Project: VIGO

EMPA
Materials Science & Technology

NM interference in an enzyme-linked immunosorbent assay (ELISA)

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1 Introduction

Nanomaterials (NM) have been shown to interfere in different in vitro assays (e.g. Belyanskaya, 2007; Casey, 2007; Guo, 2008; Monteiro-Riviere, 2006; Pulskamp, 2007; Wörle-Knirsch, 2006; for a review see also Kroll et al., 2009). To avoid false positive as well as false negative results it's thus important to elucidate possibilities of interference and to find ways to assess them experimentally. This SOP describes the theoretical considerations about potential interference reactions of NMs in an ELISA setup. Furthermore their experimental implementation is exemplified for a TNF-α ELISA measurement.

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. See Figure 1A.

NM interference could theoretically occur during all steps of an ELISA. The following questions will be considered in this SOP:

1. Do NMs possess intrinsic catalytic activity? Do they process the substrate by themselves?
2. Does the presence of NMs per se change the optical density (OD)?
3. Do NMs bind to the antibodies used? If yes, does the mere presence of NMs result in a (false positive) signal?
4. Do NMs bind to the antigen? Does this binding prevent antigen binding to the antibody (false negative result)? Or rather increase antigen affinity towards the antibodies (false positive result)?

These issues are approached as follows:

1. Spike in NMs together with the substrate
    a. Without antigen
    b. With antigen
2. Spike in NMs instead of substrate
3. Spike in NMs instead of antigen
4. Spike in NMs together with the antigen

This approach is illustrated in Figure 1.

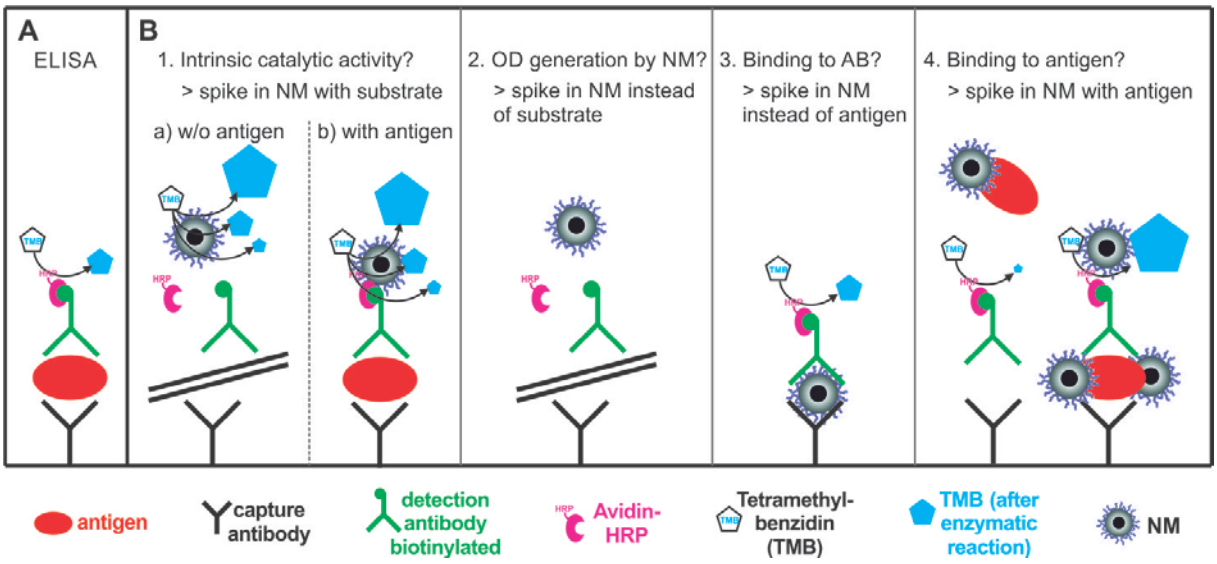

Figure 1: Schematic overview of potential NM interference sites during an ELISA procedure. A) Sandwich ELISA. B) Considerations 1-4 as described in the text.

3 Applicability and Limitations

Most likely different NMs will interfere in several unpredictable ways with the steps of an ELISA procedure. Interpreting the results will be the major challenge here. Depending on the type of interference additional experimental setups might be necessary that cannot be discussed here.

This SOP specifically addresses the interference assessment for a commercially available TNF-α ELISA (Ready-SET-Go!® from eBioscience). However all considerations are transferable to any other cytokine measurement using other ELISA kits (or "homemade" setups).

4 Related Documents

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

Document ID	Document Title
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)
- Conical tubes (15 ml and 50 ml; polypropylene or polystyrene; e.g. from Falcon)
- Flat bottom high-affinity binding 96-well plates (e.g. Corning Costar 9018 ELISA plate)

- 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

ELISA kit:

- Human TNF-α ELISA Ready-SET-Go!® *[eBioscience # 88-7346]*

For buffers and solvents not included in the ELISA kit:

- Fetal Calf Serum (FCS)
- L-glutamine
- Neomycin[1)]
- Penicillin[1)]
- Phosphate buffered saline (PBS)
- Pluronic F-127 *[CAS number: 9003-11-6]*
- Roswell Park Memorial Institute medium (RPMI-1640)
- Streptomycin[1)]
- Tween® 20 *[CAS number: 9005-64-5]*

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

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 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 TNF-α ELISA [eBioscience #88-7346] – General remarks

The ELISA procedure is performed as described by the manufacturer. Specific features concern the application of a NM dilution series at different steps of the procedure and in different solvents. Compare also chapter 2 “Principle of the Method” and Figure 1. All volumes given are for one 96-well plate with the plate layout depicted in Figure 2 and all samples run in duplicates.

