STUDY REPORT

Study Title

Assessment of Chemical Sensitization Potential Using *In Vitro* Methods

Report Title

Assessment of Chemical Sensitization Potential of Selected Isothiazolinone Compounds Using *In Vitro* Methods

Study Number

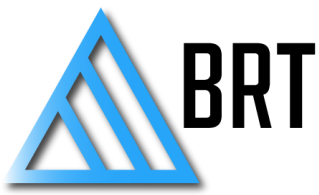
NIEHSO 20180515

Report Number

NIEHSO 20180515-1

Testing Facility
Burleson Research Technologies, Inc. (BRT)
120 First Flight Lane
Morrisville, NC 27560

03 December 2019



STUDY IDENTIFICATION

STUDY TITLE	Assessment of Chemical Sensitization Potential Using *In Vitro* Methods
STUDY NUMBER	NIEHSO 20180515
REPORT TITLE	Assessment of Chemical Sensitization Potential of Selected Isothiazolinone Compounds Using *In Vitro* Methods
REPORT NUMBER	NIEHSO 20180515-1
SPONSOR	National Toxicology Program (NTP) Immunotoxicology Testing Systems Toxicology Group Toxicology Branch 530 Davis Drive Durham, NC 27713
STUDY MONITOR	Dori. R. Germolec, Ph.D. (NTP)
TESTING FACILITY	Burleson Research Technologies, Inc. (BRT) 120 First Flight Lane Morrisville, NC 27560 Phone: 919-719-2500 Fax: 919-719-2505
STUDY DIRECTOR	Travis V. Gulledge, Ph.D. (BRT)
PRINCIPAL INVESTIGATOR FOR NTP IMMUNOTOXICOLOGY CONTRACT	Victor J. Johnson, Ph.D. (BRT)

COMPLIANCE STATEMENT

Although this study was performed as indicated in the study protocol and applicable BRT standard operating procedures (SOPs), it was investigational in nature and is not expected to conform to good laboratory practice (GLP) standards.

03 Dec 19

Travis V. Gulledge, PhD
Study Director
Burleson Research Technologies, Inc.
120 First Flight Lane
Morrisville, NC 27560
USA

Date

APPROVAL

Study Director Approval

Travis V. Gulledge, PhD
Study Director
Burleson Research Technologies, Inc.

03 Dec 19
Date

Victor J. Johnson, PhD
Principal Investigator for NTP
Immunotoxicology
Burleson Research Technologies, Inc.

03 Dec 19
Date

BRT Management Approval

Florence G. Burleson, PhD
Executive Vice President (Management)
Burleson Research Technologies, Inc.

03 Dec 19
Date

Report Review:

Gary R. Burleson, PhD
Deputy Principal Investigator
Burleson Research Technologies, Inc.

03 Dec 2019
Date

QUALITY ASSURANCE STATEMENT

STUDY NUMBER	NIEHSO 20180515
STUDY TITLE	Assessment of Chemical Sensitization Potential Using *In Vitro* Methods
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TESTING FACILITY	Burleson Research Technologies, Inc. 120 First Flight Lane Morrisville, NC 27560
STUDY DIRECTOR	Travis V. Gulledge, PhD

Phase Inspected	Date of Inspection	Date Report Sent to Study Director/Management
Protocol	26 Jun 18	26 Jun 18
Amendment 1	22 Oct 19	22 Oct 19
Draft Report and Raw Data (IT Chemicals)	01 Nov 19	01 Nov 19
Final Report (IT Chemicals)	03 Dec 19	03 Dec 19

The final report and data record audit were performed by the BRT Quality Assurance Unit.
To the best of my knowledge, this final report accurately describes the study methods and procedures used, and the reported results accurately reflect the raw data.

Sesha Jackson-Woodard
Quality Assurance Auditor

03 Dec 19
Date

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STUDY INFORMATION

Sponsor:

National Toxicology Program (NTP)
Immunotoxicology Testing Program, Systems Toxicology Group, Toxicology Branch
530 Davis Drive, Durham, NC 27713

NTP Contracting Officer's Representative (NTP COR):

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TESTING FACILITY AND KEY PERSONNEL

Testing Facility

Burleson Research Technologies, Inc. (BRT)
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PURPOSE

The purpose of this study was to assess chemical sensitization potential using *in vitro* methods. Results from the evaluation of selected isothiazolinone compounds using three *in vitro* methods are presented in this report, as requested by the Sponsor. The report number NIEHSO 20180515-1 indicates that this is the first report produced for this study. The three *in vitro* methods that have been approved by the Organization for Economic Co-operation and Development (OECD) during the rigorous European Union Reference Laboratory-European Centre for the Validation of Alternative Methods (EURL-ECVAM) testing and approval process for use in integrated approaches. Each method evaluates one specific key event identified in the skin sensitization adverse outcome pathway (AOP). The first method was the Direct Peptide Reactivity Assay (DPRA), which assesses protein reactivity of the chemicals using peptides as protein surrogates. The second method was the KeratinoSens™ assay, which assesses chemical activation of the Keap1-Nrf2-antioxidant/electrophile response element (ARE)-dependent pathway in a human-derived keratinocyte cell line. The third method was the human cell line activation test (h-CLAT), which assesses chemical activation of the immortalized human monocytic leukemia cell line, THP-1, as a dendritic cell (DC) surrogate. Information from these *in vitro* methods will expand the applicability domain for these methods and add to available information that can be used to predict the skin sensitization potential of the isothiazolinone compounds.

TEST AND CONTROL MATERIALS

Selected isothiazolinone antimicrobial pesticides were nominated by the Environmental Protection Agency (EPA) to the NTP for assessment of their potential to induce skin sensitization. BIT and CMIT/MIT were provided by Troy Corporation, OIT and BBIT were provided by Thor, MIT and DCOIT were provided by DOW Chemical Company. These chemicals were tested for sensitizing potential using the DPRA, KeratinoSens™, and h-CLAT methods. Table 1 provides a list of the five isothiazolinone compounds and one isothiazolinone mixture that were tested. Certificates of Analyses are provided in Appendix I for each compound.

Table 1: Isothiazolinones Tested Using *In Vitro* Skin Sensitization Tests

CAS #	Common Name	Chemical Name	Lot Number
2634-33-5	BIT	1,2-Benzisothiazolin-3-one	YL201811073
4299-07-4	BBIT	1,2-benzisothiazolin-3-one, 2-butyl	6445
2682-20-4	MIT	2-Methyl-4-isothiazolin-3-one	YY00H3A451
55965-84-9	CMIT/MIT Mixture		SLJ0229
26530-20-1	OIT	2-n-Octyl-4-isothiazolin-3-one	MX1837742006
64359-81-5	DCOIT	4,5-Dichloro-2-octyl-3(2h)-isothiazolone	YY00H77338

CMIT = 5-Chloro-2-methyl-4-isothiazolin-3-one

UNUSED TEST MATERIAL

Test chemical accountability was maintained by BRT. Residual test chemical final disposition will be documented in the study records at the completion of reporting for the isothiazolinone compounds.

HEALTH AND SAFETY

All compounds were considered as potential sensitizing agents and were handled with extreme care. Lab coats, nitrile gloves, Tyvec sleeve guards, and safety glasses were worn at all times when handling neat compounds. Neat compounds and working stocks were prepared in the fume hood in Lab 4. These procedures were determined to be appropriate by a certified industrial hygienist.

TEST SYSTEMS AND METHODOLOGY

Key Event 1 – Protein Binding (Direct Peptide Reactivity Assay [DPRA])

Test chemicals were evaluated for reactivity with peptides containing cysteine or lysine. HPLC analysis of peptide concentrations in solution determined the percent peptide depletion caused by each test compound, thereby indicating the degree of peptide reactivity.

Briefly, for the DPRA, test compounds were mixed with a cysteine-containing peptide and a lysine-containing peptide and interaction of the test compound with the peptides was measured by HPLC analysis. Reactive chemicals that have the potential to cause skin sensitization bind to the peptides resulting in their depletion. The percent depletion of the peptide peaks was used to classify each test chemical as a non-sensitizer, or as a low, moderate, or high sensitizer. Acceptance criteria for assay controls and test compound results were obtained from the OECD 442C guideline[1]. Details of the method are provided in Appendix II.

Key Event 2 – Events in Keratinocytes (KeratinoSens™ Assay)

Test chemicals were evaluated for activation of the Keap1-Nrf2-ARE-dependent pathway using the immortalized, human-derived keratinocyte cell line KeratinoSens™. KeratinoSens™ cells are transfected with a plasmid containing the luciferase gene whose expression is under control of the AKR1C2 gene ARE sequence upstream of the SV40 promoter. The amount of luciferase expression was measured using a luminescent substrate and a luminometer and represents a measure of keratinocyte activation.

For the KeratinoSens™ assay, the human keratinocyte cell line, KeratinoSens™, was treated with each test compound at a range of concentrations. Activation of the Keap1-Nrf2-ARE-dependent pathway was determined by measuring the amount of luminescence in each well after lysing cells and adding a luminescent luciferase substrate, using a luminometer. Cell viability was measured in parallel using the MTT assay. Increased Keap1-Nrf2-ARE-dependent light production was indicative of

keratinocyte activation and used to classify each test chemical as a non-sensitizer or sensitizer. Acceptance criteria for assay controls and test compound results were obtained from the OECD 442D guideline[2]. Details of the method are provided in Appendix III.

Key Event 3 – Events in Dendritic Cells (Human Cell Line Activation Test [h-CLAT])

THP-1 cells were treated with each test chemical at a range of concentrations determined from the dose finding assay (data from dose finding assays were maintained in the study records). The results of the dose finding assay were used to derive the CV75 concentrations which were used to calculate the CV75*1.2 starting concentration (Table 7). DC activation was determined by measuring expression of CD86 and CD54 on the cell surface by flow cytometry. Increases in the relative fluorescent intensity (RFI) greater than or equal to 150 for CD86 (EC150) and/or greater than or equal to 200 for CD54 (EC200) expression are indicative of DC activation. Cell viability was measured concurrently in the same cell population using propidium iodide (PI) staining. Acceptance criteria for assay controls and test compound results were obtained from the OECD 442E guideline[3]. Details of the method are provided in Appendix IV.

CALCULATIONS AND STATISTICAL ANALYSIS

Calculations and graphing were performed in Microsoft Excel 2016.

MAINTENANCE OF RAW DATA AND RECORDS

All raw data were labeled with the Burleson Research Technologies (Testing Facility) study number. All raw data, protocol and amendments, and Final Report and amendments generated by the Testing Facility will be archived at study completion and retained in the archive of the Testing Facility until transferred to the NTP Archive within 240 days of signing the final report. All in-life raw data not specific to this study (e.g., instrument logs, CVs, etc.) will be archived by the Testing Facility.

RESULTS

DPRA

The DPRA data for isothiazolinones were collected in a single successful assay run with all compounds initially dissolved at 100 mM in acetonitrile (DCOIT, BBIT, MIT, OIT), water (CMIT/MIT), or acetonitrile:water (BIT). These solvents were selected based on the study protocol (Appendix II) and OECD guideline. Table 2 provides the summary data for predictions of the sensitizing potential of the five isothiazolinone compounds and one mixture according to the DPRA. All of the isothiazolinones tested showed high reactivity and were classified as sensitizers. The DPRA data and calculations are provided in Appendix V. Positive and negative controls met test acceptance criteria demonstrating acceptable assay performance (Control data provided in Appendix XI).

Table 2: Summary of the sensitizing potential of isothiazolinone compounds as predicted by the DPRA.

Compound	Mean Cysteine % Depletion	Mean Lysine % Depletion	Mean % Cys+Lys Depletion	Reactivity Class	Prediction
[1]BIT	100	[1]Int	-	High Reactivity	Sensitizer
[2]CMIT/MIT	100	10.6	55.3	High Reactivity	Sensitizer
OIT	100	0	50	High Reactivity	Sensitizer
BBIT	100	0	50	High Reactivity	Sensitizer
MIT	100	0	50	High Reactivity	Sensitizer
DCOIT	100	10.4	55.2	High Reactivity	Sensitizer

Positive and negative control data met acceptance criteria and are provided in Appendix XI.
[1]Co-elution interference observed with the lysine peptide.
[2]Tested at a purity of 14.2% (10.8% CMIT + 3.4% MIT) and a weighted MW of 141.36 ((0.761*149.592) CMIT + (0.239*115.15) MIT) in accordance with the OECD guideline.

KeratinoSens™

A total of 3 assays were performed to collect data for the isothiazolinones (Table 3).

Table 3: KeratinoSens™ Assay Traceability Matrix

Assay	Outcome	Included in Report
Run 1	Assay failed due to variable gene induction in blanks	No
Run 2	Assay accepted	Yes
Run 3	Assay accepted	Yes

The compounds were prepared for the assay as outlined in Table 4. All compounds were dissolved at 200 mM in DMSO except for OIT and BBIT, which were prepared at concentrations of 50 mM, and DCOIT, which was prepared at 6.25 mM, based on the results of solubility testing. These solvents were selected based on the study protocol (Appendix III) and OECD guideline. Starting concentrations were made by diluting these stocks by 100X. The five individual isothiazolinone compounds were prepared using the molecular weights (MW) and purities reported on the certificates of analysis (Appendix I). Stock concentrations of each individual isothiazolinone compound were prepared by adding an appropriate amount of solvent determined by multiplying the amount of isothiazolinone weighed by the purity, dividing by the MW, and dividing by the intended stock concentration (millimolar). The CMIT/MIT mixture was prepared according to the OECD guideline for test chemicals without a defined MW by preparing at a default concentration of 40 mg/mL and assuming a MW of 200 g/mol and purity of 100% to prepare the "200 mM" working solution. Preparation of CMIT/MIT in this manner resulted in cytotoxicity at the top concentrations and gene induction above the 1.5-fold threshold at non-cytotoxic concentrations indicating the appropriate dose range was selected for the assay.

Table 4: KeratinoSens™ Starting Concentration, Dilution scheme, and Solvent.

Compound	Starting Concentration (µM)	Dilution Scheme	Solvent
BIT	2000	1:2	DMSO
CMIT/MIT	[1]401.8	1:2	DMSO
OIT	500	1:2	DMSO
BBIT	500	1:2	DMSO
MIT	2000	1:2	DMSO
DCOIT	62.5	1:2	DMSO

[1]CMIT/MIT was prepared according to the OECD guideline for test chemicals without a defined molecular weight (MW) by preparing at a default concentration of 40 mg/mL and assuming a MW of 200 g/mol and purity of 100% to prepare the 200 mM working stock solution. The working stock solution was diluted 100-fold to reach the final concentration of 2000 µM. Adjusting this concentration using a combined purity of 14.2% and weighted MW of 141.36 ((0.761*149.592) CMIT + (0.239*115.15) MIT), as was performed for the DPRA, translates to a starting concentration of 401.8 µM. This concentration is derived by calculating the amount of material added to the wells using the original assumptions (2000 µmol/L × 200 µg/µmol ÷ 1000 mL/1 L = 400 µg/mL) and then determining the amount of CMIT/MIT with a combined purity of 14.2% (400 µg/mL × 0.142 purity = 56.8 µg/mL) and converting to µM units with the weighted MW (56.8 µg/mL ÷ 141.36 µg/µmol × 1000 mL/1 L = 401.8 µM). A simpler way to calculate the adjusted concentration is to multiply the concentration by the combined purity and divide by a correction factor of the weighted MW to assumed MW ratio (141.36 g/mol ÷ 200 g/mol = 0.7068 correction factor). 2000 µM × 0.142 ÷ 0.7068 = 401.8 µM.

