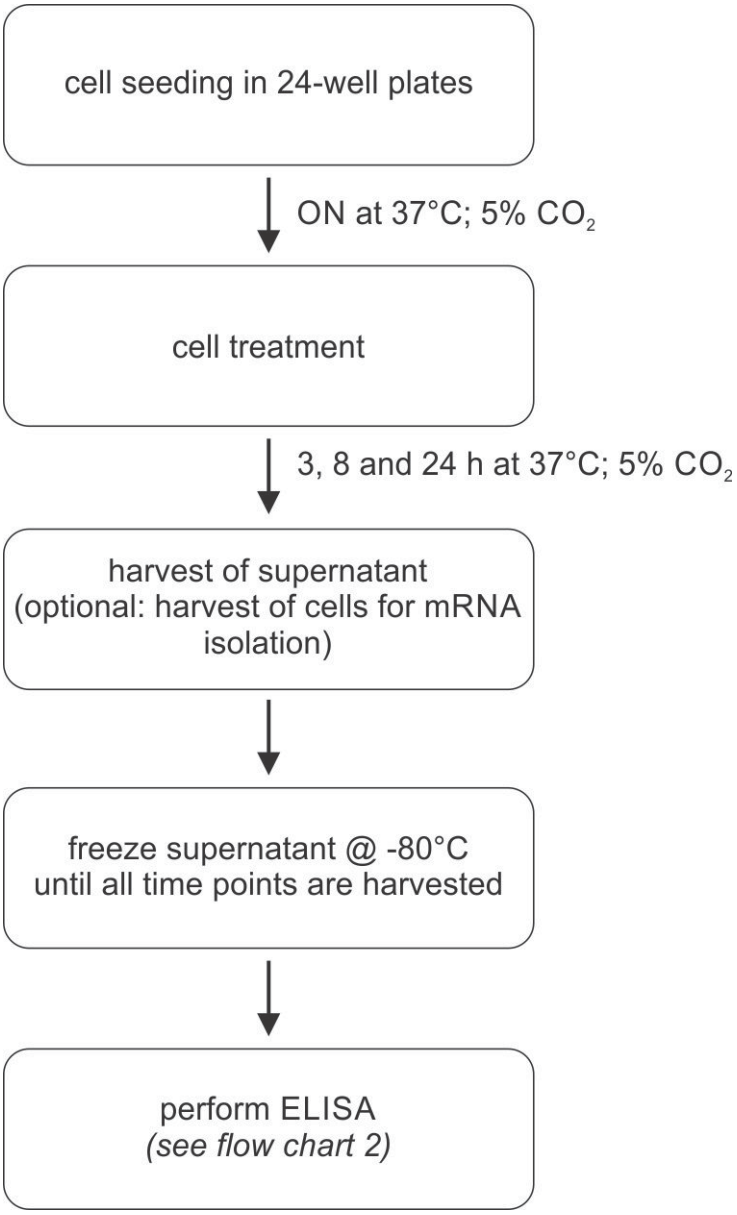

Figure 1: Brief outline of the workflow; from cell seeding until harvest of supernatant (and cells for mRNA isolation; see SOP: "Detection of cytokine expression in A549 cells – qRT-PCR").

6.3 Cell seeding

6.3.1 Cell culture

A549 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_2 in humidified air in an incubator (standard growth conditions according to SOP "Culturing A549 cells").

6.3.2 Cell seeding into 24-well plate

- One day prior to experimental start harvest and count cells as described in SOP "Culturing A549 cells".
- For 3 and 8 h measurements seed $1x10^5$ cells in 500 µl complete cell culture medium per well into a 24-well cell culture plate. Due to continuous proliferation of A549 cells, cell numbers are halved for 24 h samples. Therefore seed $5x10^4$ cells in 500 µl complete cell culture medium per well. The plate layout for cell seeding is shown in Figure 2.
- To fill two 24-well plates (3 and 8 h samples) according to Figure 2 $2x10^6$ cells are suspended in 10 ml complete cell culture medium ($2x10^5$ cells/ml). For the third time point (24 h) $5x10^5$ cells are suspended in 5 ml complete cell culture medium ($1x10^5$ cells/ml).
- Using a 1 ml micro-pipette 500 µl of these cell suspensions are distributed into each of the green wells depicted in Figure 2 (B1 to B3 and C1 to C6).

