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Standard Operating Procedure (SOP)

Title Viability assessment of cell monolayers with MTT reduction assay in 96-well plates			**Date** 2017-01-05
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Version	**valid from**	**Description of changes**
1	2014-07-03	Creation
2	2014-12-01	Adjustment of serum concentration
3	2015-10-15	Supplementary characterizations of nanocarriers; Adjustment of positive controls
4	2017-01-05	Translation

Issued by	**Reviewed by**	**Approved by**
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Scope
Workgroups of the CRC112-Z01 and BB3R projects

Directory

AIMS

Standardized viability assessment of cell monolayers with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay for the cytotoxic potential of test nanocarriers.

SCOPE

This SOP applies to the workgroups of CRC112 and BB3R projects.

INTRODUCTION

The MTT assay is applied to assess the viability of cell monolayers after treated with test nanocarriers. The method is based on the colorimetric change of the yellow tetrazolium dye into the purple formazan by oxidoreductase enzymes in viable cells.

For the study of nanocarriers, the MTT assay is performed on normal human keratinocytes (NHK) and normal human dermal fibroblasts (NHDF) isolated from foreskins. The primary cells are pooled after isolation (3 donors per cell type).

The physicochemical characterization of the nanocarriers is provided in data sheets, see Annex 1, by project partners, such as Prof. Bodmeier, Prof. Haag, Prof. Calderon and Prof. Lendlein, etc.

MATERIALS

Equipment

Designation	Manufacturer
Autoclave	Systec, Wettenberg
Cell counting chamber	Zeiss, Jena
Centrifuge (Eppendorf)	Eppendorf, Hamburg
Centrifuge (Megafuge® 1.0R)	Thermo Fisher Scientific, Waltham, MA, USA
Fluorescence microscope (BZ-8000K)	Keyence, Osaka, JAP
Fluostar Optima	BMG Labtech, Offenburg
Freezer (-20°C)	Siemens, München
Freezer (-80°C)	Thermo Fisher Scientific, Waltham, MA, USA
Incubator (BB6220)	Thermo Fisher Scientific, Waltham, MA, USA
Magnetic stirrer RCT basic	IKA-Werke, Staufen
Microtome (Hyrax M40)	Zeiss, Jena
Nitrogen tank (Arpege 70)	Air Liquide, Paris, F
Phase contrast inverted microscope (Axiovert 40C)	Zeiss, Jena

pH meter (766 Calimatic)	Knick, Nürnberg
Pipette (Eppendorf Reference®)	Eppendorf, Hamburg
Pipetting aid (Easypet®)	Eppendorf, Hamburg
Refrigerator (4°C)	Siemens, München
Shaker IKA® MTS 2	IKA, Staufen
Sterile working bench (LaminAir®)	Thermo Fisher Scientific, Waltham, MA, USA
Suction pump	VWR, Darmstadt
Vortex	Bender & Hobei, Zurich, CH
Water bath	Gesellschaft für Labortechnik, Burgwedel
Water processing unit (SG LaboStar)	SG Wasseraufberitung und Regenerierstation, Barsbüttel

Equivalent equipment of other suppliers can be used as well.

Cells

Designation	Source
NHDF, passage 3, pooled of 3 donors	Isolation from juvenile preputium [1])
NHK, passage 3, pooled of 3 donors	Isolation from juvenile preputium [1])

Abbreviations

Designation	Supplier
5-FU	5-fluorouracil
BPE	Bovine pituitary extract
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogenphosphate
MTT	3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
OD	Optical density
PBS	Phosphate buffered saline solution (pH 7.4)
SDS	Sodium dodecyl sulfate

[1]) according to SOP “Isolation of keratinocytes and fibroblasts from human specimens”

Consumables

Designation	Supplier
96-well plate (flat bottom)	TPP, Trasadingen, Switzerland
Cell culture flask (75 cm^2)	TPP, Trasadingen, Switzerland
Cell culture flask (150 cm^2)	TPP, Trasadingen, Switzerland
Centrifuge tube (15 mL)	Sarstedt, Nümbrecht
Centrifuge tube (50 mL)	Sarstedt, Nümbrecht
DMEM	Sigma-Aldrich, München
DMSO	Sigma-Aldrich, München
EDTA	Sigma-Aldrich, München
FBS Superior	Biochrom, Berlin
H_2O (pyrogen free)	Carl Roth, Karlsruhe
KCl	Sigma-Aldrich, München
KH_2PO_4	Carl Roth, Karlsruhe
KGM Bulletkit	Lonza, Köln
L-Glutamin	Biochrom, Berlin
MTT	Sigma-Aldrich, München
Na_2HPO_4	Carl Roth, Karlsruhe
NaCl	Carl Roth, Karlsruhe
Keratinocytes Growth Medium (KGM)	Lonza, Köln
L-Glutamine	Sigma-Aldrich, München
Penicillin-Streptomycin-solution	Biochrom, Berlin
Pipette tips	Eppendorf, Hamburg
Trypan blue	Biochrom, Berlin
Trypsin dry substance	Biochrom, Berlin

Equivalent consumables of other suppliers can be used as well.

