PATROLS
Advanced Tools for NanoSafety Testing

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

ANNEX 3208

PATROLS Standard Operating Procedures (SOP)

SOP_Patrols 3208_Guidance document for assessing the direct effect of NMs on fibroblast proliferation and expression of pro-fibrotic biomarkers

This is a SOP used by members of PATROLS only

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	24/03/2020	Initial Document	Sybille van den Brule
1.1.	15/04/2020	Minor revisions	Violaine Sironval Saloua Ibouraadaten Dominique Lison

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1 Introduction:

DOMAIN: Advanced *in Vitro* Model Systems for ENM Hazard Assessment

Nanotechnology promises significant scientific, economic and societal benefits, but commercialization and growth are threatened by safety uncertainties. Existing in vitro hazard testing strategies to define the human health impact of nanomaterials (NMs) commonly apply unrealistic acute, high-doses to cellular models that poorly reflect the in vivo environment.

Fibroblasts have a central role in the development of lung fibrosis and their activation triggers the distal events of the pathogenic process (migration to the site of injury, proliferation, differentiation in myofibroblasts and production of extracellular matrix proteins, e.g. collagens). Fibroblasts can be activated in response to inflammation and mediators produced by other cell types (e.g. macrophages or epithelial cells) exposed to NMs. Recently, it has also been shown that NMs can reach the lung interstitium/fibroblasts and can directly activate this cell type (reviewed in (Vietti et al., 2016)). Therefore, it appears essential to develop cell models based on fibroblasts, including acute and chronic/long term/repeated exposure, either via direct exposure of fibroblasts or via indirect exposure, i.e. by exposing fibroblasts to medium conditioned by other lung cell types exposed to NMs, or by including fibroblasts in co-cultures. This SOP describes the protocols for the in vitro culture of human lung fibroblasts (CRL1490), the direct exposure to NMs ((i) single acute (24 h) exposure, (ii) single chronic (6 days) exposure and (iii) repeated chronic (8 weeks) exposure) and the assays for evaluating the pro-fibrotic responses to NMs.

1.1 Scope and limits of the protocol

This SOP was developed as a basis for advanced fibroblast exposure models. The objective is to evaluate the predictive potential for lung fibrosis of 3 fibroblast-only in vitro models and of assays measuring proliferation and other pro-fibrotic biomarkers in response to NMs. The SOP describes the protocols for the fibroblast cell culture, the direct (single or repeated) exposure conditions and the assays to evaluate fibroblast proliferation/activation. This SOP includes positive controls for fibroblast proliferation (platelet-derived growth factor, PDGF 30 ng/ml) and activation (transforming growth factor-β, TGF-β 10 ng/ml) (see D3.2 for selection and testing of

positive controls). Micrometric crystalline silica particles (DQ12) are also used as a particulate reference for lung fibrosis, but is not considered as a positive control for the assays described in this SOP.

2 Terms and Definitions:

Nanomaterial
Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostructured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

[SOURCE: ISO/TS 80004-1: 2016, definition 2.7]

3 Abbreviations:

AA	antibiotic-antimycotic
BSA	bovine serum albumin
CNT	carbon nanotube
DHases	dehydrogenases
ENM	engineered nanomaterial
ECM	extracellular matrix
FBS	fetal bovine serum
MEM	minimal essential medium
MMP	matrix metallopeptidase
MWCNT	multi-walled carbon nanotube
NEAA	non essential amino acids
NM	nanomaterial
OPN	osteopontin
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
qRT-PCR	quantitative reverse transcription-polymlerase chain reaction
SMA (ACTA2)	smooth muscle actin
SPP1	secreted phosphoprotein
TGF	transforming growth factor
TIMP	tissue metallopeptidase inhibitor

4 Principle of the Method:

Our strategy is to repeatedly and chronically expose human lung fibroblasts directly to NMs during 8 weeks. The duration (8 weeks) is based on the observation that mice develop fibrotic responses and extracellular matrix (ECM) accumulation in response to fibrotic microparticles (e.g. crystalline silica particles) or NMs (e.g. MWCNT) 2 months after a single exposure (Vietti et al., 2013). The responses obtained after the 8-week repeated exposure are compared to the effects observed after a single (acute 24 h and 6-day) exposure. The 6-day exposure corresponds to a one week exposure duration during the 8-week repeated exposure protocol. The aim is to identify which conditions better predict the pro-fibrotic potential of NMs.

The (bio)markers assessed in this SOP were selected for their potential to predict lung fibrosis from T2.5 (Identification of mechanistic key events linked to AOPs) and D2.5 (Identification of key events with predictive value for effects due to chronic ENM exposure): cell proliferation, mRNA expression of collagen I (collagen type I alpha 1 chain COL1A1), collagen III (collagen type III alpha 1 chain, COL3A1), α-smooth muscle actin (actin alpha 2, smooth muscle ACTA2 or α-SMA), tissue metallopeptidase inhibitor 1 (TIMP1), matrix metallopeptidase 9 (MMP9) and secreted phosphoprotein 1/osteopontin (SPP1 or OPN).

Fibroblast proliferation is assessed by WST-1. WST-1 is a colorimetric assay that quantifies the cellular/mitochondrial activity and reflects cell viability by assessing the cleavage of the tetrazolium salt WST-1 by mitochondrial DHases in viable cells in its product formazan. For fibroblasts, WST-1 was shown to reflect cell number and, hence, the proliferative activity as demonstrated in (Vietti et al., 2013).

The qRT-PCR method quantifies the mRNA expression of the selected genes of interest normalized to the expression of a housekeeping gene (18S rRNA). This technique is frequently used to estimate the protein expression for these genes.

This SOP includes positive controls for fibroblast proliferation (platelet-derived growth factor, PDGF 30 ng/ml) and activation (transforming growth factor-β, TGF-β 10 ng/ml). Micrometric crystalline silica particles (DQ12) are also used as a particulate reference for lung fibrosis, but is not considered as a positive control for the assays described in this SOP. For the chronic exposure conditions (6 days and 8 weeks), a part of the wells/cells are also co-stimulated 24 h before the assays with low

concentrations of PDGF (3 ng/ml, for proliferation) or TGF-β (1 ng/ml, for the expression of pro-fibrotic biomarkers) to assess the potential of NMs/DQ12 to amplify the fibroblast responses to low concentrations of these growth factors.

5 Description of the Method:

5.1 Biological setting & test system used:

This SOP should be carried out under laboratory based conditions, with cell culture and exposure performed under sterile conditions and at a minimum of Biological Safety Level 1 conditions.

The human lung fibroblast cell line CRL1490 (CCD33Lu, ATCC® CRL1490™) is used between passage 3 and 15. Every 3 months, cells are tested for mycoplasma contamination (PCR mycoplasma test kit I/C, Bio-connect, Huissen, The Netherlands).

