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Application of Non-animal Test Methods and Defined Approaches to Skin Sensitization Assessment of Isothiazolinone Compounds

National Institutes of Health
U.S. Department of Health and Human Services

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National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
Department of Health and Human Services

June 2020

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List of Acronyms and Abbreviations ¶

ANN	Artificial neural network
AOP	Adverse outcome pathway
Avg	Average
BBIT	1,2-Benzisothiazol-3(2h)-one, 2-butyl
BCF	Bioconcentration factor
BIT	1,2-Benzisothiazolin-3-one
BP	Boiling point
CASRN	Chemical Abstracts Service Registry Number
CD54	Cluster of Differentiation 54, a cell surface protein and intercellular adhesion molecule upregulated upon dendritic cell activation
CD86	Cluster of Differentiation 86, a cell surface glycoprotein and co-stimulatory molecule upregulated upon dendritic cell activation
CMIT	5-Chloro-2-methyl-4-isothiazolin-3-one
CMIT/MIT	Mixture of 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-4-isothiazolin-3-one
Cys	Cysteine
DAs	Defined approaches
DASS	Defined approaches for skin sensitization
DCs	Dendritic cells
DCOIT	4,5-Dichloro-2-octyl-3(2h)-isothiazolinone
DPRA	Direct Peptide Reactivity Assay
EC1.5 or 3	Effective concentration of a test substance that produces a stimulation index (SI) of 1.5-fold or 3-fold compared to the vehicle control
EC150	Effective concentration of a test substance that produces a 150% increase in the expression of the CD86 cell surface marker
EC200	Effective concentration of a test substance that produces a 200% increase in expression of the CD54 cell surface marker
EPA	U.S. Environmental Protection Agency
GHS	Globally Harmonized System of Classification and Labelling of Chemicals

GPMT	Guinea pig maximization test
h-CLAT	Human cell line activation test
IC50	Concentration of a test substance that produces 50% inhibition of the response compared to vehicle controls
Imax	Maximum induction
IT	Isothiazolinone
LLNA	Murine local lymph node assay
Log_{10} P	Base 10 logarithm of the oil:water partition coefficient
Log_{10} S	Base 10 logarithm of water solubility
Lys	Lysine
MIT	2-Methyl-4-isothiazolin-3-one
MP	Melting point
MW	Molecular weight
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
OIT	2-n-Octyl-4-isothiazolin-3-one
OPERA	Open Structure-activity/property Relationship App
QSAR	Quantitative structure-activity relationships
SI	Stimulation index
SMILES	Simplified molecular information line entry system
TG	Test guideline
UN	United Nations
VP	Vapor pressure

Acknowledgements

This document has been reviewed by, and reflects comments received from, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Skin Sensitization Expert Group (SSEG). The ICCVAM SSEG is comprised of experts from seven* different ICCVAM member agencies who are listed below.

- Moiz Mumtaz, PhD, Agency for Toxic Substances and Disease Registry (ATSDR)
- Patricia Ruiz, PhD, ATSDR
- John Gordon, PhD, Consumer Product Safety Commission (CPSC)
- Joanna Matheson, PhD, CPSC
- Emily Reinke, PhD, DABT, Department of Defense
- Paul Brown, PhD, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER)
- Wei Ding, PhD, FDA, CDER
- Hon Sum Ko, MD, FDA, CDER
- Diego Rua, PhD, FDA, Center for Devices and Radiological Health (CDRH)
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- Robert Heflich, PhD, FDA, National Center for Toxicological Research
- Warren Casey, PhD, National Institute of Environmental Health Sciences (NIEHS)
- Dori Germolec, PhD, NIEHS
- Nicole Kleinstreuer, PhD, NIEHS
- Elijah Peterson, PhD, National Institute of Standards and Technology

*The U.S. Environmental Protection Agency (EPA) is one of the seven member agencies on the ICCVAM SSEG. However, EPA members were recused from this review given their separate evaluation of this information during the risk assessment process and therefore are not listed above.

The work described herein was funded in part by the National Institute of Environmental Health Sciences, National Institutes of Health under Contract No. HHSN273201500010C to Integrated Laboratory Systems, LLC.

Executive Summary ¶

Integrated approaches to assessing skin sensitization potential and assigning potency category leverage the combination of multiple methods to overcome the limitations of individual tests. Approaches that use predetermined data sources with fixed data interpretation procedures to arrive at an outcome without the use of expert judgment are referred to as defined approaches (DAs). This report provides the performance of individual in chemico, in vitro, and in silico methods for predicting skin sensitization potency in comparison to murine local lymph node assay (LLNA) results, and evaluates two versions of the Shiseido Artificial Neural Network (ANN) DA for the prediction of skin sensitization potency. The ANNs use combinations of non-animal tests that align with multiple key events in the adverse outcome pathway for skin sensitization. The test substances for this case study were six isothiazolinone (IT) compounds:

- 4,5-Dichloro-2-octyl-3(2h)-isothiazolinone (DCOIT)
- 5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT mixture)
- 2-n-Octyl-4-isothiazolin-3-one (OIT)
- 2-Methyl-4-isothiazolin-3-one (MIT)
- 1,2-Benzisothiazolin-3-one (BIT)
- 1,2-Benzisothiazol-3(2h)-one, 2-butyl (BBIT)

The IT compounds were tested using three non-animal skin sensitization tests described by internationally harmonized test guidelines issued by the Organisation for Economic Co-operation and Development (OECD): direct peptide reactivity assay (DPRA, TG442C), KeratinoSens™ (TG442D), and human cell line activation test (h-CLAT, TG442E). Skin sensitization hazard was also predicted by in silico read-across algorithms in the OECD QSAR Toolbox. The LLNA data were curated based on a report submitted by Dow and a literature search performed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, evaluated for study quality, and used to assign a representative in vivo potency value.

The skin sensitization hazard results showed that each of the individual non-animal test methods, as well as the in silico tool, classified all of the IT compounds as sensitizers, which is concordant with LLNA results. A potency evaluation using the individual in chemico and in vitro methods was also performed by ranking the substances using each test method (the in silico read-across results were used for hazard classification only and thus did not provide potency information). KeratinoSens and h-CLAT produced a similar ranking to that for the

LLNA. Peptide depletion values from DPRA were too similar to be useful for ranking the six IT compounds for skin sensitization potency.

The two versions of the Shiseido ANN DA (one relying on DPRA and h-CLAT only and the other relying on DPRA, h-CLAT, and KeratinoSens) provided quantitative values for the effective concentration at 3-fold induction (EC3) as outputs. The potency rankings based on the EC3 values predicted by the two ANN DAs were similar to one another and were also similar to those derived from the LLNA. The quantitative EC3 values predicted by the DAs were within 30-fold of the LLNA values for all IT compounds.

1.0 Introduction

Numerous non-animal alternatives for skin sensitization hazard assessment have been developed and are at various stages of evaluation (Ezendam et al. 2016, Mehling et al. 2012). Because skin sensitization is a complex process, it is unlikely that any individual alternative method will completely replace current animal tests. In fact, even the in vitro and in chemico methods that have been adopted as international test guidelines are not yet recommended as stand-alone replacements for animal test methods (OECD 2018a, b; 2019). Thus, a number of approaches to integrate the information from multiple non-animal methods as a way to overcome the limitations of individual tests and more accurately assess the potential for skin sensitization have been evaluated and compared to one another (Kleinstreuer et al. 2018). These approaches, which preclude the use of expert judgement by applying fixed data interpretation procedures to specific data streams, are referred to as "defined approaches" or "DAs." These DAs use combinations of non-animal tests that align with key events in the adverse outcome pathway for skin sensitization (OECD 2012).

1.1 Background

In partnership with the IT Task Force of the American Chemistry Council, the U.S. Environmental Protection Agency (EPA) nominated six isothiazolinone (IT) compounds (**Table 1**) to the National Toxicology Program (NTP) for testing in non-animal skin sensitization test methods. IT Task Force members donated the compounds for testing. The EPA will evaluate these data for use as a case study in ranking the potency of the six IT compounds and for performing quantitative risk assessment for these substances.

Table 1 Isothiazolinone Compounds Nominated for Testing

Common Name	Chemical Name	CASRN	Product Name	Donor	% Active Ingredient
DCOIT	4,5-Dichloro-2-octyl-3(2h)-isothiazolinone	64359-81-5	KATHON 287T Industrial Microbicide	Dow	99.3
CMIT/MIT	Mixture of 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-4-isothiazolin-3-one	55965-84-9	MERGAL MITZ	Troy Corporation	14.2
OIT	2-n-Octyl-4-isothiazolin-3-one	26530-20-1	ACTICIDE OIT	Thor	98.13
MIT	2-Methyl-4-isothiazolin-3-one	2682-20-4	KORDEK 573F Biocide	Dow	50.8
BIT	1,2-Benzisothiazolin-3-one	2634-33-5	MERGAL BIT Technical	Troy Corporation	85.2
BBIT	1,2-Benzisothiazol-3(2h)-one, 2-butyl	4299-07-4	VANQUISH 100	Lonza	98.4

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

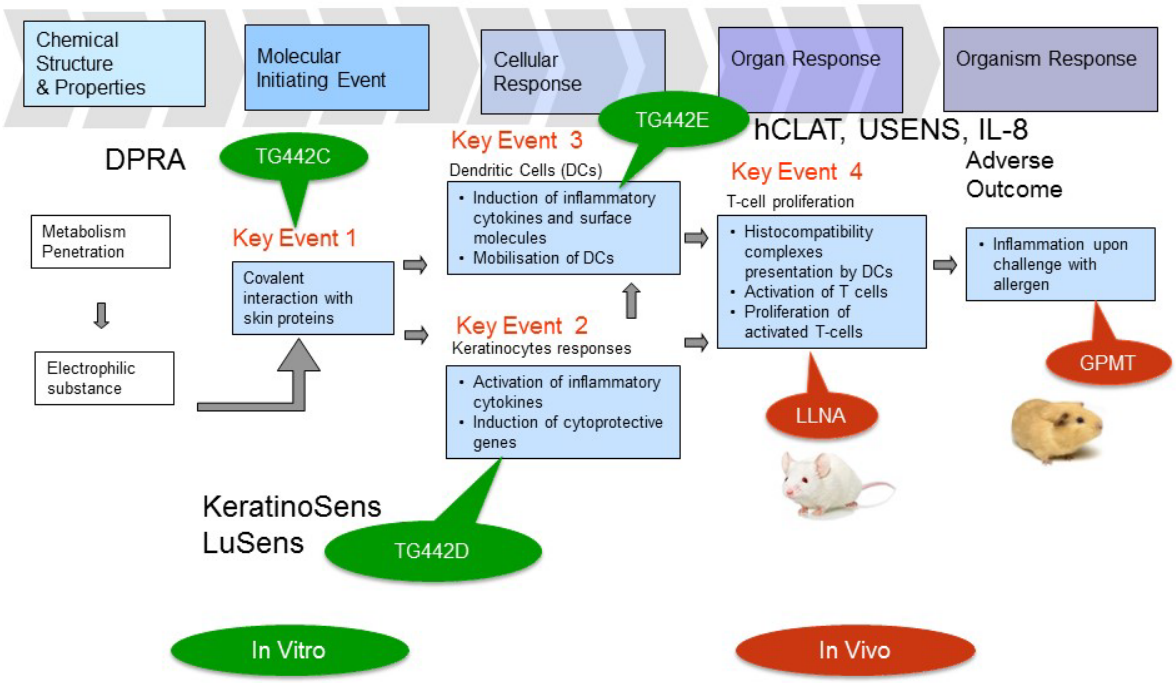
1.1.1 Adverse Outcome Pathway (AOP) for Skin Sensitization with Key Events as Targets of Alternative Method Development

An AOP is a conceptual framework constructed from existing knowledge that relates exposure of a type of toxic substance to subsequent molecular and cellular changes that in turn result in illness or injury to an individual or population (OECD 2012). The AOP for skin sensitization initiated by covalent binding to proteins (**Figure 1**) includes four key events with well-accepted biological significance: 1) binding of haptens to endogenous proteins in the skin, 2) keratinocyte activation, 3) dendritic cell activation, and 4) proliferation of antigen-specific T cells. The construction of the AOP for skin sensitization has prompted test method developers and users to align the available and conceivable methods with the key events of the AOP (Reisinger et al. 2015). Designers of defined approaches and integrated approaches to testing and assessment use the AOP as a framework to design strategies covering different multiple key events (OECD 2016). Assessment strategies using multiple methods are valuable for overcoming the limitations of the individual methods.

Figure 1 shows the association of the non-animal tests performed for this case study with the key events of the AOP. The non-animal tests include the in chemico direct peptide reactivity

assay (DPRA; OECD 2019), and the in vitro cell-based methods, KeratinoSens ™ (OECD 2018a) and human cell line activation test (h-CLAT; OECD 2018b). In silico read-across predictions (e.g. the QSAR Toolbox) cover the entire AOP because they are based on responses from in vivo methods, the murine local lymph node assay (LLNA) (OECD 2010) and guinea pig tests (OECD 1992).

Figure 1 Adverse Outcome Pathway for Skin Sensitization Caused by Covalent Binding to Proteins



Abbreviations: GPMT = Guinea Pig Maximization Test; TG = test guideline.

1.2 Objective

This report summarizes the in chemico, in vitro, in vivo, and in silico skin sensitization data and physicochemical properties for six isothiazolinone compounds and the integration of these data using defined approaches (DAs). This analysis is proposed as a case study in ranking the potency of these compounds and performing quantitative risk assessment. The report provides the performance of individual in chemico, in vitro, and in silico methods for predicting skin sensitization potency as determined by comparison to the murine local lymph node assay (LLNA). It also includes an evaluation of two DAs for the prediction of skin sensitization potency. The DAs evaluated include the Shiseido Artificial Neural Network Models "Model 1" and "Model 4" as published in Hirota et al. (2015), which rely on DPRA and h-CLAT or DPRA, h-CLAT, and KeratinoSens, and are referred to here as ANN D_hC and ANN D_hC_KS, respectively.

2.0 Methods

2.1 In Chemico and In Vitro Data Generated for This Project

Burleson Research Technologies, Inc., the NTP contract laboratory for immunotoxicity testing, tested the six isothiazolinone compounds using DPRA, KeratinoSens, and h-CLAT. Sections 2.1.1 to 2.1.3 review the tests performed by Burleson Research Technologies. The comprehensive test report, which includes detailed protocols for the methods and results, is provided in **Appendix A**.

2.1.1 DPRA

DPRA is an in chemico test that assesses the ability of a substance to form a hapten–protein complex (Gerberick et al. 2007; OECD 2019a), which is the molecular initiating event in the skin sensitization AOP (OECD 2012). Average cysteine and lysine depletion >6.38% indicates a sensitizer outcome. If the lysine peptide co-elutes with the test chemical, peptide reactivity can be assessed using cysteine depletion only. In that case, a sensitizer outcome is indicated when cysteine depletion is >13.89%. The measurement endpoints provided by the DPRA are: cysteine peptide depletion (Cys), lysine peptide depletion (Lys), average depletion of cysteine and lysine peptides (Avg.Lys.Cys), and sensitizer/nonsensitizer outcome. The DAs applied here, Shiseido ANN D_hC and ANN D_hC_KS, used the Avg.Lys.Cys values as inputs.

2.1.2 KeratinoSens

The KeratinoSens test method assesses the ability of a substance to activate cytokines and induce gene expression associated with specific cell signaling pathways in keratinocytes (Emter et al., 2010; OECD 2018a), the second key event in the skin sensitization AOP (OECD 2012). A sensitizer outcome is indicated when luciferase induction is statistically significant and at least 1.5-fold higher than control values at a concentration with cell viability >70%. The KeratinoSens assay provides the effective concentration at 1.5-fold luciferase induction (EC1.5), the effective concentration at 3-fold induction (EC3), the maximum induction (Imax) and the inhibitory concentration at 50% viability (IC50). The Imax was used in the DAs (Shiseido ANN D_hC_KS) applied here.

2.1.3 h-CLAT

h-CLAT assesses the ability of a substance to activate and mobilize dendritic cells in the skin (Ashikaga et al. 2016; OECD 2018b), the third key event of the skin sensitization AOP (OECD 2012). This test measures the induction of two cell surface markers, CD86 and CD54, which indicate dendritic cell activation. A cytotoxicity assay to determine 75% cell viability (CV75) is used to select the doses to be tested. The measurement endpoints for the

h-CLAT include the effective concentration at 150% induction for the CD86 marker (EC150) and the effective concentration at 200% induction for the CD54 marker (EC200). A sensitizer outcome is indicated when CD86 expression is at least 150% or CD54 expression is at least 200% with cell viability > 50%. All the DAs applied here used the minimum induction threshold from the CD86 and CD54 measurements. The minimum induction threshold is the lower value of these two measurements.

h-CLAT testing was also performed by the Institute for In Vitro Sciences and the data were used to assess consistency of the h-CLAT results (see **Section 3.1**); however, the defined approaches incorporated data generated at Burleson Research Technologies only. The Institute for In Vitro Sciences test report is provided as **Appendix B**. Data from the Institute for In Vitro Sciences in **Section 3** have been revised to report the effective concentrations of the active ingredients using the proportion of active ingredients in each product (**Table 1)**, but data in their report have not been revised.

2.2 Generation of In Silico Read-Across Hazard Predictions for Skin Sensitization Hazard: OECD QSAR Toolbox V4.3

QSAR Toolbox v4.3 (OECD 2019b), provided by the Organisation for Economic Co-operation and Development (OECD), was used to generate an in silico read-across hazard prediction (whether each substance was a sensitizer or nonsensitizer) based on in vivo LLNA and guinea pig data from structurally and mechanistically similar analogs. Inputs to the Toolbox were the SMILES chemical structure notation for each substance, obtained from the EPA Chemistry Dashboard (Williams et al. 2017). The automated workflow for skin sensitization was used to make the predictions. Because the automated workflow does not make predictions for substances that are not discrete chemicals, such as CMIT/MIT, predictions were made separately for CMIT and MIT. When the automated workflow could not make hazard predictions for single chemicals (e.g., due to an insufficient number of analogues), the "Skin Sensitization for DASS" profiler was implemented manually. This profiler assesses each substance, its auto-oxidation products, and skin metabolites for protein binding alerts for skin sensitization using the OASIS profiler. The results from this profiler indicate a sensitizer classification if any substance, its auto-oxidation products, or its skin metabolites are associated with a protein binding alert. The Skin Sensitization for DASS profiler will be automated in future versions of the Toolbox. The automated workflow failed to make hazard predictions for DCOIT, BIT, and CMIT. These predictions were performed by manually implementing the Skin Sensitization for DASS profiler.

2.3 Physicochemical Properties

The following physicochemical properties were collected for the IT compounds:

- Log_{10} P (octanol:water coefficient)
- Log_{10} S (water solubility) in M
- Log_{10} vapor pressure (VP) in mmHg
- Melting point (MP) and boiling point (BP), both in °C
- Molecular weight (MW) in g/mol
- Bioconcentration factor (BCF)

Experimental values for each physicochemical property were preferred, but when those were unavailable, predicted values were collected. Experimental log_{10} P values for each substance were provided by Andrew Byro, EPA. Means and ranges are shown for substances with multiple tests. All other values were obtained from OPERAv2.3, the Open Structure-activity/property Relationship App (https://github.com/NIEHS/OPERA). Only two experimental values, log_{10} VP and log_{10} S for OIT, were available. The remaining physicochemical properties for DCOIT, OIT, BIT, BBIT, and MIT were predicted. No physicochemical properties were available for CMIT/MIT because it is a mixture of two different structures. Physicochemical properties are shown in **Table 2**.

Table 2 Physicochemical Properties

Chemical	Log P	Log S (M)	BP (°C)	MP (°C)	Log VP (mmHg)	Log BCF	MW (g/mol)
DCOIT	4.4 (2.8-6.4)	-4.123	287	42.1	-3.983	1.942	281.04
CMIT/MIT	NA	NA	NA	NA	NA	NA	NA
OIT	3.4 (2.4-4.4)	-2.630	255	16.6	-4.434	1.148	213.12
MIT	-0.486	-0.435	154	131.3	0.349	0.309	115.01
BIT	1.35	-2.828	312	108.8	-4.845	0.651	151.01
BBIT	2.86	-4.002	310	87.7	-5.382	0.784	207.07

Abbreviations: NA = Not available.
All logarithms are base 10.

2.4 Evaluation of LLNA Reference Data

LLNA data were obtained from two major sources: a report submitted to EPA from Dow (Begolly 2019) (17 studies) and from publicly available scientific literature (15 studies). No LLNA studies were available for BBIT. Data from all LLNA studies are provided in **Appendix C.** With the exception of one study for MIT, all LLNA studies for all six IT substances yielded positive results. The negative MIT test, which was tested at a maximum concentration of 4.5% in water, was performed on the ultra-pure MIT product. The other four tests for MIT that yielded positive results used maximum concentrations of 0.985- 2.2% in acetone:olive oil or propylene glycol.

Using two different approaches, one from Dow and one from the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the LLNA data were evaluated to determine a single representative EC3, defined in the context of the LLNA as the concentration inducing a stimulation index (SI) of 3. This representative EC3 was used to classify each substance according to the potency categories of the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2019). Substances with EC3 ≤2% are 1A (strong) sensitizers, substances with EC3 >2% are 1B (other) sensitizers, and substances that do not produce a positive response in the LLNA are not classified.

