2 ppm Electrolytic Ozonated Water
(BES Group, BioSure, Biolux)

In Vitro Cytotoxicity Test
-MTT Assay

FINAL REPORT

Sponsor: **BES Group / Biotek Environmental Science Ltd.**

Testing Institution: **SGS Taiwan Ltd.**
Ultra Trace &Industrial Safety Hygiene

Report No.: **UG/2020/91368**

Note:

1. The analytical report is the test result issued by the testing institutions as requested by the consignor. Regarding to the legitimacy of the product, it shall be determined by the authorities according to the law.
2. Any unauthorized alteration, forgery or falsification of the content or appearance of this report is unlawful and offenders may be prosecuted to the fullest extent of the law.
3. The results shown in this test report refer only to the test article(s) tested.
4. The content of this report is invalid if it is not presented as the entire report.
5. All items in this testing report is based on the request from sponsor and we are responsible for that.

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STUDY SCHEDULE

2 ppm Electrolytic Ozonated Water (BES Group, BioSure, Biolux)
In Vitro Cytotoxicity Test- MTT Assay

Report No.:	UG/2020/91368
Test Article Received Date	2020.09.24
Experimental Starting Date:	2020.09.24
Experimental Completion Date:	2020.09.29
Study Completion Date:	See Study Director's signature date in the report
Name of Study Personnel:	Allison Lai & Momo Shih (only for mycoplasma test)

ADDRESS INFORMATION

Testing Facility/Test Site

Name: SGS TAIWAN LTD. Ultra Trace &Industrial Safety Hygiene

Address: No. 38, Wu Chyuan 7th Rd., New Taipei Industrial Park, Wu Ku Dist., New Taipei City, 24890, Taiwan.

Study Director

Name: Irene Lai

Address: No. 38, Wu Chyuan 7th Rd., New Taipei Industrial Park, Wu Ku Dist., New Taipei City, 24890, Taiwan.

Sponsor

Name: BES Group / Biotek Environmental Science Ltd.

Address: 5F/98 Xingde Rd. Sanchung New Taipei City, Taiwan 24158

Tel No.: 02-8511 1048

INFORMATION FOR TEST ARTICLE

台灣檢驗科技股份有限公司
SGS Taiwan Ltd.

INFORMATION FOR☒TEST ARTICLE/☐CONTROL ARTICLE

Sponsor Company Name	BES Group / Biotek Environmental Science Ltd.
Sponsor Address	5F/98 Xingde Rd. Sanchung New Taipei City, Taiwan 24158
Sponsor Telephone Number	02-8511 1048
Name of Test Article/ Control Article	2 ppm Electrolytic Ozonated Water (BES Group, BioSure, Biolux)
Type	☐ Medical Devices，Category): ☐ Surface Device ☐ External Communicating Device: Circulating Blood：☐Yes☐ No ☐ Implant Device ☐ Other Type：_____ ☐ Food; ☐ Cosmetics Products ; ☐ Industrial Chemicals; ☐ Pesticide Products; ☐ Drug; ☒ Others：Oral Rinse
Amount (Note 2)	A、Quantity/Unit: 50 / ml B、☒One Test (No Retention)☐Two Test (For Retention) C、Packing Condition: ☒In Bulk ☐Intact Packing
Sterilization	Has been Sterilized ☒NO ☐YES, If Yes, Please Select the Following Method, ☐EO Sterilization☐ Gamma Sterilization☐ Steam Sterilization☐ Other_____
Expiry Date (Note 3)	☐Expiry Date:_____(YYYY.MM.DD) ☒Not Provided.
Batch/Lot Number	☐ _____ ☒Not Provided.
Model Number	☐ _____ ☒Not Provided.
Description	A、Major Components: Ozone B、Purity: 99.9% C、Concentration: 2ppm D、Stability：_____ E、Color : Colorless F、Solvent and Solubility ：_____ G、External Features: ☐Regular ☐Irregular ☒Liquid ☐Powder ☐Granule ☐Flat ☐Other:_____ H、Thickness：_____mm/pcs or ☒ Not Available. I、Surface Area*： ☐ _____cm^2/ pcs, ☐Double side ☐single side ☐All side or ☒ Not Available. *According to FDA guidance, determine the appropriate amount of test material by using surface area to extractant volume ratios. Mass to extractant volume ratios should only be used if surface area cannot be calculated, or if use of mass will result in a larger sample.
Attachment(Note 4)	☐Certificate of Analysis ☐Material Safety Data Sheet ☐Stability Test Result ☐Others：_____ ☒No Attachment (Note4)
Storage Condition	☒Room Temperature☐2~8℃☐-10~-25℃☐Others：_____
Cut or not(Note5)	☐Yes ☐No ☒N.A(Liquid, Gel, Powder)
Testing parts	☐Whole Article ☒Determine by SGS ☐Specific Parts/Please Describe：_____