A summary of the KeratinoSens™ predictions for the five isothiazolinone compounds and one mixture is provided in Table 5. The results of the two accepted assays demonstrate matching predictions for all of the isothiazolinones. All of the isothiazolinones showed activation of KeratinoSens™ resulting in positive predictions for sensitization. Assay data and associated calculations for each assay run are provided in Appendix VI. Tabulated individual run induction values and viability results are shown in Appendix VII and Appendix VIII, respectively. Control data meet test acceptance criteria and are displayed in Appendix XII.

Table 5: Summary of the sensitizing potential of isothiazolinone compounds as predicted by the KeratinoSens™ assay.

Final Summary					
Compound	**Pass Viability**	**Prediction**	**$EC_{1.5}$ (µM)**	**I_{max}**	**IC_{50} (µM)**
BIT	Yes	Positive	3.14	17.64	57.80
CMIT/MIT	Yes	Positive	[1]3.41	5.61	[1]19.87
OIT	Yes	Positive	2.19	3.70	12.66
BBIT	Yes	Positive	3.84	19.61	52.98
MIT	Yes	Positive	9.54	15.84	108.25
DCOIT	Yes	Positive	1.32	4.37	4.65

Positive and negative control data met acceptance criteria and are provided in Appendix XII.
$EC_{1.5}$, I_{max}, and IC_{50} values are mean values from two independent runs passing acceptance criteria.
[1]CMIT/MIT was prepared according to the OECD guideline for test chemicals without a defined molecular weight (MW) by preparing at a default concentration of 40 mg/mL and assuming a MW of 200 g/mol and purity of 100%. Adjusting these concentrations using a combined purity of 14.2% and weighted MW of 141.36 ((0.761*149.592) CMIT + (0.239*115.15) MIT), as was performed for the DPRA, translates the calculated $EC_{1.5}$ of 16.99 µM to 3.41 µM and the calculated IC_{50} of 98.88 µM to 19.87 µM. This concentration is derived by multiplying the concentration shown by the combined purity and dividing by a correction factor of the weighted MW to assumed MW ratio (0.7068) as described in the figure legend for Table 4.

h-CLAT

A total of 5 assays were performed to collect data for the five isothiazolinone compounds and one mixture (Table 6).

Table 6: h-CLAT Traceability Matrix

Dose Finder Assay [Chemicals Tested]	**Outcome**	**Included in Report**
Run 1 [BIT, CMIT/MIT, OIT, BBIT, MIT, DCOIT]	BBIT and DCOIT did not pass the viability threshold at all concentrations testedand OIT required a repeat due to toxicity and variability between sets.	Yes
Run 2 [CMIT/MIT, OIT, BBIT, DCOIT]	Assay accepted.	Yes
Run 3 [CMIT/MIT]	CMIT/MIT repeated due to low toxicity in the main experiment runs.	Yes
Main Assay [Chemicals Tested]	**Outcome**	**Included in Report**
Run 1 [BIT, CMIT/MIT, OIT, BBIT, MIT, DCOIT]	BBIT and CMIT/MIT did not meet viability requirements	Yes
Run 2 [BIT, CMIT/MIT, OIT, BBIT, MIT, DCOIT]	CMIT/MIT did not meet viability requirements	Yes
Run 3 [BBIT, CMIT/MIT]	CMIT/MIT did not meet viability requirements	Yes
Run 4 [CMIT/MIT]	[1]Assay repeated	Yes
Run 5 [CMIT/MIT]	[1]Assay repeated	Yes
Run 6 [CMIT/MIT]	Assay accepted.	Yes
Run 7 [CMIT/MIT]	Assay accepted.	Yes

[1]DNCB viability was below 50% in Run 4 and Run 5. CD86, CD54, and IgG_1 MFI values were comparable to historical run results indicating that diffuse labeling of cytoplasmic structures was not evident. Assay was repeated to confirm results.

The compounds were prepared for the assay as outlined in Table 7. These solvents were selected based on the study protocol (Appendix IV) and OECD guideline. The five individual isothiazolinone compounds were prepared using the purities reported on the certificates of analysis (Appendix I). Stock concentrations of each individual isothiazolinone compound were prepared by adding an appropriate amount of solvent determined by multiplying the amount of isothiazolinone weighed by the purity and dividing by the intended stock concentration (mg/mL). The CMIT/MIT mixture was prepared by assuming a purity of 100% to prepare the 100 mg/mL working solution prior

to diluting 100-fold for the dose finding assay. Preparation of CMIT/MIT in this manner resulted in cytotoxicity and determination of a CV75, and therefore CV75*1.2, for subsequent evaluation of CD86 and CD54 surface expression indicating that an appropriate dose range was selected for the assay.

Table 7: Isothiazolinone Solvent and Starting (Highest Tested) Concentrations for testing in the h-CLAT.

Compound	Selected Solvent	Starting Concentration (µg/mL)	CV75 (µg/mL)
BIT	DMSO	15.7	13.1
[1]CMIT/MIT	PBS	[3]3.65	[3]3.04
OIT	DMSO	10.6	8.8
[2]BBIT	DMSO	4.0	3.3
MIT	PBS	29.5	24.6
DCOIT	DMSO	1.1	0.9

[1]CMIT/MIT was initially prepared at 1.90 µg/mL (starting concentration) for Run 1 and then increased to 2.22 µg/mL for Runs 2 & 3 to increase cytotoxicity. The dose finder assay was repeated, and the starting concentration was adjusted to 3.65 µg/mL for Runs 4-6.
[2]BBIT starting concentration was adjusted to 4.0 µg/mL for Runs 2 & 3 to decrease cytotoxicity.
[3]CMIT/MIT was prepared for the assay by assuming a purity of 100%. Using a combined purity of 14.2%, as was utilized for the DPRA, translates the calculated starting concentration of 25.7 µg/mL to 3.65 µg/mL and calculated CV75 of 21.4 µg/mL to 3.04 µg/mL.

A summary of the h-CLAT predictions for the five isothiazolinone compounds and one mixture is provided in Table 8. The results of the two accepted assays demonstrate matching predictions for all of the isothiazolinones. If more than one EC150 or EC200 value was calculated for a compound, the higher value was reported, as per the OECD guideline. All of the isothiazolinones showed activation of THP-1 cells resulting in positive predictions for sensitization. Assay data and associated calculations for each assay run are provided in Appendix IX. Individual run results are shown in Appendix X. Control data are consistent with historical run results and are shown in Appendix XIII.

Table 8: Summary of the sensitizing potential of isothiazolinone compounds as predicted by the h-CLAT.

Final Summary				
Compound	Pass Viability	Prediction	EC150 (µg/mL)	EC200 (µg/mL)
BIT	Yes	Sensitizer	7.84	7.63
[1]CMIT/MIT	Yes	Sensitizer	[2]2.81	[2]2.63
OIT	Yes	Sensitizer	7.26	0.95
BBIT	Yes	Sensitizer	3.15	3.01
MIT	Yes	Sensitizer	11.8	11.6
DCOIT	Yes	Sensitizer	No Induction	0.92

Positive and negative control data met acceptance criteria and are provided in Appendix XIII.
[1]EC150 and EC200 values reported from Runs 6 and 7.
[2]CMIT/MIT was prepared by assuming a purity of 100%. Using a combined purity of 14.2%, as was utilized for the DPRA, translates the calculated EC150 of 19.8 µg/mL to 2.81 µg/mL and the calculated EC200 of 18.52 µg/mL to 2.63 µg/mL.

CONCLUSIONS

A total of five isothiazolinone compounds and one isothiazolinone mixture were tested for skin sensitizing potential using the *in vitro* methods DPRA, KeratinoSens™, and h-CLAT. All assays demonstrate positive predictions for sensitization for all of the isothiazolinones tested.

REFERENCES

1. *Test No. 442C: In Chemico Skin Sensitisation*. (OECD, 2019). doi:10.1787/9789264229709-en
2. *Test No. 442D: In Vitro Skin Sensitisation*. (OECD, 2018). doi:10.1787/9789264229822-en
3. *Test No. 442E: In Vitro Skin Sensitisation*. (OECD, 2018). doi:10.1787/9789264264359-en

LIST OF APPENDICES

Appendix I: Certificates of Analysis for the isothiazolinone compounds.

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TROY

The Gold Standard for Performance

Troy Chemical Corporation Inc.

Date: Jan-8-2019
Customer Name:
Customer Order Number:
Customer Code:
Quantity & Weight
Remarks:

CERTIFICATE OF ANALYSIS

Product: **Mergal BIT Technical**

Lot: **YL201811073**

Characteristics	Specifications	Actual Lot Analysis
BIT, % Appearance	83.5 min Light Yellow or Off-White Powder	85.2 Pass

Date of Manufacture: Nov 2018
Expiration Date: Nov 2021

This Certificate is generated from a computerized system by the QC Manager. Authorized signature is not required.

The Gold Standard for Performance

Troy Chemical Corporation Inc.

Date: Jan-07-2019
Customer Name:
Customer Order Number:
Customer Code:
Quantity & Weight
Remarks: Expiration Date is Oct 07,2020

CERTIFICATE OF ANALYSIS

Product: MERGAL MITZ

Lot: SLJ0229

Characteristics	Specification	Actual Lot Analysis
Appearance	Colorless Liquid to Light Yellow Liquid	Colorless Liquid to Light Yellow Liquid
5CMIT, %	10.0 – 11.6	10.8
MIT, %	3.0 – 4.1	3.4
5CMIT + MIT, %	14.0 Min.	14.2
D-CMIT, %	0.1 Max.	0.0
Color, Gardner	5 Max.	0.7
Density @ 20C	1.25 – 1.33	1.31
pH	4 Max.	3

Date of Manufacture: Oct-2018

This Certificate is generated from a computerized system by the QC Manager. Authorized signature is not required.

Certificate of Analysis

THOR

Print Date: July 31, 2018

Issue Date: July 31, 2018

Product: ACTICIDE® OIT

Batch No: MX-183774-2006

Production Date: 06/2018

Expiry Date*: 30-Jun-2020

Minimum shelf-life: *24 months*

Analyzed Property	Unit	Results	Specification	Method
Appearance		OK	Clear yellow to brown liquid	QK 118
OIT	%	98.13	95 - 100	QK 101
Water content	%	0.34	0 - 0.5	QK 107

*If stored in accordance with chapters 7 & 10 of the Safety Data Sheet.

Some products are able to be retested and the expiry date extended if results warrant. Please contact your Sales Rep or Thor Specialties, Inc., directly for additional information.

The information presented above is believed to be accurate. However, said information and products are offered without warranty or guarantee except as to the composition and purity stated herein since the ultimate conditions of use and the variability of the materials treated are beyond our control.

This lot was manufactured in Querétaro, Mexico. It does not meet the eligibility requirements for NAFTA certification.

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Fax: 0034 93 833 37 13
email: thor@thor-spain.com

This Quality Assurance document has been generated by computer and is valid without signature.

LONZA GLP SERVICES
1200 BLUEGRASS LAKES PARKWAY
ALPHARETTA, GA 30004

Certificate of Analysis

Test or Reference Substance Name: Vanquish 100

Lot Number: 6445 **Expiration Date (mm/dd/yyyy):** 05/15/2019

Storage Conditions: room temperature

Compound	Assay	Analytical Technique
2-Butyl-1,2-benzisothiazolin-3-one (BBIT)	98.9 %	HPLC

Comments:

Identity confirmed by LC-MS

Master Log Number/Notebook Number and page(s): SN 383-17B10BBIT/552

Characterization of this test or reference substance was performed under EPA FIFRA Good Laboratory Practice Standards (40 CFR 160).

Study Director: Linda Hull Date: 05/17/2017
Management: [signature] Date: 05/17/2017
QA: [signature] Date: 05/17/2017
Revised June 20, 2014

Page 1 of 1

Date 2018-03-23 (YYYY-MM-DD) Time 20:10:24 (Greenwich Mean Time) Page 1 of 1

THE DOW CHEMICAL COMPANY*

ROHM AND HAAS CHEMICALS LLC
PLANT A029
6101 ORR RD
CHARLOTTE NC 28213-1521

Certificate of Analysis

Product Number Product Name 00010076308

KORDEK™ 573F Industrial Microbiocide

Delivery No. 812425714 / 000010

Shipping Units 1.000 EA

Date Shipped 2018-03-23 (YYYY-MM-DD)

Shipment No. 31671115

Customer Information

Customer Name ROHM AND HAAS CHEMICALS LLC

Container ID 106-44925203

Vehicle# USF HOLLAND

Specification Number 000000142668

Batch Number	YY00H3A451
Expiration Date	2019-03-10 (YYYY-MM-DD)
Manufacturing Date	2017-03-10 (YYYY-MM-DD)
Quantity	1.000 EA
Net Weight	242.509 LB / 110.000 KG

Test	Unit	Lower Limit	Upper Limit	Value
A.I. (MIT)	%	50.0	52.0	50.8
Appearance	-	-	-	Pass
pH		3.0	6.0	3.6

For inquiries please contact Customer Service or local sales
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Date 2017-07-26 (YYYY-MM-DD) Time 08:55:37 (Greenwich Mean Time) Page 1 of 1

ROHM AND HAAS INTERNATIONAL TRADING
SHANGHAI CO., LTD.
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DOW CHEMICAL (SHANGHAI)
COMPANY LIMITED
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WAIGAOQIAO FREE TRADE ZONE
200131 SHANGHAI

Certificate of Analysis

Product Number	00010269161
Product Name	
KATHON™ 287T Industrial Microbicide	
Delivery No.	810808143 / 000010
Order Number	106838704
Shipping Units	120.000 KG
Date Shipped	2017-07-26 (YYYY-MM-DD)
Shipment No.	30174145

Customer Information

Customer Name	DOW CHEMICAL (SHANGHAI)
Customer PO number	sample20170710
Specification Number	000000142005

Batch Number	YY00H77338
Expiration Date	2019-07-07 (YYYY-MM-DD)
Manufacturing Date	2017-07-07 (YYYY-MM-DD)
Quantity	120.000 KG
Net Weight	120.000 KG

Test	Unit	Lower Limit	Upper Limit	Value
Appearance	-	-	-	**Pass**
Color, Gardner VCS		0	4	2
Water Content	%	0.00	0.07	0.02
A.I. (DCOIT)	%	95.0	100.0	99.3
Hydrochloric Acid	%	0.00	0.10	< 0.00

For inquiries please contact Customer Service or local sales

Appendix II: DPRA Methodology.

Purpose

The Direct Peptide Reactivity Assay (DPRA) is an *in vitro* method used for assessing the sensitization potential of chemicals. The DPRA is one of a battery of *in vitro* methods proposed as alternative approaches for the assessment of contact sensitizers. The DPRA evaluates peptide reactivity, or the ability of a chemical to bind to peptides, as a measure of haptenation using high performance liquid chromatography (HPLC). Haptenation is the process by which chemicals (haptens) bind to and alter endogenous proteins (carriers), thereby creating neo-antigens (hapten-carrier complexes), which can result in immune activation. Hapten-carrier complex formation is considered one of the first steps during initiation of skin sensitization reactions.

