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Toxicology Directorate

Tiered *In Vitro* Toxicity Testing of the Novel Energetic N-propylnitroguanidine (PrNQ), March 2021

Prepared by Drs. Emily N. Reinke and Valerie H. Adams, Health Effects Division

Specialty: 500C, Toxicity Test

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14. ABSTRACT
The energetic and toxicological properties of N-propylnitroguanidine (PrNQ) are under assessment as a replacement for energetics in current use, such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). This study evaluated the toxicity of PrNQ in several in vitro test systems, including the Ames mutagenicity assay, the Microtox® Acute Toxicity Test System, and skin sensitization via the human cell line activation test (h-CLAT) and the direct peptide reactivity assay (DPRA). Data from this study are used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for further development of new munition compounds.

15. SUBJECT TERMS
N-propylnitroguanidine; PrNQ; aquatic toxicity; Ames; mutagenicity; skin sensitization; human cell line activation test; h-CLAT; direct peptide reactivity assay; DPRA

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Good Laboratory Practice Compliance Statement

The studies described in this report were conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. The test article characterization (purity) was conducted by the manufacturer and it is not known whether the testing was done in compliance with the above regulation.

2. Due to time constraints, the method of analysis for these compounds could not be validated by the Laboratory Sciences Directorate (LAB) prior to the study start in compliance with study protocol and modification requirements. At the time this report was finalized, LAB had not achieved the capability to provide concentration verification for PrNQ.

No deviations from the aforementioned regulation affected the quality or integrity of the study or the interpretation of the results.

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Acronyms and Abbreviations for TS WP18-1299 PrNQ

AERTA	Army Environmental Research and Technology Assessment
APHC	U.S. Army Public Health Center
APHC-MDV-CSD	APHC Method Development Section Client Services Division
AR	Department of the Army Regulation
ASTM	American Society for Testing and Materials
ATP	adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
AFNOR	Association Française de Normalisation
BP	boiling point
BSA	Bovine serum albumin
CAOTE	Cell-based acute oral toxicity estimate
CO_2	Carbon dioxide
CV	Cell Viability
CV75	cell viability of 75%
CDC	Center for Disease Control and Prevention
CASRN	Chemical Abstracts Service Registry Number
CD54	Cluster of Differentiation 54
CD86	Cluster of Differentiation 86
CFR	Code of Federal Regulations
CI	Confidence Interval
^oC	degrees Celsius

DA	Department of the Army
DIN	Deutsches Institut für Normung
DMSO	dimethyl sulfoxide
DNCB	2,4-dinitrochlorobenzene
DPRA	Direct peptide reactivity assay
DOD	Department of Defense
DODI	Department of Defense Instruction
ECOSAR	Ecological Structure Activity Relationship
EC	Effect concentration
EC_{50}	median (50%) effect concentration
EC150	Effective Concentration resulting in 150% increase
EC200	Effective Concentration resulting in 200% increase
EPA	U.S. Environmental Protection Agency
ESOH	Environmental safety and occupational health
ECVAM	European Centre for the Validation of Alternative Method
ECVAM DB-ALM	ECVAM Database on Alternative Methods to Animal Experimentation
FACS	Fluorescence-activated cell sorting
FDA	U.S. Food and Drug Administration
FITC	Fluorescein isothiocyanate
FL1	FITC emission wavelength 530 ± 15 nm
FL2	PI emission wavelength >650 nm

GHS	Global Harmonization System
Cat	Category
GLP	Good Laboratory Practice
g	Grams
g/m^3-h	Grams/cubic meter per hour
K_H	Henry's law constant
h-CLAT	Human cell line activation test
His	Histidine
his-	histidine or tryptophan negative
HPLC	high-performance liquid chromatography
HPLC-UV	high-performance liquid chromatography with ultraviolet detection
IC_{50}	median (50%) inhibitory concentration
IgG1	Immunoglobulin G1
ISO	International Organization for Standardization
U	International units
kg	kilogram
LA	lactic acid
L	liter
LD_{50}	median (50%) lethal (oral) dose
log K_{OC}	Log Organic carbon partition coefficient
log K_{OW}	Log Octanol-water partition coefficient

LOAEL	lowest-observed adverse effect level
MFI	mean fluorescence intensity
MPF™	microplate format
MP	melting point
µg	micrograms
µL	microliter
µM	micromolar
mg	milligram
mL	milliliter
mmHg	millimeter of mercury
mM	millimolar
Min	minutes
MW	molecular weight
MRL	minimum risk level
nm	nanometers
$NiSO_4$	nickel sulfate
NOAEL	no-observed adverse effect level
NOEL	no-observed effect level
NVN	Nederlandse voornorm
OD_{600}	optical density at wavelength of 600 nm
OECD	Organization for Economic Co-operation and Development

PBS	Phosphate Buffered Solution
PPE	Personal Protective Equipment
PI	propidium iodide
PrNQ	N-propylnitroguanidine
QSAR	Quantitative Structure-Activity Relationship
r^2	cofficient of determination
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
RDT&E	research, development, testing, and evaluation
RfD	reference dose
RFI	relative fluorescence
±S9	with or without S9 liver extract
SD	standard deviation
SERDP	DOD Strategic Environmental Research and Development Program
SOP	Standard Operating Procedures
TBD	to be determined
TOPKAT	Toxicity Prediction by Komputer Assisted Technology
TOX	Toxicology Directorate
TFA	trifluoroacetic acid
TNT	trinitrotoluene
Trp	tryptophan
Trp-	tryptophan negative

UNECE	United Nations Economic Commission for Europe
USEPA	U.S. Environmental Protection Agency
USFDA	U.S. Food and Drug Administration
VP	vapor pressure
w/v	weight per volume

Toxicology Study No. S.00058221.3-21
Tiered *In Vitro* Toxicity Testing of the Novel Energetic N-Propylnitroguanidine (PrNQ)

1. SUMMARY

1.1 Overview

The energetic and toxicological properties of N-propylnitroguanidine (PrNQ) are under assessment as a replacement for energetics in current use, such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). This study evaluated the toxicity of PrNQ in several *in vitro* test systems, including the Ames mutagenicity assay, the Microtox® Acute Toxicity Test System, and skin sensitization via the human cell line activation test (h-CLAT) and the direct peptide reactivity assay (DPRA). Data from the h-CLAT were also used to predict a Hazard Category (United Nations Economic Commission for Europe (UNECE), 2015) for acute oral toxicity. Data from this study are used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for further development of new munition compounds.

1.2 Purpose

The purpose of this study is to provide toxicology data to support environmental and occupational health assessment on PrNQ as a new or replacement energetic compound for military use. This information is critical to the research, development, testing, and evaluation (RDT&E) of munition formulation alternatives. This study addresses, in part, the environmental safety and occupational health (ESOH) requirements outlined in Department of the Army (DA) Regulation (AR) 200-1 (DA, 2007b), AR 40-5 (DA, 2020), and AR 70-1 (DA, 2018), Department of Defense (DOD) Instruction (DODI) 4715.4 (DOD, 2018), and Army Environmental Research and Technology Assessment Requirement PP-3-02-05 (AERTA, 2018), Compliant Ordnance Lifecycle for Warfighter Readiness. This program is under the direction of the DOD Strategic Environmental Research and Development Program (SERDP).

Research, development, testing, training, and use of substances potentially less hazardous to human health and the environment is vital to the readiness of the U.S. Military. Safeguarding the health of Soldiers, civilians, and the environment requires an assessment of alternatives before they are fielded. Continuous phased assessments begun early in the RDT&E process can save significant time and effort during RDT&E, as well as over the life cycle of the items developed. Residues of pyrotechnics, propellants, explosives, and incendiaries have been found in soil, air, surface, and groundwater samples, creating environmental problems and interfering with training activities.

DOD is identifying replacements for substances causing environmental and/or occupational health hazards. The purpose of this toxicology study was to evaluate PrNQ mutagenicity, acute aquatic toxicity, and skin sensitization hazard using *in vitro* methods and following Good Laboratory Practice (GLP) regulations.

1.3 Conclusions

This study reports the *in vitro* toxicity evaluation for the new energetic compound PrNQ in the Ames assay for mutagenicity, the Microtox Acute Aquatic Toxicity assay, and the h-CLAT skin sensitization assay. PrNQ was not mutagenic in the Ames assay, nontoxic with an EC_{50} above the limit of the test for acute aquatic toxicity and is not considered a skin sensitizer following negative results in the h-CLAT and DPRA. Using in silico methods, the predicted acute oral toxicity was categorized using the Globally Harmonized System (GHS) as GHS Category (Cat) 4 (UNECE, 2015). As such, PrNQ is predicted to be a low-negligible hazard for mammalian and aquatic life and is unlikely to be mutagenic. Although all equivalent toxicity endpoints have not been collected for TNT, RDX and PrNQ, key toxicity values for these three compounds are provided in Table 1.

Table 1. Comparative Toxicity for N-propylnitroguanidine (PrNQ), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and 2,4,6-trinitrotoluene (TNT).

Compound	Genotoxicity	Skin sensitization	Acute mammalian toxicity	Acute aquatic toxicity
PrNQ	negative	negative	300–2,000 mg/kg; GHS 4	GHS no category; practically nontoxic[1]
RDX	negative	indeterminant	68–500 mg/kg; GHS 3	GHS 2; moderate toxicity[1]
TNT	positive	negative	660–1,010 mg/kg; GHS 4	GHS 2; moderate toxicity[1]
Hazard Color key	Highest	Moderate-high	Low-moderate	Negligible –low

Legend:
mg/kg = milligrams per kilogram
GHS = Globally Harmonized System
Notes:
[1] US EPA hazard categories

1.4 Recommendations

PrNQ was not toxic under the conditions of the tests reported here. The compound is highly soluble, which may be a potential concern for aquatic systems. Although the estimated aquatic toxicity is low, additional aquatic toxicity testing (fish, algae, and invertebrate) should be conducted. Acute and 14-day rat studies are recommended to reduce uncertainty and better characterize toxicity.

2. REFERENCES

See Appendix A for list of references.

3. AUTHORITY

This project was funded under Military Interdepartmental Purchase Request No. W74RDV5291790. This toxicology report addresses, in part, the ESOH requirements outlined in DODI 4715.4, *Pollution Prevention* (DOD, 2018), AR 200-1, *Environmental Protection and Enhancement* (DA, 2007b); AR 40-5, *Army Public Health Program* (DA, 2020); and AR 70-1, *Army Acquisition Policy* (DA, 2018). It was conducted as part of an on-going effort by SERDP.

4. BACKGROUND

Current regulations require the assessment of human health and environmental effects arising from exposure to substances in soil, surface water, and ground water. Applied after an item has been fielded, these assessments can reveal the existence of adverse environmental and human health effects that must be addressed, often at substantial cost. It is more efficient to begin the assessment of exposure, effects, and environmental transport of military-related compounds/substances early in the RDT&E process to avoid unnecessary costs, conserve physical resources, and sustain the health of those potentially exposed. A goal of this program is to investigate new compounds for operational and/or environment, safety, and occupational health issues. The candidates under development for high density energetics include PrNQ.

National defense requires the development of unique energetic compounds to perform specialized mission requirements. These requirements include the sustainable use of these materials in the environment, particularly during training operations. The use of RDX and TNT in warheads is a concern due to their ability to contaminate groundwater and thus enter into the drinking water supply. Unexploded ordnance and low-order detonations have become sources of ground water contamination and have affected drinking water resources.

The Centers for Disease Control and Prevention (CDC), Agency for Toxic Substances and Disease Registry (ATSDR) has developed an acute oral minimum risk level (MRL) for RDX of 60 micrograms per kilograms per day (µg/kg-day) based on its epileptiform seizure neurotoxicity in humans and rodents (Burdette et al., 1988; Kasuske et al., 2009; Stone et al., 1969; Williams et al., 2011). The U.S. Environmental Protection Agency (EPA) has derived a chronic reference dose (RfD) of 3 µg/kg-day based prostatic inflammation in rodents. RDX is also classified as a possible carcinogen (U.S. Army Medical Research and Development Command (USAMRDC), 1984; Parker et al., 2006).

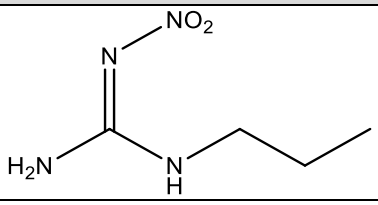
TNT is acutely toxic to rats causing ataxia, tremors, and mild convulsions; oral LD_{50} values range from 660 to 1320 mg/kg. The subchronic and chronic oral reference dose (RfD) is 0.5 µg/kg-day based on a LOAEL of 0.5 mg/kg-day for liver effects in dogs. TNT is classified in weight-of-evidence Group C, possible human carcinogen (Lima et al., 2011; Risk Assessment Information System (RAIS), 2012).

The SERDP is dedicated to finding replacements for RDX and TNT that will reduce or eliminate ESOH risks and decrease potential impacts on readiness and the costs associated with training (U.S. Army Center for Health Promotion and Preventative Medicine (USACHPPM), 2007). The energetic and toxicological properties of PrNQ are being evaluated as potential replacements for TNT and RDX. Toxicity tests can be conducted *in vivo* and *in vitro*. *In vitro* methods have the advantage of being relatively inexpensive, high-throughput, and capable of addressing many mechanistic issues at the cellular and molecular level. *In vitro* tests are ideally suitable and effective toxicity screening tools, especially when limited quantities of a compound are available. By identifying ESOH effects early in the acquisition process, unacceptable, or "regrettable," replacement compounds can be identified.

The Toxicology Directorate (TOX) of the U.S. Army Public Health Center (APHC) has been tasked with generating *in vitro* toxicity data for PrNQ to determine its potential negative human and environmental effects. The data from these studies informs recommendations for the continued development and additional toxicity testing of PrNQ that supports the appropriate hazard classification and exposure guidance. Prior to conducting *in vitro* testing, *in silico* methods are used to estimate physical and chemical properties (EpiSuite™, (EPA, 2013)) and toxicity (Ecological Structure Activity Relationship (ECOSAR) and Toxicity Prediction by Komputer Assisted Technology (TOPKAT) (EPA, 2012; BIOVIA, 2015)). The ECOSAR and TOPKAT are quantitative structure-activity relationship (QSAR) programs and provide rapid predictions for aquatic toxicity and human health. The full QSAR report was included with each PRNQ *in vitro* protocol modification; Table 2 provides the relevant data.

The Ames test for mutagenicity (Ames et al., 1975) is a method widely accepted for evaluation of mutagenic potential by the EPA (EPA, 2012), the U.S. Food and Drug Administration (FDA) (FDA, 2007), and the Organization for Economic Co-operation and Development (OECD) (OECD, 1997). Historically, the mutagenicity of test materials has been evaluated in the agar plate-based Ames assay (Ames et al., 1975). The bacteria used in this test, *Salmonella typhimurium* and *Escherichia coli*, have point mutations in the histidine (*His*) and tryptophan (*Trp*) operons, rendering the bacteria incapable of producing the amino acids histidine or tryptophan, respectively. A chemical's mutagenic potential is assessed by exposing histidine or tryptophan negative (*his*- or *trp*-) organisms to varying concentrations of a test chemical and selecting for reversion events. Reversion occurs primarily through two molecular mechanisms: single base substitutions or frameshift mutations within the *His* or *Trp* operons. Xenometrix has developed a proprietary MPF™ Ames test that provides a convenient, high-throughput capability for mutagenicity testing (Xenometrix, 2012). The test uses the same strains of bacteria required by the EPA (*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and a composite of *E. coli* pKM101/*uvrA* strains), and reduces the assay to a simple, non-agar, 384-well plate methodology that is expedient and cost effective.