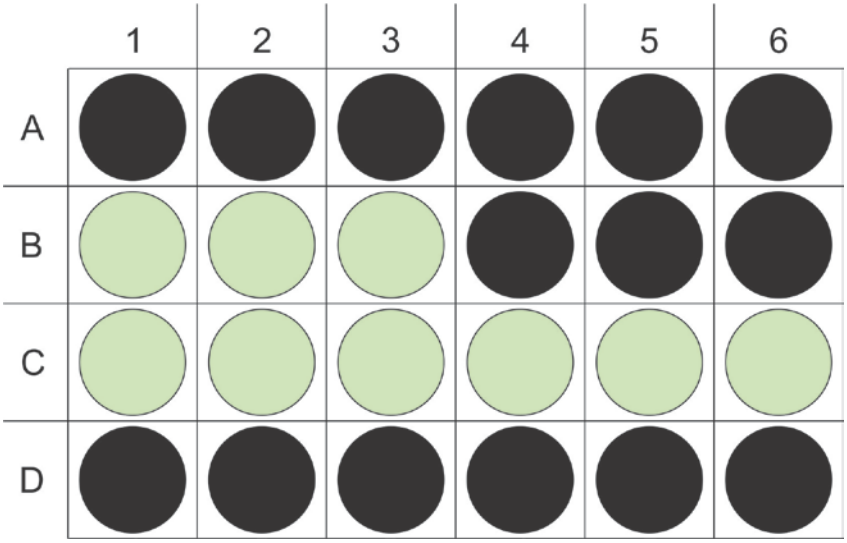

Figure 2: Cell seeding into a 24-well plate.
Cells are seeded in 500 µl complete cell culture medium per well into each of the green wells.
Cell numbers per well are:
$1x10^5$ cells per well for the 3 h and 8 h time points.
$5x10^4$ cells per well for the 24 h time point.
Black wells receive 500 µl complete cell culture medium each.

- Remaining wells (labeled in black in Figure 2) receive 500 µl complete cell culture medium only.
- Cells are kept in a humidified incubator at standard growth conditions overnight (ON).

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_2O; sub-diluted in ddH_2O; highest concentration in assay 100 µg/ml
(2) Carbon based NM: suspended and sub-diluted in 160 ppm Pluronic F-127; highest concentration in assay 80 µg/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 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_2O:

- 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_2O to tubes no. 2, 4, 5 and 6.
- Add 390 µl ddH_2O to tube 3.

1. 1 ml NM stock suspension in ddH_2O → 1 mg/ml (1)
2. 350 µl of 1 mg/ml stock suspension (1) are mixed with 350 µl of ddH_2O → 500 µg/ml (2)
3. 260 µl of 500 µg/ml (2) are mixed with 390 µl ddH_2O → 200 µg/ml (3)
4. 350 µl of 250 µg/ml (3) are mixed with 350 µl ddH_2O → 100 µg/ml (4)
5. 350 µl of 100 µg/ml (4) are mixed with 350 µl ddH_2O → 50 µg/ml (5)
6. 390 µl ddH_2O → 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_2O):
    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_2O (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.

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% $NaHCO_3$	450	µl
100% FCS	1.7	ml
ddH_2O	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® 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)

6.4.2 Dilution of TNF-α (chemical positive control)

Prepare a 10 µg/ml sub-dilution of the TNF-α stock (100 µg/ml) in 0.1%BSA in PBS:

- mix 9 µl of 0.1%BSA in PBS with 1 µl of the stock.

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

- 200 ng/ml: 2 ml medium + 4 µl stock (100 µg/ml)
- 20 ng/ml: 2 ml medium + 4 µl sub-dilution (10 µg/ml)

Apply NM as well as TNF-α as described below.

6.4.3 Application of stimuli

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

One day after cell seeding:

- Remove complete cell culture medium.
- 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 TNF-α 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.