Materials

- Analytical balance; capable of accurately measuring up to 20 grams with at least 0.1 mg readability
- Analytical micropipettes
- Chemical fume hood
- Glass beakers
- Volumetric flasks
- Vacuum filtration units (VWR #97066-204 or equivalent)
- Vacuum pump
- Vacuum degassing flask with stopper and stop cock
- Dimethyl sulfoxide (DMSO)
- Isopropanol
- Acetone
- Benchtop vortex mixer
- Benchtop ultrasonic water bath
- High pressure liquid chromatograph with light-excluding autosampler capable of delivering 0.35 mL/min flow rate
- UV detector capable of measuring UV absorbance at 220 nm
- pH meter with electrode and calibration buffers

- HPLC Column, (Agilent Zorbax SB-C18 2.1 mm x 100 mm x 3.5 µm; Part #861753-902)
- Guard Column, (Phenomenex Security Guard C18 4 mm x 2 mm; Part #AJO-4286 or equivalent)
- 1.8 mL autosampler vials with closures (VWR Cat #89523-478 or equivalent)
- 4 mL glass vials with Teflon or polyethylene-lined closure (VWR Cat #66009-557 or equivalent)
- Trifluoroacetic acid (CAS# 76-05-1, Sigma-Aldrich #299537 or equivalent)
- Sodium Phosphate, monobasic monohydrate (CAS# 10049-21-5, Sigma-Aldrich #S9638 or equivalent)
- Sodium Phosphate, dibasic heptahydrate (CAS# 7782-85-6, Sigma-Aldrich #S9390 or equivalent)
- Ammonium Acetate (CAS# 631-61-8, Sigma-Aldrich #238074 or equivalent)
- Ammonium Hydroxide (CAS# 1336-21-6, Sigma-Aldrich #320145 or equivalent)
- Acetonitrile, HPLC Grade (CAS# 75-05-8, Sigma-Aldrich #439134 or equivalent)
  Note: Each new lot should be tested for impact on cysteine peptide stability.
- HPLC grade or Millipore Milli-Q grade water
- Cysteine peptide (Ac-RFAACAA-COOH), Store at ≤-20˚C (RS synthesis, Louisville, KY)
- Lysine peptide (Ac-RFAAKAA-COOH), Store at ≤-20˚C, (RS synthesis, Louisville, KY)
- Cinnamic Aldehyde, ~95% purity, (CAS# 104-55-2, Sigma-Aldrich #W228613 or equivalent)

Procedure

1. Buffer Preparation

Record all details of preparations .

1.1 Prepare 100 mM sodium phosphate monobasic solution using a volumetric flask:

1.1.1. Dissolve sodium phosphate monobasic monohydrate to 100 mM in HPLC-grade water (e.g. 13.8 g of sodium phosphate monobasic monohydrate in 1 L of HPLC-grade water).

1.1.2. Store at 2-8°C for up to 3 months.

1.2. Prepare 100 mM sodium phosphate dibasic solution using a volumetric flask:

1.2.1. Dissolve sodium phosphate dibasic heptahydrate to 100 mM in HPLC-grade water (e.g. 26.8 g of sodium phosphate dibasic heptahydrate in 1 L of HPLC-grade water).

1.2.2. Store at 2-8°C for up to 3 months.

1.3. Prepare 100 mM Phosphate buffer solution in a glass beaker:

NOTE: Oxygen will react with the thiol groups of the peptide resulting in disulfide bonds and precipitation of the peptide. It is important to use degassed buffer for reconstitution of the peptides.

1.3.1. Combine 0.1 M monobasic solution with 0.1 M dibasic solution at a 1:4.56 ratio (e.g. 18 mL of 0.1 M monobasic with 82 mL of 0.1 M dibasic).

1.3.2. Adjust the pH to 7.5 ± 0.05 using monobasic (to acidify) or dibasic (to basify) solution.

1.3.3. Transfer to a vacuum degassing flask. Degas under vacuum by sonicating for 10-15 minutes. Degas solution prior to each use in the assay.

1.3.4. Store at 2-8°C. Expiration date will be dependent on the monobasic and dibasic solutions used.

1.4. Prepare 100 mM Ammonium Acetate buffer solution in a glass beaker:

NOTE: Prepare buffer using a chemical fume hood

1.4.1. Dissolve ammonium acetate to 100 mM in HPLC-grade water (e.g. 1.542 g of ammonium acetate in 200 mL of HPLC-grade water).

1.4.2. Adjust pH to 10.2 by dropwise addition of ammonium hydroxide.

1.4.3. Store at 2-8°C for up to 2 weeks.

1.5. Prepare HPLC Mobile Phase A (aqueous solution)

NOTE: Prepare the HPLC solution using a chemical fume hood.

1.5.1. Dissolve Trifluoroacetic Acid (TFA) to 0.1% in HPLC-grade water (e.g. 1 mL of TFA to 1 L of HPLC-grade water).

1.5.2. Store at room temperature for up to 2 weeks.

1.6. Prepare HPLC Mobile Phase B (organic solution)

NOTE: Prepare the HPLC solution using a chemical fume hood.

1.6.1. Dissolve TFA to 0.085% in HPLC-grade acetonitrile (e.g. 850 µL of TFA to 1L of HPLC-grade acetonitrile).

1.6.2. Store at room temperature for up to 2 weeks.

2. Test Chemical Preparation Pre-Work

2.1. Test Chemical Solvent Selection (document using Attachment VI)

2.1.1. Dissolve test chemical in acetonitrile at 100 mM concentration. Vortex to mix. If the chemical is not completely dissolved, sonicate for up to one minute.

2.1.2. If the chemical will not dissolve in step 2.1.1, make a 100 mM solution using HPLC-grade water as the solvent. Vortex to mix. If the chemical is not completely dissolved, sonicate for up to one minute.

Note: Water is not a good solvent choice for anhydrides, which are reactive with water.

2.1.3. If the chemical will not dissolve in steps 2.1.1-2.1.2, make a 1:1 acetonitrile:water mixture and use as the solvent to make a 100 mM solution of test chemical. Vortex to mix. If the chemical is not completely dissolved, sonicate for up to one minute.

Note: This solvent mixture is typically effective for organic salts.

2.1.4. If the chemical will not dissolve in steps 2.1.1-2.1.3, make a 100 mM solution using isopropanol as the solvent. Vortex to mix. If the chemical is not completely dissolved, sonicate for up to one minute.

2.1.5. If the chemical will not dissolve in steps 2.1.1-2.1.4, make a 100 mM solution using either acetone or a 1:1 acetone:acetonitrile mixture as the solvent. Vortex to mix. If the chemical is not completely dissolved, sonicate for up to one minute.

2.1.6. If the chemical will not dissolve in steps 2.1.1-2.1.5, weigh enough chemical and dissolve in 1 part dimethyl sulfoxide (DMSO). Dilute this solution by 9 parts acetonitrile for a final 1:10 DMSO:acetonitrile solution. Vortex to mix. If the chemical is not completely dissolved, sonicate for up to one minute.

2.1.7. If chemical will not dissolve in steps 2.1.1-2.1.6, weigh the same amount of test chemical used in step 2.1.6., and add 1 part of DMSO to the chemical. Dilute this solution by adding 1 part of acetonitrile for a final 1:1 DMSO:acetonitrile solution. Vortex to mix. If the chemical is not completely dissolved, sonicate for up to one minute.

2.2. Pre-weigh control and test chemicals (document using Attachment VII)

2.2.1. Calculate the weight of positive control (cinnamic aldehyde) or test chemical needed to prepare 3.0 mL of a 100 mM solution using formula A:

$$\left(\frac{0.100\ \text{mol}}{\text{L}}\right) \text{x}\ (0.003\text{L})\ \text{x}\ \left(\text{MW in}\frac{\text{g}}{\text{mol}}\right) \text{x}\ \frac{100}{\%\ \text{Purity}} = \frac{\text{MW}}{\%\ \text{Purity}}\ \text{x}\ 30 = \text{Target Weight (mg)}$$

2.2.2. Weigh the target amount (± 10% of target) of positive control or test chemical directly into a 4 mL glass vial and record the actual weight, identity, molecular weight, and purity.

2.2.3. Tightly close each vial and store under appropriate conditions until ready to perform testing. See supplier information for proper storage information of each chemical.

Preparation of Test samples and Reference Control B replicates 1-3 should be timed to ensure that injection of the first set of replicates will start within 24 ± 2 hours of mixing. The order listed in the following sections for sample preparation are suggested but can be modified to accommodate this incubation period. Standards and controls do not have a specific time window. Total run length should not exceed 30 hours between the first and third injections for sample replicates.

3. Controls and Standards Preparation (document using Attachment VIII and IX)

3.1. Pre-weigh cysteine or lysine peptide for stock solutions (0.667 mM)

3.1.1. Pre-weigh an appropriate amount of cysteine into a test tube to prepare a solution that is 0.501 mg/mL (0.667 mM). Record the exact amount added to the test tube, or glass sample vial.

Note: Each sample replicate requires 750 µL of stock solution.

3.1.2. Pre-weigh an appropriate amount of lysine into a test tube to prepare a solution that is 0.518 mg/mL (0.667 mM). Record the exact amount added to the test tube or glass sample vial.

Note: Each sample replicate requires 750 µL of stock solution.

3.2. Dissolve the previously weighed cysteine and/or lysine in the appropriate buffer solution (**degassed** phosphate buffer for cysteine, and ammonium acetate buffer for lysine). The cysteine solution may be sonicated for 30-60 seconds to ensure complete solubilization of the peptide.

3.3. Prepare a dilution buffer that will be used to dilute the stock 0.667 mM peptide solution into standards.

3.3.1. Prepare dilution buffer by diluting acetonitrile to 20% in peptide buffer (**degassed** phosphate buffer for cysteine, ammonium acetate buffer for lysine).

3.4. Prepare standards 1-7 (STD1-7) in labeled HPLC autosampler glass vials as follows:

3.4.1. STD1: Aliquot 800 µL of the peptide solution followed by 200 µL of acetonitrile. Mix with minimal air entrainment by carefully micro pipetting.

3.4.2. STD2-7: Pipette 500 µL of dilution buffer (prepared in step 3) into each of 6 autosampler glass vials.

3.4.3. Transfer 500 µL of STD1 to STD2. Mix by carefully pipetting.

3.4.4. Transfer 500 µL of STD2 to STD3. Mix by carefully pipetting.

3.4.5. Transfer 500 µL of STD3 to STD4. Mix by carefully pipetting.

3.4.6. Transfer 500 µL of STD4 to STD5. Mix by carefully pipetting.

3.4.7. Transfer 500 µL of STD5 to STD6. Mix by carefully pipetting.

3.4.8. STD7 will contain only the dilution buffer.

3.5. Record the time that standard preparation is complete.

3.6. For cysteine samples only, loosen caps and sonicate for 30-60 seconds. Carefully tighten caps and place the vials in the autosampler until analysis.

3.7. Reference Controls

3.7.1. Reference Controls A and B: verifies that the peptide solutions can be accurately quantified from the standard curve and are stable during the

analysis time. Label 3 vials as Reference A and the remaining 6 vials as Reference B for each peptide set and prepare following the table below:

Cysteine Peptide	Lysine Peptide
• 750 µL cysteine solution • 250 µL acetonitrile	• 750 µL lysine solution • 250 µL acetonitrile

3.7.2. Reference Control C: verifies that solvent does not impact the percent peptide depletion. Triplicate samples should be prepared for each solvent used following the table below:

Cysteine Peptide	Lysine Peptide
• 750 µL cysteine solution • 200 µL acetonitrile • 50 µL of solvent	• 750 µL of lysine solution • 250 µL of solvent

3.7.3. Record the time that solutions are completed.

3.7.4. For cysteine samples only, loosen caps and sonicate for 30-60 seconds. Carefully tighten caps and place all samples in the HPLC autosampler until analysis.

3.8. Co-elution Controls

3.8.1. Create one co-elution control for each test chemical following the table below:

Cysteine Co-Elution	Lysine Co-Elution
• 750 µL of phosphate buffer • 200 µL of acetonitrile • 50 µL of test chemical	• 750 µL of ammonium acetate buffer • 250 µL of test chemical

3.8.2. Record the time that all samples are completed and place all samples in the autosampler until analysis.

3.9. Positive Controls

3.9.1. Dissolve pre-weighed cinnamic aldehyde in 3.0 mL of acetonitrile.

3.9.2. Prepare the samples in triplicate following the table below:

Cysteine Peptide	Lysine Peptide
• 750 µL cysteine solution • 200 µL acetonitrile • 50 µL of cinnamic aldehyde	• 750 µL of lysine solution • 250 µL of cinnamic aldehyde

3.9.3. Record the time that solutions are completed.

3.9.4. For cysteine samples only, loosen caps and sonicate for 30-60 seconds. Carefully tighten caps and place all samples in the autosampler until analysis.

4. Test Chemical Preparation (document using Attachment VII)

4.1. Dissolve pre-weighed test chemicals in the appropriate solvent determined in section **2. Test Chemical Preparation Pre-Work**.

4.2. Prepare each test chemical in triplicate following the table below:

Cysteine Peptide Test Sample	Lysine Peptide Test Sample
• 750 µL Cysteine peptide solution • 200 µL Acetonitrile • 50 µL of Test Chemical Solution	• 750 µL Lysine peptide solution • 250 µL Test Chemical Solution

4.3. Record the time that each sample is completed.

4.4. Visually inspect samples for precipitation and record if precipitate is observed. If necessary, samples that have precipitate may be centrifuged at 300 x g for 5 minutes to pellet precipitate and transferred to a new vial to prevent clogging of the HPLC tubing or columns prior to analysis.

4.5. For cysteine samples only, loosen caps and sonicate for 30-60 seconds. Carefully tighten caps and place in the autosampler until analysis. Beginning the run sequence must be timed such that the first replicate of the first test chemical in the test sample set begins within 24 ± 2 hours of mixing with the peptide.

5. HPLC Analysis (document using Attachment X)

5.1. Setup the HPLC system

5.1.1. If the HPLC system has not been used in over a week, install the C18 column in its proper orientation, and turn on the instrument. If the instrument is already on, proceed to step 5.2.

5.1.2. Use the appropriate login credentials to open LabSolutions and purge the lines or rinse with ethanol according to the instructions in SOP BRT 254-XX.

5.1.3. Record the preparation and expiration date of the isopropanol rinsing solution. If expired, prepare a new solution, and replace the expired solution.

5.1.4. Equilibrate the column for 2 hours at 50% HPLC Mobile Phase A and 50% HPLC Mobile Phase B at an oven temperature of 30°C and 0.35 mL/min flow rate.

5.1.5. Condition the column by running the gradient (shown in step 5.2.3 below) at least twice.

5.2. Setup the LabSolutions software

5.2.1. Create a new folder for the HPLC run. Copy the "DPRA Method" file into the new folder. Create a batch file to analyze each sample using the "DPRA Method" file, name each vial according to the run sequence (an example run sequence is shown in step 2 below), assign samples to the correct tray and vial position, and set the sample injection volume to 7 µL.

5.2.2. Assign the first two rows to vial "-1", to run the gradient without injecting sample. This will condition the column twice before proceeding to the first sample.

5.2.3. The flow conditions in the "DPRA Method" file should be set for 20 minutes total as outlined in the following table:

Time (min)	Flow (mL/min)	%A	%B
0	0.35	90	10
10	0.35	75	25
11	0.35	10	90
13.5	0.35	90	10
20	End Run		

5.2.4. Select "Start Realtime Batch" on the left panel.

5.2.5. Select the "Column Equilibration" file for the Startup procedure and set the "Pumping Period" to 120 min to equilibrate the column prior to the analysis.

5.2.6. Select the "Low Flow" file for the Shutdown procedure and set "Cool Down Time" to an appropriate amount of time to ensure that the procedure

does not end before the instrument can be shut down according to SOP BRT 254-XX or maintained in a low flow state if the next analysis will be performed in ≤ 1 week.

5.3. Begin the HPLC run

5.3.1. Set the start time and date to begin sample analysis so that the first test chemical replicate is injected 24 ± 2 hours after it was mixed with peptide. Take into account 2 hours of column equilibration, 40 minutes of column conditioning, 140 minutes for standards, 60 minutes for Reference Control A, 60 minutes for Reference Control B rep 1-3, and 20 minutes for the first replicate of each Reference Control C (at least 1, but possibly up to 7). Additionally, co-elution controls may be run prior to test chemical samples and the time to run these controls should also be considered when determining the start time.