The Dow report included two to four studies for each of five substances, totaling 17 LLNA studies. Dow determined a representative EC3 for each substance by selecting the tests that were performed using acetone or acetone:olive oil as the solvent. Their rationale was that they considered the best way to rank these substances for potency to be using tests with the same or similar solvents because it is well known that EC3 values can vary with solvent (Dumont et al. 2016; Anderson et al. 2011). Two representative tests with similar EC3 values, 0.20% and 0.25% were selected for OIT. Of the studies evaluated, the representative EC3 values were also the most potent values available for each substance (**Table 3**). The Dow approach classified all substances with LLNA results as GHS 1A sensitizers.

The NICEATM approach used the 17 studies provided by Dow and 15 studies from the scientific literature to determine a representative EC3 for each substance. Again, no studies were found for BBIT. A total of 32 studies were available with three to 13 studies for each of the other five substances. One MIT test with EC3 = 1.9% from Gerberick et al. (2005) was excluded because it was the same test reported by Basketter et al. (2003); it had the same stimulation index values with erroneous test concentrations and EC3 value (Roberts 2013). The remaining individual LLNA tests were evaluated for inclusion in determining a single representative mean EC3 using the approach designed by the OECD Expert Group for Defined Approaches for Skin Sensitization. To be included in the evaluation, studies were required to have these attributes:

- The test substance was applied topically to both ears of the mice.
- Lymphocyte proliferation was measured in the lymph nodes draining the site of test substance application.
- Lymphocyte proliferation was measured during the induction phase of skin sensitization.
- A vehicle control was included.
- Either individual or pooled animal data were collected.
- Concentrations tested and corresponding SI values were available.

- ^{3}H-methyl thymidine or other radiolabeled marker was administered in vivo rather than ex vivo
- Sodium lauryl sulfate was not applied to enhance the response.
- Extrapolated EC3 values passed the criteria from Ryan et al. (2007) as follows:
    - The lowest measured SI value was less than five.
    - The extrapolated EC3 was less than 10-fold of the closest tested concentration.
    - The slope ratio was less than two and non-negative. This value is the ratio of the slope from the high dose to the mid-dose to that from the mid-dose to the lowest dose.¶

The NICEATM evaluation rejected five studies because they did not meet the criteria for extrapolated EC3 values. Four studies were rejected because the lowest SI was greater than 5: these included three CMIT/MIT tests with EC3 = 0.021, 0.012, and 0.003% with lowest SI = 6.3, 10.43, and 8.1, respectively, and one DCOIT test (no EC3 calculated because lowest SI = 32.14). One CMIT/MIT test with EC3 = 0.002564% was rejected because the slope ratio was negative. Two to nine studies were then available for each of the five substances with LLNA studies. A representative EC3 for each substance was calculated by determining the mean EC3 for each substance (**Table 3**). The NICEATM approach classified all substances with LLNA results into as GHS 1A sensitizers, except for BIT, which was classified as a GHS 1B sensitizer.

Table 3 Representative LLNA EC3 Values

Chemical	Dow LLNA EC3 (%)	Dow GHS Classification	NICEATM LLNA EC3 (%)[a]	n for NICEATM LLNA EC3	NICEATM GHS Classification
DCOIT	0.004	1A	0.008 (0-0.053)	2	1A
CMIT/MIT	0.002	1A	0.018 (0.0011-0.034)	9	1A
OIT	0.2-0.25 (n=2)	1A	0.361 (0.029-0.69)	4	1A
MIT	0.863	1A	1.154 (0-3.476)	3[b]	1A
BIT	1.54	1A	10.57 (0-23.36)	7	1B
BBIT	NA	NA	NA	0	NA

Abbreviations: NA = not available (no LLNA data for BBIT)

[a] Numbers in parentheses are the 95% confidence intervals for the mean EC3

[b] NICEATM identified four acceptable LLNA studies for MIT, but one was negative and did not provide an EC3 value

2.5 Brief Description of Defined Approaches Used for This Project

The Shiseido artificial neural network (ANN) models are non-linear statistical models that combine multiple in vitro parameters covering various key events of the skin sensitization AOP and predict the LLNA EC3 as an output. The ANN models consist of an input layer (descriptors from in vitro results), a hidden layer, and an output layer (EC3 predictions). Two of the four Shiseido ANN models described in Hirota et al. (2015) were evaluated here, chosen based on availability of the input data and published performance of the models. The first model (ANN D_hC, "Model 1" in Hirota et al. 2015) used quantitative values from the DPRA (Avg.Lys.Cys) and the h-CLAT (minimum induction threshold) to predict the EC3 value that would be produced in the LLNA. The second model (ANN D_hC_KS, "Model 4" in Hirota et al. 2015) used the same structure with an additional value from the KeratinoSens (Imax) used as the third input. The ANN DAs were coded in R (available upon request), and in brief, logistic activation functions were used for the hidden and output layers, 10,000 iterations were used for training, and learning rate, scaling functions, and momentum parameters were inferred from Hirota et al. (2015). For each IT compound, each model was run 100 times and mean EC3 prediction and 95% confidence intervals were calculated. Additional details on DAs and performance-based validation on a set of 128 reference chemicals can be found in Kleinstreuer et al. (2018). All data used as information sources for the DAs, as well as the DA output predictions, are included in **Appendix D**.

2.6 Data Analyses

2.6.1 Comparison of Individual Non-Animal Methods Against LLNA

Concordance of the hazard classifications for in chemico and in vitro data amongst the non-animal tests was evaluated as well as concordance of the non-animal methods with the LLNA data. Concordance of potency was compared by ranking the IT compounds from most potent to least potent using both the LLNA EC3 values and the measured endpoints from the in chemico and in vitro methods. The in silico read-across predictions were not used for potency ranking because they are not quantitative.

2.6.2 Comparison of Defined Approaches Against LLNA

Concordance of the defined approaches with the LLNA data, with respect to hazard classifications and potency predictions, was evaluated. Concordance of potency was compared by ranking the IT compounds from most potent to least potent using both the measured LLNA EC3 values and the predicted EC3 values from the ANN DAs. Root mean square error (RMSE) and mean absolute error (MAE) were reported for the measured vs. predicted EC3 values.

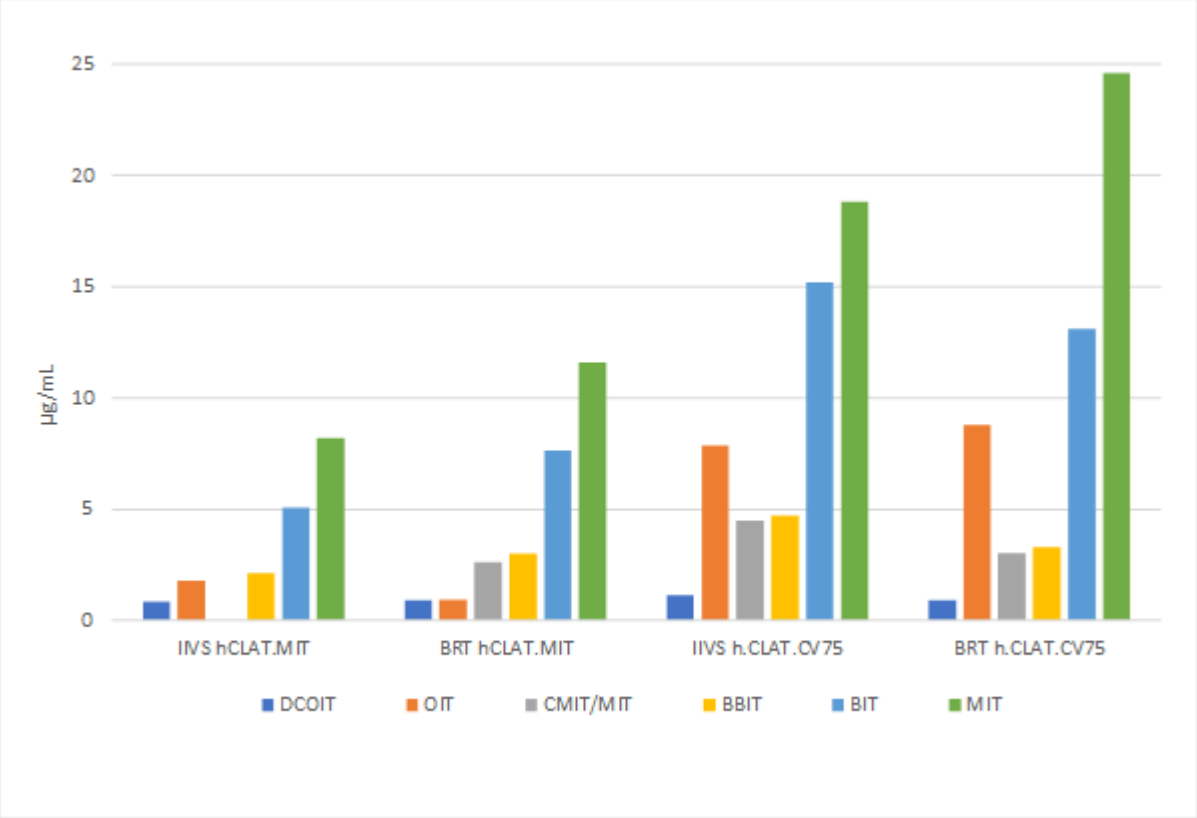
3.0 Results

3.1 Skin Sensitization Hazard Comparison of Individual Non-animal Methods with Respect to LLNA Results

The hazard classification results for each of the non-animal test methods, DPRA, KeratinoSens, h-CLAT, and for the in silico read-across, were the same for each of the six isothiazolinone compounds. All tests performed by Burleson Research Technologies classified all six compounds as sensitizers. With the exception of BBIT, which had no LLNA data, the hazard classification of the non-animal methods was concordant with that of the LLNA.

The h-CLAT results from Burleson Research Technologies and the Institute for In Vitro Sciences were comparable (**Figure 2**). With the exception of CMIT/MIT, which yielded negative results for cell surface marker expression at the Institute for In Vitro Sciences in 2/3 tests, the results from the two laboratories ranked the chemicals in the same order for both cell surface marker expression and cytotoxicity (CV75). h-CLAT data from the Institute for In Vitro Sciences are provided because they were available to show the consistency of h-CLAT data between laboratories. Reports of h-CLAT data hereafter are those from Burleson Research Technologies, which also performed the DPRA and KeratinoSens tests and is the official immunotoxicity testing contract facility for NTP.

Figure 2 Comparison of h-CLAT Results from Two Laboratories



Abbreviations: BRT = Burleson Research Technologies; IIVS = Institute for In Vitro Sciences

[a] All results have been corrected for % active ingredient

3.2 Skin Sensitization Potency Comparison of Individual Non-animal Methods with Respect to LLNA Results

Because the in chemico and in vitro methods are not to be used for potency classification (OECD 2018a, b; 2019), no GHS criteria for these methods have been proposed for classification of 1A and 1B sensitizers. However, the sensitization endpoint measurements from these methods (**Table 4**) were used as indicators of potency to rank the six IT compounds and compare with the LLNA EC3 rankings (**Table 5**).

Table 4 Skin Sensitization Measurement Endpoints for LLNA and Non-animal Methods

Chemical	Dow LLNA EC3 (%)	NICEATM EC3 (%)[a]	DPRA Mean Depletion (%)	Keratino-Sens EC1.5 (µM)[b]	Keratino-Sens Imax	h-CLAT Minimum Induction Threshold (µg/mL)[b]
DCOIT	0.004	0.008 (0-0.053)	55.2	1.32	4.37	0.92
CMIT/MIT	0.002	0.018 (0.0011-0.034)	55.3	3.41	5.61	2.63
OIT	0.2-0.25	0.361 (0.029-0.69)	50	2.19	3.70	0.95
MIT	0.863	1.154 (0-3.476)	50	9.54	15.84	11.6
BIT	1.54	10.57 (0-23.36)	NA	3.14	17.64	7.63
BBIT	NA	NA	50	3.84	19.61	3.01

Abbreviations: NA = not available

[a] Numbers in parentheses are the 95% confidence intervals for the mean EC3

[b] Results corrected for % active ingredient

Peptide depletion values from DPRA were not useful for ranking potency because all of the compounds reacted very strongly with the cysteine peptide and minimally, or not at all, with the lysine peptide (**Appendix A, Table 2**). The exception was BIT, which co-eluted with the lysine peptide. Based on a statistical comparison with the NICEATM LLNA ranking, KeratinoSens EC1.5 and h-CLAT yielded similar ranks (Wilcoxon signed rank test, p-values of 0.85 and 0.59, respectively). Both methods ranked DCOIT as the most potent and MIT as the least potent (**Table 5**).

The representative LLNA EC3 values used by Dow and NICEATM yielded the same ranks except for the positions of DCOIT and CMIT/MIT, which were 2 and 1 for the Dow approach and 1 and 2 for the NICEATM approach (**Table 5**). The ranks based on KeratinoSens EC1.5 and h-CLAT were roughly similar to that for the LLNA, which ranked DCOIT as very potent and BIT and MIT as least potent.

Table 5 Potency Rank by Test Method

Chemical	Dow LLNA	NICEATM LLNA	KeratinoSens	h-CLAT
DCOIT	2	1	1	1
CMIT/MIT	1	2	4	3
OIT	3	3	2	2
MIT	4	4	6	6
BIT	5	5	3	5
BBIT	NA	NA	5	4

NA = not available (no LLNA data for BBIT)

3.3 Comparison of Defined Approaches and LLNA Results for Hazard and Potency

The hazard classification result for each of the DAs was the same for each of the six isothiazolinone compounds, where all six compounds were classified as sensitizers. With the exception of BBIT, which had no LLNA data, the hazard classification of the DAs was concordant with that of the LLNA. The potency classification (**Table 6**) of 1A for all compounds was concordant across the DAs and with the LLNA data, with the exception of the NICEATM LLNA for BIT, which yielded a 1B classification, and BBIT, which had no LLNA data.

Table 6 Potency Classification Prediction for Isothiazolinones

Chemical	Dow LLNA	NICEATM LLNA	DA: ANN D_hC[a] Potency	DA: ANN D_hC_KS[b] Potency
DCOIT	1A	1A	1A	1A
CMIT/MIT	1A	1A	1A	1A
OIT	1A	1A	1A	1A
MIT	1A	1A	1A	1A
BIT	1A	1B	1A	1A
BBIT	NA	NA	1A	1A

[a] Model 1 from Hirota et al. 2015: DPRA + h-CLAT
[b] Model 4 from Hirota et al. 2015: DPRA + h-CLAT + KeratinoSens

3.4 Comparison of Predicted Potency to LLNA

The two ANN DAs provide quantitative EC3 predictions as outputs, shown below in comparison to the LLNA EC3 values from Dow or NICEATM (**Table 7**). When comparing the five IT compounds with in vivo data and quantitative DA predictions, the RMSE between the Dow LLNA EC3 values and the DA EC3 values was 0.49 for the model using only DPRA and h-CLAT (ANN D_hC) and 0.57 for the model using DPRA, h-CLAT, and

KeratinoSens (ANN D_hC_KS). The MAE between the Dow EC3s and the DA EC3s was 0.36 for ANN_D_hC and 0.38 for ANN D_hC_KS. The RMSE between the NICEATM LLNA EC3 values and the ANN DA EC3 values was 4.32 for the ANN D_hC model and 4.58 for the ANN D_hC_KS model, and the MAEs were 2.14 and 2.28, respectively. The differences in these comparative values were driven by the different representative LLNA EC3 values for BIT between the Dow data and the NICEATM data, where the DA EC3 predictions for BIT were more similar to the Dow data.

The quantitative EC3 predictions derived from the ANN DAs were similar to the NICEATM LLNA EC3 values, with overlapping 95% confidence intervals (CI) in most cases, with the exception of CMIT/MIT, where the upper bound of the in vivo CI was 3.5-fold less than the lower bound of the in silico CI (for the ANN D_hC DA). Because the in vivo EC3 values for CMIT/MIT were low in comparison to those for the most potent component, CMIT (EC3=0.009 and 0.01% from the NICEATM LLNA database [NICEATM 2013]), EC3 values weighted by the amount of each component were calculated (**Appendix E**). The weighted EC3 values of 0.21% (Dow approach) and 0.28% (NICEATM approach) were closer to the predicted values from the ANN DAs. While the in vivo and in silico CI for BIT did overlap, the average EC3 predictions derived from the DAs were closer to the in vivo estimate provided by Dow than that calculated by NICEATM. The largest discrepancy between the two ANN DAs was seen for the CMIT/MIT mixture, with a 4-fold difference between the average EC3 predictions.

Table 7 Quantitative EC3 Prediction for Isothiazolinones

Chemical	Dow LLNA EC3 (%)	NICEATM LLNA EC3 (%)[a]	DA: ANN D_hC[b] EC3 (%)[a]	DA: ANN D_hC_KS[c] EC3 (%)[a]
DCOIT	0.004	0.008 (0-0.053)	0.0566 (0.0555 – 0.0578)	0.023 (0.02 – 0.026)
CMIT/MIT	0.002[d]	0.018 [e] (0.0011-0.034)	0.121 (0.119 – 0.123)	0.492 (0.4 – 0.605)
OIT	0.2-0.25	0.361 (0.029-0.69)	0.0569 (0.0559 – 0.058)	0.015 (0.013 – 0.017)
MIT	0.863	1.154 (0-3.476)	1.775 (1.732 – 1.818)	0.826 (0.759 – 0.9)
BIT	1.54	10.57 (0-23.36)	0.934 (0.909 – 0.959)	0.341 (0.317 – 0.367)
BBIT	NA	NA	0.148 (0.146 – 0.151)	0.061 (0.055 - 0.068)

[a] Numbers in parentheses are the 95% confidence intervals
[b] Model 1 from Hirota et al. 2015: DPRA + h-CLAT
[c] Model 4 from Hirota et al. 2015: DPRA + h-CLAT + KeratinoSens
[d] Weighted EC3 = 0.21% using CMIT data from NICEATM LLNA database that were selected using the same criteria used by Dow: vehicle was acetone or acetone:olive oil
[e] Weighted EC3 = 0.28% using the average of CMIT values from NICEATM LLNA database

The predicted EC3 values in **Table 7** from the ANN DAs were used to rank the six isothiazolinones by potency (**Table 8**) and compared to the potency rank derived from the LLNA studies based on the Dow submission or NICEATM literature review (also from **Table 7**). The DAs ranked DCOIT and OIT as the most potent IT compounds in the class, followed by CMIT/MIT, BBIT, and BIT (with differing ranks for this middle group between the two DAs) and lastly MIT. With the exception of BBIT, which had no LLNA data, the ranks for ANN D_hC and D_hC_KS were similar to in vivo results, based on a statistical comparison with the NICEATM LLNA rank (Wilcoxon signed rank test, p-values of 0.59 in each case).

Table 8 Potency Rank Comparison

Chemical	Dow LLNA	NICEATM LLNA	DA: ANN D_hC[a]	DA: ANN D_hC_KS[b]
DCOIT	2	1	1	2
CMIT/MIT	1	2	3	5
OIT	3	3	2	1
MIT	4	4	6	6
BIT	5	5	5	4
BBIT	NA	NA	4	3

[a] Model 1 from Hirota et al. 2015: DPRA + h-CLAT
[b] Model 4 from Hirota et al. 2015: DPRA + h-CLAT + KeratinoSens

3.5 Consideration of Uncertainties for the In Vivo, In Chemico, and In Vitro Data, and for the Defined Approaches

3.5.1 Uncertainties Related to the In Vivo Data

The LLNA is a standardized test method described in an internationally harmonized OECD test guideline for skin sensitization assessment. This method has been validated as relevant and reproducible for skin sensitization hazard and potency. It is applicable for testing most substances unless there are properties associated with a substance that may interfere with the accuracy of the LLNA (e.g., certain metals and surfactants).

The in vivo nature of the test incorporates the absorption, distribution, metabolism, excretion, and pharmacodynamic elements of the adverse outcome pathway between chemical exposure and key event 4, T-cell proliferation. The inherent reproducibility of the LLNA has been shown by multiple analyses (e.g. Hoffman et al. 2018, Dumont et al. 2016) to be in the range of 70-80% for hazard prediction and 60-70% for potency prediction, depending on the summary statistic used for comparison (e.g., median, mean, etc.). The NICEATM EC3 values reported in **Table 7** for the IT compounds represent the means of EC3 values from tests that meet criteria designed to identify the most reliable EC3 values (**Section 2.4**). Presenting the 95% confidence intervals around the mean EC3 provides a quantitative measure of uncertainty in the results. Dow EC3 values were derived to limit EC3 values to those in the same or similar solvents and were the most potent EC3 values available for each substance. Qualitative uncertainties regarding the LLNA data include:

- The LLNA incorporates all four key events of the AOP, but not the adverse outcome of skin sensitization.
- Mice, the experimental model used in the LLNA, are not humans, the species of interest.

3.5.2 Uncertainties Related to the In Chemico and In Vitro Data

The DPRA, KeratinoSens, and h-CLAT assays are standardized test methods described in internationally harmonized OECD test guidelines. These test methods have been validated as relevant and reproducible for regulatory use when used with other information (i.e., they are not intended to be used as stand-alone tests). The reproducibility of these tests and the accuracy, sensitivity, and specificity with respect to LLNA hazard classifications are provided in the OECD test guidelines:

- DPRA: reproducibility was approximately 85% within laboratories and 80% between laboratories; accuracy = 80% (126/157), sensitivity = 80% (88/109), and specificity = 77% (37/48) (OECD 2019).
- KeratinoSens: reproducibility was approximately 85% within and between laboratories; accuracy = 77% (155/201), sensitivity = 78% (71/91), and specificity = 76% (84/110) (OECD 2018a).
- h-CLAT: reproducibility was approximately 80% within and between laboratories; accuracy = 85% (121/142), sensitivity = 93% (94/101), and specificity = 66% (27/41) (OECD 2018b).

