Project:

Preparation of blood and PBMC for cytokine secretion

Analysis of nanoparticles potential to induce inflammatory cytokines, chemokines and interferons in vitro

AUTHORED BY:	DATE:
Neill Liptrott	22.02.2016

REVIEWED BY:	DATE:
Matthias Roesslein	24.02.2016
Matthias Roesslein	30.03.2017

APPROVED BY:	DATE:
Matthias Roesslein	24.02.2016
Matthias Roesslein	30.03.2017

DOCUMENT HISTORY

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

Version	Approval Date	Description of the Change		Author / Changed by
1.0	22.02.2016	All	Initial Document	Neill Liptrott

Version	Approval Date	Description of the Change		Author / Changed by
1.3	30.03.2017	All	Minor additions and corrections	Neill Liptrott

Table of Content

1 Introduction 4
2 Principle of the Method 4
3 Applicability and Limitations (Scope) 5
4 Related Documents 5
5 Equipment and Reagents 5
5.1 Reagents for whole blood cultures 5
5.2 Reagents for PBMC cultures 6
5.3 Controls 6
5.4 Equipment and materials 7
6 Procedure 8
6.1 Collection and handling of whole blood for culture 8
6.2 Preparation of PBMC 8
6.3 Preparation of Nanoparticles 9
6.4 Reagent and control preparation 10
6.5 Experimental procedure for whole blood culture 11
6.6 Experimental procedure for PBMC culture 13
6.7 Flow chart 14
7 Quality Control, Quality Assurance, Acceptance Criteria 14
8 Health and Safety Warnings, Cautions and Waste Treatment 14
9 Abbreviations 15
10 References 15
11 Annex – example plate layout 16

1 Introduction

Cytokine storm is a condition characterized by high plasma levels of inflammatory cytokines, chemokines and interferons which can be commonly induced by pathogens or their components (endotoxin, lipoproteins, DNA, RNA etc.). Cytokine storm can also be induced in response to certain drugs (e.g. recombinant proteins, therapeutic antibodies, macromolecular nucleic acid based therapeutics). It is accompanied by fever, hypo- or hypertension and may progress to a more severe life-threatening condition called systemic inflammatory response syndrome (SIRS). For example, cytokine storm was severe side effect in phase I clinical trial of experimental monoclonal antibody therapeutics TGN1412, which resulted in 6 healthy donor volunteers enrolled into the study becoming critically ill and requiring intense care (1). All patients had high serum levels of TNFα, IFNγ and other pro-inflammatory messengers (1). Cytokine storm to this drug was not observed in preclinical studies involving rats and cynomolgus monkeys (1), but was easily detectable in vitro using cytokine release assay in human primary blood cells (2). Nanoparticles can be used for delivery of therapeutic proteins, antibodies and nucleic acids, or contain biologicals (antibodies, proteins or nucleic acids) as targeting agents. In addition, some nanoparticles can be made of biological molecules (e.g. self-assembling peptides or siRNAs). These warrants studying both nanotechnology platforms and their macromolecular payload and targeting agents for the ability to induce inflammatory cytokines. Human whole blood and peripheral blood mononuclear cells (PBMC) are considered reliable and predictive model for this purpose. The data obtained from such in vitro studies is intended to supplement other preclinical data to create nanoparticle safety profile and ensure transition of nanomedicines toward clinical development.

2 Principle of the Method

The purpose of the protocol described herein is to use whole blood or peripheral blood mononuclear cells derived from healthy donor volunteers and culture these specimens in the presence of controls and nanoparticles in order to identify nanoparticle potential to induce cytokine storm. The culture supernatants prepared according to this protocol can be analyzed by commercial assays specific to human cytokines, chemokines and interferons, EU-NCL uses commercial PBL ELISA kits to test for the presence of type I interferons as well as multiplex kits. There is no harmonized approach to what type of assay to use as well as to the choice between singleplex and multiplex analysis. One should rely on the scientific judgement and the critical path of the project focusing on the certain type of nanoparticles to determine the type of the cytokines and method for analysis of supernatants. It takes 24 hours to culture whole blood or PBMC to collect supernatants, and additional 5-6 hours to complete ELISA or multiplex. If ELISA or multiplex analysis cannot be conducted immediately after incubation of

