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Standard Operating Procedure

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NRCWE Protocol for determination of LDH, IL-6, IL-8 adsorption onto MNM

General description

It has been suggested that MNM can adsorb various molecules including interleukin-6 (IL-6), interleukin-8 (IL-8) and lactate dehydrogenase (LDH) on to their surface. The measurement of the amount of cytokines and LDH released in *in vitro* experiments is therefore uncertain and can lead to an underestimation of the cytotoxicity of the MNM tested.

This protocol describes the assay set up to investigate IL-6, IL-8 and LDH scavenging potential of NANoREG MNM during *in vitro* exposure and the links to physicochemical parameters (dimensions, surface roughness, zeta-potential, BSA-coating, specific surface area, pH, solubility, redox potential) in HAMs F12+10% FBS as an example of a typical growth medium.

Preparation of the stock dispersion

The stock-dispersion is prepared following the NANoREG version of the NANOGENOTOX dispersion protocol.

Preparing the protein mix and final HAMs F12 test medium

Introduction

MNM at increasing concentrations were mixed with the test medium containing fixed concentrations of IL-6, IL-8 and LDH.

Preparation of the HAMs test medium:

1) Fill a clean (cell growth) bottle with approximately 50 mL of HAMs F12 medium.

2) Add 1.5 mL penicillin/streptomycin (10000 U/mL; 10 mg/mL; In Vitro, item number: BI-03-031-1B).

3) Add 15 mL of inactivated Fetal Bovine Serum 10% (USA grade; In Vitro, item number: BI-04-001-1A).

4) Add HAMs F12 (Life Technologies, catalogue number: 21765037) q.s. 150 mL. This medium is called medium A.

5) Fill a syringe with medium A.

6) Mount a 0.8 µm filter on the syringe.

7) Filter medium A through the syringe filter into a clean bottle.

8) Fill a clean bottle with approximately 50 mL of medium A.

9) Add 50 µL IL-6 standard (NIBCS code: 89/548; final concentration=500 pg/mL).

10) Add 400 µL IL-8 standard (NIBCS code: 89/520; final concentration=4000 pg/mL).

11) Add 2 µL LDH (Sigma-Aldrich, product number: 10127230001, final concentration=100 ng/mL).

12) Add medium A q.s. 100 mL. This is the final test medium, containing penicillin/streptomycin, FBS, HAMs F12, IL-6, IL-8, and LDH.

The final test medium can be stored in the refrigerator for about 1 week.

Preparation of the dispersions:

1) Mix the dispersion of MNM with the HAMs F12 final test medium immediately after sonication. Follow the dosing scheme below to obtain final concentrations of MNM of 0, 0.010, 0.020, 0.040, 0.080, 0.160, 0.320, and 0.640 mg/mL.

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Dosing scheme with amounts of medium and make-up BSA-water to be dosed into each well

Dose tested [mg/mL]	2.56 mg/mL MNM dispersion [µL]	0.05% BSA-water [µL]	Test medium [µL]
0.000	0.0	250.0	750
0.010	3.9	246.1	750
0.020	7.8	242.2	750
0.040	15.6	234.4	750
0.080	31.3	218.8	750
0.160	62.5	187.5	750
0.320	125.0	125.0	750
0.640	250.0	0.0	750

2) Pipette the indicated amount of MNM dispersion into a 24-well plates as described in the table below. Run the experiment in duplicate.

The final target concentrations of the proteins in each well are:

[IL-6]= 500∗0.750 = 375 pg/well,
[IL-8] = 4000∗0.750 = 3000 pg/well
[LDH]= 100∗0.750 = 75 ng/well.

Placement of the dispersions in a 24-wells plate

	1	2	3	4	5	6
A		0	0	80	80	
B		10	10	160	160	
C		20	20	320	320	
D		40	40	640	640	

4) Incubate the plates for 24 hours in a cell-incubator (97% relative humidity, 37ºC, 5% CO_2).

5) Transfer the content of each well into a tube and centrifuge 30 minutes at 20,000 x g (RCF).

6) Transfer the supernatant to another tube to measure the remaining concentration of IL-6, IL-8 and LDH.

7) Measure LDH in the supernatant no later than 24 hours later (see

Measurement of LDH in the **samples** below) and store the remaining surfactant in the freezer at -20ºC until measurement of IL-6 and IL-8 (see

Measurement of IL-6 in the **samples** and

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Measurement of IL-8 **in the samples** below).

Preparation of the controls

1) Mix 1.5 mL of final test medium with 0.5 mL of 0.05% w/v BSA-water.

The control is necessary to assess the potential interaction of BSA with the MNM in the dispersion protocol.

Measurement of LDH in the samples

The LDH measurement has to be done no later than 24 hours after the centrifugation.

Materials

The LDH Cytotoxicity Detection Kit (Roche, Cat. No. 11 644 793 001) contains:

- Solution 1: catalyst (vial with the blue cap, dissolved in 1 mL MilliQ-water)
- Solution 2: dye solution (vial with the red cap, ready to use)

Store the kit at -15 to -25ºC. The reconstituted catalyst solution is stable for 4 weeks when stored at +2 to +8ºC. Once thawed, the dye solution is stable for several weeks when stored at +2 to +8ºC.

