STUDY REPORT

DETERMINATION OF THE IN VITRO CYTOTOXICITY OF AEROSOL GENERATED FROM THE TEST ITEM, HEATED TOBACCO PRODUCT HNB TOBACCO - REGULAR, AND THE REFERENCE ITEM, 3R4F, IN THE NEUTRAL RED UPTAKE ASSAY

REPORT NUMBER: JC-CPC240031-3Z1

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1. Approval and Verification

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2. List of Abbreviations

FBS	Fetal bovine serum
SDS	Sodium dodecyl sulphate
DMSO	Dimethylsulphoxide
CMF-PBS	Phosphate-buffered saline without calcium chloride and magnesium chloride
CHO cells	Chinese hamster ovary cells
PP	Particulate phase of the smoke
GVP	Gas-vapour phase of the smoke
TPM	Total particulate matter of the smoke
DMEM	Dulbecco's modification of Eagle's medium

3. General Information

3.1 Applicant and Test Item

Applicant Name	LONO International Co., Limited
Applicant Add.	FLAT 1512, 15/F, LUCKY CENTRE, NO.165-171 WAN CHAI ROAD, WAN CHAI, HONG KONG
Sample No.	CPC240031-3
Sample Name	HNB tobacco - Silver

3.2 Study Schedule

Sample Received Date	Jan. 10, 2024
Testing Period	Jan. 10, 2024 - Jan. 26, 2024
Report Issue Date	Jan. 29, 2024

4. Abstract

In this study, the in vitro cytotoxicity of TPM aerosol fractions derived from heated tobacco product HNB tobacco - Silver and 3R4F reference cigarettes will be evaluated using the CHO cells in the NRU assay. The heated tobacco product HNB tobacco - Silver is regarded as the test specimen and the 3R4F reference cigarettes are regarded as the reference specimen.

CHO cells are exposed to different concentrations of TPM samples in a 96-well tissue culture plate. As a comparison, the blank, negative/solvent, and positive controls are assayed at the same time. The neutral red dye can accumulate in the lysosomes/endosomes of living CHO cells, while it could not accumulate in dead/injured CHO cells. Cytotoxicity is then indicated by a decreased ability of the injured cell to take up the neutral red dye into the lysosome.

In this study, the cytotoxicity of the tested heated tobacco product is compared with the standard cigarette 3R4F by Neutral Red Uptake Assay using CHO cell line. As the results shown, the IC_{50} value of TPM sample of 3R4F reference cigarettes is 253.82 μg/mL. According to the average relative cell viability data, the tested TPM sample of heated tobacco product HNB tobacco - Silver of LONO International Co., Limited has much lower cell growth inhibition rates than 3R4F does under the test conditions, and also has no obvious dose-dependent effect, which indicates that the tested heated tobacco product HNB tobacco - Silver product has a much lower cytotoxicity than standard traditional cigarette 3R4F does at cell level.

5. Introduction

The Neutral Red Uptake (NRU) assay is an in vitro test that can be used to evaluate the cytotoxicity of mainstream smoke-derived aerosols, e.g. total particulate matter (TPM) and gas vapour phase (GVP). It is based upon the uptake of the supra-vital dye Neutral Red by CHO cells where it accumulates in the lysosomes/endosomes of living cells, while it does not in dead/dying cells. Cell viability is then quantified by the spectrophotometric determination of the amount of dye recovered from each population of tested cells. In this study, CHO cells will be exposed to aerosol fractions derived from the mainstream aerosol of heated tobacco product HNB tobacco - Silver and

mainstream smoke of 3R4F reference cigarettes, namely the total particulate matter (TPM) fraction.

Cytotoxicity, expressed as the percentage of viable cells relative to the solvent control cell population, will be quantified for the test substance by its IC_{50} value, which is defined as the effective concentration which reduces the number of viable cells by 50%.

6. Objective

The objectives of this study are firstly to determine in vitro cytotoxicity on the basis of TPM derived from heated tobacco product HNB tobacco - Silver, following the exposure to mammalian cells and secondly are to demonstrate whether there is a difference when it is compared to the in vitro cytotoxicity of the mainstream smoke fractions derived from the 3R4F research cigarette.