Test/Control Article No.
UG/2020/91368
It will be labeled by SGS sample receiving personnel.

版次：3.7 試驗物質/對照物質資料表 Information For Test Article/Control Article
發行日期：2020.05.05 page 1of 2

台灣檢驗科技股份有限公司
SGS Taiwan Ltd.

Extraction	Ratio： ☒No Extraction ☐By Surface Area：☐(3±10%)cm²/mL☐(6±10%)cm²/mL ☐By Weight：☐(0.1±10%)g/mL☐(0.2±10%)g/mL ☐Other：_____
	Condition： ☐(37±1)℃(24±2)Hours ☐(50±2)℃(72±2)Hours ☐Other：_____
Source	A、Location of Origin：_____ B、Manufacturer/Supplier：BES Group / Biotek Environmental Science Ltd. C、Manufacture Date：_____
Article Return (Note6)	☐No ☐Yes, only for un-used test article ☒Other：Ozone generator
Others	The half-life of ozone in water is about 20-30 minutes, so it must be used immediately when newly generated. The applicant provides a BES ozone generator, the necessary items, to ensure the correct and expedient generation of test samples.

Note1. All information in the form including the blanks which can't be provided are disclosure and should taken responsibility by the sponsor .
Note2. If the test follow GLP, please provide the test article with the same lot for retention. If sponsor doesn't provide the retention of test article/control article, the retention of a reserved test article/control article from each batch of test article /control article is the responsibility of the Sponsor.
Note3. Should prepare extra amount of the same lot test/control article for the retention. For retention, if the effective period is less than 5 years, the test article/control article will be retained till the expiry date. If the expiry date is longer than 5 years, the test article/control article will be retained for 5 years only. If the expiry date remained incomplete, it mentions that sponsor will agree that test facility will determine the earliest date, e.g. Exp. Date: 2015(YYYY), sponsor didn't identify the MM/DD, the expiration date should be 2015.01.01.
Note4. Sponsor should determinate, document and confirm the identity, strength, purity, stability, composition, method of synthesis, fabrication, derivation or other characteristics of the test article/control article before study. If the sponsor cannot provide the information, determination and documentation of the test article/control article are the responsibility of the Sponsor.
Note5. The test article/control article which has been destroyed or cutting will be discarded after the end of experiment unless applicant/sponsor have extra requirement. If the sponsor does not agree or have special requirements, should take the initiative to inform test facility and state the application prior to test. If related losses incurred because applicant/sponsor didn't inform, we will not be liable.
Note6. If sponsor wants to return the sample, the retention, inconsistencies and biological safety related issue of test article/control article are the responsibility of the Sponsor.
Note7. Note 'N/A' or 'N.A'if not applicable. Do not leave blank.
Note8. Test article and control article should be filled individually in "INFORMATION FOR TEST ARTICLE/CONTROL ARTICLE".
Note9. GLP test：(1) Corrections and additions to a final report should be in the form of amendments. Amendments should clearly specify the reason for the corrections or additions. Lot number, test article photos and raw data cannot be amended by sponsor's requirement. (2)One protocol only can generate one report except for translation version. If the report with more than two languages version, we only issue English version protocol. We only issue amendment or additional language GLP report within three years and non-GLP report within one year. (3)The GLP compliance statement will state that we follow TFDA GLP and TAF OECD GLP norm unless sponsor's requirement. SGS Taiwan Ltd. UTIS have acquired Good Laboratory Practice Statement of Compliance from TFDA and TAF .
Note10. Please write down it carefully and in detail. "INFORMATION FOR TEST ARTICLE/CONTROL ARTICLE" will be placed in the protocol and report along with a copy of this official document. If the information is not clear, we will exclude them from GLP statement.
Note11. The results shown in this test report refer only to the test article(s) tested.