6.2 Flow chart

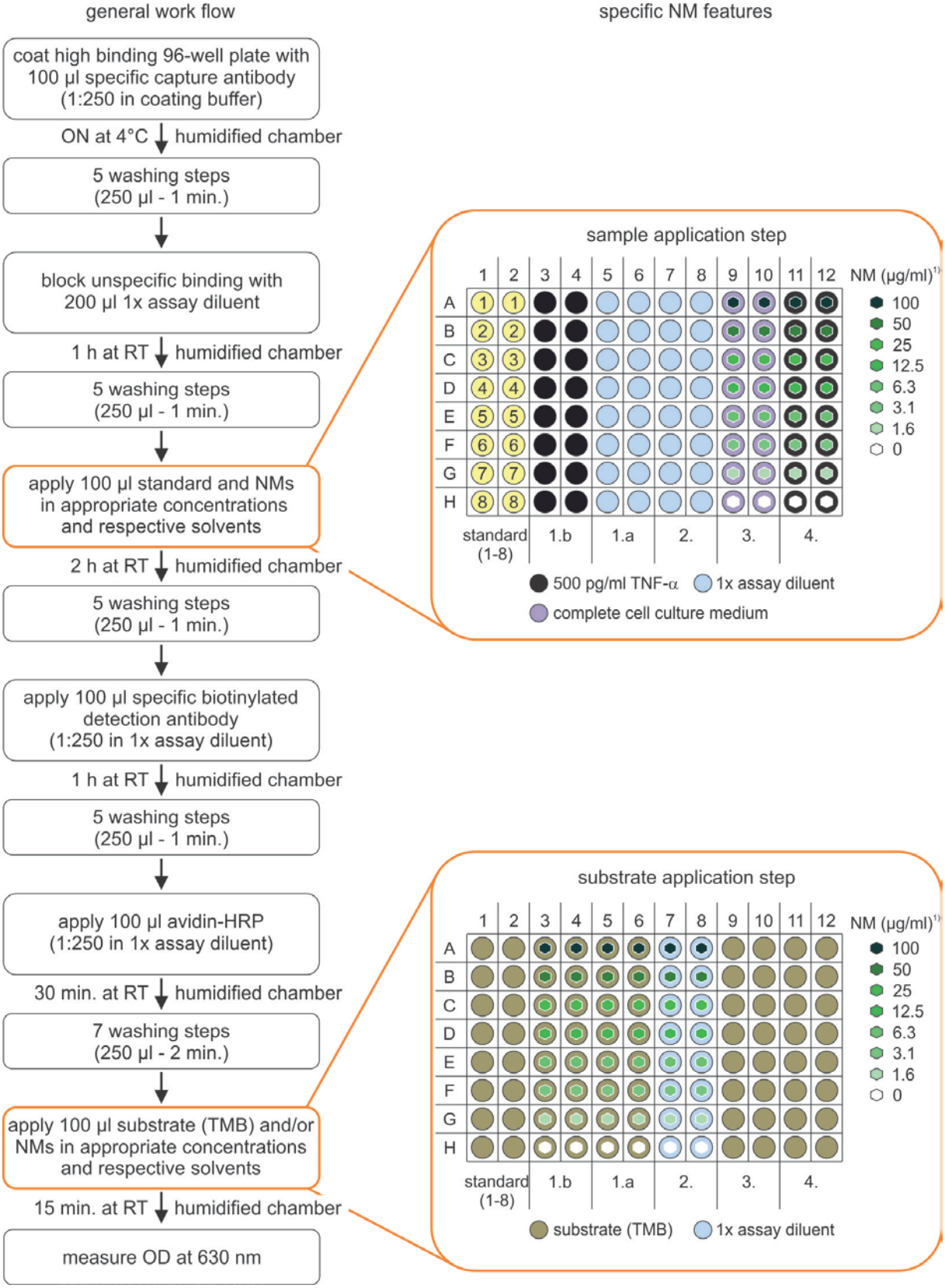

Figure 2: Brief outline of the ELISA work flow. Specific NM related features are depicted on the right and concern two steps: 1. sample application and 2. substrate application. Considerations 1b, 1a, 2, 3 and 4 relate to the ones described in chapter 2 and illustrated in **Figure 1**.

[1)] NM concentrations given here refer to metal oxide NMs. Carbon based NM concentrations are detailed in the text.

6.3 To get started

Day 1:

- Prepare appropriate amount of 1x coating buffer from the 10x stock:
  1.5 ml 10x coating buffer + 13.5 ml ddH_2O

Day 2:

- Prepare appropriate amount of 1x assay diluent from the 5x stock:
  20 ml 5x assay diluent + 80 ml ddH_2O
- Prepare appropriate amount of wash buffer:
  0.56 g Tween-20® / 1 l PBS
- Standard curve: Prepare serial 1:2 dilutions of the recombinant TNF-α 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. 0.5 µl of the stock suspension (1 µg/ml) are mixed with 1000 µl of 1x assay diluent → 500 pg/ml (1)
  2. 300 µl of 500 pg/ml (1) are mixed with 300 µl 1x assay diluent → 250 pg/ml (2)
  3. 300 µl of 250 pg/ml (2) are mixed with 300 µl 1x assay diluent → 125 pg/ml (3)
  4. 300 µl of 125 pg/ml (3) are mixed with 300 µl 1x assay diluent → 62.5 pg/ml (4)
  5. 300 µl of 62.5 pg/ml (4) are mixed with 300 µl 1x assay diluent → 31.3 pg/ml (5)
  6. 300 µl of 31.3 pg/ml (5) are mixed with 300 µl 1x assay diluent → 15.6 pg/ml (6)
  7. 300 µl of 15.6 pg/ml (6) are mixed with 300 µl 1x assay diluent → 7.8 pg/ml (7)
  8. 300 µl 1x assay diluent → solvent control (8)

- Recombinant TNF-α protein as sample and solvent for NMs: Prepare 4 ml of a 500 pg/ml dilution.
  - Add 2 µl TNF-α stock solution (1 µg/ml) to 4 ml 1x assay diluent → 500 pg/ml

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

6.4 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

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.