1) Reconstitute the lyophilisate (solution 1) in 1 mL water for 10 minutes and mix thoroughly. Solution 2 is ready to use.

2) Pipette 100 µL of the supernatant solution into each well of a 96-well plate (Nunc MaxiSorp 96-well plate).

3) Shortly before use, add the total volume of bottle 1 to the total volume of bottle 2 and mix well.

4) Add 100 µL of the reaction mixture freshly prepared to each well.

5) Incubate for up to 30 minutes at room temperature on the shaker. During the incubation period the plates are wrapped in tinfoil.

6) Measure the absorbance of the samples at 492 nm with a reference wavelength of 630 nm.

Measurement of IL-6 in the samples

The BD Pharmingen kit (Cat. No. 555220) is used. The reagents provided by the kit and need are listed below.

Procedure

Standards preparation and handling

1) After warming lyophilized standard to room temperature, carefully open the vials to avoid loss of material. Run in duplicate.

2) Reconstitute lyophilized standard with 1.0 mL of deionized water to yield a stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions.

3) Vortex gently to mix.

4) After reconstitution, immediately aliquot standard stock in polypropylene vials at 50 µL per vial and freeze at -80ºC for up to 6 months.

Materials and chemicals needed for the IL-6 measurements

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Capture antibody	Anti-human IL-6 monoclonal antibody
Detection antibody	Biotinylated anti-human IL-6 monoclonal antibody
Enzyme Reagent	Streptavidin-horseradish peroxidase conjugate
Standard	Recombinant human IL-6. lyophilized
Coating buffer	
Na_2CO_3	1.59 g
$NaHCO_3$	7.13 g
MilliQ-water	q.s. 1000 mL
pH adjusted to 9.5	
Phosphate Buffered Saline (PBS)	
NaCl	80.0 g
Na_2HPO	11.6 g
KH_2PO_4	2.0 g
KCl	2.0 g
MilliQ-water	q.s. 10 L
pH adjusted to 7.0	
Assay Diluent	
PBS with 10% FBS	
Wash buffer	
PBS with 0.05% Tween-20	
Substrate solution	
Tetramethylbenzidine (TMB)	
Hydrogen peroxide	
Stop solution	
2 N H_2SO_4	
Additional materials required	
Nunc MaxiSorp 96-well plates	
Microplate reader capable of measuring absorbance at 450 nm	
Precision pipettes	
Graduated cylinder. 1 L	
Deionized or distilled water	
Tubes to prepare standard dilutions	
Laboratory timer	
Plate sealers or parafilm	

5) Pipette 100 µL of assay diluent into each well except the one for the highest standard concentration (300 pg/mL).

6) Add 100 µL of the 300 pg/mL standard to the 300 pg/mL well and the 150 pg/mL well.

7) Mix the well contents by rinsing the pipette tip.

8) Add 100 µL of the 150 pg/mL standard to the 75 pg/mL well.

9) Repeat these dilutions until the last well (4.7 pg/mL), out of which the extra 100 µL should be discarded.

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NB: The quantity of standard recombinant human IL-6 lyophilized provided by the supplier may vary from kit to kit. Look at the instruction/analysis certificate provided with the kit to make the standard curve accordingly. The highest concentration should be 300 pg/mL.

Assay procedure

1) Mix 40 µL of capture antibody with 9960 µL of coating buffer to obtain 10 mL of capture antibody diluted to 1/250.

2) Coat wells of a 96-well plate with 100 µL per well of the capture antibody previously diluted. 10 mL of solution is needed to coat a 96-well plate.

3) Seal plate and incubate overnight in the refrigerator.

4) Empty wells and wash 3 times with the wash buffer. Between each wash, invert plate and blot on adsorbent paper to remove any residual buffer.

5) Block plates with 200 µL assay diluent.

6) Incubate 1 hour at room temperature on the shaker.

7) Empty/wash 3 times as in step 3.

8) Prepare standard and sample dilutions in assay diluent as described in Standards preparation and handling.

9) Pipette 100 µL of each standard, sample, and control into appropriate wells in duplicate. Assay diluent is used as a blank.

10) Seal plate and incubate for 2 hours at room temperature on the shaker.

11) Empty wells and wash 4 times with the wash buffer. Between each wash, invert plate and blot on adsorbent paper to remove any residual buffer.

12) Prepare working detector (solution B), maximum 15 minute before use, as following:

Solution A: 12.45 mL assay diluent + 50 µL detection antibody
Solution B: 12.45 mL solution A + 50 µL enzyme reagent

13) Add 100 µL of working detector (detection antibody + enzyme reagent) to each well.

14) Seal plate and incubate for 1 hour at room temperature.

15) Empty wells and wash 4 times. In this final wash step, soak wells in wash buffer for 30 seconds for each wash.