whole blood or PBMC with nanoparticles, the culture supernatants can be frozen at -20 °C. Different cytokines have different stability at room temperature (RT) and upon repeated freeze/thaw (FT) cycles. Please refer to individual manufacturer's instruction of commercial kits for the information about RT and FT stabilities. When such information is not available as in the case of some commercial kits, analyze supernatants immediately and prepare multiple aliquots for repeat analysis in order to avoid multiple FT cycles.

3 Applicability and Limitations (Scope)

This assay is aimed at the determination of the potential for nanoparticle preparations to induce cytokines and chemokines from primary human immune cells. When utilising PBMC preparation one must near in mind that granulocytes will not be represented within the cell population. In order to include these cells within the analysis then whole blood must be used. The analysis of the most generaly proinflammatory cytokines is conducted here however one must consider that not all cytokines/chemokines are represented and it may be possible for nanoparticles to induce anti-inflammatory cytokines depending on their mode of interactions. Careful consideration must be given to the required output and how it relates to the determination of compatibility of the tested nanoparticles.

4 Related Documents

Table 1:

Document ID	Document Title
EUNCL_ITA_030	*Determination of cytokine concentrations*

5 Equipment and Reagents

5.1 Reagents for whole blood cultures

5.1.1 Human blood anti-coagulated with Li-heparin and obtained from at least 3 healthy donors

5.1.2 PBS, GE Life Sciences, SH30256.01

5.1.3 RPMI-1640, Invitrogen, cat#11835-055

5.1.4 Fetal bovine serum, GE Life Sciences, HyClone, cat# SH30070.03

5.1.5 Penicillin streptomycin solution, Invitrogen, cat#15140-148

5.1.6 L-glutamin, GE Life Sciences, SH30034.01

5.2 Reagents for PBMC cultures

In addition to all materials listed in section 5.1 you will also need the following reagents:

5.2.1 Ficoll Paque Premium, GE Healthcare, cat#17-5442-02 and

5.2.2 Hank's balanced salt solution (HBSS) (Invitrogen, 24020-117)

Please refer to the table below for guidance in selecting whole blood or PBMC

Culture	**Whole Blood**	**PBMC**
Primary Purpose	Inflammatory cytokines and type II interferon	Type I interferons
Nanoparticles	Any payload except for nucleic acid based API or targeting	Payload or targeting composed of therapeutic nucleic acid (siRNA, ASN, aptamer)

Note: pro-inflammatory cytokines and type II interferons can also be detected in PBMC cultures. In addition to the cells present in PBMC, type II interferon is also produced by neutrophils, which are present only in whole blood culture, therefore the latter is the preferable model to study type II interferon.

5.3 Controls

5.3.1. Ultrapure LPS from K12 *E.coli*, Invivogen, cat#tlrl-peklps

5.3.2. ODN2216 is CpG DNA oligonucleotide with mixed backbone and the following sequence 5'-ggGGGACGATCGTCGggggG-3, where lowercase letters show phosphorothioate linkage and capital letters refer to phosphodiester linkage between nucleotides; we order custom synthesis from IDT, equivalent supplier can be used.

5.3.3. Phytohemagglutinin (PHA-M), Sigma cat# L8902

Refer to the table below for guidance on concentrations and purpose of these controls

Description\Control	LPS	ODN2216	PHA-M
Primary Purpose	Positive control for Inflammatory Cytokines (TNFα, IL1β, IL-6, IL-8, IL-10, IL-12)	Positive control for Type I interferons IFNα and IFNβ	Positive Control for Type II interferon IFNγ
Final concentration in assay	20ng/mL	5μg/mL	10μg/mL

Note: other agents can be used as the assay positive control. Apply your scientific judgement for selecting other controls.

