

## **FINAL REPORT**

### Study Title

***In Vitro* Micronucleus Test using Reconstructed Skin Micronucleus (RSMN)  
Assay in EpiDerm™**

### Test Article

B486 (AD74TF)

### Author

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### Study Completion Date

04 January 2016

### Testing Facility

BioReliance Corporation  
9630 Medical Center Drive  
Rockville, MD 20850

### BioReliance Study Number

AD74TD, AD74TF.358RNGLP.BTL

### Sponsor

Cosmetics Europe – The Personal Care Association  
Avenue Herrmann-Debroux 40  
B-1160 Brussels, Belgium

## CERTIFICATION

Study No. AD74TD, AD74TF.358RNGLP.BTL was conducted using the Good Laboratory Practice (GLP) Regulations for nonclinical laboratory studies as a guideline; however, the study did not fall within the scope of GLP requirements. The study was conducted in accordance with Standard Operating Procedures and as an exploratory study. I, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.



Shambhu Roy, Ph.D.  
Study Director

05 Jan 2016  
Date

## SUMMARY

The purpose of this study was to evaluate the potential genotoxicity of test articles B546 (AD74TD) and B486 (AD74TF) to the EpiDerm™ Skin Model after repeat exposure to the test article for 72±3 hours. However, the RSMN assay using test article B546 (AD74TD) was not conducted per Sponsor request since additional test article to run the assay was not available and it was determined that the chemical was not appropriate for validation due to uncertainty of its genotoxicity/carcinogenicity. A copy of the study protocol is included in Appendix I.

Cytotoxicity was determined by measuring the relative percent binucleation (Table 1 and Table 2) compared to the solvent control. Genotoxic potential was determined by measuring the frequency of micronucleated binucleated cells in the test article treated cultures compared to the solvent control for statistical significance (Table 3).

Cytotoxicity was also determined by relative live cell count of the test article compared to the solvent control (Table 4). Cytokinesis-blocked proliferation index (CBPI) is reported (Table 5) for completeness but was not used in this study. At 10 mg/mL, there was complete toxicity (no cells were available) and at 8 mg/mL there was only 1 valid tissue based on cell counts. Therefore, the concentrations selected for analysis were 2, 4, 5, and 6 mg/mL. No toxicity was observed at any of these concentrations based on percent binucleation. Based on relative live cell count around 50% cytotoxicity was observed at 5 and 6 mg/mL.

Conclusion: Overall, a statistically significant increase in micronuclei was observed at 6 mg/mL B486. The Cochran Armitage trend test was positive ( $p < 0.05$ ). Therefore B486 was concluded to be positive for the induction of micronuclei in the RSMN assay after 72 hours exposure.

**TABLE 1**  
**Cell Counts to Determine Relative Percent Binucleation**

Test Article Conc. Mg/mL	Solvent for Test Article	Toxicity # >2N G1	Toxicity # >2N G2	Toxicity >2N G3	Toxicity # 1N G1	Toxicity # 1N G2	Toxicity # 1N G3	Toxicity # 2N G1	Toxicity # 2N G2	Toxicity # 2N G3	Toxicity Total Cells Counted G1	Toxicity Total Cells Counted G2	Toxicity Total Cells Counted G3
0	ETOH/Water (70/30)	27	30	22	101	89	97	372	382	381	500	501	500
2.00	ETOH/Water (70/30)	17	17	24	105	116	105	378	367	371	500	500	500
3.00	ETOH/Water (70/30)	32	19	18	100	99	83	368	382	399	500	500	500
4.00	ETOH/Water (70/30)	20	20	20	90	92	99	390	388	381	500	500	500
4.50	ETOH/Water (70/30)	29	21	26	101	98	78	370	381	396	500	500	500
5.00	ETOH/Water (70/30)	17	18	15	97	110	111	386	372	374	500	500	500
6.00	ETOH/Water (70/30)	28	20	17	95	92	90	377	388	393	500	500	500
8.00	ETOH/Water (70/30)	3	3	7	90	92	98	427	405	395	500	500	500
10	ETOH/Water (70/30)	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC
MMC, 3 µg/mL	Acetone	10	10	3	87	123	113	403	367	384	500	500	500
MMC, 5 µg/mL	Acetone	1	4	2	157	82	140	342	414	359	500	500	501