Table 2. PrNQ Physical Chemical Properties, Structure and QSAR Toxicity Estimates

EPISuite							
Mass g/mol	**MP (ºC)**	**BP (ºC)**	**Aqueous solubility (mg/L; 25ºC)**	**log K_{OW}**	**log K_{OC}**	**Henry's Law Constant (atm-m^3/mol) @ 25ºC**	**VP (mmHg; 25°C)**
146.15	100.00	251.91	7.81E+04	0.13	0.97	1.74E-11	1.48E-11
TOPKAT							
Oral LD_{50}	**Chronic Oral LOAEL**	**Inhalation LC_{50}**	**Dermal**	**Ocular**	**Devel/ Repro**	**Ames**	**Carcinogen**
235.3 mg/kg	0.704 mg/kg-day	5.5 g/m^3-h	Negative irritant, Possible sensitizer	Possible irritant	Negative	Indeterminant	Indeterminant
ECOSAR				**Molecular structure**			
Model Class	**Green Algae EC_{50} (mg/L)**	**Daphnia LC_{50} (mg/L)**	**Fish LC_{50} (mg/L)**				
aliphatic amine	97.5	71.4	772.5				

The Microtox test system uses a strain of naturally occurring bioluminescent bacteria, *Aliivibrio fischeri* (formerly *Vibrio fischeri* and still referred to as *V. fischeri* by the supplier of the reagents, Modern Water, also known as *Photobacterium phosphoreum*, NRRL number B-11177). The marine bacterial bioluminescence is tied directly to cellular respiration, which is fundamental to cellular metabolism and associated life processes. These nonpathogenic, marine, bioluminescent bacteria are sensitive to a broad range of toxicants resulting in a decreased rate of respiration and a corresponding decrease in the rate of luminescence. Reduction of the microorganism's light emission is proportional to the toxicity expressed as EC_{50} (the midpoint of the effective concentration). This test has been shown to be an effective screening tool in assessing toxicity of varied chemical compounds comparing with other bioassays. The bacterial bioluminescence aquatic toxicity test has been validated by the industrial, academic, and governmental testing communities and achieved official "Standards Status" in several countries including an ASTM Standard (D-5660; withdrawn), ISO 11348-3 and Standard Method 8050 in the U.S., AFNOR T90-320 in France, NVN 6516 (withdrawn) in the Netherlands, and DIN 38412 in Germany.

There are multiple skin sensitization assays that can be utilized to determine skin sensitization hazard in a tiered testing strategy that forms a defined approach. The USEPA has recently accepted two defined approaches for submission and registration to predict hazard. In one, the h-CLAT is the first in a tiered strategy, where a positive result allows for a hazard determination to be made and no further testing is required (USEPA, 2018). During a skin sensitizing reaction, activated dendritic cells migrate to the lymph node where the major histocompatibility complexes which they are presenting activate T-cells and T-cell proliferation. Secondary exposure to the chemical will then result in inflammation and an allergic reaction. Using adverse

outcome pathway analysis, four key events for skin sensitization were identified (OECD, 2012). *In vitro* assays for each step have been developed and validated. The h-CLAT is an *in vitro* assay for key event 3 that measures the test chemical mediated dendritic cell activation via increased expression of CD54 and CD86 on the cell surface (OECD, 2012). The presence of CD54 and CD86 proteins on the cell surface is detected with flow cytometry using fluorescently labelled antibodies specific for CD54 and CD86 (Ashikaga et al., 2010; European Center for the Validation of Alternative Methods, Database on Alternative Methods to Animal Experimentation (ECVAM DB-ALM), 2014; OECD, 2018). The threshold criteria for a positive reaction in h-CLAT requires either a 2-fold induction of CD54 or a 1.5-fold or induction of CD86 compared to solvent controls. The h-CLAT can also be utilized to predict acute oral toxicity from the cytotoxicity data produced in the course of conducting the assay.

In the absence of a positive result in the h-CLAT, a second assay should be conducted, either in a tiered approach or as a best-of-three approach (Kleinstreuer et al., 2018; USEPA, 2018). The direct peptide reactivity assay (DPRA) fulfills either of these approaches. The DPRA assesses the first key event in the adverse outcome pathway, formation of haptens through covalent binding of chemicals to proteins, which induces an immune response, leading to downstream effects such as the activation of dendritic cells as described in the previous paragraph. The DPRA is an *in chemico* assay the measures the decay of cysteine or lysine containing polypeptide sequences via high-performance liquid chromatography with ultraviolet detection (HPLC-UV). The threshold for a positive reaction requires a decrease in amino acid signal of approximately 6.5% as compared to control samples.

This report describes the toxic effect of PrNQ in the Ames mutagenicity assay, the Microtox assay, and the skin sensitization defined approach. Table 3 identifies the critical events and dates of these studies.

Table 3. Critical Events

Critical Event	Date of Event (Ames)	Date of Event (Microtox)	Date of Event (h-CLAT)	Date of Event (DPRA)
Type-Protocol Modification Approved	20 June 2019	6 September 2018	25 June 2019	25 June 2019
Study Start Date	20 June 2019	20 August 2018	25 June 2019	25 June 2019
Experimental Start Date	20 June 2019	20 August 2018	25 June 2019	16 August 2020
Experimental Completion Date	8 August 2020	21 August 2018	5 July 2019	September 2020
Study Completion Date	TBD	October 2019	TBD	TBD

Legend:
H-CLAT = human cell line activation test
DPRA = direct peptide reactivity assay
TBD = to be determined

5. MATERIALS

5.1 Test Substance

Synthesis of PrNQ (CASRN 35091-64-6) was performed at Army Research Laboratory, Aberdeen Proving Ground, Maryland. Purity analysis for PrNQ was not available.

PrNQ was readily soluble at 500 mg/mL in dimethyl sulfoxide (DMSO), aqueous solubility was determined for the Ames assay and used for setting the high dose in the Microtox and h-CLAT tests (APHC, 2019b; 2017b). Concurrent with each test, the most dilute serial dilution was frozen at -80 ºC for concentration verification by the APHC Method Development Section Client Services Division (APHC-MDV-CSD).

5.2 Test System (Ames)

Xenometrix MPF™ kits were obtained from ANIARA (Mason, Ohio). The kit contained quality assurance certificates for the *Salmonella* and *E.coli* strains. Appendix D provides a list of the kit reagents and supplementary reagents with expiration dates. All reagents were stored in refrigerators and freezers in accordance with directions in the kit as described in the Toxicology Standing Operating Procedure (TOX SOP) 068 (APHC, 2017b). BacTiter-Glo kits were obtained from Promega Corporation (Madison, Wisconsin), and contain a reagent that becomes luminescent when activated by incubation with adenosine triphosphate (ATP) in lysed bacteria.

5.3 Test System (Microtox™)

The Microtox Acute Toxicity Test reagent and associated media and solutions were obtained from Modern Water, Inc. (New Castle, Delaware). The reagent is a freeze-dried preparation of a specially selected strain of the marine bacterium *A. fischeri*. Appendix D provides a list of media,

solutions, and other necessary test materials with expiration dates and lot numbers. All reagents were stored according to manufacturer instructions as described in the TOX SOP 037 and study protocol (APHC, 2017a; 2017c). The positive control (zinc sulfate) was purchased from Sigma-Aldrich (St. Louis, Missouri). Each vial of lyophilized *A. fischeri* was tested against the standard following reconstitution. Only vials with a calculated EC_{50} of 2–10 mg/L at 15 min were qualified for further use.

5.4 Skin Sensitization Assays

5.4.1 Test System (h-CLAT)

THP-1 cells were acquired from the American Type Tissue Collection (Manassas, Virginia). Appendix D provides a list of media, solutions and other necessary test materials with expiration dates and lot numbers. All tissue culture reagents were acquired from Gibco, a subsidiary of ThermoFisher (Waltham, Massachusetts). Cells were cultured in Gibco Roswell Park Memorial Institute (RPMI) 1640 containing 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.05 mM 2-mercaptoethanol. All cells, reagents, and chemicals were stored according to manufacturer's instructions (APHC, 2019a). The positive controls for the reactivity check were 2,4-dinitrochlorobenzene (DNCB) and nickel sulfate ($NiSO_4$). DNCB was used as the positive control for the full test. Lactic acid (LA) was the negative control for the reactivity check. All chemicals were obtained from Sigma Aldrich.

5.4.2 Test System (DPRA)

Polypeptide sequences were synthesized and obtained from RS Synthesis (Lexington, Kentucky). Sequences were as followed: Ac-RFAAKAA-COOH (776.2 gram mole (g/mol)) and AC-RFAACAA-COOH (751.9 g/mol). Appendix D provides a list of chemicals, buffers, and other necessary test materials with expiration dates and lots numbers. All chemicals and buffers were stored according to manufacturer instructions and were obtained from Sigma-Aldrich. (APHC, 2019a). Cinnamaldehyde (100 mM stock) was the positive control for the assay. All chemicals were obtained from Sigma Aldrich.

5.5 Quality Assurance

APHC policy requires that all experiments and studies conducted by any element of TOX will be compliant with the applicable GLP Standard guideline (APHC, 2016). For this study, the test article dictates that the following GLP guideline applies:

Code of Federal Regulations (CFR), Title 40, *Protection of Environment,* Part 792, *Good Laboratory Practice Standards* (CFR, 1989).

According to this policy and that these results may be used in regulatory decisions involving the USEPA, these assays were conducted in compliance with GLP standards and followed the appropriate regulatory testing guidelines.

In compliance with the GLP requirements, the APHC Quality Systems Office audited critical phases of this study. Appendix B provides a Quality Assurance Statements, which provides the

dates of these audits along with the audited phases and the dates that the results of the audits were reported to Management and the Study Director. Appendix C provides the additional Quality Assurance/GLP requirement of archives location as well as the names of personnel contributing to the performance of this study.

6. METHODS

6.1 Ames

The experimental design and general procedures of this study were conducted under the APHC TOX SOP 068 for the Xenometrix MPF™ Ames Test Kit (APHC, 2017b). The test kit is designed to determine the mutagenicity of a test material in compliance with the USEPA (USEPA, 2012) and OECD guidelines for the Bacterial Reverse Mutation Test (OECD, 1997), APHC TOX Type Protocol: *"The Ames Test For Mutagenicity # 2758-70-iv19-04-01*" (APHC, 2019b), and modifications. The modifications to the protocol are approved and signed by the Study Director. The electronic and hard copy versions of the protocol modifications are saved and archived with the protocol and the raw data.

6.1.1 Sample Preparation

Solutions of 50 mg/mL PrNQ in DMSO were first serially diluted in DMSO [six (6) half-log, (1:3.12), serial dilutions] and then further diluted 25X in Ames Exposure Medium without apparent precipitation. These final diluted solutions represent the exposure concentrations with final concentrations ranging from 6.8 to 2000 µg/mL (nominal).

6.1.2 Validation of Cell Growth

The frozen bacterial stocks were thawed according to TOX SOP 068 (APHC, 2017b) and expanded in Xenometrix growth medium with shaking at approximately 37 °C overnight with or without ampicillin, as appropriate for each strain. The following morning, evidence of sufficient bacterial growth was assessed measuring the absorbance at 600 nanometers (nm) (OD_{600}; BioMate™ 3S spectrophotometer, Fisher Scientific) $OD_{600} \geq 2.0$ indicated sufficient density for the Xenometrix Ames Assay, although lower OD_{600} values may also be acceptable. Per personal conversation with the manufacturer, sub-threshold growth were corrected by proportionately increasing the volume of each bacterial suspension to the final incubation solution to provide an appropriate density of bacteria.

6.1.3 Compound Incubation

The bacterial suspensions were diluted with Xenometrix incubation media and aliquoted to wells containing serial dilutions of 25X concentrates of the test material. The suspensions were incubated with shaking for 90 min at approximately 37 °C with and without the inclusion of S9 liver extract (±S9); inclusion of S9 determines if a mutagen is generated from the parent compound due to metabolism by liver enzymes. At the end of the incubation, each well was diluted 11-fold with purple Indicator Medium and 50 microliter (µL) aliquots were distributed appropriately into 384-well plates. Plates were incubated for 2 days at approximately 37 °C and then scored for presence of revertant colonies (i.e., evidence of a positive mutagenic event).

Positive wells were indicated by a color change (purple to yellow) or a visible bacterial colony. The indicator media turns from purple to yellow due to a pH change resulting from active metabolism of revertant bacteria.

6.1.4 Criteria for a Positive Mutagenic Event

Positive controls appropriate for each strain were included alongside the test material to assure the assay was valid for each test; positive controls are tested in triplicate for each strain with and without the S9 microsomal fraction. The assay as a whole is considered valid if both the number of control background reversions and the number of positive control reversions are within prescribed limits (APHC, 2019b; 2017b).

There are several criteria for determining a positive result: the number of reversions induced by the test compound is at least 2-fold above the background control, there is a concentration-related increase over the range tested, and/or there is a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response. Results from test substances that do not meet the above criteria are considered nonmutagenic (Xenometrix, 2012).

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgment about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated. Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions and/or frame shifts in the genome of *Salmonella.* Negative results indicate that under the test conditions the test substance is not mutagenic in the tested species.

6.1.5 Determination of Cytotoxicity

Coincident with the test incubation, a plate is prepared for determination of cytotoxicity of the test material using ATP luminescence. After a 90-min incubation at approximately 37 °C, samples from the cytotoxicity plate are aliquoted to a 96-well plate. An equal volume of luminescent reagent is added to each well according to the method described for the BacTiter-Glo Microbial Cell Viability Assay and bioluminescence is measured via plate reader.

6.1.6 Data Analysis

Raw scores for color change (purple to yellow) were entered into an excel spreadsheet containing statistical algorithms to calculate mean, S.D. baseline and fold change. These data were then graphed using GraphPad Prism® version 5.04 (GraphPad Software, San Diego California).

6.2 Microtox

6.2.1 Experimental Design

The experimental design and general procedures of this study were conducted under the APHC TOX SOP 037 for the Microtox Acute Toxicity Assay (APHC, 2017a). The test kit is designed to determine the aquatic toxicity of a test material in compliance with the APHC TOX Type Protocol: *"Microtox Toxicity Testing System*" (APHC, 2017c), and modifications. The modifications to the protocol are approved and signed by the Study Director. The electronic and hard copy versions of the protocol modifications are saved and archived with the protocol and the raw data.

6.2.2 Range Finding

PrNQ was dissolved in DMSO at the assay test limit (200 mg/mL). The solubility of PrNQ determined previously in the Ames test (APHC, 2017b). PrNQ was serially diluted 1:2 in DMSO and further diluted 1:100 in diluent. A total of eight concentrations were tested for the range finding. Reconstituted *A. fischeri* was added to each test concentration (10 µL) and samples were incubated and tested for luminescence at 5, 15, and 30 min using the Microtox Model 500 Analyzer (Modern Water, Inc. (London, United Kingdom)). The EC_{50} from the range finding determined the final test concentration range (See Appendix F for final chemical specific ranges).

6.2.3 Cytotoxicity Test

In instances where the range-finding test does not produce an EC_{50}, the cytotoxicity test is used to verify the range-finding data using the methodology described in paragraph 6.2.2.

6.2.4 Data Analysis

Raw luminescence data were recorded at 5, 15, and 30 min by the Microtox analyzer. As no EC_{50} was found, no further analysis was necessary. All data (prints and files) were archived.

6.2.5 Derivation of Ecotoxicity Hazard Values

Data from Microtox tests were aligned with the aquatic toxicity criteria of the USEPA, the OECD and the GHS to categorize the potential ecotoxicity of PrNQ (Table 4) ((EPA, 2017; OECD, 2001; UNECE, 2015).

Table 4. Ecotoxicity Assessment Scale

LC_{50} or EC_{50} Concentration Range (mg/L)	Hazard Categories (USEPA, 2017)	Hazard Classes (OECD, 2001)	Acute Aquatic Toxicity (UNECE, 2015)
< 0.01	Super Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. 1
0.01 to 0.1	Extremely Toxic		
0.1 to 1	Highly Toxic		
1 to 10	Moderately Toxic	Acute Toxicity II (toxic to aquatic life)	Acute Cat. 2
10 to 100	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. 3
100 to 1,000	Practically Nontoxic	—	—
> 1,000	Relatively Harmless	—	—

Legend:
LC_{50} = median (50%) lethal concentration
EC_{50} = median (50%) effect concentration
mg/L = milligrams per liter
USEPA = U.S. Environmental Protection Agency
OECD = Organization for Economic Co-operation and Development
UNECE = United Nations Economic Commission for Europe

6.3 Skin Sensitization Assays

6.3.1 h-CLAT

The assay was conducted in compliance with the APHC TOX Type Protocol: *In Vitro* Skin Sensitization Parts 1-3 (APHC, 2019a). In the absence of an SOP, testing was performed according to ECVAM DB-ALM protocol number 158 and OECD Guideline 442E (ECVAM DB-ALM, 2014; OECD, 2018).

6.3.1.1 Buffers

FACS buffer was prepared with PBS and 0.1% (w/v) BSA the day before use and stored at +4 ± 2 °C. Blocking solution was made up in 1% (w/v) globulins in PBS stocks as needed, with stock being used within 1 week and stored at
+4 ± 2 °C. Blocking solution for use on the day of the experiment was diluted to a 0.1% solution in FACS buffer immediately prior to use. Propidium iodide (PI) was diluted to 12.5 µg/mL in PBS on the day of the experiment and maintained on ice.