Note: Sub-dilutions of the NMs are prepared in advance and stored at 4°C until final dilutions in respective solvents are needed. Final dilutions are prepared shortly before application. This assures that the same stock suspensions and respective sub-dilutions are used for all steps. Furthermore it safes time (which is scarce) at the moment of NM application.

(1) Metal oxide NM:

Prepare serial sub-dilutions of the stock suspension (1 mg/ml) in ddH_2O:

- Label eight microreaction tubes (1.5 ml total volume) with 1 to 8 (relates to steps 1-8 below).
- Add 250 µl ddH_2O to tubes 2 to 8.

1. Add 500 µl of the stock suspension (1 mg/ml) to tube no. 1 (1).
2. 250 µl of 1 mg/ml stock suspension (1) are mixed with 250 µl of ddH_2O → 500 µg/ml (2)
3. 250 µl of 500 µg/ml (2) are mixed with 250 µl ddH_2O → 250 µg/ml (3)
4. 250 µl of 250 µg/ml (3) are mixed with 250 µl ddH_2O → 125 µg/ml (4)
5. 250 µl of 125 µg/ml (4) are mixed with 250 µl ddH_2O → 62.5 µg/ml (5)
6. 250 µl of 62.5 µg/ml (5) are mixed with 250 µl ddH_2O → 31.3 µg/ml (6)
7. 250 µl of 31.3 µg/ml (6) are mixed with 250 µl ddH_2O → 15.6 µg/ml (7)
8. 250 µl ddH_2O → solvent control (8)

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

Preparation of final dilutions to be used in **sample application step** (see Figure 2):

i. **In complete cell culture medium** (relates to consideration 3)**:**
   - Label eight microreaction tubes (1.5 ml total volume) as follows:
     1. 100 µg/ml (medium)
     2. 50 µg/ml (medium)
     3. 25 µg/ml (medium)
     4. 12.5 µg/ml (medium)
     5. 6.25 µg/ml (medium)
     6. 3.13 µg/ml (medium)
     7. 1.56 µg/ml (medium)
     8. 0 µg/ml (medium)
   - Add 360 µl complete cell culture medium to each tube.

Note: These 8 tubes can be prepared together with the sub-dilutions of the NMs. Final dilution steps have to be done shortly before application to the 96-well plate.

- Mix with 40 µl of the respective sub-dilution, solvent or stock suspension as follows:
  1. 360 µl medium are mixed with 40 µl stock suspension 1 (1 mg/ml) → 100 µg/ml (1)
  2. 360 µl medium are mixed with 40 µl sub-dilution 2 (500 µg/ml) → 50 µg/ml (2)
  3. 360 µl medium are mixed with 40 µl sub-dilution 3 (250 µg/ml) → 25 µg/ml (3)
  4. 360 µl medium are mixed with 40 µl sub-dilution 4 (125 µg/ml) → 12.5 µg/ml (4)
  5. 360 µl medium are mixed with 40 µl sub-dilution 5 (62.5 µg/ml) → 6.25 µg/ml (5)
  6. 360 µl medium are mixed with 40 µl sub-dilution 6 (31.3 µg/ml) → 3.13 µg/ml (6)
  7. 360 µl medium are mixed with 40 µl sub-dilution 7 (15.6 µg/ml) → 1.56 µg/ml (7)
  8. 360 µl medium are mixed with 40 µl ddH_2O (solvent) → 0 µg/ml (8)

ii. In 500 pg/ml TNF-α (relates to consideration 4)**:**

- Label eight microreaction tubes (1.5 ml total volume) as follows:
  1. 100 µg/ml (TNF)
  2. 50 µg/ml (TNF)
  3. 25 µg/ml (TNF)
  4. 12.5 µg/ml (TNF)
  5. 6.25 µg/ml (TNF)
  6. 3.13 µg/ml (TNF)
  7. 1.56 µg/ml (TNF)
  8. 0 µg/ml (TNF)
- Add 360 µl 500 pg/ml TNF-α to each tube.

Note: These 8 tubes can be prepared together with the sub-dilutions of the NMs. Final dilution steps have to be done shortly before application to the 96-well plate.

- Mix with 40 µl of the respective sub-dilution, solvent or stock suspension as follows:
  1. 360 µl TNF-α are mixed with 40 µl stock suspension 1 (1 mg/ml) → 100 µg/ml (1)
  2. 360 µl TNF-α are mixed with 40 µl sub-dilution 2 (500 µg/ml) → 50 µg/ml (2)
  3. 360 µl TNF-α are mixed with 40 µl sub-dilution 3 (250 µg/ml) → 25 µg/ml (3)
  4. 360 µl TNF-α are mixed with 40 µl sub-dilution 4 (125 µg/ml) → 12.5 µg/ml (4)
  5. 360 µl TNF-α are mixed with 40 µl sub-dilution 5 (62.5 µg/ml) → 6.25 µg/ml (5)
  6. 360 µl TNF-α are mixed with 40 µl sub-dilution 6 (31.3 µg/ml) → 3.13 µg/ml (6)
  7. 360 µl TNF-α are mixed with 40 µl sub-dilution 7 (15.6 µg/ml) → 1.56 µg/ml (7)
  8. 360 µl TNF-α are mixed with 40 µl ddH_2O (solvent) → 0 µg/ml (8)