Cell culture medium

Description	Ingredients			Remarks
FBM	DMEM	500	mL	
	L-Glutamine	5	mL	
	Penicillin/Streptomycin	5	mL	4°C, 6 weeks
FGM	FBM	510	mL	
	Fetal Calf Serum	37.5	mL	4°C, 6 weeks
KGM	KBM	500	mL	
	KGM supplements			4°C, 6 weeks

For NHDF, FGM is applied as cell culture medium, and FBM as nanocarrier testing medium.
For NHK, KGM is applied as both cell culture and nanocarrier testing medium.

General information

Unless stated otherwise, all procedures should be performed under sterile laminar flow conditions, cells are incubated in the incubator at 37°C, 5 % CO_2 and 95 % humidity, and all the mediums are pre warmed in water bath at 37°C for 30 min.

Colored nanocarriers

For assessment of colored nanocarriers, before undergoing the procedure, first mix the testing nanocarrier at 0.05% (w/v) with freshly prepared MTT solution. If the solution turns blue or purple, the nanocarriers are supposed to reduce MTT directly.
And to further study the interference of the colorant with the MTT reading, an additional color control should be performed. When performing the standard absorbance measurement, each interfering nanocarrier is applied in triplicates per exposure time, which undergoes the same procedures but is incubated with medium instead of MTT solution during the MTT incubation step to generate a non-specific color control. The final viability of the test nanocarriers is then calculated by subtracting the value of the color control.
When the MTT reading does not meet the accepting criteria, an alternative method, e.g. Neutral Red Uptake assay (OECD Test Guideline 432), could be performed for the aim of cytotoxicity evaluation.

Solutions

PBS and Trypsin-EDTA solutions

PBS and Trypsin-EDTA solutions are prepared according to the SOP as on page 5, autoclaved, and stored at 4°C.

Positive Control SDS solutions

Dilute 5 mg of SDS with 10 mL of distilled H_2O into a 0.05% (w/v) stock solution, and a 1:10 dilution of the stock solution and respective testing medium is applied as positive control.

Solvent Control solutions

Dilute pyrogen free H_2O or PBS, depending on the solvent used in the nanocarrier respectively, in 1:10 with respective testing medium. The solution is applied as solvent control,.

MTT solutions

Dilute MTT powder with PBS into a 5 mg/mL solution, aliquot and store at -20°C as stock solution. Dilute MTT stock solution 1:10 with respective testing medium.

Testing nanocarriers solutions

Dilute the nanocarrier solution with pyrogen free H_2O or PBS, depending on the solvent used in the nanocarrier respectively, into 0.5% and 0.05% (w/v) solutions. Dilute these nanocarriers 1:10 with the respective testing medium to a final concentrations of 0.05% and 0.005% (w/v).

STEP-TO-STEP PROTOCOL

Seeding cells | Day 0

- 1.5 mL of Trypsin-EDTA is added into the cell culture flask 75 cm^2 and put into the incubator for 3 min.
- 3.5 mL of KGM or FGM is added into the flask, and the cell suspension is transferred into a 50 mL centrifuge tube. The cell culture bottom is then washed twice with 5 mL PBS each, and all washed cell suspensions are transferred to the same tube
- Centrifugate at 1000 rpm for 5 min. discard the supernatant and resuspend the cells with 10 mL of PBS, then count the cells according to the SOP on page 5 and repeat the centrifugation.
- Adjust the cell suspension to 1 × 10^5 cells / mL with relative cell culture medium.
- Pipet 100 µL of the cell suspension into each inner wells of the 96-well plate (see scheme day 0), which corresponds to 10,000 cells / well. And pipet 100 µL of the relative cell culture medium, see Page 7, into the outer wells. The plates are then placed into the incubator for 24 h.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**
B	**B**	CS	CS	CS	CS	CS	CS	CS	CS	CS	CS	**B**
C	**B**	CS	CS	CS	CS	CS	CS	CS	CS	CS	CS	**B**
D	**B**	CS	CS	CS	CS	CS	CS	CS	CS	CS	CS	**B**
E	**B**	CS	CS	CS	CS	CS	CS	CS	CS	CS	CS	**B**
F	**B**	CS	CS	CS	CS	CS	CS	CS	CS	CS	CS	**B**
G	**B**	CS	CS	CS	CS	CS	CS	CS	CS	CS	CS	**B**
H	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**

Scheme day 0: cell seeding

B: Blank (Cell culture medium); CS: cell suspension (1 x 10^5 cells / ml)

Nanocarrier Stimulation | Day 1

- Prepare all the solutions as described in the solutions section.
- Check the cell morphology under the microscope.
- Aspirate all the medium from the plate with the suction pump and pipet tips (yellow) in a gentle manner.