Sponsor's Representative Signature/ Date： Esther Cheng, 2020.09.10

SIGNATURE OF PERSONNEL

2 ppm Electrolytic Ozonated Water (BES Group, BioSure, Biolux)
In Vitro Cytotoxicity Test- MTT Assay

Approval
Signatory:

Irene Lai / SGS Taiwan Ltd. Date 2020.10.16

Laboratory Head:

Shin Jyh Chen/ SGS Taiwan Ltd. Date 2020.10.16

＊Approval signatory is the study director of this study.

ABSTRACT

In vitro cytotoxicity test was performed in this study to evaluate the biological compatibility of "2 ppm Electrolytic Ozonated Water (BES Group, BioSure, Biolux)", which was provided by BES Group / Biotek Environmental Science Ltd.. Dilution of test article and treatment of mouse lung fibroblast cells (L929 cells) with test article solutions were performed according to ISO 10993-5. Cell viability determined by MTT assay showed that the highest concentration of the solution (100% test article solution) corresponds to ISO 10993-5 described weight/volume ratio of 0.2g±10%/1mL had in average <30% inhibitory effects to the viability of cells. The results suggested that the test article solution did not induce cytotoxic effect in L929.

PURPOSE

According to the nature and duration of the anticipated contact with human tissues when in use the test article should be carefully tested for biocompatibility to avoid potential physiological damage by toxic substances produced or contaminated during manufacturing. In this study, the test article was subjected to *in vitro* cytotoxicity test to evaluate toxicity of substances that could be extracted or released from the test article according to the ISO 10993-5:2009, Biological evaluation of medical device－Part 5: Tests for *in vitro* cytotoxicity guidance. Therefore, the test system was mouse lung fibroblast cells (L929 cells). The original source was from Food Industry Research and Development Institute, Strain No. BCRC RM60091. Based on recommendations described in ISO 10993-5, quantitative determination of cell viability by MTT assay was carried out. This result provided practical information for assessing the *in vitro* cytotoxicity of the test article.

EXPERIMENTAL DESIGN

1. Test System

A. Cell line: Mouse lung fibroblast L929 cells. The original source was supplied by Food Industry Research and Development Institute, Strain No.: BCRC RM60091.
Subculture passage number: P17. Bank No.: 20180404-2E-72-31

B. Morphology: Fibroblast-like

C. Culture properties: Adherent

D. Incubation condition: Incubate in MEM medium with 10% horse serum at 37±1°C in the presence of 5±1% CO_2.

2. Reagents

	Reagents	Brand	Cat No.:	Lot No.:
A.	100X L-Glutamine solution	HyClone	SH30034.01	AF29484084
B.	100X Penicillin-Streptomycin solution	HyClone	SV30010	J190044
C.	100X Sodium pyruvate solution	HyClone	SH30239.01	AF29484086
D.	10X Phosphate buffer solution (PBS)	BIOMAN SCIENTIFIC CO., LTD	PBS105000	20051805
E.	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)	SIGMA	M5655	MKCL1832
F.	Dimethyl sulfoxide (DMSO)	SIGMA	34943	I1270
G.	Horse serum	GIBCO	16050-122	2208943
H.	Minimum Essential Medium (MEM)	GIBCO	10370021	2177692
I.	0.25% Trypsin solution	HyClone	SH30042.01	J200011
J.	HDPE	FDSC	RM-C	C-161
K.	ZDEC	FDSC	RM-A	A-191K