5.4 Equipment and materials

5.4.1. Pipettes covering a range of 0.05 to 10 mL

5.4.2. 96-well round bottom plates

5.4.3. Polypropylene tubes, 50 and 15 mL

5.4.4. Microcentrifuge tubes

5.4.5. Centrifuge

5.4.6. Refrigerator, 2-8 ºC

5.4.7. Freezer, -20 ºC

5.4.8. Cell culture incubator with 5% CO2 and 95% humidity.

5.4.9. Biohazard safety cabinet approved for level II handling of biological material

5.4.10. Inverted microscope

5.4.11. Vortex

5.4.12. Hemocytometer

6 Procedure

6.1 Collection and handling of whole blood for culture

Collect whole blood from healthy donor volunteers who have been not on medication and clear from infection for at least 2 weeks prior to blood donation. Use Li-heparin tubes and discard first 10 cc. For the best results, whole blood should be used within 1 hour after collection. Prolonged storage (> 2h) of whole blood will lead to decrease in cell function.

6.2 Preparation of PBMC

6.2.1. - Place freshly drawn blood into 15 or 50 mL conical centrifuge tubes,

- add an equal volume of room-temperature PBS and

- mix well.

6.2.2 - Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by

- placing the tip of the pipet containing Ficoll-Paque at the bottom of the blood sample tube.
- Alternatively, the blood/PBS mixture may be slowly layered over the Ficoll-Paque solution.
- Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture. For example, 15 mL Ficoll-Paque per 20 mL of diluted blood in a 50 mL tube

Note: To maintain Ficoll-blood interface it is helpful to hold the tube at a 45˚ angle.

6.2.3 Centrifuge 30 min at 900 x g, 18-20˚C, without brake.

Note: depending on the type of centrifuge one also may need to set up acceleration speed to minimum

6.2.4 Using a sterile pipet, remove the upper layer containing plasma and platelets and discard it.

6.2.5 Using a fresh sterile pipet, transfer the mononuclear cell layer into another centrifuge tube.

6.2.6 Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400 x g, 18-20˚C.

- The HBSS volume should be ~3 times the volume of mononuclear layer.

Note: Usually 4 mL of blood/PBS mixture results in ~ 2 mL of mononuclear layer and

requires at least 6 mL of HBSS for the wash step. We use 10 mL of HBSS per each

2 mL of cells.

6.2.7 Discard supernatant and repeat wash step one more time.

6.2.8 Re-suspend cells in complete RPMI-1640 medium.

- Dilute cells 1:5 or 1:10 with trypan blue,
- count cells and determine viability using trypan blue exclusion.
- If viability is at least 90%, proceed to step 6.6.1.

6.3 Preparation of Nanoparticles

When experiment is done in 24 well plates, the assay requires 5 mL of nanoparticles dissolved/re-suspended in complete culture medium at concentration 5 times higher than the highest final test concentration. The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration were reviewed elsewhere (3) and are summarized in Box 1 below.

Box 1. Example Calculation of Nanoparticle concentration for In Vitro Test

In this example, we assume the mouse dose is known to be 123 mg/kg.

$$human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\frac{mg}{kg}}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of body weight, (e.g. a 70 kg human has approximately 5.6 L (8% of 70) of blood). This allows us to get a very rough estimation of what the maximum blood concentration may be.

$$in\ vitro\ concentration_{human\ maxtrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg\ \times 10\frac{mg}{kg}}{5.6\ L}$$

$$= \frac{700\ mg}{5.6\ L} = 0.125\ mg/mL$$

The assay will evaluate 4 concentrations: 10 X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration and two 1: 5 serial dilutions of the theoretical

plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 10 mg/mL will be prepared and diluted 10-fold (1 mg/mL), followed by two 1: 5 serial dilutions (0.2 and 0.04 mg/mL). When 200 µL of each of these samples are combined in a culture plate well with 800 µL of cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2mg/mL . Each nanoparticle concentration is plated 3 times. Additional 600 µL is required for cell free control. When cell free control is prepared for the whole blood plate, an aliquot of the blood diluted in PBS from step 6.5.1 is spun down for 10 minutes at 2,500 x g and 800 µL of this cell free supernatant is combined with 200 µL of test nanoparticles.