To develop the SOP, some experimental conditions were optimized. This is described below.

a) Cell density for single acute (24 h) exposure

CRL1490 were cultured in 96-well plates at different cell densities in culture medium containing 10 % fetal bovine serum to identify the lowest cell density that gives pre-confluence after 24 h culture. Pre-confluence was observed with 10^4 cells/well, i.e. 2.94×10^4 cells/cm² (data not shown). This cell number was selected for all single acute experiments.

b) Proliferative response of CRL1490 after single acute (24 h) exposure to MWCNT

Given that the fibroblast direct exposure model is based on the observation that certain NMs, such as multiwall carbon nanotubes (MWCNT), can directly activate lung fibroblasts, especially their proliferation, CRL1490 were exposed to increasing concentrations of NC7000 (Nanocyl tangled MWCNT) and Mitsui-7 (needle-like MWCNT) and their cell viability/WST-1 activity (reflecting cell proliferation) was assessed 24 h later. Cells were also exposed to a positive control (PDGF 30 ng/ml)

during 24 h. Both NC7000 and Mitsui-7 stimulated fibroblast proliferation in a dose-dependent manner (figure 1), indicating that CRL1490 are reactive to NMs and an appropriate cell line for the objective of the model.

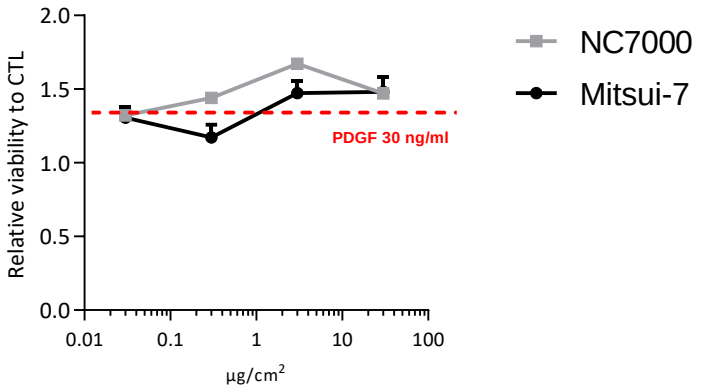

Figure 1: Pre-confluent CRL1490 were exposed to PDGF 30 ng/ml and 0.03, 0.3, 3 and 30 µg/cm² NC7000 and Mitsui-7. Cell viability/WST-1 activity was measured 24 h later to assess fibroblast proliferation.

c) Cell density and serum concentration for single chronic (6 days) exposure

Different seeding cell densities and serum concentrations were evaluated to identify the best conditions for a 6-day fibroblast culture with no morphology change and/or post-confluence. We selected $4x10^5$ cells/flask (25 cm^2 culture flask), i.e. $1.6x10^4$ cells/cm^2 and 1 % serum as appropriate (figure 2).

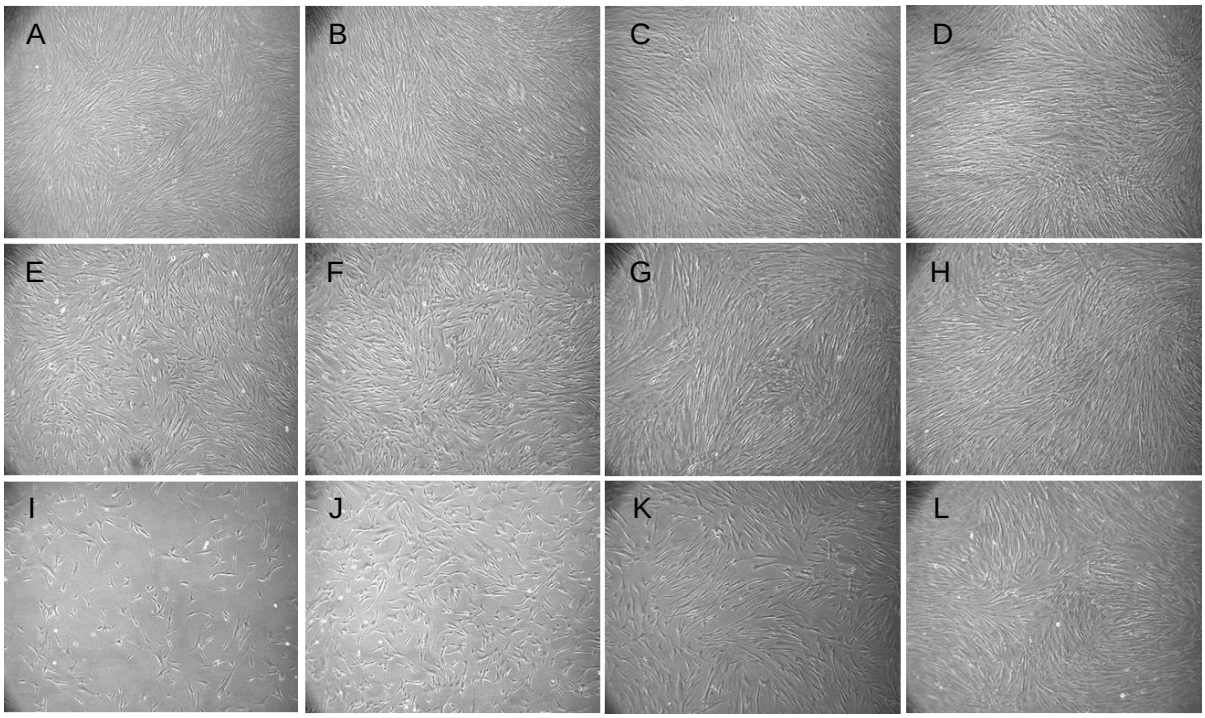

Figure 2: (A-D) 8x10^5, (E-H) 4x10^5 and (I-L) 2x10^5 cells/well were cultured in 25 cm² culture flasks 24 h in complete culture medium (10 % FBS) and then 3 d (A, B, E, F, I, J) and 6 d (C, D, G, H, K, L) in culture medium containing 1 % FBS (A, E, I, C, G, K) or 3 % FBS (B, F, J, D, H, L).

d) Fibroblast morphology after 8-week culture in the selected conditions

CRL1490 were cultured during 8 weeks according to the exposure conditions described in the present SOP with the exception that 25 cm² culture flasks were used instead of 6-well plates. Briefly, cells were seeded at the determined cell density, cultured 24 h in complete cell culture medium containing 10 % serum and, then, cultured 6 d in exposure medium containing 1 % serum. After this period (1 week), cells were passaged and cultured the same way during 7 additional weeks (passage every week). Viable cells counted at each passage were between 5x10^5 and 10^6 cells/25 cm² flask (figure 3A), i.e. 2-4x10^4 cells/cm². At the end of the 8 weeks, cells appeared to grow normally and had an unchanged morphology (compare figure 2 with figure 3B), indicating that these conditions can be used for the repeated chronic (8-week) exposure experiments.