7. Experimental Design

7.1 Identification and Description

In this study, the heated tobacco product HNB tobacco - Silver is regarded as the test specimen and the 3R4F research cigarettes are regarded as the reference specimen (Table 1).

Table 1. Identification and Description of the Test and Reference Specimen

Item	Short Name	Batch Number/ Production Date	Additional Description
Test Specimen	HNB tobacco - Silver	/	/
Reference Specimen	3R4F	V348Z61B5	Kentucky reference cigarette

For this study, five heated tobacco product HNB tobacco - Silver test specimens and twenty 3R4F reference specimen are employed to generate TPM per item, leading to the final NRU valid test.

7.2 Test Design

In this study, the in vitro cytotoxicity of TPM derived from heated tobacco product HNB tobacco - Silver is evaluated and compared to the in vitro cytotoxicity of TPM derived from the 3R4F research cigarettes using CHO cell line in the NRU assay. On the same day, one batch of TPM from the test and reference specimen are generated and tested

concurrently, including both of the positive and solvent controls, in the NRU assay. The NRU tests, meeting the assay acceptance criteria is required for each item and each fraction.

Eight concentrations of the TPM aerosol fractions from the heated tobacco product HNB tobacco - Silver, the TPM smoke fractions from the 3R4F research cigarettes are assayed in order to cover a viability range from the maximum to little or no toxicity. Each test also includes solvent, cell control and positive control. Cells are exposed to test substances for 24 hours. Following this exposure incubation period, cells are cultured for an additional 3 hours with Neutral Red dye, before being washed and treated with a solution of ethanol, water and acetic acid, mixed in a 50, 49 and 1 volume ratio, to extract the dye. The optical density (OD) (λ = 540 nm) is then measured for the cells containing the extraction solution in each plate by a microplate reader.

7.3 Validity of the Test System

According to the method validation report of Neutral Red Uptake Assay in appendix 3, the test system is confirmed to be valid for the NRU Assay. Thus, the test system employed during the NRU tests is considered to be valid.

7.4 Testing Procedure

7.4.1 Preparation and Collection of Samples

The tested samples are smoked as following conditions: Puff volume: (55.0±0.3) mL; Puff interval: (30.0±0.5) s; Puffing duration: (2.0±0.02)s; Waveform: sinusoidal wave; Average flow rate: (40~50) mL/s. The 3R4F cigarette samples are smoked as following conditions: Puff volume: (55.0±0.3) mL; Puff interval: 30 s; Puffing duration: (2.0±0.02) s; Waveform: sinusoidal wave; Average Flow Rate: (40-50) mL/s. Mainstream smoke are passed through a 44-mm Cambridge filter pad for PP collection. If the collection Cambridge filter pad is not extracted immediately, it can be stored in an airtight flask at -70°C or below for 12 h. Record the TPM weight collected from the filter pad and the pipette appropriate amount of DMSO to the flask such that the final concentration of PP is 50 mg/mL and shake for 30 minutes to extract the pad.

7.4.2 Culture of the CHO Cells

CHO cell line is widely used in scientific research all over the world and it is one of the most commonly used mammalian cell lines in cytotoxicity testing. CHO cells are cultured in a carbon dioxide incubator at 37±1°C with humidified atmosphere of 5% CO_2. Use DMEM culture medium containing 10% FBS and 0.5% penicillin-streptomycin solution for CHO cells culture.

7.4.3 Exposing CHO Cells to Smoke Fractions and Measurement of Absorbance Values

1) Dispense the prepared single cell suspension into 96-well tissue culture plates and culture in a 37°C incubator with humidified atmosphere of 5% CO_2.

2) TPM samples grouping and dose setting: There are four groups on a 96-well tissue culture plate: blank control group, solvent control group, positive control group and TPM group. Add different solution into these four groups as follow:

   Blank control: cell culture medium;

   Solvent control: DMSO (PP) or CMF-PBS buffer (GVP);

   Positive control: SDS [CAS NO. 151-21-3] solution (200 μg/mL);

   TPM: different concentrations of TPM-DMSO extracted solution.