These in chemico and in vitro tests use human cellular and molecular targets to provide information on the activation of a key event by a test substance without the potential interference of upstream effects. The results of the DPRA were not helpful for distinguishing potencies of the IT compounds; all produced similar results. Confidence in the KeratinoSens and h-CLAT results is increased because they provided similar potency ranks for the IT chemicals and they have higher reproducibility than the in vivo results (Kleinstreuer et al. 2018). The qualitative uncertainties for DPRA, h-CLAT, and KeratinoSens results include the following:

- These methods assess the first three key events of the skin sensitization AOP, but not the fourth key event, T-cell proliferation, or the adverse outcome.
- The in chemico and in vitro tests do not mimic the absorption, distribution, metabolism, and excretion of a test substance that occur in vivo.

3.5.3 Uncertainties Related to the ANN

The ANN DAs incorporate information from two to three of the in chemico or in vitro tests. Model 1 uses DPRA and h-CLAT data and Model 4 uses DPRA, h-CLAT, and KeratinoSens data. Because DPRA was not effective in ranking the IT compounds for potency, and Model 4 includes both KeratinoSens and h-CLAT data, which ranked the IT compounds similarly for potency, confidence in Model 4 results is higher than that for Model 1. Model 4 also covers three key events of the AOP, rather than two. The reported ANN EC3 values are means

resulting from 100 runs of each model. The 95% confidence intervals around the mean ANN EC3 values provide a quantitative measure of uncertainty in the results based on the variation inherent in the machine learning algorithm. The variability of the in vitro methods is not explicitly incorporated, but during the OECD validation studies all methods were shown to have ≥80% within- and between-lab reproducibility. The performance of the ANN DAs with respect to predicting LLNA potency classification (strong, weak, and nonsensitizing) for a diverse group of 126 chemicals were provided in Kleinstreuer et al. (2018):

- Model 1 (ANN D_hC): accuracy = 65.1% (82/126), over-predicted = 21.4% (27/126), and under-predicted = 13.5% (17/126)
- Model 4 (ANN D_hC_KS): accuracy = 69.8% (88/126), over-predicted = 23.0% (29/126), and under-predicted = 7.1% (9/126)

Kleinstreuer et al. (2018) also provides performance of the ANN DAs for predicting human potency classification (strong, weak, and nonsensitizing):

- Model 1 (ANN D_hC): accuracy = 61.1% (77/126), over-predicted = 22.2% (28/126), and under-predicted = 16.7% (21/126)
- Model 4 (ANN D_hC_KS): accuracy = 62.7% (79/126), over-predicted = 25.4% (32/126), and under-predicted = 11.9% (15/126)
- In comparison, the LLNA performance against this set was: accuracy = 59.4% (76/128), over-predicted = 19.5% (25/128), and under-predicted = 21.1% (27/128)

The qualitative uncertainties for ANN Model 4 include the following:

- The ANN models were trained to predict T-cell proliferation results in mice (EC3 values), and not the adverse outcome in humans, the species of interest.

4.0 Conclusions

All of the non-animal methods, DPRA, KeratinoSens, h-CLAT, and the in silico read-across OECD QSAR Toolbox, were concordant with the LLNA in yielding a sensitizer hazard classification for each of the six isothiazolinone compounds. Peptide depletion values from DPRA were not useful for ranking the six IT compounds for skin sensitization potency because they were too similar to one another. KeratinoSens and h-CLAT produced a similar ranking to that based on the LLNA. The quantitative EC3 values generated from the DAs were comparable to those derived from the LLNA data. The DAs ranked DCOIT and OIT as the most potent IT compounds in the class, followed by BBIT, CMIT/MIT, BIT and MIT.

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Appendix A:
Burleson Research Technologies, Inc.
In Vitro Testing Results ¶

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
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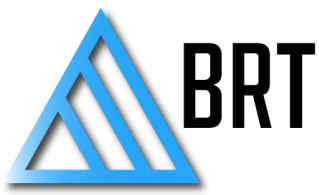
03 December 2019



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

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APPROVAL

Study Director Approval

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QUALITY ASSURANCE STATEMENT

STUDY NUMBER	NIEHSO 20180515
STUDY TITLE	Assessment of Chemical Sensitization Potential Using *In Vitro* Methods
REPORT NUMBER	NIEHSO 20180515-1
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SPONSOR	National Toxicology Program Immunotoxicology Testing Program Systems Toxicology Group Toxicology Branch 530 Davis Drive, Morrisville, NC 27560
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

TABLE OF CONTENTS

STUDY INFORMATION

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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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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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on behalf of its wholly owned subsidiary, Rohm & Haas Chemicals LLC.

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.
A Subsidiary of The Dow Chemical Company

DOW CHEMICAL (SHANGHAI)
COMPANY LIMITED
D BLOCK, 1/F,185 TAI GU RD
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.

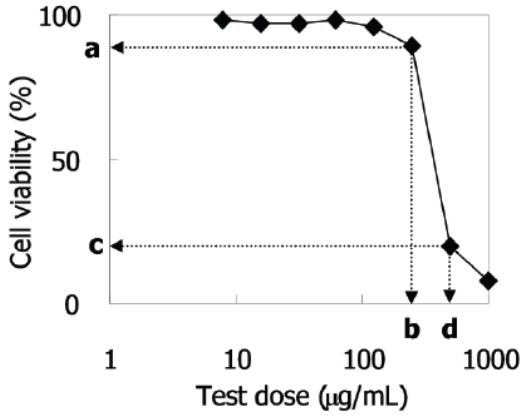
5.8.2. Add 10 µL of a 12.5 µg/mL PI solution to each tube.

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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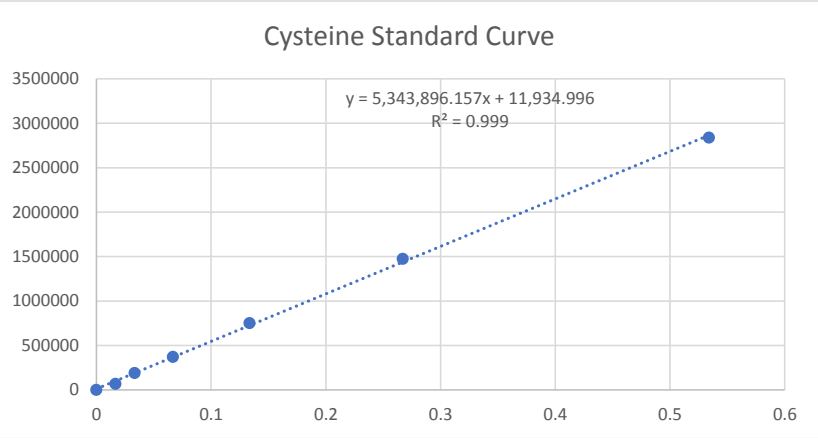
NIEHSO 20180515
Standard Curves Isothiazolinones

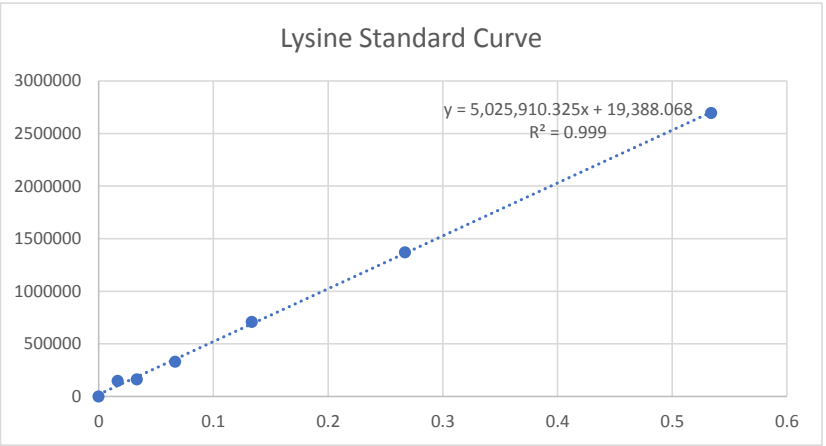
		X values	*Y values*
		Peptide conc. (mM)	**Peptide peak area at 220 nm**
CYSTEINE	STD1	0.534	2840659
	STD2	0.267	1476041
	STD3	0.1335	751941
	STD4	0.0667	373124
	STD5	0.0334	191226
	STD6	0.0167	68592
	STD7	0	0

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		

Appendix B:
Institute for In Vitro Sciences, Inc.
h-CLAT Testing Results ¶

FINAL REPORT

HUMAN CELL LINE ACTIVATION TEST (h-CLAT)

Laboratory Study Number:

18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Study Completion Date:

30 October 2019

Authors:

Rishil J. Kathawala, Ph.D.
Kyrstin Driscoll, B.S.

Sponsor

National Institute of Environmental Health Sciences (NIEHS)
NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
601 Keystone Park Drive, Suite 200
Morrisville, NC 27560

Performing Laboratory:

Institute for In Vitro Sciences, Inc.
30 W. Watkins Mill Road, Suite 100
Gaithersburg, MD 20878

Laboratory Project Number:

10426

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STATEMENT OF COMPLIANCE

The Human Cell Line Activation Test (h-CLAT) of the test substances: ACTICIDE OIT, Vanquish 100, Mergal MITZ, Mergal BIT Technical, KORDEKTM 573F BIOCIDE, KATHON 287T Industrial Microbicide was conducted in compliance with the principles presented in the EPA FIFRA (40 CFR part 160) series on Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test substances or assay controls have not been determined by the testing facility. However, the Sponsor (test substances) and the manufacturers (controls) provided Certificates of Analysis that are included in Appendix C.

The stability of the test substances or assay controls under the storage conditions at the testing facility and under the actual test conditions has not been determined by the testing facility and is not included in the final report.

Analyses to determine the uniformity, concentration, or stability of the test article mixtures, if applicable, were not performed by the testing facility.

Study Director: [redacted] 30 October 2019

Rishil J. Kathawala, Ph.D. Date

QUALITY ASSURANCE STATEMENT

Study Title: The Human Cell Line Activation Test (h-CLAT)

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Study Director: Rishil J. Kathawala, Ph.D.

A random sampling approach was used to select at least one in-process, laboratory phase to inspect for this study. The Quality Assurance Unit inspections specific to this study are listed in the table below. Procedures, documentation, equipment records, etc., were examined in order to assure that the study was performed in accordance with the EPA FIFRA (40 CFR part 160) series on Good Laboratory Practice and to assure that the study was conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director and Management
Protocol and Initial Paperwork	24 April 2019	24 April 2019
Preliminary Assay- Solvent Selection (18AO64, 19AA12, AA13)	20 May 2019	20 May 2019
Draft Report, Data and Protocol Amendment I	12-13 September 2019 & 16-18 September 2019	18 September 2019
Final Report and Protocol Amendment II & III	24 October 2019 29 October 2019	24 October 2019 29 October 2019

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

[redacted] 30 Oct 2019

Megan Conahan, B.S., RQAP-GLP Date

Quality Assurance

SIGNATURE PAGE

HUMAN CELL LINE ACTIVATION TEST (h-CLAT)

Initiation Date:	23 April 2019
Laboratory Start Date:	20 May 2019
Laboratory Completion Date:	21 August 2019
Completion Date:	30 October 2019
Sponsor:	National Institute of Environmental Health Sciences (NIEHS) NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
Sponsor's Representative:	Judy Strickland, Ph.D., DABT Integrated Laboratory Systems, Inc., Contractor supporting the NICEATM
Testing Facility:	Institute for In Vitro Sciences, Inc. 30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
Archive Location:	Institute for In Vitro Sciences, Inc. Gaithersburg, MD 20878
Director, Laboratory Services:	Gertrude-Emilia Costin, Ph.D., M.B.A., ATS

Study Director: [redacted] 30 October 2019

Rishil J. Kathawala, Ph.D. Date

TEST SUBSTANCE RECEIPT

IIVS Test Substance Number	Sponsor Designated Synonym^	Trade Name	Lot/Batch Number	Physical Description	Receipt Date	Storage Conditions*
18AO64	OIT	ACTICIDE OIT	MX-183774-2006	clear light yellow non-viscous liquid	18 December 2018	room temperature
19AA05	BBIT	Vanquish 100	Lot# 6445	clear orange semi-viscous liquid	10 January 2019	room temperature
19AA12	CMIT/MIT Mixture	Mergal MITZ	Lot# SLJ0229	clear colorless non-viscous liquid	14 January 2019	room temperature
19AA13	BIT	Mergal BIT Technical	Lot# YL201811073	white powder	14 January 2019	room temperature
19AA98	MIT	KORDEK™ 573F BIOCIDE	Batch# YY00H3A451	clear colorless non-viscous liquid	7 February 2019	room temperature
19AB24	DCOIT	KATHON 287T Industrial Microbicide	YY00H77338	off-white waxy solid	15 February 2019	room temperature

* - Protected from exposure to light

^ - Chemical name for Sponsor designated synonym is as follows:

OIT is also known as 2-n-Octyl-4-isothiazolin-3-one
BBIT is also known as 2-butyl-1,2-benzisothiazolin-3-one; synonym: 1,2-Benzisothiazol-3(2h)-one, 2-butyl
CMIT/MIT is also known as 5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one
BIT is also known as 1,2-Benzisothiazolin-3-one
MIT is also known as 2-Methyl-4-isothiazolin-3-one
DCOIT is also known as 4,5-Dichloro-2-octyl-3(2h)-isothiazolone

INTRODUCTION

The Human Cell Line Activation Test (h-CLAT) was used to assess the skin sensitization potential of the test substance(s) by monitoring the upregulation of cell surface markers, CD54 and CD86, on the surface of human acute monocytic leukemia cells (THP-1). The upregulation of CD54 and CD86 in response to

a skin sensitizer is correlated to dendritic cell activation, which is the third key event of the skin sensitization pathway.

MATERIALS AND METHODS

The assay procedures were performed as outlined in the study protocol (See Appendix A).

DEVIATIONS

A deviation occurred in the definitive trail B6 of the study. Per protocol, following the three rinses of FACS buffer, the cells are to be suspended in 600 microliters of 0.01% (w/v) blocking suspension and incubated at 2-8°C for 15±1 minutes. However, the sample of positive control DNCB stained with anti-FITC isotype antibody was incubated at 2-8°C for 15 minutes and for approximately an additional 40 minutes at room temperature. In addition, the protocol mentions that cells will be suspended in a final addition of 200 microliter of FACS buffer prior to running them on the flow cytometer. Given the limited availability of the sample of positive control DNCB, the cells were resuspended in a final volume of 100 microliters of FACS buffer to have an appropriate density of cells for the flow cytometer reading. This was a deviation from the study protocol, however, given that blocking step majorly involved non-specific binding, this deviation would not be of significant impact.

RESULTS AND DISCUSSION

Solubility Determination

Prior to the preliminary (dose range finding) assay, the test substances were tested in a solubility test to determine an appropriate solvent. The following observations were determined during the solubility test:

The test substance, ACTICIDE OIT, was found to be soluble at 500 mg/mL in DMSO with vortexing for 1 min. The description of the dilution was noted to be a clear colorless non-viscous solution. The test substance dilution was observed to remain in solution in the primary solvent as well as culture medium.

The test substance, Mergal MITZ, was found to be soluble at 500 mg/mL in DMSO with vortexing for 1 min. The description of the dilution was noted to be a clear colorless non-viscous solution The test substance dilution was observed to remain in solution in the primary solvent as well as culture medium.

The test substance, Mergal BIT Technical, was found to be soluble at 500 mg/mL in DMSO with vortexing for 3 min. The description of the dilution was noted to be a clear colorless non-viscous solution The test substance dilution was observed to remain in solution in the primary solvent as well as culture medium.

The test substance, Vanquish 100, was found to be soluble at 250 mg/mL in DMSO with vortexing for 1 min. The description of the dilution was noted to be a clear light yellow non-viscous solution. The test substance dilution was observed to remain in solution in the primary solvent as well as culture medium.

The test substance, KORDEK™ 573F BIOCIDE, was found to be soluble at 250 mg/mL in DMSO with vortexing for 1 min. The description of the dilution was noted to be a clear colorless non-viscous solution. The test substance dilution was observed to remain in solution in the primary solvent as well as culture medium.

The test substance, KATHON 287T Industrial Microbicide, was found to be soluble at 500 mg/mL in DMSO with vortexing for 30 sec. The description of the dilution was noted to be a clear light yellow non-viscous solution. The test substance dilution was observed to remain in solution in the primary solvent as well as culture medium.

The neat test substance, KATHON 287T Industrial Microbicide, was heated in a glass water bath at 53°C on a hot plate (IIVS0967) for ~ 5 minutes immediately prior to addition of the solvent, as per sponsor instructions.

Dose Range Finding Assay

A preliminary (dose range finding assay) was performed to determine the viability of the THP-1 cells after 24 ± 0.5 hour exposure to 8 test substance concentrations. The CV75, which is the concentration leading to 75% cell viability was calculated for each test substance.

Definitive Assays

Based on the results of the dose range finding assay, the doses were chosen for the test substances for the definitive assays. At least two valid definitive trials were performed.

Seven serial doses using a typical dilution factor of 1.2 were prepared such that eight doses were tested in the definitive assay. If there was insufficient cytotoxicity in the dose finding assay (i.e. CV75 > highest prepared dose), the highest soluble concentration of test article, up to a maximum stock concentration of 500 mg/mL in either saline or DMSO was selected.

If the first two independent assays were not concordant, a third assay was performed and the final prediction was based on the mode of the conclusions from the three individual runs (i.e. 2 out of 3).

The positive control, 2,4-Dinitrochlorobenzene, was tested in the definitive assays only.

Evaluation of Test Results

The relative fluorescence intensity (RFI) was calculated for each test substance and control treated cell population. RFI ≥ 200 at any tested concentration for CD54, and/or RFI ≥ 150 at any tested concentration for CD86 was considered a sensitizer by the h-CLAT.

The EC200 and EC150 values, which are the calculated test substance concentrations leading to an RFI of 200 or 150, were calculated for each test substance.

If the RFI of CD86 is equal to or greater than 150 at any tested dose with >50% cell viability in at least

two independent assays and/or if the RFI of CD54 is equal to or greater than 200 at any tested dose with >50% cell viability in at least two independent assays, the prediction will be considered as positive (sensitizer). Otherwise, the prediction will be considered as negative.

Summary

Table 1 presents the results from the Dose Finding Assay.

Table 2 presents the results for the valid definitive trials.

Table 3 presents the results for the positive control (2,4-Dinitrochlorobenzene.)

An assay met acceptance criteria when:

- The cell viability values of the solvent controls were > 90%.
- For the solvent controls, RFI values of both CD86 and CD54 were less than the positive criteria (CD86 RFI < 150 and CD54 RFI < 200).
- For the positive control (DNCB), RFI values of both CD86 and CD54 were predicted to be positive (CD86 RFI ≥ 150 and CD54 RFI ≥ 200), and cell viability was > 50%.
- For the medium and solvent controls, the MFI ratio of both CD86 and CD54 to isotype control was > 105%.
- The cell viability of the test substance-treated cultures was > 50% in at least four doses.

All acceptance criteria for a valid assay were met for the definitive trials presented in this report. The test substances, ACTICIDE OIT, Vanquish 100, Mergal BIT Technical, KORDEK™ 573F BIOCIDE and KATHON 287T Industrial Microbicide, were considered sensitizers according to the h-CLAT. Mergal MITZ was considered a non-sensitizer according to the h-CLAT.

Table 1

Test Substance Results for h-CLAT Dose Range Finding Assay

IIVS Test Substance Number	Sponsor Designated Synonym	CV75 (µg/mL)
18AO64	OIT	8.0
19AA05	BBIT	4.8
19AA12	CMIT/MIT Mixture	31.8
19AA13	BIT	17.8
19AA98	MIT	37.3
19AB24	DCOIT	1.1

Table 2

Test Substance Results for h-CLAT Definitive Assay

IIVS Test Substance Number	Sponsor Designated Synonym	Trade Name	CV75 (µg/mL)	Trial	CD54 EC_{200} (µg/mL)	CD86 EC_{150} (µg/mL)	Sensitization Potential	Overall Sensitization Potential
18AO64	OIT	ACTICIDE OIT	8.0	B1 Assay Date: 11 Jun 2019	<2.7	>9.6[1]	Sensitizer	Sensitizer
				B2 Assay Date: 18 Jun 2019	<2.7	>9.6[1]	Sensitizer	
19AA12	CMIT/MIT Mixture	Mergal MITZ	31.8	B1 Assay Date: 11 Jun 2019	>22.1[1]	>22.1[1]	Non-sensitizer	Non-sensitizer
				B2 Assay Date: 18 Jun 2019	16.91	>18.4[1]	Sensitizer	
				B3 Assay Date: 2 Jul 2019	>18.4[1]	>18.4[1]	Non-sensitizer	
19AA13	BIT	Mergal BIT Technical	17.8	B1 Assay Date: 11 Jun 2019	6.0	>17.8[1]	Sensitizer	Sensitizer
				B2 Assay Date: 18 Jun 2019	<6.0	>17.8[1]	Sensitizer	
19AA05	BBIT	Trade name Vanquish 100	4.8	B3* Assay Date: 2 Jul 2019	<1.6	2.12	Sensitizer	Sensitizer
				B4 Assay Date: 10 Jul 2019	2.13	3.86	Sensitizer	
19AA98	MIT	KORDEK™ 573F BIOCIDE	37.3	B3* Assay Date: 2 Jul 2019	<12	35.87	Sensitizer	Sensitizer
				B4 Assay Date: 10 Jul 2019	16.11	>45[1]	Sensitizer	
19AB24	DCOIT	KATHON 287T Industrial Microbicide	1.1	B3# Assay Date: 10 Jul 2019	0.71	>1.37[1]	Sensitizer	Sensitizer
				B6# Assay Date: 19 Aug 2019	0.84	>1.37[1]	Sensitizer	

*B1 and B2 definitive trials did not meet assay acceptance criteria for positive control and therefore those trials were not considered valid.