**TABLE 2**  
**Cytotoxicity of B486 as Determined by Relative Percent Binucleation**

Test Article Conc. Mg/mL	G1 Bi%	G2 Bi%	G3 Bi%	Avg. Bi%	Std Dev	G1 Relative % Binuc.	G2 Relative % Binuc.	G3 Relative % Binuc.	Avg. Relative % Binuc.	SD of Relative % Binuc.
0	74.4	76.2	76.2	75.61583	1.05	98.39	100.84	100.77	100.00	1.39
2.00	75.6	73.4	74.2	74.40000	1.11	99.98	97.07	98.13	98.39	1.47
3.00	73.6	76.4	79.8	76.60000	3.10	97.33	101.04	105.53	101.30	4.11
4.00	78.0	77.6	76.2	77.26667	0.95	103.15	102.62	100.77	102.18	1.25
4.50	74.0	76.2	79.2	76.46667	2.61	97.86	100.77	104.74	101.13	3.45
5.00	77.2	74.4	74.8	75.46667	1.51	102.10	98.39	98.92	99.80	2.00
6.00	75.4	77.6	78.6	77.20000	1.64	99.71	102.62	103.95	102.10	2.16
8.00	82.1	81.0	79.0	80.70513	1.58	108.60	107.12	104.48	106.73	2.09
10	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC
MMC, 3 µg/mL	80.6	73.4	76.8	76.93333	3.60	106.59	97.07	101.57	101.74	4.76
MMC, 5 µg/mL	68.4	82.8	71.7	74.28556	7.55	90.46	109.50	94.76	98.24	9.99

**TABLE 3**  
**Micronucleus Analysis of EpiDerm<sup>TM</sup> Treated with B486**

Test Article Conc. Mg/mL	G1 MN%	G2 MN%	G3 MN%	Avg. MN%	Std Dev	G1 MN	G2 MN	G3 MN	Total MN	G1 Total BN	G2 Total BN	G3 Total BN	MN/1000 BN	Total MN	Total BN	P value
0	0.10	0.10	0.10	0.10	0.00	1	1	1	3	1000	1000	1000	1.00	3	3000	
2.00	0.00	0.10	0.10	0.07	0.06	0	1	1	2	1000	1000	1000	0.67	2	3000	
3.00																
4.00	0.00	0.10	0.30	0.13	0.15	0	1	3	4	1000	1000	1000	1.33*	4	3000	p<0.5
4.50																
5.00	0.30	0.30	0.30	0.30	0.00	3	3	3	9	1000	1000	1000	3.00**	9	3000	p<0.07
6.00	0.50	0.50	0.40	0.47	0.06	5	5	4	14	1000	1000	1000	4.67**	14	3000	p<0.01
8.00																
10	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC
MMC, 3 µg/mL																
MMC, 5 µg/mL	3.30	2.90	2.50	2.90	0.40	33	29	25	87	1000	1000	1000	29.00**	87	3000	p<0.00

\*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ , Fisher's exact test, relative to the solvent control.

The Cochran Armitage test was positive for a dose-response ( $p < 0.05$ )

**TABLE 4**  
**Cytotoxicity of B486 as Determined by Cell Count Relative to Vehicle Control**

Test Article Conc. Mg/mL	G1 Total cell count X 10 <sup>5</sup>	G2 Total cell count X 10 <sup>5</sup>	G3 Total cell count X 10 <sup>5</sup>	Avg. Total cell count X 10 <sup>5</sup>	SD Total Cell Count X 10 <sup>5</sup>	G1 % Relative Total Cell Count	G2 % Relative Total Cell Count	G3 % Relative Total Cell Count	Avg. % Relative Total Cell Count	Std. Dev. Relative Total Cell Count
0	4.34	4.06	5.32	4.57	0.66	94.90	88.78	116.33	100.00	14.47
2.00	4.27	3.78	3.71	3.92	0.31	93.37	82.65	81.12	85.71	6.67
3.00	3.85	4.13	4.06	4.01	0.15	84.18	90.31	88.78	87.76	3.19
4.00	3.71	3.64	3.57	3.64	0.07	81.12	79.59	78.06	79.59	1.53
4.50	2.17	2.38	2.38	2.31	0.12	47.45	52.04	52.04	50.51	2.65
5.00	2.10	2.03	2.10	2.08	0.04	45.92	44.39	45.92	45.41	0.88
6.00	2.31	2.03	2.59	2.31	0.28	50.51	44.39	56.63	50.51	6.12
8.00	1.47	2.10	1.19	1.59	0.47	32.14	45.92	26.02	34.69	10.19
10	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC
MMC, 3 µg/mL	2.94	3.29	3.92	3.38	0.50	64.29	71.94	85.71	73.98	10.86
MMC, 5 µg/mL	3.92	3.43	3.08	3.48	0.42	85.71	75.00	67.35	76.02	9.23