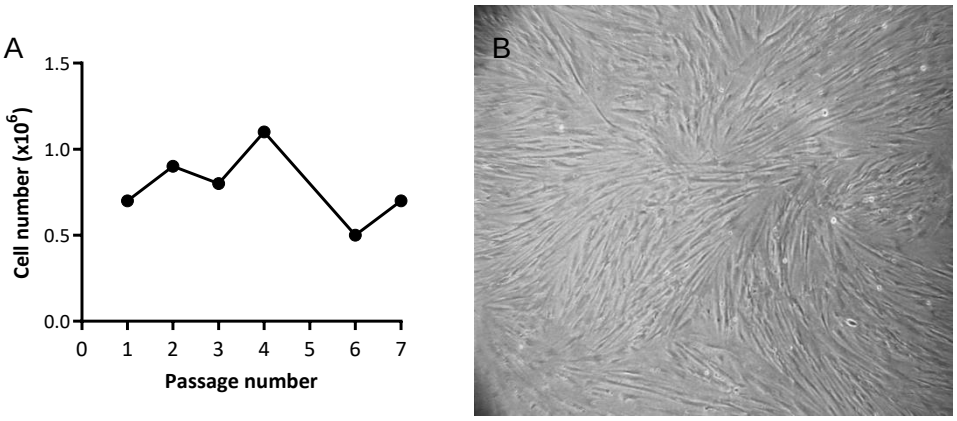

Figure 3: CRL1490 cultured during 8 weeks with a total of 7 passages (every week). (A) Viable cell number at each passage, (B) cell morphology after 8-week culture and 7 passages.

e) Selection of NM concentrations for single chronic (6-day) and repeated chronic (8-week) exposures

CRL1490 were exposed 6 days to increasing concentrations of NMs (0.1, 1 and 10 µg/cm²) in exposure medium containing 1 % serum (medium used for the 6-day and 8-week exposure conditions). Cell viability, to assess cell cytotoxicity and proliferation, was evaluated by the WST-1 assay every 2 days during this period. NM402, CeO_2 and $BaSO_4$ were not cytotoxic at any of the concentrations tested (figures 4B-D) and, thus, these concentrations were selected for further chronic exposure experiments. Mitsui-7 were cytotoxic at 1 and 10 µg/cm² after 6 days (figure 4 A) while similar concentrations of these MWCNT increased cell proliferation after 24 h (figure 1). Given that 10 µg Mitsui-7/cm² dropped cell viability to 0, this concentration was not considered for further chronic experiments. Mitsui-7 was therefore tested also at a lower concentration (0.01 µg/cm²). To be comparable, NM402 was also tested at 0.01 µg/cm².

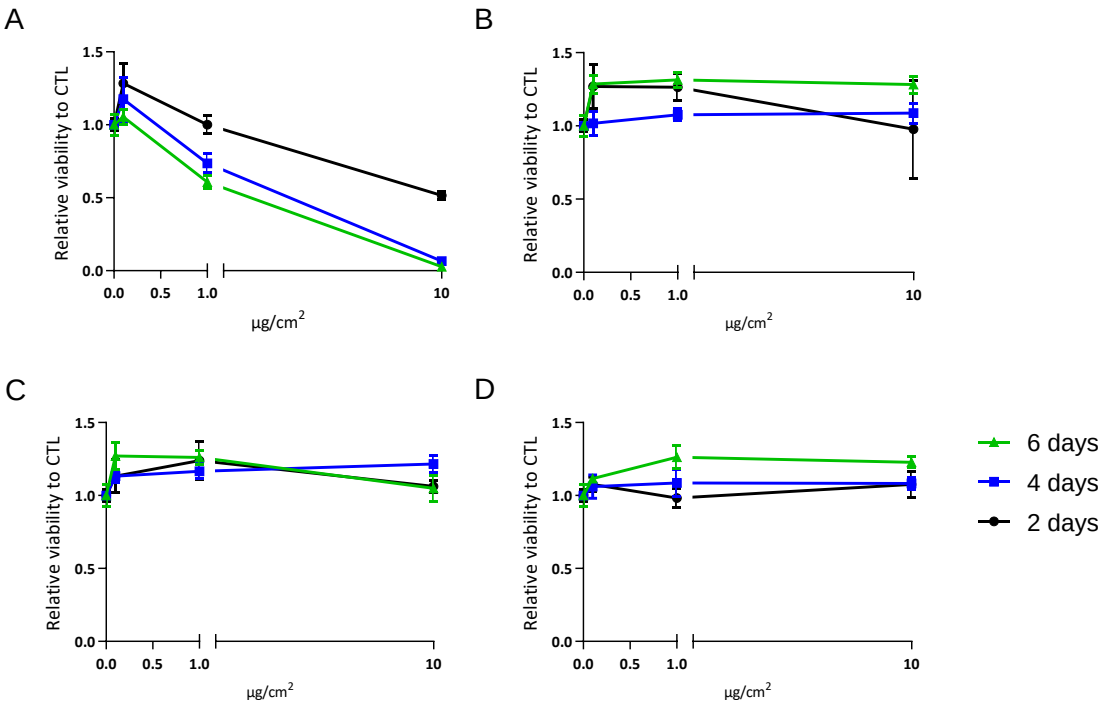

Figure 4: CRL1490 were exposed during 6 days to 0.1, 1 and 10 µg/cm² (A) Mitsui-7, (B) NM402, (C) CeO_2 and (D) $BaSO_4$ and cell viability was measured after 2, 4 and 6 days.

5.2 Chemicals and reagents used:

Cell culture and exposure:

- Antibiotic-antimycotic (AA): 15240-062, Invitrogen, Paisley, USA
- Bovine serum albumin (BSA): A9418-50G, Sigma Aldrich, St Louis, USA
- Ethanol ≥99.8%: 20821.310, VWR, Oud-Heverlee, Belgium
- Fetal bovine serum (FBS): 10270-106, Invitrogen, Paisley, USA
- Minimal essential medium (MEM): 31095-052, Invitrogen, Paisley, USA
- Non essential amino acids (NEAA): 11140035, Invitrogen, Paisley, USA
- PDGF-BB: 100-14B, PeproTech, London, UK
- Phosphate-buffered saline (PBS): 14190-169, Invitrogen, Paisley, USA
- Sodium pyruvate (100 mM): 11360-039, Invitrogen, Paisley, USA
- TGF-β1: 100-B-001/CF, R&D Systems, Minneapolis, USA
- Trypan blue solution: T8154, Sigma Aldrich, St Louis, USA
- Trypsin 0.25 %-EDTA: 25200-072, Invitrogen, Paisley, USA

Culture media, PBS and trypsin should be pre-warmed at room temperature before use.