6.3.1.2 Tissue Culture

Tissue culture media was prepared as described in paragraph 5.4 and maintained at +4 ± 2 °C. Media was pre-warmed at room temperature prior to use for each cell plating and passage. Cells were maintained at 1.5 x 10^5 – 8 x 10^5 cells/mL 37 °C, 5% CO_2. Cells were passaged every 2–3 days for no more than 30 passages or 60 days. Prior to passage or test plating, cell density was determined by counting with the TC-20 automated cell counter (Bio-Rad, Inc., Hercules, California). Cell viability was determined by Trypan blue staining (Bio-Rad, Inc.). For all testing (reactivity check, range finding and h-CLAT), cells were plated into 24-well plates at a density of 1 x 10^6 cells/well in 0.5 mL (i.e., 2 x 10^6 cells/mL). For maintenance, cells were plated at 1.5-2.0 x 10^5 cells/mL in 25–40 mL media depending on the timing of subsequent tests.

6.3.1.3 Reactivity Check

The reactivity check prior to full testing is used to confirm cell viability and induction of CD54 and CD86. Two weeks post thaw, a reactivity check of cells sampled from each propagation flask was performed using the control compounds DNCB, $NiSO_4$ and LA. DNCB was prepared as a 20 mg/mL stock solution in DMSO and stored at +4 ± 2 °C in the dark. $NiSO_4$ was prepared as a 10 mg/mL stock solution in saline and stored at room temperature protected from light. LA was freshly prepared as a 100 mg/mL solution in saline. From these stock solutions additional dilutions were made so that the tested concentrations were 3.3–4 µg/mL DNCB, 100 µg/mL $NiSO_4$, and 1,000 µg/mL for LA. A 100% cytotoxic DNCB concentration (0.2 mg/mL) was added to one well as a positive control. Negative controls (diluted DMSO and saline) were also included. After all dosing solutions were distributed to the test wells, the test plate was incubated (approximately 37°C / 5% CO_2) for 24 hours. Cells were then collected, processed, stained with PI and antibodies [anti-IgG1 (isotype control), anti-CD54 and anti-CD86], and analyzed by flow cytometry (see paragraphs 6.3.6 and 6.3.7). Criteria for a successful reactivity check requires the positive controls DNCB and $NiSO_4$ induce CD54 and CD86 (Relative Fluorescence Intensity (RFI) criteria exceed: CD54 ≥ 200 and CD86 ≥ 150) and the negative control, LA, does not induce CD54 or CD86 or reduce viability by more than 50% [target ~75% viability (CV75)]. When the cell sample meets these criteria, the remainder of cells from its propagation flask are used for testing chemicals. Propagation flasks can be resampled if the first sample fails the reactivity check to confirm no or poor reactivity. A second fail is cause to discard that flask and thaw a new lot of cells.

6.3.1.4 Range Finding

The range finding test is used to bracket the appropriate dose range for the full test using only the percent viability endpoint. PrNQ (500 mg/mL in DMSO) was prepared as the stock for eight serial dilutions (1:2, diluent = DMSO). Each dilution was subsequently diluted 1:250 into tissue culture media (=final serial dilution). Pooled THP-1 cells were plated at 1 x 10^6 cells/well (24-well plate). An equal volume (0.5 mL) of each final serial dilution was added to the appropriate test wells. Negative (vehicle) control and cytotoxicity (‘dead cell’) positive controls were also included on each test plate. Cells were incubated for 24 hours (approximately 37°C /5% CO_2). After the 24 hour incubation, cells were collected and processed for staining with PI. Briefly, cells were transferred to 5 mL tubes, centrifuged (200 x g; +4 °C), and supernatants were discarded. Each pellet was resuspended in 0.6 mL cold FACS buffer and 0.2 mL of each

sample was transferred to new tubes and washed by centrifuging (200 x g; +4 °C), decanting the supernatant, and resuspending the pellet in 0.2 mL FACS buffer. The wash step was repeated one time. The final pellets were resuspended in 0.4 mL FACS buffer and stained with 20 µL 12.5 µg PI/mL solution. Samples were maintained on ice in the dark and analyzed by flow cytometry (see paragraph 6.3.7). Percent viability (ratio of live cells to total acquired cells) was utilized to determine the CV75. Where CV75 was not achieved due to compound toxicity, additional range finding tests with lower compound concentrations were conducted until the CV75 was identified. If cytotoxicity was not observed at the maximum concentration, then, by default, the maximum dose is used as the highest dose in the h-CLAT.

6.3.1.5 h-CLAT Test

In the full test, the CV75 from the range finding test is used to develop the dose range and represents the 2nd highest dose of an 8-dose treatment. For the PrNQ range finding assay, cytotoxicity was observed; therefore, the experimentally determine CV75 (0.009 mg/mL PrNQ) was used to calculate the top dose (0.011 mg/mL final). PrNQ was solubilized in DMSO at 5.5 mg/mL (500x). Eight 1:1.2 serial dilutions of PrNQ were subsequently diluted 1:250 in complete media and added in equal volume to test wells containing 0.5 mL medium and 1 x 10^6 cells per well (24-well plate). Three concentrations of DNCB were prepared from the 20 mg/mL stock solution and added to the appropriate wells containing 1 x 10^6 cells (final concentrations 0.003, 0.004 and 0.0048 mg/mL DNCB in medium). A DMSO vehicle control was prepared as was a "dead cell" control containing 10 µL of the 20 mg/mL DMSO stock. Cells were incubated for 24 hours and processed for IgG1, CD54, and CD86 staining and analysis by flow cytometry, see paragraphs 6.3.6 and 6.3.7.

6.3.1.6 Antibody Staining

Cells from each test well were transferred to individual 5 mL tubes and collected by centrifugation (250 x g/5 min/+4 °C). The supernatants were discarded and the pellets were resuspended in 1 mL cold FACS buffer and washed 2x. Cell were then incubated with blocking solution (0.6 mL 0.1% blocking buffer) for 15 min at +4 ± 2 °C. Following blocking, samples were prepared in triplicate (i.e., split into 3 aliquots) of 180–200 µL each in a round-bottom 96-well plate, centrifuged (250 x g/5 min/+4 °C) and blocking buffer decanted. Samples were resuspended in 50 µL FACS buffer containing either IgG1, CD54, or CD86 antibodies as per the ECVAM protocol and gently vortexed, incubated at +4 ± 2 °C in the dark for 30 min and washed twice in FACS buffer (ECVAM DB-ALM, 2014). Samples were transferred to FACS analysis tubes between washes. Following the final wash, all samples were resuspended in 0.4 mL FACS buffer, stained with 20 µL PI and mixed by vortexing. All samples were maintained on ice or at +4 °C throughout the staining process.

6.3.1.7 Flow Cytometry

The fluorescence intensities of the labeled cells were analyzed by flow cytometry using a BD FACSVerse™ flow cytometer and captured/analyzed with BD FACSuite™ v1.0.5. The acquisition channels were FL1 (fluorescein isothiocyanate (FITC) emission wavelength 530 ± 15 nanometers (nm)) and FL2 (PI emission wavelength >650 nm). PI stained untreated cells were used to determine the correct voltages for the forward scatter and side scatter channels.

The dead cell and media controls were used to gate live (PI negative) versus dead (PI positive) cells. For each sample, 10,000 live or 30,000 total counts (whichever count was acquired first) in the PI channel were acquired, and the geometric mean fluorescence intensity (MFI) for FITC was calculated. The cell viability for each test concentration was determined from the isotype (IgG1) stained subpopulations, which were co-stained with PI as per paragraph 6.3.6.

6.3.1.8 Data Analysis

If the RFI for any concentration exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150), the EC200 and EC150 were calculated using the validated calculation spreadsheet. If the EC200 or EC150 fell below the lowest dose, the values were extrapolated according to the ECVAM protocol (ECVAM DB-ALM, 2014). Two independent experiments were completed for PrNQ; the data from these two experiments were sufficient to determine sensitization and a third experiment was not necessary.

6.3.1.9 Criteria for a Valid Assay

The following criteria establish a valid assay:

- Cell viability of medium and DMSO controls was more than 90%.
- RFI values for the DNCB control for both CD54 and CD86 exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150).
- RFI values for the DMSO solvent control did not exceed positive criteria.
- The MFI ratio of both CD54 and CD86 to isotype control for DMSO and media controls exceeded 105%.
- The cell viability of at least four doses was greater than 50%.

6.3.1.10 Cell-based Acute Oral Toxicity Estimate (CAOTE)

The IC_{50} is equivalent to the EC50 (level at which cytotoxicity was induced by 50%) and was extrapolated from the cell viability of the range-finding experiments. From this IC_{50}, the following prediction algorithm was used to predict the acute rodent toxicity:

$$\log LD_{50}\ (mg/kg) = 0.372 \log IC_{50}\ (\mu g/mL) + 2.024$$

This algorithm is based upon a rat-only weight regression as demonstrated in the validation project for the Neutral Red Uptake assay, an alternative cytotoxicity assay. This model was applied to the THP-1 cells of the uptake in order to determine a hazard category and not to provide an LD_{50} point estimate of rodent acute oral toxicity. The calculated LD_{50} was assigned a GHS category for acute oral toxicity (UNECE, 2015; ICCVAM, 2006).

6.3.2 DPRA

The assay was conducted in compliance with the APHC TOX Type Protocol: *In Vitro* Skin Sensitization Parts 1–3 (APHC, 2019b). In the absence of an SOP, testing was performed according to ECVAM DB-ALM protocol number 154 and OECD Guideline 442C (ECVAM DB-ALM, 2012; OECD, 2015).

6.3.2.1 Buffers

A 100 mM, pH 7.5 sodium phosphate buffer was prepared using both sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate 1 day prior to use and stored at +4 °C. Ammonium acetate (100 mM, pH 10.2) buffer was also prepared 1 day prior to use and stored for up to 2 weeks at +4 °C. All buffers were prepared with NanoPure water (ThermoScientific, Waltham, Massachusetts).

6.3.2.2 Sample Preparation

100 mM solutions of PrNQ and cinnamaldehyde (0.5 mL for lysine or 1.25 mL for cysteine experiments) were prepared in acetonitrile. Peptides were prepared in a stock solution of 0.667 mM in sodium phosphate (cysteine) or ammonium acetate (lysine) buffers. Controls consisted of peptide and buffer only and co-elution (test chemical only). Samples were prepared in triplicate according to Table 5.

Table 5. ECVAM DB-ALM, *Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing DB-ALM Protocol No. 154* (2012): Prescribed Sample Preparation.

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM test chemical	1:50 Ratio, Lysine Peptide 0.5 mM Peptide, 25 mM test chemical
• 750 µL Cysteine peptide solution (or pH 7.5 phosphate buffer for Co-elution controls) • 200 µL Acetonitrile • 50 µL Test chemical solution (or solvent for reference controls)	• 750 µL Cysteine peptide solution (or pH 10.2 ammonium acetate buffer for Co-elution controls) • 250 µL Test chemical solution (or solvent for reference controls

In accordance with the protocol, the standard curve was prepared using a 20% acetonitrile buffer solution and the samples were prepared in a 25% acetonitrile buffer solution. The dilution buffer is prepared in advance and the peptide is diluted to 0.534 mM (10:8 dilution) and a serial 1:2 dilution was performed for a total of six standards plus a buffer-only sample.

Standard curve and reference controls were incubated in the dark at room temperature for approximately 21 hours prior to the start of sample analysis, this allowed for test chemical and positive control sample analysis to begin approximately 24 hours after incubation started. Each peptide was analyzed two times.

6.3.2.3 Sample Analysis

The sample analyses were performed by HPLC UV-Vis detection. Chromatographic separation was achieved using a reverse phase C-18 HPLC column under gradient conditions. The aqueous mobile phase consists of 0.1% trifluoroacetic acid (TFA) in deionized water; the organic mobile phase consists of 0.085% TFA in acetonitrile. Column flow was maintained at 0.35 mL/min; the gradient was linear from 10% to 25% acetonitrile over 10 min, followed by a

step to 90% acetonitrile. Injection volume was
10 µL. Cysteine and Lysine calibration standards, ranging in concentration from 0.000 to 0.534 mM, were prepared at seven concentrations by serial dilution of a 0.667 mM stock standard, in phosphate or ammonium acetate buffer, respectively. Quantitation was performed at 220 nm.

6.3.2.4 Data Analysis

The concentration of the peptide was determined from the absorbance at 220 nm by determining the area under the curve and matching it to a standard curve. Depletion of the peptide was determined by dividing the peak area of the test chemical by the mean peak area of the reference controls. The percent depletion was calculated by subtracting from 1 and multiplying by 100.

6.3.2.5 Criteria for a Valid Assay

The following criteria establish a valid assay:

- Standard curve $r^2 > 0.990$.
- Mean peptide concentration in Reference Control A = 0.50 ± 0.05 mM.
- Percent depletion in the positive control must fall into the following boundaries:
  - Cysteine: 60.8–100%.
  - Lysine: 40.2–69.4%.
- Maximum standard deviations for positive control and test chemical replicates cannot exceed the following:
  - Cysteine: 14.9%.
  - Lysine: 11.6%.
- The CV of the peptide peak areas for Reference Controls B and C must be < 15.0%.

6.4 Concentration Verification of PrNQ

At the end of each test day for each assay, samples of the final serial dilution were collected and stored at -80 °C for analysis by the APHC-MDV-CSD. At the time of this report, verified test concentrations were not available, so the nominal value has been used for reporting.

7. RESULTS AND DISCUSSION

7.1 Ames Assay

Appendix E provides the raw data and calculations for the Ames data.

7.1.1 Cytotoxicity of PrNQ

In conjunction with the mutagenicity tests, the cytotoxicity of PrNQ was evaluated. Cytotoxicity is indicated when bacterial viability is reduced by greater than 20% compared to controls. PrNQ was not cytotoxic to TA98, TA100, and *E.coli*. Cytotoxicity was observed in TA1535 +S9 and in TA1537 both –S9 and +S9. Some strain and absolute viability differences were observed in the

presence of S9 indicating that metabolites of PrNQ may be slightly more toxic than PrNQ. The data are presented in Figure 1, left panel.

7.1.2 Mutagenicity of PrNQ

PrNQ was not mutagenic in any of the tested strains, either with or without S9 activation (Figure 1, right panel). The data for the PrNQ/*E.coli* +S9 test were confounded due to the positive control chemical not provoking revertants. The assay data were reviewed for a possible cause. Since the TA1537 test was conducted alongside the *E.coli* test and the TA1537 positive control chemical (2-aminoantracene) did generate revertants, it was determined that the assay as a whole worked and only the *E.coli* positive control chemical was inactive and the lack of PrNQ revertants in the *E.coli* +S9 test demonstrated PrNQ was not mutagenic to *E.coli*.

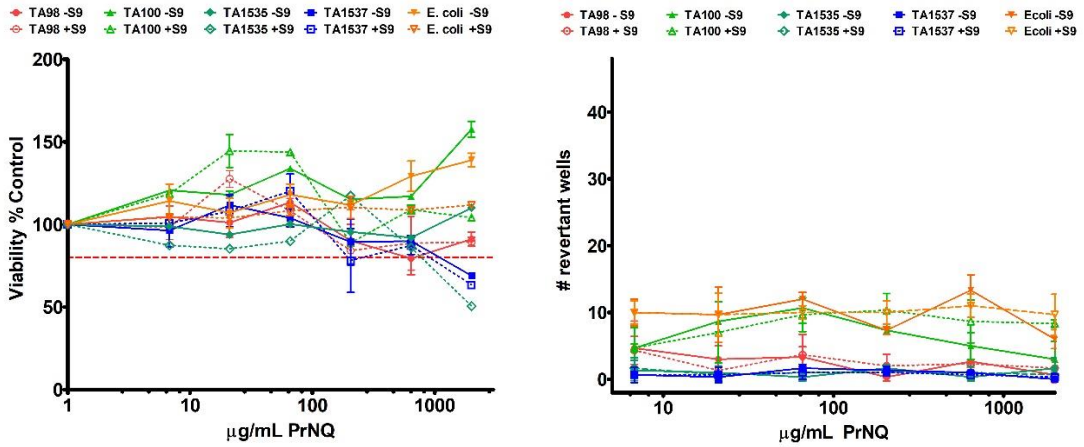


Figure 1. The Cytotoxicity and Number of Revertants of PrNQ in the Ames Assay

Left panel. The viability of the individual bacterial strains with and without S9 activation after exposure to PrNQ. For most of the test conditions, PrNQ was not cytotoxic and the viability threshold of >80% (dashed red line) was maintained. TA1537 was most sensitive to PrNQ, with cytotoxicity observed at and above 205 µg/mL PrNQ and cytotoxicity > 20% observed at the highest PrNQ concentration in both S9- and S9+ conditions. PrNQ was also cytotoxic to TA 1535 S9+ at 2,000 µg/mL.

Right panel. PrNQ was not mutagenic and did not increase the number of revertants for any of the strains in either the S9- or S9+ conditions.

7.2 Microtox Toxicity and Risk Assessment

Toxicity of PrNQ to marine bacteria, *A. fischeri*, was measured by the Microtox acute toxicity test system at 5, 15, and 30 min. For each test compound, three individual experiments were performed in duplicate. Table 6 presents the toxicity data (EC_{50} and the 95% Confidence Interval) and risk assessment. Appendix F: Figures – Microtox presents the analyzed data. This evaluation suggests that PrNQ is nontoxic to fish.