6.4 Reagent and control preparation

6.4.1 Complete RPMI-1640 medium

The complete RPMI medium should contain the following reagents:

- 10% FBS (heat inactivated)
- 2 mM L-glutamine
- 100U/mL penicillin
- 100 µg/ml streptomycin

Store at 2-8 °C protected from light for no longer than 1 month. Before use warm the media in a water bath.

6.4.2 Lipopolysaccharide (LPS, 1mg/mL stock)

- Add 1 mL of sterile water to 1 mg of LPS to the vial and vortex to mix.
- Aliquot 20 µL and
- store at a nominal temperature of -20 °C.
- Avoid repeated freeze- thaw cycles.
- On the day of experiment thaw one aliquot and use such as its final concentration in PBMC or WB culture is 20ng/mL.

6.4.3 Phytohemagglutinin (PHA-M, 1mg/mL stock)

- Add 1 mL of sterile PBS or cell culture medium per 1 mg of PHA-M to the vial and
- gently rotate to mix. Store daily use aliquots at a nominal temperature of -20˚C.

- Avoid repeated freezing/thawing. On the day of experiment dilute stock PHA-M solution in cell culture medium so that its final concentration in the positive control sample is 10μg/mL

6.4.4 ODN 2216 (1mg/mL stock)

- This oligonucleotide is supplied as lyophilized powder.
- Reconstitute in pyrogen free, nuclease free water to a final concentration of 1mg/mL.
- Prepare single use 5 μL aliquots and store at -20°C. On the day of experiment thaw an aliquot at room temperature and
- dilute in culture media so that final concentration in the test sample is 5 μg/mL

6.4.5 Negative Control

- Use PBS as a negative control.
- Process this control the same way as your test samples

6.4.6 Heat inactivated fetal bovine serum

Thaw a 50mL aliquot of fetal bovine serum and equilibrate to room temperature. Place the tube in a water bath set up to 56 C and incubate with mixing for 35 min. The heat inactivation takes 30 min and the initial 5 min is used to bring the entire content of the vial to 56C. Chill the serum and use to prepare complete culture media.

6.4.7 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

6.5 Experimental procedure for whole blood culture

6.5.1. Dilute whole blood 4 times with complete culture media (e.g 3 mL of whole blood and 9 mL of complete culture media).

6.5.2 Dispense 800µL of diluted blood from step 8.1. per well in 24 well plate. Refer to section 10 for plate layout.

Note: if positive control supernatants are used to prepare inhibition enhancement controls, do not forget to add extra replicates of the positive control sample

6.5.3 Dispense 200 µL of blank media (baseline), negative control, positive control, vehicle control and test samples into corresponding wells on 24 well plate containing 800µL of diluted blood from step 8.1. Prepare triplicate wells for each sample. Prepare cell free control by dispending 200 µL of nanoparticles into 800 µL of cell free supernatant prepared by spinning an aliquot of whole blood from step 8.1 for 10 min at 2500 x g. Gently shake plates to allow all components to mix

Note: The cell free sample will be processed the same way as the whole blood samples and will serve as control for false-positive results. To test for potential false-negative result supernatant from positive control can be spiked with nanoparticle at a final nanoparticle concentration identical to that in test sample. Alternatively, a cell free supernatant containing nanoparticles can be spiked with cytokine standard in individual ELISA assay and analyzed against relevant quality control. If nanoparticle inhibits detection of cytokine a decrease in the cytokine level will be seen when compared to the level of cytokine in positive control or in quality control samples. Additionally, if one wants to understand whether nanoparticle may potentiate or inhibit cellular response to the assay positive control (LPS, PHA-M or ODN2216), the positive control should be co-cultured with nanoparticles in the presence of cells.

6.5.4 Repeat steps 6.5.1 to 6.5.3 for cells obtained from each individual donor. There is no limit to the number of donors used in this test. It is advised to test each nanoparticle formulation using blood derived from at least three donors.