Fibroblast proliferation (WST-1 assay):

- WST-1: 11 644 807 001, Roche Diagnostics GmbH, Mannheim, Germany

Expression of pro-fibrotic biomarkers (RNA extraction and qRT-PCR):

- Autoclaved mQ H_2O
- β-mercaptoethanol : M6250-100ML, Sigma Aldrich, St Louis, USA
- dNTP Mix (20 µmoles): NU-0010-50, Eurogentec, Seraing, Belgium
- M-MLV Reverse Transcriptase (200 U/µl): 28025013, Invitrogen, Paisley, USA
- Power SYBR Green PCR Master Mix: 4368708, Applied Biosystems, Foster City, USA
- Random Hexamers 700 pmol/µl: Eurogentec, Seraing, Belgium
- RNAse free DNase set: 79254, Qiagen, Hilden, Germany
- RNeasy mini kit: 74104, Qiagen, Hilden, Germany
- UltraPure DNase/RNase-free distilled water: 10977035, Invitrogen, Paisley, USA

5.3 Apparatus and equipment used:

Cell culture and exposure:

- 1.5 and 2 mL plastic tubes: 616201 and 623201, Greiner bio-one, Vilvoorde, Belgium
- 15 and 50 mL sterile plastic tubes: 188271 and 227261, Greiner bio-one
- 75 cm^2 culture flasks: 658175, Greiner bio-one
- 96-well and 6-well cell culture plates: 655180 and 657160, Greiner bio-one
- Analytical balance
- Biological safety cabinet
- Burker cell
- Centrifuge
- Incubator (37 °C, 5 % CO_2, humidified atmosphere)
- Invertoscope: Leitz Labovert, Leica Microsystems, Belgium
- Light microscope, oculars 10x and objective 40x: Leica DMLB, Leica Microsystems, Belgium
- Pipettes (20, 100, 200 µl and 1 mL)

- VC750 ultrasonic processor: Sonics & Materials, Newtown, USA

Fibroblast proliferation (WST-1 assay)

- Centrifuge
- Pipettes (20, 100, 200 µl and 1 mL)
- SpectraMax iD3 multi-mode microplate reader: Molecular Devices, San José, USA

Expression of pro-fibrotic biomarkers (RNA extraction and qRT-PCR)

- 1 ml syringes: 612-0106, VWR, Oud-Heverlee, Belgium
- 1.5 and 2 mL plastic tubes: 211-2164 and 211-2165, VWR.
- 25G needles: 613-0902, VWR
- Centrifuge
- NanoDrop UV-Vis Spectrophotometer
- Optical adhesive films: 4311971, Applied Biosystems, Foster City, USA
- PCR microplates: 652210, Greiner bio-one
- Pipettes (20, 100, 200 µl and 1 mL)
- StepOnePlus Real-Time PCR System + StepOne software v2.3: Applied Biosystems
- Waterbath or thermoblock

5.4 Reporting of protected elements:

To the best of our knowledge, this SOP does not have any associated patent restrictions, specific licenses, material transfer agreements or commercial purchase requirements required to perform the protocol described.

5.5 Health and safety precautions:

Standard health and safety precautions associated with working within a laboratory environment and performing mammalian cell culture, as described by the European Agency for Safety and Health at Work (https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines), should be adopted when conducting this SOP. In addition, all health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must also be followed.

5.6 Nanomaterials used / handling procedures:

NMs and particles should be kept in their original container, closed and protected from light and humidity. Table 1 lists the particles and NMs used in this SOP. This list is indicative and this SOP can be used with any NM.

Table 1: List of particles/NMs used at LTAP in the frame of this SOP.

Material	code	CAS-Number	Source
Crystalline silica particles	DQ12 IUF-silica	14808-60-7	IUF
Needle-like MWCNT	Mitsui-7 JRCNM40011a	-	JRC
Tangled MWCNT	NM-402 JRCNM04002a	-	JRC
$BaSO_4$	NM220	7727-43-7	Fraunhöfer
CeO_2	NM212	1306-38-3	Fraunhöfer

NMs are prepared as 2.56 mg/ml stock suspensions in BSA-water 0.05% w/v containing 0.5 % v/v EtOH and sonicated according to the SOP developed in NANOGENOTOX (NGTOX batch dispersion SOPv1.2). After dispersion, directly use NM stock suspensions (within 30 min) for dilutions and fibroblast exposure. Serially dilute stock suspensions in the dispersion medium (BSA-water 0.05% w/v, 0.5 % EtOH v/v) to a concentration 10 fold higher than the final exposure concentration because suspensions are diluted 10 x in the cell culture medium. The exact same dispersion medium (BSA-water 0.05% w/v, 0.5 % EtOH) is used as negative control in all experiments.

DQ12 is also prepared as a 2.56 mg/ml stock suspension in exposure cell culture medium and diluted in exposure cell culture medium to a concentration 10 fold higher than the final exposure concentration.

The exposure concentrations are expressed in µg/cm² (surface area of culture well). So, if we consider a 96-well plate (well surface 0.34 cm²), and an exposure volume of 0.2 ml, a final exposure concentration of 10 µg/cm² corresponds to 17 µg/ml (10 µg/cm² x 0.34 cm² / 0.2 ml), i.e. 170 µg/ml to be diluted 10 x in the exposure medium (see table 2 for calculations for all concentrations). Fibroblasts are exposed once or repeatedly to 0.1-10 µg NM/cm² (to cover a broad range of concentrations) and 10 µg DQ12/cm². Recommendations from PATROLS Milestone 5 could not be followed

since this takes into account the alveolar (epithelial) surface, which is not relevant to fibroblasts located in the intestitium. NM402 and Mitsui-7 are additionally tested at 0.01 µg NM/cm² and Mitsui-7 are not tested at 10 µg NM/cm² (strongly cytotoxic 6 days after exposure).

Table 2: Calculations for NM/particle dilutions before exposure.

Final concentration (µg/cm²)	plate	Well surface (cm²)	Exposure volume (ml)	Final concentration (µg/ml)	concentration to be diluted 10 x (µg/ml)
0.01	96-well	0.34	0.2	0.017	0.17
0.1	96-well	0.34	0.2	0.17	1.7
1	96-well	0.34	0.2	1.7	17
10	96-well	0.34	0.2	17	170
0.01	6-well	9.6	5.64	0.017	0.17
0.1	6-well	9.6	5.64	0.17	1.7
1	6-well	9.6	5.64	1.7	17
10	6-well	9.6	5.64	17	170

5.7 Reagent preparation:

- Complete cell culture medium: MEM, 1 % NEAA, sodium pyruvate 1 mM, 10 % FBS, 1 % AA.
- Exposure cell culture medium (for 24 h exposure to NM): MEM, 1 % NEAA, sodium pyruvate 1 mM, 1 % AA.
- Exposure cell culture medium (for 6-day and 8-week exposure to NM): MEM, 1 % NEAA, sodium pyruvate 1 mM, 1 % FBS, 1 % AA.
- Dispersion medium: BSA-water 0.05% w/v, 0.5 % EtOH v/v (see NGTOX batch dispersion SOPv1.2).
- WST-1 medium: (MEM, 1 % NEAA, sodium pyruvate 1 mM, 1 % AA) + WST-1 reagent; 9:1 v/v.
- TGF-β1 (1 µg/ml): suspension of powder as recommended by the manufacturer.
- PDGF-BB (10 µg/ml): suspension of powder as recommended by the manufacturer.

5.8 Procedure:

5.8.1 Fibroblast culture

Human lung fibroblasts (CRL-1490) are cultured in 75 cm^2 flasks in 20 ml complete cell culture medium at 37 °C under 5 % CO2.