- 100 µL of the corresponding medium or solutions are then pipetted into each well (see scheme day 1).
- The plates are then placed into the incubator for 24 h or 48 h.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**
B	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
C	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
D	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
E	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
F	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
G	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
H	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**

Scheme day 1

B: Blank (only testing medium without cells); UC: Untreated Control (only testing medium with cells); SC: Solvent Control; PC: Positive Control; C1-6: Testing nanocarrier solutions (a: 0.05%, b: 0.005%)

MTT Evaluation | Day 02 or 3

- Prepare the MTT solution as described in the solutions section.
- Check the cell morphology under the microscope.
- Aspirate all the medium from the plate with the suction pump and pipet tips (yellow) in a gentle manner, and wash each well once with 100 µL of PBS.
- Add 100 µL of the MTT solution into each well, then place the plates into the incubator for 4 h.
- Remove the MTT solution by gentle suction and place the plates over a sterilized paper towel for 1 min.
- Add 50 µL of DMSO into each well, and put the plate onto the plate shaker at 500 rpm for 10 min.
- Measure the absorption at the wavelength of 540 nm, name all the relative excel and prism files with the same experiment ID, and calculate the viability.

Photometer – Setting

Device	FLUOstar OPTIMA (or equivalent device)
Programme	MTT Absorbance, TPP96
Mode	Disk Mode
Positioning Delay	0.7 s
No. Kinetic Windows	1

Excitation Filter	A540
Emission Filter	Empty
No. Multichromatics	1
Shaking width	1 mm; 600 rpm
No. Cycles	1
No. Flashes per well and cycle	2

A comparable device with adequate adjustment can also be used.

Data Analysis

- The blank value is subtracted from all measured values to gain a corrected OD value.
- From each plate, the mean corrected value for the solvent control is set equal to 100%. The viability rates of the test nanocarriers are calculated as follows:

$$\text{Viability (\%)} = \frac{\text{Corrected Testing Nanocarrier Value}}{\text{Corrected Sovent Control}} \times 100\%$$

- An Excel and GraphPad Prism 5 spreadsheet is provided which is used for the calculation of the results. For use details of the spreadsheet see Annex 2.

Accepting Criteria

The results are acceptable if:

- The corrected OD values of the untreated controls are within the range:

Cell Type	Corrected OD Value
NHK	0.15–1.0
NHDF	0.3–1.0

- The difference of viability among triplicates is is < 20% in the same run.
- The values of the viability from column 2 and 11 (untreated controls) show a deviation of ≤ 15%.
- The viability of positive controls is below 15 %.

Annex 1

Sample Data Sheet **Working Group**_____ **Project Name** _____

Table 1 General Information

Sample Name	**Date of Delivery**
Technician or PhD Name	**Tel. No.**
Lab Journal No.	**Batch No.**

Chemical Structure, Formulation/Solvent, Guest, Marker:

Table 2 Delivered Amount, Concentration and Solubility

Amount (mg or mL)
C(carrier)
Dissolved in
Sterilization
Storage Recommendation
History of Sample

Table 3 Characterization (optional)

IR	**DLS**
NMR	**REM**
UV	**TEM**

Annex 2

Table 1 (Excel) raw data OD

Experiment ID:

	1	2	3	4	5	6	7	8	9	10	11	12
A	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**
B	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
C	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
D	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
E	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
F	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
G	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
H	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**

Table 2 (Excel) corrected OD (raw data OD – blank OD)

Experiment ID:

	1	2	3	4	5	6	7	8	9	10	11	12
A	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**
B	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
C	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
D	**B**	UC	SC	PC	C1a	C2a	C3a	C4a	C5a	C6a	UC	**B**
E	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
F	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
G	**B**	UC	SC	PC	C1b	C2b	C3b	C4b	C5b	C6b	UC	**B**
H	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**
MEAN OD	**B**	**UC**	**SC**	**PC**	**C1a**	**C2a**	**C3a**	**C4a**	**C5a**	**C6a**	**UC**	**B**
	B (1+12)	**UC (2+11)**			**C1b**	**C2b**	**C3b**	**C4b**	**C5b**	**C6b**		**B**

Table 3 (Excel) viability (%)

Sample Name	Corrected OD	Viability (%)
SC		**100**
PC		
C1a		
C1b		
C2a		
C2b		
C3a		
C3b		
C4a		
C4b		
C5a		
C5b		
C6a		
C6b		

Table 4 (Prism)

Graph Collum, Plot Mean ± SEM

Experiment ID:

	C1b	C1a	C2b	C2a	C3b	C3a	C4b	C4a	C5b	C5a	C6b	C6a	PC	SC
	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
1														
2														
3