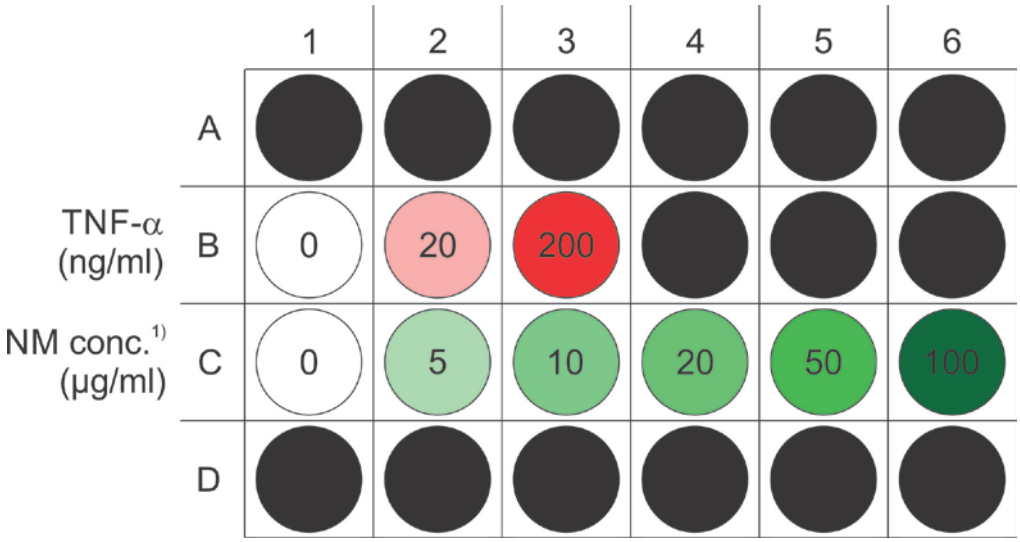

Figure 3: Application of stimuli. NMs as well as TNF-α are applied in 500 µl complete cell culture medium per well after two washing steps in PBS.
[1)] NM concentrations given here refer to metal oxide NMs. Carbon based NM concentrations are detailed in the text.

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 expression in A549 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_2O
- 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_2O

6.6.2 Flow chart 2

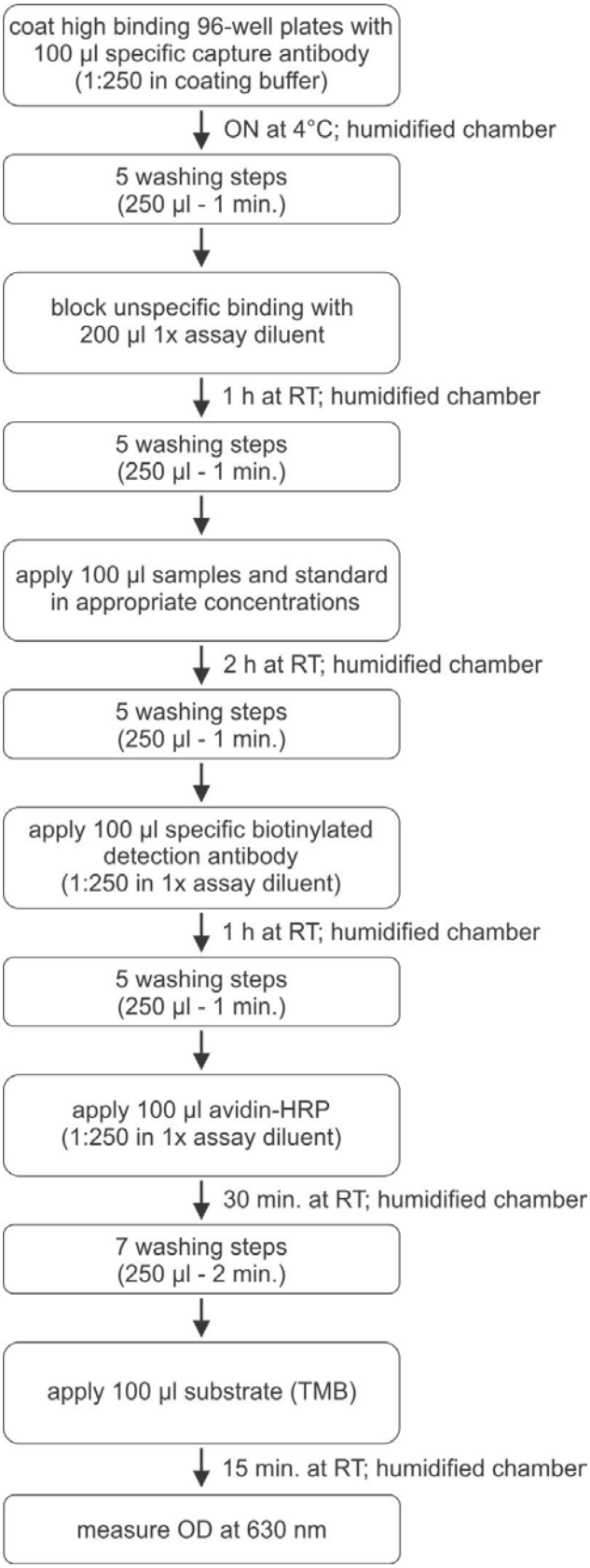

Figure 4: Brief outline of the ELISA workflow.

6.6.3 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.

    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 1:10 in complete cell culture medium:
  30 µl sample + 270 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.