For sub-culturing, cells are passaged at least once a week at pre-confluence (1:50 dilution):

1) After washing cells with 10 ml PBS, add 3 ml trypsin 0.25 %-EDTA.

2) Incubate flasks 5 min at 37 °C.

3) Before collecting cells, hit flasks on the side to completely detach cells and check under the invertoscope.

4) Neutralize trypsin by adding 7 ml complete cell culture medium.

5) Transfer suspensions to a 15 or 50 ml tube and centrifuge for 10 min at 400 *g*.

6) Suspend cell pellets in 5 ml fresh complete cell culture medium.

7) Dilute 100 µl cell suspension to 20 ml with fresh complete medium and transfer to a new flask.

For exposure to NM, trypsinize cells as described above (steps 1 to 6) and then:

1) Determine cell number and viability with Trypan Blue Solution (20 µl cells + 180 µl Trypan Blue) in a Burker cell under a light microscope.

2) Seed cells at a concentration of 29400 viable cells/cm^2 in 96 well-culture plates for acute (24 h) exposure, i.e. 10000 cells/well in 200 µl complete medium, and at a concentration of 16000 cells/cm^2 in 6 well-culture plates for 6-day and 8-week exposure, i.e. 153600 cells/well in 1 ml complete medium (d-1) (6 wells/condition for the 6-day experiments and 1 well/condition for the 8-week experiments, see point 5.8.2 for details).

3) Incubate cells 24 h at 37°C under 5 % CO_2 (d0).

5.8.2 Fibroblast exposure

Test each condition at least in triplicates for the acute (24 h) exposure. For 6-day and 8-week exposures, use triplicate or replicate wells for RNA extraction and single wells for proliferation (only during the last week of exposure for the 8-week exposure

experiments; before the proliferation/cell viability assay, cells are sub-cultured at least in triplicate wells before the WST-1 assay). Examples of plate layouts are given in subsections for each exposure conditions.

5.8.2.1 Single acute (24 h) exposure

An overview of the protocol for the single acute (24 h) exposure experiments is given in figure 5. In this protocol, fibroblasts are exposed 24 h to NMs/DQ12 or positive controls (TGF-β 10 ng/ml and PDGF 30 ng/ml). An example of 96-well plate layout is given in figure 6.

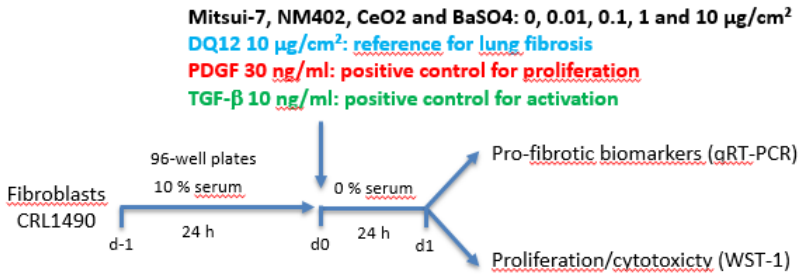

Figure 5: Overview of the single acute (24 h) exposure protocol.

	A	B	C	D	E	F	G	H	I	J	K	L
1												
2		CTL	PDGF 30 ng/ml	TGF 10 ng/ml	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10	
3		CTL	PDGF 30 ng/ml	TGF 10 ng/ml	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10	
4		CTL	PDGF 30 ng/ml	TGF 10 ng/ml	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10	
5		DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10				
6		DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10				
7		DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10				
8												

Figure 6: Example of 96-well plate layout for the single acute (24 h) exposure: one plate for the WST-1 assay and one plate for qRT-PCR. Conditions should be tested at least in triplicates: CTL (untreated), PDGF 30 ng/ml (positive control for WST-1 assay), TGF-□ 10 ng/ml (positive control for pro-fibrotic biomarkers), DQ12 10 µ/cm² (reference for lung fibrosis), Mitsui-7 0.01, 0.1 and 1 µg/cm², NM402 0.01, 0.1, 1 and 10 µg/cm², CeO_2 0.1, 1 and 10 µg/cm², $BaSO_4$ 0.1, 1 and 10 µg/cm².

After 24 h incubation at 37 °C in 96-well plates (d0),

1) Wash cells once with 200 µl PBS.

2) Add 180 µl exposure medium.

3) Dilute stock solutions of positive controls (TGF-β and PDGF) in dispersion medium to a concentration 10 x higher (100 ng/ml and 300 ng/ml, respectively) than the final exposure concentration.

4) Add 20 µl dispersion medium ± NMs/DQ12/positive controls at a concentration 10 x higher than the final exposure concentration (see table 2 for NM/DQ12 and point 3 for positive controls). Gently mix by pipetting 3 times while adding the 20 µl.

5) Incubate cells 24 h at 37°C under 5% CO_2 (d1).

6) For measuring the expression of the pro-fibrotic biomarkers, wash triplicate wells with 200 µl PBS and then add 200 µl of RLT buffer (RNeasy mini kit). Store cell lysates at -80 °C until extraction (point 5.8.4). For measuring fibroblast proliferation, perform WST-1 assay (point 5.8.3).

5.8.2.2 Single 6-day exposure

An overview of the protocol for the single 6-day exposure experiments is given in figure 7. In this protocol, fibroblasts are exposed 6 d to NMs/DQ12 in 6-well plates. At the end of the exposure duration, the cells from one well are sub-cultured in 96-well plates for assessing cell proliferation. Positive controls (TGF-β 10 ng/ml and PDGF 30 ng/ml) are added 24 h before the assays (WST-1 and qRT-PCR). Low concentrations of TGF-β (1 ng/ml) and PDGF (3 ng/ml), called co-stimulation conditions, are also added 24 h before the assays to some wells to assess the potential of NMs/DQ12 to amplify the fibroblast responses to low concentrations of these growth factors. An example of 6-well and 96-well plate layouts is given in figure 8.

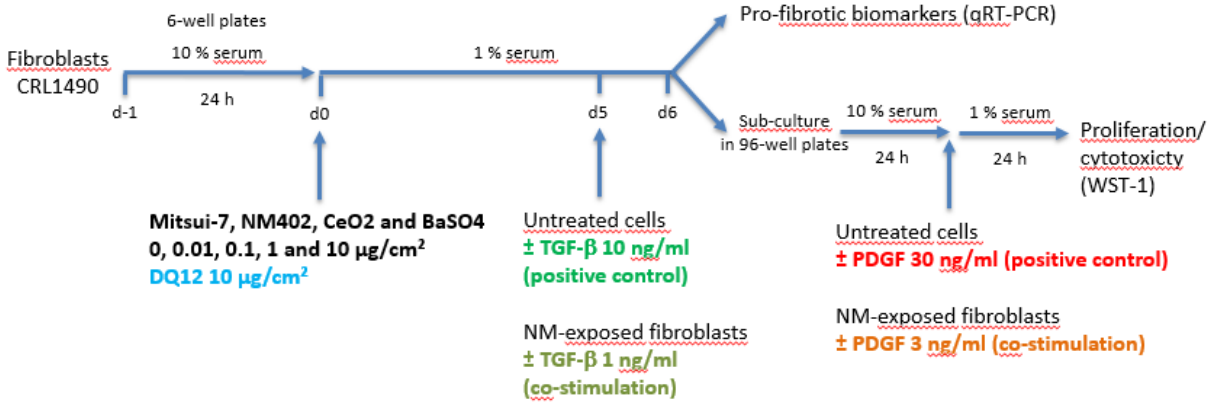

Figure 7: Overview of the single chronic (6 days) exposure protocol.

