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TEST REPORT

EVALUATION OF EYE IRRITATION ON QUANTUM ION USING *IN VITRO* RECONSTRUCTED HUMAN CORNEA-LIKE EPITHELIUM TISSUE MODEL EPIOCULAR™ EYE IRRITATION TEST

Job No. J736/20

Report No. R736/20/B19/44

Sponsor:
Eva Energy Sdn Bhd,
12, Jalan Bandar 20,
Pusat Bandar Puchong,
47160 Puchong,
Selangor

Test Facility:
Industrial Biotechnology Research Centre (IBRC),
Building 19, SIRIM Berhad

Study Initiation Date:
29 June 2020

Experimental Start Date:
05 August 2020

Experimental End Date:
11 August 2020

Study Completion Date:
18 August 2020

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APPROVED SIGNATORIES

We, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected throughout the study.

(SUZAINI BADRUDIN)
Reviewer
Industrial Biotechnology Research Centre

26 AUG 2020
Date

(NURHAYATI ARIFFIN)
Analyst
Industrial Biotechnology Research Centre

26 AUG 2020
Date

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SUMMARY

EVALUATION OF EYE IRRITATION ON QUANTUM ION USING *IN VITRO* EPIOCULAR™ EYE IRRITATION TEST (EIT)

In vitro eye irritation test on Quantum Ion using *in vitro* EpiOcular™ Eye Irritation Test (EIT) was performed according to Standard Operating Procedure developed at MatTek Corporation. This *in vitro* standard method was validated by European Centre of the Validation of Alternative Methods (ECVAM) as *in vitro* test method based on reconstructed human cornea-like epithelium (RhCE) technology. The test was conducted in line with the requirement of OECD Guidelines for Testing of Chemicals No 492.

The test was conducted to determine whether the test item causes irritation to the *in vitro* eye model EpiOcular™.

In vitro EpiOcular™ eye irritation test assessed irritancy level of the Quantum Ion via topical exposure of the sample on three-dimensional reconstructed human cornea-like epithelial (RhCE) model EpiOcular™, followed by cell viability test. The test was conducted in duplicate; two RhCE tissues. After (30 ± 2) minutes of exposure, tissues were thoroughly rinsed, blotted to remove the test item and followed by (12 ± 2) minutes post-treatment immersion (Post-Soak) at room temperature. Then, the medium was changed and tissues were incubated for another (120 ± 15) minutes. Next, MTT assay was performed by transferring the tissues into 24-well plates containing MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) medium (1 mg/mL). After (180 ± 10) minutes of incubation, blue formazan salt formed via chemical conversion of MTT in cellular mitochondria was extracted with 2.0 mL isopropanol / tissue. Optical density of the extracted formazan was determined at 570 nm using spectrophotometer. Relative cell viability was calculated for each tissue as % of the mean of the negative control tissues. The eye irritation potential was classified according to the remaining cell viability obtained after test item treatment.

The data indicates that Quantum Ion did not reduce viability of the EpiOcular™ tissue to below 60 % of the negative control. Under the condition of this test, Quantum Ion is considered as **Non-Irritant** to *in vitro* eye model EpiOcular™.

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BACKGROUND

According to United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS), eye irritation refers to the production of changes in the eye following application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. The EpiOcular Eye Irritation Test predicts the acute eye irritation potential of a topically applied chemical or formulation by measurement of its cytotoxic effect of the EpiOcular cornea epithelial model.

The EpiOcular™ OCL-200 Eye Irritation Test (OCL-200-EIT) RhCE tissue construct is similar to the *in vivo* corneal epithelium three-dimensional structure and is produced using cells from the species of interest. The EpiOcular™ tissue construct is a non-keratinized epithelium prepared from normal human keratinocytes. It offers features appropriate for a model of ocular irritation.