   The test concentrations are: 0, 100, 160, 200, 300, 350, 400, 450, 500 μg/mL, and each concentration performs three replicates.

3) Add the tested substances into 96-well tissue culture plates: Prepare the desired concentrations of the negative (DMSO), TPM sample (PP) or positive control (SDS) solution by mixing with 200 μL of fresh cell growth medium per well. Remove the culture medium from each well and replace with the prepared desired concentrations of blank control, solvent control, positive control and TPM sample solutions.

4) Incubate the prepared 96-well plate in a 37°C incubator with humidified atmosphere of 5% CO_2 for 24 h.

5) When finished incubation, treat the CHO cells with neutral red dye solution (50 μg/mL), and fix the cells, then extract the neutral red dye solution. The absorbance values are then measured by Thermo K3 microplate reader.

8. Results and Discussion

8.1 Collection of TPM Samples

The information of samples is shown in Table 2, and the data for samples collection are shown in the Table 3. The tested heated tobacco product HNB tobacco - Silver has been smoked 60 puffs totally. The weight of the PP of the smoke collected is 208.6 mg. The 20 standard cigarettes 3R4F have also been smoked, with 11 puffs per cigarette. The 3R4F sample weight of PP collected is 340.7 mg. The model of the smoking machine is Cerulean CETI8. Smoking machine settings for test item: Puff volume: Puff volume: (55.0±0.3) mL; Puff interval: (30.0±0.5) s; Puffing duration: (2.0±0.02)s; Waveform: sinusoidal wave; Average flow rate: (40~50) mL/s. The 3R4F cigarette samples are smoked as following conditions: Puff volume: (55.0±0.3) mL; Puff interval: 30 s; Puffing duration: (2.0±0.02) s; Waveform: sinusoidal wave; Average Flow Rate: (40-50) mL/s.

8.2 Calculation of Average Relative Cell Viability Rate

Expose the CHO cells in 96 well-plates by different concentrations of PP sample, then experience the process of dyeing, fixation and extraction, the extracted solutions were determined its absorbance values by using the microplate reader at OD_{540nm}. After the determination, the blank average absorbance values were deducted from each average absorbance values of solvent control group, cell control group, positive control group and sample exposed group to get a corrected value. Then we can calculate the average cell viability rates by following formula:

$$X = \frac{OD_n - OD_0}{OD_c - OD_0} \times 100\%$$

In the formula:

X: The average cell viability rates of CHO cells at different tested TPM concentrations;

OD_n: The average absorbance value of TPM group;

OD_0: The average absorbance value of blank control group;

OD_c: The average absorbance value of cell control group.

After treating CHO cells with different concentrations of PP samples in Table 4 below, the average cell viability rates are calculated by the statistical software SPSS Statistics17, according to the raw absorbance data. The average cell viability rates of PP samples treated cells are shown in Table 4. The average cell viability rates of 3R4F decreased significantly with the increase of PP concentrations, showing an obviously dose-response relationship. When the PP concentration is 100 μg/mL, about 8.39% of cells are inhibited. When the PP concentration is increased to 300 μg/mL, the cell viability rate precipitously decreased to 44.34%; When the PP concentration is higher than 300 μg/mL, the cell viability rates decreased sharply, reached 7.59% with 500 μg/mL PP treated. While the PP concentrations of the tested sample are the same as 3R4F, the average cell viability rate is significantly higher than that of 3R4F, and no significant dose-response effect existed. For example, when the PP concentration is 100 μg/mL, the average cell viability rate of the tested sample is 93.79%; when the PP concentration increased to 500 μg/mL, the average viability rate of the tested sample is 93.20%, which is changed very slightly.

Additionally, the IC_{50} value of the 3R4F reference sample is 253.82 μg/mL in Table 4. When PP concentration of the tested sample is 500 μg/mL, the cell inhibition rate is still only 6.80% and much less than 50%, so IC_{50} value of the tested sample could not be calculated. The results show that the average cell viability rates of the tested sample are much higher than that of the standard traditional cigarette 3R4F, indicating that the cytotoxicity of the tested sample is far below that of the standard cigarette 3R4F.