**TABLE 4 (CONTD.)**  
**Cytotoxicity of B486 as Determined by Cell Count Relative to Vehicle Control**

Test Article Conc. Mg/mL	G1 Viable cell count x 10 <sup>5</sup>	G2 Viable cell count x 10 <sup>5</sup>	G3 Viable cell count x 10 <sup>5</sup>	Average Viable cell x 10 <sup>5</sup>	Std. Dev. Viable cell	G1 % Relative Viable Cell Count	G2 % Relative Viable Cell Count	G3 % Relative Viable Cell Count	Average % Relative Viable Cell Count	Std. Dev. % Relative Viable Cell Count	G1 % Viability	G2 % Viability	G3 % Viability	Average % Viability	Std. Dev. % Viability
0	4.13	3.71	4.41	4.08333	0.35	101.14	90.86	108.00	100.00	8.63	95.16	91.38	82.89	89.81	6.28
2.00	3.36	3.57	3.22	3.38333	0.18	82.29	87.43	78.86	82.86	4.31	78.69	94.44	86.79	86.64	7.88
3.00	3.36	3.36	3.99	3.57000	0.36	82.29	82.29	97.71	87.43	8.91	87.27	81.36	98.28	88.97	8.59
4.00	3.64	3.50	3.43	3.52333	0.11	89.14	85.71	84.00	86.29	2.62	98.11	96.15	96.08	96.78	1.15
4.50	2.10	2.24	2.38	2.24000	0.14	51.43	54.86	58.29	54.86	3.43	96.77	94.12	100.00	96.96	2.95
5.00	2.03	1.89	2.03	1.98333	0.08	49.71	46.29	49.71	48.57	1.98	96.67	93.10	96.67	95.48	2.06
6.00	2.17	1.75	1.68	1.86667	0.27	53.14	42.86	41.14	45.71	6.49	93.94	86.21	64.86	81.67	15.06
8.00	1.40	1.82	1.12	1.44667	0.35	<del>34.29</del>	44.57	<del>27.43</del>	<del>35.43</del>	8.63	95.24	86.67	94.12	92.01	4.66
10	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC
MMC, 3 µg/mL	2.94	3.15	3.78	3.29000	0.44	72.00	77.14	92.57	80.57	10.71	100.00	95.74	96.43	97.39	2.29
MMC, 5 µg/mL	3.78	3.22	2.80	3.26667	0.49	92.57	78.86	68.57	80.00	12.04	96.43	93.88	90.91	93.74	2.76

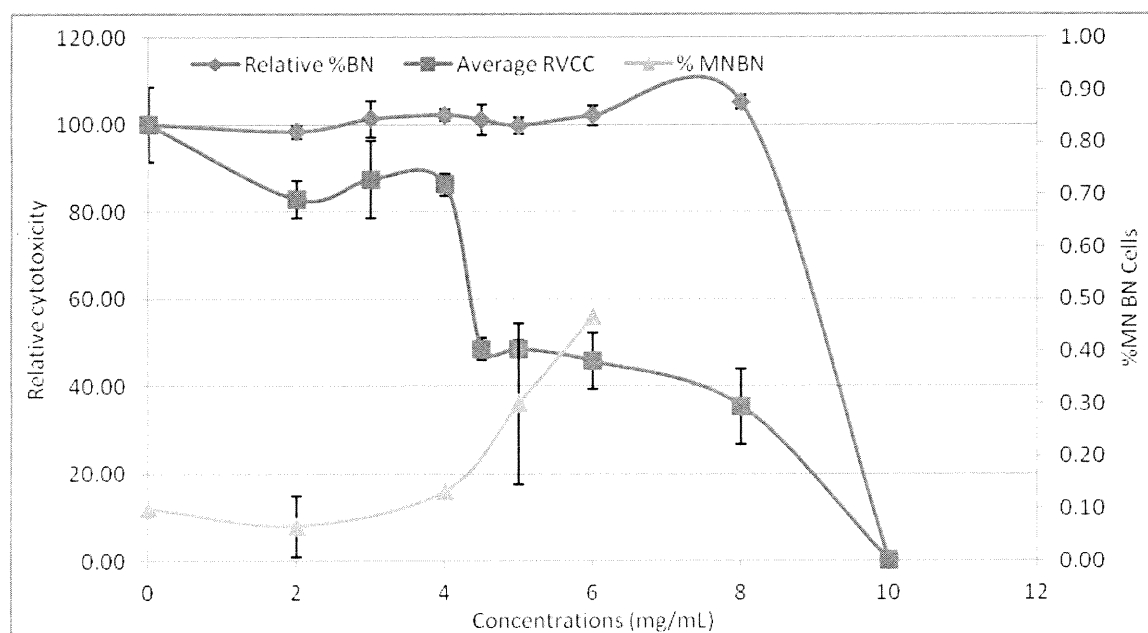
**TABLE 4 (CONTD.)**  
**Cytotoxicity of B486 as Determined by Cell Count Relative to Vehicle Control**