5.4. Shutdown the HPLC system

5.4.1. After sample analysis is complete, visually inspect samples for precipitation and record if precipitate is observed.

5.4.2. 100% acetonitrile should be run over the column for 1 hour (or longer if necessary) to completely clear the column. If this step is not performed, a peak could co-elute in the cinnamic aldehyde positive control sample with the lysine peptide peak.

5.4.3. If the column will be stored for more than one week, fill with acetonitrile (without TFA), cap both ends, and store at room temperature. Purge acid-containing mobile phases from the system with a 1:1 mixture of acetonitrile:water. Shut down the HPLC instrument according to SOP BRT 254-XX. If further analyses will be performed in ≤ 1 week, reduce the flow rate to 0.05 mL/min with 50% HPLC Buffer A:50% HPLC Buffer B and decrease column temperature to 25°C.

5.5. Run sequence example:

• **STD1** • **STD2** • **STD3** • **STD4** • **STD5** • **STD6** • **STD7 (Dilution Buffer)** • **Reference Control A, rep 1** • **Reference Control A, rep 2** • **Reference Control A, rep 3**	**Calibration Standards and Reference Controls:** • Verify linearity of response • Verify precision and accuracy of pipetting **System Suitability** $R^2 > 0.990$ Mean peptide concentration of reference control A = 0.50 ± 0.05 mM
• **Reference Control B, rep 1** • **Reference Control B, rep 2** • **Reference Control B, rep 3**	**Reference Controls** • Verify stability of reference controls over analysis time
• **Reference Control C, rep 1 (acetonitrile)** • **Reference Control C, rep 1 (water, etc.)** • **Cinnamic Aldehyde, rep 1** • **Sample 1, rep 1** • **Sample 2, rep 1, cont. to Nth sample**	**First set of replicates** • Start first test chemical replicate 24 hours ± 2 hours after mixing
• **Reference Control C, rep 2 (acetonitrile)** • **Reference Control C, rep 2 (water, etc.)** • **Cinnamic Aldehyde, rep 2** • **Sample 1, rep 2** • **Sample 2, rep 2** • **Sample 3, rep 2, cont. to Nth sample**	**Second set of replicates**
• **Reference Control C, rep 3 (acetonitrile)** • **Reference Control C, rep 3 (water, etc.)** • **Cinnamic Aldehyde, rep 3** • **Sample 1, rep 3** • **Sample 2, rep 3** • **Sample 3, rep 3, cont. to Nth sample**	**Third set of replicates**
• **Reference Control B, rep 4** • **Reference Control B, rep 5** • **Reference Control B, rep 6**	**Reference Controls** 1. Verify stability of reference controls over analysis time: CV of peptide peak areas of the nine reference controls B and C in acetonitrile must be < 15.0%
• **Co-elution Control 1**	**Co-elution Controls**

• **Co-elution Control 2** • **Co-elution Control 3, cont. to Nth sample**	• Verify co-elution of test chemicals with peptide

6. Data Analysis

6.1. The LabSolutions software integrates peaks automatically. In the Post-Run analysis, manually check each chromatogram to ensure peptide peaks are integrated appropriately. All peaks must be consistently integrated via "valley to valley." Reject test chemical peaks integrated by LabSolutions that have retention times different from the expected peptide retention time. Select the check box in the "Report Output" column for each sample (excluding column conditioning) and click "Start" to begin post-batch analysis.

6.2. Print chromatograms for the study records.

6.3. Record the area of each integrated peptide peak into Excel. If co-elution of the test chemical with the peptide peak is suspected, confirm that the test chemical peak is present at the same retention time in the co-elution control chromatogram (remember to account for differences in the chromatogram y-axis scales) and record the result as "**Interference**."

6.4. Generate the calibration curve based on the standard concentrations and the peak areas. Acceptable curves should have an $R^2 > 0.990$.

6.4.1. If the standard curve does not meet criteria, the run must be repeated.

6.5. Record the individual peptide concentrations calculated for all reference controls.

6.6. For Reference Control A, calculate the mean peptide concentration, SD and CV for the 3 replicates. The mean should be 0.50 ± 0.05 mM.

6.6.1. If the mean value is not met, repeat the experiment as it is generally indicative of a pipetting or sample preparation error.

6.7. For the nine Reference Controls B and C in acetonitrile, calculate the mean peptide peak area, SD, and CV across each control set.

6.7.1. For Reference Control C only, calculate the mean peptide peak area for the 3 Reference Control C replicates for each solvent used.

6.8. Calculate the mean peptide concentration, SD and CV for the 3 Reference Control C replicates for each solvent used. The mean should be 0.50 ± 0.05 mM. Verify the UV absorbance spectrum and retention time are consistent for the Reference Control C injections.

6.9. For the positive control and for each test chemical, calculate the percent peptide depletion in each replicate from the peptide peak area of the replicate injection and the mean peptide peak area of the three relevant Reference Controls C using the following formula:

$$\left(1 - \frac{\text{Peptide Peak Area in Replicate Injection}}{\text{Mean Peptide Peak Area in Reference Controls C}}\right) \times 100$$

6.9.1. Record the percent peptide depletion for each injected positive control and test chemical replicate. Additionally, record the mean percent peptide depletion of the three replicate determinations, SD, and CV. Where appropriate, report results to one decimal place.

7. Acceptance Criteria

7.1. System Suitability:
Calibration linearity $R^2 > 0.990$
Mean peptide concentration of Reference Controls A = 0.50 ± 0.05 mM.
Mean peptide concentration of solvent Reference Controls C = 0.50 ± 0.05 mM.
CV of the mean peptide peak area for Reference Controls B and Reference Controls C combined must be < 15.0%.

7.2. Positive Control:

	Percent Cysteine Depletion		Percent Lysine Depletion	
Positive Control	Lower Bound	Upper Bound	Lower Bound	Upper Bound
Cinnamic Aldehyde	60.8	100.0	40.2	69.4

- ✓ Standard deviation for percent cysteine depletion must be < 14.9%
- ✓ Standard deviation for percent lysine depletion must be < 11.6%

3. Test Chemical Acceptance Criteria:
   - ✓ Standard deviation for percent cysteine depletion must be < 14.9%
   - ✓ Standard deviation for percent lysine depletion must be < 11.6%

8. Chemical Classification Method

8.1. The mean percent peptide depletion of replicates is calculated for each test chemical and the positive control. **Negative depletion values should be considered as "0" when calculating the mean**.

8.2. The maximum standard deviation for the test chemical replicates should be < 14.9% for the percent cysteine depletion and < 11.6% for the percent lysine depletion. The mean peptide concentration of the three Reference Controls C in the appropriate solvent

should be 0.50 ± 0.05 mM. If these criteria are not met, the run should be repeated for that specific test chemical.

8.3. A reactivity category is assigned to each test chemical by using the cysteine 1:10/lysine 1:50 prediction model as shown:

Table I. Cysteine 1:10/Lysine 1:50 prediction model

Mean of Cysteine and Lysine % depletion	Reactivity Class	Prediction
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

8.4. There might be cases where the test chemical absorbs at 220 nm and has the same retention time as the peptide (co-elution). First, rule-out baseline noise by determining if the peak area of the "interfering" chemical peak is > 10% of the mean peptide peak area in the appropriate Reference Control. If the test chemical is confirmed to be interfering, the peak of the peptide cannot be integrated and the calculation of the percent peptide depletion is not possible. If co-elution of the test chemical occurs with cysteine only or cysteine and lysine peptides, then the analysis must be reported as "**interference**" for that test chemical. In cases where co-elution occurs only with lysine, then the cysteine 1:10 prediction model should be used as shown below.

Table II. Cysteine 1:10 prediction model

Cysteine (Cys) % depletion	Reactivity Class	Prediction
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

8.5. If the percent peptide depletion is < -10.0%, this could be a situation of co-elution, inaccurate peptide addition, or baseline "noise." If this occurs, the co-elution control for that test chemical should be carefully analyzed (as described above). If the retention time and shape of the peptide peak appear normal, the peak can be integrated. However, if the peak does not have the proper shape or retention time due to co-elution, then the peak cannot be integrated. If this issue occurred only with lysine, use the cysteine only model. If this issue occurred with cysteine only or both peptides, the result should be reported as "**Inconclusive**." If retention times do not completely overlap and underlying peaks can be de-convoluted, record with notation "co-elution – percent depletion estimated".

8.6. There might be other cases where the overlap in retention time between the test chemical and either of the peptides is incomplete. If lysine is the co-eluting peptide, the cysteine-only prediction model should be used. If cysteine is the co-eluting peptide, the Percent Peptide Depletion values can still be estimated and used in the cysteine 1:10/lysine 1:50 Prediction Model; however, assignment of the test chemical to a reactivity class must be

made with additional notation. If the result is "High Reactivity," it should be reported as such, but noted to be an estimation. If the result is "Moderate Reactivity" or "Low Reactivity," it should be noted as "≥ Moderate Reactivity" or "≥ Low Reactivity," respectively. If the result is "Minimal Reactivity," the result should be reported as "**Inconclusive**."

Appendix III: KeratinoSens™ Assay Methodology.

Purpose

The KeratinoSens™ is an *in vitro* test method, which measures activation of the Keap1-Nrf2-antioxidant/electrophile response element (ARE)-dependent pathway through luciferase gene induction in an immortalized adherent cell line derived from HaCaT human keratinocytes transfected with a selectable plasmid. The Keap1-Nrf2-ARE pathway has been reported to be an important regulator of protective responses to electrophiles and oxidative stress by controlling expression of detoxification, antioxidant, and stress response enzymes and proteins. Several *in vivo* studies have demonstrated the involvement of the Keap1-Nrf2-ARE pathway in skin sensitization; and therefore, information from the KeratinoSens™ is considered relevant for assessing the skin sensitization potential of chemicals.

Materials

- Sterile hood for cell culture work
- CO_2 incubator
- Multi-channel and single-channel pipettes for volumes between 1 µL and 1000 µL
- 96-well plate luminometer/spectrophotometer
- DMEM, low glucose, cell culture medium (Gibco, Cat#: 10567-014)
- Heat inactivated-fetal bovine serum (HI-FBS) (Gibco, Cat#: 10438-026 or equivalent)
- Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, Cat#: 14190-144)
- Trypsin-EDTA Solution (Gibco, Cat#: 25300 or equivalent)
- G418 (Geneticin) (Gibco, Cat#: 10131-027)
- EDTA (Sigma, Cat#: ED3SS)
- Dimethylsulfoxide (DMSO) (Sigma, Cat#: D1435 or equivalent)
- Luciferase substrate (Steady-Glo®) (Promega, Cat#: E2520)
- Passive Lysis 5X Buffer (Promega, Cat#: E1941)
- MTT (Sigma, Cat#: M2128)
- Isopropanol (Sigma, Cat#: 59300 or equivalent), 10% SDS Solution (Promega, Cat#: V6551)
- Cinnamic aldehyde (Sigma, Cat#: 239968)

Note: Ensure the correct Cat# is selected from the chemical cabinet

- White 96-well culture plates (Greiner Bio-One, Cat#: 655 083 or equivalent)
- Transparent 96-well culture plates (Orange Scientific, Cat#: 5530100 or equivalent)
- Adhesive plate sealer (ThermoFisher Scientific, Cat#: 236366)
- Culture dishes or flasks
- CryoTubes (Nunc, Cat#: 368632 or equivalent)
- Sterile reagent reservoirs
- Orbital plate shaker
- Cellometer and AO/PI for cell counts (or another validated cell counting method)

Procedure

1. Reagent Preparation

1.1. Cell culture medium: To 500 mL of DMEM add:

1.1.1. 50 mL of FBS (Final FBS concentration 9.1%)

1.1.2. 5.5 mL G418 (Final concentration 500 µg/mL)

Store at 2-8°C and use within one month.

1.2. Thawing/plating cell culture medium: To 500 mL of DMEM add:

1.2.1. 50 mL of FBS (Final FBS concentration 9.1%)

Store at 2-8°C and use within one month.

1.3. Freezing medium: To 35 mL of DMEM add:

1.3.1. 10 mL of FBS (Final FBS concentration 20%)

1.3.2. 5 mL of sterile DMSO

Discard leftover medium.

1.4. Test chemical medium: To 495 mL of DMEM add:

1.4.1. 5 mL of FBS (Final FBS concentration 1%)

Store at 2-8°C and use within one month.

1.5. DPBS-0.05% EDTA solution:

1.5.1. Weigh out 10 g ± 0.05 g of EDTA.

1.5.2. Dissolve in 100 mL of diH_2O.

1.5.3. Adjust pH to 8 ± 0.02 by adding NaOH.

1.5.4. Sterilize by filtration through a 0.2 µm filter.

1.5.5. Add 2.5 mL to 500 mL of DPBS.

Store at 2-8°C and use within 3 months.

1.6. MTT solution: For each 96-well plate prepare:

1.6.1. Weigh out 15 mg ± 0.05 mg of MTT.

1.6.2. Dissolve with 3 mL of DPBS in an appropriate container.

1.6.3. Add 2.7 mL of this solution to 20 mL of test chemical medium.

Discard leftover solution.

2. Routine cell culture procedures:

2.1. Thawing cells:

2.1.1. To thaw cells, warm rapidly in a 37±1°C water bath.

2.1.2. Move contents to a 15 mL conical tube and slowly resuspend in 10 mL of thawing/plating cell culture medium.

2.1.3. Centrifuge cells at ~125 x g for 5 minutes at 4°C and discard the supernatant to remove DMSO.

2.1.4. Resuspend cell pellet in an appropriate volume of thawing/plating cell culture medium.

Note: G418-containing medium is only added in the next passage.

2.1.5. Plate cells in a 100 mm or T75 tissue culture dish.

2.2. Maintenance/Cell passage:

2.2.1. Maintain cells in cell culture medium at 37±1°C in the presence of 5% CO_2.

2.2.2. Allow cells to reach 80-90% confluency before passaging.

2.2.3. Remove media and wash cells twice with DPBS-0.05% EDTA solution.

2.2.4. Add 1-2 mL of Trypsin-EDTA per 100 mm dish (or equivalent volume for flask surface area) and place into the 37±1°C incubator.

2.2.5. Monitor cells regularly for detachment (usually after 5-10 minutes).

2.2.6. After cells are detached, inactivate the Trypsin by adding 9-10 mL of cell culture medium (or equivalent volume for flask surface area) and transfer to a sterile conical tube.

2.2.7. Perform a cell count.

2.2.8. Calculate and record the total cell number, viability, and the doubling time from the previous passage.

2.2.9. Ratio split the cells ~1:3, ~1:6, or ~1:12 for a 2, 3, or 4 day passage, respectively, and record the total number of cells plated.

2.3. Freezing cells:

2.3.1. Harvest cells as described above and perform a cell count to calculate and record doubling time, viability, and total cell number.

2.3.2. Pellet cells (~125 x g for 5 minutes at 4°C) and aspirate the supernatant.

2.3.3. Resuspend the cells at a density of 3-4 x 10^6 cells/mL in freezing medium.

2.3.4. Quickly aliquot 1 mL into CryoTubes, cap, and place into a cell freezing container.

2.3.5. Store the cell freezing container at ≤ -70°C for 24±1 hours and then transfer the CryoTubes to a liquid nitrogen storage tank.

2.4. Cell plating for testing:

2.4.1. Cells propagated from the original stock may be employed for routine testing up to a maximum of 25 passages.

2.4.2. Prior to harvesting for cell plating, cells should be ratio split ~1:6 and/or ~1:12 into 100 mm dishes or T75 flasks.

2.4.3. In the ***morning*** 3 days after plating, replace spent cell culture medium from the ~1:12 ratio split cultures with fresh, warmed cell culture medium.