3. Equipments

	Equipments	Brand	Model	Equipment No.:
A.	Balance	OHAUS	PA214C	BAL-17
B.	Biological safety cabinet	NuAire	NU-543-600	BSC-07
C.	CO_2 Incubator	ASTEC	SCA-165DS	INB-01
D.	Microscope	OLYMPUS	CKX41	MIS-02
E.	Centrifuge	EPPENDORF	5804R	CEN-07
F.	Water bath	KANSIN	WB212-B2	WAB-02
G.	Microplate Spectrophotometer	BioTek™	Eon	MPS-02

4. Preparation of Test Article and Controls

A. Test Article

The test article was handled under sterile environment and operated with aseptic technique during preparation. MEM complete medium contain 10% horse serum was used as dilution buffer. The test article was added in the MEM complete medium contain 10% horse serum to the final concentration 0.2g ± 10% /1mL. In this study, 0.1995 g of test article was added in 0.998 mL dilution buffer. The test article solution was performed serial dilution with MEM complete medium contain 10% horse serum. Finally the 100% test article solution, 50% test article solution, 25% test article solution, and 12.5% test article solution were used in this study. The pH adjustment; filtration and centrifugation are not conducted. The solution was used within 24±2 hours after preparation.

B. Controls

(1) Blank control: MEM complete medium contain 10% horse serum as blank control.
(2) Positive control: MEM complete medium contain 10% DMSO was as positive control. Finally the 100% positive control, 50% positive control, 25% positive control, and 12.5% positive control were used in this study.
(3) Negative control: The 1X PBS was added in the MEM complete medium to the final concentration 3 cm^2 ± 10% /l mL was as negative control.

5. *In vitro* cytotoxicity test-MTT assay

A. Cell incubation

(1) Preparation of MEM complete cell culture medium
For 500mL complete cell culture medium as example, complete cell culture medium was prepared by mixing 435 mL of MEM, 5 mL of 100X Penicillin- Streptomycin solution, 5 mL of 100X L-Glutamine solution, 5 mL of 100X Sodium pyruvate solution and 50 mL of horse serum. The completed medium was stored at 4±2°C.

(2) Cell culture
Mouse lung fibroblast cells were used here for cytotoxicity test. The L929 cells were grown on a 10-cm dish containing 10 mL of MEM complete medium and incubated at 37±1°C in the presence of 5±1% CO_2. Detachment of the cells was performed by washing the cells with 1X PBS followed by treatment with 1.0 mL/dish of trypsin solution for 3 minutes at 37±1°C. Enzymatic activity of trypsin solution was terminated by adding MEM complete medium and then transferred to new 10-cm dish for subculture.

B. MTT assay

(1) 100 µL of L929 cell suspension (1×10^5cells/mL) was transferred into each well of a 96-well cell culture plate. The cells were then incubated at 37±1°C for 24±2 hours in a humidified atmosphere containing 5±1% CO_2.
(2) Culture medium was replaced with 100 µL of test article solution and the different dilutions or controls. The cells will be then incubated at 37±1°C for another 24±2 hours in a humidified atmosphere containing 5±1% CO_2. Treatments of the cells with the solution and the following end concentration of the solution (100%, 50%, 25%, and 12.5 %) were performed in triplicates.

(3) Morphology of the cells was observed under microscope.
(4) Following evaluation of cell conditions, the culture medium was aspirated from the plates. 50 µL of the MTT solution was then added to each well and the plate was further incubated for 2 hours ± 10 mins at 37±1 °C.
(5) MTT solution was replaced with 100 µL of DMSO. The plate was incubated at room temperature for 10 minutes and subsequently subjected to a microplate reader equipped with a 570 nm filter for colorimetric measurement (reference 650 nm).
(6) The triplicate results of MTT assay were presented as mean ± standard deviation (SD) If the mean of cytotoxicity was less than 30%, the result showed "<30%".
(7) The average of inhibition of cell viability was used to give final interpretation of cytotoxicity.
(8) All the experiment procedure was according to SGS SOP: TESP-UB-1038.

6. Quality criteria

A. Positive control and negative control

(1) Positive and negative controls should be included in every cytotoxicity test.
(2) Positive control was MEM complete medium contain 10% DMSO; Negative reference material was 1X PBS was in the MEM complete medium to the final concentration 0.2g ± 10% /l mL.
(3) The inhibition of cell viability of 100% positive control was greater than 30% (>30%) and negative control was less than 30% (≦30%).