Test Article Conc. Mg/mL	G1 Dead cell count x 10 <sup>5</sup>	G2 Dead cell count x 10 <sup>5</sup>	G3 Dead cell count x 10 <sup>5</sup>	Average Dead cell	Std. Dev. Dead cell	G 1 Live	G 2 Live	G 3 Live	G 1 Dead	G 2 Dead	G 3 Dead
0	0.21	0.35	0.91	0.49000	0.37	59	53	63	3	5	13
2.00	0.91	0.21	0.49	0.53667	0.35	48	51	46	13	3	7
3.00	0.49	0.77	0.07	0.44333	0.35	48	48	57	7	11	1
4.00	0.07	0.14	0.14	0.11667	0.04	52	50	49	1	2	2
4.50	0.07	0.14	0.00	0.07000	0.07	30	32	34	1	2	0
5.00	0.07	0.14	0.07	0.09333	0.04	29	27	29	1	2	1
6.00	0.14	0.28	0.91	0.44333	0.41	31	25	24	2	4	13
8.00	0.07	0.28	0.07	0.14000	0.12	20	26	16	1	4	1
10	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC
MMC, 3 µg/mL	0.00	0.14	0.14	0.09333	0.08	42	45	54	0	2	2
MMC, 5 µg/mL	0.14	0.21	0.28	0.21000	0.07	54	46	40	2	3	4

**TABLE 5**  
**Cytotoxicity of B486 as Determined by CBPI Relative to Vehicle Control**

Test Article Conc. Mg/mL	G1 CBPI	G2 CBPI	G3 CBPI	Avg- CBPI	SD of CBPI	G1 % Relative CPBI	G2 % Relative CPBI	G3 % Relative CPBI	Avg. % Relative CPBI	Std Dev % Relative CPBI
0	1.85	1.88	1.85	1.86141	0.02	98.91	102.42	98.68	100.00	2.10
2.00	1.82	1.80	1.84	1.82133	0.02	95.66	93.10	97.28	95.35	2.11
3.00	1.86	1.84	1.87	1.85800	0.02	100.30	97.51	101.00	99.60	1.84
4.00	1.86	1.86	1.84	1.85267	0.01	99.84	99.37	97.75	98.98	1.10
4.50	1.86	1.85	1.90	1.86600	0.03	99.37	98.21	104.02	100.53	3.07
5.00	1.84	1.82	1.81	1.82133	0.02	97.51	94.73	93.80	95.35	1.93
6.00	1.87	1.86	1.85	1.85867	0.01	100.53	99.37	99.14	99.68	0.75
8.00	1.83	1.82	1.82	1.82423	0.01	96.67	95.42	94.96	95.68	0.88
10	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC	TOXIC
MMC, 3 µg/mL	1.85	1.77	1.78	1.80000	0.04	98.21	89.85	90.55	92.87	4.64
MMC, 5 µg/mL	1.69	1.84	1.72	1.75218	0.08	79.87	97.98	84.11	87.32	9.47

**GRAPH 1**  
**Test article: B486 (AD74TF)**



## **APPENDIX I: Study Protocol**



# Protocol

Study Title	<b><i>In Vitro</i> Micronucleus Test using Reconstructed Skin Micronucleus (RSMN) assay in EpiDerm<sup>TM</sup></b>
Study Director	Shambhu Roy, Ph.D.
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
Sponsor	Cosmetics Europe – The Personal Care Association Avenue Herrmann-Debroux 40 B-1160 Brussels, Belgium
Sponsor's Authorized Representative	Stefan Phfuler
BioReliance Study Number	AD74TD, AD74TF.358RNGLP.BTL

**1. KEY PERSONNEL**

Study Director                      Shambhu Roy, PhD  
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Sponsor's Authorized              Stefan Pfuhler  
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Cincinnati, OH 45252  
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Email: pfuhler.s@pg.com

**2. TEST SCHEDULE**

Proposed Experimental Initiation Date	05 Nov 2014
Proposed Preliminary Results Date	14 Nov 2014
Proposed Experimental Completion Date	To be determined
Proposed Report Date	To be determined

**3. REGULATORY REQUIREMENTS**

This study will be performed using the Good Laboratory Practice (GLP) Regulations for nonclinical laboratory Studies as a guideline; however, this study will not meet GLP requirements.

**4. PURPOSE**

The purpose of this study is to evaluate the potential dermal genotoxicity of the test article to the EpiDerm™ Skin Model after repeat exposure to the test article for 48±3 hours. The cytotoxicity will be determined by measuring the relative percent binucleation or relative live cell count of the cultures exposed to the test article compared to the solvent control, (whichever toxicity method is more sensitive). Genotoxicity potential will be determined by measuring the frequency of micronucleated binuclear cells in the test article treated cultures compared to the solvent control for statistical significance.