Preparation of final dilutions to be used in **substrate application step** (see Figure 2):

i. **In substrate (TMB)** (relates to considerations 1a & 1b)**:**

- Label eight microreaction tubes (1.5 ml total volume) as follows:
  1. 100 µg/ml (TMB)
  2. 50 µg/ml (TMB)
  3. 25 µg/ml (TMB)
  4. 12.5 µg/ml (TMB)
  5. 6.25 µg/ml (TMB)
  6. 3.13 µg/ml (TMB)
  7. 1.56 µg/ml (TMB)
  8. 0 µg/ml (TMB)
- Add 540 µl TMB to each tube.

Note: These 8 tubes can be prepared together with the sub-dilutions of the NMs. Final dilution steps have to be done shortly before application to the 96-well plate.

- Mix with 60 µl of the respective sub-dilution, solvent or stock suspension as follows:
  1. 540 µl TMB are mixed with 60 µl stock suspension 1 (1 mg/ml) → 100 µg/ml (1)
  2. 540 µl TMB are mixed with 60 µl sub-dilution 2 (500 µg/ml) → 50 µg/ml (2)
  3. 540 µl TMB are mixed with 60 µl sub-dilution 3 (250 µg/ml) → 25 µg/ml (3)
  4. 540 µl TMB are mixed with 60 µl sub-dilution 4 (125 µg/ml) → 12.5 µg/ml (4)
  5. 540 µl TMB are mixed with 60 µl sub-dilution 5 (62.5 µg/ml) → 6.25 µg/ml (5)
  6. 540 µl TMB are mixed with 60 µl sub-dilution 6 (31.3 µg/ml) → 3.13 µg/ml (6)
  7. 540 µl TMB are mixed with 60 µl sub-dilution 7 (15.6 µg/ml) → 1.56 µg/ml (7)
  8. 540 µl TMB are mixed with 60 µl ddH_2O (solvent) → 0 µg/ml (8)

ii. 1x assay diluent (relates to consideration 2)**:**

- Label eight microreaction tubes (1.5 ml total volume) as follows:
  1. 100 µg/ml (1x diluent)
  2. 50 µg/ml (1x diluent)
  3. 25 µg/ml (1x diluent)
  4. 12.5 µg/ml (1x diluent)
  5. 6.25 µg/ml (1x diluent)
  6. 3.13 µg/ml (1x diluent)
  7. 1.56 µg/ml (1x diluent)
  8. 0 µg/ml (1x diluent)
- Add 360 µl 1x assay diluent to each tube.

Note: These 8 tubes can be prepared together with the sub-dilutions of the NMs. Final dilution steps have to be done shortly before application to the 96-well plate.

- Mix with 40 µl of the respective sub-dilution, solvent or stock suspension as follows:
  1. 360 µl 1x assay diluent are mixed with 40 µl stock suspension 1 (1 mg/ml) → 100 µg/ml (1)
  2. 360 µl 1x assay diluent are mixed with 40 µl sub-dilution 2 (500 µg/ml) → 50 µg/ml (2)
  3. 360 µl 1x assay diluent are mixed with 40 µl sub-dilution 3 (250 µg/ml) → 25 µg/ml (3)
  4. 360 µl 1x assay diluent are mixed with 40 µl sub-dilution 4 (125 µg/ml) → 12.5 µg/ml (4)
  5. 360 µl 1x assay diluent are mixed with 40 µl sub-dilution 5 (62.5 µg/ml) → 6.25 µg/ml (5)
  6. 360 µl 1x assay diluent are mixed with 40 µl sub-dilution 6 (31.3 µg/ml) → 3.13 µg/ml (6)
  7. 360 µl 1x assay diluent are mixed with 40 µl sub-dilution 7 (15.6 µg/ml) → 1.56 µg/ml (7)
  8. 360 µl 1x assay diluent are mixed with 40 µl ddH_2O (solvent) → 0 µg/ml (8)

(2) Carbon based NM:

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

- Label eight microreaction tubes (1.5 ml total volume) with 1 to 8 (relates to steps 1-8 below).
- Add 250 µl Pluronic F-127 to tubes 2 to 8.

1. Add 500 µl of the stock suspension (500 µg/ml) to tube no. 1 (1).
2. 250 µl of 500 µg/ml stock suspension (1) are mixed with 250 µl of Pluronic F-127 → 250 µg/ml (2)
3. 250 µl of 250 µg/ml (2) are mixed with 250 µl Pluronic F-127 → 125 µg/ml (3)
4. 250 µl of 125 µg/ml (3) are mixed with 250 µl Pluronic F-127 → 62.5 µg/ml (4)
5. 250 µl of 62.5 µg/ml (4) are mixed with 250 µl Pluronic F-127 → 31.3 µg/ml (5)
6. 250 µl of 31.3 µg/ml (5) are mixed with 250 µl Pluronic F-127 → 15.6 µg/ml (6)
7. 250 µl of 15.6 µg/ml (6) are mixed with 250 µl Pluronic F-127 →7.8 µg/ml (7)
8. 250 µl Pluronic F-127 → solvent control (8)

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

Preparation of final dilutions to be used in **sample application step** (see Figure 2):

i. **In complete cell culture medium** (relates to consideration 3)**:**

- Label eight microreaction tubes (1.5 ml total volume) as follows:
  1. 80 µg/ml (medium)
  2. 40 µg/ml (medium)
  3. 20 µg/ml (medium)
  4. 10 µg/ml (medium)
  5. 5 µg/ml (medium)
  6. 2.5 µg/ml (medium)
  7. 1.25 µg/ml (medium)
  8. 0 µg/ml (medium)
- Add 336 µl complete cell culture medium to each tube.