[#]B1 trial for 19AB24 did not meet assay acceptance criteria for positive control and therefore those trials were not considered valid. In B2 trial, CD86 and Isotype control antibodies were inadvertently plated in reverse, leading to higher Isotype control values and negative RFI. Therefore, results from this B2 trial were not considered valid. B4 and B5 definitive trials did not meet assay acceptance criteria for positive control and therefore those trials were not considered valid.

[1] - ">" values reflect a negative response (i.e., insufficient induction for a positive response).

Table 3

Positive Control Results for the Definitive Assay

Date	Trial	CD54 RFI	CD86 RFI	Cell Viability (%)	Results
11 Jun 2019	B1	668.50	158.07	78.97	Pass
18 Jun 2019	B2	1442.44	171.78	74.00	Pass
2 Jul 2019	B2/B3	751.74	158.24	83.31	Pass
10 Jul 2019	B3/B4	1050.98	197.64	78.22	Pass
19 Aug 2019	B6	1462.78	166.83	81.26	Pass

APPENDIX A (Protocol, Protocol Attachment 1, Protocol Amendment I, Protocol Amendment II & Protocol Amendment III)

HUMAN CELL LINE ACTIVATION TEST (h-CLAT)

1.0 PURPOSE

The purpose of this study is to identify potential skin sensitizers and non-sensitizers in accordance with the United Nations Globally Harmonized System (UN GHS). The skin sensitization potential of a test article is evaluated by measuring the changes in the expression of cell surface markers CD54 and CD86 associated with the process of dendritic cell activation in the human leukemia cell line, THP-1, following exposure to a test article. The changes of surface marker expression are measured by flow cytometry following cell staining with fluorescently labelled antibodies for CD54 and CD86.

2.0 SPONSOR

2.1 Name: National Institute of Environmental Health Sciences (NIEHS)
NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

2.2 Address: Judy Strickland, Ph.D., DABT
Integrated Laboratory Systems, Inc.,
Contractor supporting the NICEATM

601 Keystone Park Drive, Suite 200
Morrisville, NC 27560
(919) 281-1110 x245
strickl2@niehs.nih.gov

2.3 Representative: Judy Strickland, Ph.D., DABT

3.0 IDENTIFICATION OF TEST ARTICLES AND ASSAY CONTROLS

3.1 Test Article(s): See Protocol Attachment 1

3.2 Assay Controls: Positive: 2,4-dinitrochlorobenzene (DNCB)
(2 mg/mL in DMSO)
Solvent/Vehicle: Saline or Cell Culture Medium for aqueous-soluble or surfactant test articles
Dimethyl Sulfoxide (DMSO) for DNCB and DMSO soluble test articles

3.3 Determination of Strength, Purity, etc.

3.3.1 For GLP studies only, the Institute for In Vitro Sciences, Inc. (IIVS) will attempt to secure documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions from the Sponsor. If the Sponsor is unable to provide such information, IIVS will retain documentation

supporting attempts to obtain this information with the study file and an exception will be noted in the Statement of Compliance in the Final Report.

3.3.2 IIVS will be responsible for the documentation of the analytical purity and composition of the controls and solvents used in the assay. This may be accomplished by maintaining a certificate of analysis from the supplier.

3.3.3 The stability of the test article(s) and dosing solutions under the storage conditions at the testing facility and under the actual experimental conditions will not be determined by IIVS.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Institute for In Vitro Sciences, Inc.
4.2	Address:	30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
4.3	Study Director:	Greg Mun, B.A.

5.0 TEST SCHEDULE

5.1	Proposed Experimental Initiation Date:	29 April 2019
5.2	Proposed Experimental Completion Date:	17 May 2019
5.3	Proposed Report Date:	21 June 2019

6.0 TEST SYSTEM

The h-CLAT is an *in vitro* assay which measures the changes in the expression of cell surface markers CD54 (ICAM-1) and CD86 associated with the process of dendritic cell activation in the human acute monocytic leukemia cell line, THP-1 (American Type Culture Collection, ATCC, Manassas, VA, TIB-202™). Dendritic cell activation is considered one of the key biological events in the adverse outcome pathway for skin sensitization, where CD54 and CD86 are subsequently involved in dendritic cell migration to the lymph nodes and T-cell priming. THP-1 cells, seeded at a density of 2.0×10^6 cells/mL in culture medium in 24-well plate format define the Test System. After treatment of the test or control articles to the Test System, the expression of cell surface markers are measured by flow cytometry following cell staining with fluorescein isothiocyanate (FITC) labelled antibodies. Cytotoxicity measurement, using propidium iodide (PI) staining, is conducted concurrently to assess whether upregulation of surface marker expression occurs at sub-cytotoxic concentrations.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The experimental design of this study consists of a dose range finding assay and at least two definitive assays to determine the changes in the expression of the cell surface markers CD54 and

CD86. The Relative Fluorescence Intensity (RFI) is used as an indicator of CD54 and CD86 expression. RFI is calculated from the Geometric Mean Fluorescence Intensity (MFI) data acquired by flow cytometry software. The flow cytometry data acquisition will be performed using a MACSQuant Analyzer (Miltenyi) with a three laser system capable of both FITC and PI acquisition. The procedures are based on those presented in the OECD Test Guideline 442E and the EURL ECVAM DB-ALM Protocol No. 158.

7.1 Medium and Reagents

7.1.1 Culture Medium (RPMI-1640 with 10% heat inactivated Fetal Bovine Serum and 0.05 mM 2-mercaptoethanol)

7.1.2 Saline (0.9% NaCl)

7.1.3 DMSO, CAS 67-68-5

7.1.4 DNCB, CAS 97-00-7

7.1.5 Calcium and Magnesium Free Dulbecco's Phosphate Buffered Saline (CMF-DPBS)

7.1.6 FACS Buffer (CMF-DPBS with 0.1% (w/v) Bovine Serum Albumin - Fraction V) to be fully dissolved before use

7.1.7 Blocking Suspension (1% w/v globulins- Cohn fraction II, III, Human in FACS Buffer)

7.1.8 FITC Mouse anti-Human CD54, Clone 6.5B5 (DAKO/Agilent)

7.1.9 FITC Mouse anti-Human CD86, Clone FUN-1 (BD Pharmingen)

7.1.10 FITC Mouse IgG1 K Isotype Control (DAKO/Agilent)

7.1.11 PI solution (12.5 µg/mL of propidium iodide in CMF-DPBS)

7.2 Environmental Conditions

Throughout this protocol, ranges for test material and test system exposure or incubation conditions (e.g., temperature, humidity, CO_2) are presented. These ranges describe the equipment performance specifications under static conditions (i.e., in the absence of frequent opening of equipment doors, accessing chambers, changing loads, etc.), as presented in the relevant equipment SOPs.

7.3 Maintenance of THP-1 Cell Line

Cryopreserved THP-1 cells, tested for and cleared of mycoplasma contamination, will be stored in liquid nitrogen. The stock ampule(s) will be thawed and slowly diluted in

approximately 9 mL of culture medium kept at 2-8°C. To wash the cells of cryopreservative, the cells will be collected by centrifugation (200-300g, in a centrifuge set for 5 minutes and 4°C). The rinse will be repeated with the same volume of medium and centrifuge settings. After the second rinse, the cells will be resuspended in an appropriate volume of culture medium warmed to approximately 37°C for the culture vessel used (typically either T25 or T75 flasks without a growth surface). The cells will be maintained at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (standard culture conditions) with at least one agitation per each day. Cells will typically be refed every 2-3 days with culture medium warmed to approximately 37°C until the cells are confluent enough to be passaged or transferred to a larger culture vessel.

Cells will be routinely passaged every 2 to 3 days and seeded at a density of 0.1×10^6 to 0.2×10^6 cells/mL. The cells will routinely be maintained at densities ranging from 0.1 to 0.8×10^6 cells/mL. The cell density should not exceed 1.0×10^6 cells/mL. Cells can be propagated up to two months after thawing but not in excess of 30 passages post thawing.

At least two weeks after thawing, the cells will undergo a reactivity check. Only the cells which pass the reactivity check will be used in subsequent studies. Routine cell culture activities and reactivity check assay will be documented in the cell culture records and briefly summarized in the study report.

Prior to an assay, cells will be seeded in culture flasks at densities of 0.1 to 0.2×10^6 cells/mL and pre-cultured for approximately 72 or 48 hours, respectively. The culture conditions and cell density defined for this pre-assay culture conditioning should be maintained as consistently as possible to ensure optimal CD54 and CD86 induction and expression. On the day of testing, cells will be harvested from the culture flasks and seeded into 24-well plates, as described in section 7.4.3 for the dose range finding assay, or section 7.5.3 for the definitive assays.

7.4 Dose Range Finding Assay

A dose range finding assay will be conducted to determine the doses to be used in the definitive assays. The highest dose in the definitive assays will be selected by the Study Director which may be 1.2-fold higher than the calculated CV75 concentration (i.e., the test article concentration resulting in 75% cell viability relative to the solvent control).

7.4.1 Solvent Selection

A solubility test may be performed prior to the dose range finding assay in order to determine the most appropriate solvent. The evaluation of solvents should start with saline (0.9% NaCl) or cell culture medium, followed by DMSO. Other solvents may be attempted, and if used, must not adversely affect cell viability in the assays. If other solvents are used, solvent controls will be tested concurrently with the test article dilutions in the assays. Solubility is required for this assay. If solubility cannot be achieved, the Sponsor will be contacted regarding how to proceed. Test articles which do not form solutions (e.g., are noted as cloudy or form precipitates) may be sonicated and/or heated at 37±1°C in an attempt to further

solubilize the test article. In some cases, or under the guidance of the Sponsor, the sonication and/or heating may affect the stability of the test article and therefore heating and sonication would not be used for test article preparation. Solubility of the test articles should be evaluated at a maximum concentration of 100 mg/mL in saline or 500 mg/mL in DMSO or another appropriate solvent.

NOTE: The OECD Test Guideline specifies that in the absence of cytotoxicity in an initial dose range finding assay, test articles prepared in saline to a stock concentration of 100 mg/mL may be retested using a higher stock concentration up to a maximum of 500 mg/mL. Accordingly, at the Study Director's discretion, the dose range finding assay may be conducted using a maximum saline stock concentration of up to 500 mg/mL.

7.4.2 Preparation of Dilutions

The test and control articles will be prepared on the day of testing and applied to the test system within one hour of preparation to minimize potential for chemical degradation or breakdown. Based upon the results of the solubility test, the test articles will be dissolved to the maximum appropriate concentration determined in the solubility test, or up to a maximum final concentration of 100 mg/mL in saline (or up to 500 mg/mL in saline; see NOTE in section 7.4.1), or to a maximum final concentration of 500 mg/mL in DMSO. Other concentrations and solvents can be used if determined appropriate by the Study Director and/or Sponsor.

From the initial test article dilution, 2-fold serial dilutions will be prepared using the same solvent to obtain eight serial stock dilutions. These stock dilutions will then be further diluted 50-fold (for test articles diluted in saline) or 250-fold (for test articles diluted in DMSO) in the culture medium (2X dosing dilutions). These dosing dilutions are prepared at 2X the desired final concentration so that when 500 µL of each dosing dilution are added to 500 µL of cell suspension in the 24-well plate, a 1X final dose concentration is achieved.

The solvent control will be culture medium for test articles diluted in saline, or DMSO in culture medium for test articles diluted in DMSO. A single concentration of the solvent control(s) will be prepared in culture medium and dosed on the cells in the same manner as the test article(s) so that the final concentration of DMSO on the cells is 0.2%.

The positive control will be DNCB prepared at a stock concentration of 2 mg/mL in DMSO. The working solution of DNCB will be prepared by making an 8 µg/mL dilution of the stock in culture medium. The working solution of DNCB will be dosed on the cells in the same manner as the test article(s).

7.4.3 Preparation of the Test System

On the day of dosing, cells will be collected by centrifugation (200-300g, in a centrifuge set to 5 minutes at room temperature). The cells will be resuspended in

fresh culture medium to a density of 2.0×10^6 cells/mL, and 500 µL of the cell suspension will be seeded into the appropriate wells of a 24-well plate (resulting in 1.0×10^6 cells/well). The plates will be maintained at standard culture conditions.

7.4.4 Test System Exposure

The 2X dosing dilutions will be applied to the cells by pipetting 500 µL of each of the 2X dosing dilutions directly to the appropriate wells containing 500 µL of cell suspension. The treated plates will be sealed with plate sealers prior to incubation (to avoid evaporation or cross-contamination of volatile test articles), and will be incubated for 24±0.5 hours at standard culture conditions with at least 1 agitation per each day.

7.4.5 Cytotoxicity Assessment - Propidium Iodide (PI) Staining

After 24±0.5 hours of exposure, the samples will be removed from the 24-well plates and added to labeled micro-centrifuge tubes. The cells will be collected by centrifugation (200-300g, in a centrifuge set for 5 minutes and 4°C). The supernatants will be carefully decanted into a waste container. The remaining cell pellets will be resuspended with 1 mL of FACS buffer and centrifuged again using the above centrifuge settings and decanting the supernatant. The rinsing process is performed 2 additional times using 1 mL of FACS buffer.

After the three rinses, each cell pellet will be resuspended in 600 µL of FACS buffer and 200 µL of the suspension will be transferred to the appropriate wells of a 96-well round-bottom plate. Propidium Iodide will be added to the appropriate samples of the 96-well plate to make a final concentration of 0.625 µg/mL of PI in the plate.

7.4.6 Cytotoxicity Measurement and Calculation of CV75

The PI uptake will be analyzed using flow cytometry. Cells stained with PI represent the non-viable cell population and will be gated out to identify the viable populations. Approximately 10,000 living (PI negative) cells will be acquired. When the cell viability is low, up to approximately 30,000 cells including dead cells can be acquired. Alternatively, the data acquisition can be finished one minute after the initiation. The cell viability will be calculated (e.g. PI negative events versus total events).

The CV75 value, a concentration expected to result in 75% cell viability, will be calculated using the following formula:

$$\text{Log of CV75} = \frac{(75\text{-}C)\text{Log}(B) - (75\text{-}A)\text{Log}(D)}{A\text{-}C}$$

Where:
A is the minimum concentration with cell viability over 75%
C is the maximum concentration with cell viability below 75%
B and D are the viabilities associated with A and C, respectively

The CV75 value will be used to calculate the test article concentrations tested in the definitive assays. The range of doses used in the definitive assays may be modified at the Study Director's discretion.

The dose range finding assay may be repeated if the results of the dose range finding assay aren't sufficient to select doses for the definitive assays.

7.5 Definitive Assay

7.5.1 Test Article Dose Selection

Seven serial doses using a typical dilution factor of 1.2 to 1.5 will be prepared such that eight doses will be tested in the definitive assay. If there was insufficient cytotoxicity in the dose finding assay (i.e. CV75 > highest prepared dose), the highest soluble concentration of test article, up to a maximum stock concentration of 500 mg/mL in either saline or DMSO may be selected. At the Study Director's discretion and justification, the range of doses and the dilution factor to be used in the definitive assay may be modified.

7.5.2 Preparation of Stock and 2X Dosing Dilutions

The same solvent used in the dose range finding assay will be used to dissolve the test article in the definitive assays. The test article will be prepared as stock concentrations corresponding to 100-fold (for saline) or 500-fold (for DMSO). Seven serial dilutions using a dilution factor of 1.2-1.5 will be made using the same solvent to obtain eight serial dilutions. These dilutions will then be further diluted 50-fold (for test articles diluted in saline) or 250-fold (for test articles diluted in DMSO) in the culture medium (2X dosing dilutions). These dosing dilutions are prepared at 2X the desired final concentration so that when 500 µL of each dosing dilution are added to 500 µL of cell suspension in the 24-well plate, a 1X final dose concentration is achieved. The test article dilutions should be exposed to the cells within one hour of preparation.

The solvent controls and the positive controls will be prepared in the same manner as for the dose range finding assay (section 7.4.2).

7.5.3 Preparation of the Test System

On the day of dosing, the cells to be used in the assay will be prepared in the same manner as for the dose range finding assay (section 7.4.3).

7.5.4 Test System Exposure

The 2X dosing solutions will be applied to the cells by pipetting 500 µL of each of the 2X dosing solutions directly into the appropriate wells containing 500 µL of cell suspension. The treated plates will be sealed with plate sealers prior to incubation (to avoid evaporation or cross-contamination of volatile test article), and will be incubated for 24±0.5 hours at standard culture conditions with at least 1 agitation per each day.

For each test article, two independent trials with agreeing results are needed to make a prediction. In the case of incongruent results and/or at the Study Director's discretion, a third run (or more) may be completed.

7.5.5 Staining and Analysis

After 24±0.5 hours of exposure, the samples will be placed into labeled micro-centrifuge tubes and the cells will be collected by centrifugation as described in section 7.4.5. The supernatants will be carefully decanted into a waste container. The remaining cell pellets will be resuspended with 1 mL of FACS buffer and centrifuged. The rinsing process is performed 2 additional times using 1 mL of FACS buffer. Finally, cells will be resuspended in 600 µL of 0.01% (w/v) blocking suspension (prepared in FACS buffer from a 1% (w/v) stock suspension immediately before use) and incubated at 2-8°C for 15±1 minutes.

After the blocking step, the samples will be divided into 3 aliquots of 180 µL each into the designated wells of a 96-well round-bottom plate. The cells will be collected by centrifugation as described in section 7.4.5 and the supernatants will be aspirated without disturbing the cell pellet. A master mixture of each antibody (CD54, CD86 and mouse IgG isotype control) will be prepared based on the number of samples needing to be stained with each antibody so that each sample receives 50 µL of the appropriate antibody dose. For each test article dilution or control there will be three cell populations each treated with a different antibody mixture. There will be a separate cell population treated with FITC anti-CD54, FITC anti-CD86, and FITC isotype control. The antibody mixtures will be prepared in FACS buffer using the following ratios:

3 µL of CD54 to 50 µL total
6 µL of CD86 to 50 µL total
3 µL of isotype control to 50 µL total
Fifty microliters of each antibody mixture will be added to the appropriate wells of the 96-well plate. The plate will be gently agitated by hand to mix the reagents and then incubated in the dark at 2-8°C for 30±1 minutes. Following incubation, 150 µL of FACS buffer will be added to each well and the plate will be centrifuged as described in section 7.4.5. The wash step is repeated twice with 200 µL of FACS buffer. Finally, cells will be resuspended in 200 µL of FACS buffer. PI will be added to the appropriate wells of the 96-well plate to make a final concentration of 0.625 µg/mL of PI in the plate.

The expression of CD54, CD86, isotype control and PI uptake will be analyzed using flow cytometry. Cells stained with PI will be gated out to identify the viable populations. Approximately 10,000 living (PI negative) cells will be acquired. When the cell viability is low, up to 30,000 cells including dead cells can be acquired. Alternatively, the data acquisition can be finished one minute after the initiation. The cell viability will be calculated (e.g. PI negative events versus total events). In addition the MFI of the antibody stained cell populations will be calculated. The MFI values will be used to calculate the RFI values to determine skin sensitization predictions.

7.5.6 Data Analysis

The following plots are prepared using the flow cytometry software (MACSQuantify™ Version 2.10 / MACSQuant® Analyzer used for operation and data collection and FlowLogic 7.2.1 for data analysis):

- Side Scatter (SSC) versus Forward Scatter (FSC)
  FSC is a measure of cell size. SSC is a measure of cell granularity. This plot is created to confirm a single population of cells is present without excessive debris.

-2 Histogram Plots (Cell Count versus PI) (Cell Count versus FITC)
  These plots are used to determine the percentage of each cell population expressing PI (for cell viability) or FITC (for upregulation of CD54 and CD86).

A gate will be visually placed halfway between the peak of the PI negative fraction and the PI positive fraction on the histogram using the DNCB-treated isotype control cells. The PI negative fraction corresponds to living cells which are used for subsequent analysis. The MFI of the living populations of each cell sample is determined by the software and used in the following formula to determine the RFI values for each test article treated sample.

$$RFI = \frac{\text{MFI of test article treated cells} - \text{MFI of test article treated isotype control cells}}{\text{MFI of solvent treated control cells} - \text{MFI of solvent treated isotype control cells}}$$

The isotype controls consist of the same test article concentrations tested for the CD54 and CD86 staining, but these samples will be treated with isotype control consisting of mouse IgG. Use of the isotype control will allow for the distinction between specific CD54 and CD86 antibody binding and non-specific background antibody binding.

7.5.7 Prediction Model

Each test article will be tested in at least two independent definitive assays to derive a single prediction (skin sensitizer or non-sensitizer). The definitive assays may be performed on the same day provided that for each assay: a) independently

harvested cells will be used (i.e. cells collected from different culture flasks), and b) independent fresh stock solutions of the 2X dosing dilutions of the test articles and antibodies will be prepared.

If the RFI of CD86 is equal to or greater than 150 at any tested dose with >50% cell viability in at least two independent assays and/or if the RFI of CD54 is equal to or greater than 200 at any tested dose with >50% cell viability in at least two independent assays, the prediction will be considered as positive (sensitizer). Otherwise, the prediction will be considered as negative. In case the first two independent assays are not concordant, a third assay will be performed and typically the final prediction will be based on the mode of the conclusions from the three individual runs (i.e. 2 out of 3).

Test articles with limited solubility may still be tested at lower soluble concentrations or as suspensions. In such a case, a negative result will be considered inconclusive, whereas a positive result will be used to support the identification of the test article as a skin sensitizer.