## 5. TEST ARTICLE INFORMATION

Identification	B546 (AD74TD)
Storage Conditions	Room Temperature Protect from light
Purity	Not provided. There will be no correction factor.
Molecular Weight	Not provided

Identification	B486 (AD74TF)
Storage Conditions	Room Temperature Protect from light
Purity	Not provided. There will be no correction factor.
Molecular Weight	Not provided

### Characterization of Test Article

Characterization of the Test Article is the responsibility of the Sponsor.

### Test Article Reserve Sample

Since the in-life portion of this study is less than four weeks in duration, a reserve sample will not be retained.

### Characterization of Dose Formulations

Dose formulations will not be analyzed.

### Stability of Test Article in Vehicle (Solvent)

Stability of Test Article in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

### Disposition of Test Article and Dose Formulations

All unused test article will be disposed prior to report finalization unless the test article is used on another study.

Residual dose formulations will be discarded after use.

## 6. TEST SYSTEM

This study will use the MatTek Corporation's EpiDerm™ Skin Model. The Features that make the EpiDerm™ Skin Model advantageous in the study of potential dermal toxicity are:

- it is derived from human skin
- the target cells are epithelial
- the tissue has a functional *stratum corneum*

- the test materials are applied directly to the culture surface, at air interface so that undiluted and/or end use dilutions can be tested directly.

## 7. EXPERIMENT DESIGN AND METHODOLOGY

Confirmatory trial will be conducted using 72 hr treatment (3 treatments with 24 hr interval)

### PREPARATION AND DELIVERY OF TEST ARTICLE

Test article formulation will be prepared fresh on each day of dosing **based on instructions provided by the sponsor**. Test article may be sub aliquoted before formulation, as needed. Ten  $\mu\text{L}$  of test article formulation will be applied to the middle of the EpiDerm™ tissue. The plate containing tissues will be tilted couples of times immediately following the test article application to ensure a full coverage of the topical surface of the tissue.

### PREPARATION AND DELIVERY OF POSITIVE AND VEHICLE CONTROLS

#### **Preparation of Mitomycin C Master Positive Control**

Mitomycin C (MMC) will be used as the positive control. A master (stock) concentration of the positive control will be prepared and stored frozen. Stock formulation of MMC at 0.5 mg/mL will be prepared by adding 4.0 ml of room temperature, sterile, tissue culture grade water to the vial containing 2 mg of MMC and vortexed until complete solubility is achieved. Multiple aliquots of 200  $\mu\text{L}$ /vial of MMC will be prepared from stock formulation in glass vials and stored at -15 to -25°C. The positive control performance will be monitored and a new preparation of MMC will be made when past the manufacturer's expiration date or with low MN frequency).

On the day of use, a vial of MMC stock from the freezer will be removed and will be bring to room temperature (do not thaw in a 37°C water bath). MMC stock will be vigorously vortex to thaw the content until all dark purple flecks (precipitates) are dissolved. If dark flecks are still visible after vortexing, the vial may be briefly sonicated. An initial dilution of 100 $\mu\text{g}/\text{ml}$  (see example chart in next section) will be prepared by adding 800 $\mu\text{L}$  acetone to the vial. The content will be mixed well, and checked again for the dark precipitate. If the dark precipitate persists, the aliquot will be discarded and another vial will be thawed, or a fresh batch of MMC stock will be made. It is possible that some cloudiness in the first dilution of 10 $\mu\text{g}/\text{ml}$  can occur, however if an easily visible chunky white precipitate forms, the formulation will be discarded and dilution will be started over again with a different aliquot of MMC stock.

#### **MMC (AD76YV) dose preparation scheme for the Micronucleus assay:**

Begin with a vial containing 100 $\mu\text{g}$  MMC stock (200  $\mu\text{L}$  MMC stock x 0.5  $\mu\text{g}/\mu\text{L}$ )

Initial dilution: Add 0.8 mL of acetone to the stock vial=100  $\mu\text{g}/\text{mL}$

Dilution 1      0.5 mL of Initial diluted with 4.5 mL of Acetone=10  $\mu\text{g}/\text{mL}$

Dilution 2      1.5 mL of Dose 1 diluted with 3.5 mL of Acetone=3  $\mu\text{g}/\text{mL}$

The MMC dose(s) will be prepared fresh each day of dosing the tissues.

**Vinblastine (AD76YW) dose preparation scheme for the Micronucleus assay (if used):**

Step 1: 0.3 mg/mL in sterile water (stock)

Step 2: 10 µg/mL in acetone using step 1 formulation.