Note: These 8 tubes can be prepared together with the sub-dilutions of the NMs. Final dilution steps have to be done shortly before application to the 96-well plate.

- Mix with 64 µl of the respective sub-dilution, solvent or stock suspension as follows:
  1. 336 µl medium are mixed with 64 µl stock suspension 1 (500 µg/ml) → 80 µg/ml (1)
  2. 336 µl medium are mixed with 64 µl sub-dilution 2 (250 µg/ml) → 40 µg/ml (2)
  3. 336 µl medium are mixed with 64 µl sub-dilution 3 (125 µg/ml) → 20 µg/ml (3)
  4. 336 µl medium are mixed with 64 µl sub-dilution 4 (62.5 µg/ml) → 10 µg/ml (4)
  5. 336 µl medium are mixed with 64 µl sub-dilution 5 (31.3 µg/ml) → 5 µg/ml (5)
  6. 336 µl medium are mixed with 64 µl sub-dilution 6 (15.6 µg/ml) → 2.5 µg/ml (6)
  7. 336 µl medium are mixed with 64 µl sub-dilution 7 (7.8 µg/ml) → 1.25 µg/ml (7)
  8. 336 µl medium are mixed with 64 µl Pluronic F-127 (solvent) → 0 µg/ml (8)

ii. In 500 pg/ml TNF-α (relates to consideration 4)**:**

- Label eight microreaction tubes (1.5 ml total volume) as follows:
  1. 80 µg/ml (TNF)
  2. 40 µg/ml (TNF)
  3. 20 µg/ml (TNF)
  4. 10 µg/ml (TNF)
  5. 5 µg/ml (TNF)
  6. 2.5 µg/ml (TNF)
  7. 1.25 µg/ml (TNF)
  8. 0 µg/ml (TNF)
- Add 336 µl 500 pg/ml TNF-α to each tube.

Note: These 8 tubes can be prepared together with the sub-dilutions of the NMs. Final dilution steps have to be done shortly before application to the 96-well plate.

- Mix with 64 µl of the respective sub-dilution, solvent or stock suspension as follows:
    1. 336 µl TNF-α are mixed with 64 µl stock suspension 1 (500 µg/ml) → 80 µg/ml (1)
    2. 336 µl TNF-α are mixed with 64 µl sub-dilution 2 (250 µg/ml) → 40 µg/ml (2)
    3. 336 µl TNF-α are mixed with 64 µl sub-dilution 3 (125 µg/ml) → 20 µg/ml (3)
    4. 336 µl TNF-α are mixed with 64 µl sub-dilution 4 (62.5 µg/ml) → 10 µg/ml (4)
    5. 336 µl TNF-α are mixed with 64 µl sub-dilution 5 (31.3 µg/ml) → 5 µg/ml (5)
    6. 336 µl TNF-α are mixed with 64 µl sub-dilution 6 (15.6 µg/ml) → 2.5 µg/ml (6)
    7. 336 µl TNF-α are mixed with 64 µl sub-dilution 7 (7.8 µg/ml) → 1.25 µg/ml (7)
    8. 336 µl TNF-α are mixed with 64 µl Pluronic F-127 (solvent) → 0 µg/ml (8)

Preparation of final dilutions to be used in **substrate application step** (see Figure 2):

i. **In substrate (TMB)** (relates to considerations 1a & 1b)**:**

- Label eight microreaction tubes (1.5 ml total volume) as follows:
    1. 80 µg/ml (TMB)
    2. 40 µg/ml (TMB)
    3. 20 µg/ml (TMB)
    4. 10 µg/ml (TMB)
    5. 5 µg/ml (TMB)
    6. 2.5 µg/ml (TMB)
    7. 1.25 µg/ml (TMB)
    8. 0 µg/ml (TMB)
- Add 672 µl TMB to each tube.

Note: These 8 tubes can be prepared together with the sub-dilutions of the NMs. Final dilution steps have to be done shortly before application to the 96-well plate.

- Mix with 128 µl of the respective sub-dilution, solvent or stock suspension as follows:
    1. 672 µl TMB are mixed with 128 µl stock suspension 1 (1 mg/ml) → 80 µg/ml (1)
    2. 672 µl TMB are mixed with 128 µl sub-dilution 2 (500 µg/ml) → 40 µg/ml (2)
    3. 672 µl TMB are mixed with 128 µl sub-dilution 3 (250 µg/ml) → 202 µg/ml (3)
    4. 672 µl TMB are mixed with 128 µl sub-dilution 4 (125 µg/ml) → 10 µg/ml (4)
    5. 672 µl TMB are mixed with 128 µl sub-dilution 5 (62.5 µg/ml) → 5 µg/ml (5)
    6. 672 µl TMB are mixed with 128 µl sub-dilution 6 (31.3 µg/ml) → 2.5 µg/ml (6)
    7. 672 µl TMB are mixed with 128 µl sub-dilution 7 (15.6 µg/ml) → 1.25 µg/ml (7)
    8. 672 µl TMB are mixed with 128 µl ddH_2O (solvent) → 0 µg/ml (8)

ii. **1x assay diluent** (relates to consideration 2)**:**

- Label eight microreaction tubes (1.5 ml total volume) as follows:
  1. 80 µg/ml (1x diluent)
  2. 40 µg/ml (1x diluent)
  3. 20 µg/ml (1x diluent)
  4. 10 µg/ml (1x diluent)
  5. 5 µg/ml (1x diluent)
  6. 2.5 µg/ml (1x diluent)
  7. 1.25 µg/ml (1x diluent)
  8. 0 µg/ml (1x diluent)
- Add 336 µl 1x assay diluent to each tube.