6.5.5 Incubate 24 hours in a humidified 37C, 5% CO_2 incubator.

6.5.6 Collect cultured blood into 1.5mL centrifuge tubes and spin in a microcentrifuge at a 18,000 x g for 5 minutes.

6.5.7 Transfer supernatants into fresh tubes and either proceed with ELISA analysis or aliquot and store at -20 °C.

Note: To avoid multiple freeze/thaw cycles, it is better to prepare multiple (200 or 300 µL) aliquots for each supernatant. Refer to the manufacturer's instruction for any freeze/thaw limits relevant to the kits used.

6.6 Experimental procedure for PBMC culture

6.6.1. Adjust PBMC concentration to 1.3 x 10^6 viable cells/mL using complete RPMI medium.

6.6.2 Dispense 200 µL of blank media (baseline), negative control, vehicle control, positive control and test samples into corresponding wells on 24 well plate. Refer to section 10 for plate layout.

Note: if positive control supernatants are used to prepare inhibition enhancement controls, do not forget to add extra replicates of the positive control sample

6.6.3 Dispense 800µL of PBMC from step 9.1 per well in 24 well plate containing 200µL of nanoparticles or complete culture medium to wells intended for cell free control. Refer to section 10 for plate layout. Gently shake plates to allow all components to mix.

Note: for each nanoparticle concentration prepare cell free control by plating 800 µL of complete culture medium and 200µL of nanoparticle sample. The resulting sample will be processed the same way as PBMC samples and will serve as control for false-positive results. To test for potential false-negative result supernatant from positive control can be spiked with nanoparticle at the final nanoparticle concentration identical to that in the test sample. Alternatively, cell-free control supernatants can be spiked with relevant cytokine standard used in ELISA or multiplex. If nanoparticle inhibits detection of cytokine a decrease in the cytokine level will be seen when compared to the level of cytokine in positive control or quality control, respectively. Additionally, if one wants to understand whether nanoparticle may potentiate or inhibit cellular response to the assay positive control (LPS, PHA-M or ODN2216), the positive control should be co-cultured with nanoparticles in the presence of cells.

6.6.4 Repeat steps 6.6.1-6.6.3 for cells obtained from each individual donor. There is no limit to the number of donors used in this test. It is advised to test each nanoparticle formulation using blood derived from at least three donors.

6.6.5 Incubate 24 hours in a humidified 37C, 5% CO2 incubator.

6.6.6 Collect cultured blood into 1.5mL centrifuge tubes and spin in a microcentrifuge at a maximum speed for 5 minutes. Transfer supernatants into fresh tubes and either proceed with cytokine analysis or aliquot and store at -20 °C.

Note: To avoid multiple freeze/thaw cycles, it is better to prepare multiple (200 or 300 µL) aliquots for each supernatant. Refer to the commercial plates manufacturer's instruction for any freeze/thaw limits relevant to the kits used.