Step 3: 1 µg/mL in acetone using step 2 formulation.

Step 4: 0.6 µg/mL in acetone using step 3 formulations and this will be used to dose tissue.

**CYTOCHALASIN B (AD76YX) PREPARATION (Master CytoB stock)**

For 3 mg/mL (3 µg/µL) CytoB stock will be prepared by adding 3.3 mL of DMSO to a 10 mg vial of CytoB and will be vortex until completely solubilized. Using a calibrated micropipette, 100 µL (or other appropriate volume) of CytoB will be aliquoted into labeled cryovials. Each aliquot will be capped tightly and stored at -5 to -40°C. The expiration date will be 1 year or less based on the expiration date specified for CytoB original vial. On the day of use, 3 µg/mL cytoB in NMM will be prepared and used in experiment.

**RECEIPT OF ASSAY KIT(S)**

The assay kits will be received, prepared and cultured per SOP OPGT1021.R00.

**Confirmatory Reconstructed Skin Micronucleus (RSMN)**

The confirmatory micronucleus assay will be conducted for 72 hours (±3 hr) treatment. The number and spacing of the dose levels for the study will be as follows:

AD74TD (B546): 20, 40, 50, 60, 70, 80, 90, and 100 mg/mL

AD74TF (B486): 2, 3, 4, 4.5, 5, 6, 8, and 10 mg/mL

Concurrent negative (solvent) and positive control (MMC at 3 and 5 µg/mL) will also be used.

**Treatment of Tissues (Confirmatory Assays)**

Test article dosing solutions and 3 µg/mL cytoB in New Maintenance Medium (NMM) will be prepared fresh on the day of use. Prior to dosing, the medium will be aspirated and the models will be re-fed with fresh, warm (~37°C) NMM containing 3.0 µg/mL cytoB and then dosed on the dermal surface with 10 µL of the test article dosing formulation with appropriate target concentration, vehicle control, or positive control. The dosing solution will be placed on the surface of the models, in the center, tilting the plate gently to help ensure that the surface of the model is covered by the dosing solution **(First treatment)**.

After dosing, the lid will be replaced on the dish and the models will be incubated under standard conditions ( $37 \pm 1^\circ\text{C}$  in a humidified atmosphere of  $5 \pm 1\%$   $\text{CO}_2$  in air).

After 24 hours ( $\pm 3$ ) of incubation of first treatment), the medium will be carefully pipetted out and the models will be re-fed with fresh warm NMM containing  $3 \mu\text{g/mL}$  cytoB, dosed again on the dermal surface with  $10 \mu\text{L}$  dosing solution, and incubated under standard conditions for further 24 hours ( $\pm 3$ ) (**Second treatment**).

After 24 hours ( $\pm 3$ ) of incubation of second treatment, the medium will be carefully pipetted out and the models will be re-fed with fresh warm NMM containing  $3 \mu\text{g/mL}$  cytoB, dosed again on the dermal surface with  $10 \mu\text{L}$  dosing solution, and incubated under standard conditions for further 24 hours ( $\pm 3$ ) (**Third treatment**) followed by harvesting.

Visual observations will be made to all tissues and observations will be recorded on observation sheet prior to dosing. Tissues with significant defects such as blisters, detachment and significant moisture will be not included in experiment.

## TERMINATION OF TREATMENT AND TRYPSINIZATION

### Cell Harvest

72 hours ( $\pm 3$ ) after initial treatment, the models will be trypsinized to obtain a single-cell suspension from the basal layer. To avoid over-trypsinization and to maintain consistency in the single-cell suspensions, the tissue model will be trypsinized in groups of six models or fewer per technician, keeping the remaining models under standard conditions. Each EpiDerm<sup>TM</sup> model will be placed in a 12- well plate containing 5.0 mL CMF-DPBS at room temperature for 5 to 15 minutes. Each model will be removed from its well, inverted to decant the CMF-DPBS, blotted on a paper towel, then placed in a new well containing 5.0 mL of EDTA (0.1%, 1 g/L) and incubated at room temperature for approximately 15 min. Each model will be then removed, inverted to decant excess EDTA, blotted, then placed in a new well containing 1.0 mL of warm (approximately  $37^\circ\text{C}$ ) trypsin-EDTA solution. Warm trypsin (0.5 mL) will be added inside each model then incubated for 10 to 15 min at  $37^\circ\text{C}$ . After incubation, each model will be held over a new well of a 12-well plate containing 1.0 mL of fresh warm trypsin-EDTA. The model will be carefully separated from the supporting membrane by gently lifting the edge of the model. Both the detached model and the supporting membrane will be transferred to the new well. The tissue model insert will be thoroughly rinsed using the trypsin-EDTA in the well to collect any remaining basal cells left on the supporting membrane in the well, after which the insert will be discarded. The model will be gently agitated to release additional attached cells from the detached model, which should now have primarily only the stratum corneum and that is resistant to further trypsinization. At this point, the remaining tissue will be discarded. The cell clumps will be disrupted by repeatedly drawing the cell suspension in trypsin-EDTA into a pipette no larger than 2.0 mL and gently expelling the solution on the dish surface. The single cell suspension (approximately 1.5 mL) will be transferred to a 15-mL conical tube containing 1.5 to 2.0 mL of warm McCoy's 5A with 10% FBS to inactivate the trypsin. A sample (10 to 50