Based on the depth of injury model, the EpiOcular™ Eye Irritation Test is intended to differentiate those materials that are non-irritants (would not require a warning label in the European chemical classification systems) from those that would require labeling as either Globally Harmonized System (GHS) 1 or 2. Liquids and solids are treated with different exposure and post-exposure incubations. Minimum of two construct tissues are used for each treatment and control group. Relative tissue viability is determined against the negative control-treated constructs by the NAD(P)H-dependent microsomal enzyme reduction of MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide (and to a lesser extent, by the succinate dehydrogenase reduction of MTT) in control and test article-treated cultures (Berridge, et al., 1996). Thus, the toxicity of the test item or the ocular irritation potential is evaluated by the relative viability of the treated tissues relative to the negative control-treated tissues.

From validation study and its independent peer review, it was concluded that the EpiOcular™ EIT able to correctly identify chemicals (both substances and mixtures), not requiring classification and labelling for eye irritation or serious eye damage according to UN GHS, and the test method was recommended as scientifically valid for that purpose. The EpiOcular™ EIT is thus referred to as the Validated Reference Method (VRM) in the present Test Guideline.

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1.0 OBJECTIVE

The objective of this study was to predict eye irritation potential of Quantum Ion using an *in vitro* eye irritation test using EpiOcular™ model.

2.0 STUDY TIMETABLE

2.1 **Receipt of Reconstructed Human Cornea-like Epithelium (RhCE) model EpiOcular™ tissues:**
05 August 2020

2.2 **Tissue Conditioning**
05 August 2020

2.3 **Pre-Treatment**
06 August 2020

2.4 **Treatment**
06 August 2020

2.5 **Rinsing**
06 August 2020

2.6 **Post-Soak**
06 August 2020

2.7 **Post Incubation**
06 August 2020

2.8 **MTT Viability Test**
06 August 2020

2.9 **Optical Density Reading**
07 August 2020

2.10 **Data Analysis**
08 August 2020 – 11 August 2020

3.0 MATERIALS

3.1 **Test Item**

3.1.1 Test item: Quantum Ion

3.1.2 Sample marking: Copper Ion

3.1.3 Date received: 29 June 2020

3.1.4 Physical appearance: Liquid

3.1.5 Colour: Colourless

3.1.6 Physical Chemical Properties Data: Not provided

3.1.7 Quantity received : 250 ml + 50 ml + 50 ml

3.1.8 pH: Not provided

3.1.9 Storage condition: Room temperature

3.1.10 Solubility: Not provided

3.1.11 Stability: Not provided

3.1.12 Expiration date: Not provided

3.2 **Test System**

3.2.1 Test System: Reconstructed Human Cornea-like Epithelium (RhCE) model EpiOcular™

The Reconstructed Human Cornea-like Epithelium (RhCE) model EpiOcular™ (OCL-200, MatTek, Ashland, USA) consists of normal, human-derived epidermal keratinocytes which have been cultured to form a stratified, squamous epithelium similar to that found in the cornea.

Cultured on specially prepared cell culture inserts using serum-free culture medium, the cells differentiate to form a multi-layered structure which closely parallels the corneal epithelium

3.2.2 Product Number: OCL-200 version 2.0

3.2.3 Lot No: 28060

3.2.4 Production date: 30 July 2020

3.2.5 Date of Shipping: 31 July 2020

3.2.6 Receipt of EpiOcular™: 05 August 2020

3.2.7 Visual quality control of the Eye: All tissues in good condition

3.2.8 The EpiOcular™ System is manufactured according to defined quality assurance procedures. All biological components of the EpiOcular™ and the culture medium are tested by manufacturer for viral, bacterial, fungal and mycoplasma contamination. MatTek determines the ET-50 value following exposure to Triton X-100 (0.3%) for each EpiOcular™ lot. The ET-50 must fall within a range established based on a historical database of results or acceptability ranges for quality control based on OECD Guidelines.