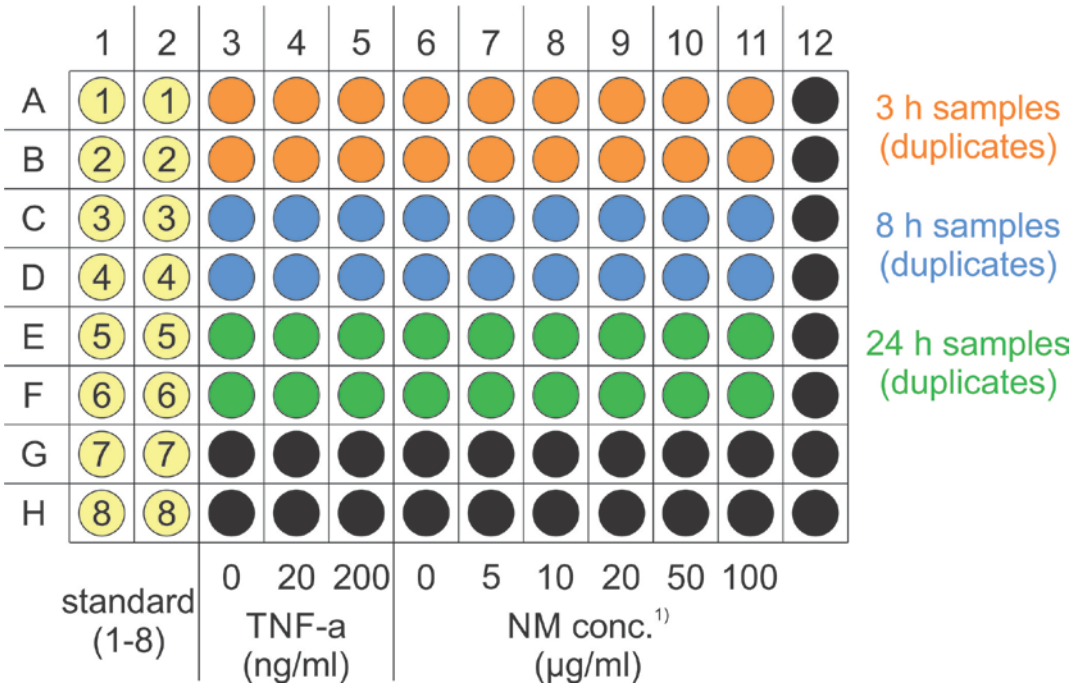

Figure 5: Distribution of standard and sample dilutions. Yellow: duplicates of the 8 dilutions of the recombinant standard protein. 100 µl/well are distributed into wells A1 to H8. 100 µl/well of the sample dilutions are distributed into wells A3 to F11 (orange, blue and green). Black: empty wells.
[1)]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

6.7 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).

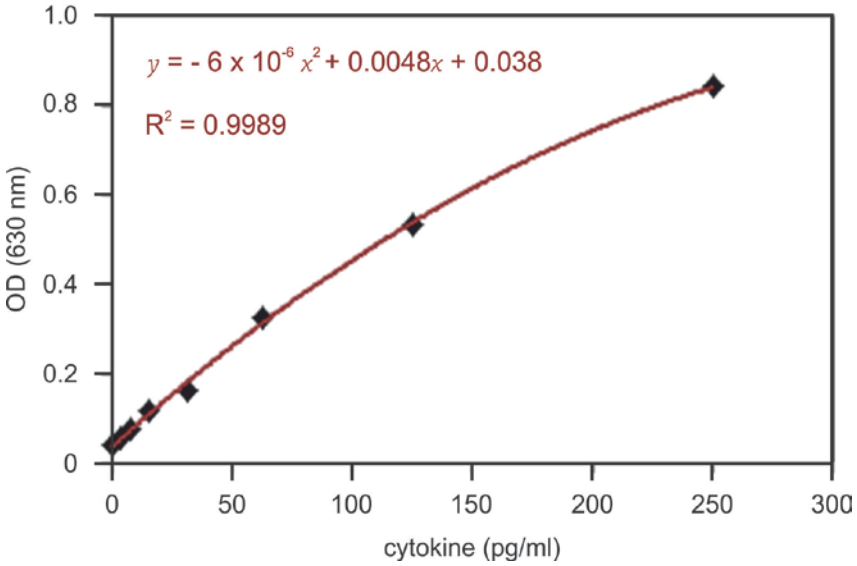

Figure 6: Example of standard curve measurement and polynomic curve fitting. Resulting quadratic equation (1) and correlation coefficient (R^2) are given.

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 = -6×10^{-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

The highest concentration of the recombinant standard protein (250 pg/ml IL-8) should result in OD (650 nm) values of at least 0.8. Values lower than 0.8 indicate improper binding of antibodies to the

plate or unfolding of the recombinant standard protein. 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 TNF-α treatment should result in considerable IL-8 induction. As a rough estimate check the following fold changes compared to the respective untreated control:

	3 h	8 h	24 h
IL-8 induction	8-fold	7-fold	5-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).

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

9 Abbreviations

BSA	bovine serum albumin
ddH_2O	double-distilled water
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	massenger ribonucleic acid
NM	nanomaterial
OD	optical density
ON	overnight
PBS	phosphate buffered saline
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-α	tumor necrosis factor alpha

10 References