Table 6. Microtox Toxicity and Risk Assessment

Compound	Microtox EC_{50} (mg/L) [95% CI]			Hazard Categories (USEPA, 2017)	Hazard Classes (OECD, 2001)	Acute Aquatic Toxicity (GHS, 2005)
	5 min	15 min[a]	30 min			
PrNQ	>2,000	>2,000	>2,000	Relatively harmless	---	---

Legend:
EC_{50} = median (50%) effect concentration
mg/L = milligrams per liter
CI = Confidence Interval
USEPA = U.S. Environmental Protection Agency
OECD = Organization for Economic Co-operation and Development
GHS = Global Harmonization System
PrNQ = N-Propylnitroguanidine
Note:
[a] The value of EC_{50} at 15 min is used for the risk assessment.

7.3 Skin Sensitization Assays

7.3.1 h-CLAT

7.3.1.1 Reactivity Check

The THP-1 cells were checked and verified for reactivity to DNCB, $NiSO_4$, and lack of reactivity to LA. Cells reacted as expected with DNCB and $NiSO_4$ eliciting positive reactions for both CD54 and CD86 while LA was negative. See Appendix G for data.

7.3.1.2 Range-finding Assay

Two independent dose finding assays were completed in order to determine the CV75 of PrNQ in THP-1 cells. Cytotoxicity was not observed in the dosing range, therefore a CV75 could not be experimentally determined. Therefore, the top dose for the full assay was set at the test maximum of 500 mg/mL. Appendix G contains the taw data for the Range Finding.

7.3.1.3 h-CLAT

Two independent h-CLAT assessments were completed for PrNQ. PrNQ was negative for both CD86 and CD54 expression, indicating a negative test, and is not likely to be a sensitizer, a confirmatory assessment in the DPRA is required according to the Defined Approach. Appendix G contains the raw data. Data are reported for the nominal concentrations of the compounds due to the fact that as of the writing of this report, concentration verification has not yet been completed by APHC-MDV-CSD.

7.3.1.4 Acute Oral Hazard Designation

Mammalian acute oral toxicity was predicted using data collected in the h-CLAT. Insufficient toxicity was observed in the experiments to calculate a meaningful LD_{50}. Thus, PRNQ was categorized in GHS Cat 5- (LD_{50} range 2,000 – 5,000 mg/kg) and is estimated to have low oral toxicity (UNECE, 2015).

7.3.2 DPRA

Two independent experiments were completed for PrNQ for both cysteine and lysine. Compared to reference controls, neither the cysteine nor the lysine peptides were depleted by more than 1%. This leads to a conclusion that PrNQ is not sensitizing based on the prediction model. Appendix H contains the raw data.

8. CONCLUSIONS

In this study, PrNQ was evaluated for mutagenicity, acute aquatic toxicity, and skin sensitization using QSAR and *in vitro* approaches. PrNQ mutagenicity was inadequately modeled by TOPKAT and was negative in the in the bacterial mutagenicity test up to 2 mg/mL– the limit dose for the assay. The ECOSAR predicted low aquatic toxicity (96-hour EC_{50} in green algae of 97.5 mg/L, a 48-hour LC_{50} in *Daphnia* of 71.38 mg/L, and a 96-hour LC_{50} in fish of 772.5 mg/L); these estimates were supported by the Microtox aquatic toxicity assay with an LC_{50} > 2,000 mg/L. Although TOPKAT modeled PrNQ as a skin sensitizer, it was negative in the *in vitro* skin sensitization assays. Mammalian toxicity was predicted as moderate (GHS Cat 3) by TOPKAT (235.3 mg/kg) and >2,000 mg/kg in the CAOTE. Using these data, PrNQ is conservatively assigned GHS Cat 4 for acute mammalian toxicity.

9. RECOMMENDATIONS

PrNQ had negligible toxicity in the *in vitro* assays conducted as part of the APHC tiered assessment. As the data reported here is considered preliminary, workers should continue to use universal chemical hygiene precautions and wear PPE. The fate and transport characteristics for PrNQ indicate it could enter ground water. Additional testing appropriate to the stage of PrNQ development must be completed to fully address ESOH risk. See Table 1 for the preliminary toxicological comparison of PrNQ to RDX and TNT.

10. POINTS OF CONTACT

Drs. Emily N. Reinke and Valerie H. Adams were the Study Directors and are the points of contact for this project. They may be reached at DSN 584-3980 or commercial 410-436-3980.

Toxicology Study No. S.00058221.3-21, March 2021

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25 March 2021

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30 March 2021

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30 March 2021

Mark S. Johnson, Ph.D., D.A.B.T.
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Date

APPENDIX A

REFERENCES

Ames, B.N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat Res* 31(6):347–63.

Army Environmental Requirements and Technology Assessments (AERTA). 2018. *Requirement PP-3-02-05 Compliant Ordnance Lifecycle for Warfighter Readiness*. U.S. Army Environmental Command. Aberdeen Proving Ground, Maryland.

Ashikaga, T., H. Sakaguchi, S. Sono, N. Kosaka, M. Ishikawa, Y. Nukada, M. Miyazawa, Y. Ito, N. Nishiyama, and H. Itagaki. 2010. A Comparative Evaluation of *in vitro* Skin Sensitisation Tests: The Human Cell-Line Activation Test (h-CLAT) *versus* the Local Lymph Node Assay (LLNA). *Altern Lab Anim,* 38(4):275–84.

BIOVIA. 2015. Toxicity Prediction Komputer Assisted Technology (TOPKAT) Version 6.2 6.2. Dassault Systems, San Diego, California.

Burdette, L.J., L.L. Cook, and R.S. Dyer. 1988. Convulsant properties of cyclotrimethylenetrinitramine (RDX): Spontaneous, audiogenic, and amygdaloid kindled seizure activity. *Toxicol Appl Pharmacol,* 92(3):436–44.

Code of Federal Regulations (CFR). 1989. *Title 40 CFR Part 792, Good Laboratory Practice Standards.* U.S. National Archives and Records Administration, Washington, D.C. http://www.ecfr.gov/cgi-bin/text-idx?c=ecfr&SID=03a4fd2c79bd074423f47bc15f186e00&rgn=div5&view=text&node=40:33.0.1.1.3&idno=40

Department of the Army (DA). 2007b. Regulation 200-1, *Environmental Protection and Enhancement*. https://armypubs.army.mil/

Department of the Army (DA). 2018. Regulation 70-1, *Army Acquisition Policy.* https://armypubs.army.mil/

Department of the Army (DA). 2020. Regulation 40-5, *Army Public Health Program*. https://armypubs.army.mil/

Department of Defense (DOD). 2018. Instruction 4715.23*, Integrated Recycling and Solid Waste Management (Cancels DoDI 4715.4 Pollution Prevention)*. http://www.dtic.mil/whs/directives/corres/pdf/471504p.pdf

European Center for the Validation of Alternative Methods, Database on Alternative Methods to Animal Experimentation (ECVAM DB-ALM). 2012. *DB-ALM Testing Protocol No. 154*: *Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing.* European Union Reference Laboratory for Alternatives to Animal Testing.

European Center for the Validation of Alternative Methods, Database on Alternative Methods to Animal Experimentation (ECVAM DB-ALM). 2014. *DB-ALM Protocol No. 158*: *human Cell Line Activation Test (h-CLAT).* European Union Reference Laboratory for Alternatives to Animal Testing.

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). 2006. NIH Publication No: 07-4518, *In Vitro Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity*. http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_brd.htm

Kasuske, L., J.M. Schofer, and K. Hasegawa. 2009. Two marines with generalized seizure activity. *J Emerg Nurs,* 35(6):542–3.

Kleinstreuer, N.C., S. Hoffmann, N. Alepee, D. Allen, T. Ashikaga, W. Casey, E. Clouet, et al. 2018. Non-animal methods to predict skin sensitization (II): an assessment of defined approaches(*). *Crit Rev Toxicol, 48(5)*:1–16.

Lima, D.R., M.L. Bezerra, E.B. Neves, and F.R. Moreira. 2011. Impact of ammunition and military explosives on human health and the environment. *Rev Environ Health.* 26(2):101–10.

Organization for Economic Co-operation and Development (OECD). 1997. OECD Guideline for Testing of Chemicals No. 471 *Bacterial reverse mutation assay*. www.oecd.org/dataoecd/18/31/1948418.pdf ·

Organization for Economic Co-operation and Development (OECD). 2001. OECD Series on Testing and Assessment No. 33*, Harmonized Integrated Classification System for Human and Environmental Hazards of Chemical Substances and Mixtures*. Paris: OECD Publishing.

Organization for Economic Co-operation and Development (OECD). 2012. OECD Series on Testing and Assessment No. 168. *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins Part 1: Scientific Evidence*. Paris: OECD Publishing.

Organization for Economic Co-operation and Development (OECD). 2015. OECD Guideline for Testing of Chemicals No. 442C: In Chemico Skin Sensitisation. OECD Guidelines for the Testing of Chemicals, Section 4, Paris: OECD Publishing. https://www.oecd-ilibrary.org/environment/test-no-442c-in-chemico-skin-sensitisation_9789264229709-en

Organization for Economic Co-operation and Development (OECD). 2018. OECD Guideline for Testing of Chemicals No. 442E: *In Vitro* Skin Sensitisation: *In Vitro* Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation. OECD Guidelines for the Testing of Chemicals, Section 4, Paris: OECD Publishing.. https://www.oecd-ilibrary.org/content/publication/9789264264359-en.

Parker, G.A., G. Reddy, and M.A. Major. 2006. Reevaluation of a twenty-four-month chronic toxicity/carcinogenicity study of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in the B6C3F1 hybrid mouse. *Int J Toxicol,* 25(5):373–8.

Risk Assessment Information System (RAIS). 2012. Formal Toxicity Summary for 2,4,6-TRINITROTOLUENE. Prepared by: D.M. Opresko, Oak Ridge National Laboratory, Tennessee. http://rais.ornl.gov/tox/profiles/2_4_6_trinitrotoluene_f_V1.html.

Stone, W.J., T.L. Paletta, E.M. Heiman, J.I. Bruce, and J.H. Knepshield. 1969. Toxic effects following ingestion of C-4 plastic explosive. *Arch Intern Med,* 124(6):726–30.

United Nations Economic Commission for Europe (UNECE). 2015. *The Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*. New York and Geneva: United Nations.

U.S. Army Center for Health Promotion and Preventative Medicine (USACHPPM). 2007. Toxicology Report No. MA03N3-CA0700, *Environmental Health Evaluation for CA 07-00 (IM Fill DEMN) Formulation of New, Environmentally Benign Missile Propellants To Eliminate TNT-Based Fills*. Prepared by: M.S. Johnson and H.T. Bausum, Aberdeen Proving Ground, Maryland..

U.S. Army Medical Research and Development Command (USAMRDC). 1984. IITRI Project No. L6121, Study No. 7, Contract No. DAMD17-79-C-9161, *Twenty-Four Month Chronic Toxicity/Carcinogenicity Study of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) in the B6C3F1 Hybrid Mouse*. Prepared by: P.M. Lish, B.S. Levine, E.M. Marianna-Furedi, J.M. Sagartz and V.S. Rac.

U.S. Army Public Health Center (APHC). 2016. Director's Policy Memorandum No. 70-1, Good Laboratory Practice Policy. Aberdeen Proving Ground, Maryland.

U.S. Army Public Health Center (APHC). 2017a. TOX SOP 037*, Operation and Maintenance of the Microtox Analyzer*. Aberdeen Proving Ground, Maryland.

U.S. Army Public Health Center (APHC). 2017b. TOX SOP 068*, Xenometrix AMES Test Kit*. Aberdeen Proving Ground, Maryland.

U.S. Army Public Health Center (APHC). 2017c. Type Protocol Report No. 0FMA-92-iv-17-03-01*, Microtox Toxicity Testing System*. Aberdeen Proving Ground, Maryland.

U.S. Army Public Health Center (APHC). 2019a. Type Protocol Report No. 49-iv19-03-01, *In Vitro Skin Sensitization Parts 1-3*. Aberdeen Proving Ground, Maryland.

U.S. Army Public Health Center (APHC). 2019b. Type Protocol Report No. 70-iv19-04-01, *The Ames Test for Mutagenicity*. Aberdeen Proving Ground, Maryland.

U.S. Environmental Protection Agency (USEPA). Office of Research and Development. 2012. Sustainable Futures/P2 Framework Manual, EPA-748-B12-001*, Section 6 Estimating Aquatic Toxicity Using ECOSAR*.

U.S. Environmental Protection Agency (USEPA). 2013. *Estimation Programs Interface Suite™ EPI Suite for Microsoft® Windows, v 4.11.* U.S. Environmental Protection Agency, Washington, D.C.

U.S. Environmental Protection Agency (USEPA). Office of Prevention, Pesticides, and Toxic Substances. 2017. *Technical Overview of Ecological Risk Assessment, Analysis phase: Ecological effects characterization, ecotoxicity categories for terrestrial and aquatic organisms.* Washington, D.C.

U.S. Environmental Protection Agency (USEPA). Office of Chemical Safety and Pollution Prevention. 2018. *Interim Science Policy: Use of Alternative Approaches for Skin Sensitization as a Replacement for Laboratory Animal Testing*. Washington, D.C.

U.S. Food and Drug Adminstration (FDA). Center for Food Safety and Applied Nutrition. 2007. *Guidance for Insustry and Other Stakeholders: Toxicological Principles for the Safety Assessment of Food Ingredients.* College Park, Maryland.

Williams, L.R., V. Aroniadou-Anderjaska, F. Qashu, H. Finne, V. Pidoplichko, D.I. Bannon, and M.F. Braga. 2011. RDX Binds to the GABAA Receptor-Convulsant Site and Blocks GABAA Receptor-Mediated Currents in the Amygdala: A Mechanism for RDX-Induced Seizures. *Environ Health Perspect,* 119(3):357–63.

Xenometrix. 2012. *Ames MPF™ Penta I Microplate Format Mutagenicity Assay Instructions for use. Version 4.5_S*. Available from http://www.aniara.com/pdf/literature/ICT-ANIARA-MPF-PENTA-AD.pdf.