Note: These 8 tubes can be prepared together with the sub-dilutions of the NMs. Final dilution steps have to be done shortly before application to the 96-well plate.

- Mix with 64 µl of the respective sub-dilution, solvent or stock suspension as follows:
  1. 336 µl 1x assay diluent are mixed with 64 µl stock suspension 1 (500 µg/ml) → 80 µg/ml (1)
  2. 336 µl 1x assay diluent are mixed with 64 µl sub-dilution 2 (250 µg/ml) → 40 µg/ml (2)
  3. 336 µl 1x assay diluent are mixed with 64 µl sub-dilution 3 (125 µg/ml) → 20 µg/ml (3)
  4. 336 µl 1x assay diluent are mixed with 64 µl sub-dilution 4 (62.5 µg/ml) → 10 µg/ml (4)
  5. 336 µl 1x assay diluent are mixed with 64 µl sub-dilution 5 (31.3 µg/ml) → 5 µg/ml (5)
  6. 336 µl 1x assay diluent are mixed with 64 µl sub-dilution 6 (15.6 µg/ml) → 2.5 µg/ml (6)
  7. 336 µl 1x assay diluent are mixed with 64 µl sub-dilution 7 (7.8 µg/ml) → 1.25 µg/ml (7)
  8. 336 µl 1x assay diluent are mixed with 64 µl Pluronic F-127 (solvent) → 0 µg/ml (8)

6.5 ELISA performance as such

- Prepare a 1:250 dilution of the TNF-α capture antibody in 1x coating buffer.
  10 ml 1x coating buffer + 40 µl TNF-α capture antibody
- Coat high affinity binding 96-well plate with 100 µl/well of this TNF-α 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 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.

- **Sample application step:**
  - Make sure to have the final dilutions of NMs in complete cell culture medium and in 500 pg/ml TNF-α ready (see 6.4).
    Note: All NM dilutions have to be vortexed directly before application to the cells.
  - Make sure to have the TNF-α dilutions for the standard curve ready (see 6.3).
  - Apply 100 µl of standard, 500 pg/ml TNF-α, 1x assay diluent and NM dilutions (in complete cell culture medium or in 500 pg/ml TNF-α) per well according to pipetting scheme in Figure 3 and incubate in a humidified chamber for 2 h at RT.

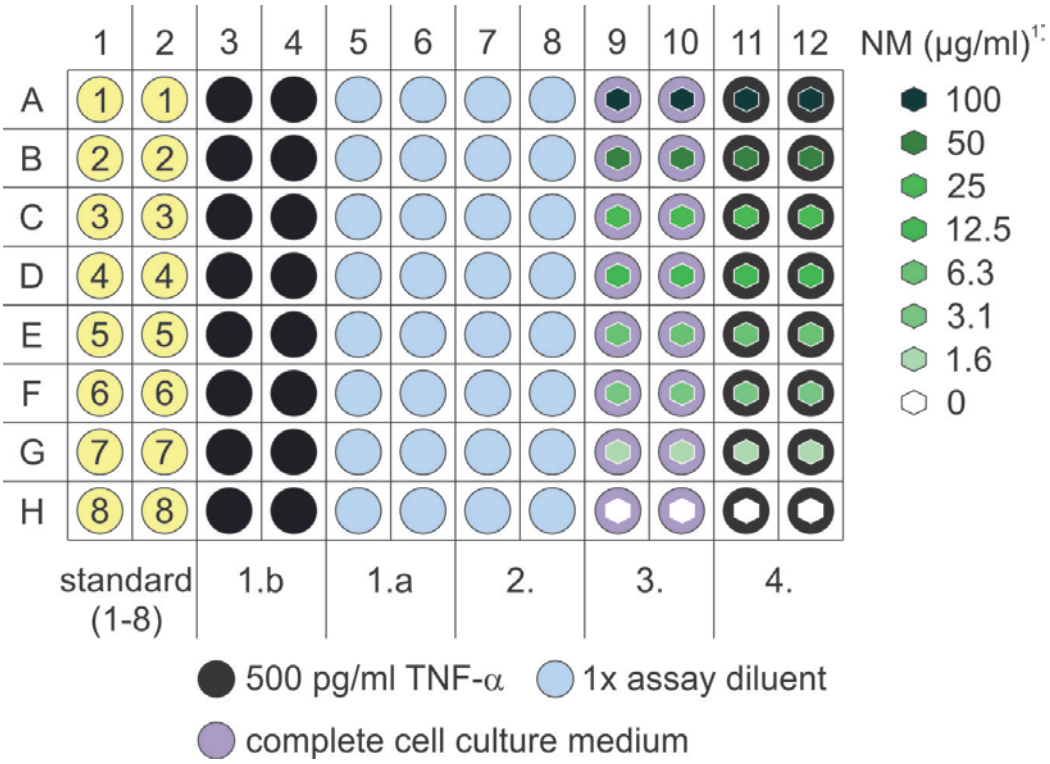

Figure 3: Sample application to the 96-well plate. Standard (1-8) refers to the dilution series of TNF-α. Considerations 1 to 4 are depicted underneath the plate. Here considerations 3 and 4 become relevant: 3) Binding to antibody? NMs spiked in instead of antigen (in complete cell culture medium and as a concentration series; wells A9 to H10). 4) Binding to antigen? NMs spiked in with the antigen (500 pg/ml recombinant TNF-α is used as the antigen; wells A11 to H12).
[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 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.