2.4.4. Observe the cells to ensure appropriate confluency (ideally between 80-90%) prior to harvesting cells.

2.4.5. Harvest cells split at a ~1:6 ratio (on Monday) or ~1:12 ratio (on Tuesday) as described above.

2.4.6. Perform a cell count and centrifuge cells at ~125 x g for 5 minutes at 4°C and resuspend cells in thawing/plating cell culture medium.

Note: To seed 4 (96-well) plates, it is recommended to add 2.4x10^6 viable cells (based on cell counts) to 2 (50 mL) conical tubes prior to centrifuging. Resuspend each cell pellet with 30 mL of thawing/plating cell culture medium for a final concentration of 80,000 viable cells/mL, as described below.

2.4.7. Adjust cell concentration to 80,000 viable cells/mL and plate 125 µL/well into 3 white 96-well plates and 1 clear 96-well plate using a sterile reagent reservoir and pipette for a total of 10,000 cells/well. An additional clear

96-well plate may be plated to reduce variability in the MTT assay if necessary. **Do not add cells to well H12 as it will serve as the no cell blank.**

Note: If more than 1 conical tube of cells is prepared as recommended, combine the resuspended cells into one homogenous solution (for example, in a sterile reagent reservoir). Avoid cell sedimentation during this step by moving quickly and pouring enough cell solution for one plate at a time.

2.4.8. Leave plates undisturbed in the cell culture hood for 30±5 min to allow cell adherence before placing in the incubator. **Movement of the plates when placing in the incubator may cause cells to settle to one side of the well.**

2.4.9. Incubate plates for 24±1 hours in the incubator set to 37±1°C with 5% CO_2.

3. Test chemical solubility testing:

3.1. Dissolve test chemical in DMSO at 200 mM concentration. Chemicals with no defined molecular weight should be prepared to a concentration of 40 mg/mL or 4% (w/v).

NOTE: DMSO solutions can be considered self-sterilizing

3.2. If chemical is not soluble in DMSO, dissolve in test chemical medium at the maximum visible soluble concentration (up to 40 mg/mL) and sterilize by filtration through a 0.2 µm filter.

3.3. Dilute the 200 mM DMSO solution of test chemical 100 fold in test chemical medium.

3.4. Prepare additional 1:2 serial dilutions in transparent tubes or clear 96-well plate and incubate for 1-2 hours protected from light.

3.5. Observe for signs of precipitation or phase separation. If testing a mixture, visually verify that all constituents are dissolved or form a stable dispersion before proceeding. If precipitation/phase separation occurs, the test chemical should be tested at the highest soluble concentration.

Note: With Study Director and NTP Contract Principal Investigator approval, alternative concentrations may be used with justification such as in cases of

cytotoxicity or poor solubility. Alternatives will be documented in the study records and indicated in the final report.

4. Test chemical and 100X master plate preparation:

4.1. Weigh between 20 – 40 mg of test chemicals or the positive control (cinnamic aldehyde) into a 4 mL glass vial and record the actual weight, identity, lot number, molecular weight, and purity.

4.2. Dissolve each test chemical with solvent to 200 mM and inspect closely for any signs of precipitation or phase separation. If precipitation or phase separation is observed alert Study Director and make appropriate corrections.

4.3. Further dilute the 200 mM cinnamic aldehyde solution to 6.4 mM by adding 32 µL of the 200 mM solution to 968 µL of DMSO.

4.4. Begin preparing the 100X master plate (layout shown below) by adding 100 µL of DMSO to rows A-G in columns 1-11.

4.5. Add 100 µL of DMSO to columns 1-10 and 12 of column H.

4.6. Add 200 µL of 7 prepared test chemical solutions to column 12 of rows A-G. Serially dilute the 7 test chemicals by transferring 100 µL from column 12 to column 11 with a multichannel pipette and mix by repeated pipetting at least 3 times. Change tips and continue transferring until column 1 is reached.

4.7. Add 200 µL of the 6.4 mM cinnamic aldehyde solution to well H11. Serially dilute cinnamic aldehyde by transferring 100 µL from well H11 to H10 and mix by repeated pipetting at least 3 times. Change tips and continue transferring until column 7 is reached.

Note: Alternative volumes may be used to prepare test chemicals if available material is limited. All changes will be documented in the study records and approved by the Study Director.

Example 100X DMSO Master Plate Setup. Concentrations for each unknown chemical (UC) or cinnamic aldehyde (CA) shown are in mM:

	1	2	3	4	5	6	7	8	9	10	11	12
A	UC1 0.098	UC1 0.195	UC1 0.39	UC1 0.78	UC1 1.56	UC1 3.125	UC1 6.25	UC1 12.5	UC1 25	UC1 50	UC1 100	UC1 200
B	UC2 0.098	UC2 0.195	UC2 0.39	UC2 0.78	UC2 1.56	UC2 3.125	UC2 6.25	UC2 12.5	UC2 25	UC2 50	UC2 100	UC2 200
C	UC3	UC3	UC3	UC3	UC3	UC3	UC3	UC3	UC3	UC3	UC3	UC3

	0.098	0.195	0.39	0.78	1.56	3.125	6.25	12.5	25	50	100	200
D	UC4 0.098	UC4 0.195	UC4 0.39	UC4 0.78	UC4 1.56	UC4 3.125	UC4 6.25	UC4 12.5	UC4 25	UC4 50	UC4 100	UC4 200
E	UC5 0.098	UC5 0.195	UC5 0.39	UC5 0.78	UC5 1.56	UC5 3.125	UC5 6.25	UC5 12.5	UC5 25	UC5 50	UC5 100	UC5 200
F	UC6 0.098	UC6 0.195	UC6 0.39	UC6 0.78	UC6 1.56	UC6 3.125	UC6 6.25	UC6 12.5	UC6 25	UC6 50	UC6 100	UC6 200
G	UC7 0.098	UC7 0.195	UC7 0.39	UC7 0.78	UC7 1.56	UC7 3.125	UC7 6.25	UC7 12.5	UC7 25	UC7 50	UC7 100	UC7 200
H	Blank DMSO	Blank DMSO	Blank DMSO	Blank DMSO	Blank DMSO	Blank DMSO	CA 0.4	CA 0.8	CA 1.6	CA 3.2	CA 6.4	No Cells Blank

5. Test material exposure procedure:

5.1. Use a pipette to dispense 240 µL of test chemical medium from a sterile reagent reservoir to each well of a clear 96-well plate. If chemicals are dissolved in water, add 230 µL of test chemical medium to the row for that chemical and 10 µL of DMSO.

5.2. Dilute the 100X master plate to a 4X master plate by transferring 10 µL from each corresponding well into the wells now containing 240 µL.

5.3. Remove media from plated cells (that have incubated for 24±1 hours) by aspiration and replace with 150 µL of **warm** test chemical medium using a sterile reagent reservoir.

5.4. Distribute 50 µL from the 4X master plate to the 3 white replicate assay plates and the clear, cell viability assay plate.

5.5. Seal each plate with a plate seal to avoid evaporation of volatile compounds and to avoid cross-contamination between wells by volatile compounds.

5.6. Incubate the plates for 48±2 hours in the incubator at 37±1°C and 5% CO_2.

6. Endpoint Measurement:

6.1. Luciferase activity

6.1.1. After the 48±2 hour incubation, aspirate supernatants from the white assay plates and discard.

6.1.2. Wash cells once with room temperature DPBS by pipetting gently against the wall of the wells.

6.1.3. Add 50 µL of room temperature 1X passive lysis buffer and incubate at room temperature protected from light for 10±1 minutes.

6.1.4. Turn on the luminometer and load with the appropriate settings and plate layout.

6.1.5. Add 50 µL of room temperature Steady-Glo® reagent.

6.1.6. Place the plate in the luminometer and begin reading within 10 minutes.

6.2. MTT assay

6.2.1. After the 48±2 hour incubation, aspirate and replace the medium on cells in the clear plate with 200 µL of MTT solution in test chemical medium.

6.2.2. Seal plates and return to the incubator for 4 hours ± 5 minutes.

6.2.3. Note: After this step, plates can be frozen (≤ -20°C) over the weekend and thawed on the following Monday.

6.2.4. Aspirate medium containing MTT solution and add 50 µL of isopropanol to each well.

6.2.5. Plate can be placed on an orbital shaker for 30±2 minutes and absorbance measured at 570 nm with a spectrophotometer.

Note: Alternatively, MTT containing medium can be removed and cells solubilized with 200 µL of 10% SDS solution. Seal the plate and place in the incubator protected from light for an overnight incubation to dissolve the cells. Plates can be incubated protected from light over the weekend (or up to 3 days) prior to the next step if necessary. Remove plate and rock on an orbital shaker for 10±1 minutes. Read the absorbance at 600 nm for each well on a spectrophotometer.

7. Data Analysis

7.1. Copy the file "**KeratinoSens_Evaluation-Sheet**."

7.1.1. Fields that should be filled are marked in yellow.

7.1.2. The "*Summary sheet*" has the compound and plate identifiers inserted.

7.1.3. On sheet "*rep1*", the plate readout of the triplicate analysis can directly be inserted in the yellow areas.

7.1.4. The second and third repetitions are added to sheets "*rep2*" and "*rep3*".

7.1.5. The cytotoxicity results are pasted into the sheets "*Cytotoxicity (1)-(3)*".

Note: With Study Director approval, an outlier can be removed.

7.1.6. After entering results into the file, the gene induction and the wells with statistically significant induction over a given threshold are automatically calculated.

7.1.7. The I_{max} and $EC_{1.5}$ value (concentration for induction above threshold) both with linear and log-linear extrapolation are calculated.

7.1.8. The results from the different repetitions are then summarized in the "*Summary sheet*." This sheet also generates a plot summarizing the gene induction and cytotoxicity dose-response in all repetitions for each chemical.

7.1.9. The data are also automatically plotted in graphs on the different repetition sheets. The values should be visually checked for uneven dose-response curves or large variations, which may lead to incorrect extrapolations that may need to be corrected manually.

7.1.10. In the rare cases where a statistically non-significant luciferase induction ≥ 1.5 fold is observed followed by a higher concentration with a statistically significant induction, results from this repetition are only considered as valid and positive if the statistically significant induction of ≥ 1.5 fold was obtained for a non-cytotoxic concentration, or if the induction of all three replicate wells of the first concentration above the $EC_{1.5}$ value are clearly above 1.5 fold.

7.1.11. For test chemicals generating a 1.5 fold or higher induction already at the lowest tested concentration (i.e. 0.98 μM), the $EC_{1.5}$ value of < 0.98 is set based on visual inspection of the dose-response curve.

8. Acceptance Criteria

8.1. Each test chemical and positive control must be tested in at least two independent repetitions containing three replicates each (i.e. n=6) with concordant results. If discordant results between the first two independent runs are obtained, a third repetition containing three replicates (i.e. n=9) should be performed.

Note: Each independent repetition is performed on a different day with fresh stock solutions of test chemicals and independently harvested cells. Cells may come from the same passage, however.

8.2. Positive Control:

8.2.1. Cinnamic aldehyde must be positive with gene induction statistically significant above the threshold of 1.5 in at least one of the tested concentrations (4 to 64 µM).

8.2.2. The average induction of the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8. The EC1.5 value should be between 7 µM and 30 µM. At least one of these criteria must be met, otherwise the run is rejected. If only one criterion is fulfilled, the dose-response of cinnamic aldehyde should be carefully examined, and results may be accepted only if there is a clear dose-response with increasing luciferase activity induction at increasing concentrations.

8.2.3. *Vehicle Control:* The average CV of the DMSO control luminescence readings should be below 20% in each repetition. The variability is calculated as 100 x [standard deviation (18 DMSO wells) / average (18 DMSO wells)]. Results should be rejected if variability is higher.

Note: One well of the 6 solvent control wells per plate can be removed as an outlier in the case that one well is > 25% lower or higher than the average of the other 5 wells. This may occasionally happen for well H1 at the corner of the plate.

8.3. *KeratinoSens™ Positive Prediction:* The following 4 conditions must be met in 2 of 2 or at least 2 of 3 repetitions, otherwise the prediction is considered negative:

8.3.1. The I_{max} is ≥ 1.5 fold and statistically significantly different as compared to the solvent/vehicle control.

8.3.2. The cellular viability is > 70% at the lowest concentration with induction of luciferase activity ≥ 1.5 fold.

8.3.3. The $EC_{1.5}$ value is < 1000 µM (or 200 µg/mL for test chemicals with no defined MW).

8.3.4. There is an apparent overall dose-response for luciferase induction (or a biphasic response).

8.4. If the three first conditions are met, but a clear dose-response for the luciferase induction cannot be observed, then the result of that repetition should be considered inconclusive and further testing may be required.

8.5. A negative result obtained with test chemicals that do not dissolve or form a stable dispersion at concentrations of 1000 µM (or 200 µg/mL for test chemicals with no defined MW) should also be considered inconclusive.

8.6. In rare cases, test chemicals which induce the luciferase activity very close to the cytotoxic levels can be positive in some repetitions at non-cytotoxic levels (i.e. $EC_{1.5}$ determining concentration < the IC_{30}), and in other repetitions only at cytotoxic levels (i.e. $EC_{1.5}$ determining concentration > the IC_{30}). Such test chemicals should be retested with a **narrower dose-response** analysis (such as a 1:1.333 serial dilution) using a lower dilution factor to determine if induction has occurred at cytotoxic levels or not. These results should be analyzed using the "**KeratinoSens_Evaluation-Sheet_Oct_21_2014_different dilution series**" file.

8.7. In other rare cases, chemicals may be extremely cytotoxic. Cells should remain >70% viable at least at two consecutive test concentrations. If this is not the case, chemicals should be retested at lower concentrations than the standard dose-range.

Appendix IV: h-CLAT Methodology.

PURPOSE

The Human Cell Line Activation Test (h-CLAT) is an *in vitro* method used for assessing the sensitization potential of chemicals. This assay measures dendritic cell (DC) activation in response to chemical exposure using the immortalized human monocytic leukemia cell line, THP-1, as a DC surrogate. In this assay, THP-1 cells are cultured for 24 hours with various doses of the chemical of interest then analyzed by flow cytometry for cell surface expression of the activation markers, CD86 and CD54. Activation of DCs is considered a key event in the skin sensitization pathway. As such, h-CLAT is one of a battery of *in vitro* assays proposed as alternative approaches for the assessment of contact hypersensitivity.

MATERIALS

- THP-1 cell line (ATCC # TIB-202)
- Incubator with CO_2 supply
- Biological safety cabinet
- Centrifuge
- Flow Cytometer
- Culture flasks (Non-tissue culture treated, 250 mL, BD Falcon # 353133 or equivalent)
- 24-well flat-bottom plate (BD Falcon # 351147 or equivalent)
- 96-well flat-bottom plate (BD Falcon # 351172 or equivalent)
- 96-well round-bottom plate (BD Falcon # 353910 or equivalent)
- Volumetric flask
- Glass vial or tube
- RPMI-1640 with GlutaMax (Gibco # 61870-036 or equivalent)
- Heat-inactivated fetal bovine serum (FBS)
- 2-mercaptoethanol (Gibco # 21985-023 or equivalent)
- Penicillin-Streptomycin (Gibco # 15140-122 or equivalent)
- Dimethylsulfoxide (DMSO) (Sigma # D5879 or equivalent)
- Calibration beads for flow cytometer

- Phosphate-buffered saline (PBS) without magnesium, calcium or phenol red (Gibco # 10010-23 or equivalent)
- Bovine Serum Albumin (BSA) Fraction V solution (30% in DPBS) (Sigma # A9576 or equivalent)
- Globulins Cohn fraction II, Human (MP Bio # 08823101 or equivalent)
- Propidium Iodide (PI) (BD Biosciences # 556463 or equivalent)
- Anti-human CD86 antibody (BD Biosciences # 555657)
- Anti-human CD54 antibody (Dako # F7143)
- Fluorescent labeled mouse IgG control for CD86/CD54 antibodies (Dako # X0927)
- 2,4-dinitrochlorobenzene (DNCB), CAS # 97-00-7
- Nickel Sulfate ($NiSO_4$), CAS # 10101-97-0
- Lactic Acid (LA), CAS # 50-21-5

PROCEDURE

1. Reagent Preparation

1.1. THP-1 culture medium: supplement RPMI-1640 with 10% FBS (v/v), 0.05 mM 2-mercaptoethanol and 100 U/mL penicillin + 100 U/mL streptomycin. Store at 2-8°C for up to 1 month.