Toxicology Study No. 00058221.3, December 2020

Appendix B

QUALITY ASSURANCE STATEMENT
AMES ASSAY

For: Toxicology Study No. No. S.00058221.3, Protocol No. 2758-70-iv19-04-01A Ames mutagenesis screen of Novel Energetic N-propylnitroguanidine (PrNQ) the following critical phases were inspected/audited by the Quality Systems and Regulatory Compliance Office (QSARC):

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Type Protocol Good Laboratory Practice Standard Review	8/13/2019	8/19/2019
Test Article Specific Protocol Modification Review	07/01/2019	07/01/2019

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Analytical Chemistry Support – QA review of Dosing Solution Concentration Verification	12/06/2016	12/06/2016
In-Vitro AMES Assay - Reagents, Working Solutions Bacteria Storage	05/02/2018	05/05/2018
In-Vitro AMES Assay – Compliance with GLP requirements for Test Facility SOPs	05/02/2018	05/05/2018
In-Vitro AMES Assay – Calibration Verification of Equipment Used during assay	05/02/2018	05/05/2018
Study Raw Data Good Laboratory Practice Standard Review	12/14/2020	12/14/2020
Final Study Good Laboratory Practice Standard Report Review	12/14/2020	12/14/2020

Note 1: *All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.*

Note 2: *This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and of the procedures followed*

Note 3: *In addition to the study specific critical phase inspections listed here, general facility and process based inspections not specifically related to this study are done monthly and are also listed here in accordance with QA Standard Operating Procedure.*

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Michael P. Kefauver
GLP Quality Assurance Specialist, QSARC

12/14/2020
Date

Toxicology Study No. S.0058221-19, October 2019

Appendix B

QUALITY ASSURANCE STATEMENT

FOR: Toxicology Study No. S.0058221-19, Protocol No. 0FMA-92-iv17-03-01 J; Microtox Toxicity Testing of the Energetic Replacement N-propylnitroguanidine (PrNQ) the following critical phases were inspected/audited Quality Systems and Regulatory Compliance Office (QSARC):

Study Specific Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Type Protocol Good Laboratory Practice Standard Review	03/01/2018	03/01/2018
Test Article Specific Type Protocol Modification Review	04/25/2019	04/25/2019
Analytical Chemistry Support – QA review of Dosing Solution Concentration Verification	12/06/2016	12/06/2016
Microtox - Reagent and Test System Storage and Labeling requirements	05/02/2018	05/05/2018
Microtox - Data Processing and Raw Data Documentation Procedures	05/02/2018	05/05/2018
Microtox - Compliance with GLP requirements for Test Facility SOPs	05/02/2018	05/05/2018
Microtox - Calibration Verification of Equipment - Balance and Pipettes	05/02/2018	05/05/2018
Microtox Test Study Endpoint Criteria Compliance	10/28/2018	10/28/2018
Study Raw Data Good Laboratory Practice Standard Review	10/29/2019	10/29/2019
Final Study Good Laboratory Practice Standard Report Review	10/29/2019	10/29/2019

Note 1: *All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.*

Note 2: *This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and of the procedures followed*

Note 3: *In addition to the study specific critical phase inspections listed here, general facility and process based inspections not specifically related to this study are done monthly and are also listed here in accordance with QA Standard Operating Procedure.*

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02/06/2020

Michael P. Kefauver
Good Laboratory Practice Standard
Quality Assurance Specialist, QSARC

Date

Toxicology Study No. 00058221.3, March 2021

Appendix B

QUALITY ASSURANCE STATEMENT
H-CLAT ASSAY

For: Toxicology Study No. 0058223.2, Protocol No. 49-iv19-03-01E, h-CLAT Skin Sensitization Testing of the Novel Energetic N-propylnitroguanidine(PrNQ) the following critical phases were inspected/audited by the Quality Systems and Regulatory Compliance Office (QSARC):

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Type Protocol Good Laboratory Practice Standard Review	03/18/2019	03/18/2019
Test Article Specific Protocol Modification Review	05/21/2019	05/21/2019

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
In-Vitro Skin Sensitization h-CLAT Assay - Preparation of Stock and Working Solutions	7/10/2019	9/23/2019
In-Vitro Skin Sensitization h-CLAT Assay - Cell Suspension and Exposure	7/10/2019	9/24/2019
In-Vitro Skin Sensitization h-CLAT Assay - Reagents, Working Solutions and Cell Suspension Storage	7/10/2019	9/23/2019
In-Vitro Skin Sensitization h-CLAT Assay – Compliance with GLP requirements for Test Facility SOPs	7/10/2019	9/24/2019
In-Vitro Skin Sensitization h-CLAT Assay – Calibration Verification of Equipment Used during assay	7/10/2019	9/23/2019
Study Raw Data Good Laboratory Practice Standard Review	10/29/2019	10/29/2019
Final Study Good Laboratory Practice Standard Report Review	10/29/2019	10/29/2019

Note 1: *All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.*

Note 2: *This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and of the procedures followed*

Note 3: *In addition to the study specific critical phase inspections listed here, general facility and process based inspections not specifically related to this study are done monthly and are also listed here in accordance with QA Standard Operating Procedure.*

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Date

Appendix C

ARCHIVES AND STUDY PERSONNEL

C-1. ARCHIVES

All raw data, documentation, records, protocols, contributing scientist reports, and a copy of the final report generated as a result of this study will be archived in the storage facilities of the Toxicology Directorate, APHC, for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

Records on the test system will be archived by the Toxicology Directorate for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

The present study used the Toxicology Study No. S.00058221.3-21, Protocol Nos. 0FMA-92-iv17-03-01S, 2758-70-iv19-04-01A, and 49-iv19-03-01F.

The protocol, raw data, summary data, and the final report pertaining to this study will be physically maintained within Building E-2100, APHC. These data may be scanned to a computer disk. Scanned study files will be stored electronically with the study data in the archive.

Archived SOPs can be found in the Master Control database at APHC. Maintenance and calibration logbooks may be found in Room 1026, Building E-2100, APHC, Aberdeen Proving Ground, MD, 21010.

Archivist: Martha Thompson.

C-2. PERSONNEL

Management: Mark Johnson, Ph.D., D.A.B.T., Toxicology Director; Michael J. Quinn, Ph.D., Division Chief, Health Effects Division (HEF).

Study Directors: Valerie H. Adams Ph.D., D.A.B.T and Emily N. Reinke, Ph.D., D.A.B.T HEF.

Technical staff: Lindsay A. Holden, Ph.D., HEF and Taryn Brown, ORISE Fellow.

Quality Assurance: Michael P. Kefauver, Chemist, Quality Systems Office.

APPENDIX D

STUDY REAGENTS

Table D-1. Ames Test Reagents

Ames Reagents	**Source**	**Lot #**	**Exp Date**
AG-TA98 *hisD3052 Salmonella typhimurium*	Xenometrix	27a	2/28/21
AG-TA100 *hisG46 Salmonella typhimurium*	Xenometrix	23b	1/1/2021
AG-TA1535 his G46 *Salmonella typhimurium*	Xenometrix	17c	5/28/21
AG-TA1537 hisC3076 *Salmonella typhimurium*	Xenometrix	21d	4/28/21
E. coli wp2 [pKM101] *trpE65 Escherichia coli*	Xenometrix	P19	7/31/21
E. coli wp2 *uvrA trpE65 Escherichia coli*	Xenometrix	U20	7/31/21
S9-Postmitochondrial Supernatant (Aroclor)	Xenometrix	3930	9/28/20
Ampicillin	Xenometrix	191	8/31/21
N4-aminocytidine	Xenometrix	NA079541202	n/a
2-aminoanthracene	Sigma-Adrich	STBG0530V	9/28/20
2-nitrofluorene	Xenometrix	1550-1901	1/28/22
4-nitroquinoline-N-oxide	Sigma-Aldrich	034K1332	5/29/20
9-aminoacridine	Xenometrix	1018-1810	10/28/21
Ames MPF Exposure Medium	BioReliance	52701	12/17/19
Ames MPF Exposure Medium	Moltox	52878	7/23/20
Ames MPF Exposure Medium	Xenometrix	MC04819P	6/30/21
Ames MPF Reversion Indicator Medium	Xenometrix	MC06879P	6/30/21
Ames MPF Growth Medium	Xenometrix	MC02717P	4/30/21
E.coli Reversion Indicator Medium	Xenometrix	ND09642P	11/30/21
E.coli Exposure Medium	Xenometrix	MC04819P	6/30/21
Ames MPF S9 Buffer Salts, ,	Xenometrix	MC09053P	1/31/22
S9-100/1537 Booster Solution	Xenometrix	X1909BSA	9/28/22
Glucose-6-phosphate/ S9-G-6-P	Xenometrix	X1810SGPA	10/31/20
NADP/ S9-NADP	Xenometrix	X1810SNAA	10/31/21

Table D-2. Microtox Test Reagents

Microtox Reagents	Source	Lot #	Date Expiration
Modern Water Microtox Diluent	Modern Water	17E4130	05/2020
Modern Water Microtox Acute Reagent	Modern Water	17C4076	03/2019
Dimethyl Sulfoxide	Sigma-Aldrich	17H4227	09/2019
Zinc Sulfate	Sigma-Aldrich	RNBG1729	07/2019
Phenol	Sigma-Aldrich	SLBC2469V	N/A
Modern Water Microtox Reconstitution Solution	Modern Water	16D4031	4/2019

Table D-3. h-CLAT Test Reagents

Reagent	Supplier	Product Number	Lot Number	Expiration Date
THP-1	ATCC	TIB-202	62996831	N/A
RPMI-1640	Gibco	22400	2052771	03-30-2020
FBS	Gibco	16140	1939739	11-30-2022
2-Mercaptoethanol	Gibco	21985	1922541	11-30-2020
Penicillin-Streptomycin[a]	HyClone	SV30010	J170027	06-30-2019[a]
Saline	Sigma	S8776	RNBD7305	N/A
DMSO (TC)	Sigma	D2438	RNBG4916	02-2020
Globulins	Sigma	G2388	017K7650V	N/A
BSA Fraction V	EMD Millipore	12660	3035346	N/A
D-PBS	Gibco	14190	1897013 1855254	07-30-2020 03-30-2020
Propidium Iodide	Sigma	P4864	MKBR1007V	N/A
CD54 Antibody, ICAM-1 Clone 6.5B5, FITC	Dako	F714301-8	20051521	09-2020
CD86 Antibody, Hu Fun-1, FITC	BD	555657	6348610	11-30-2021
IgG1 (mouse), FITC	Dako	X092701-2	20036430	07-31-2019
Flow Cytometer Beads	BD	650622	81165	05-31-2020
Sheath Fluid	BD	342003	0000221830	02-04-2022
2,4-dinitrochlorobenzene (DNCB)	Sigma	237329	BCBN7826V	N/A
Nickel Sulfate ($NiSO_4$)	Sigma	656895	MKBT0269V	N/A
Lactic Acid (LA)	Sigma	W261106	MKBR4746V	N/A

Note:

[a] Penicillin-Streptomycin did have an expiration date preceding the usage in the testing during the reactivity check and early growth of the cell stock; however, media stock had been appropriately temperature maintained and was within a month of expiration. Professional experience has shown that this reagent maintains full effectiveness past the expiration date.

Table D-4. DPRA Test Reagents

Reagent	Supplier	Product Numberke	Lot Number	Expiration Date
Lysine Peptide	RS Synthesis	N/A	RS2464040620-2	
Cysteine Peptide	RS Synthesis	N/A	RS2464040620-1	
Acetonitrile	Fisher Chemical	A955-4	172559	01/30/24
Cinnamaldehyde	Sigma-Aldrich	W228613	MKBX8146V	02/2021
Sodium phosphate monobasic monohydrate	Sigma-Aldrich	S9638-250G	SLBP0799V	
Sodium phosphate dibasic heptahydrate	Sigma-Aldrich	S9390-500G	SLBQ2859V	
Ammonium acetate	Sigma-Aldrich	238074-500G	MKBW7225V	
Ammounium hydroxide	Sigma-Aldrich	32145-500ML	19296MJ	

APPENDIX E

PrNQ AMES ASSAY RAW DATA

TA98

Compound:	PRNQ		
TA 98 -S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	4	9	1
21.1	1	3	5
65.9	2	5	3
205.5	0	0	1
641.0	4	4	0
2000	0	0	2
Pos. Control	48	47	48

Spontaneous	
TA 98 -S9	
3	2
2	5
1	2
4	
2	
3	

Compound:	PRNQ		
TA 98 +S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	5	2	6
21.1	1	2	1
65.9	3	1	7
205.5	1	4	1
641.0	2	3	2
2000	1	3	1
Pos. Control	48	48	48

Spontaneous	
TA 98 +S9	
1	0
5	1
2	2
3	
5	
3	

PRNQ
TA 98 -S9 — Assay Date: 7/12/2019

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)	
0	9	2.67		1.22	3.89				
6.7648322	3	4.67		4.04		1.75	1.20	0.0931	
21.106276	3	3.00		2.00		1.13	0.77	0.3655	
65.851582	3	3.33		1.53		1.25	0.86	0.2283	
205.45694	3	0.33		0.58		0.13	0.09	0.0055	Cytotoxic effect?
641.02564	3	2.67		2.31		1.00	0.69	0.5000	
2000	3	0.67		1.15		0.25	0.17	0.0164	Cytotoxic effect?
Pos. Control	3	47.67		0.58					

PRNQ
TA 98 +S9 — Assay Date: 7/12/2019

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)
0	9	2.44		1.74	4.18			
6.7648322	3	4.33		2.08		1.77	1.04	0.0746
21.106276	3	1.33		0.58		0.55	0.32	0.1578
65.851582	3	3.67		3.06		1.50	0.88	0.1984
205.45694	3	2.00		1.73		0.82	0.48	0.3547
641.02564	3	2.33		0.58		0.95	0.56	0.4590
2000	3	1.67		1.15		0.68	0.40	0.2465
Pos. Control	3	48.00		0.00				

TA100

Compound:	PrNQ		
TA 100 -S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	3	1	2
21.1	1	2	2
65.9	2	0	2
205.5	1	1	3
641.0	1	1	2
2000	3	1	2
Pos. Control	48	48	48

Spontaneous
TA 100 -S9
3
3
0

Compound:	PrNQ		
TA 100 +S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	1	0	0
21.1	4	3	4
65.9	4	4	2
205.5	2	0	1
641.0	1	1	1
2000	1	0	3
Pos. Control	24	30	24

Spontaneous
TA 100 +S9
2
2
1

PrNQ
TA 100 -S9 — Assay Date: 6/19/2019

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)
0	3	2.00		1.73	3.73			
6.76483215	3	2.00		1.00		1.00	0.54	0.5000
21.1062763	3	1.67		0.58		0.83	0.45	0.3838
65.8515821	3	1.33		1.15		0.67	0.36	0.3043
205.456936	3	1.67		1.15		0.83	0.45	0.3976
641.025641	3	1.33		0.58		0.67	0.36	0.2807
2000	3	2.00		1.00		1.00	0.54	0.5000
Pos. Control	3	48.00		0.00				

PrNQ
TA 100 +S9 — Assay Date: 6/19/2019

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)	
0	3	1.67		0.58	2.24				
6.76483215	3	0.33		0.58		0.20	0.15	0.0237	Cytotoxic effect?
21.1062763	3	3.67		0.58		2.20	1.63	0.0066	
65.8515821	3	3.33		1.15		2.00	1.49	0.0445	
205.456936	3	1.00		1.00		0.60	0.45	0.1870	
641.025641	3	1.00		0.00		0.60	0.45	0.0581	
2000	3	1.33		1.53		0.80	0.59	0.3708	
Pos. Control	3	26.00		3.46					

TA1535

Compound:	PRNQ		
TA 1535 -S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	3	1	0
21.1	2	1	0
65.9	0	0	1
205.5	2	1	2
641.0	0	0	1
2000	0	2	3
Pos. Control	48	48	48

Spontaneous	
TA 1535 -S9	
2	0
1	1
3	1
0	
3	
0	

Compound:	PRNQ		
TA 1535 +S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	2	0	3
21.1	0	2	0
65.9	0	1	2
205.5	1	1	1
641.0	1	2	0
2000	1	1	0
Pos. Control	32	44	48

Spontaneous	
TA 1535 +S9	
1	1
2	0
2	1
2	
0	
1	

PRNQ
TA 1535 -S9 — Assay Date: 8/7/2019

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)
0	9	1.22		1.20	2.42			
6.76483215	3	1.33		1.53		1.09	0.55	0.4492
21.1062763	3	1.00		1.00		0.82	0.41	0.3902
65.8515821	3	0.33		0.58		0.27	0.14	0.1278
205.456936	3	1.67		0.58		1.36	0.69	0.2800
641.025641	3	0.33		0.58		0.27	0.14	0.1278
2000	3	1.67		1.53		1.36	0.69	0.3060
Pos. Control	3	48.00		0.00				

PRNQ
TA 1535 +S9 — Assay Date: 8/7/2019

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)
0	9	1.11		0.78	1.89			
6.76483215	3	1.67		1.53		1.50	0.88	0.2069
21.1062763	3	0.67		1.15		0.60	0.35	0.2304
65.8515821	3	1.00		1.00		0.90	0.53	0.4224
205.456936	3	1.00		0.00		0.90	0.53	0.4082
641.025641	3	1.00		1.00		0.90	0.53	0.4224
2000	3	0.67		0.58		0.60	0.35	0.1961
Pos. Control	3	41.33		8.33				

TA1537

Compound:	PRNQ		
TA 1537 -S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	2	0	0
21.1	0	0	1
65.9	2	1	2
205.5	2	1	1
641.0	1	1	1
2000	0	0	0
Pos. Control	48	48	48

Spontaneous	
TA 1537 -S9	
2	1
0	1
1	0
1	
1	
1	

Compound:	PRNQ		
TA 1537 +S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	2	0	0
21.1	2	0	0
65.9	1	0	2
205.5	1	1	1
641.0	1	0	1
2000	0	0	1
Pos. Control	29	24	27

Spontaneous	
TA 1537 +S9	
2	1
1	2
0	1
0	
3	
1	

PRNQ
TA 1537 -S9 — Assay Date: 7/31/2020

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)	
0	9	0.89	1.00	0.60	1.49				
6.76483215	3	0.67		1.15		0.67	0.45	0.3321	
21.1062763	3	0.33		0.58		0.33	0.22	0.0962	
65.8515821	3	1.67		0.58		1.67	1.12	0.0394	
205.456936	3	1.33		0.58		1.33	0.89	0.1448	
641.025641	3	1.00		0.00		1.00	0.67	0.3814	
2000	3	0.00		0.00		0.00	0.00	0.0163	Cytotoxic effect?
Pos. Control	3	48.00		0.00					

PRNQ
TA 1537 +S9 — Assay Date: 7/31/2020

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)
0	9	1.22		0.97	2.19			
6.76483215	3	0.67		1.15		0.55	0.30	0.2145
21.1062763	3	0.67		1.15		0.55	0.30	0.2145
65.8515821	3	1.00		1.00		0.82	0.46	0.3701
205.456936	3	1.00		0.00		0.82	0.46	0.3547
641.025641	3	0.67		0.58		0.55	0.30	0.1899
2000	3	0.33		0.58		0.27	0.15	0.0861
Pos. Control	3	26.67		2.52				

E.coli

Compound:	PRNQ		
EC Combo -S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	16	11	11
21.1	15	17	15
65.9	14	17	14
205.5	10	19	15
641.0	16	12	12
2000	14	12	12
Pos. Control	40	42	43

Spontaneous	
EC Combo -S9	
15	14
12	17
19	15
18	
14	
21	

Compound:	PRNQ		
EC Combo +S9			
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
6.8	17	9	12
21.1	15	15	17
65.9	7	11	12
205.5	8	6	9
641.0	12	16	19
2000	14	15	13
Pos. Control	25	10	16

Spontaneous	
EC Combo +S9	
15	16
13	10
15	13
13	
13	
16	

PRNQ
EC Combo -S9 — Assay Date: 7/31/2020

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)	
0	9	16.11		2.85	18.96				
6.76483215	3	12.67		2.89		0.79	0.67	0.0503	
21.1062763	3	15.67		1.15		0.97	0.83	0.4014	
65.8515821	3	15.00		1.73		0.93	0.79	0.2727	
205.456936	3	14.67		4.51		0.91	0.77	0.2600	
641.025641	3	13.33		2.31		0.83	0.70	0.0803	
2000	3	12.67		1.15		0.79	0.67	0.0374	Cytotoxic effect?
Pos. Control	3	41.67		1.53					

PRNQ
EC Combo +S9 — Assay Date: 7/31/2020

Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)	
0	9	13.78		1.92	15.70				
6.76483215	3	12.67		4.04		0.92	0.81	0.2596	
21.1062763	3	15.67		1.15		1.14	1.00	0.0728	
65.8515821	3	10.00		2.65		0.73	0.64	0.0109	Cytotoxic effect?
205.456936	3	7.67		1.53		0.56	0.49	0.0003	Cytotoxic effect?
641.025641	3	15.67		3.51		1.14	1.00	0.1258	
2000	3	14.00		1.00		1.02	0.89	0.4275	
Pos. Control	3	17.00		7.55					

APPENDIX F

PrNQ MICROTOX TEST RAW DATA

Table F-1. PrNQ Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration[a] (mg/mL; 100x test concentration)		
	Test 1	Test 2	Test 3
1.56			
3.13			
6.25			
12.5			
25			
50			
100			
200			

Legend:
PrNQ = N-Propylnitroguanidine
mg/mL = milligrams per milliliter
Note:
[a] Corrected Working Concentrations were unavailable at the time of this report. Concentration verification had not been performed by PHC-MDV-CSD.