For test articles considered to be sensitizers, two effective concentrations (EC) values, the EC150 for CD86 and EC200 for CD54 will be calculated using the following formulas. Two consecutive concentrations starting from the lowest dose and with RFI values greater than and less than 200 or 150 respectively, will be used in the EC calculations. The EC values represent the calculated test article concentration at which an RFI of 150 or 200 is achieved.

$$EC150\ (\text{for CD86}) = B_{dose} + [(150-B_{RFI})/(A_{RFI} - B_{RFI})(A_{dose}-B_{dose})]$$

$$EC200\ (\text{for CD54}) = B_{dose} + [(200-B_{RFI})/(A_{RFI} - B_{RFI})(A_{dose}-B_{dose})]$$

Where:
A_{dose} is the lowest concentration in µg/mL with RFI ≥150 (CD86) or 200 (CD54)
B_{dose} is the highest concentration in µg/mL with RFI <150 (CD86) or 200 (CD54)
A_{RFI} is the RFI value associated with A_{dose}
B_{RFI} is the RFI value associated with B_{dose}

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The assay will be accepted if all of the following acceptance criteria are met:

8.1 The cell viability values of the solvent control(s) are > 90%.

8.2 For the solvent control(s), RFI values of both CD86 and CD54 are less than the positive criteria (CD86 RFI <150 and CD54 RFI <200).

8.3 For the positive control (DNCB), RFI values of both CD86 and CD54 are predicted to be positive (CD86 RFI ≥150 and CD54 RFI ≥200), and cell viability is >50%.

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

8.5 The cell viability of the test article-treated cultures should be >50% in at least four doses.

9.0 EVALUATION OF TEST RESULTS

Negative results are acceptable only for test articles exhibiting cell viability <90% at the highest dose tested. Negative results with cell viabilities of ≥90% at the highest dose tested are not valid, and may require retesting at higher doses, unless the highest allowable doses were tested (i.e., up to 5000 µg/mL in saline, 1000 µg/mL in DMSO, or the highest soluble concentration).

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. A copy of the protocol used for the study, any amendments and any significant deviations from the protocol will appear as a part of the final report.

11.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

12.0 TEST MATERIAL RETENTION

Unless indicated otherwise, all test articles provided by the sponsor will be retained for one year after completion of the final report. These test articles may be disposed after this 1 year retention period according to IIVS SOP. Unless indicated otherwise, dose solutions used for testing or analysis before or during the course of the assay will be discarded after testing.

13.0 PROTOCOL AMENDMENTS

When it becomes necessary to change the approved protocol for a specific study, the change and the reason for it should be put in writing and signed by the Study Director as soon as practical. When the change may impact the study design and/or execution, verbal agreement to make this change should be made between the Study Director and Sponsor. This document is then provided to the Sponsor and is attached to the protocol as an amendment.

14.0 REFERENCES

Ashikaga, T., *et al.* (2006) Development of an in vitro skin sensitization test using human cell lines: The human Cell Line Activation Test (h-CLAT) I. Optimization of the h-CLAT protocol. *Toxicol. In Vitro* 33 20:767–773.

DB-ALM (INVITTOX) Protocol 158: human Cell Line Activation Test (h-CLAT).

OECD (2018) *In vitro* skin sensitisation assays addressing the key event on activation of dendritic cells on the adverse outcome pathway for skin sensitisation 442E.

15.0 APPROVAL

[redacted]

4/17/2019
SPONSOR REPRESENTATIVE DATE

Judy Strickland, Ph.D., DABT
(Print or Type Name)

[redacted] 23 April 2019
IIVS STUDY DIRECTOR DATE

PROTOCOL ATTACHMENT 1

IIVS Test Article Designation	Sponsor Designation	Sponsor Designated Synonym
18AO64	ACTICIDE OIT	OIT
19AA05	2-Butyl-1,2-benzothiazolin-3-one (BBIT). Trade name: Vanquish 100	BBIT
19AA12	Mergal MITZ	CMIT/MIT Mixture
19AA13	Mergal BIT Technical	BIT
19AA98	KORDEK™ 573F BIOCIDE	MIT
19AB24	KATHON 287T industrial Microbicide	DCOIT

REGULATORY REQUIREMENTS:

Will this study be conducted according to **GLPs**? ☒ YES or ☐ NO

If **YES**, please indicate which agency(ies) guidelines are to be followed:
☐ OECD; ☐ FDA; ☐ Other:
☐ EPA TSCA (40 CFR part 792); ☒ EPA FIFRA (40 CFR part 160)

IIVS Study No.: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000
IIVS Project No.: 10426

PROTOCOL AMENDMENT I

SPONSOR:	National Institute of Environmental Health Sciences (NIEHS) NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
IIVS STUDY NO.:	18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

AMENDMENT(S):

1) Location: §4.0 TESTING FACILITY AND KEY PERSONNEL
§4.3 Study Director

Amendment: Replace "Greg Mun, B.A." with "Rishil J. Kathawala, Ph.D."

Reason: Rishil J. Kathawala is assuming the Study Director responsibility.

APPROVAL: [redacted] 20 May 2019

ASSU[redacted]ECTOR DATE

APPROVAL: [redacted] 5/20/19

TESTING FACILITY MANAGEMENT DATE

IIVS Study No.: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000
IIVS Project No.: 10426

PROTOCOL AMENDMENT II

SPONSOR:	National Institute of Environmental Health Sciences (NIEHS) NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
IIVS STUDY NO.:	18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

AMENDMENT(S):

1) Location: §2.0 SPONSOR
§2.3 Representative

§15.0 APPROVAL
SPONSOR REPRESENTATIVE

Amendment: Replace "Judy Strickland, Ph.D., DABT"

with "Judy Strickland, Ph.D., DABT
Integrated Laboratory Systems, Inc.,
Contractor supporting the NICEATM"

Reason: sponsor request

APPROVAL: [redacted] 9 October 2019

STUDY DIRECTOR DATE

IIVS Study No.: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000
IIVS Project No.: 10426

PROTOCOL AMENDMENT III

SPONSOR:	National Institute of Environmental Health Sciences (NIEHS) NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
IIVS STUDY NO.:	18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

AMENDMENT(S):

1) Location: PROTOCOL ATTACHMENT 1, Sponsor Designation in the table

Amendment:

Replace

19AA05	2-Butyl-1,2-benzothiazolin-3-one (BBIT). Trade name: Vanquish 100	BBIT

with

19AA05	2-Butyl-1,2-benzisothiazolin-3-one (BBIT). Trade name: Vanquish 100	BBIT

Reason: sponsor update

2) Location: PROTOCOL AMENDMENT II

Amendment: Add

Protocol page 1 and 12

To

1) Location:

Reason: protocol amendment II generation error

APPROVAL: [redacted] 28 October 2019

STUDY DIRECTOR DATE

APPENDIX B (Analyzed Data)

h-CLAT Dose Range Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Data Folder Name	Dose Range 052119
Plate Seeding Date	5/20/2019
Collection Date	5/21/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

TA ID	Well ID	Well Name	Living #Events	Living %Parent	Concentrations on the cells (µg/mL)						New Dose Range on the Cells (ug/mL)	New Dose Range in Primary Solvent (mg/mL)	New Dose Range in Media (µg/mL)
Controls	D1	Media	9997	98.21									
	C1	DMSO	9997	97.90									
	B1	DNCB	27088	68.09									
18AO64	A2	TA1D1	8164	11.75	1000	Solvent	DMSO				9.6	4.8	19.2
	B2	TA1D2	9810	75.61	500			Above Concentration*	7.8	Viability 77.18	8.0	4.0	16.0
	C2	TA1D3	6397	9.91	250			Below Concentration*	15.6	Viability 13.32	6.7	3.3	13.3
	D2	TA1D4	4194	6.69	125			Log CV75	0.903		5.6	2.8	11.1
	E2	TA1D5	2884	5.62	62.5			CV75	8.0	ug/mL on the cells	4.6	2.3	9.3
	F2	TA1D6	3437	7.24	31.3						3.9	1.9	7.7
	G2	TA1D7	7284	13.32	15.6						3.2	1.6	6.4
	H2	TA1D8	9992	77.18	7.81						2.7	1.3	5.4
19AA12	A3	TA2D1	6811	12.86	1000	Solvent	DMSO				38.2	19.1	76
	B3	TA2D2	5914	11.15	500			Above Concentration*	31.3	Viability 76.37	31.8	15.9	64
	C3	TA2D3	5971	10.61	250			Below Concentration*	62.5	Viability 25.87	26.5	13.3	53
	D3	TA2D4	5007	9.18	125			Log CV75	1.503		22.1	11.1	44
	E3	TA2D5	9943	25.87	62.5			CV75	31.8	ug/mL on the cells	18.4	9.2	37
	F3	TA2D6	9975	76.37	31.3						15.4	7.7	31
	G3	TA2D7	9995	96.92	15.6						12.8	6.4	26
	H3	TA2D8	9996	97.79	7.81						10.7	5.3	21
19AA13	A4	TA3D1	8970	13.25	1000	Solvent	DMSO				21.4	10.7	43
	B4	TA3D2	6564	10.12	500			Above Concentration*	15.6	Viability 85.07	17.8	8.9	36
	C4	TA3D3	5588	9.34	250			Below Concentration*	31.3	Viability 31.46	14.8	7.4	30
	D4	TA3D4	4028	7.36	125			Log CV75	1.250		12.4	6.2	25
	E4	TA3D5	3325	7.05	62.5			CV75	17.8	ug/mL on the cells	10.3	5.1	21
	F4	TA3D6	9965	31.46	31.3						8.6	4.3	17
	G4	TA3D7	9992	85.07	15.6						7.2	3.6	14
	H4	TA3D8	10000	94.48	7.81						6.0	3.0	12

New top stock concentration for the cytotoxicity assays

* - Refers to the concentration above or below 75% cell viability

Note: Cell dot plot showed dead events for this concentration. PI stained histogram peak appeared to have shifted to the left and fell under the living events gate when observing on flow cytometer. CB 5/22/19

h-CLAT Dose Range Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000; 19AB83.177000; 19AD20.177000

Data Folder Name	Dose Range 061319
Plate Seeding Date	6/12/2019
Collection Date	6/13/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

TA ID	Well ID	Well Name	Living #Events	Living %Parent	Concentrations on the cells (ug/mL)							New Dose Range on the Cells (ug/mL)	New Dose Range in Primary Solvent (mg/mL)	New Dose Range in Media (ug/mL)
Controls	D1	Media	10000	97.91										
	C1	DMSO	10000	97.77										
	B1	DNCB	22861	51.69										
19AA05	A2	TA1D1	9706	14.13	500	Solvent	DMSO					5.8	2.9	11.5
	B2	TA1D2	5072	21.03	250			Above Concentration*	3.9	Viability	82.07	4.8	2.4	9.6
	C2	TA1D3	10002	30.81	125			Below Concentration*	7.8	Viability	58.55	4.0	2.0	8.0
	D2	TA1D4	10002	34.81	62.5			Log CV75	0.682			3.3	1.7	6.7
	E2	TA1D5	3010	5.86	31.3			CV75	4.8	ug/mL on the cells		2.8	1.4	5.6
	F2	TA1D6	10005	23.06	15.6							2.3	1.2	4.6
	G2	TA1D7	10002	58.55	7.81							1.9	1.0	3.9
	H2	TA1D8	10000	82.07	3.91							1.6	0.8	3.2
19AA98	A3	TA2D1	6210	11.18	500	Solvent	DMSO					45	22.4	90
	B3	TA2D2	5558	10.44	250			Above Concentration*	31.3	Viability	82.02	37	18.7	75
	C3	TA2D3	5376	10.60	125			Below Concentration*	62.5	Viability	54.61	31	15.6	62
	D3	TA2D4	10002	54.61	62.5			Log CV75	1.572			26	13.0	52
	E3	TA2D5	10000	82.02	31.3			CV75	37.3	ug/mL on the cells		22	10.8	43
	F3	TA2D6	10000	96.01	15.6							18	9.0	36
	G3	TA2D7	10000	97.31	7.81							15	7.5	30
	H3	TA2D8	10000	97.77	3.91							12	6.2	25
19AB24	A4	TA3D1	7331	13.68	500	Solvent	DMSO							
	B4	TA3D2	8923	13.33	250			Above Concentration*	NA	Viability	NA			
	C4	TA3D3	8411	12.95	125			Below Concentration*	3.91	Viability	12.91			
	D4	TA3D4	7622	10.68	62.5			Log CV75	NA					
	E4	TA3D5	3405	6.04	31.3			CV75	<3.91	ug/mL on the cells				
	F4	TA3D6	3614	5.98	15.6									
	G4	TA3D7	2762	4.70	7.81									
	H4	TA3D8	7753	12.91	3.91									
19AB83	A5	TA4D1	10000	77.62	1000	Solvent	DMSO					1000	500	2000
	B5	TA4D2	10000	96.70	500			Above Concentration*	NA	Viability	NA	833	417	1667
	C5	TA4D3	10000	95.90	250			Below Concentration*	NA	Viability	NA	694	347	1389
	D5	TA4D4	10001	97.49	125			Log CV75	NA			579	289	1157
	E5	TA4D5	10001	97.63	62.5			CV75	NA	ug/mL on the cells		482	241	965
	F5	TA4D6	10000	97.30	31.3							402	201	804
	G5	TA4D7	10000	97.19	15.6							335	167	670
	H5	TA4D8	10000	97.68	7.81							279	140	558
19AD20	A6	TA5D1	6540	10.08	1000	Solvent	DMSO					11.5	5.7	22.9
	B6	TA5D2	7527	11.08	500			Above Concentration*	7.81	Viability	95.72	9.6	4.8	19.1
	C6	TA5D3	5749	9.87	250			Below Concentration*	15.63	Viability	24.47	8.0	4.0	15.9
	D6	TA5D4	6562	9.97	125			Log CV75	0.980			6.6	3.3	13.3
	E6	TA5D5	5810	9.45	62.5			CV75	9.6	ug/mL on the cells		5.5	2.8	11.1
	F6	TA5D6	5331	8.22	31.3							4.6	2.3	9.2
	G6	TA5D7	10015	24.47	15.6							3.8	1.9	7.7
	H6	TA5D8	10000	95.72	7.81							3.2	1.6	6.4
													New top stock concentration for the definitive assays	

* - Refers to the concentration above or below 75% cell viability

h-CLAT Dose Range Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Data Folder Name	Dose Range 062519
Plate Seeding Date	6/24/2019
Collection Date	6/25/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

TA ID	Well ID	Well Name	Living #Events	Living %Parent	Concentrations on the cells (ug/mL)	New Dose Range on the Cells (ug/mL)	New Dose Range in Primary Solvent (mg/mL)	New Dose Range in Media (ug/mL)
Controls	D1	Media	9997	98.11				
	C1	DMSO	9998	98.13				
	B1	DNCB	19526	67.98				
19AB24	A2	TA1D1	9816	14.92	3.00	1.37	0.69	2.74
	B2	TA1D2	9938	61.57	1.50	1.14	0.57	2.28
	C2	TA1D3	9991	95.70	0.75	0.95	0.48	1.90
	D2	TA1D4	9991	96.43	0.38	0.79	0.40	1.59
	E2	TA1D5	9992	96.36	0.19	0.66	0.33	1.32
	F2	TA1D6	9994	97.03	0.09	0.55	0.28	1.10
	G2	TA1D7	9995	96.50	0.05	0.46	0.23	0.92
	H2	TA1D8	9996	96.99	0.02	0.38	0.19	0.76

Solvent	DMSO		
Above Concentration*	0.75	Viability	95.70
Below Concentration*	1.50	Viability	61.57
Log CV75	0.058		
CV75	1.1	ug/mL on the cells	

New top stock concentration for the definitive assays

* - Refers to the concentration above or below 75% cell viability

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 061119
Plate Seeding Date	6/10/2019
Collection Date	6/11/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

Well ID	Well Name	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
A2	Media CD54	10001	9865	96.73	7.91
B2	Media CD86	10006	9987	96.33	22.70
C2	Media Isotype	10003	9881	96.23	6.16
E1	DMSO CD54	10004	9906	97.26	8.17
F1	DMSO CD86	10004	9982	97.25	22.49
G1	DMSO Isotype	10003	9848	96.38	5.63
D1	DNCB CD54	10015	9912	77.20	23.19
C1	DNCB CD86	10007	9979	79.20	32.86
B1	DNCB Isotype	18430	18167	78.97	6.21

Acceptance Criteria for a Valid Assay

Cell viabilities for medium and solvent controls are > 90%

Control	Viability	Criteria Met?
Medium	96.23	Yes
DMSO	96.38	Yes

Solvent control RFI values are negative responses

Control	RFI	Criteria Met?
DMSO CD54	145.14	Yes
DMSO CD86	101.93	Yes

MFI ratio of CD54/86 to isotype control for medium and solvent controls are > 105%

Control	Ratio	Criteria Met?
Medium CD54	128.41	Yes
Medium CD86	368.51	Yes
DMSO CD54	145.12	Yes
DMSO CD86	399.47	Yes

DNCB RFI values are positive and cell viability is > 50%

Control	RFI	Criteria Met?
DNCB CD54	668.50	Yes
DNCB CD86	158.07	Yes

Control	Viability	Criteria Met?
DNCB	78.97	Yes

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 061119
Plate Seeding Date	6/10/2019
Collection Date	6/11/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
18AO64	A4	TA1D1 CD54	9.6	10015	9748	76.97	18.14	455.91
	B4	TA1D2 CD54	8.0	10007	9805	90.69	15.61	338.58
	C4	TA1D3 CD54	6.7	10006	9882	87.77	15.96	345.67
	D4	TA1D4 CD54	5.6	10007	9906	88.43	17.62	426.77
	E4	TA1D5 CD54	4.6	10007	9904	85.02	19.62	516.93
	F4	TA1D6 CD54	3.9	10006	9897	88.19	19.55	529.92
	G4	TA1D7 CD54	3.2	10009	9913	86.90	17.65	460.63
	H4	TA1D8 CD54	2.7	6808	6704	83.96	13.76	321.26
	A5	TA1D1 CD86	9.6	10013	9953	78.13	23.70	101.66
	B5	TA1D2 CD86	8.0	10012	9961	89.50	22.01	88.97
	C5	TA1D3 CD86	6.7	10003	9970	87.96	29.29	131.14
	D5	TA1D4 CD86	5.6	10008	9989	90.46	28.32	127.76
	E5	TA1D5 CD86	4.6	10008	9981	84.12	24.41	106.29
	F5	TA1D6 CD86	3.9	10009	9985	90.03	25.57	115.54
	G5	TA1D7 CD86	3.2	10006	9976	88.69	23.16	102.08
	H5	TA1D8 CD86	2.7	10010	9976	84.61	23.09	103.74
	A6	TA1D1 Isotype Control	9.6	10012	9729	80.33	6.56	
	B6	TA1D2 Isotype Control	8.0	10007	9825	89.87	7.01	
	C6	TA1D3 Isotype Control	6.7	10010	9884	87.52	7.18	
	D6	TA1D4 Isotype Control	5.6	10005	9886	89.55	6.78	
	E6	TA1D5 Isotype Control	4.6	10008	9857	84.94	6.49	
	F6	TA1D6 Isotype Control	3.9	10006	9828	89.67	6.09	
	G6	TA1D7 Isotype Control	3.2	10010	9844	88.40	5.95	
	H6	TA1D8 Isotype Control	2.7	10005	9774	85.06	5.60	

Highest Concentration below 200	NA	RFI	NA
Lowest Concentration above 200	2.7	RFI	321.26
EC200	<2.7		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	9.6	RFI	101.66
Lowest Concentration above 150	NA	RFI	NA
EC150	>9.6		
Is the TA a sensitizer?	No		

s viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10004	9906	97.26	8.17
CD86	F1	10004	9982	97.25	22.49
Isotype	G1	10003	9848	96.38	5.63

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 061119
Plate Seeding Date	6/10/2019
Collection Date	6/11/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA12	A7	TA2D1 CD54	38.2	3727	3692	26.07	8.74	64.57
	B7	TA2D2 CD54	31.8	6320	6268	26.53	8.19	60.63
	C7	TA2D3 CD54	26.5	6794	6747	23.28	8.42	98.03
	D7	TA2D4 CD54	22.1	10036	9918	54.66	10.91	198.03
	E7	TA2D5 CD54	18.4	10014	9873	77.25	8.93	157.87
	F7	TA2D6 CD54	15.4	10007	9831	90.27	7.87	129.53
	G7	TA2D7 CD54	12.8	10003	9847	95.02	8.36	120.08
	H7	TA2D8 CD54	10.7	10002	9800	96.26	7.39	111.02
	A8	TA2D1 CD86	38.2	4672	4638	22.22	21.37	84.64
	B8	TA2D2 CD86	31.8	6524	6496	24.71	19.46	75.98
	C8	TA2D3 CD86	26.5	6859	6844	23.59	22.45	97.98
	D8	TA2D4 CD86	22.1	10053	10036	53.53	21.89	94.96
	E8	TA2D5 CD86	18.4	10013	10000	75.56	19.27	85.11
	F8	TA2D6 CD86	15.4	10016	10003	89.20	17.26	75.21
	G8	TA2D7 CD86	12.8	10005	9968	95.09	19.86	86.30
	H8	TA2D8 CD86	10.7	10004	9966	95.95	15.14	62.69
	A9	TA2D1 Isotype Control	38.2	4538	4488	23.63	7.10	
	B9	TA2D2 Isotype Control	31.8	6635	6572	24.01	6.65	
	C9	TA2D3 Isotype Control	26.5	7128	7062	22.53	5.93	
	D9	TA2D4 Isotype Control	22.1	10048	9879	51.27	5.88	
	E9	TA2D5 Isotype Control	18.4	10020	9728	74.53	4.92	
	F9	TA2D6 Isotype Control	15.4	10012	9685	89.33	4.58	
	G9	TA2D7 Isotype Control	12.8	10010	9818	94.58	5.31	
	H9	TA2D8 Isotype Control	10.7	10005	9700	96.38	4.57	