9. Testing Conclusion

In this study, the cytotoxicity of the tested heated tobacco product is compared with the standard cigarette 3R4F by Neutral Red Uptake Assay (using CHO cell line). The results of this study are shown in Figure 1. As the assay results shown, the tested heated tobacco product HNB tobacco - Silver of LONO International Co., Limited has much lower cell growth inhibition rate than 3R4F does, and also has no obvious dose-dependent effect. This study indicated that the tested heated tobacco product HNB tobacco - Silver has a much lower cytotoxicity than standard traditional cigarette 3R4F does at cell level.

10. References

Neutral Red Uptake Assay for Mainstream Tobacco Smoke. Health Canada Official Method T-502, Second Edition 2004-11-01.

OECD Test Guideline No.432, In Vitro 3T3 NRU Phototoxicity Test, 18 June 2019.

11. Results Tables and Figures

Table.2 Sample ID

Laboratory Sample ID	Sample Description
CPC240031-3	HNB tobacco - Silver
3R4F	Kentucky Reference 3R4F

Table.3 Smoking Data

Sample ID	Smoking Date	Repeat No.	Cigarettes Smoked	Puff Counts (per cig.)	Blank Pad Weight (g)	Pad Weight after TPM Collection (g)	MSTPM Weight (mg)	Smoking Machine
CPC240031-3	Jan. 16, 2024	1	5	12	40.2143	40.4229	208.6	CERULEAN CETI 8
3R4F	Jan. 16, 2024	1	20	11	39.2032	39.5439	340.7	CERULEAN CETI 8

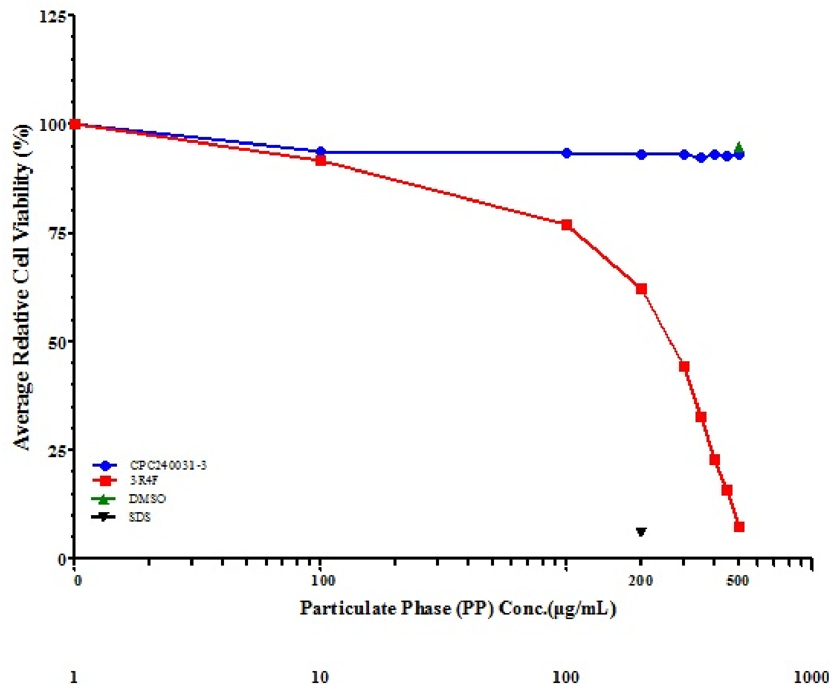
Notes: Samples were extracted by DMSO to reach a final concentration of 50 mg/mL.