- **Substrate application step:**
  - Make sure to have the final dilutions of NMs in substrate (TMB) and in 1x assay diluent ready (see 6.4).
    Note: All NM dilutions have to be vortexed directly before application to the cells.
  - Apply 100 µl of TMB and NM dilutions (in TMB or in 1x assay diluent) per well according to pipetting scheme in Figure 4 and incubate in a humidified chamber for 15 min. at RT.

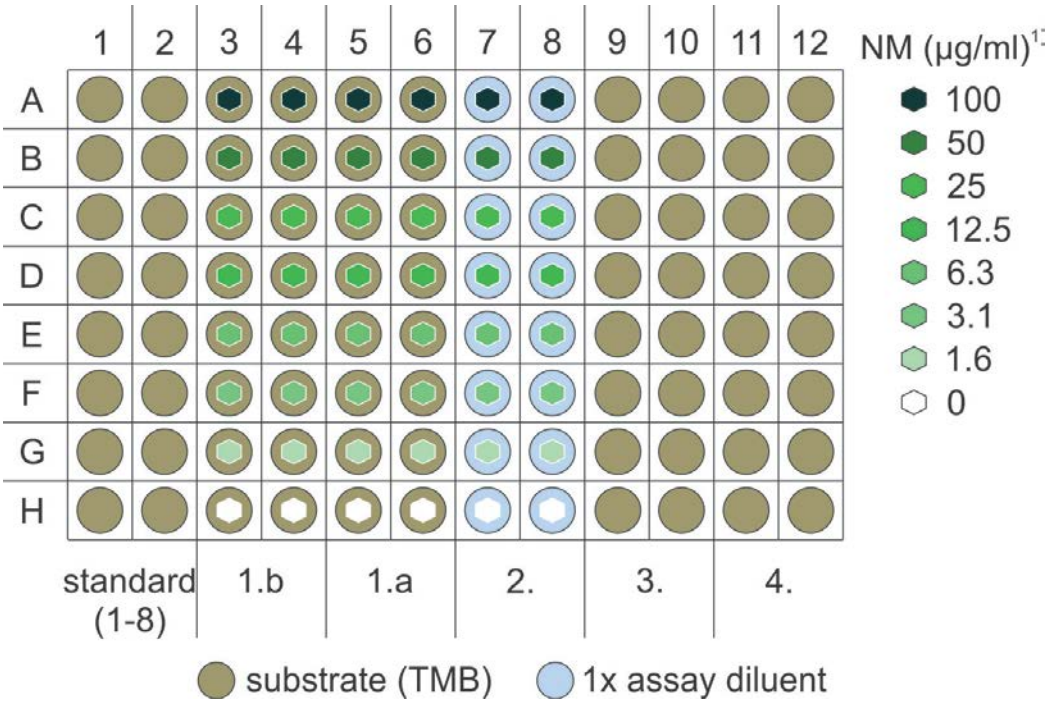

Figure 4: Substrate application to the 96-well plate. Substrate only is added to wells A1 to H2 and A9 to H12. Considerations 1 to 4 are depicted underneath the plate. Here considerations 1 and 2 become relevant: 1) Intrinsic catalytic activity? NMs spiked in with the substrate. a) w/o antigen (wells A5 to H6) b) with antigen (wells A3 to H4). 2) OD generation by NM? NMs spiked in instead of the substrate (diluted in 1x assay diluent; wells A7 to H8).
[1)] NM concentrations given here refer to metal oxide NMs. Carbon based NM concentrations are detailed in the text.

- Read plate at 650 nm.

6.6 Data evaluation

6.6.1 Standard curve – Polynomic regression

The mean is calculated from the OD-values of the standard curve duplicates. These mean values are plotted against their corresponding concentrations (see Figure 5).

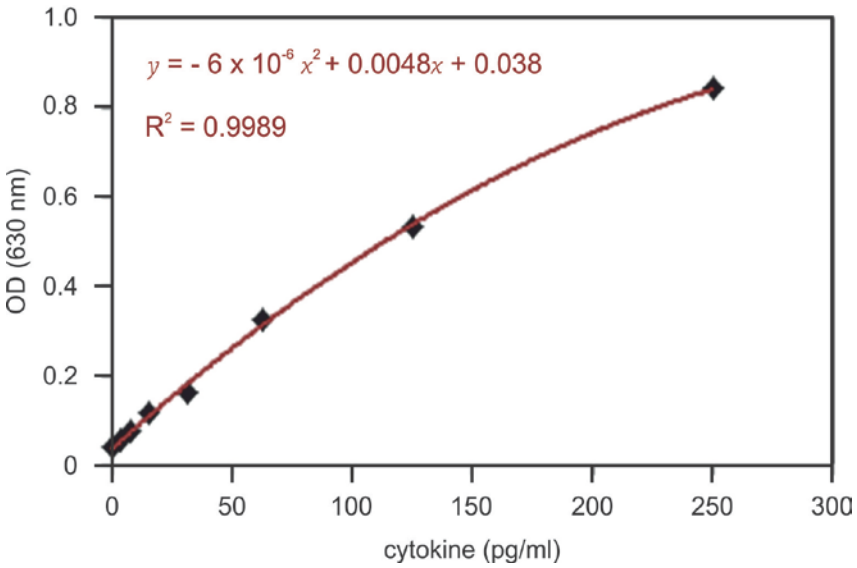

Figure 5: Example of standard curve measurement and polynomic curve fitting. Resulting quadratic equation (1) and correlation coefficient (R^2) are given.