Highest Concentration below 200	22.1	RFI	198.03
Lowest Concentration above 200	NA	RFI	NA
EC200	>22.1		
Is the TA a sensitizer?	No		
Highest Concentration below 150	22.1	RFI	94.96
Lowest Concentration above 150	NA	RFI	NA
EC150	>22.1		
Is the TA a sensitizer?	No		

s viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

Viability <50% CB 6/14/19

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10004	9906	97.26	8.17
CD86	F1	10004	9982	97.25	22.49
Isotype	G1	10003	9848	96.38	5.63

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 061119
Plate Seeding Date	6/10/2019
Collection Date	6/11/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA13	A10	TA3D1 CD54	21.4	10028	9964	38.66	18.66	349.21
	B10	TA3D2 CD54	17.8	10035	9935	48.68	33.99	1035.04
	C10	TA3D3 CD54	14.9	10009	9899	75.45	27.86	816.14
	D10	TA3D4 CD54	12.4	10013	9871	87.15	20.03	542.91
	E10	TA3D5 CD54	10.3	10014	9871	90.19	18.24	456.30
	F10	TA3D6 CD54	8.6	10008	9859	94.35	13.91	333.07
	G10	TA3D7 CD54	7.2	10012	9810	95.42	12.28	264.96
	H10	TA3D8 CD54	6.0	10005	9770	96.55	10.48	198.03
	A11	TA3D1 CD86	21.4	10037	10012	38.02	22.88	77 64
	B11	TA3D2 CD86	17.8	10029	10004	52.88	26.47	111.33
	C11	TA3D3 CD86	14.9	10010	9980	75.78	30.00	135.65
	D11	TA3D4 CD86	12.4	10017	9982	89.05	29.52	138.08
	E11	TA3D5 CD86	10.3	10013	9972	90.17	25.64	112.63
	F11	TA3D6 CD86	8.6	10008	9977	94.92	23.71	108.30
	G11	TA3D7 CD86	7.2	10008	9955	95.85	23.34	105.52
	H11	TA3D8 CD86	6.0	10004	9912	96.57	21.12	92.94
	A12	TA3D1 Isotype Control	21.4	9060	9001	41.65	9.79	
	B12	TA3D2 Isotype Control	17.8	10029	9879	56.01	7.70	
	C12	TA3D3 Isotype Control	14.9	10012	9826	79.59	7.13	
	D12	TA3D4 Isotype Control	12.4	10011	9784	89.15	6.24	
	E12	TA3D5 Isotype Control	10.3	10014	9832	90.08	6.65	
	F12	TA3D6 Isotype Control	8.6	10009	9786	94.85	5.45	
	G12	TA3D7 Isotype Control	7.2	10003	9781	96.10	5.55	
	H12	TA3D8 Isotype Control	6.0	10001	9797	96.77	5.45	

Highest Concentration below 200	6.0	RFI	198.03
Lowest Concentration above 200	7.2	RFI	264.96
EC200	6.02		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	17.8	RFI	111.33
Lowest Concentration above 150	NA	RFI	NA
EC150	>17.8		
Is the TA a sensitizer?	No		

s viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10004	9906	97.26	8.17
CD86	F1	10004	9982	97.25	22.49
Isotype	G1	10003	9848	96.38	5.63

Viability <50% CB 6/14/19

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 061819
Plate Seeding Date	6/17/2019
Collection Date	6/18/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

Well ID	Well Name	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
A2	Media CD54	9992	9827	98.38	7.66
B2	Media CD86	9992	9953	98.50	22.21
C2	Media Isotype	9996	9841	98.60	6.17
E1	DMSO CD54	9997	9831	98.50	7.36
F1	DMSO CD86	9999	9964	98.60	24.42
G1	DMSO Isotype	9994	9754	98.17	5.64
D1	DNCB CD54	9969	9787	71.02	31.26
C1	DNCB CD86	9970	9921	70.59	38.71
B1	DNCB Isotype	16393	15836	74.00	6.45

Acceptance Criteria for a Valid Assay

Cell viabilities for medium and solvent controls are > 90%

Control	Viability	Criteria Met?
Medium	98.60	Yes
DMSO	98.17	Yes

Solvent control RFI values are negative responses

Control	RFI	Criteria Met?
DMSO CD54	115.44	Yes
DMSO CD86	117.08	Yes

MFI ratio of CD54/86 to isotype control for medium and solvent controls are > 105%

Control	Ratio	Criteria Met?
Medium CD54	124.15	Yes
Medium CD86	359.97	Yes
DMSO CD54	130.50	Yes
DMSO CD86	432.98	Yes

DNCB RFI values are positive and cell viability is > 50%

Control	RFI	Criteria Met?
DNCB CD54	1442.44	Yes
DNCB CD86	171.78	Yes

Control	Viability	Criteria Met?
DNCB	74.00	Yes

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 061819
Plate Seeding Date	6/17/2019
Collection Date	6/18/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
18AO64	A4	TA1D1 CD54	9.6	9968	9780	70.07	24.34	1104.65
	B4	TA1D2 CD54	8.0	9980	9778	87.25	16.54	631.40
	C4	TA1D3 CD54	6.7	9994	9847	86.57	19.25	790.70
	D4	TA1D4 CD54	5.6	9988	9880	84.98	20.85	854.07
	E4	TA1D5 CD54	4.6	9989	9885	82.51	29.44	1372.67
	F4	TA1D6 CD54	3.9	9990	9884	82.35	29.96	1421.51
	G4	TA1D7 CD54	3.2	9991	9881	82.33	27.04	1223.84
	H4	TA1D8 CD54	2.7	9993	9875	83.87	24.65	1109.30
	A5	TA1D1 CD86	9.6	9960	9926	69.60	22.27	90.15
	B5	TA1D2 CD86	8.0	9990	9961	88.10	24.53	100.37
	C5	TA1D3 CD86	6.7	9988	9959	84.59	22.40	89.19
	D5	TA1D4 CD86	5.6	9986	9967	83.43	25.46	102.77
	E5	TA1D5 CD86	4.6	9993	9961	80.63	22.79	90.31
	F5	TA1D6 CD86	3.9	9993	9952	81.14	21.06	82.80
	G5	TA1D7 CD86	3.2	9994	9960	81.80	22.04	85.46
	H5	TA1D8 CD86	2.7	9989	9953	80.37	21.21	83.28
	A6	TA1D1 Isotype Control	9.6	9965	9566	68.51	5.34	
	B6	TA1D2 Isotype Control	8.0	9984	9710	87.04	5.68	
	C6	TA1D3 Isotype Control	6.7	9991	9732	83.84	5.65	
	D6	TA1D4 Isotype Control	5.6	9984	9812	83.43	6.16	
	E6	TA1D5 Isotype Control	4.6	9993	9803	78.57	5.83	
	F6	TA1D6 Isotype Control	3.9	9990	9759	79.42	5.51	
	G6	TA1D7 Isotype Control	3.2	9990	9787	82.11	5.99	
	H6	TA1D8 Isotype Control	2.7	9996	9781	81.93	5.57	

Highest Concentration below 200	NA	RFI	NA
Lowest Concentration above 200	2.7	RFI	1109.30
EC200	<2.7		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	9.6	RFI	90.15
Lowest Concentration above 150	NA	RFI	NA
EC150	>9.6		
Is the TA a sensitizer?	No		

Is viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	9997	9831	98.50	7.36
CD86	F1	9999	9964	98.60	24.42
Isotype	G1	9994	9754	98.17	5.64

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 061819
Plate Seeding Date	6/17/2019
Collection Date	6/18/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA12	A7	TA2D1 CD54	38.2	5534	5455	19.54	5.55	42.44
	B7	TA2D2 CD54	31.8	7532	7474	19.50	7.65	82.56
	C7	TA2D3 CD54	26.5	9792	9683	25.31	8.29	226.16
	D7	TA2D4 CD54	22.1	9878	9750	38.94	11.69	377.33
	E7	TA2D5 CD54	18.4	9971	9689	73.90	8.20	240.70
	F7	TA2D6 CD54	15.4	9990	9737	86.92	7.11	158.14
	G7	TA2D7 CD54	12.8	9993	9703	95.39	6.11	95.35
	H7	TA2D8 CD54	10.7	9995	9775	96.79	6.25	79.07
	A8	TA2D1 CD86	38.2	5374	5351	19.21	17.18	65.81
	B8	TA2D2 CD86	31.8	6694	6675	19.11	22.00	83.97
	C8	TA2D3 CD86	26.5	9368	9347	25.36	20.01	83.12
	D8	TA2D4 CD86	22.1	9892	9870	37.62	24.92	105.01
	E8	TA2D5 CD86	18.4	9969	9947	73.72	16.86	68.16
	F8	TA2D6 CD86	15.4	9977	9963	87.42	16.63	65.18
	G8	TA2D7 CD86	12.8	9992	9970	95.56	17.52	69.49
	H8	TA2D8 CD86	10.7	9987	9964	97.06	18.35	71.67
	A9	TA2D1 Isotype Control	38.2	5462	5381	19.79	4.82	
	B9	TA2D2 Isotype Control	31.8	7184	7107	19.42	6.23	
	C9	TA2D3 Isotype Control	26.5	8712	8576	26.09	4.40	
	D9	TA2D4 Isotype Control	22.1	9873	9669	40.47	5.20	
	E9	TA2D5 Isotype Control	18.4	9958	9513	72.45	4.06	
	F9	TA2D6 Isotype Control	15.4	9979	9591	86.62	4.39	
	G9	TA2D7 Isotype Control	12.8	9993	9640	94.77	4.47	
	H9	TA2D8 Isotype Control	10.7	9993	9715	96.82	4.89	

Highest Concentration below 200	15.4	RFI	158.14
Lowest Concentration above 200	18.4	RFI	240.70
EC200	16.91		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	18.4	RFI	68.16
Lowest Concentration above 150	NA	RFI	NA
EC150	>18.4		
Is the TA a sensitizer?	No		

s viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

Viability <50% CB 6/24/19

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	9997	9831	98.50	7.36
CD86	F1	9999	9964	98.60	24.42
Isotype	G1	9994	9754	98.17	5.64

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 061819
Plate Seeding Date	6/17/2019
Collection Date	6/18/2019
Cell Thaw Date	4/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	5/16/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA13	A10	TA3D1 CD54	21.4	9937	9889	38.42	14.17	278.49
	B10	TA3D2 CD54	17.8	9953	9842	47.68	32.05	1445.93
	C10	TA3D3 CD54	14.9	9963	9843	66.16	33.91	1655.81
	D10	TA3D4 CD54	12.4	9988	9884	80.33	29.55	1356.40
	E10	TA3D5 CD54	10.3	9987	9835	92.04	15.13	555.81
	F10	TA3D6 CD54	8.6	9993	9833	93.04	13.90	463.95
	G10	TA3D7 CD54	7.2	9990	9801	95.85	10.65	320.35
	H10	TA3D8 CD54	6.0	9995	9855	97.03	10.28	286.63
	A11	TA3D1 CD86	21.4	9930	9913	42.12	19.81	55.54
	B11	TA3D2 CD86	17.8	9945	9895	52.19	19.20	64.00
	C11	TA3D3 CD86	14.9	9967	9923	70.73	20.85	82.11
	D11	TA3D4 CD86	12.4	9990	9952	83.27	26.37	107.29
	E11	TA3D5 CD86	10.3	9986	9941	91.94	23.04	93.02
	F11	TA3D6 CD86	8.6	9987	9950	93.53	24.57	99.31
	G11	TA3D7 CD86	7.2	9997	9958	96.19	20.08	79.55
	H11	TA3D8 CD86	6.0	9997	9947	96.83	19.34	74.49
	A12	TA3D1 Isotype Control	21.4	9925	9831	39.05	9.38	
	B12	TA3D2 Isotype Control	17.8	9952	9692	46.33	7.18	
	C12	TA3D3 Isotype Control	14.9	9973	9657	68.48	5.43	
	D12	TA3D4 Isotype Control	12.4	9979	9748	79.40	6.22	
	E12	TA3D5 Isotype Control	10.3	9988	9749	91.57	5.57	
	F12	TA3D6 Isotype Control	8.6	9988	9783	93.92	5.92	
	G12	TA3D7 Isotype Control	7.2	9987	9742	95.96	5.14	
	H12	TA3D8 Isotype Control	6.0	9998	9819	97.29	5.35	

Highest Concentration below 200	NA	RFI	NA
Lowest Concentration above 200	6.0	RFI	286.63
EC200	<6.0		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	17.8	RFI	64.00
Lowest Concentration above 150	NA	RFI	NA
EC150	>17.8		
Is the TA a sensitizer?	No		

Is viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	9997	9831	98.50	7.36
CD86	F1	9999	9964	98.60	24.42
Isotype	G1	9994	9754	98.17	5.64

Viability <50% CB 6/24/19

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 070219
Plate Seeding Date	7/1/2019
Collection Date	7/2/2019
Cell Thaw Date	6/10/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	6/15/2019

Well ID	Well Name	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
A2	Media CD54	10000	9800	97.58	7.16
B2	Media CD86	10001	9923	98.12	18.74
C2	Media Isotype	10000	9801	98.15	5.15
E1	DMSO CD54	10000	9851	97.70	7.50
F1	DMSO CD86	10000	9964	97.93	19.71
G1	DMSO Isotype	10000	9791	98.29	4.91
D1	DNCB CD54	10000	9900	81.56	24.52
C1	DNCB CD86	10000	9963	81.25	28.47
B1	DNCB Isotype	18676	18317	83.31	5.05

Acceptance Criteria for a Valid Assay

Cell viabilities for medium and solvent controls are > 90%

Control	Viability	Criteria Met?
Medium	98.15	Yes
DMSO	98.29	Yes

Solvent control RFI values are negative responses

Control	RFI	Criteria Met?
DMSO CD54	128.86	Yes
DMSO CD86	108.90	Yes

MFI ratio of CD54/86 to isotype control for medium and solvent controls are > 105%

Control	Ratio	Criteria Met?
Medium CD54	139.03	Yes
Medium CD86	363.88	Yes
DMSO CD54	152.75	Yes
DMSO CD86	401.43	Yes

DNCB RFI values are positive and cell viability is > 50%

Control	RFI	Criteria Met?
DNCB CD54	751.74	Yes
DNCB CD86	158.24	Yes

Control	Viability	Criteria Met?
DNCB	83.31	Yes

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 070219
Plate Seeding Date	7/1/2019
Collection Date	7/2/2019
Cell Thaw Date	6/10/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	6/15/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA12	A4	TA1D1 CD54	38.2	4958	4908	27.42	6.19	18.92
	B4	TA1D2 CD54	31.8	7756	7677	26.20	6.98	25.87
	C4	TA1D3 CD54	26.5	6663	6598	21.16	6.06	34.36
	D4	TA1D4 CD54	22.1	10003	9874	38.74	10.25	175.68
	E4	TA1D5 CD54	18.4	10000	9859	75.13	9.89	184.56
	F4	TA1D6 CD54	15.4	10001	9833	89.06	9.07	154.05
	G4	TA1D7 CD54	12.8	10000	9828	95.85	7.57	112.74
	H4	TA1D8 CD54	10.7	10000	9837	96.06	7.79	109.65
	A5	TA1D1 CD86	38.2	5237	5195	27.26	16.17	70.74
	B5	TA1D2 CD86	31.8	7046	7002	26.03	16.23	67.03
	C5	TA1D3 CD86	26.5	6809	6789	20.69	17.25	81.62
	D5	TA1D4 CD86	22.1	10002	9979	38.63	18.83	88.72
	E5	TA1D5 CD86	18.4	10002	9980	73.32	17.98	86.96
	F5	TA1D6 CD86	15.4	10000	9971	90.05	16.71	78.58
	G5	TA1D7 CD86	12.8	10000	9978	94.97	15.37	72.43
	H5	TA1D8 CD86	10.7	10001	9968	96.08	17.88	87.36
	A6	TA1D1 Isotype Control	38.2	4181	4125	25.43	5.70	
	B6	TA1D2 Isotype Control	31.8	7201	7128	25.25	6.31	
	C6	TA1D3 Isotype Control	26.5	6079	6010	20.80	5.17	
	D6	TA1D4 Isotype Control	22.1	10003	9878	38.06	5.70	
	E6	TA1D5 Isotype Control	18.4	10001	9838	74.04	5.11	
	F6	TA1D6 Isotype Control	15.4	10000	9821	90.03	5.08	
	G6	TA1D7 Isotype Control	12.8	10000	9793	95.79	4.65	
	H6	TA1D8 Isotype Control	10.7	10000	9818	96.86	4.95	

Highest Concentration below 200	18.4	RFI	184.56
Lowest Concentration above 200	NA	RFI	NA
EC200	>18.4		
Is the TA a sensitizer?	No		

Highest Concentration below 150	18.4	RFI	86.96
Lowest Concentration above 150	NA	RFI	NA
EC150	>18.4		
Is the TA a sensitizer?	No		

Is viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Viability < 50% MM 7/3/19

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10000	9851	97.70	7.50
CD86	F1	10000	9964	97.93	19.71
Isotype	G1	10000	9791	98.29	4.91

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 070219
Plate Seeding Date	7/1/2019
Collection Date	7/2/2019
Cell Thaw Date	6/10/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	6/15/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA05	A7	TA2D1 CD54	5.8	10001	9932	80.90	38.80	1207.34
	B7	TA2D2 CD54	4.8	10002	9957	81.32	38.81	1180.69
	C7	TA2D3 CD54	4.0	10000	9954	84.65	28.79	797.30
	D7	TA2D4 CD54	3.3	10002	9959	91.83	25.50	693.44
	E7	TA2D5 CD54	2.8	10001	9931	88.55	19.22	468.73
	F7	TA2D6 CD54	2.3	10001	9932	94.19	16.60	383.40
	G7	TA2D7 CD54	1.9	10000	9903	94.01	13.82	299.23
	H7	TA2D8 CD54	1.6	10000	9915	96.20	12.11	240.15
	A8	TA2D1 CD86	5.8	10001	9988	82.77	30.12	152.64
	B8	TA2D2 CD86	4.8	10001	9983	79.27	29.07	140.81
	C8	TA2D3 CD86	4.0	10000	9993	86.45	33.25	169.66
	D8	TA2D4 CD86	3.3	10000	9987	89.47	34.27	180.61
	E8	TA2D5 CD86	2.8	10001	9985	88.96	33.08	175.68
	F8	TA2D6 CD86	2.3	10000	9979	93.32	30.79	162.97
	G8	TA2D7 CD86	1.9	10000	9977	94.71	25.83	133.51
	H8	TA2D8 CD86	1.6	10000	9977	95.11	23.43	118.51
	A9	TA2D1 Isotype Control	5.8	10000	9914	83.72	7.53	
	B9	TA2D2 Isotype Control	4.8	10001	9928	79.65	8.23	
	C9	TA2D3 Isotype Control	4.0	10003	9928	86.12	8.14	
	D9	TA2D4 Isotype Control	3.3	10000	9932	89.17	7.54	
	E9	TA2D5 Isotype Control	2.8	10000	9915	89.57	7.08	
	F9	TA2D6 Isotype Control	2.3	10000	9913	92.69	6.67	
	G9	TA2D7 Isotype Control	1.9	10000	9885	95.92	6.07	
	H9	TA2D8 Isotype Control	1.6	10000	9885	95.65	5.89	

Highest Concentration below 200	NA	RFI	NA
Lowest Concentration above 200	1.6	RFI	240.15
EC200	<1.6		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	1.9	RFI	133.51
Lowest Concentration above 150	2.3	RFI	162.97
EC150	2.12		
Is the TA a sensitizer?	Yes		

Is viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10000	9851	97.70	7.50
CD86	F1	10000	9964	97.93	19.71
Isotype	G1	10000	9791	98.29	4.91

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AA98, AB24.177000

Plate Name	Definitive 070219
Plate Seeding Date	7/1/2019
Collection Date	7/2/2019
Cell Thaw Date	6/10/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	6/15/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA98	A10	TA3D1 CD54	45	10000	9949	68.87	25.29	716.22
	B10	TA3D2 CD54	37	10000	9934	77.32	40.80	1337.45
	C10	TA3D3 CD54	31	10000	9919	89.90	20.34	555.60
	D10	TA3D4 CD54	26	10000	9910	94.01	17.82	469.11
	E10	TA3D5 CD54	22	10000	9863	95.10	14.85	380.69
	F10	TA3D6 CD54	18	10000	9871	95.96	15.75	409.27
	G10	TA3D7 CD54	15	10000	9875	95.92	15.83	413.90
	H10	TA3D8 CD54	12	10000	9858	97.37	12.83	310.42
	A11	TA3D1 CD86	45	10000	9978	69.32	20.06	90.00
	B11	TA3D2 CD86	37	10000	9972	78.98	29.22	155.81
	C11	TA3D3 CD86	31	10000	9967	90.98	24.33	124.19
	D11	TA3D4 CD86	26	10000	9980	92.87	23.62	121.28
	E11	TA3D5 CD86	22	10001	9972	96.38	18.56	91.69
	F11	TA3D6 CD86	18	10000	9954	96.61	20.68	104.93
	G11	TA3D7 CD86	15	10001	9955	97.59	18.61	91.22
	H11	TA3D8 CD86	12	10000	9950	97.43	16.85	81.49
	A12	TA3D1 Isotype Control	45	10000	9887	69.81	6.74	
	B12	TA3D2 Isotype Control	37	10001	9834	81.19	6.16	
	C12	TA3D3 Isotype Control	31	10000	9836	90.32	5.95	
	D12	TA3D4 Isotype Control	26	10000	9854	93.60	5.67	
	E12	TA3D5 Isotype Control	22	10000	9796	95.93	4.99	
	F12	TA3D6 Isotype Control	18	10000	9857	96.15	5.15	
	G12	TA3D7 Isotype Control	15	10000	9800	97.25	5.11	
	H12	TA3D8 Isotype Control	12	10000	9783	97.68	4.79	