3.3 **Reagent**

3.3.1 Assay Medium: EPI-100-NMM-SIT / Assay Medium
Lot No. : 072720ALB
Sterility : Sterile
Expiration Date : 24/08/2020
Storage : Refrigerator (5 ± 3 °C)
Manufacturer : Mattek Corporation

3.3.2 Phosphate Buffered Saline without Calcium and Magnesium
Lot No.: 070720RAHA
Expiration Date : 07/07/2021
Manufacturer : Mattek Corporation

3.3.3 Phosphate Buffered Saline without Calcium and Magnesium, contain the following:

i. Sodium Chloride 8 g/L
Lot No.: K50722004912
Manufacturer : MERCK

ii. Potassium chloride 0.2 g/L
Lot No.: 1409BI4J35501
Manufacturer : Bio Basic Canada Inc

iii. Anhydrous potassium dihydrogen orthophosphate 0.2 g/L
Lot No.: 1211ACN1K12062801
Manufacturer : Bio Basic Canada Inc

iv. Anhydrous disodium hydrogen orthophosphate 1.15 g/L
Lot No.: 1306ACK2NA12020101
Manufacturer : Bio Basic Canada Inc

3.3.4 MTT – 2mL (5mg/mL)
Lot No. : MKBW0025V
CAS No. : 298-93-1
Manufacturer : SIGMA

3.3.5 MTT Diluent – 8mL
As above 3.3.1

3.3.6 Extractant Solution – Isopropanol
Lot No. : 632261
Storage : Room Temperature
CAS No. : 67-63-0
Manufacturer : Fisher Scientific

3.3.7 Positive Control: Methyl Acetate
Part No. : TC-MA
Lot No. : 070620ALA
Expiration Date : 02/10/2020
Storage : Room Temperature
Manufacturer : MatTek Corporation

3.3.8 Negative Control: Sterile pure water

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4.0 METHOD

Upon receipt of the reconstructed human cornea-like epithelial model EpiOcular™, the tissue kits and the solutions were stored according to the manufacturer's directions for unpacking and storage.

4.1 Assessment of Direct Test Item Reduction by MTT

50 µL of the test item was added to 1 mL of the 1 mg/mL MTT and incubated at (37 ± 1) ° C, (5 ± 1) % CO_2, 95 % Relative Humidity (RH) for 3 hours. Untreated MTT medium was used as control.

4.2 Assessment of Non-Coloured Material

4.2.1 Isopropanol

50 µL of the test item was added to 2 mL of isopropanol and agitated for 3 hours at room temperature. Isopropanol was run in parallel as negative control.

4.2.2 Sterile pure water

50 µL of the test item was added to 1 mL of water and incubated at (37 ± 1) ° C, (5 ± 1) % CO_2, 95 % RH for 60 minutes. Sterile pure water was run in parallel as negative control.

4.3 Tissue Conditioning – Day 0

Under sterile conditions, the sealed 24-well plates containing the EpiOcular™ tissues, was opened. Visual inspection of each insert containing the EpiOcular™ tissue was done prior to tissue conditioning.

1.0 mL of assay medium was dispensed into each well of six-well plates, the EpiOcular™ tissue cultures were transferred into the wells and the plates were incubated for one hour at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH. At the end of the first hour of pre-incubation, the assay medium was renewed for further pre-incubation. Each insert was aseptically transferred into well containing 1.0 mL assay medium and pre-incubation was conducted at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH for overnight (16 – 24 hours).

4.4 Dosing protocol – Day 1

4.4.1 Pre-Treatment

After overnight incubation, each tissues were pre-wetted with 20 µL of Phosphate Buffered Saline without Calcium and Magnesium (PBS without Ca^{2+} and Ma^{2+}). The tissues were incubated at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH for 30 ± 2 minutes.

4.4.2 Treatment

50 µl of test item was applied directly on the tissue. Negative and positive controls were run in parallel in an identical way to the dosed cultures. The cultures were incubated at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH for 30 ± 2 minutes.

4.4.3 Rinsing

At the end of the exposure period, the EpiOcular™ tissue cultures were removed from the incubator and extensively rinsed with phosphate buffered saline to eliminate any residual test item.

4.4.4 Post-Soak

After rinsing, each EpiOcular™ tissues was immediately immersed in 5 ml of Assay Medium which was previously warmed to room temperature in a pre-labeled 12-well plate. The cultures were incubated at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH for (12 ± 2) minutes.

