SIRIM BIOTEST

TEST REPORT

Sponsor	WATERCO FAR EAST SDN BHD	**Technical initiation**	9 July 2001
Address	Lot 3B, 30/60 Km 10, Jalan Kepong, 52000 Kuala Lumpur.	**Technical completion**	11 July 2001
		Report Date	16 July 2001
Contact	Ms Lim Foong Seim	**Job Number**	046/01

Test Article	Solid composite material	**Study**	Direct Contact Assay

REFERENCE: This study was based on the procedure and method described in British Standard (BS) 6920-2.5:2000 Suitability of non-metallic products for use in contact with water intended for human consumption with regard to their effect on the quality of the water – Part 2: Methods of test – Section 2.5: The extraction of substances that may be of concern to public health.

GENERAL PROCEDURE: The degree of cytotoxicity in a mammalian cell culture, CCL 81 Vero cells, in response to the test articles (GF1 and GF2) was determined. Validation solution (zinc sulfate) and a blank were included in the study to verify the proper functioning of the test system. The extracts of the test articles, the validation solution and the blank were tested in replicates of six at the Neat concentration. The cultures were incubated at 37 ± 1 °C in a humidified atmosphere of 5 % carbon dioxide and 95 % air. Assessment was carried out after 24-hour incubation through microscopic observation.

RESULTS:

Effects on culture	**Validation solution**	**Blank**	**Test articles**	
			GF1	**GF2**
Monolayer	Dissociated	Confluent	Confluent	Confluent
Cellular damage/lysis	Positive	Negative	Negative	Negative

CONCLUSION: The test articles exhibited no cytotoxic response.

AUTHORIZED PERSONNEL:

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Consultant.

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SIRIM BIOTEST

TECHNICAL REPORT

SUBMITTED TO

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DIRECT CONTACT ASSAY

1 SUMMARY

A study was conducted to evaluate the cytotoxic potential of solid composite materials from Ms Lim Foong Seim, Waterco (Far East) Sdn Bhd, Selangor Darul Ehsan. A Direct Contact Assay was used to assess the cytotoxic potential of two solid composite materials using the African green monkey kidney (Vero) cell line as the target cells. The test was performed according to the procedure described in BS 6920-2.5:2000. NO OTHER TESTS WERE UNDERTAKEN ON THIS TEST ARTICLE.

2 TEST ARTICLE DATA

2.1 Name and code of article: GF

2.2 Characteristics: Solid-composite material

2.3 Condition of receipt by test lab, including packaging in contact with test product: Seven (7) test articles supplied in plastic package.

2.4 Condition of storage between sample receipt and the start of testing: Room temperature.

2.5 Description of test article:

2.5.1 Material type: Solid composite.

2.5.2 Color: Beige.

2.5.3 Shape/form: Slightly slanted square disc.

2.5.4 Dimension (mm): Length 100-108, width 61-68, thickness less than 10.

2.5.5 Appearance: One rough surface, one smooth surface with tapered edges.

2.5.6 Opacity: Translucent.

3 REAGENTS

3.1 Pure water. Water used in the test, produced by reverse osmosis.

3.2 Growth medium. Sterilized ingredients of 10 ml of 199 concentrate (x10) with Earle's salts but without sodium bicarbonate (Sigma), 7 ml of heat-inactivated fetal bovine serum, 1 ml of gentamicin solution (Sigma), 4 ml of sodium hydrogen carbonate, and 90 ml of pure water were mixed and stored refrigerated in the absence of light. Before use, 300 µl of a 200mM L-glutamine solution (Flowlab) was added to each 100 ml.

3.3 Sodium hydrogen carbonate solution. A 44 g/l sodium hydrogen carbonate (Sigma) solution was sterilized in an autoclave at a temperature of 121 °C for 10 minutes and stored refrigerated.

3.4 Heat inactivated fetal bovine serum. Fetal bovine serum (Flowlab) was heat inactivated at 56 °C for 30 minutes and stored frozen in aliquots of 20 ml.

3.5 Phosphate buffered saline solution. A mixture of 8.0 g of sodium chloride (Riedel-deHaën), 0.02 g of potassium chloride (Sigma), 0.20 g of potassium dihydrogen orthophosphate (BDH), and 1.15 g of disodium hydrogen orthophosphate (Merck) was dissolved in 1 l of pure water. The solution was sterilized in an autoclave at a temperature of 121 °C for 10 minutes.

3.6 Trypsin-EDTA solution. A 0.1 % (w/v) trypsin (Sigma) and 0.02 % (w/v) EDTA (Sigma) was dissolved in phosphate buffered saline. The solution was filter sterilized and stored frozen in a freezer.

3.7 Concentrated growth medium, prepared as described in 3.2, but omitting the pure water. The medium was stored refrigerated in the absence of light.

3.8 Cell line. Established Vero cell line of African green monkey kidney cells (ATCC number CCL 81) was used.

4 APPARATUS

4.1 Tissue culture wares. Pre-sterilized plastic containers of tissue culture grade were used: 25 cm^2 tissue culture grade flask (Corning), 75 cm^2 tissue culture grade flask (Nunc), 24-well tissue culture plate (Falcon), and 15-ml conical tube (Falcon).