A

	A	B	C
1	CTL	CTL	CTL
2	CTL + TGF 1 ng/ml 1 day before the end of exposure	CTL + TGF 1 ng/ml 1 day before the end of exposure	CTL (for WST-1)

	A	B	C
1	NM or DQ12	NM or DQ12	NM or DQ12
2	NM or DQ12 + TGF 1 ng/ml 1 day before the end of exposure	NM or DQ12+ TGF 1 ng/ml 1 day before the end of exposure	NM or DQ12 (for WST-1)

	A	B	C
1	CTL + TGF 10 ng/ml 1 day before the end of exposure	CTL + TGF 10 ng/ml 1 day before the end of exposure	CTL + TGF 10 ng/ml 1 day before the end of exposure
2			

B

	A	B	C	D	E	F	G	H	I	J	K	L
1												
2		CTL	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10	PDGF 30 ng/ml		
3		CTL	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10	PDGF 30 ng/ml		
4		CTL	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10	PDGF 30 ng/ml		
5		CTL	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10			
6		CTL	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10			
7		CTL	Mitsui 0.01	Mitsui 0.1	Mitsui 1	NM402 0.01	NM402 0.1	NM402 1	NM402 10			
8												

	A	B	C	D	E	F	G	H	I	J	K	L
1												
2		CTL	DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10	PDGF 30 ng/ml		
3		CTL	DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10	PDGF 30 ng/ml		
4		CTL	DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10	PDGF 30 ng/ml		
5		CTL	DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10			
6		CTL	DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10			
7		CTL	DQ12 10	CeO_2 0.1	CeO_2 1	CeO_2 10	$BaSO_4$ 0.1	$BaSO_4$ 1	$BaSO_4$ 10			
8												

Figure 8: Examples of 6-well and 96-well plate layouts for the single 6-dayexposure conditions. (A) Cells are first cultured and exposed to the different conditions in 6-well plates during 6 days. As examples, one control plate (untreated fibroblasts), one plate with cells exposed to a NM or DQ12 at one concentration and one positive control plate (TGF-□ 10 ng/ml, positive control for the pro-fibrotic biomarkers) are shown. Wells in grey are dedicated to qRT-PCR with triplicates with no additional treatment and replicates additionally exposed to TGF-□ 1 ng/ml (co-stimulation conditions). Wells in white are sub-cultured in 96-well plates at the end of the exposure duration for assessing fibroblast proliferation. (B) 96-well plates with sub-cultured cells for the WST-1 assay. Triplicates receive no

additional treatment and other triplicates are additionally exposed to PDGF 3 ng/ml (in blue, co-stimulation conditions).

After 24 h incubation at 37 °C in 6-well plates (d0, 6 wells/condition),

1) Wash cells once with 1 ml PBS.

2) Add 5.076 ml exposure medium.

3) Add 564 µl dispersion medium ± NMs/DQ12 at a concentration 10 x higher than the final exposure concentration (see table 2). Gently mix by pipetting 3 times.

4) Incubate cells 5 d at 37°C under 5 % CO_2 (d5).

5) Dilute stock solutions of TGF-β in exposure medium to 574 ng/ml and 57.4 ng/ml.

6) Add 100 µl TGF-β 574 ng/ml (final concentration 10 ng/ml) to at least 3 untreated wells as positive control for activation.

7) Add 100 µl TGF-β 57.4 ng/ml (final concentration 1 ng/ml) to 2 of the wells (co-stimulation conditions) and 100 µl exposure medium to the other half .

8) Incubate cells 24 h at 37°C under 5 % CO_2 (d6).

9) For measuring the expression of the pro-fibrotic biomarkers, wash cells with 1 ml PBS and then add 350 µl of RLT buffer (RNeasy mini kit). Store cell lysates at -80 °C until extraction.

R/ For the NM402 10 µg/cm² exposure conditions, directly add 350 µl of RLT buffer without washing cells before (weaker cell adherence probably due to a slight cytotoxicity).

10) For measuring fibroblast proliferation,

a) Wash the wells (one well/condition) with 1 ml PBS.

R/ For the NM402 10 µg/cm² exposure conditions, directly add 350 µl trypsin without washing cells before (weaker cell adherence probably due to a slight cytotoxicity).

b) Add 350 µl trypsin 0.25 %-EDTA.
c) Incubate cells 5 min at 37 °C.
d) Neutralize trypsin by adding 1 ml complete cell culture medium.
e) Transfer suspensions to a 1.5 ml tube and centrifuge for 7 min at 400 *g*.
f) Suspend cell pellets in 200 µl fresh complete cell culture medium.
g) Determine cell number and viability with Trypan Blue Solution (20 µl cells + 80 µl Trypan Blue) in a Burker cell under a light microscope.

h) Seed cells at least in 2 x triplicates at a concentration of 29400 viable cells/cm², i.e. 10000 cells/well into 96 well-culture plates in 200 µl complete medium.
i) Incubate cells 24 h at 37°C under 5 % CO_2.
j) Wash cells with 200 µl PBS.
k) Add 200 µl exposure medium/PDGF 30 ng/ml (positive control for proliferation) to 3 untreated wells, 200 µl exposure medium to half of the wells and 200 µl exposure medium/PDGF 3 ng/ml to the other half of the wells.
l) Incubate cells 24 h at 37°C under 5 % CO_2.
m) Perform WST-1 assay (point 5.8.3)

5.8.2.3 Repeated 8-week exposure

An overview of the protocol for the repeated 8-week exposure experiments is given in figure 9. In this protocol, fibroblasts are exposed 6 d to NMs/DQ12, repeatedly on a total period of 8 weeks. After each 6-day exposure period, fibroblasts are sub-cultured in new wells, left 24 h for adherence and then, again, exposed to NMs/DQ12 during 6 days. As for the single 6-day exposure, positive controls (TGF-β 10 ng/ml and PDGF 30 ng/ml) are added 24 h before the assays (WST-1 and qRT-PCR). Low concentrations of TGF-β (1 ng/ml) and PDGF (3 ng/ml), called co-stimulation conditions, are also added 24 h before the assays to half of the wells to assess the potential of NMs/DQ12 exposure to amplify the fibroblast responses to these growth factors.

Contrary to the 6-day exposure protocol, only one culture well (6-well plate) is used for each condition during the 6 first weeks, due to too many cell counts to perform if each condition was replicated in 6 culture wells. During the 7th week, each condition (well) is divided in 3 wells (triplicates) to increase the number of cells. Finally, during the 8th week, cells from the triplicates are pooled and divided in 6 wells (per condition) exactly as for the 6-day exposure experiment. An example of plate layouts is given in figure 10.