Highest Concentration below 200	NA	RFI	NA
Lowest Concentration above 200	12	RFI	310.42
EC200	<12		
Is the TA a sensitizer?	Yes		
Highest Concentration below 150	31	RFI	124.19
Lowest Concentration above 150	37	RFI	155.81
EC150	35.87		
Is the TA a sensitizer?	Yes		

Is viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10000	9851	97.70	7.50
CD86	F1	10000	9964	97.93	19.71
Isotype	G1	10000	9791	98.29	4.91

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AB98, AB24.177000; 19AB83.177000; 19AD20.177000

Plate Name	Definitive 071119
Plate Seeding Date	7/10/2019
Collection Date	7/11/2019
Cell Thaw Date	6/10/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	6/25/2019

Well ID	Well Name	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
A2	Media CD54	10002	9876	98.22	7.35
B2	Media CD86	10002	9970	98.70	25.74
C2	Media Isotype	10005	9839	98.57	6.10
E1	DMSO CD54	10001	9922	98.96	7.73
F1	DMSO CD86	10003	9990	98.80	22.22
G1	DMSO Isotype	10001	9870	98.69	5.69
D1	DNCB CD54	10013	9926	76.31	27.78
C1	DNCB CD86	10016	9995	76.30	39.01
B1	DNCB Isotype	16150	15879	78.22	6.34

Acceptance Criteria for a Valid Assay

Cell viabilities for medium and solvent controls are > 90%

Control	Viability	Criteria Met?
Medium	98.57	Yes
DMSO	98.69	Yes

Solvent control RFI values are negative responses

Control	RFI	Criteria Met?
DMSO CD54	163.20	Yes
DMSO CD86	84.16	Yes

MFI ratio of CD54/86 to isotype control for medium and solvent controls are > 105%

Control	Ratio	Criteria Met?
Medium CD54	120.49	Yes
Medium CD86	421.97	Yes
DMSO CD54	135.85	Yes
DMSO CD86	390.51	Yes

DNCB RFI values are positive and cell viability is > 50%

Control	RFI	Criteria Met?
DNCB CD54	1050.98	Yes
DNCB CD86	197.64	Yes

Control	Viability	Criteria Met?
DNCB	78.22	Yes

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AB98, AB24.177000; 19AB83.177000; 19AD20.177000

Plate Name	Definitive 071119
Plate Seeding Date	7/10/2019
Collection Date	7/11/2019
Cell Thaw Date	6/10/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	6/25/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA05	A4	TA1D1 CD54	5.8	10017	9960	77.75	28.34	976.47
	B4	TA1D2 CD54	4.8	10014	9947	82.11	18.51	532.84
	C4	TA1D3 CD54	4.0	10013	9948	86.71	16.86	475.49
	D4	TA1D4 CD54	3.3	10003	9942	90.44	14.37	351.47
	E4	TA1D5 CD54	2.8	10006	9909	93.07	11.18	229.90
	F4	TA1D6 CD54	2.3	10002	9910	93.77	11.41	228.43
	G4	TA1D7 CD54	1.9	10002	9867	95.05	9.29	159.31
	H4	TA1D8 CD54	1.6	10005	9897	95.91	9.26	144.12
	A5	TA1D1 CD86	5.8	10015	9989	79.44	35.18	161.89
	B5	TA1D2 CD86	4.8	10015	10001	84.70	33.52	156.56
	C5	TA1D3 CD86	4.0	10005	9994	86.38	32.25	151.78
	D5	TA1D4 CD86	3.3	10011	9995	90.26	30.98	143.86
	E5	TA1D5 CD86	2.8	10009	9991	91.67	24.57	109.38
	F5	TA1D6 CD86	2.3	10008	9997	94.91	28.44	131.22
	G5	TA1D7 CD86	1.9	10004	9980	95.19	21.61	94.19
	H5	TA1D8 CD86	1.6	10006	9993	95.46	24.70	111.19
	A6	TA1D1 Isotype Control	5.8	10006	9902	79.34	8.42	
	B6	TA1D2 Isotype Control	4.8	10013	9934	83.21	7.64	
	C6	TA1D3 Isotype Control	4.0	10009	9918	87.58	7.16	
	D6	TA1D4 Isotype Control	3.3	10004	9934	90.50	7.20	
	E6	TA1D5 Isotype Control	2.8	10005	9891	93.18	6.49	
	F6	TA1D6 Isotype Control	2.3	10002	9924	94.75	6.75	
	G6	TA1D7 Isotype Control	1.9	10004	9842	95.54	6.04	
	H6	TA1D8 Isotype Control	1.6	10003	9907	96.43	6.32	

Highest Concentration below 200	1.9	RFI	159.31
Lowest Concentration above 200	2.3	RFI	228.43
EC200	2.13		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	3.3	RFI	143.86
Lowest Concentration above 150	4.0	RFI	151.78
EC150	3.86		
Is the TA a sensitizer?	Yes		

Is viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10001	9922	98.96	7.73
CD86	F1	10003	9990	98.80	22.22
Isotype	G1	10001	9870	98.69	5.69

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AB98, AB24.177000; 19AB83.177000; 19AD20.177000

Plate Name	Definitive 071119
Plate Seeding Date	7/10/2019
Collection Date	7/11/2019
Cell Thaw Date	6/10/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	6/25/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AA98	A7	TA2D1 CD54	45	10016	9926	72.18	29.61	1175.00
	B7	TA2D2 CD54	37	10014	9873	88.37	21.83	770.10
	C7	TA2D3 CD54	31	10007	9821	91.42	16.41	542.65
	D7	TA2D4 CD54	26	10010	9810	94.40	13.79	404.90
	E7	TA2D5 CD54	22	10004	9774	94.88	10.96	296.57
	F7	TA2D6 CD54	18	10006	9834	95.86	11.88	299.51
	G7	TA2D7 CD54	15	10005	9708	97.11	7.76	162.75
	H7	TA2D8 CD54	12	10002	9801	97.76	9.21	183.82
	A8	TA2D1 CD86	45	10022	9995	71.79	22.52	102.12
	B8	TA2D2 CD86	37	10011	9989	89.74	27.48	129.22
	C8	TA2D3 CD86	31	10006	9983	92.39	22.78	105.51
	D8	TA2D4 CD86	26	10003	9982	94.56	23.58	109.20
	E8	TA2D5 CD86	22	10006	9981	95.05	20.50	94.31
	F8	TA2D6 CD86	18	10006	9970	96.06	20.66	90.08
	G8	TA2D7 CD86	15	10005	9966	97.38	16.68	74.05
	H8	TA2D8 CD86	12	10003	9967	97.71	18.14	76.71
	A9	TA2D1 Isotype Control	45	10015	9715	71.31	5.64	
	B9	TA2D2 Isotype Control	37	10012	9728	89.32	6.12	
	C9	TA2D3 Isotype Control	31	10010	9676	91.86	5.34	
	D9	TA2D4 Isotype Control	26	10003	9683	94.71	5.53	
	E9	TA2D5 Isotype Control	22	10004	9606	95.19	4.91	
	F9	TA2D6 Isotype Control	18	10006	9778	96.22	5.77	
	G9	TA2D7 Isotype Control	15	10002	9532	97.72	4.44	
	H9	TA2D8 Isotype Control	12	10003	9753	97.48	5.46	

Highest Concentration below 200	15	RFI	162.75
Lowest Concentration above 200	18	RFI	299.51
EC200	16.11		
Is the TA a sensitizer?	Yes		
Highest Concentration below 150	45	RFI	102.12
Lowest Concentration above 150	NA	RFI	NA
EC150	>45		
Is the TA a sensitizer?	No		

Is viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10001	9922	98.96	7.73
CD86	F1	10003	9990	98.80	22.22
Isotype	G1	10001	9870	98.69	5.69

h-CLAT Definitive Assay

Study Number: 18AO64, 19AA05, AA12-AA13, AB98, AB24.177000; 19AB83.177000; 19AD20.177000

Plate Name	Definitive 071119
Plate Seeding Date	7/10/2019
Collection Date	7/11/2019
Cell Thaw Date	6/10/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	6/25/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AB24	A10	TA3D1 CD54	1.37	10065	9973	52.34	14.59	322.06
	B10	TA3D2 CD54	1.14	10021	9939	77.36	15.54	399.02
	C10	TA3D3 CD54	0.95	10016	9909	92.82	13.24	331.37
	D10	TA3D4 CD54	0.79	10012	9905	94.13	14.21	386.76
	E10	TA3D5 CD54	0.66	10009	9861	97.17	7.88	100.00
	F10	TA3D6 CD54	0.55	10002	9892	97.99	7.65	72.55
	G10	TA3D7 CD54	0.46	10001	9783	98.13	6.83	52.94
	H10	TA3D8 CD54	0.38	10002	9884	98.05	7.48	70.10
	A11	TA3D1 CD86	1.37	10068	10034	53.71	21.95	84.27
	B11	TA3D2 CD86	1.14	10018	9995	75.80	26.52	115.67
	C11	TA3D3 CD86	0.95	10007	9987	92.91	24.90	111.43
	D11	TA3D4 CD86	0.79	10005	9984	94.87	22.14	95.70
	E11	TA3D5 CD86	0.66	10003	9982	97.85	21.01	91.77
	F11	TA3D6 CD86	0.55	10006	9977	98.00	20.36	85.84
	G11	TA3D7 CD86	0.46	10001	9979	98.08	17.76	72.66
	H11	TA3D8 CD86	0.38	10002	9973	98.42	18.77	76.95
	A12	TA3D1 Isotype Control	1.37	10070	9920	48.85	8.02	
	B12	TA3D2 Isotype Control	1.14	10023	9870	75.19	7.40	
	C12	TA3D3 Isotype Control	0.95	10010	9862	93.16	6.48	
	D12	TA3D4 Isotype Control	0.79	10008	9850	95.00	6.32	
	E12	TA3D5 Isotype Control	0.66	10005	9848	98.06	5.84	
	F12	TA3D6 Isotype Control	0.55	10006	9856	98.56	6.17	
	G12	TA3D7 Isotype Control	0.46	10003	9825	98.54	5.75	
	H12	TA3D8 Isotype Control	0.38	10005	9873	98.66	6.05	

Highest Concentration below 200	0.66	RFI	100.00
Lowest Concentration above 200	0.79	RFI	386.76
EC200	0.71		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	1.37	RFI	84.27
Lowest Concentration above 150	NA	RFI	NA
EC150	>1.37		
Is the TA a sensitizer?	No		

s viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	10001	9922	98.96	7.73
CD86	F1	10003	9990	98.80	22.22
Isotype	G1	10001	9870	98.69	5.69

h-CLAT Definitive Assay

Study Number: 18O64, 19AA05, AA12-13, AA98, AB24.177000;19AB83.177000;19AD20.177000

Plate Name	Definitive 082019
Plate Seeding Date	8/19/2019
Collection Date	8/20/2019
Cell Thaw Date	7/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	8/13/2019

Well ID	Well Name	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
A2	Media CD54	9998	9909	98.59	7.54
B2	Media CD86	9997	9983	98.36	14.49
C2	Media Isotype	9997	9902	98.16	5.88
E1	DMSO CD54	9999	9858	98.69	6.97
F1	DMSO CD86	9999	9987	98.77	13.37
G1	DMSO Isotype	9992	9843	98.34	5.17
D1	DNCB CD54	9975	9910	82.09	31.29
D2	DNCB CD86	6127	6114	88.23	18.64
C1	DNCB Isotype	15262	15037	81.26	4.96

Acceptance Criteria for a Valid Assay

Cell viabilities for medium and solvent controls are > 90%

Control	Viability	Criteria Met?
Medium	98.16	Yes
DMSO	98.34	Yes

Solvent control RFI values are negative responses

Control	RFI	Criteria Met?
DMSO CD54	108.43	Yes
DMSO CD86	95.24	Yes

MFI ratio of CD54/86 to isotype control for medium and solvent controls are > 105%

Control	Ratio	Criteria Met?
Medium CD54	128.23	Yes
Medium CD86	246.43	Yes
DMSO CD54	134.82	Yes
DMSO CD86	258.61	Yes

DNCB RFI values are positive and cell viability is > 50%

Control	RFI	Criteria Met?
DNCB CD54	1462.78	Yes
DNCB CD86	166.83	Yes

Control	Viability	Criteria Met?
DNCB	81.26	Yes

h-CLAT Definitive Assay

Study Number: 18O64, 19AA05, AA12-13, AA98, AB24.177000;19AB83.177000;19AD20.177000

Plate Name	Definitive 082019
Plate Seeding Date	8/19/2019
Collection Date	8/20/2019
Cell Thaw Date	7/26/2019
Did cells pass the reactivity check?	Yes
Reactivity Date	8/13/2019

	Well ID	Well Name	Final Test Article Concentration (µg/mL)	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean	Calculated RFI
19AB24	A4	TA1D1 CD54	1.37	9978	9907	82.22	16.72	537.22
	B4	TA1D2 CD54	1.14	9994	9927	95.34	15.28	516.11
	C4	TA1D3 CD54	0.95	9992	9887	97.28	10.68	297.78
	D4	TA1D4 CD54	0.79	9996	9890	97.57	8.64	160.00
	E4	TA1D5 CD54	0.66	9997	9920	98.24	7.94	121.67
	F4	TA1D6 CD54	0.55	9997	9935	98.35	8.03	135.56
	G4	TA1D7 CD54	0.46	9995	9903	98.56	7.30	101.11
	H4	TA1D8 CD54	0.38	9996	9926	98.47	7.47	109.44
	A5	TA1D1 CD86	1.37	9963	9955	82.76	18.23	136.34
	B5	TA1D2 CD86	1.14	69660	69575	95.66	16.99	134.15
	C5	TA1D3 CD86	0.95	77687	77567	97.03	15.03	118.41
	D5	TA1D4 CD86	0.79	80181	80075	97.73	15.17	114.76
	E5	TA1D5 CD86	0.66	65317	65255	98.30	15.31	116.59
	F5	TA1D6 CD86	0.55	62205	62139	98.60	14.18	104.76
	G5	TA1D7 CD86	0.46	32514	32466	98.60	12.95	91.10
	H5	TA1D8 CD86	0.38	47173	47122	98.26	14.97	115.49
	A6	TA1D1 Isotype Control	1.37	38423	38079	78.44	7.05	
	B6	TA1D2 Isotype Control	1.14	71911	71147	94.41	5.99	
	C6	TA1D3 Isotype Control	0.95	76699	75592	96.71	5.32	
	D6	TA1D4 Isotype Control	0.79	78334	77488	97.45	5.76	
	E6	TA1D5 Isotype Control	0.66	62668	62170	97.93	5.75	
	F6	TA1D6 Isotype Control	0.55	74747	74106	98.28	5.59	
	G6	TA1D7 Isotype Control	0.46	60800	60085	98.23	5.48	
	H6	TA1D8 Isotype Control	0.38	51086	50651	98.16	5.50	

Highest Concentration below 200	0.79	RFI	160.00
Lowest Concentration above 200	0.95	RFI	297.78
EC200	0.84		
Is the TA a sensitizer?	Yes		

Highest Concentration below 150	1.4	RFI	136.34
Lowest Concentration above 150	NA	RFI	NA
EC150	>1.37		
Is the TA a sensitizer?	No		

s viability ≥ 50% for at least 4 concentrations?	Yes
Is viability of highest concentration < 90%?	Yes

Solvent DMSO

	Well ID	Viable Events	Positive Events	% Viable	Living FITC Geometric Mean
CD54	E1	9999	9858	98.69	6.97
CD86	F1	9999	9987	98.77	13.37
Isotype	G1	9992	9843	98.34	5.17

APPENDIX C (Certificates of Analysis)

SIGMA-ALDRICH

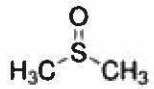
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Dimethyl sulfoxide - for HPLC, ≥99.7%

Product Number:	**34869**
Batch Number:	**SHBJ7917**
Brand:	SIGALD
CAS Number:	67-68-5
MDL Number:	MFCD00002089
Formula:	C2H6OS
Formula Weight:	78.13 g/mol
Quality Release Date:	09 JAN 2018
Expiration Date:	JUN 2021



Test	Specification	Result
Appearance (Color)	Colorless	Colorless
Appearance (Form)	Liquid	Liquid
UV Absorbance 350nm	≤ 0.01	< 0.01
UV Absorbance 300nm	≤ 0.10	0.07
UV Absorbance 280nm	≤ 0.30	0.18
UV Absorbance 270nm	≤ 0.70	0.38
Purity (GC)	≥ 99.70 %	99.98 %
Water (by Karl Fischer)	≤ 0.2 %	< 0.1 %
Residue on Evaporation	≤ 0.002 %	< 0.001 %
Expiration Date Period 1260 Days	----------	----------

[redacted]
Michael Grady, Manager
Quality Control
Sheboygan Falls, WI US

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Sodium chloride solution, 0.9% in water, BioXtra, suitable for cell culture
Product Number	S8776
Product Brand	SIGMA
CAS Number	7647-14-5
Molecular Formula	NaCl
Molecular Weight	58.44

TEST	SPECIFICATION	LOT RNBH2274 RESULTS
Storage:		ROOM TEMPERATURE
Print Date:		21 DEC 2018
Date of QC Release:		21 DEC 2018
Place of Manufacture:		Irvine, United Kingdom
Production Date:		DEC 2018
Appearance (Turbidity)	Clear	Clear
Appearance (Colour)	Colorless	Colorless
Appearance (Form)	Solution	Solution
pH	-	7.0
Osmolality	278 - 308 mOs/kg	290 mOs/kg
Salt Toxicity Test	Pass	Pass
Cell Line	Cell Line - Cell Types	ED1
Key Element Conc - ICP (Sodium)	3.3 - 3.7 g/l	3.6 g/l
Sterility	Pass	Pass
Endotoxin Level	<= 1.0 EU/ml	< 1.0 EU/ml

[redacted]

Jane Findlay, Manager
Quality Control
Irvine, United Kingdom

SIGMA-ALDRICH

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	1-CHLORO-2,4-DINITROBENZENE
	>= 99 %
Product Number:	237329
Batch Number:	BCBS4201V
Brand:	Aldrich
CAS Number:	97-00-7
Formula:	$ClC_6H_3(NO_2)_2$
Formula Weight:	202.55
Quality Release Date:	04 JUL 2016

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	FAINT YELLOW TO YELLOW	FAINT YELLOW
APPEARANCE (FORM)	POWDER OR CRYSTALS	CRYSTALS
PURITY (GC AREA %)	≥ 99.0 %	99.0 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

[redacted]

Dr. Claudia Geltner
Manager Quality Control
Buchs, Switzerland

SIGMA-ALDRICH
3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	1-CHLORO-2,4-DINITROBENZENE
	>= 99 %
Product Number:	237329
Batch Number:	BCBW5262
Brand:	Aldrich
CAS Number:	97-00-7
Formula:	$ClC_6H_3(NO_2)_2$
Formula Weight:	202.55
Quality Release Date:	07 FEB 2018

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	FAINT YELLOW TO YELLOW	YELLOW
APPEARANCE (FORM)	POWDER OR CRYSTALS	CRYSTALS
PURITY (GC AREA %)	≥ 99.0 %	99.8 %
INFRARED SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

[redacted]

Dr. Reinhold Schwenninger
Quality Assurance
Buchs, Switzerland

Date 2019-06-07 (YYYY-MM-DD) Time 20:31:17 (Greenwich Mean Time) Page 1 of 1

DOW

DP SPECIALTY ELECTRONIC MATERIALS US, INC.

Certificate of Analysis

Product Number 00010406546
Product Name

KORDEK™ 573F Industrial Microbiocide

Delivery No. /000000

Shipping Units 1.000 KG

Shipment No.

Customer Information

Customer Name

Specification Number 000000226833

Batch Number	YY00H3A451
Expiration Date	2019-09-10 (YYYY-MM-DD)
Manufacturing Date	2017-03-10 (YYYY-MM-DD)
Quantity	1.000 KG

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

For inquiries please contact Customer Service or local sales

Date 2019-07-19 (YYYY-MM-DD) Time 08:55:37 (Greenwich Mean Time) Page 1 of 1

IM AND HAAS INTERNATIONAL TRADING SHANGHAI CO., LTD. A Subsidiary of The Dow Chemical Company	DOW CHEMICAL (SHANGHAI) COMPANY LIMITED D BLOCK, 1/F,185 TAI GU RD 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	2020-01-07 (YYYY-MM-DD)
Manufacturing Date	2017-07-07 (YYYY-MM-DD)
Quantity	120.000 KG
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

Date 2017-07-26 (YYYY-MM-DD) Time 08:55:37 (Greenwich Mean Time) Page 1 of 1

:OHM AND HAAS INTERNATIONAL TRADING
SHANGHAI CO., LTD.
A Subsidiary of The Dow Chemical Company

DOW CHEMICAL (SHANGHAI)
COMPANY LIMITED
D BLOCK, 1/F,185 TAI GU RD
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
.et 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
® ™ Trademark of The Dow Chemical Company ("Dow") or an affiliated company of Dow

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.