4.4.5 Post-Incubation

After (12 ± 2) minutes incubation, the medium was changed and EpiOcular™ tissues were incubated in 1ml Assay medium for a further (120 ± 15) minutes at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH.

4.5 **MTT Viability Test**

4.5.1 The MTT assay was performed by transferring each EpiOcular™ tissues to 24-well plates containing 300 µL MTT medium (1 mg/mL) in each well. After (180 ± 10) minutes of MTT incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2.0 mL isopropanol / tissue. The extraction plates were sealed with parafilm and agitated for at least 2 hours at room temperature.

4.5.2 At the end of the extraction period, EpiOcular™ tissue was pierced with an injection needle and the extract allowed to run into the well from which insert was taken. The insert was then discarded. The extraction solution was pipetted up and down to ensure complete mixing. Finally, 200 µL were transferred into a 96 well microtiter plate for absorbance measurement (OD=optical density) at 570 nm without using a reference filter. 200 µL of isopropanol was used as blank.

Relative cell viability was calculated for each tissue as % of the mean of the negative control tissues.

5.0 DATA ANALYSIS

5.1 **Assessment of Direct Test Item Reduction by MTT**

The test item is presumed to have reduced the MTT if the MTT solution colour turns blue / purple.

5.2 **Assessment of Non- Coloured material**

5.2.1 Isopropanol

The test item has to be considered as possibly interacting with the MTT measurement if the OD of the test item solution is more than 0.08 (approximately 5 % of the mean viability of the negative control) after subtraction with the OD of isopropanol. An additional test on colourant controls has to be performed.

5.2.2 Sterile Pure Water

The test item has to be considered as possibly interacting with the MTT measurement if the test item becomes coloured after contact with sterile pure water.

5.3 **Workbook EpiOcular-EIT**

A blank, password protected MS EXCEL workbook EpiOcular-EIT-SPREAD.XLS was provided by MatTek Corporation. The workbook consists of two spreadsheets named: Import and Spread.

5.4 **Raw Data of Optical Densities (ODs)**

Raw data of optical densities (ODs) generated by the microplate reader (without blank subtraction) were copied from the reader software and then pasted into the Import spreadsheet of the Excel workbook. The blank corrections, calculation of results and statistical parameters are done automatically in the Spread spreadsheet of the workbook.

5.5 **Calculation**

5.5.1 After data entry, the spreadsheet performs the following calculations:

5.5.2 Blank correction

5.5.3 For each individual tissue treated with a test substance (TS), the positive control (PC) and the negative control (NC) the individual relative tissue viability was calculated according to the following formulas

$$\text{Relative Viability TS (\%)} = [\text{Corrected } OD_{TS} / \text{Corrected Mean of } OD_{NC}] \times 100$$

$$\text{Relative Viability NC (\%)} = [\text{Corrected } OD_{NC} / \text{Corrected mean of } OD_{NC}] \times 100$$

$$\text{Relative Viability PC (\%)} = [\text{Corrected } OD_{PC} / \text{Corrected mean of } OD_{NC}] \times 100$$

5.5.4 For each test item, negative control and positive control, the mean relative viability of the two individual tissues was calculated and used for classification according to the Prediction Model (Refer to 9.0).

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6.0 ACCEPTABILITY RANGES FOR QUALITY CONTROL

	Lower Acceptance Limit	Upper Acceptance Limit
EpiOcular™ EIT (OCL-200)	ET_{50} = 12.2 min	ET_{50} = 37.5 min

7.0 ACCEPTABILITY RANGES FOR NEGATIVE CONTROL OPTICAL DENSITY (OD) VALUES OF THE TEST METHODS

	Lower Acceptance Limit	Upper Acceptance Limit
EpiOcular™ EIT (OCL-200)	≥ 0.8*	≤ 2.5

*This acceptance limit considers the possibility of extended shipping / storage time (e.g.,> 4days) which has been shown not to impact on the performance of the test method.

8.0 ACCEPTANCE CRITERIA FOR POSITIVE CONTROL

The assay meets the acceptance criterion if the mean viability of Positive Control tissues expressed as percent of the negative control tissues is ≤ 50 %. The standard deviation shall be below 20 % for all substances and controls.