Table F-2. Microtox EC50 for PrNQ

PrNQ EC_{50} (mg/L; 95% CI)		
5 minute	15 minute	30 minute
>2,000	>2,000	>2,000

Legend:
PrNQ = N-Propylnitroguanidine
EC_{50} = median (50%) effect concentration
Mg/L = milligrams per liter
CI = Confidence Interval

APPENDIX G

PrNQ SKIN SENSITIZATION TEST RAW DATA

h-CLAT Data

Table G-1. Reactivity Check

Test article	Concentration (mg/mL)	Viability (% alive)	% Change (CD86)	% Change (CD54)	Positive (CD86/CD54)	Pass/Fail
Media		95.64	1	1	N/N	Pass
Saline		95.68	1	1	N/N	Pass
DMSO		95.9	1	1	N/N	Pass

DNCB	0.0033	83.58	335.80	518.64	Y/Y	Pass
	0.0040	79.72	223.15	664.07	Y/Y	Pass
	0.0048	65.56	119.08	227.63	Y/Y	
$NiSO_4$	0.10	78.01	297.63	2887.9	Y/Y	Pass
Lactic Acid	1	95.64	83.66	95.31	N/N	Pass

Legend:
mg/mL = milligrams per milliliter
N = No
Y = Yes
DMSO = dimethyl sulfoxide
DNCB = 2,4-dinitrochlorobenzene
$NiSO_4$ = nickel sulfate

Reactivity Check Raw Data

Experiment:
Reactivity Check 5-21-19
Protocol 49-iv19-03-01 A-E

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Dead:All Events	10,000	***	***	100.00	1,982
Dead:Live Cells	609	6.09	***	6.09	1,120
Live Cells:All Events	10,000	***	***	100.00	950
Live Cells:Live Cells	9,612	96.12	***	96.12	913
IgG Media:All Events	10,456	***	***	100.00	978
IgG Media:Live Cells	10,000	95.64	***	95.64	939
IgG Saline:All Events	10,451	***	***	100.00	967
IgG Saline:Live Cells	10,000	95.68	***	95.68	923
IgG DMSO:All Events	10,427	***	***	100.00	923
IgG DMSO:Live Cells	10,000	95.90	***	95.90	884
IgG Lactic Acid:All Events	10,456	***	***	100.00	931
IgG Lactic Acid:Live Cells	10,000	95.64	***	95.64	892
IgG Nickel Sulfate:All Events	12,817	***	***	100.00	1,027
IgG Nickel Sulfate:Live Cells	9,999	78.01	***	78.01	929
IgG DNCB 1:All Events	14,585	***	***	100.00	1,437
IgG DNCB 1:Live Cells	10,000	68.56	***	68.56	1,149
IgG DNCB 2:All Events	12,544	***	***	100.00	1,277
IgG DNCB 2:Live Cells	10,000	79.72	***	79.72	1,129
IgG DNCB 3:All Events	11,965	***	***	100.00	1,206
IgG DNCB 3:Live Cells	10,000	83.58	***	83.58	1,075
CD86 Media:All Events	10,504	***	***	100.00	1,799
CD86 Media:Live Cells	10,003	95.23	***	95.23	1,638
CD54 Media:All Events	10,503	***	***	100.00	1,241
CD54 Media:Live Cells	10,000	95.21	***	95.21	1,181
CD86 Saline:All Events	10,539	***	***	100.00	1,828
CD86 Saline:Live Cells	10,000	94.89	***	94.89	1,682
CD54Saline:All Events	10,520	***	***	100.00	1,235
CD54Saline:Live Cells	10,000	95.06	***	95.06	1,179
CD86 DMSO:All Events	10,597	***	***	100.00	1,992
CD86 DMSO:Live Cells	10,000	94.37	***	94.37	1,817
CD54 DMSO:All Events	10,521	***	***	100.00	1,235
CD54 DMSO:Live Cells	10,000	95.05	***	95.05	1,179
CD86 Lactic Acid:All Events	10,483	***	***	100.00	1,667
CD86 Lactic Acid:Live Cells	10,000	95.39	***	95.39	1,527
CD54 Lactic Acid:All Events	10,579	***	***	100.00	1,192
CD54 Lactic Acid:Live Cells	10,000	94.53	***	94.53	1,136
CD86 Nickel Sulfate:All Events	13,362	***	***	100.00	3,954
CD86 Nickel Sulfate:Live Cells	10,014	74.94	***	74.94	3,188
CD54 Nickel Sulfate:All Events	16,274	***	***	100.00	7,606
CD54 Nickel Sulfate:Live Cells	9,985	61.36	***	61.36	8,322
CD86 DNCB 1:All Events	15,254	***	***	100.00	3,965
CD86 DNCB 1:Live Cells	10,000	65.56	***	65.56	2,260
CD54 DNCB 1:All Events	15,883	***	***	100.00	2,210
CD54 DNCB 1:Live Cells	10,002	62.97	***	62.97	1,968
CD86 DNCB 2:All Events	13,327	***	***	100.00	4,070
CD86 DNCB 2:Live Cells	10,000	75.04	***	75.04	3,211
CD54 DNCB 2:All Events	13,597	***	***	100.00	2,992
CD54 DNCB 2:Live Cells	9,995	73.51	***	73.51	3,088
CD86 DNCB 3:All Events	12,327	***	***	100.00	4,736
CD86 DNCB 3:Live Cells	10,009	81.20	***	81.20	4,208
CD54 DNCB 3:All Events	12,469	***	***	100.00	2,648
CD54 DNCB 3:Live Cells	10,000	80.20	***	80.20	2,605

Operator: Emily Reinke, Ph.D.

Printed: 5/21/2019 11:49:14 AM

Software: BD FACSuite v1.0.5

Table G-2. Range Finding

6/25/2019		PI- Dose Finding			
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	CV75	mg/mL
DMSO		0	94.76		
PrNQ	3.90625	0.0078125	94.8		
Run #1	7.8125	0.015625	94.8		
	15.625	0.03125	94.48		
	31.25	0.0625	95.07		
	62.5	0.125	95.27		
	125	0.25	94.86		
	250	0.5	94.22		
	500	1	93.89		
6/26/2019		PI- Dose Finding			
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	CV75	mg/mL
DMSO		0	96.29		
PrNQ	3.90625	0.0078125	96.07		
Run #2	7.8125	0.015625	96.18		
	15.625	0.03125	96.74		
	31.25	0.0625	95.44		
	62.5	0.125	95.86		
	125	0.25	95.92		
	250	0.5	96.01		
	500	1	95.25		

Legend:
PI = Propidium iodide
DMSO = dimethyl sulfoxide
PrNQ = N-Propylnitroguanidine
mg/mL = milligrams per milliliter
CV75 = 75% cell viability

Range Finding Experiment 1 Raw Data

Experiment:
hCLAT Range-Finding Ammonia Borane, PrNQ, HEATN 6-25-19

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Protocol 49-iv19-03-01 A, F-G

Statistics

Name	Events	% Parent	% Grandparent	% Total
Live Cells:All Events	2,764	***	***	100.00
Live Cells:Live Cells	2,623	94.90	***	94.90
Dead Cells:All Events	1,379	***	***	100.00
Dead Cells:Live Cells	165	11.97	***	11.97
Media 1:All Events	10,488	***	***	100.00
Media 1:Live Cells	10,000	95.35	***	95.35
DMSO 1:All Events	10,569	***	***	100.00
DMSO 1:Live Cells	10,000	94.62	***	94.62
Ammonia Borane 1:All Events	10,492	***	***	100.00
Ammonia Borane 1:Live Cells	9,997	95.28	***	95.28
Ammonia Borane 2:All Events	10,522	***	***	100.00
Ammonia Borane 2:Live Cells	10,000	95.04	***	95.04
Ammonia Borane 3:All Events	10,552	***	***	100.00
Ammonia Borane 3:Live Cells	10,000	94.77	***	94.77
Ammonia Borane 4:All Events	10,574	***	***	100.00
Ammonia Borane 4:Live Cells	10,000	94.57	***	94.57
Ammonia Borane 5:All Events	10,762	***	***	100.00
Ammonia Borane 5:Live Cells	9,999	92.91	***	92.91
Ammonia Borane 6:All Events	14,182	***	***	100.00
Ammonia Borane 6:Live Cells	10,009	70.58	***	70.58
Ammonia Borane 7:All Events	17,781	***	***	100.00
Ammonia Borane 7:Live Cells	4,103	23.08	***	23.08
Ammonia Borane 8:All Events	14,348	***	***	100.00
Ammonia Borane 8:Live Cells	2,663	18.56	***	18.56
Media 2:All Events	10,673	***	***	100.00
Media 2:Live Cells	9,998	93.68	***	93.68
DMSO 2:All Events	10,551	***	***	100.00
DMSO 2:Live Cells	9,998	94.76	***	94.76
Dead 2:All Events	15,054	***	***	100.00
Dead 2:Live Cells	3,209	21.32	***	21.32
PrNQ 1:All Events	10,549	***	***	100.00
PrNQ 1:Live Cells	10,000	94.80	***	94.80
PrNQ 2:All Events	10,548	***	***	100.00
PrNQ 2:Live Cells	10,000	94.80	***	94.80
PrNQ 3:All Events	10,584	***	***	100.00
PrNQ 3:Live Cells	10,000	94.48	***	94.48
PrNQ 4:All Events	10,519	***	***	100.00
PrNQ 4:Live Cells	10,000	95.07	***	95.07
PrNQ 5:All Events	10,497	***	***	100.00
PrNQ 5:Live Cells	10,000	95.27	***	95.27
PrNQ 6:All Events	10,542	***	***	100.00
PrNQ 6:Live Cells	10,000	94.86	***	94.86
PrNQ 7:All Events	10,613	***	***	100.00
PrNQ 7:Live Cells	10,000	94.22	***	94.22
HEATN 1 :All Events	10,580	***	***	100.00
HEATN 1 :Live Cells	10,000	94.52	***	94.52
HEATN 2:All Events	10,584	***	***	100.00
HEATN 2:Live Cells	10,000	94.48	***	94.48
HEATN 3:All Events	10,552	***	***	100.00
HEATN 3:Live Cells	10,000	94.77	***	94.77
HEATN 4:All Events	10,580	***	***	100.00
HEATN 4:Live Cells	9,985	94.38	***	94.38
HEATN 5:All Events	10,656	***	***	100.00
HEATN 5:Live Cells	10,000	93.84	***	93.84
HEATN 6:All Events	10,615	***	***	100.00
HEATN 6:Live Cells	10,000	94.21	***	94.21
HEATN 7:All Events	10,599	***	***	100.00
HEATN 7:Live Cells	10,000	94.35	***	94.35
HEATN 8:All Events	10,589	***	***	100.00
HEATN 8:Live Cells	10,000	94.44	***	94.44
PrNQ 8:All Events	10,651	***	***	100.00
PrNQ 8:Live Cells	10,000	93.89	***	93.89

Operator: Emily Reinke, Ph.D.

Printed: 6/25/2019 10:47:05 AM

Software: BD FACSuite v1.0.5

Page 1 of 1

Range Finding Experiment 2 Raw Data

Experiment:
Range-Finding Ammonia Borane,PrNQ, HEATN 6-26-19 #2
Protocol 49-iv19-03-01 A, F-G

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total
Live Cells:All Events	8,642	***	***	100.00
Live Cells:Live Cells	8,400	97.20	***	97.20
Dead Cells:All Events	4,409	***	***	100.00
Dead Cells:Live Cells	1,941	44.02	***	44.02
Media 1:All Events	10,315	***	***	100.00
Media 1:Live Cells	9,999	96.94	***	96.94
DMSO 1:All Events	10,338	***	***	100.00
DMSO 1:Live Cells	10,000	96.73	***	96.73
Ammonia Borane 1:All Events	10,350	***	***	100.00
Ammonia Borane 1:Live Cells	10,000	96.62	***	96.62
Ammonia Borane 2:All Events	10,384	***	***	100.00
Ammonia Borane 2:Live Cells	10,000	96.30	***	96.30
Ammonia Borane 3:All Events	10,350	***	***	100.00
Ammonia Borane 3:Live Cells	10,000	96.62	***	96.62
Ammonia Borane 4:All Events	10,377	***	***	100.00
Ammonia Borane 4:Live Cells	10,003	96.40	***	96.40
Ammonia Borane 5:All Events	10,594	***	***	100.00
Ammonia Borane 5:Live Cells	10,005	94.44	***	94.44
Ammonia Borane 6:All Events	15,083	***	***	100.00
Ammonia Borane 6:Live Cells	9,992	66.25	***	66.25
Ammonia Borane 7:All Events	21,438	***	***	100.00
Ammonia Borane 7:Live Cells	6,869	32.04	***	32.04
Ammonia Borane 8:All Events	17,057	***	***	100.00
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Media 2:All Events	10,390	***	***	100.00
Media 2:Live Cells	10,000	96.25	***	96.25
DMSO 2:All Events	10,385	***	***	100.00
DMSO 2:Live Cells	10,000	96.29	***	96.29
PrNQ 1 :All Events	10,409	***	***	100.00
PrNQ 1 :Live Cells	10,000	96.07	***	96.07
PrNQ 2:All Events	10,396	***	***	100.00
PrNQ 2:Live Cells	9,999	96.18	***	96.18
PrNQ 3:All Events	10,337	***	***	100.00
PrNQ 3:Live Cells	10,000	96.74	***	96.74
PrNQ 4:All Events	10,479	***	***	100.00
PrNQ 4:Live Cells	10,001	95.44	***	95.44
PrNQ 5:All Events	10,432	***	***	100.00
PrNQ 5:Live Cells	10,000	95.86	***	95.86
PrNQ 6:All Events	10,425	***	***	100.00
PrNQ 6:Live Cells	10,000	95.92	***	95.92
PrNQ 7:All Events	10,416	***	***	100.00
PrNQ 7:Live Cells	10,000	96.01	***	96.01
PrNQ 8:All Events	10,499	***	***	100.00
PrNQ 8:Live Cells	10,000	95.25	***	95.25
HEATN 1:All Events	10,398	***	***	100.00
HEATN 1:Live Cells	10,000	96.17	***	96.17
HEATN 2:All Events	10,399	***	***	100.00
HEATN 2:Live Cells	10,000	96.16	***	96.16
HEATN 3:All Events	10,366	***	***	100.00
HEATN 3:Live Cells	10,003	96.50	***	96.50
HEATN 4:All Events	10,405	***	***	100.00
HEATN 4:Live Cells	10,001	96.12	***	96.12
HEATN 5:All Events	10,463	***	***	100.00
HEATN 5:Live Cells	10,000	95.57	***	95.57
HEATN 6:All Events	10,485	***	***	100.00
HEATN 6:Live Cells	10,004	95.41	***	95.41
HEATN 7:All Events	10,502	***	***	100.00
HEATN 7:Live Cells	10,000	95.22	***	95.22
HEATN 8:All Events	10,529	***	***	100.00
HEATN 8:Live Cells	10,000	94.98	***	94.98

Operator: Emily Reinke, Ph.D.