B. Blank

(1) Measure the absolute value of optical density, OD. The acceptance criterion of blank was ≧ 0.2.
(2) Blanks were placed both at the left side and the right side of the 96-well plate (contained 12 replicates).
(3) The difference between the OD average of left blanks and the right blanks were less than 15% compares to the total average mean.
Calculation of the difference:

$$\left| 1 - \frac{\text{the OD average of left (right) blanks}}{\text{total average of the blanks}} \right| \times 100\%$$

C. Test article

The 50% test article extract should have at least the same, lower, or no significant difference ($p>0.05$) of the inhibition of cell viability than the 100% test article extract; otherwise the test should be repeated.

7. Calculation

$$\text{Cell viability (\%)} = \frac{\text{POS/NEG/T(OD)}}{\text{BK(OD)}} \times 100(\%)$$

$$\text{Inhibition of viability (\%)} = 100(\%) - \text{Cell viability (\%)}$$

POS: positive control; NEG: negative control; BK: blank; T: the test article.

DATA MANAGEMENT

The quantitative data was showed as mean and standard deviation (SD). The achievement of a reduction of cell viability by more than 30% (>30%) of the 100% test article solution is considered as cytotoxic effect.

RESULTS

1. **Appearance**
   The test article solution was not obviously different from the blank control.

2. **Inhibition of cell viability**
   The acquired readings of OD absorbance of blank control were averaged and set as 0% inhibition of cell viability. In proportion to blank control, we determined inhibition of cell viability of negative control, the highest concentration of positive control, the highest concentration of the test article solution as <30%, 95.35%±0.85% and <30% respectively. The relative values of inhibition of cell viability were shown in Table 3.

CONCLUSION

The inhibition of cell viability was averaged and listed in Table 3. According to ISO 10993-5, the averaged result which concluded that the "2 ppm Electrolytic Ozonated Water (BES Group, BioSure, Biolux)" solution did not induce cytotoxic to L929 cells.

REFERENCES

1. Abiraman S, Varma HK, Kumari TV, Umashankar PR, John A (2002) Preliminary *in vitro* and in vivo characterizations of a sol-gel derived bioactive glass-ceramic system. Bull. Mater. Sci. 25(5), 419-429.
2. Mendes SC, Reis RL, Bovell YP, Cunha AM, Blitterswijk CA, Bruijn JD (2001) Biocompatibility testing of novel starch-based materials with potential application in orthopaedic surgery: a preliminary study. Biomaterials. 22, 2057-2064.
3. ISO 10993-5:2009 Biological evaluation of medical device－Part 5: Tests for *in vitro* cytotoxicity.
4. ANSI/AAMI/ISO 10993-5:2009/(R)2014 Biological evaluation of medical device－Part 5: Tests for *in vitro* cytotoxicity.
5. ISO 10993-12:2012 Biological evaluation of medical devices-Part 12: Sample preparation and reference materials.
6. ANSI/AAMI/ISO 10993-12:2012 Biological evaluation of medical devices-Part 12: Sample preparation and reference materials.
7. SGS SOP: EOMP-USL-0003 Operating procedures and analysis of the Balance. Version 1.3
8. SGS SOP: EOMP-USL-0020 High-speed centrifuges operating procedures. Version 1.1
9. SGS SOP: EOMP-USL-0027 Operating procedures of the biosafety cabinet and laminar flow, UV-lamp verification and aerobic plate counts. Version 2.5
10. SGS SOP: EOMP-USL-0029 Operating Procedures of Microplate Spectrophotometer-BioTek EON. Version 1.1
11. SGS SOP: EOMP-USL-0030 Maintenance and operating procedures of the microscope. Version 1.1
12. SGS SOP: EOMP-USL-0035 CO_2 incubator Operating procedures. Version 1.0
13. SGS SOP: EOMP-USL-0036 Operating Procedures of MilliQ. Version 1.0
14. SGS SOP: EOMP-USL-0037 Manual of freezer-refrigerators. Version 1.0
15. SGS SOP: EOMP-USL-0038 Microorganism incubator operating Procedures Version 1.0
16. SGS SOP: TESP-UB-0217 Operating Procedures of the cells' activation and verification. Version 2.1
17. SGS SOP: TESP-UB-1038 *In Vitro* Cytotoxicity Test- MTT Assay. Version 1.0