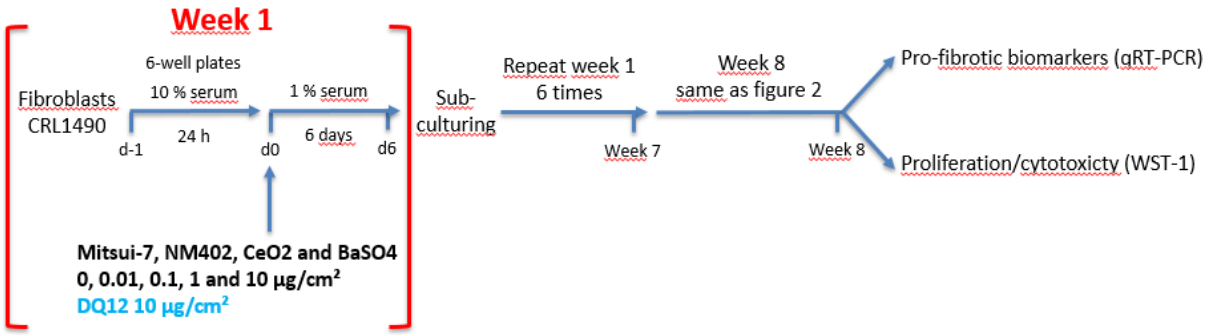

Figure 9: Overview of the repeated chronic (8 weeks) exposure protocol.

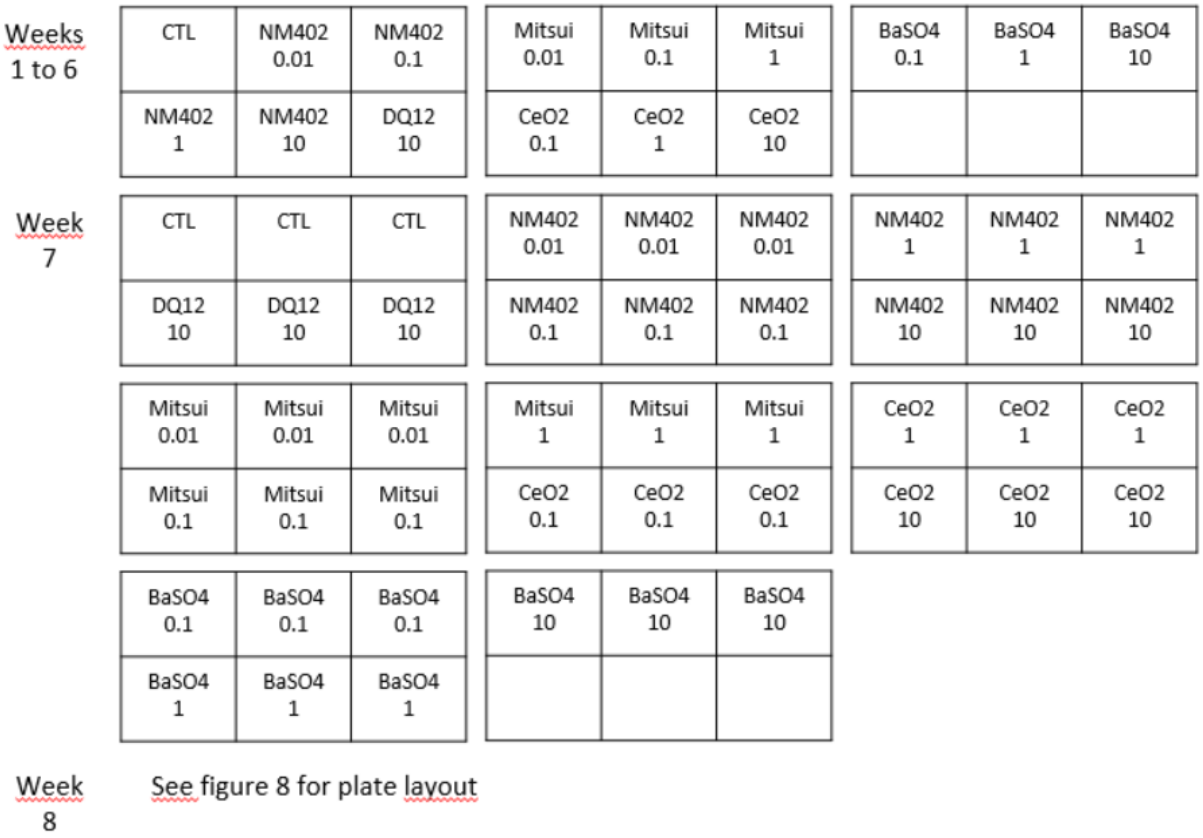

Figure 10: Example of 6-well plate layouts for the repeated 8-week exposure conditions.

After 24 h incubation at 37 °C in 6-well plates (d0, 1 well/condition),

1) Wash cells once with 1 ml PBS.

2) Add 5.076 ml exposure medium.

3) Add 564 µl dispersion medium ± NMs/DQ12 at a concentration 10 x higher than the final exposure concentration (see table 2). Gently mix by pipetting 3 times.

4) Incubate cells 6 d at 37°C under 5 % CO_2 (d6).

5) Wash cells with 1 ml PBS.

6) Add 350 µl trypsin 0.25 %-EDTA.

7) Incubate cells 5 min at 37 °C.

8) Neutralize trypsin by adding 1 ml complete cell culture medium.

9) Transfer suspensions to a 1.5 ml tube and centrifuge for 7 min at 400 *g*.

10) Suspend cell pellets in 200 µl fresh complete cell culture medium.

11) Determine cell number and viability with Trypan Blue Solution (20 µl cells + 80 µl Trypan Blue) in a Burker cell under a light microscope.

12) Seed cells at a concentration of 16000 viable cells/cm² in new 6 well-culture plates, i.e. 153600 cells/well in 5.076 ml complete medium (1 well/condition).

13) Incubate cells 24 h at 37°C under 5 % CO_2.

14) Repeat steps 1) to 13) 5 times (i.e. until week 7) except during the last week, divide each condition in 3 wells to increase cell number (see figure 10).

15) Repeat steps 1) to 13) except that triplicates are pooled, counted and seeded in 6 wells (same plate layout as for the 6-day exposure experiment).

15) Perfom steps 1) to 10) from point 5.8.2.2.

5.8.3 Assessing fibroblast proliferation

At the end of exposure,

1) Wash cells with 200 µl PBS.

2) Add 200 µl WST-1/medium to all culture wells and to 3 empty wells (blanks).

3) Incubate cells at 37°C during 1 to 2 h.

4) Measure absorbance at 450 nm (corrected at 690 nm) with a spectrophotometer.

5) When untreated cell absorbance reaches at least 0.5, centrifuge cell culture plates 5 min at 5000 *g*.

6) Transfer 150 µl supernatants to new 96-well plates (explained in section 5.8.5).