Table.4 Average Relative Cell Viability Rates for TPM Samples

Cigs Sample No.	Average Relative Cell Viability (%)											IC_{50} (μg/mL)
	PP Concentration (μg/mL)									Negative Control (DMSO)	Positive Control (SDS)	
	0	100	160	200	300	350	400	450	500			
CPC240031-3	100	93.79	93.30	93.25	92.98	92.44	92.93	92.66	93.20	94.82	6.10	/
Std. Dev.	0.0139	0.0042	0.0082	0.0110	0.0068	0.0113	0.0122	0.0076	0.0040	0.0066	0.0073	/
3R4F	100	91.61	76.87	62.39	44.34	32.69	22.81	15.87	7.59	95.89	5.61	253.82
Std. Dev.	0.0128	0.0099	0.0118	0.0202	0.0140	0.0155	0.0131	0.0155	0.0123	0.0102	0.0090	/

Note: The IC_{50} value of 3R4F is calculated by SPSS Statistics17.

Fig.1: Comparison of Cytotoxicity between CPC240031-3 Aerosol and 3R4F Reference Cigarette and 3R4F Reference Cigarettes Smoke



Note : The Fig.1 above is made by GraphPad Prism 5 of which the X axis scale is log10 and Y axis scale is linear.

12. Test Sample Photo

CPC240031-3: HNB tobacco - Silver

13. Appendix

13.1 Appendix 1 Raw Data File

13.2 Appendix 2 Equipment, Reagents and Mediums for NRU Assay

13.3 Appendix 3 Method Validation Report of Neutral Red Uptake Assay

Appendix 1

Raw Data File of Absorbance at 540 nm

Sample ID	Repeat No.	TPM Concentration (μg/mL)									Control Group		
		0	100	160	200	300	350	400	450	500	DMSO (2.0 μL)	SDS (200 μg/mL)	Blank
3R4F	A	0.664	0.601	0.508	0.443	0.304	0.268	0.201	0.158	0.075	0.629	0.078	0.046
	B	0.653	0.624	0.537	0.450	0.338	0.242	0.186	0.147	0.105	0.654	0.089	0.044
	C	0.684	0.619	0.523	0.404	0.317	0.231	0.169	0.121	0.091	0.641	0.067	0.039
CPC240031-3	D	0.645	0.629	0.609	0.634	0.621	0.624	0.618	0.612	0.623	0.621	0.072	0.043
	E	0.679	0.622	0.624	0.619	0.625	0.622	0.603	0.610	0.614	0.631	0.089	0.041
	F	0.661	0.619	0.628	0.607	0.609	0.599	0.633	0.627	0.622	0.637	0.085	0.049

Note:

A, B, C - Three replicates of each tested concentration of reference item 3R4F PP sample, and positive, solvent, blank controls.

D, E, F - Three replicates of each tested concentration of test item CPC240031-3 PP sample, and positive, solvent, blank controls.

Appendix 2

Equipment, Reagents and Mediums for NRU Assay

<table>
<tr><th>Apparatus and Equipment</th><th>Model and Size</th><th>Reagents and Medium</th></tr>
<tr><td>Smoking Machine</td><td>CERULEAN CETI 8</td><td rowspan="15">
1. Growth Medium:
90% nutrient mixture (DMEM with L-glutamine) + 10% fetal bovine serum (FBS) + 1% penicillin-streptomycin solution.
2. Positive control solution:
Prepare 1 mg/mL of SDS solution in sterile deionised water.
3. Neutral red dye solution:
Prepare 50 μg/mL of neutral red dye solution in DMEM.
4. Fixative solution:
Prepare 1% (v/v) formalin solution in sterile deionised water as fixative.
5. Neutral red dye extractant:
50% (v/v) ethanol solution + 1% (v/v) glacial acetic acid + 49% (v/v) sterile deionised water.
6. 0.25%(w/v) trypsin solution :
Purchase from Mikx.
7. 10×PBS :
Purchase from Mikx.
8. DMEM basic:
Purchase from Gibco.
9. FBS:
Purchase from Gibco.
10. The major reagents and mediums in NRU Assay are prepared as listed above. Other reagents are prepared as per Health Canada Official Method T-502.
</td></tr>
<tr><td>Super Clean Bench</td><td>AIRTECH SW-CJ-2FD</td></tr>
<tr><td>Inverted Microscope</td><td>Xingming guangxue XDS-1</td></tr>
<tr><td>CO_2 Incubator</td><td>Blue Pard BPN-80CH</td></tr>
<tr><td>Microplate Reader</td><td>Thermo K3</td></tr>
<tr><td>Vertical Autoclave</td><td>Blue Pard LDZX-50KBS</td></tr>
<tr><td>One Hundred Thousandth Analytical Balance</td><td>SHIMADZU AUW120D</td></tr>
<tr><td>Electronic Balance, accurate to 0.1mg</td><td>Sartorius BSA224S</td></tr>
<tr><td>Electro-Thermostatic Water Bath</td><td>Blue Pard HWS-26</td></tr>
<tr><td>DHG Series Heating and Drying Oven</td><td>DHG-9030A</td></tr>
<tr><td>Ultra Low Temperature Freezer, －(80±1°C)</td><td>MeiLing DW-HW50</td></tr>
<tr><td>Pipette</td><td>BRAND Transferpette S：(100~1000μL) , (10~200μL), (10~100μL), (0.5~10μL), (0~2.5μL)</td></tr>
<tr><td>Microtitre Plate Shaker</td><td>Kylin-Bell Mini Shaker MH-2</td></tr>
<tr><td>Low Speed Tabletop Centrifuge</td><td>XiangYi L500-A</td></tr>
</table>