4.2 Haemacytometer counting chamber (Assistant).

4.3 Sterile 0.2 µm syringe filter unit (Schleicher & Schuell).

4.4 Inverted microscope with phase contrast (Nikon Eclipse TE200).

4.5 Carbon dioxide water-jacketed incubator (Forma Scientific, model 3111).

4.6 Biological safety cabinet Class II (Clyde Apac model BHA 120).

4.7 Glasswares, were prepared as follows.
Pipettes were washed with a detergent (Decon 90) in a washer unit. After cleaning, the wares were thoroughly rinsed firstly in tap water, followed by a final rinse in pure water and left to dry at room temperature. The pipettes were placed in a pipette canister and sterilized in an autoclave at 121 °C for 20 minutes.

5 TEST PROCEDURE

The procedure was divided into three stages as follows:
(a) The cell line was grown and maintained as healthy monolayer.
(b) The test articles were subjected to extraction.
(c) The extract was used to prepare batches of growth media.
(d) The effect of the media on the morphology of cells was observed.

5.1 Maintenance of Vero cell

Cell cultures were grown in tissue culture grade flasks using the growth medium. Cultures were examined daily to ensure they remain healthy. Any changes in morphology (such as increase in size) or their adhesive properties were noted.

5.2 Preparation of cell suspension

Vero cells were grown as a monolayer in 75 cm^2 tissue culture flasks, at 37 ± 1 °C in a humidified atmosphere of 5 % carbon dioxide and 95 % air. When the cells reached confluency they were removed from the flask by trypsinisation. The medium was decanted and the cells rinsed with phosphate buffered saline. The culture was bathed with trypsin-EDTA solution and left for 30 s at room temperature. After removing excess trypsin-EDTA solution, the cells were incubated for a further 5 min. The flask was gently tapped several times to disaggregate the cells and the cells were suspended in growth medium. Cells were counted to determine their number using a haemacytometer. The number of viable cells was determined by trypan blue exclusion.

5.3 Extraction procedure

Two pieces of test articles were selected for extraction. The total surface area of each test article was determined using a ruler and a caliper. The first test article, designated as GF1, and the second test article, designated as GF2, each has a total surface area of 15130.62 mm^2 and 15030.4 mm^2 respectively.

The test articles were rinsed in pure water and separately placed in a glass beaker. Pure water was added to each beaker up to the 1 l mark. A validation solution of 800 mg/l zinc sulfate was prepared and included in the study. One empty beaker filled with pure water was also included as the blank.

The four beakers were sealed with a fresh piece of aluminium foil and incubated in the absence of light at 23 ± 2 °C for 24 hours. Before use, the test extracts, validation solution and blank were filter sterilized.

5.4 Growth procedure

Growth medium was prepared by thoroughly mixing 1.1 ml of concentrated growth medium together with 4.9 ml of each extract of the test articles, including the validation solution and blank, in conical tubes. Approximately 1.8 x 10^6 cells was added to each tube. The mixture was gently mixed and 1 ml aliquots of the resulting cell suspension were immediately transferred into each well on a 24 well plate. The test extracts, validation solution and blank were each assessed in replicates of six.

The plate was incubated at 37 ± 1 ^{0}C in a humidified atmosphere of 5 % carbon dioxide and 95 % air for 24 hours.

6 ASSESSMENT OF RESULTS

Results of the study were determined by assessing cell damage after 24-hour incubation. Cells were examined using the inverted microscope and the results presented below and in Table 1.

Validation solution - Monolayer dissociated and total lysis of cells for all replicates. This indicates a cytotoxic response.

Blank - Healthy intact monolayer and no morphological changes for all replicates. This indicates no cytotoxic response.

Test articles - Vero cultures exposed to extracts from both test articles did not demonstrate any cytotoxic effect. The monolayers were healthy and maintained their confluent appearance for all replicates. No sign of cellular damage (rounding off, granulation, lysis) were noted. This indicates no cytotoxic response.

Table 1. Results of direct contact assay following a 24-hour exposure of Vero cells with the test articles.

Effects on culture	Validation solution	Blank	Test articles	
			GF1	GF2
Monolayer	Dissociated	Confluent	Confluent	Confluent
Cellular damage/lysis	Positive	Negative	Negative	Negative

7 CONCLUSION

The test articles exhibited no cytotoxic response.

REFERENCES

BS 6920-2.5:2000. Suitability of non-metallic products for use in contact with water intended for human consumption with regard to their effect on the quality of the water – Part 2: Methods of test – Section 2.5: The extraction of substances that may be of concern to public health.

GENERAL

Sponsor:	Waterco Far East Sdn Bhd, Lot 3B, 30/60 Km 10, Jalan Kepong, 52000 Kuala Lumpur.
Testing facility:	SIRIM-Biotest Safety and Toxicology Laboratory, SIRIM Berhad, Shah Alam, Selangor.
Test code:	TS103
Test conducted by:	Mohd Helme Helan, *B.Appl.Sc (Hons.)*
Test article:	GF
Title:	Direct contact assay
Consultant:	Dr Anuar Osman, *DVM, M.Sc, Ph.D.*
Start of experiment:	9 July 2001

Test reported by: Date:

Dr Anuar Osman, *DVM, M.Sc, Ph.D.* 16/07/01

Report verified by: Date:

Dr Seri Intan Mokhtar, *B.Sc (Hons), Ph.D.* 16.07.01