7) Measure absorbance at 450 nm (corrected at 690 nm) with a spectrophotometer.

5.8.4 Assessing the expression of pro-fibrotic biomarkers

This section describes the methods used to quantify the mRNA expression of pro-fibrotic biomarkers by qRT-PCR. Genes of interest are collagen I (collagen type I alpha 1 chain COL1A1), collagen III (collagen type III alpha 1 chain, COL3A1), α-smooth muscle actin (actin alpha 2, smooth muscle ACTA2 or α-SMA), tissue

metallopeptidase inhibitor 1 (TIMP1), matrix metallopeptidase 9 (MMP9) and secreted phosphoprotein 1/osteopontin (SPP1 or OPN). 18S rRNA is used as a housekeeping gene to normalize the expression of the genes of interest. Primers used for the PCR are listed in table 3. The protocol below describes a method for total RNA extraction, the reverse transcription (RT) of RNA in cDNA and the real time PCR using specific reagents and PCR machine. However, this part of the protocol can use reagents from other manufacturers and other real-time PCR machine without affecting the results.

1) Add RLT buffer to cell lysates to a final volume of 350 µl when necessary and extract total RNA according manufacturer (RNeasy mini kit). Use an elution volume of 40 µl per column.

2) Measure RNA concentration with a spectrophotometer or Nanodrop (measure absorbance at 260 nm, 1 $OD_{260\ nm}$ = 40 ng/µl).

3) Perform a RT with M-MLV with 350 pmol random hexamers in a final volume of 25 µL. Use either the maximum total RNA possible (for the single acute 24 h exposure) or 1 µg total RNA (for the single 6-day and the repeated 8-week exposure).

4) Dilute cDNA 10 times in autoclaved mQ H_2O.

5) Perform a real-time PCR for each of the genes listed in table 3 by using SYBR Green technology on a StepOnePlus Real-Time PCR System according to the following program: 10 min 95 °C, (15 s 95 °C, 1 min 60 °C) x40. Five µl of diluted cDNA or standards were amplified with 300 nM of the primers using Power SYBR Green PCR Master Mix in a total volume of 25 µl.

Table 3: list of primers used in this SOP.

Gene	Forward or reverse primer	Primer sequence	Amplicon length (bp)
Collagen type I alpha 1 chain (COL1A1)	Forward	GTGTGGCCCAGAAGAACTGGT	102
	Reverse	CGCCATACTCGAACTGGAATC	
Collagen type III alpha 1 chain (COL3A1)	Forward	GATGGAGAATCAGGTAGACCCG	124
	Reverse	GTCCATCGAAGCCTCTGTGTC	
actin alpha 2, smooth muscle (ACTA2, α-SMA)	Forward	GACGAAGCACAGAGCAAAAGAG	102
	Reverse	AGAGTGGTGCCAGATCTTTTCC	
Tissue metallopeptidase inhibitor 1 (TIMP1)	Forward	ATTCCGACCTCGTCATCAGG	109
	Reverse	TGGAACCCTTTATACATCTTGGTCA	
Matrix metallopeptidase 9 (MMP9)	Forward	CTTCCAGTACCGAGAGAAAGCCTA	105
	Reverse	TAGGTCACGTAGCCCACTTGGT	
Secreted phosphoprotein 1 (SPP1) or osteopontin (OPN)	Forward Reverse	CCCACAGTAGACACATATGATGGC TCTGTAGCATCAGGGTACTGGATG	107
18S rRNA	Forward	CGGCTACCACATCCAAGGAA	200
	Reverse	ATACGCTATTGGAGCTGGAATTACC	

5.8.5 Testing for nanomaterial interferences:

According to our expertise and literature, most of the interferences occurring between the WST-1 assay and NMs are due to interferences of the NMs with the absorbance wavelength used for the WST-1. This can easily be overcome by removing most of the NMs by washing cells before the assay and transferring the supernatants to new plates. However, the interferences can be more systematically assessed (i) by measuring dose-dependent NM absorbance at the assay wavelength in the exposure conditions, (ii) by incubating the WST-1 reagent with increasing concentrations of NMs, (iii) by incubating viable cells with increasing concentrations of NMs and the WST-1 reagent simultaneously, and (iv) by incubating formazan (the reduced form of the WST-1 reagent) with increasing concentrations of NMs. Washing cells from NMs still in suspension and transferring the supernatants is important before performing the WST-1 assay as it prevents most of the interference with the absorbance wavelength.

According to our knowledge, not much is known about the possible interferences between NMs and the methods used in this SOP to evaluate the expression of the pro-fibrotic mediators (RNA extraction and qRT-PCR). The interaction of NMs such

as CNT with nucleic acids is known (Nandy et al., 2012); however, it is not known whether this interferes significantly with the above processes and how this could be prevented.

5.9 Quality control & acceptance criteria:

Positive controls were included in the SOP:

- PDGF 30 ng/ml must significantly increase the fibroblast proliferation after 24 h exposure.

- TGF-β 10 ng/ml must significantly increase the expression of Collagen I and α-SMA RNA.

6 Data Analysis and Reporting of Data:

WST-1 assay

The analysis uses absorbance values measured on cell culture supernatants (see point 5.8.3). Data are expressed as a ratio to the control/untreated condition.

$Abs_{sample} = (Abs_{450nm} - Abs_{690nm})_{sample} - mean(Abs_{450nm} - Abs_{690nm})_{blank}$

$Ratio\ in\ cell\ activity = Abs_{treated} / mean\ Abs_{untreated}$

Expression of pro-fibrotic biomarkers

Data are analyzed by the StepOne software v2.3.

The results are expressed as a ratio of gene expression to the expression of the reference gene (18 S rRNA).

Statistical analysis

Statistical analyses are performed with GraphPad Prism (GraphPad Software, San Diego, USA) and statistical significance is considered at $p \leq 0.05$ (two-side). Results for positive controls (TGF-β 10 ng/ml and PDGF 30 ng/ml) and DQ12 are compared to control/untreated conditions by a t-test. Results for NMs are compared to control/untreated conditions via a one-way ANOVA, followed by a Dunnett's multiple comparison test and a trend test.

7 Publications:

Not applicable to this SOP.

8 References

NANDY, B., SANTOSH, M. & MAITI, P. K. 2012. Interaction of nucleic acids with carbon nanotubes and dendrimers. *J Biosci,* 37**,** 457-74.

VIETTI, G., IBOURAADATEN, S., PALMAI-PALLAG, M., YAKOUB, Y., BAILLY, C., FENOGLIO, I., MARBAIX, E., LISON, D. & VAN DEN BRULE, S. 2013. Towards predicting the lung fibrogenic activity of nanomaterials: experimental validation of an in vitro fibroblast proliferation assay. *Part Fibre. Toxicol,* 10**,** 52.

VIETTI, G., LISON, D. & VAN DEN BRULE, S. 2016. Mechanisms of lung fibrosis induced by carbon nanotubes: towards an Adverse Outcome Pathway (AOP). *Part Fibre. Toxicol,* 13**,** 11.