Appendix 3

Method Validation Report of Neutral Red Uptake Assay

Editor Signature Bing Lu **Date** 11 May 2020

Reviewer Signature Yujun Lin **Date** 11 May 2020

Approver Signature VanessaHuang **Date** 11 May 2020

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1. Purpose

This research aims to verify whether the laboratory meets various requirements and can carry out the test of this standard by confirming the ability of laboratory personnel, equipment, reagents, environmental conditions and testing procedures.

2. Introduction

CHO cells uptake the Neutral Red Dye by ion diffusion and the dye can bind to the anions in lysosomal matrix. The accumulation of Neutral Red Dye in the CHO cells requires a complete cell membrane. When the cell membrane and lysosomal membrane are injured, the accumulation of Neutral Red Dye in the cell will be decreased. That is to say the uptake of Neutral Red Dye in cells is positively correlated with the cells' viability rate in the culture system.

3. Instrument and Equipment Calibration

3.1 Requirements for Test Equipment

Super clean bench, inverted microscope, carbon dioxide incubator, microplate reader, autoclave sterilizer, pipette, microtitre plate shaker, electronic scales, and ultra-low temperature refrigerator, other laboratory routine equipment.

3.2 Equipped Instrument

The laboratory is equipped with all the instruments listed in 3.1.

3.3 Equipment Verification

The equipment is qualified according to the requirements.

4. Verification in Different Environment

4.1 Requirements of the Test Environment

The biosafety laboratory equipped with a ventilating device, requires a working condition without microbial pollutions and won’t be affected by any other pathogens as well as should be clean, dry, and smoke-free. The buffer

room can access the aseptic operation area, located in a separate compartment inside the room. The washing area and sterilization room is in the outside.

4.2 Facilities and Monitoring of the Environment

The laboratory environmental conditions meet the test requirements listed in 4.1.

4.3 Environmental Verification

The cell room has been equipped with air conditioner, ultraviolet light, etc., which meets the test condition requirements.

5. Personnel capacity

5.1 Staffing of the Project

Two qualified operators at least.

5.2 Personnel Training and Assessment

Lu Bing and Wang Haipeng have passed the examination and are proficient in the test.

6. Verification of Reference Materials and Reagents

6.1 Standard (substance) Solutions and Reagents Required for the Method

DMEM cell culture medium (containing L-glutamine, without pyruvic acid sodium), Neutral Red dye, dimethyl sulfoxide (DMSO), fetal bovine serum, phosphate buffer (without calcium and magnesium), penicillin, streptomycin mixture (1000 units/mL), trypan blue, trypsin, formalin, anhydrous ethanol and glacial acetic acid (99.9%), sodium dodecyl sulphate (SDS), disposable aseptic filter (0.2 μm), sterile deionized water; Chinese hamster ovary cells (CHO cells). Cell culture medium and solutions: cell growth medium, Neutral Red Dye solution, trypsin solution, fixed solution, Neutral Red extraction solution, SDS solution.