TROY

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.

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: [redacted] Date: 05/17/2017
Management: [redacted] Date: 05/17/2017
QA: [redacted] Date: 05/17/2017

Revised June 20, 2014

EXACT COPY OF RAW DATA
SIGNATURE: [redacted]
DATE: 05-20-2019

TSOP027 ATTACHMENT 1

LONZA GLP SERVICES
1200 BLUEGRASS LAKES PARKWAY
ALPHARETTA, GA 30004

Certificate of Analysis

Test or Reference Substance Name: Vanquish 100 EPA Reg. No. 1258-1249
CAS No.: 4299-07-4 **Lot Number:** 6445
Manufacturing Date: 12/8/2015
Test Date: 05/15/2019 **Expiration Date** 05/15/2021
Storage: Room temperature

Compound	Assay	Analytical Technique
2-butyl-1,2-benzisothiazolin-3-one (BBIT)	98.4	HPLC

Comments:
N/A

Master Log Number/Notebook Number and page(s): SN 439-19B10BBIT/609 pages 3,4 and 5

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

Study Director: [redacted] Date: 05/20/2019
Management: [redacted] Date: 05/20/2019
QA: [redacted] Date: 5/20/2019
Revised August 06, 2018

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.

US Agent:
THOR SPECIALTIES, INC.
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Appendix C:
LLNA Data

Chemical	CAS#	individual EC3s [%]	individual EC3s [%] EXT	Max Dose Tested	Passes Extrap Crit 1	Passes Extrap Crit 2	Passes Extrap Crit 3	Keep or Reject	Experimental schedule ac	Strain (female 8-12 weeks old unless otherwise specified)	purity %	n° animals/ group	1 Conc. (%)	1 SI
1,2-Benzisothiazolin-3-one (BIT)	2634-33-5	32.4		50				Keep	According to 429	CBA/Ca (6-8 weeks)	100	4	3	1.56
1,2-Benzisothiazolin-3-one (BIT)	2634-33-5	4.8		50				Keep	According to 429	CBA/Ca (6-8 weeks)	100	4	3	2.72
1,2-Benzisothiazolin-3-one (BIT)	2634-33-5	2.3	ext	50	Y	Y	Y	Keep	According to 429	CBA	NA	NA	10	3.8
1,2-Benzisothiazolin-3-one (BIT)	2634-33-5	29		30				Keep	According to 429	CBA/Ca	89.8	NA	3	1.5
1,2-Benzisothiazolin-3-one (BIT)	2634-33-5	1.8		9.9				Keep	According to 429	BALB/cAnNCrl	19.77	NA	0.40	1.1
1,2-Benzisothiazolin-3-one (BIT)	2634-33-5	2.2		10.0				Keep	According to 429	BALB/cAnNCrl	19.9	NA	0.40	2.3
1,2-Benzisothiazolin-3-one (BIT)	2634-33-5	1.5		10.0				Keep	Non-guideline; dosed 4 days	CBA/J	19.2	4 or 5	0.50	2.78
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.021	ext	0.7	N	Y	Y	Reject	According to 429	CBA/J	14	NA	0.028	6.30
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.002564	ext	0.1	Y	Y	Neg slope	Reject	According to 429	CBA/J	14	5	0.003	3.40
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.006467		0.1				Keep	Not noted	CBA/J	14	NA	0.003	1.50
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT) (KathonTM)	55965-84-9	0.012	ext	0.7	N	N	Y	Reject	According to 429	CBA/J	7.02	NA	0.18	10.43
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT) (KathonTM)	55965-84-9	0.003	ext	0.1	N	Y	Y	Reject	4 days treatment, labelling and excision from 18 to 24 hours after fourth treatment	CBA/J (6-9 weeks old)	NA	5	0.005	8.1
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT) (KathonTM)	55965-84-9	0.0082		0.075				Keep	According to 429	CBA/Ca (6-12 weeks)	1.5	4	0.00375	1.30
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT) (KathonTM)	55965-84-9	0.063		0.075				Keep	According to 429	CBA/Ca (6-12 weeks)	1.5	4	0.00375	0.8
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.0076		0.075				Keep	According to 429	CBA/Ca	1.5	4	0.0015	1.2
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.0049		0.0375				Keep	According to 429	CBA/Ca	1.5	4	0.00075	0.9
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.0075		0.075				Keep	According to 429	CBA/Ca	1.5	4	0.0015	1.5
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.0075		0.075				Keep	According to 429	CBA/Ca	1.5	4	0.0015	1.0
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.0068		0.075				Keep	According to 429	CBA/Ca	1.5	4	0.0015	0.9
5-Chloro-2-methyl-4-isothiazolin-3-one/2-Methyl-4-isothiazolin-3-one (CMIT/MIT)	55965-84-9	0.048		0.075				Keep	According to 429	CBA/Ca	1.5	4	0.0015	2.0
4,5-Dichloro-2-octyl-3(2h)-isothiazolone (DCOIT)	64359-81-5	0.0113		1				Keep	According to 429	CBA/Ca/Ola/Hsd	99.3	NA	0.01	2.6
4,5-Dichloro-2-octyl-3(2h)-isothiazolone (DCOIT)	64359-81-5	0.004092		0.13088				Keep	According to 429	CBA/J	99.3	5	0.0013	0.87
4,5-Dichloro-2-octyl-3(2h)-isothiazolone (DCOIT) (RH-287T)	64359-81-5		ext	1	N			Reject	Non-guideline	CBA/J or CBA/Ca	99.3	NA	0.10	32.14
Methylisothiazolinone (act.19.7%)	2682-20-4	1.9		5				Reject (error)	According to 429	CBA	NA	NA	0.25	1.5
Methylisothiazolinone (act.19.7%)	2682-20-4	2.2		9.85				Keep	According to 429	CBA/Ca (6-12 weeks)	19.7	4	0.99	1.9
Methylisothiazolinone (act.19.7%)	2682-20-4	0.4		0.985				Keep	According to 429	CBA/Ca (6-12 weeks)	19.7	4	0.049	1.5
Methylisothiazolinone	2682-20-4	0.863		1.8				Keep	According to 429	CBA/J	10.37	5	0.15	2.08
Methylisothiazolinone (Ultra Pure MIT)	2682-20-4	NC		4.5				Keep	According to 429	CBA/J	NA		0.75	0.7
2-n-Octyl-4-isothiazolin-3-one (OIT)	26530-20-1	0.6624		1.125				Keep	According to 429	CBA/J	99.76		0.01006	0.75
2-n-Octyl-4-isothiazolin-3-one (OIT)	26530-20-1	0.2		1				Keep	Non-guideline; dosed 4 days	CBA/J	99.32	5	0.03	2.4
2-n-Octyl-4-isothiazolin-3-one (OIT)	26530-20-1	0.25		1				Keep	Non-guideline; dosed 4 days	CBA/J	99.32	6	0.01	1.1
2-n-Octyl-4-isothiazolin-3-one (OIT) (RH-893)	26530-20-1	0.3305		1				Keep	Non-guideline	CBA/J or CBA/Ca	99.76		0.1	1.43

Bold text Selected by Dow as primary study

From OECD project or other source

2 Conc. (%)	2 SI	3 Conc. (%)	3 SI	4 Conc. (%)	4 SI	5 Conc. (%)	5 SI	6 Conc. (%)	6 SI	7 Conc. (%)	7 SI	8 Conc. (%)	8 SI
10	1.22	30	2.79	50	4.53								
10	3.84	30	4.45	50	4.97								
30	4.4	50	4.9										
10	1.5	30	3.1										
2.0	3.2	9.9	7.2										
2.0	2.8	10.0	12.6										
1.0	2.64	2.5	3.64	5	2.72	10	3.35						
0.14	26.6	0.7	68.7										
0.005	4.7	0.007	4.2	0.009	6.7	0.036	20.5	0.1	45.5				
0.005	1.9	0.007	3.4	0.009	3.3	0.036	6.7	0.1	7.7				
0.4	12.37	0.7	18.44										
0.05	27.8	0.1	48.2										
0.0075	2.60	0.015	7.00	0.0375	10.90	0.075	14.0						
0.0075	0.8	0.015	0.8	0.0375	1.5	0.075	3.7						
0.0075	2.9	0.015	9.3	0.0375	17.7	0.075	23.5						
0.0015	1.2	0.0075	4.4	0.015	9.1	0.0375	8.5						
0.0075	3	0.015	4.7	0.0375	10.3	0.075	28						
0.0075	3	0.015	9.5	0.0375	6.4	0.075	10.3						
0.0075	3.3	0.015	8.4	0.0375	14.0	0.075	17.6						
0.0075	0.8	0.015	2.1	0.0375	2.3	0.075	4.7						
0.1	30.2	1.0	42										
0.0038	2.67	0.013	13.33	0.0368	13.99	0.13088	36.26						
1.0	41.47												
0.5	1.5	1	1.8	2.5	3.8	5	2.5						
1.97	2.6	4.93	7.0	9.85	7.6								
0.099	1.5	0.197	1.8	0.493	3.8	0.985	2.5						
0.45	2.4	0.76	2.23	1.35	6.64	1.57	4.73	1.8	6.62				
1	1.4	1.25	1.3	1.5	0.9	1.5	0.7	3	0.6	4.5	0.8		
0.03204	0.95	0.10366	1.06	0.30621	2.23	1.125	4						
0.1	2.8	0.3	3.2	1	5.7								
0.03	1.5	0.1	2.1	0.3	3.3	1	3.8						
1	7.56	ND	ND										

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Keep or Reject	Keep

	Row Labels	Min of individual EC3s [%]	Max of individual EC3s [%]2	Average of individual EC3s [%]3	Count of individual EC3s [%]
BIT	**2634-33-5**	**1.5400**	**32.4**	**10.570**	**7**
OIT	**26530-20-1**	**0.2000**	**0.6624**	**0.361**	**4**
MIT	**2682-20-4**	**0.4000**	**2.2**	**1.154**	**4**
CMIT/MIT	**55965-84-9**	**0.0049**	**0.063**	**0.018**	**9**
DCOIT	**64359-81-5**	**0.0041**	**0.0113**	**0.008**	**2**

No LLNA data for BBIT (1,2-Benzisothiazolin-3-one, 2-butyl), 4299-07-4

Potency Ranks	NICEATM Ave LLNA EC3	Dow LLNA EC3	BRT KeratinoSens EC1.5	BRT h-CLAT MIT	IIVS h-CLAT MIT
BIT	5	5	3	4	3
OIT	3	3	2	2	2
MIT	4	4	5	5	4
CMIT/MIT	2	1	4	3	5 (Neg)
DCOIT	1	2	1	1	1

Appendix D:
In Vivo, *In Vitro*, *In Silico* and Defined Approach Results

Column Key for IT_invitro_insilico

In vitro data	Data from DPRA, hCLAT, and KeratinoSens in vitro assays
In silico/physchem data	In silico predictions from OECD Toolbox, and physchem properties + source (experimental or predicted)
In vivo data	LLNA data, based on Dow submission and NICEATM literature review
DA outputs	Outputs from four defined approaches, including hazard/potency classifications and EC3 predictions

SMILES from EPA Chemistry Dashboard (https://comptox.epa.gov)
Physicochemical properties from OPERAv2.3.
database)
Predicted values have "OPERA" for source
Water solubility (S) in mol/L, VP in mmHg, MP and BP in degrees C, and MW in g/mol.
logP values with source MRID numbers were provided by email on May 7, 2019, from Andrew Byro, PhD, EPA OPP, Antimicrobials Division; EPA Comptox dashboard only contained predicted values for MIT, BIT, DCOIT, and BBIT; EPA Comptox experimental logP for OIT was 2.45 vs 3.4 from Dr. Byro).
Physicochemical properties are not available for CMIT/MIT; but are provided for the individual chemicals

Int = interference observed
NA = Not available (physicochemical properties for CMIT/MIT) or no depletion (DPRA) or no induction (h-CLAT)
NT = Not tested
OECD = QSAR Toolbox v4.3 automated workflow or Profiler for DASS (performed manually).
Prediction for CMIT/MIT = sensitizer because both individual chemicals had sensitizer predictions

PC Codes were provided by Dr. Byro, EPA

Num or Code	PC Code	Chemical	CASRN	SMILES	DPRA.Call	DPRA.percCysdep	DPRA.percLysdep
DCOIT	128101	4,5-Dichloro-2-octyl-3(2h)-isothiazolone	64359-81-5	CCCCCCCCN1SC(Cl)=C(Cl)C1=O	1	100	10.4
OIT	099901	2-n-Octyl-4-isothiazolin-3-one	26530-20-1	CCCCCCCCN1SC=CC1=O	1	100	0
BBIT	098951	1,2-Benzisothiazolin-3-one, 2-butyl	4299-07-4	CCCCN1SC2=C(C=CC=C2)C1=O	1	100	0
BIT	098901	1,2-Benzisothiazolin-3-one	2634-33-5	O=C1NSC2=C1C=CC=C2	1	100	Int
MIT	107103	2-Methyl-4-isothiazolin-3-one	2682-20-4	CN1SC=CC1=O	1	100	0
CMIT/MIT	107104/107103	Mixture (5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-Methyl-4-isothiazolin-3-one	55965-84-9	-	1	100	10.6
CMIT	107104	5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	CN1SC(Cl)=CC1=O	NT	NT	NT

DPRA.mean	DPRA.potency	logDPRA	IIVS hCLAT.Call	IIVS hCLAT.CD86.EC150	IIVS hCLAT.CD54.EC200	IIVS h.CLAT.CV75	IIVS hCLAT.MIT	IIVS logMinhCLAT	BRT hCLAT.Call	BRT hCLAT.CD86.EC150	BRT hCLAT.CD54.EC200	BRT h.CLAT.CV75	BRT hCLAT.MIT	BRT logMinhCLAT
55.2	High	1.71940	1	NA	0.83	1.1	0.83	-0.081	1	NA	0.92	0.9	0.92	-0.036
50	High	1.69897	1	NA	1.80	7.85	1.80	0.255	1	7.26	0.95	8.80	0.95	-0.022
50	High	1.69897	1	3.81	2.10	4.73	2.10	0.322	1	3.15	3.01	3.30	3.01	0.479
NA	High	NA	1	NA	5.10	15.2	5.10	0.708	1	7.84	7.63	13.1	7.63	0.883
50	High	1.69897	1	18.22	8.18	18.80	8.18	0.913	1	11.8	11.6	24.6	11.6	1.064
55.3	High	1.74273	0	NA	NA	4.51	NA	NA	1	2.81	2.63	3.04	2.63	0.420
NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

KS.Call	KS.EC1.5	KS.EC3	KS.IC50	KS.Imax	logKS.Imax	OECD	LogP	LogP.Source	LogS	LogS.Source	BP	BP.Source
1	1.32	2.82	4.65	4.37	0.64048	1	4.4 (2.8 -6.4)	43458503, 47802208, 40808501	-4.123	OPERA	287	OPERA
1	2.19	6.61	12.66	3.70	1.30833	1	3.4 (2.4 - 4.4)	00148769, 43637502, 41687801	-2.630	PhysProp	255	OPERA
1	3.84	15.96	52.98	19.61	1.29248	1	2.86	44364902	-4.002	OPERA	310	OPERA
1	3.14	16.93	57.80	17.64	2.87017	1	1.35	43584001, 41927501	-2.828	OPERA	312	OPERA
1	9.54	28.32	108.25	15.84	1.19976	1	-0.486	50396603, 41741401	-0.435	OPERA	154	OPERA
1	3.41	8.26	19.87	5.61	0.74896	1	NA	NA	NA	NA	NA	NA
NT	NT	NT	NT	NT	NT	1	0.401	43825703, 50396603, 41741401	-0.468	OPERA	193	OPERA

MP	MP.Source	LogVP	LogVP.Source	LogBCF	LogBCF.Source	MW
42.1	OPERA	-3.983	OPERA	1.942	OPERA	281.04
16.6	OPERA	-4.434	PhysProp	1.148	OPERA	213.12
87.7	OPERA	-5.382	OPERA	0.784	OPERA	207.07
108.8	OPERA	-4.845	OPERA	0.651	OPERA	151.01
131.3	OPERA	0.349	OPERA	0.309	OPERA	115.01
NA	NA	NA	NA	NA	NA	NA
137.1	OPERA	-0.836	OPERA	0.926	OPERA	148.97

Num or Code	PC Code	Chemical	CASRN	SMILES	Dow.LLNA.EC3	NICEATM.Avg.LLNA.EC3	log.Dow.LLNA.EC3	log.Avg.LLNA.EC3
DCOIT	128101	4,5-Dichloro-2	64359-81-5	CCCCCCCCN1SC(Cl)=C(Cl)C1=O	0.004092	0.008	-2.38806437	-2.09691001
OIT	099901	2-n-Octyl-4-is	26530-20-1	CCCCCCCCN1SC=CC1=O	0.225	0.361	-0.64781748	-0.4424928
BBIT	098951	1,2-Benzisoth	4299-07-4	CCCCN1SC2=C(C=CC=C2)C1=O	NA	NA	NA	NA
BIT	098901	1,2-Benzisoth	2634-33-5	O=C1NSC2=C1C=CC=C2	1.5	10.57	0.17609126	1.02407499
MIT	107103	2-Methyl-4-is	2682-20-4	CN1SC=CC1=O	0.863	1.154	-0.0639892	0.06220581
CMIT/MIT	107104/107103	Mixture (5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-Methyl-4-isothiazolin-3-one	55965-84-9	-	0.002564	0.018	-2.59108198	-1.74472749

Num or Code	PC Code	Chemical	CASRN	SMILES	DA.Kao.STS.Call	DA.Kao.STS.Pot	DA.ITSv2.Pot	DA.ITSv2.Call	ANN_D_hC.Avg.pred.LLNA.EC3	ANN_D_hC_KS.Avg.pred.LLNA.EC3
DCOIT	128101	4,5-Dichloro-2-octyl-3(2h)-isothiazolone	64359-81-5	CCCCCCCCN1SC(Cl)=C(Cl)C1=O	1	1A	1A	1	0.0576	0.0196
OIT	099901	2-n-Octyl-4-isothiazolin-3-one	26530-20-1	CCCCCCCCN1SC=CC1=O	1	1A	1A	1	0.0581	0.0129
BBIT	098951	1,2-Benzisothiazolin-3-one, 2-butyl	4299-07-4	CCCCN1SC2=C(C=CC=C2)C1=O	1	1A	1A	1	0.1435	0.0528
BIT	098901	1,2-Benzisothiazolin-3-one	2634-33-5	O=C1NSC2=C1C=CC=C2	1	1A	1A	1	0.9856	0.3181
MIT	107103	2-Methyl-4-isothiazolin-3-one	2682-20-4	CN1SC=CC1=O	1	1B	1B	1	1.8399	0.8037
CMIT/MIT	107104/ 107103	Mixture (5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-Methyl-4-isothiazolin-3-one	55965-84-9	-	1	1B	1B	1	2.7642	1.8103

Appendix E:
Calculation of Weighted LLNA EC3 for CMIT/MIT

Appendix E

Calculation of Weighted LLNA EC3 for CMIT/MIT

Composition of CMIT/MIT mixture = 10.8% CMIT + 3.4% MIT = 14.2% active ingredient

Calculation of a weighted average CMIT/MIT EC3 uses the CMIT EC3 and the MIT EC3 and assumes that the sensitization effects of these ingredients are additive and that no other ingredients in the mixture impact the sensitization potential. The EC3 of each component is multiplied by its fraction (%) in the mixture, the two products are added, and then the sum is divided by the fraction of active ingredients (14.2%) in the CMIT/MIT mixture.

The NICEATM LLNA database (https://ntp.niehs.nih.gov/whatwestudy/niceatm/test-method-evaluations/immunotoxicity/llna/index.html) has two EC3 values for CMIT: 0.009% (vehicle = dimethyl formamide) and 0.01% (vehicle = acetone:olive oil). The NICEATM approach averages these for EC3 = 0.0095% for CMIT. The Dow approach would use EC3 = 0.01% for CMIT because the vehicle for this test was acetone:olive oil.

Calculation of the weighted LLNA EC3 for CMIT/MIT using the NICEATM approach and comparison to NICEATM in vivo data:

- Weighted EC3 = [(0.0095 * 10.8) + (1.154 * 3.4)]/14.2 **= 0.28%** EC3 for CMIT/MIT
- Measured LLNA EC3 for CMIT/MIT = **0.018%**

Calculation of the weighted LLNA EC3 for CMIT/MIT using the Dow approach, and comparison to Dow in vivo data:

- Weighted EC3 = [(0.01 * 10.8) + (0.863 * 3.4)]/14.2 = **0.21%** EC3 for CMIT/MIT
- Measured LLNA EC3 = **0.002%**

Chemical	Dow LLNA EC3 (%)	NICEATM LLNA EC3 (%)	Weighted EC3 (%)	DA: ANN D_hC[b] EC3 (%)[a]	DA: ANN D_hC_KS[c] EC3 (%)[a]
CMIT		0.0095			
CMIT/MIT	0.002	0.018 (0.0011-0.034)	0.21 (Dow) 0.28 (NICEATM)	0.121 (0.119 – 0.123)	0.492 (0.4 – 0.605)
MIT	0.863	1.154 (0-3.476)		1.775 (1.732 – 1.818)	0.826 (0.759 – 0.9)

[a] Numbers in parentheses are the 95% confidence intervals

[b] Model 1 from Hirota et al. 2015: DPRA + h-CLAT

[c] Model 4 from Hirota et al. 2015: DPRA + h-CLAT + KeratinoSens

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