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Software: BD FACSuite v1.0.5
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h-CLAT Experiment 1 Raw Data

Experiment:
h-CLAT Ammonia Borane,PrNQ,HEATN 6-28-19 #1

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Protocol 49-iv19-03-01 Mod F

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	1,751	***	***	100.00	964
Live Cells:Live Cells	1,647	94.06	***	94.06	918
Dead Cells:All Events	2,563	***	***	100.00	1,818
Dead Cells:Live Cells	71	2.77	***	2.77	1,639
IgG Media:All Events	10,930	***	***	100.00	1,048
IgG Media:Live Cells	10,261	93.88	***	93.88	995
IgG DMSO:All Events	10,711	***	***	100.00	1,031
IgG DMSO:Live Cells	10,000	93.36	***	93.36	960
IgG DNCB 1:All Events	12,863	***	***	100.00	1,319
IgG DNCB 1:Live Cells	10,000	77.74	***	77.74	1,136
IgG DNCB 2A:All Events	834	***	***	100.00	1,214
IgG DNCB 2A:Live Cells	628	75.30	***	75.30	965
IgG DNCB 3:All Events	10,535	***	***	100.00	1,386
IgG DNCB 3:Live Cells	7,935	75.32	***	75.32	1,157
IgG PrNQ 1:All Events	10,768	***	***	100.00	1,059
IgG PrNQ 1:Live Cells	10,000	92.87	***	92.87	982
IgG PrNQ 2:All Events	10,862	***	***	100.00	1,019
IgG PrNQ 2:Live Cells	10,000	92.06	***	92.06	936
IgG PrNQ 3:All Events	10,620	***	***	100.00	1,018
IgG PrNQ 3:Live Cells	10,000	94.16	***	94.16	953
IgG PrNQ 4:All Events	10,773	***	***	100.00	1,051
IgG PrNQ 4:Live Cells	10,000	92.82	***	92.82	977
IgG PrNQ 5:All Events	10,842	***	***	100.00	1,053
IgG PrNQ 5:Live Cells	10,000	92.23	***	92.23	967
IgG PrNQ 6:All Events	10,744	***	***	100.00	1,024
IgG PrNQ 6:Live Cells	10,000	93.08	***	93.08	949
IgG PrNQ 7:All Events	10,782	***	***	100.00	1,050
IgG PrNQ 7:Live Cells	10,000	92.75	***	92.75	968
IgG PrNQ 8:All Events	10,818	***	***	100.00	1,046
IgG PrNQ 8:Live Cells	10,000	92.44	***	92.44	964
CD86 Media:All Events	10,818	***	***	100.00	2,055
CD86 Media:Live Cells	10,000	92.44	***	92.44	1,796
CD54 Media:All Events	10,991	***	***	100.00	1,298
CD54 Media:Live Cells	10,000	90.98	***	90.98	1,173
CD86 DMSO:All Events	10,894	***	***	100.00	2,183
CD86 DMSO:Live Cells	10,000	91.79	***	91.79	1,887
CD54 DMSO:All Events	10,853	***	***	100.00	1,266
CD54 DMSO:Live Cells	10,000	92.14	***	92.14	1,166
CD86 DNCB 1:All Events	13,638	***	***	100.00	6,805
CD86 DNCB 1:Live Cells	9,999	73.32	***	73.32	5,863
CD54 DNCB 1:All Events	13,914	***	***	100.00	2,710
CD54 DNCB 1:Live Cells	9,995	71.83	***	71.83	2,589
CD86 DNCB 2A:All Events	13,418	***	***	100.00	6,653
CD86 DNCB 2A:Live Cells	10,000	74.53	***	74.53	5,663
CD86 DNCB 3:All Events	15,324	***	***	100.00	3,827
CD86 DNCB 3:Live Cells	10,000	65.26	***	65.26	2,353
CD54 DNCB 2A:All Events	13,914	***	***	100.00	2,703
CD54 DNCB 2A:Live Cells	9,945	71.47	***	71.47	2,530
CD54 DNCB 3:All Events	15,436	***	***	100.00	2,619
CD54 DNCB 3:Live Cells	10,000	64.78	***	64.78	2,480
CD86 PrNQ 1:All Events	10,988	***	***	100.00	2,406
CD86 PrNQ 1:Live Cells	10,000	91.01	***	91.01	2,086
CD54 PrNQ 1:All Events	10,814	***	***	100.00	1,199
CD54 PrNQ 1:Live Cells	10,000	92.47	***	92.47	1,117
CD86 PrNQ 2:All Events	10,801	***	***	100.00	2,196
CD86 PrNQ 2:Live Cells	10,000	92.58	***	92.58	1,976
CD54 PrNQ 2:All Events	10,812	***	***	100.00	1,184
CD54 PrNQ 2:Live Cells	10,000	92.49	***	92.49	1,109
CD86 PrNQ 3:All Events	10,965	***	***	100.00	2,335
CD86 PrNQ 3:Live Cells	10,000	91.20	***	91.20	2,035
CD54 PrNQ 3:All Events	10,908	***	***	100.00	1,239
CD54 PrNQ 3:Live Cells	10,000	91.68	***	91.68	1,150
CD86 PrNQ 4:All Events	10,803	***	***	100.00	2,133
CD86 PrNQ 4:Live Cells	10,001	92.58	***	92.58	1,882
CD54 PrNQ 4:All Events	10,855	***	***	100.00	1,209
CD54 PrNQ 4:Live Cells	9,979	91.93	***	91.93	1,131
CD86 PrNQ 5:All Events	10,860	***	***	100.00	2,098
CD86 PrNQ 5:Live Cells	10,000	92.08	***	92.08	1,854
CD54 PrNQ 5:All Events	10,928	***	***	100.00	1,248
CD54 PrNQ 5:Live Cells	10,000	91.51	***	91.51	1,149
CD86 PrNQ 6:All Events	10,923	***	***	100.00	2,110
CD86 PrNQ 6:Live Cells	10,000	91.55	***	91.55	1,853
CD54 PrNQ 6:All Events	10,897	***	***	100.00	1,217
CD54 PrNQ 6:Live Cells	10,000	91.77	***	91.77	1,122
CD86 PrNQ 7:All Events	10,856	***	***	100.00	2,183
CD86 PrNQ 7:Live Cells	10,000	92.11	***	92.11	1,940
CD54 PrNQ 7:All Events	10,818	***	***	100.00	1,223
CD54 PrNQ 7:Live Cells	10,001	92.45	***	92.45	1,126
CD86 PrNQ 8:All Events	10,975	***	***	100.00	2,184
CD86 PrNQ 8:Live Cells	10,000	91.12	***	91.12	1,951
CD54 PrNQ 8:All Events	11,092	***	***	100.00	1,277
CD54 PrNQ 8:Live Cells	10,000	90.16	***	90.16	1,156

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Table G-3. h-CLAT Experiment 1 Data Analysis

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
Media		0	93.88	995	1796	1	100		1173	1	100	
DMSO		0	93.36	960	1887	1.16	116		1166	1.16	116	
DNCB Control	1.67	0.0033	77.74	1136	5863	5.10	510		2589	8.16	816	
	2	0.004	78.58	1082	5663	4.94	494		2530	8.13	813	
	2.4	0.0048	75.32	1157	2353	1.29	129		2480	7.43	743	
PrNQ	139.5	0.279	92.87	982	2086	1.19	119		1117	0.66	66	
6/28/2019	167.4	0.335	92.06	936	1976	1.12	112		1109	0.84	84	
	200.9	0.402	94.16	953	2035	1.17	117		1150	0.96	96	
	241.1	0.482	92.82	977	1882	0.98	98		1131	0.75	75	
	289.4	0.579	92.23	967	1854	0.96	96		1149	0.88	88	
	347.2	0.694	93.08	949	1853	0.98	98		1122	0.84	84	
	416.7	0.833	92.75	968	1940	1.05	105		1126	0.77	77	
	500	1.00	92.44	964	1951	1.06	106		1156	0.93	93	

Legend:
mg/mL = milligrams per milliliter
IgG = Immunoglobulin G
FITC = fluorescein isothiocyanate
RFI = Relative Fluorescence Intensity
DMSO = dimethyl sulfoxide
DNCB = 2,4-dinitrochlorobenzene
PrNQ = N-Propylnitroguanidine

h-CLAT Experiment 2 Raw Data

Experiment: hCLAT Ammonia Borane PrNQ HEATN #2 7-5-19
Protocol 49-iv19-03-01 Mod F

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	835
Live Cells:Live Cells	9,491	94.91	***	94.91	805
Dead Cells:All Events	10,000	***	***	100.00	1,805
Dead Cells:Live Cells	197	1.97	***	1.97	1,327
IgG Media:All Events	10,534	***	***	100.00	836
IgG Media:Live Cells	10,000	94.93	***	94.93	806
IgG DMSO:All Events	10,628	***	***	100.00	887
IgG DMSO:Live Cells	10,000	94.09	***	94.09	835
IgG DNCB 1:All Events	12,413	***	***	100.00	937
IgG DNCB 1:Live Cells	9,998	80.54	***	80.54	899
IgG DNCB 2A:All Events	12,499	***	***	100.00	969
IgG DNCB 2A:Live Cells	9,960	79.69	***	79.69	910
IgG DNCB 3:All Events	14,316	***	***	100.00	1,231
IgG DNCB 3:Live Cells	10,000	69.85	***	69.85	1,016
IgG PrNQ 1:All Events	10,585	***	***	100.00	834
IgG PrNQ 1:Live Cells	10,000	94.47	***	94.47	795
IgG PrNQ 2:All Events	10,615	***	***	100.00	829
IgG PrNQ 2:Live Cells	10,000	94.21	***	94.21	794
IgG PrNQ 3:All Events	10,698	***	***	100.00	884
IgG PrNQ 3:Live Cells	10,000	93.48	***	93.48	829
IgG PrNQ 4:All Events	10,643	***	***	100.00	860
IgG PrNQ 4:Live Cells	10,000	93.96	***	93.96	819
IgG PrNQ 5:All Events	10,624	***	***	100.00	851
IgG PrNQ 5:Live Cells	10,000	94.13	***	94.13	806
IgG PrNQ 6:All Events	10,794	***	***	100.00	889
IgG PrNQ 6:Live Cells	10,000	92.64	***	92.64	818
IgG PrNQ 7:All Events	10,588	***	***	100.00	865
IgG PrNQ 7:Live Cells	10,000	94.45	***	94.45	812
IgG PrNQ 8:All Events	10,577	***	***	100.00	836
IgG PrNQ 8:Live Cells	10,000	94.54	***	94.54	795
CD86 Media:All Events	10,640	***	***	100.00	1,568
CD86 Media:Live Cells	10,000	93.98	***	93.98	1,432
CD54 Media:All Events	10,556	***	***	100.00	1,083
CD54 Media:Live Cells	10,000	94.73	***	94.73	1,027
CD86 DMSO:All Events	10,591	***	***	100.00	1,574
CD86 DMSO:Live Cells	10,000	94.42	***	94.42	1,428
CD54 DMSO:All Events	10,598	***	***	100.00	1,067
CD54 DMSO:Live Cells	10,000	94.36	***	94.36	1,013
CD54 DNCB 1:All Events	13,037	***	***	100.00	2,263
CD54 DNCB 1:Live Cells	10,000	76.70	***	76.70	2,310
CD86 DNCB 2A:All Events	12,952	***	***	100.00	4,781
CD86 DNCB 2A:Live Cells	10,000	77.21	***	77.21	4,245
CD54 DNCB 2A:All Events	12,498	***	***	100.00	1,979
CD54 DNCB 2A:Live Cells	9,999	80.00	***	80.00	2,135
CD86 DNCB 3 SKIPPED:All Events	***	***	***	***	***
CD86 DNCB 3 SKIPPED:Live Cells	***	***	***	***	***
CD54 DNCB 3 SKIPPED:All Events	***	***	***	***	***
CD54 DNCB 3 SKIPPED:Live Cells	***	***	***	***	***
CD86 PrNQ 1:All Events	10,740	***	***	100.00	1,778
CD86 PrNQ 1:Live Cells	10,000	93.11	***	93.11	1,595
CD54 PrNQ 1:All Events	10,709	***	***	100.00	1,115
CD54 PrNQ 1:Live Cells	10,000	93.38	***	93.38	1,051
CD86 PrNQ 2:All Events	10,706	***	***	100.00	1,707
CD86 PrNQ 2:Live Cells	10,000	93.41	***	93.41	1,548
CD54 PrNQ 2:All Events	10,645	***	***	100.00	1,057
CD54 PrNQ 2:Live Cells	10,000	93.94	***	93.94	1,008
CD86 PrNQ 3:All Events	10,620	***	***	100.00	1,729
CD86 PrNQ 3:Live Cells	10,000	94.16	***	94.16	1,577
CD54 PrNQ 3:All Events	10,679	***	***	100.00	1,114
CD54 PrNQ 3:Live Cells	10,000	93.64	***	93.64	1,043
CD86 PrNQ 4:All Events	10,644	***	***	100.00	1,689
CD86 PrNQ 4:Live Cells	10,000	93.95	***	93.95	1,536
CD54 PrNQ 4:All Events	10,645	***	***	100.00	1,094
CD54 PrNQ 4:Live Cells	10,000	93.94	***	93.94	1,033
CD86 PrNQ 5:All Events	10,725	***	***	100.00	1,818
CD86 PrNQ 5:Live Cells	10,000	93.24	***	93.24	1,631
CD54 PrNQ 5:All Events	10,755	***	***	100.00	1,113
CD54 PrNQ 5:Live Cells	9,990	92.89	***	92.89	1,038
CD86 PrNQ 6:All Events	10,678	***	***	100.00	1,727
CD86 PrNQ 6:Live Cells	10,000	93.65	***	93.65	1,551
CD54 PrNQ 6:All Events	10,901	***	***	100.00	1,148
CD54 PrNQ 6:Live Cells	10,000	91.73	***	91.73	1,050
CD86 PrNQ 7:All Events	10,698	***	***	100.00	1,703
CD86 PrNQ 7:Live Cells	10,000	93.48	***	93.48	1,535
CD54 PrNQ 7:All Events	10,760	***	***	100.00	1,143
CD54 PrNQ 7:Live Cells	10,000	92.94	***	92.94	1,064
CD86 PrNQ 8:All Events	10,755	***	***	100.00	1,665
CD86 PrNQ 8:Live Cells	10,000	92.98	***	92.98	1,508
CD54 PrNQ 8:All Events	10,803	***	***	100.00	1,162
CD54 PrNQ 8:Live Cells	10,000	92.57	***	92.57	1,081

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Software: BD FACSuite v1.0.5
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Table G-4. h-CLAT Experiment 2 Data Analysis

	Stock Concentration (mg/mL)	**Concentration (mg/mL)**	**Viability (IgG)**	**FITC IgG**	**FITC CD86**	**RFI**	**% change**	**EC150**	**FITC CD54**	**RFI**	**% change**	**EC200**
Media		0	94.93	806	1432	1	100		1027	1	100	
DMSO		0	94.09	835	1428	0.947	94.7		1013	0.805	80.5	
DNCB Control	1.67	0.0033	80.54	899	3779	3.11	311		2310	7.93	793	
	2	0.004	79.69	910	4245	3.60	360		2135	6.88	688	
PrNQ	139.5	0.279	94.47	795	1595	1.35	135		1051	1.44	144	
7/5/2019	167.4	0.335	94.21	794	1548	1.27	127		1008	1.20	120	
	200.9	0.402	93.48	829	1577	1.26	126		1043	1.20	120	
	241.1	0.482	93.96	819	1536	1.21	121		1033	1.20	120	
	289.4	0.579	94.13	806	1631	1.39	139		1038	1.30	130	
	347.2	0.694	92.64	818	1551	1.24	124		1050	1.30	130	
	416.7	0.833	94.45	812	1565	1.27	127		1064	1.42	142	
	500	1.00	94.54	795	1508	1.20	120		1081	1.61	161	