6.2 Consumables

The Lab has been equipped with the required reagents and materials: DMEM cell medium (containing L-glutamine,

without pyruvic acid sodium), Neutral Red dye, DMSO, fetal bovine serum (FBS), Sterile phosphate buffered Saline (without calcium and magnesium)(CMF-PBS), penicillin, streptomycin mixture (1000 units/mL), trypan blue, trypsin, formalin, anhydrous ethanol and glacial acetic acid (99.9%), SDS, disposable aseptic filter (0.2 μm), sterile deionized water.

Chinese hamster ovary cell line (CHO) is already equipped.

Cell culture medium and solution are almost ready: cell growth medium, Neutral Red solution, trypsin solution, fixed solution, Neutral Red extraction solution, SDS solution.

7. Method Verification

The cell suspension was almost ready as follows:

The cells were cultured in a carbon dioxide incubator. The morphology, growth, and confluence of the cells were observed daily under an inverted microscope to check for the absence of mycoplasma contamination. When cell growth reached 80% confluence, remove the growth medium, and add CMF-PBS to rinse and then discard the washing solution and repeat the washing step once. After discarding the washing solution, add 0.25% (W/V) trypsin solution to the monolayer incubating at 37±1°C for about 1 min to harvest the cells, and then add the growth medium to the culture dish to end up the digestion, and mix well to make a single cell suspension.

Preparation of TPM samples: Collect and prepare TPM samples of tested e-cigarette smoke as per the appendix 1 of Health Canada Official Method T-502, second edition (2004-11-01). The test procedures have been verified:

1) Preparation of 96-well tissue culture plate: Dispense 200 μL of the cell suspension into the corresponding wells of the 96-well tissue culture plate. And the prepared 96-well tissue culture plate was incubated at 37±1°C, 5% CO_2 atmosphere in the carbon dioxide incubator for 24 h.

2) Grouping of subjects, and dose setting: Four test groups were set on the 96-well tissue culture plate, labeled: blank control group, solvent control group, positive control group, and TPM group. In the blank control group, only cell

growth medium was added; in the solvent control group, DMSO was added; in the positive control group, SDS solution was added; in the TPM group, different doses of TPM sample solution was added. The concentration range of the TPM group was designed to produce a cell inhibition rate of 10-90%. Each sample shall be tested at least three wells in parallel.

3) Exposing CHO cells to TPM samples: Prepare the desired concentrations of the negative (DMSO), TPM sample (PP or GVP or GVP+PP) or positive control (SDS) solution by mixing with 200 μL of fresh cell growth medium per well. The tested concentrations of TPM sample are required to ensure to produce approximately 10% to 90% inhibition of Neutral Red Uptake, and after a series of range finder experiment performed as per Health Canada Official Method T-502, it has been observed that for TPM prepared from typical e-cigarettes and the reference cigarette 3R4F, concentrations of 0, 100, 160, 200, 300, 350, 400, 450, and 500 μg/mL will generally give a satisfactory response. Thus, the concentrations of TPM are tested up to 500 μg/mL and the tested concentration of SDS is 200 μg/mL as positive control, the tested concentration of DMSO is depended on the highest dose of TPM sample as negative/solvent control.

4) Treating CHO cells with Neutral Red Dye for cellular uptake: Remove the medium from the wells after 24 h incubation. Add 200 μL of 50 μg/mL freshly prepared neutral red dye solution to each well. Incubate the cells at 37±1°C, 5% CO_2 atmosphere in the carbon dioxide incubator for 3 h.

5) Fixation: Remove the Neutral Red solution from the plate, and add 200 μL of freshly prepared 1% formalin solution to each well within 2 minutes.

6) Extraction of Neutral Red Dye: After removal of 1% Formalin solution from the wells, add 200 μL of freshly prepared 50% ethanol solution containing 1% acetic acid to each well. Shake the plate on a microtitre plate shaker for 10 min.

7) Determination of absorbance of Neutral Red Dye: Read the absorbance from the wells containing the extracted

neutral red solution on a microplate reader at a wavelength of 540 nm.