μL) of cell suspension will be diluted with equal volume of Trypan blue solution and cells will be counted to obtain a cell count and determine the proportion of live cells of each treatment compared with the control.

#### **Cell Fixation**

The cell suspension will be centrifuged (100×g for 5 min) at room temperature, and the supernatant will be carefully removed. The cell pellet will be loosened by gentle flicking the base of the centrifuge tube and 1.0 mL of warm (~37°C) KCl (0.56%) solution will be added slowly down the side of the tube while gently mixing on vortex at low speed. The cell suspension will be allowed to sit at room temperature for approximately 3.0 min, then 3.0 mL of fresh, cold (4°C), methanol/acetic acid (3:1) fixative will be added slowly to the cells, and the cell suspension will be centrifuged at 100×g for 5.0 min, and supernatant will be removed. The cell pellet will be gently re-suspended in cold fixative. In a second fixation, 3.0 mL of cold fresh 40:1: methanol/acetic acid will be added to the cell suspension and centrifuged immediately at 100×g for 5.0 min and slides prepared. If slides cannot be dropped (cells dispensed) on the day of harvest, the fixed cells may be stored in 99:1 fixative (MeOH/Acetic Acid (99:1.v/v) at 2-8°C for a few days, then centrifuged and dropped on slides).

#### **Slide Preparation**

After centrifugation, all but a small portion (approximately 50–200μL) of the supernatant will be removed and discarded. The cell pellet will be loosened by gently flicking the centrifuge tube. One to two drops (i.e. 15–20μL) of the cell suspension will be applied on to a clean, dry microscope slide. At least two slides will be prepared from each EpiDerm™ model, whenever possible. Slides will be air dried at room temperature. The slides will be stained with acridine orange and identified by a BioReliance code system.

#### **Evaluation of Cytotoxicity**

After staining, an assay qualification check will be conducted by evaluating one each of the vehicle and positive control slides (uncoded) to determine if the assay meets criteria for acceptable % BN and causes a significant increase in the MN frequency for the positive control. If the assay appears to be valid based on this qualification analysis, the relative live cell count data and % BN for all concentrations will be analyzed uncoded. The relative cell counts from uncoded samples are used in the final analysis of the study.

#### **Cytotoxicity based on Viable Cell Count Determination:**

Using trypan blue exclusion or other live vs. dead cell count method, the relative viable cell count will be determined for each tissue. Either the relative viable cell count or the relative binucleation, whichever is more sensitive, will be used to select the slides for micronucleus scoring. The % relative viable cell count will be calculated according to the following formula:

$$\% \text{ Relative Viable Cell Count (RVCC)} = \frac{\text{Viable Cell count (x10}^5\text{) the tissue}}{\text{average cell count (x10}^5\text{) of the solvent controls}} \times 100$$

Cytotoxicity based on viable cell count =  $100 - 100 \times (\text{viable cell count}_{T-1}) / (\text{Average viable cell count}_{C-1})$

T = Treated tissue

C = Control tissue

**Cytotoxicity based on Binucleation:**

The fluorescence microscope will be used to determine the percent binucleation and the micronucleus frequency of each tissue. 20 to 40X objectives in "dry" condition will be used for scoring for binucleation.

The % binucleation is determined based on at least a 500 cell count. If fewer than 500 scorable cells are available for determining binucleation, then the slide will not be evaluated for MN and will be considered 0% survival (100% cytotoxic) and averaged into the calculation of the toxicity for the test article concentration.

Number of mono, bi, and multi nucleated (> 2 nuclei) cells will be determined from at least 500 cell counts. The percent of binucleated cells will be determined as follows:

$$\% \text{ binucleated cells} = \frac{\text{Number of binucleated cells}}{\text{Total of Mono + Bi + multi nucleated cells}} \times 100$$

Following calculation will be used to determine the % relative binucleation for each tissue:

$$\% \text{ Relative Binucleation of the tissue} = \frac{\% \text{ Binucleation of the TA treated tissue} \times 100}{\text{average \% Binucleation of solvent control tissue}}$$

The relative cytotoxicity based on binucleated cells will be calculated for each tissue as follows:

$$\text{Relative cytotoxicity based on BN cells} = 100 - 100 \times \{(\text{Total binucleated cell}_{T-1}) / (\text{Average binucleated cell}_{C-1})\}$$

Where,

T= Test chemical treated tissue

C= Average vehicle control tissue

The % viable cells and % binucleated cells for each tissue will be presented. Cytotoxicity will be expressed relative to the solvent control for each tissue as well as at each dose level (average, if more than one tissue used).