	Mean of Viability (%)	Standard Deviation of Viability (SD %)
EpiOcular™ EIT (OCL-200)	≤ 50	<20

9.0 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

An irritant is predicted if the mean relative tissue viability of two individual tissues exposed to the test substance is reduced below 60 % of the mean viability of the negative controls.

***In vitro* result**	***In vivo* prediction**
mean tissue viability ≤ 60 %	Irritant (I) (GHS Categories 1 or 2) Further testing in other test method is required
mean tissue viability > 60 %	Non-Irritant (NI) (GHS No Category)

10.0 RESULT AND DISCUSSION

10.1 Assessment of Direct Test Item Reduction by MTT

There was no change in MTT colour therefore the test item did not interact with MTT.

10.2 Assessment of Non- Coloured material

10.2.1 Isopropanol

There was no colour change and the OD of the test item solution in isopropanol less than 0.08 (approximately 5 % of the negative control). No further testing on colourant controls has to be performed.

10.2.2 Sterile Deionized Water

There was no colour change therefore no additional test has to be performed.

10.3 Viability Measurement

10.3.1 Raw data of optical densities (ODs) of Blank

Refer to Table 1

10.3.2 Blank Correction

Refer to Table 2

10.4 The Quality Control value meets the acceptance range criteria

EpiOcular™ EIT (OCL-200)	Minute
ET_{50}	17.18

10.5 The negative control OD value meets the acceptance range criteria

EpiOcular™ EIT (OCL-200)	Lower OD	Upper OD
Negative Control	1.358	1.483

10.6 The positive control OD value meets the acceptance range criteria

EpiOcular™ EIT (OCL-200)	Mean of Viability (%)	Standard Deviation of Viability (SD %)
Positive Control	39.1	3.67

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10.7 **Classification**

Test substance, negative control and positive control are qualified according to prediction model in clause 9.0. The mean relative viability of the two individual tissues was calculated and used for classification.

Refer to Table 3

10.8 **Graph**

10.8.1 The spreadsheet shows a graph of the results (% of relative viability ± standard deviation)

Refer to Figure 1

10.9 **Prediction Model**

	Mean of Viability (%)	**Standard Deviation of Viability (SD %)**	***In vitro* result**	***In vivo* prediction**
Quantum Ion	90.5	3.81	mean tissue viability > 60 %	Non-Irritant (NI)
Negative Control	100.0	6.22	mean tissue viability > 60 %	Non-Irritant (NI)
Positive Control	39.1	3.67	mean tissue viability ≤ 50 %	Irritant (I)

11.0 CONCLUSION

Under the condition of this test, Quantum Ion is considered as **Non-Irritant** to *in vitro* eye model EpiOcular™.

12.0 RETENTION OF RECORDS AND TEST ITEM

One report will be forwarded to the Sponsor. The other report, together with all generated raw data is maintained at the Industrial Biotechnology Research Centre Archives.

13.0 REFERENCES

13.1 OECD (2015), Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage –OECD Guidelines for Testing of Chemicals No. 492

13.2 Protocol for: In *Vitro* EpiOcular™ Eye Irritation Test (OCL-200-EIT) – Reconstructed Human Cornea-like Epithelium (RhCE) Model EpiOcular (OCL-200-EIT). For use with MatTek Corporation

13.3 (LWI-238-44) - In Vitro EpiOcular ™ Eye Irritation Test (OCL-200-EIT)

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Table 1 Optical Densities (ODs) of Blank

Optical Densities (ODs) of Blank	Mean Optical Densities (ODs) of Blank
0.0441	0.0406
0.0427	
0.0408	
0.0397	
0.0394	
0.0390	
0.0399	
0.0391	

Table 2 Blank Corrected Data

	Tissue	Raw data		Blank corrected data		Mean	% of Viability
		1	2	1	2		
Quantum Ion	1	1.3296	1.3364	1.289	1.296	1.292	91.0
	2	1.3204	1.3188	1.280	1.278	1.279	90.0
Negative Control	1	1.5246	1.5224	1.484	1.482	1.483	104.4
	2	1.3921	1.4049	1.352	1.364	1.358	95.6
Positive Control	1	0.5524	0.5652	0.512	0.525	0.518	36.5
	2	0.6379	0.6272	0.597	0.587	0.592	41.7