8) Calculation of 50% of lethal cell dose (IC_{50}): SPSS Statistics17 software was used to calculate the 50% cell lethal dose (IC_{50}) in our laboratory with the absorbance tested above.

8. Conclusion

Neutral Red Uptake Assay is applicable to the assessment of the potential cytotoxicity of cigarette and or e-cigarette smoke fractions. And the testing procedures of the assay have reached all the standards of the test requirements, which have been verified from the aspects of equipment, environmental conditions, personnel ability and experimental methods. As the consequence that the experimental result obtained from our laboratory are valid.

9. Attachment (Records)

Annex 1 Instrument and Equipment Calibration Records

Number	Verification items	Verification Result	Verification Result	Verification Result	Notes
1	Device name	Clean bench	Microplate reader	Inverted microscope	
2	Model	AIRTECH SW-CJ-2FD	Lab K3	XDS-1	
3	Use record	Yes	Yes	Yes	
4	Calibration period	2020.02.21-2021.02.20	2020.02.21-2021.02.20	2020.02.21-2021.02.20	
5	Calibration result judgment	Qualified	Qualified	Qualified	
6	Verification result judgment	Qualified	Qualified	Qualified	
7	Equipment account	Yes	Yes	Yes	

Annex 2 Environment Verification Records

Number	Environmental Conditions of Instruments	Environmental Conditions of the Method	Environment Control Equipment	Verification Results	Notes
1	20±4°C	20±4°C	Air Conditioner	Qualified	
2	40%-80%	40%-80%	Humidifier	Qualified	

Annex 3 Personnel Competency Verification Records

Number	Verification Items	Yes	No	Verification Results	Notes
1	Establishment of personnel files	√		Qualified	
2	Methods record of training and assessment	√		Qualified	
3	Instrument training record	√		Qualified	
4	Instrument authorization	√		Qualified	

Annex 4 Verification Records of Reference Materials and Reagents

Number	Verification Items	Yes	No	Verification Results	Notes
1	Material acceptance record	√		Qualified	
2	Certificate of reference material	√		Qualified	
3	Registration of the use of reference materials	√		Qualified	
4	Standard preparation / calibration record	√		Qualified	

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Annex 5 Methods Verification Record

Cigs Sample No.	Cell Viability Rate (%)											IC_{50} (μg/mL)
	PP Concentration (μg/mL)									Positive Control (SDS)	Negative Control (DMSO)	
	0	100	160	200	300	350	400	450	500			
CPC200164-1	100	98.88	97.57	98.73	96.20	93.28	88.56	90.07	90.27	5.57	96.25	/
Std. Dev.	0.0574	0.0674	0.0571	0.0475	0.0313	0.0125	0.0134	0.0139	0.0473	0.0064	0.0346	/
3R4F	100	97.40	92.37	73.31	27.74	8.18	8.91	4.67	4.16	/	/	244.339
Std. Dev.	0.0361	0.0732	0.0912	0.0675	0.0495	0.0186	0.0127	0.0068	0.0107	/	/	/

The results showed that in the sample CPC200164-1 group, the cell viability rates of different concentrations were higher than 90% except at 400 μg/mL, the cell viability rate was 88.56%, and there was no significant difference. Additionally, when treated with 100 ~ 350 μg/mL of sample solution, the cell viability rates were ranged from 98.88% to 96.20%, which were very close to the cell control group. However, in the reference traditional cigarette 3R4F group, the cell viability rate was lower than 50% from 300 μg/mL, and the cell viability rate reached 4.16% at the highest tested dose 500 μg/mL decreasing sharply with TPM dose, showing an obvious cytotoxicity potential to the cells. The IC_{50} of 3R4F was calculated to be 244.339 μg/mL, falling on the dose range of 200 to 300 μg/mL, which was well consistent with the cell viability rate results of 3R4F. The cell viability rate of the negative control is 96.25% closing to 100%, showing a very low cytotoxicity to the CHO cells. While the cell viability rate of the positive control was only 5.57%. As a conclusion, both of the negative and positive control groups were consistent with expected, indicating that the experimental results were valid.