6.7 Flow chart

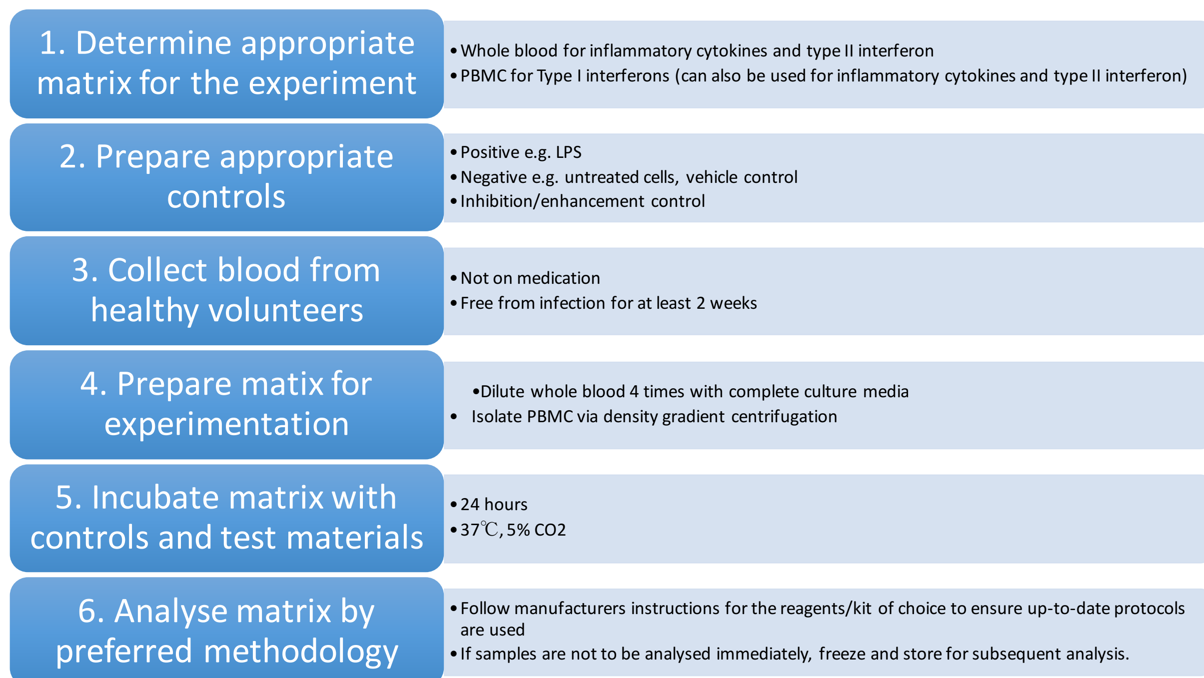

Figure 1: **Brief outline of the workflow.**

7 Quality Control, Quality Assurance, Acceptance Criteria

7.1 %CV and PDFT for each calibration standard and quality control should be within 20%.

7.2 % CV for each test sample including supernatants from whole blood cultures treated with positive control, negative control and nanoparticle sample should be within 20 %. At least one replicate of positive and negative control should be acceptable for run to be accepted.

7.2 If both replicates of positive control or negative control fail to meet acceptance criterion described in 8.3 the run should be repeated.

7.4 Within the acceptable run if two of three replicates of unknown sample fail to meet acceptance criterion described in 7.3 this unknown sample should be re-analyzed.

8 Health and Safety Warnings, Cautions and Waste Treatment

Universal precautions must be used when handling human peripheral blood.

- No sharps are to be used in the preparation of cell isolates of plasma from human healthy volunteer blood.
- All liquid waste must be discarded into 1% Virkon solution and left overnight (18 hours) in a class II BSC with the lid loose to allow for venting.

9 Abbreviations

FBS	fetal bovine serum
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute
VC	vehicle control
PBMC	peripheral blood mononuclear cells
FT-	freeze/thaw
IL	interleukin
LPS	lipopolysaccharide
ODN	oligodeoxyribonucleotide
PHA-M	phytohemagglutinin
TNF	tumor necrosis factor
IFN	interferon

10 References

1. Suntharalingam GS et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. New England Journal of Medicine2006, 355: 1018-1028

2. Stebbings R *et.al.* Cytokine storm in the phase I trial of the monoclonal antibody TGN1412: better understanding the cause to improve the preclinical testing of immunotheraputics. J Immunology, 2007, 179: 3325-3331

3. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release. 2013 Dec10;172(2):456-66

11 Annex – example plate layout

Example of 24 well plate Template for culturing Whole Blood or PBMC.

	1	2	3	4	5	6
A	NC (PBS)	PC (LPS 20 ng/ml)	TS 1	TS2	VC 1	VC 2
B	NC (PBS)	PC (LPS 20 ng/ml)	TS 1	TS2	VC 1	VC 2
C	NC (PBS)	PC (LPS 20 ng/ml)	TS 1	TS2	VC 1	VC 2
D	TS 1 Cell free	TS2 Cell free	TS 1 Cell free	TS2 Cell free	VC 1 Cell free	VC 2 Cell free

Row D does not have cells; NC- negative control, PC – positive control; TS 1 and 2 – test nanoparticle at concentrations 1 and 2, respectively; VC1 and 2 – vehicle control at concentrations 1 and 2, respectively