16) Add 100 µL of substrate solution to each well.

17) Incubate plate for 30 minutes at room temperature in the dark.

18) Add 50 µL of stop solution to each well.

19) Read absorbance at 450 nm within 30 minutes of stopping reaction with 570 nm as reference wavelength.

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Measurement of IL-8 in the samples

The BD Pharmingen kit (Cat. No. 555244) is materials and chemicals needed are described below.

Materials and chemicals needed for the IL-8 measurements

Capture antibody	Anti-human IL-8. monoclonal antibody
Detection antibody	Biotinylated anti-human IL-8. monoclonal antibody
Enzyme Reagent	Streptavidin-horseradish peroxidase conjugate
Standard:	Recombinant human IL-8
Coating buffer	
Na_2CO_3	1.59 g
$NaHCO_3$	7.13 g
MilliQ-water	q.s. 1000 mL
pH adjusted to 9.5	
Phosphate Buffered Saline (PBS)	
NaCl	80.0 g
Na_2HPO	11.6 g
KH_2PO_4	2.0 g
KCl	2.0 g
MilliQ-water	q.s. 10 L
pH adjusted to 7.0	
Assay Diluent	
PBS with 10% FBS	
Wash buffer	
PBS with 0.05% Tween-20	
Substrate solution	
Tetramethylbenzidine (TMB)	
Hydrogen peroxide	
Stop solution	
2 N H_2SO	
Other materials:	
ELISA-plates (NUNC Maxisorp)	
Diverse pipettes. tips and other utensils	

Procedure

Standards preparation and handling

1) After warming to room temperature, carefully open vial to avoid loss of material.

2) Reconstitute the lyophilized standard with 1.0 mL of deionized water to yield a stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions.

3) Vortex the solution gently to mix.

4) After reconstitution, immediately aliquot standard stock in polypropylene vials at 50 µl per vial and freeze at -80ºC for up to 6 months. If necessary, store at 2-8ºC for up to 8 hours prior to aliquoting/freezing. Do not leave reconstituted standard at room temperature.

5) Pipette 100 µL of assay diluent into each standard well except the highest (200 pg/mL). Run in duplicate.

6) Add 100 µL of the 200 pg/mL standard to the 200 pg/mL standard well and the 100 pg/mL well.

7) Mix the well contents by rinsing the pipette tip.

8) Add 100 µL of the 100 pg/mL standard to the 50 pg/mL well.

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9) Repeat until the 3.1 pg/mL standard well, out of which the extra 100 µL should be discarded.

NB: The quantity of standard recombinant human IL-8 lyophilized provided by the supplier may vary from kit to kit. Look at the instruction/analysis certificate provided with the kit to make the standard curve accordingly. The highest concentration should be 200 pg/mL.

Assay procedure

1) Mix 40 µL of capture antibody with 9960 µL of coating buffer to obtain 10 mL of capture antibody diluted to 1/250.

2) Coat wells of a 96-well plate with 100 µL per well of the capture antibody previously diluted. 10 mL of solution is needed to coat a 96-well plate.

3) Seal plate and incubate overnight in the refrigerator.

4) Empty wells and wash 3 times with the wash buffer.

5) After the last wash, invert the plate and blot on absorbent paper to remove any residual buffer.

6) Block plates with 200 µL of assay diluent. Incubate 1 hour on the shaker at room temperature.

7) Empty/wash 3 times as in step 2.

8) Prepare standards and samples dilutions as described in Standards preparation and handling.

9) Pipette 100 µL of each standard, sample and control into appropriate wells, in duplicate. The assay diluent is used as a blank.

10) Seal plate and incubate 2 hours at room temperature on the shaker.

11) Empty/wash 4 times as in step 3.

12) Prepare working detector (solution B), maximum 15 minute before use, as following:

Solution A: 12,475 mL assay diluent + 25 µL detection antibody

Solution B: 12,45 mL solution A + 50 µL enzyme reagent

13) Add 100 µL of prepared working detector (solution B) to each well.

14) Seal plate and incubate 1 hour on the shaker at room temperature.

15) Empty/wash 4 times as in step 2. In this final wash step, soak wells in wash buffer for 30 seconds to 1 minute for each wash.

16) Add 100 µL of substrate solution to each well. Incubate plate for 30 minutes at room temperature in the dark.

17) Add 50 µL of stop solution to each well.

18) Read absorbance at 450 nm within 30 minutes of stopping reaction with 570 nm as a reference wavelength.

Used ELISA incubation plan in a 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank			0	0	0	0	0	0	0	0	
B	Blank			10	10	10	10	10	10	10	10	
C	Blank			20	20	20	20	20	20	20	20	

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D	Blank			40	40	40	40	40	40	40	40	
E	Blank			80	80	80	80	80	80	80	80	
F	Blank			160	160	160	160	160	160	160	160	
G	+			320	320	320	320	320	320	320	320	
H	+			640	640	640	640	640	640	640	640	

+ is the positive control.

Blanks are the assay diluent.

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