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

$$y = ax^2 + bx + c \tag{1}$$

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

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

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 5 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.

6.6.2 Exemplary interpretation of interference results

Figure 6 shows the results of an interference measurement using carbon based nanomaterials. No antigen was added (during the sample application step) under conditions 1a, 2 and 3. Therefore OD values are very low. In contrast 500 pg/ml recombinant TNF-α were applied during the sample application step under conditions 1b and 4. Resulting OD values around 1 (or even higher) are expected.

Higher concentrations of NMs (starting from 20 µg/ml) lead to an increase in OD under conditions 1a, 1b and 2. Considering only conditions 1a and 1b, where NMs were added to the substrate (in the absence or presence of antigen, respectively) this would indicate a certain intrinsic catalytic activity of the particles. However, under condition 2 an equivalent increase in OD is detected. This condition reflects the "intrinsic" OD resulting from the particles only. As the slope of all three curves is similar the increase in OD under all three conditions is likely to result from the particles' OD rather than from their catalytic activity.

No changes in OD are detectable under condition 3 where NMs were substituted for the antigen to assess NM binding to the antibodies. This indicates that the particular particles did not bind to the antibodies.

Higher concentrations of NMs (starting again from 20 µg/ml) result in a reduction of OD values under condition 4. Here NMs were added together with the antigen. The reduction in OD suggests that NMs are able to bind to the antigen thereby reducing the binding affinity of the antigen to the antibody/antibodies.

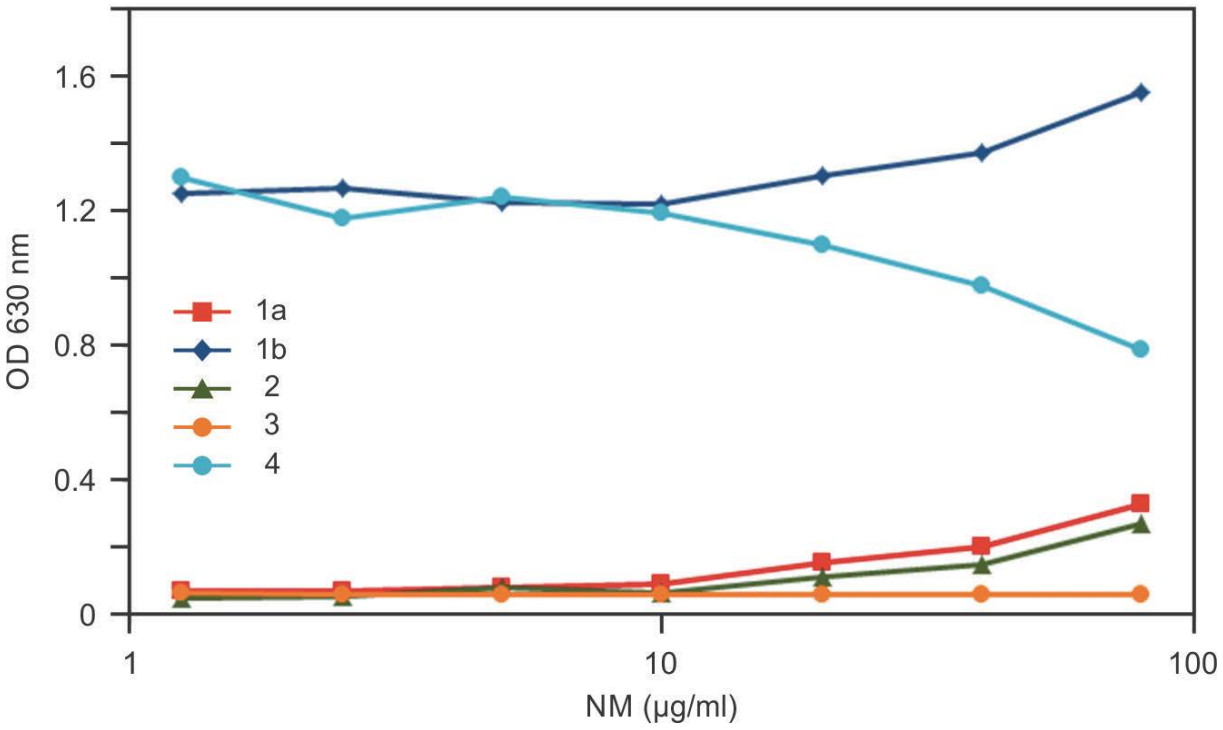

Figure 6: Example of an interference measurement using carbon based nanomaterials. Considerations 1 to 4 relate to the ones described in chapter 2 and illustrated in Figure 1. In brief: 1) Intrinsic catalytic activity? NMs spiked in with substrate. a) no antigen added during sample application. b) antigen present during sample application. 2) OD generation by NM? NMs spiked in instead of substrate. 3) Binding to AB? NMs spiked in instead of antigen. 4) Binding to antigen? NMs spied in together with antigen.

7 Quality Control, Quality Assurance, Acceptance Criteria

The highest concentration of the recombinant standard protein (500 pg/ml) 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 and will lower the detection limit of the whole assay.

The correlation coefficient R^2 (as depicted in Figure 5) 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.

8 Health and Safety Warnings, Cautions and Waste Treatment

9 Abbreviations

ddH_2O	double-distilled water
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
HRP	horseradish peroxidase
NM	nanomaterial
OD	optical density
ON	overnight
PBS	phosphate buffered saline
ppm	parts per million
PSN	Penicillin, Streptomycin, Neomycin
RPMI	Roswell Park Memorial Institute medium
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor alpha

10 References

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