TABLES

Table 1 – The difference between the optical density average of left blanks and the right blanks

Left row	Right row	
0.939	0.887	-
0.914	0.917	
0.916	0.881	
0.874	0.839	
0.871	0.830	
0.846	0.867	
Left Avg. 0.893	Right Avg. 0.870	Difference % 1.31%

Table 2 – Optical density of Groups

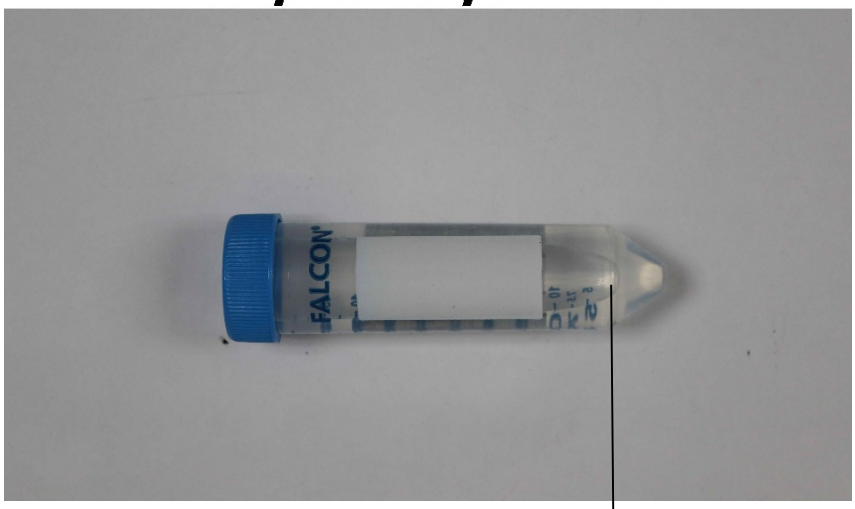
Groups	Exp.01	Exp. 02	Exp. 03	Avg.
Blank control	0.831	0.834	0.841	0.835
Negative control	0.831	0.833	0.828	0.830
Positive control (100%)	0.038	0.033	0.047	0.039
Positive control (50%)	0.287	0.308	0.293	0.296
Positive control (25%)	0.593	0.600	0.583	0.592
Positive control (12.5%)	0.746	0.759	0.745	0.750
UG/2020/91368 (100%)	0.721	0.705	0.706	0.710
UG/2020/91368 (50%)	0.803	0.754	0.729	0.762
UG/2020/91368 (25%)	0.845	0.805	0.787	0.812
UG/2020/91368 (12.5%)	0.840	0.773	0.779	0.797

Table 3- Cytotoxic effect of test article extract in inhibition of L929 cell viability (%)

Groups	Exp. 01	Exp.02	Exp. 03	Avg.	SD	*p*
Blank control	<30%	<30%	<30%	<30%	0.61%	—
Negative control	<30%	<30%	<30%	<30%	0.30%	
Positive control (100%)	95.51%	96.11%	94.43%	95.35%	0.85%	
Positive control (50%)	65.68%	63.17%	64.96%	64.60%	1.30%	
Positive control (25%)	<30%	<30%	30.23%	<30%	1.02%	
Positive control (12.5%)	<30%	<30%	<30%	<30%	0.94%	
UG/2020/91368 (100%)	<30%	<30%	<30%	<30%	1.07%	0.083
UG/2020/91368 (50%)	<30%	<30%	<30%	<30%	4.51%	
UG/2020/91368 (25%)	<30%	<30%	<30%	<30%	3.56%	—
UG/2020/91368 (12.5%)	<30%	<30%	<30%	<30%	4.44%	

TEST ARTICLE PHOTO

UG/2020/91368



Only tested the test article inside the tube.