1.2. Freezing medium: supplement THP-1 culture medium with 10% (v/v) sterile DMSO.

1.3. Flow cytometry buffer (FACs): supplement PBS with 0.1% (w/v) BSA. Store at 2-8°C for up to 1 month.

1.4. Blocking solution: supplement flow cytometry buffer + 0.01% (w/v) globulin. To prepare, use a 1% globulin in PBS solution which must be prepared at least the day before use. Store at 2-8°C for up to 7 days. On the day of analysis, dilute the 1% solution 1:100 with FACS buffer just prior to use.

2. Cell Culture

2.1. Cells should be maintained in suspension at densities of 0.1-0.8 x 10^6 cells/mL. Pass cells every 2-3 days.

2.2. Cell density should not exceed 1 x 10^6 cells/mL.

2.3. To sub-culture, collect cells, centrifuge (250 x g, 5 min, 4°C) to pellet and resuspend in fresh medium.

2.4. Determine the appropriate suspension volume based on cell counts.

2.5. Cells can be propagated up to two months after thawing but should be discarded after 30 passages.

2.6. Seed THP-1 cells at 0.1 and 0.2 x 10^6 cells/mL for 48 or 72 hour pre-culture periods, **respectively**.

**For each new batch of THP-1 cells, the doubling time should be within the normal range established using historical data at BRT and the reactivity test should be performed prior to use.*

3. Calculating Doubling Time

3.1. Record the date and time of each passage and number of viable cells seeded per flask.

3.2. Perform a cell count to determine cell viability, and total cells/mL.

3.3. Calculate the doubling time using the following equation:

$$Doubling\ time = ((T1 - T0) \times 24) \times \frac{\log_{10}(2)}{\log_{10}(Conc_y) - \log_{10}(Conc_x)}$$

Where *T0* is the date and time cells were plated, *T1* is the date and time of cells were harvested, $Conc_x$ is the number of cells plated, and $Conc_y$ is the number of viable cells harvested.

3.4. Record the doubling time.

4. Reactivity Check

4.1. Perform the reactivity check two weeks after thawing each new cell batch prior to performing experiments.

4.2. Prepare the positive control chemicals, DNCB and $NiSO_4$, and the negative control, LA, the day of the reactivity test.

4.2.1. DNCB: weigh 10 mg and add DMSO up to 2 mL (5 mg/mL). Mix and dilute by adding 3 mL of DMSO to a final concentration of 2 mg/mL.

4.2.2. NiSO4: weigh 20 mg and add PBS up to 2 mL (10 mg/mL).

4.2.3. LA: weigh 200 mg and add PBS up to 2 mL (100 mg/mL).

4.3. Each stock solution should be kept in the dark until the working solution is prepared.

4.4. Dilute stock solutions in culture medium as follows: DNCB 1:250 (8 µg/mL final), $NiSO_4$ 1:50 (200 µg/mL final), and LA 1:50 (2000 µg/mL final).

4.5. Collect pre-cultured cells and re-suspend in fresh culture medium at a density of 2×10^6 cells/mL.

4.6. Add 500 µL of cell suspensions to each well of a 24-well flat-bottom plate.

4.7. Add 500 µL of the working solution to cell suspensions in the well.

4.8. Incubate for 24±1 hours.

4.9. Collect cells and analyze for CD86/CD54 expression by flow cytometry.

4.10. Acceptance Criteria for the reactivity check:

4.10.1. Cell viability for non-treated cells should be > 90%

4.11. Both DNCB and NiSO4 should produce a positive response for both CD86 (RFI ≥ 150%) and CD54 (RFI ≥ 200%)

4.12. LA should not produce a response for either CD86 (RFI < 150%) or for CD54 (RFI < 200%)

If the acceptance criteria are not met for one or both positive control chemicals, proceed with a dose finder assay for the positive control(s) and rerun the reactivity check at the calculated CV75 if different from the concentration used here.

5. Dose Finding Assay

Note: The dose finding assay should be performed on the same culture of THP-1 cells that will be tested in the main experiment due to potential differences in calculated CV75 values from cells thawed on different days.

5.1. Determine the solubility of each chemical and prepare stock/working solutions:

5.1.1. The preferred solvent is PBS. Chemicals should be dissolved at 100 mg/mL (1 mL solvent + 0.1 g test chemical). Surfactant should always be solubilized with PBS. If a surfactant is not soluble at 100 mg/mL, the highest soluble concentration should be used (minimum 1 mg/mL).

5.1.2. RPMI 1640 can be used if solubility is comparable to PBS.

5.1.3. If the chemical is not soluble in PBS/RPMI at 100 mg/mL, the chemical should be dissolved in DMSO at 500 mg/mL (1 mL DMSO + 0.5 g test chemical). If chemical is not soluble at 500 mg/mL, the highest soluble concentration should be used (minimum 1 mg/mL).

5.2. Prepare 7 more doses by 1:2 serial dilutions from the 100 mg/mL or 500 mg/mL stocks.

5.3. If PBS is the solvent, dilute each stock solution 1:50 with culture medium. If DMSO is the solvent, dilute each stock solution 1:250 with culture medium.

Doses	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
2X Working Solution (mg/mL)	2.0	1.0	0.5	0.25	0.125	0.063	0.031	0.016

5.4. Harvest cells that have been in culture for 48-72 ± 2 hours (depending on cell seeding density), centrifuge to pellet (250 x g, 4°C, 5 min), and prepare a single cell suspension in fresh media at 2 x 10^6 cells/mL.

5.5. Add 500 µL of cell suspension to each well of a 24-well flat-bottom plate or 80 µL of cell suspension to each well of a 96-well flat-bottom plate.

**Duplicate plates should be set up to run two independent PI experiments per chemical (see below).*

5.6. Add equal volumes of working solution to the cells (500 µL for final volume of 1 mL in 24-well plate or 80 µL for final volume of 160 µL in 96-well plate). Shake the plate gently by hand to mix and place in the incubator (5% CO_2).

5.7. Culture for 24 ± 1 hours.

5.8. <u>PI experiment</u>**:** (run two independent experiments per chemical)

5.8.1. After incubation, transfer cells into sample tubes and collect by centrifugation (250 x g, 4°C, 5 min). Discard supernatant, wash cells

twice with 200 µL of flow cytometry buffer and re-suspend cells in 200 µL of buffer.

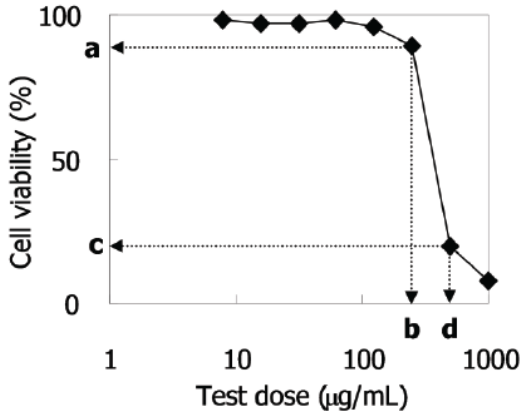
5.8.2. Add 10 µL of a 12.5 µg/mL PI solution to each tube.

5.8.3. Analyze cell viability by flow cytometry. Acquire 10,000 events within the live cell gate (PI negative) or acquire events for 1 minute.

5.9. Estimate the CV75 value for each chemical as follows:

5.9.1. Calculate the percent viability for each chemical concentration

5.9.2. Plot as Cell viability (%) vs Test dose (µg/mL)



Example of the result of PI assay

5.9.3. Calculate the CV75 using the following equation:

$$Log\ CV75 = \frac{(75 - c) \times Log(b) - (75 - a) \times Log(d)}{a - c}$$

5.9.4. Plate duplicates are calculated separately and the CV75 values are averaged.

5.9.5. If the lowest dose has <75% viability, rerun the experiment using a lower dose range

5.9.6. If the default highest concentration for PBS soluble compounds does not result in significant reduction of cell viability, rerun the experiment with a higher dose range **by dissolving chemical to 500 mg/mL** (if soluble) and testing at 5000 µg/mL with 1:1.2 serial dilutions. If the test chemical is

not soluble at 500 mg/mL in PBS, determine the highest soluble concentration in PBS to repeat the dose finder.

5.9.7. If the range of doses directly above and below the CV75 is large (>500 µg/mL), after performing 1:2 serial dilutions, the experiment with a narrower range (i.e. smaller dilution factor than 1:2) if necessary. If a chemical is dissolved to 500 mg/mL and tested with 1:1.2 serial dilutions and the CV75 is below the lowest concentration tested, alert the Study Director and document steps taken in the study record.

6. Endpoint Measurement Assay

6.1. Calculate the 1.2 x CV75.

6.1.1. Prepare the highest dose stock solution for each chemical. For PBS/media soluble chemicals, prepare a stock at 100 times the calculated value. For DMSO, prepare a stock at 500 times this value.

6.1.2. Prepare 7 more stock solutions by serial 1:1.2 dilutions of the highest dose stock (e.g. 500 µL of chemical stock to 100 µL of solvent).

6.2. For chemicals in PBS/RPMI, dilute the 8 stock solutions 1:50 (50 µL stock to 2450 µL media) and for chemicals in DMSO, dilute the 8 stock solutions 1:250 (10 µL stock to 2490 µL media).

6.3. Harvest the cells that have been in culture for 48-72 ± 2 hours (depending on cell seeding density), centrifuge to pellet (250 x g, 4°C, 5 min), and prepare a single cell suspension in fresh media at 2 x 10^6 cells/mL.

6.4. Add 500 µL of cell suspension to each well of a 24-well flat-bottom plate or 80 µL of cell suspension to each well of a 96-well flat-bottom plate.

6.5. Add equal volumes of working solution to the cells (500 µL for final volume of 1 mL in 24-well plate or 80 µL for final volume of 160 µL in 96-well plate). Shake the plate gently by hand to mix and place in the incubator (5% CO_2).

6.6. Media and vehicle controls (0.2% DMSO) should be included.

6.7. DNCB should be included as a positive control in each assay at a final concentration of 4 µg/mL, yielding approximately 70-90% viability.

Alternately, the CV75 of DNCB can be determined by the dose finding assay and included here as a positive control.

6.8. Culture for 24 ± 1 hours.

6.9. After incubation, transfer cells to sample tubes, centrifuge to pellet (250 x g, 4°C, 5 min), wash twice with 1 mL of flow cytometry buffer, and resuspend in 600 µL of blocking buffer.

6.10. Incubate at 2-8°C for 15 ± 1 min.

6.11. After blocking, split cells into three aliquots of ~180 µL.

6.12. For each antibody, label a single aliquot of cells: anti-CD86 (3 µg/mL), anti-CD54 (3 µg/mL), or mouse IgG (3 µg/mL).

6.13. Incubate at 2-8°C for 30 ± 2 min in the dark.

6.14. Wash cells twice with 150 µL of flow buffer and resuspend in a final volume of 100 µL per tube.

6.15. Just before analysis, add 5 µL of a 12.5 µg/mL PI solution to each tube.

6.16. Set up the following acquisition parameters:

6.16.1. 2D dot plot consisting of forward scatter (FSC) and side scatter (SSC) to check that a single population appears without contamination or excessive debris.

6.16.2. 2D dot plot consisting of FSC vs FL-2 to determine viability with a gate set on the viable cell population. 10,000 events should be collected within this gate.

6.16.3. 2D dot plot of viable cells consisting of FSC vs FL-1 to determine surface marker expression with gate set to detect increases in expression compared to vehicle-treated cells.

6.17. Acquire 10,000 events within the live cell gate (PI negative). If the events are extremely low acquire events for 1 minute.

7. Data Analysis

7.1. The relative fluorescence intensity (RFI) is calculated as follows for each chemical concentration where MFI = mean fluorescence intensity:

$$RFI = \frac{MFI\ of\ chemical\ treated\ cells\ - MFI\ of\ chemical\ treated\ isotype\ cells}{MFI\ of\ solvent\ treated\ cells - MFI\ of\ solvent\ treated\ isotype\ cells} \times 100$$

7.2. For each treatment, the cell viability is recorded from the isotype control cells. When viability is <50%, the RFI is not used because of the diffuse labeling of cytoplasmic structures generated following cell membrane destruction.

7.3. Calculate the EC150 for CD86 and the EC200 for CD54 (the concentrations at which the test chemicals induce an RFI of 150 or 200, respectively):

7.3.1. Plot RFI vs Test dose (µg/mL) for the Higher and Lower doses flanking RFI = 150 for CD86 data or RFI = 200 for CD54 data.

7.3.2. Interpolate using a linear equation to determine the concentration at which the RFI is equal to 150 or 200 as appropriate.

7.4. Prediction model: If the RFI of CD86 is equal to or greater than 150% at any tested dose (>50% of cell viability) AND/OR if the RFI of CD54 is equal to or greater than 200% at any tested dose (>50% of cell viability) in at least 2 independent runs, the chemical prediction is considered positive. Otherwise it is considered negative. If the two independent runs are not concordant, a third run should be performed and the final prediction will be based on the 2 out of 3 run results which agree. Up to six runs are permitted to reach a conclusion for each chemical. If no prediction can be made after the sixth, the result is inconclusive and the chemical is classified accordingly.

8. Acceptance Criteria

8.1. Cell viability in medium and vehicle controls should be > 90%

8.2. The RFI values for the DNCB control should be over the positive criteria (CD86 ≥ 150, CD54 ≥ 200).

8.3. The RFI values for the vehicle control should be below the positive criteria (CD86 < 150, CD54 < 200).

8.4. For media and DMSO controls, the MFI ratio of CD86 and CD54 to the isotype control should be > 105%.

8.5. Abnormal values:

8.5.1. RFI values cannot be less than zero for any reason and such values should be omitted from the prediction.

8.5.2. If an abnormal value (for instance, strongly induced CD86 or CD54 expression at only one non-cytotoxic concentration) is observed, check whether there are abnormal conditions in the run and record them.

8.6. Requirements for data acceptance:

8.6.1. For the test chemical resulting in a negative outcome, viability at the 1.2 x CV75 must be < 90%.

8.6.2. For the test chemical resulting in a positive outcome, viability at the 1.2 x CV75 of > 90% is acceptable.

8.6.3. If the chemical is tested at the maximal concentration, the data is accepted regardless of cell viability at this dose.

Cell viability of at least 4 doses in each assay should be > 50%

Appendix V: Assay data and calculations for the DPRA.