Table 3 Classification of Test Item

	Mean of OD	SD of OD	Mean of Viability [%]	SD of Viability	CV [%]	*In vitro* result	Classification
Quantum Ion	1.286	0.013	90.5	3.81	4.21	**Non-Irritant**	Qualified
Negative Control	1.420	0.125	100.0	6.22	6.22	**Non-Irritant**	Qualified
Positive Control	0.555	0.074	39.1	3.67	9.39	**Irritant**	Qualified

OD- Optical Density, SD- Standard Deviation, CV- Coefficient of Variation

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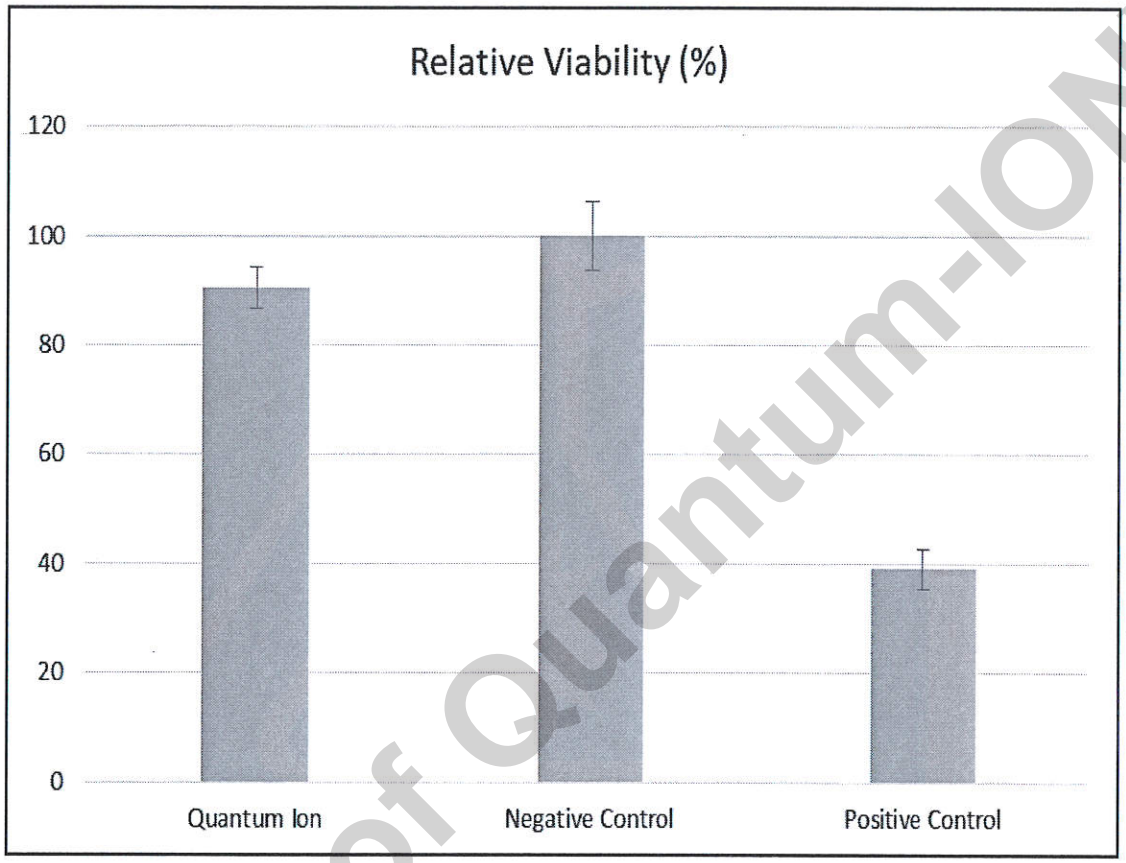

Figure 1 Relative Viability

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