The treatment related cytotoxicity will be measured either by the relative viable cell count or the relative binucleation, whichever is more sensitive.

### Percent Micronucleus Determination

The percent micronucleus is determined based on at least 500 BN (typically 1000 binucleated (BN) cells/tissue). Tissues with < 500 BN cells are considered unscorable. The MN scoring is conducted separately from the % BN count. Count the number of binucleated cells with and without micronucleus. Use 40 to 60X objectives in “dry” condition for the MN slide scoring.

Use the following calculation to determine the % micronucleus of a slide:

$$\% \text{ micronucleus} = \frac{\# \text{ of BN cells with at least one micronucleus} \times 100}{\text{Total \# of binucleated cells}}$$

### CRITERIA FOR DETERMINATION OF A VALID TEST

A number of validity criteria have been defined to ensure that only valid experiments are used in the assessment of a substance.

- The yield of viable cells in the vehicle control should be higher than  $5 \times 10^4$  cells per tissue. Any vehicle treated tissue with less than  $5 \times 10^4$  cells per tissue is eliminated. If less than two tissues meet the criterion, the entire experiment is not valid.
- The binucleation rate in each vehicle control tissue must be at least 25%. Any control tissue with < 25% binucleation in the vehicle is eliminated. If less than two tissues meet the criterion, the entire experiment is not valid.
- The positive control must cause a statistically significant increase in the % MN compared with the average of the vehicle control tissues (one sided fisher's exact  $p < 0.05$ ). If not met, the entire experiment is not valid.
- Controls and at least three concentrations\* of the test article must meet the criteria below:
  - Controls and each valid concentration will have at least two tissues per treatment.
  - 500 total cells (minimum) per tissue are analyzed for proliferation (1N, 2N,  $\geq 3N$ ). Any tissue with < 500 cells is considered 100% toxic and averaged into the group toxicity.
  - The % relative viable cell count is 40% or more in each test article-treated tissue (no more than  $55\% \pm 5\%$  toxicity) compared to the average of vehicle control tissues.

- The % MN in the solvent controls (average) should not exceed the historical vehicle control range for the testing lab.

\*An experiment with only two valid dose-groups can be considered a valid experiment if one or both of the concentrations was clearly positive and cytotoxicity was acceptable.

If the vehicle or positive control fails to meet any criterion, the entire experiment is not valid.

## **ASSESSMENT**

### **Statistical Analysis**

Statistical analysis will employ a Fisher's exact test for pair-wise comparisons between each treated and vehicle control treatment groups. The Cochran-Armitage trend test will also be conducted. One-sided tail probabilities for an increase or positive trend will be used to evaluate statistical significance ( $p < 0.05$ ).

Positive or Negative results, both for test articles and experiments, are determined according to the following criteria:

#### **Assessment of an experiment:**

Results from concentrations higher than the lowest precipitating dose will not be considered in the assessment as remaining test material on the tissue surface has been shown to impact the tissue quality.

An experiment is considered positive if it has one or more concentrations that are statistically significant (experiment has to meet validity criteria). When a positive response is observed the Cochran-Armitage trend test ( $p < 0.05$ ) will be used in the overall judgment of the response.

An experiment is considered negative if no statistically significant or dose dependent increases in the frequency of MN-BN are observed.

Isolated statistically significant findings will not be considered relevant if the group mean does not exceed the upper 95% CI of the historical vehicle control group means.

Biological relevance of the findings will be considered, and will take into account the above mentioned points (dose-dependence, strength of the effect in relation to the 95%CI of the vehicle control) as well as reproducibility between tissues and experiments

#### **Assessment of a test article:**

- Positive call - A test article will be called positive overall if it has at least one experiment with two or more statistically significant concentrations, or one concentration that is statistically significant and biologically relevant (one sided Fisher's Exact test) in two independent studies.

- Negative call - A test article will be called negative overall if in two independent studies the test article produces no statistically significant or biologically relevant increase in the frequency of MNBN
- Equivocal call - Despite extensive testing, a test article may produce results that are neither clearly positive nor clearly negative. In those rare instances the test article may be considered to have produced equivocal responses.

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**APPROVALS**

Study Director Approval

  
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BioReliance Study Director

27 Oct 2014  
Date