Lysine Peptide Results	Peptide Peak Area at 220 nm	Percent Peptide Depl.	Mean Percent Peptide Depl.	SD of Percent Peptide Depl.	CV of Percent Peptide Depl.
[1]DCOIT (Acetonitrile)	2280077	10.2	11.6	1.2	10.3
	2237068	11.9			
	2220649	12.6			
BBIT (Acetonitrile)	2768341	-9	[5]-15.6	5.8	N/A
	2992082	-17.8			
	3048823	-20			
MIT (Acetonitrile)	2605260	-2.6	[5]-1.5	2	N/A
	2608307	-2.7			
	2519253	0.8			
[1]OIT (Acetonitrile)	2516838	0.9	1.3	0.8	N/A
	2483912	2.2			
	2516820	0.9			
CMIT/MIT (Water)	2199116	10.4	10.6	3.6	34
	2104410	14.3			
	2280085	7.1			
[2]BIT (Acetonitrile:Water)	N/A	N/A	N/A	N/A	N/A
	N/A	N/A			
	N/A	N/A			

Cysteine Peptide Results	Peptide Peak Area at 220 nm	Percent Peptide Depl.	Mean Percent Peptide Depl.	SD of Percent Peptide Depl.	CV of Percent Peptide Depl.
[2]DCOIT (Acetonitrile)	0	100	100	0	0
	0	100			
	0	100			
BBIT (Acetonitrile)	0	100	100	0	0
	0	100			
	0	100			
MIT (Acetonitrile)	0	100	100	0	0
	0	100			
	0	100			
[3]OIT (Acetonitrile)	0	100	100	0	0
	0	100			
	0	100			
[4]CMIT/MIT (Water)	0	100	100	0	0
	0	100			
	0	100			
BIT (Acetonitrile:Water)	0	100	100	0	0
	0	100			
	0	100			

[1]Precipitation observed in both the co-elution control and replicate test samples. [2]Co-elution interference observed. [3]Precipitation observed in test chemical samples only. [4]Precipitation observed in co-elution sample only. [5]Negative values are reported as "0" when calculating mean depletion.
Solvents utilized for dissolving each compound or mixture is shown in parenthesis.
CV's are not calculated for samples with negative depletion values.

Appendix VI: Assay data and calculations for KeratinoSens™.

Compound	Run 1					Run 2				
	Pass Viability	Prediction	$EC_{1.5}$ (µM)	I_{max}	IC_{50} (µM)	Pass Viability	Prediction	$EC_{1.5}$ (µM)	I_{max}	IC_{50} (µM)
BIT	Yes	Positive	3.45	19.28	54.56	Yes	Positive	2.86	16.01	69.22
CMIT/MIT	Yes	Positive	4.09	5.14	20.47	Yes	Positive	2.84	6.09	19.03
OIT	Yes	Positive	2.57	3.87	12.94	Yes	Positive	1.86	3.54	12.30
BBIT	Yes	Positive	4.24	17.75	55.41	Yes	Positive	3.48	21.47	50.26
MIT	Yes	Positive	8.87	16.18	112.19	Yes	Positive	10.26	15.49	103.76
DCOIT	Yes	Positive	1.31	4.36	3.67	Yes	Positive	1.34	4.38	5.70

Notes: Chemicals OIT and BBIT were tested at a top concentration of 500 µM and DCOIT at 62.5 µM rather than 2000 µM, the maximum recommended concentration, due to solubility limitations.
The $EC_{1.5}$ and IC_{50} concentrations of CMIT/MIT have been adjusted as described in the figure legend for Table 4.

Appendix VII: Tabulated individual run data for KeratinoSens™.

BIT	*0.98*	*1.95*	*3.91*	*7.81*	*15.63*	*31.25*	*62.50*	*125.00*	*250.00*	*500.00*	*1000.00*	*2000.00*
rep1	1.18	1.37	1.54	2.04	2.85	5.82	19.28	-0.01	-0.01	-0.01	0.00	-0.01
rep2	1.32	1.33	1.70	2.22	2.66	5.59	16.01	0.00	-0.01	-0.01	-0.01	-0.01
induction BIT	**1.25**	**1.35**	**1.62**	**2.13**	**2.75**	**5.71**	**17.64**	**0.00**	**-0.01**	**-0.01**	**-0.01**	**-0.01**
Stdev	**0.10**	**0.03**	**0.11**	**0.13**	**0.13**	**0.16**	**2.31**	**0.00**	**0.00**	**0.00**	**0.01**	**0.00**
[1]CMIT/MIT	*0.20*	*0.39*	*0.78*	*1.57*	*3.14*	*6.28*	*12.56*	*25.11*	*50.23*	*100.45*	*200.90*	*401.80*
rep1	1.07	1.01	1.04	1.15	1.32	1.91	5.14	3.61	0.00	-0.01	-0.01	-0.01
rep2	1.02	1.13	1.14	1.27	1.55	2.19	4.96	6.09	-0.01	-0.01	-0.01	-0.01
induction CMIT/MIT	**1.04**	**1.07**	**1.09**	**1.21**	**1.44**	**2.05**	**5.05**	**4.85**	**-0.01**	**-0.01**	**-0.01**	**-0.01**
Stdev	**0.04**	**0.09**	**0.07**	**0.08**	**0.16**	**0.20**	**0.13**	**1.76**	**0.01**	**0.00**	**0.00**	**0.00**
OIT	*0.24*	*0.49*	*0.98*	*1.95*	*3.91*	*7.81*	*15.63*	*31.25*	*62.50*	*125.00*	*250.00*	*500.00*
rep1	1.07	1.05	1.16	1.37	1.79	3.87	3.69	0.02	0.00	0.00	-0.01	0.00
rep2	1.12	1.08	1.21	1.53	1.96	3.26	3.54	0.02	0.00	-0.01	-0.01	-0.02
induction OIT	**1.09**	**1.07**	**1.18**	**1.45**	**1.87**	**3.56**	**3.61**	**0.02**	**0.00**	**-0.01**	**-0.01**	**-0.01**
Stdev	**0.03**	**0.02**	**0.04**	**0.11**	**0.12**	**0.43**	**0.10**	**0.00**	**0.00**	**0.01**	**0.01**	**0.01**
BBIT	*0.24*	*0.49*	*0.98*	*1.95*	*3.91*	*7.81*	*15.63*	*31.25*	*62.50*	*125.00*	*250.00*	*500.00*
rep1	1.05	1.08	1.14	1.24	1.44	2.10	2.77	6.24	17.75	0.00	-0.01	-0.01
rep2	1.00	1.01	1.15	1.26	1.57	1.95	3.05	6.58	21.47	-0.01	-0.01	-0.01
induction BBIT	**1.02**	**1.05**	**1.15**	**1.25**	**1.51**	**2.02**	**2.91**	**6.41**	**19.61**	**-0.01**	**-0.01**	**-0.01**
Stdev	**0.04**	**0.05**	**0.01**	**0.02**	**0.09**	**0.11**	**0.20**	**0.24**	**2.64**	**0.01**	**0.00**	**0.01**
MIT	*0.98*	*1.95*	*3.91*	*7.81*	*15.63*	*31.25*	*62.50*	*125.00*	*250.00*	*500.00*	*1000.00*	*2000.00*
rep1	1.07	1.12	1.26	1.43	1.94	3.81	8.61	16.18	0.01	0.00	-0.01	0.00
rep2	1.14	1.16	1.31	1.38	1.76	2.89	6.82	15.49	0.00	0.00	-0.01	-0.01
induction MIT	**1.10**	**1.14**	**1.28**	**1.41**	**1.85**	**3.35**	**7.72**	**15.84**	**0.00**	**0.00**	**-0.01**	**-0.01**
Stdev	**0.05**	**0.03**	**0.03**	**0.04**	**0.13**	**0.65**	**1.27**	**0.49**	**0.01**	**0.00**	**0.00**	**0.01**
DCOIT	*0.03*	*0.06*	*0.12*	*0.24*	*0.49*	*0.98*	*1.95*	*3.91*	*7.81*	*15.63*	*31.25*	*62.50*
rep1	0.96	1.05	1.02	1.11	1.18	1.31	1.86	4.36	0.86	0.35	0.00	-0.01
rep2	1.04	1.00	1.04	1.08	1.14	1.25	1.93	4.38	1.36	0.41	-0.01	-0.01
induction DCOIT	**1.00**	**1.02**	**1.03**	**1.10**	**1.16**	**1.28**	**1.90**	**4.37**	**1.11**	**0.38**	**-0.01**	**-0.01**
Stdev	**0.06**	**0.03**	**0.01**	**0.02**	**0.03**	**0.04**	**0.05**	**0.01**	**0.35**	**0.04**	**0.01**	**0.01**

Numbers in blue are concentrations (µM) tested for each isothiazolinone.

[1]CMIT/MIT concentrations have been adjusted as described in the figure legend for Table 4.

Appendix VIII: Tabulated viability results for KeratinoSens™.

Viability Average												
[1]*conc*	***12***	***11***	***10***	***9***	***8***	***7***	***6***	***5***	***4***	***3***	***2***	**1**
BIT	117.06	101.04	107.45	121.92	137.40	149.05	32.46	0.25	0.51	0.73	1.41	0.27
CMIT/MIT	89.91	87.46	91.29	101.10	99.26	97.19	106.98	9.08	1.35	1.73	2.09	0.88
OIT	89.29	86.72	93.58	101.51	101.53	112.26	11.87	-0.42	0.64	1.56	0.57	1.64
BBIT	88.18	89.44	94.74	102.80	103.05	112.76	132.56	142.55	9.43	0.40	1.60	0.84
MIT	92.45	88.62	92.37	99.15	103.99	114.25	127.99	21.44	-0.11	0.66	0.40	0.17
DCOIT	87.59	85.50	89.82	92.70	95.27	112.77	147.32	60.87	3.56	1.03	0.55	1.85

[1]All compounds were tested at a top concentration of 2000 µM except for OIT and BBIT which were tested at 500 µM and DCOIT which was tested at 62.5 µM due to solubility limitations. The starting concentration of CMIT/MIT has been adjusted from 2000 µM to 401.8 µM as described in the figure legend for Table 4.

Results shown are mean values from two independent experiments.

Appendix IX: Assay data and calculations for the h-CLAT.

Compound	Run 1 (21 May 19)				Run 2 (24 May 19)				[1]Run 3 (24 May 19)			
	Pass Viability	Classification	EC150 (µg/mL)	EC200 (µg/mL)	Pass Viability	Classification	EC150 (µg/mL)	EC200 (µg/mL)	Pass Viability	Classification	EC150 (µg/mL)	EC200 (µg/mL)
BBIT	No	Sensitizer	2.16	1.64	Yes	Sensitizer	2.4	2.6	Yes	Sensitizer	3.2	3.0
MIT	Yes	Sensitizer	*	*	Yes	Sensitizer	11.8	11.6				
CMIT/MIT	No				No				No			
OIT	Yes	Sensitizer	NI	0.482	Yes	Sensitizer	7.26	0.949				
BIT	Yes	Sensitizer	5.16	1.85	Yes	Sensitizer	7.84	7.63				
DCOIT	Yes	Sensitizer	NI	0.44	Yes	Sensitizer	NI	0.92				
Compound	[2]Run 4 (31 May 19)				[2,3]Run 5 (31 May 19)				Run 6 (02 Aug 19)			
	Pass Viability	Classification	EC150 (µg/mL)	EC200 (µg/mL)	Pass Viability	Classification	EC150 (µg/mL)	EC200 (µg/mL)	Pass Viability	Classification	EC150 (µg/mL)	EC200 (µg/mL)
BBIT												
MIT												
[4]CMIT/MIT	Yes	Sensitizer	2.91	2.66	Yes	Sensitizer	NI	3.07	Yes	Sensitizer	2.81	2.63
OIT												
BIT												
DCOIT												
Compound	Run 7 (06 Aug 19)											
	Pass Viability	Classification	EC150 (µg/mL)	EC200 (µg/mL)								
BBIT												
MIT												
[4]CMIT/MIT	Yes	Sensitizer	NI	1.96								
OIT												
BIT												
DCOIT												

NI = No induction, *Did not meet guideline criteria to calculate the EC150 or EC200. The RFI value at the lowest dose was above the positive criteria and no higher doses (up to the fourth lowest dose) resulted in an RFI value ≥10% of the RFI value at the lowest dose. [1]Two independent runs were performed on 24 May 19. [2]DNCB control did not meet viability criteria (cells were <50% viable). [3]Two independent runs were performed on 31 May 19. DNCB control did not meet viability criteria (cells were <50% viable). [4]CMIT/MIT concentrations have been adjusted as described in the figure legend for Table 7.

Appendix X: Individual run data for the h-CLAT.

[1]BBIT	Run 1 (24 May 19)			[2]Run 2 (24 May 19)			Run 3 (N/A)		
Treatment	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**
4.0 µg/ml	194.91	354.04	92.99	159.85	277.24	94.26			
3.3 µg/ml	186.12	258.31	94.72	153.65	229.21	94.97			
2.8 µg/ml	168.77	208.77	95.69	142.31	179.70	94.55			
2.3 µg/ml	145.70	181.66	96.21	111.18	148.60	95.07			
1.9 µg/ml	128.73	135.49	96.72	115.05	150.59	95.90			
1.6 µg/ml	117.08	115.50	97.10	105.82	125.28	95.81			
1.3 µg/ml	124.80	124.52	97.17	113.18	131.15	95.69			
1.1 µg/ml	125.21	122.34	97.19	103.78	126.70	96.85			

[1]BBIT was initially tested at a starting concentration of 9.0 µg/ml (results not shown) and was adjusted to 4.0 µg/mL to decrease cytotoxicity.
[2]Two independent runs were performed on 24 May 19.

MIT	Run 1 (21 May 19)			Run 2 (24 May 19)			Run 3 (N/A)		
Treatment	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**
29.5 µg/ml	238.57	1288.26	72.30	361.27	1323.23	76.56			
24.6 µg/ml	252.35	982.88	81.53	312.30	885.86	87.96			
20.5 µg/ml	233.91	801.70	84.70	247.98	578.94	91.39			
17.1 µg/ml	204.34	637.47	88.96	179.01	393.20	92.46			
14.2 µg/ml	169.60	539.99	91.22	161.72	298.24	93.94			
11.9 µg/ml	142.77	420.66	93.06	150.97	204.94	95.75			
9.88 µg/ml	136.64	387.76	94.30	127.85	168.23	96.14			
8.23 µg/ml	233.20	947.69	80.04	110.14	135.90	97.63			

[1]CMIT/MIT	[2]Run 1 (31 May 19)			[2,3]Run 2 (31 May 19)			Run 3 (02 Aug 19)		
Treatment	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**
3.65 µg/ml	204.83	346.60	50.11	126.65	647.00	78.00	227.23	364.22	46.88
3.04 µg/ml	-	-	75.67	103.84	183.04	88.83	172.15	292.45	71.55
2.53 µg/ml	121.38	181.57	90.76	70.02	139.96	95.19	124.67	178.65	89.33
2.11 µg/ml	114.57	137.12	95.44	52.52	97.62	96.39	94.42	118.26	94.17
1.76 µg/ml	106.43	115.64	97.02	64.94	72.20	97.39	98.54	110.03	96.39
1.47 µg/ml	94.68	112.38	97.91	67.48	73.63	97.05	-	-	97.43
1.22 µg/ml	88.42	73.85	97.85	89.96	135.84	94.64	108.08	133.55	97.98
1.02 µg/ml	83.32	94.36	97.90	80.53	141.81	97.11	96.07	94.92	97.32

[1]CMIT/MIT concentrations have been adjusted as described in the figure legend for Table 7. CMIT/MIT was initially tested at a starting concentration of 1.90 µg/ml and 2.22 µg/ml (results not shown). The dose finder was repeated, and the starting concentration was adjusted to 3.65 µg/ml. [2]DNCB control did not meet viability acceptance criteria (cells were <50% viable in Runs 1 & 2). [3]Two independent runs were performed on 31 May 19. Cells with a "-" indicate values that were excluded due to abnormal run conditions. High IgG_1 staining caused negative RFI values for one sample. Forward scatter vs side scatter plots were abnormal for another sample causing IgG_1 staining to be low and RFI values high. These values were excluded from the analysis.

[1]CMIT/MIT	Run 4 (06 Aug 19)			Run 5 (N/A)			Run 6 (N/A)		
Treatment	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**
3.65 µg/ml	101.42	315.96	17.18						
3.04 µg/ml	107.60	327.36	26.27						
2.53 µg/ml	119.32	292.46	49.57						
2.11 µg/ml	106.58	222.11	70.94						
1.76 µg/ml	88.51	169.68	85.43						
1.47 µg/ml	88.11	152.21	91.73						
1.22 µg/ml	92.85	91.93	94.62						
1.02 µg/ml	84.85	76.23	94.94						

[1] CMIT/MIT concentrations have been adjusted as described in the figure legend for Table 7. CMIT/MIT was initially tested at a starting concentration of 1.90 µg/ml and 2.22 µg/ml (results not shown). The dose finder was repeated, and the starting concentration was adjusted to 3.65 µg/ml.

OIT	Run 1 (21 May 19)			Run 2 (24 May 19)			Run 3 (N/A)		
Treatment	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**
10.6 µg/ml	143.85	1298.12	19.87	231.58	878.24	47.32			
8.83 µg/ml	161.10	1278.27	43.79	200.82	499.58	75.93			
7.36 µg/ml	134.17	683.06	67.40	150.90	296.35	92.38			
6.13 µg/ml	107.97	686.54	64.96	140.09	330.63	94.19			
5.11 µg/ml	131.03	1086.83	50.28	132.16	366.24	93.94			
4.26 µg/ml	115.89	901.41	57.90	131.60	386.09	93.53			
3.55 µg/ml	113.61	953.97	53.90	142.98	332.24	94.06			
2.96 µg/ml	122.01	881.35	51.05	129.91	340.91	94.89			

BIT	Run 1 (21 May 19)			Run 2 (24 May 19)			Run 3 (N/A)		
Treatment	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**
15.7 µg/ml	41.23	967.54	29.96	181.88	871.82	62.30			
13.1 µg/ml	134.36	1446.29	51.78	218.13	515.48	78.16			
10.9 µg/ml	155.60	764.98	82.06	205.18	279.01	87.24			
9.09 µg/ml	143.65	716.72	73.82	175.49	239.99	92.05			
7.57 µg/ml	152.55	630.08	88.22	144.58	198.29	94.50			
6.31 µg/ml	162.18	627.51	91.25	142.12	156.67	95.28			
5.26 µg/ml	153.56	646.68	85.15	140.09	159.57	96.14			
4.38 µg/ml	120.41	568.69	92.27	120.95	168.07	96.58			

DCOIT	Run 1 (21 May 19)			Run 2 (24 May 19)			Run 3 (N/A)		
Treatment	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**	**CD86 RFI**	**CD54 RFI**	**IgG_1 Viability**
1.1 µg/ml	98.80	653.54	45.41	110.56	576.08	91.55			
0.92 µg/ml	73.83	517.56	74.90	99.69	192.00	95.66			
0.76 µg/ml	84.27	513.38	86.67	95.52	106.28	96.50			
0.64 µg/ml	76.27	356.13	90.07	96.37	96.93	96.14			
0.53 µg/ml	92.01	235.63	93.22	88.54	84.15	96.25			
0.44 µg/ml	91.37	201.42	93.35	95.86	78.68	97.19			
0.37 µg/ml	101.47	147.47	94.42	91.30	76.80	96.81			
0.31 µg/ml	100.86	117.81	95.24	95.75	85.51	97.01			

Appendix XI: DPRA control data

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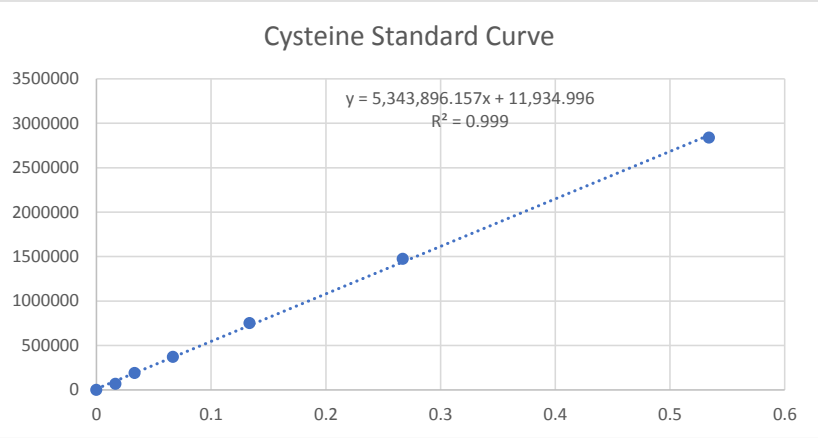
NIEHSO 20180515
Standard Curves Isothiazolinones

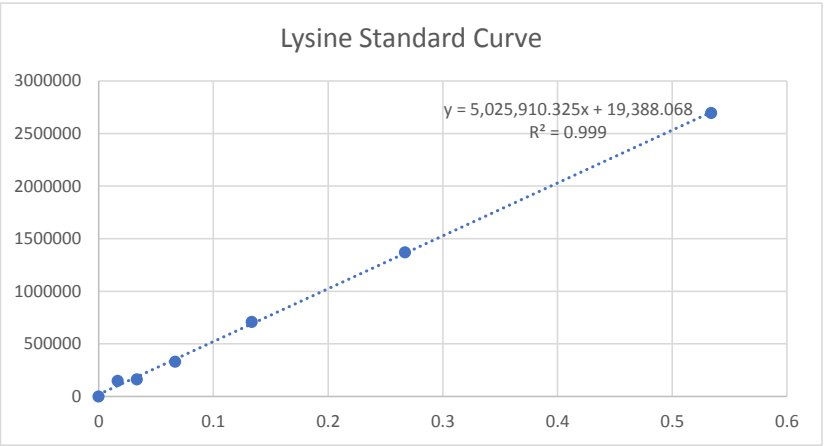
		X values	*Y values*
		Peptide conc. (mM)	**Peptide peak area at 220 nm**
CYSTEINE	STD1	0.534	2840659
	STD2	0.267	1476041
	STD3	0.1335	751941
	STD4	0.0667	373124
	STD5	0.0334	191226
	STD6	0.0167	68592
	STD7	0	0

R^2	0.999
Intercept	11934.996
Slope	5343896.157



		X values	*Y values*
		Peptide Conc. (mM)	**Peptide Peak Area at 220 nm**
LYSINE	STD1	0.534	2696205
	STD2	0.267	1371600
	STD3	0.1335	710365
	STD4	0.0667	330827
	STD5	0.0334	162227
	STD6	0.0167	148232
	STD7	0	0

R^2	0.999
Intercept	19388.068
Slope	5025910.325



NIEHSO 20180515

21 Feb 19

Lysine Set Isothiazolinones

Lysine Peptide	Peptide Peak Area at 220 nm	Peptide Conc. (mM)	Mean Peptide Conc. (mM)	SD of Mean Peptide Conc. (mM)	CV of Peptide Conc.	Mean Peptide Conc. (mM)	SD of Mean Peptide Conc. (mM)	CV of Peptide Conc.
Reference Control A, Rep 1	2625406	0.519	0.512	0.008	1.5			
Reference Control A, Rep 2	2552608	0.504						
Reference Control A, Rep 3	2591189	0.512						
Reference Control B, Rep 1	2495230	0.493	0.506	0.018	3.5	0.501	0.012	2.4
Reference Control B, Rep 2	2526371	0.499						
Reference Control B, Rep 3	2661642	0.526						
Reference Control B, Rep 4	2583600	0.510	0.503	0.007	1.3			
Reference Control B, Rep 5	2515143	0.497						
Reference Control B, Rep 6	2542525	0.502						
Reference Control C, Rep 1 (acetonitrile)	2481795	0.490	0.495	0.012	2.5			
Reference Control C, Rep 2 (acetonitrile)	2577399	0.509						
Reference Control C, Rep 3 (acetonitrile)	2461731	0.486						
Reference Control C, Rep 1 (water)	2457271	0.485	0.484	0.003	0.6			
Reference Control C, Rep 2 (water)	2439300	0.481						
Reference Control C, Rep 3 (water)	2466603	0.487						
Reference Control C, Rep 1 (acetonitrile:water	2498028	0.493	0.501	0.008	1.5			
Reference Control C, Rep 2 (acetonitrile:water	2537780	0.501						
Reference Control C, Rep 3 (acetonitrile:water	2571221	0.508						

	Peptide Peak Area at 220 nm	Percent Peptide Depl.	Mean Percent Peptide Depl.	SD of Percent Peptide Depl.	CV of Percent Peptide Depl.
†Cinnamic aldehyde	1092677	56.4	56	0.4	0.7
	1112399	55.6			
	1101011	56.1			

NIEHSO 20180515
Cysteine Isothiazolinones

Cysteine Peptide	Peptide Peak Area at 220 nm	Peptide Conc. (mM)	Mean Peptide Conc. (mM)	SD of Mean Peptide Conc. (mM)	CV of Peptide Conc.	Mean Peptide Conc. (mM)	SD of Mean Peptide Conc. (mM)	CV of Peptide Conc.
Reference Control A, Rep 1	2723699	0.507	0.511	0.004	0.7			
Reference Control A, Rep 2	2758690	0.514						
Reference Control A, Rep 3	2740334	0.511						
Reference Control B, Rep 1	2741861	0.511	0.511	0.001	0.1	0.501	0.010	2.0
Reference Control B, Rep 2	2741275	0.511						
Reference Control B, Rep 3	2736107	0.510						
Reference Control B, Rep 4	2595731	0.484	0.494	0.009	1.8			
Reference Control B, Rep 5	2680486	0.499						
Reference Control B, Rep 6	2679757	0.499						
Reference Control C, Rep 1 (acetonitrile)	2728741	0.508	0.498	0.009	1.8			
Reference Control C, Rep 2 (acetonitrile)	2666627	0.497						
Reference Control C, Rep 3 (acetonitrile)	2628979	0.490						
Reference Control C, Rep 1 (water)	2721926	0.507	0.500	0.006	1.2			
Reference Control C, Rep 2 (water)	2657314	0.495						
Reference Control C, Rep 3 (water)	2673254	0.498						
Reference Control C, Rep 1 (acetonitrile:water	2653276	0.494	0.493	0.008	1.6			
Reference Control C, Rep 2 (acetonitrile:water	2690767	0.501						
Reference Control C, Rep 3 (acetonitrile:water	2603938	0.485						

	Peptide Peak Area at 220 nm	Percent Peptide Depl.	Mean Percent Peptide Depl.	SD of Percent Peptide Depl.	CV of Percent Peptide Depl.
Cinnamic aldehyde	794094	70.3	71.1	0.8	1.1
	754423	71.8			
	772787	71.1			

Appendix XII: KeratinoSens™ control data

Quality control: Induction values Reference							Criteria		Quality control: Variability blank	
cinnamic aldehyde	*4.00*	*8.00*	*16.00*	*32.00*	*64.00*	**EC 1.5**	**EC 1.5**	**Ind. 64 uM**	**% standard deviation blanks**	
rep1	1.23	1.27	1.76	2.33	5.78	11.72	**TRUE**	**TRUE**	10.0	**ACCEPTED**
rep2	1.20	1.41	1.55	2.01	3.75	13.26	**TRUE**	**TRUE**	7.6	**ACCEPTED**
Average	**1.21**	**1.34**	**1.65**	**2.17**	**4.76**	**12.49**				
Standard Deviation	**0.03**	**0.10**	**0.15**	**0.23**	**1.44**					

Appendix XIII: h-CLAT control data

	Compound	Treatment	CD86 MFI	CD54 MFI	IgG_1 MFI	CD86 RFI	CD54 RFI	IgG1 Viability	CD86 Ratio	CD54 Ratio
Run 1 (21 May 19)	Controls	Media	4801.19	5918.51	2639.10			95.01	181.93	224.26
		0.2% DMSO	4453.29	5086.71	2458.65	92.26	80.14	95.25	181.13	206.89
		DNCB	11183.91	12812.46	3952.86	362.52	337.12	74.66		
	Compound	**Treatment**	**CD86 MFI**	**CD54 MFI**	**IgG_1 MFI**	**CD86 RFI**	**CD54 RFI**	**IgG1 Viability**	**CD86 Ratio**	**CD54 Ratio**
[1]Run 2 (24 May 19)	Controls	Media	3487.50	3259.54	2478.45			97.38	140.71	131.52
		0.2% DMSO	3453.31	3252.75	2283.59	115.92	124.08	97.08	151.22	142.44
		DNCB	8626.79	5820.20	2759.59	501.59	315.80	80.83		
	Compound	**Treatment**	**CD86 MFI**	**CD54 MFI**	**IgG_1 MFI**	**CD86 RFI**	**CD54 RFI**	**IgG1 Viability**	**CD86 Ratio**	**CD54 Ratio**
[1]Run 3 (24 May 19)	Controls	Media	3602.21	3400.79	2472.67			96.84	145.68	137.54
		0.2% DMSO	3626.88	3373.52	2331.61	114.67	112.26	96.11	155.55	144.69
		DNCB	9155.53	6951.71	2875.69	484.83	391.21	78.05		
	Compound	**Treatment**	**CD86 MFI**	**CD54 MFI**	**IgG_1 MFI**	**CD86 RFI**	**CD54 RFI**	**IgG1 Viability**	**CD86 Ratio**	**CD54 Ratio**
[1]Run 4 (31 May 19)	Controls	Media	3149.41	2713.40	2353.14			97.81	133.84	115.31
		0.2% DMSO	3026.80	2690.17	2315.54	89.32	103.99	97.13	130.72	116.18
		DNCB	6090.92	15599.26	2832.55	458.11	3407.82	[2]29.27		
	Compound	**Treatment**	**CD86 MFI**	**CD54 MFI**	**IgG_1 MFI**	**CD86 RFI**	**CD54 RFI**	**IgG1 Viability**	**CD86 Ratio**	**CD54 Ratio**
[1]Run 5 (31 May 19)	Controls	Media	3048.43	2659.30	2228.32			98.03	136.80	119.34
		0.2% DMSO	2940.93	2596.58	2154.49	95.89	102.58	98.09	136.50	120.52
		DNCB	7728.77	18193.24	2807.88	625.72	3480.14	[2]39.93		

[1]Two independent runs were performed on 24 May 19 and 31 May 19.
[2]DNCB viability was <50% in Run 4 and Run 5. CD86, CD54, and IgG_1 MFI values were comparable to historical run results indicating that diffuse labeling of cytoplasmic structures was not evident. Assay was repeated to confirm results.

Appendix XIII: h-CLAT control data (Continued)

	Compound	Treatment	CD86 MFI	CD54 MFI	IgG_1 MFI	CD86 RFI	CD54 RFI	IgG1 Viability	CD86 Ratio	CD54 Ratio
Run 6 (02 Aug 19)	Controls	Media	3625.18	3063.42	2733.33			97.01	132.63	112.08
		0.2% DMSO	3670.80	3018.67	2611.62	118.76	123.31	97.66	140.56	115.59
		DNCB	11617.00	12031.87	3034.03	810.34	2210.50	75.93		
	Compound	**Treatment**	**CD86 MFI**	**CD54 MFI**	**IgG_1 MFI**	**CD86 RFI**	**CD54 RFI**	**IgG1 Viability**	**CD86 Ratio**	**CD54 Ratio**
Run 7 (06 Aug 19)	Controls	Media	5397.16	4590.34	2857.41			95.13	188.88	160.65
		0.2% DMSO	4717.38	3682.11	2816.97	74.83	49.92	97.21	167.46	130.71
		DNCB	12318.17	15357.41	3033.97	488.54	1424.44	58.05		