Legend
h-CLAT = human cell line activation test
mg/mL = milligrams per milliliter
IgG = Immunoglobulin G
FITC = fluorescein isothiocyanate
RFI = Relative Fluorescence Intensity
DMSO = dimethyl sulfoxide
DNCB = 2,4-dinitrochlorobenzene
PrNQ = N-Propylnitroguanidine

Table G-5. Acute Oral Hazard Estimation Example

Test #1				Test #2			
Concentration (µg/mL)	Log Concentration	Viability	1,000-viability	Concentration (µg/mL)	Log Concentration	Viability	1,000-viability
7.81	0.893	94.8	905.2	7.81	0.893	96.07	903.93
15.63	1.194	94.8	905.2	15.63	1.194	96.18	903.82
31.3	1.49	94.48	905.52	31.3	1.49	96.74	903.26
62.5	1.80	95.07	904.93	62.5	1.80	95.44	904.56
125	2.10	95.27	904.73	125	2.10	95.86	904.14
250	2.40	94.86	905.14	250	2.40	95.92	904.08
500	2.70	94.22	905.78	500	2.70	96.01	903.99
1,000	3	93.89	906.11	1000	3	95.25	904.75
	Log Concentration	Viability	Desired LD		Log Concentration	Viability	Desired LD
>50%	2.70	94.22	50	>50%	2.70	96.01	50
<50%	3	93.89		<50%	3	95.25	
	Slope =	-1.01			Slope =	-2.52	
	Intercept	97.18			Intercept	102.8	
X	43.04			X	20.92		
IC50	1.09E+43			IC50	8.38E+20		
LOG LD_{50} (mg/kg)	18.033			LOG LD_{50} (mg/kg)	9.81		
LD_{50} (mg/kg)	1.08E+18			LD_{50} (mg/kg)	6418274863		

Legend:
µg/mL = micrograms per milliliter
mg/kg = milligrams per kilogram

DPRA Data

Raw Data Cysteine #1

Pr NQ and OFHDA First HC4

Summary

Sequence Details			
Name:	DP-DPRA-OFHDA_OFHMBS_PrQ-13_AUG20	*Created On:*	26/Feb/19 14:30:42
Directory:	DNK\Data	*Created By:*	US Army
Data Vault:	ChromeleonLocal	*Updated On:*	17/Aug/20 08:24:34
No. of Injections:	37	*Updated By:*	US Army

By Component Cystene

No.	Injection Name	Ret.Time min Cystene UV_VIS_1	Area mAU*min Cystene UV_VIS_1	Height mAU Cystene UV_VIS_1	Amount mM peptide Cystene UV_VIS_1	Rel.Area % Cystene UV_VIS_1	Peak Type Cystene UV_VIS_1
1	ACN blk	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	STD 7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	STD 6	10.942	1.073	10.461	0.018	100.00	M
6	STD 5	10.933	1.958	21.030	0.032	100.00	M
7	STD 4	10.933	4.252	42.545	0.069	96.44	M
8	STD 3	10.927	8.076	82.462	0.132	98.18	M
9	STD 2	10.923	15.715	154.266	0.267	98.27	M
10	STD 1	10.913	28.306	268.935	0.534	96.50	M
11	ref control A	10.897	26.874	255.608	0.500	96.89	M
12	ref control A	10.917	27.037	254.258	0.504	97.02	M
13	ref control A	10.910	26.822	253.686	0.499	96.94	M
14	ref control B	10.917	27.256	256.986	0.509	96.56	M
15	ref control B	10.908	27.188	254.557	0.507	96.43	M
16	ref control B	10.903	27.072	255.791	0.505	96.58	M
17	ref control C	10.910	26.862	253.837	0.500	97.04	M
18	Co-el OFHDA	10.900	0.152	1.068	0.004	100.00	M
19	Co-el PrQ	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
20	Cinnamaldehyde	10.928	6.066	59.331	0.098	98.05	M
21	OFHDA	10.903	24.705	234.889	0.451	96.47	M
22	PrQ	10.922	25.850	243.911	0.476	96.42	M
23	reference control C	10.910	26.558	251.530	0.493	96.96	M
24	Cinnamaldehyde	10.800	0.066	1.539	0.003	1.13	M
25	OFHDA	10.902	24.560	232.748	0.447	96.71	M
26	PrQ	10.903	25.818	244.939	0.471	96.58	M
27	reference control C	10.902	26.120	247.313	0.482	96.31	M
28	Cinnamaldehyde	10.922	5.516	54.287	0.089	98.90	M
29	OFHDA	10.918	24.168	229.861	0.439	97.23	M
30	PrQ	10.912	25.210	240.936	0.462	97.18	M
31	reference control C	10.900	26.103	247.237	0.482	96.56	M
32	Cinnamaldehyde	10.925	5.394	53.301	0.087	98.80	M
33	Reference control B	10.905	26.104	248.058	0.482	97.18	M
34	Reference control B	10.900	26.069	248.302	0.481	96.74	M
35	Reference control B	10.895	26.015	247.318	0.480	96.57	M
36	10% ACN	10.922	0.372	1.521	0.007	100.00	M
37	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Raw Data Lysine #1

Summary PRNQ and OFHDA first "K"

ce Details

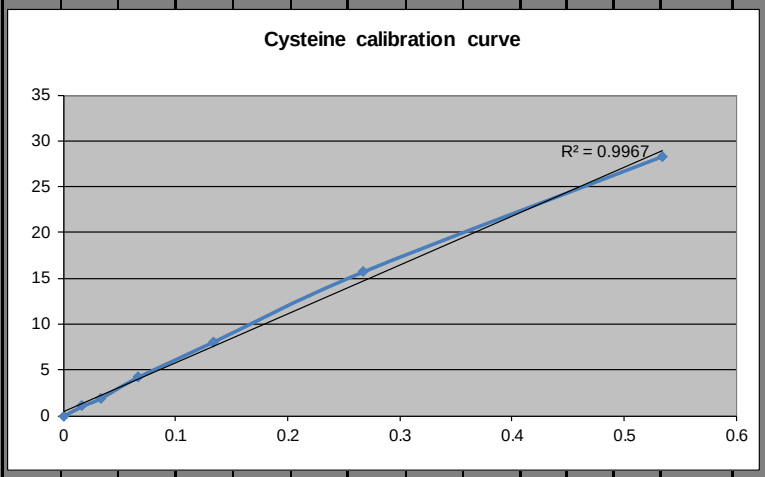
	DP-DPRA-OFHDA_PrQ-K-18_AUG20	Created On:	26/Feb/19 14:30:42
:	DNK\Data	Created By:	US Army
lt:	ChromeleonLocal	Updated On:	21/Aug/20 10:00:11
ections:	37	Updated By:	US Army

ponent Lysene

Injection Name	Ret.Time min Lysene UV_VIS_1	Area mAU*min Lysene UV_VIS_1	Height mAU Lysene UV_VIS_1	Amount mM peptide Lysene UV_VIS_1	Rel.Area % Lysene UV_VIS_1	Pea Ly UV
ACN blk	n.a.	n.a.	n.a.	n.a.	n.a.	
10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	
10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	
STD 7	8.275	31.347	266.218	0.534	100.00	E
STD 6	8.297	15.599	154.287	0.267	100.00	E
STD 5	8.307	7.747	84.787	0.134	100.00	E
STD 4	8.332	3.834	44.309	0.067	100.00	E
STD 3	8.328	1.724	21.859	0.031	100.00	E
STD 2	8.343	0.942	11.372	0.018	100.00	E
STD 1	n.a.	n.a.	n.a.	n.a.	n.a.	
ref control A	8.270	29.717	256.860	0.506	100.00	E
ref control A	8.263	29.746	258.696	0.507	100.00	E
ref control A	8.267	29.883	256.997	0.509	100.00	E
ref control B	8.265	29.537	254.245	0.503	100.00	E
ref control B	8.272	29.588	256.325	0.504	100.00	E
ref control B	8.273	29.409	255.046	0.501	99.75	E
ref control C	8.267	30.186	259.348	0.514	100.00	E
Co-el OFHDA	10.770	0.129	0.814	0.004	27.49	
Co-el PrQ	n.a.	n.a.	n.a.	n.a.	n.a.	
Cinnamaldehyde	8.320	6.872	72.020	0.119	96.97	E
OFHDA	8.203	29.445	217.510	0.502	98.99	E
PrQ	8.263	29.826	256.090	0.508	100.00	E
reference control C	8.270	29.660	257.456	0.505	99.59	E
Cinnamaldehyde	8.312	7.000	73.650	0.121	96.69	E
OFHDA	8.207	29.758	219.681	0.507	99.06	E
PrQ	8.277	29.892	256.848	0.509	100.00	E
reference control C	8.278	29.663	255.136	0.505	100.00	E
Cinnamaldehyde	8.315	7.390	76.066	0.128	98.50	E
OFHDA	8.198	29.770	218.174	0.507	98.63	E
PrQ	8.270	29.876	255.688	0.509	99.51	E
reference control C	8.277	29.666	255.296	0.506	100.00	E
Cinnamaldehyde	8.303	7.793	78.493	0.134	97.02	E
Reference control B	8.277	29.344	253.270	0.500	100.00	E
Reference control B	8.263	29.288	254.585	0.499	100.00	E
Reference control B	8.268	29.438	254.817	0.502	100.00	E
10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	
10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	

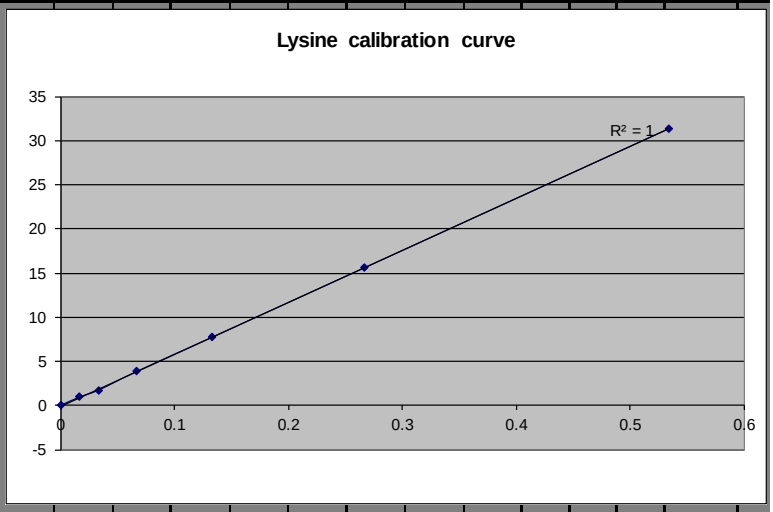
DPRA Cysteine #1 Calculations

Vehicle	Code	Conc/ replicate	Cysteine: Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Precipitates	Re-solub. Or Centrif.	Absorbs at 220 nm	Retent. Time similar to pept.	Co-elution
	STANDARD																			
		STD1	28	0.5																
		STD2	16	0.3																
		STD3	8.1	0.1																
		STD4	4.3	0.1																
		STD5	2	0																
		STD6	1.1	0																
		Dil Buff	0	0																
	REF CTRL A																			
		r1	26.874	0.49541066																
		r2	27	0.498470249						**0.50**	**0.00**	**0.00**								
		r3	27	0.494434595																
	REF CTRL B																			
		r1	27.256	0.502580986																
		r2	27.188	0.501304593																
		r3	27.072	0.499127216			**27.172**	**0.093**	**0.0034**	**0.25**	**0.28**	**1.14**								
		r4		-0.009027338																
		r5		-0.009027338																
		r6		-0.009027338																
Acetonitrile	**REF CTRL C**																			
		r1	26.862	0.495185415																
		r2	27	0.489479187			**26.508**	**0.382**	**0.0144**	**0.4885**	**0.0072**	**0.0147**								
		r3	26	0.480938617																
Acetonitrile	**POSITIVE CONTROL**																			
	Cinnamic Aldehyde	r1	6.0066	0.103719454	0.773401406	0.773401406														
		r2	6	0.094510654	0.791909259	0.791909259	**5.6389**	**0.3243**	**0.0575**	**0.0968**	**0.0061**	**0.0629**	**0.7873**	**0.0122**	**0.0155**					
		r3	5	0.092220655	0.796511701	0.796511701														
Acetonitrile	**CHEMICAL 1**																			
	PrNQ 1	r1	25.85	0.476189684	0.024810432	0.024810432														
		r2	26	0.471853703	0.033524892	0.033524892	**25.56**	**0.3241**	**0.0127**	**0.4707**	**0.0061**	**0.0129**	**0.0358**	**0.0122**	**0.3419**	N	Y/N	Y/N	N	Y/N
		r3	25	0.464176574	0.048954391	0.048954391														



DPRA Lysine#1 Calculations

Vehicle	Code	Conc/ replicate	Lysine: Peak Area at 220 nm	Lysine: Peptide Concentration (mM)	Lysine: Peptide Depletion (%)	Lysine: CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Precipitates	Re-solub. Or Centrif.	Absorbs at 220 nm	Retent. Time similar to pept.	Co-elution
	STANDARD																			
		STD1	31	0.5																
		STD2	16	0.3																
		STD3	7.7	0.1																
		STD4	3.8	0.1																
		STD5	1.7	0																
		STD6	0.9	0																
		Dil Buff	0	0																
	REF CTRL A																			
		r1	29.717	0.506615991						0.5077	0.0015	0.003								
		r2	29.746	0.507108787																
		r3	29.883	0.509436826																
	REF CTRL B																			
		r1	29.537	0.503557254																
		r2	29.588	0.504423896																
		r3	29.409	0.501382152			**29.434**	**0.1135**	**0**	**0.5018**	**0.0019**	**0.0038**								
		r4	29.344	0.500277608																
		r5	29.288	0.499326001																
		r6	29.438	0.501874948																
Acetonitrile	**REF CTRL C**																			
		r1	29.66	0.505647391																
		r2	29.663	0.50569837			**29.67**	**0.0142**	**0.0005**	**0.5058**	**0.0002**	**0.0005**								
		r3	29.686	0.506089208																
Acetonitrile	**POSITIVE CONTROL**																			
	Cinnamic Aldehyde	r1	6.872	0.118411261	0.768382973	0.768382973														
		r2	7	0.120586363	0.764068802	0.764068802	**7.0873**	**0.2698**	**0.0381**	**0.1221**	**0.0046**	**0.0376**	**0.7611**	**0.0091**	**0.0119**					
		r3	7.39	0.127213627	0.750924064	0.750924064														
Acetonitrile	**CHEMICAL 1**																			
	PrNQ 1	r1	29.826	0.508468226	-0.00526913	0														
		r2	29.802	0.508060395	-0.004460223	0	**29.835**	**0.0378**	**0.0013**	**0.5086**	**0.0006**	**0.0013**	**0**	**0**	######	N	N	N	N	N
		r3	29.876	0.509317875	-0.006954353	0														



QC data Cysteine and Lysine #1

Vehicle	Code	Criterion	Criterion met?		
	STANDARD				
		Lysine: r2 > 0,99	Lys R2	0.999955464	**YES**
			Lys Intercept	-0.096243936	
		Cysteine: r2 > 0,99	Lys Slope	58.84781466	
			Cys R2	0.996684435	**YES**
			Cys Intercept	0.480932599	
			Cys Slope	53.27513012	
	REF CTRL A				
		Range Mean Conc Cys [0.45 to 0.55]			**YES**
		Range Mean Conc Lys [0.45 to 0.55]			**YES**
	REF CTRL B				
		Peak Area CV ctrl B < 15% - Cys			**YES**
		Peak Area CV ctrl B < 15% - Lys			**YES**
Acetonitrile	**REF CTRL C**	Peak Area CV ctrl C < 15% - Cys			**YES**
		Peak Area CV ctrl C < 15% - Lys			**YES**
		Range Mean Conc Cys [0.45 to 0.55]			**YES**
		Range Mean Conc Lys [0.45 to 0.55]			**YES**
Acetonitrile	**POSITIVE CONTROL**				
	Cinnamic Aldehyde	60,8 < Mean % Depl Cys < 96,6			**YES**
		40,2< Mean % Depl Lys < 69,4			**Not Met**
		SD % Depl Cys < 14,9			**YES**
		SD % Depl Lys < 11,6			**YES**
Acetonitrile	**CHEMICAL 1**				
	PrNQ 1	SD % Depl Cys < 14,9			**YES**
		SD % Depl Lys < 11,6			**YES**

Cinnamaldehyde is referred to as cinnamic aldehyde in this table. The cinnamaldehyde depletion for lysine was greater than the preferred mean percentage range. Still consider that this was a successful assay. Additionally, lysine is not required to make a call for sensitization.

Raw Data Cysteine #2

Summary

Sequence Details

Name:	DP-DPRA-PrQ-IAMMDA52-OFHBS-28_AUG20	*Created On:*	26/Feb/19 14:30:42
Directory:	DNK\Data	*Created By:*	US Army
Data Vault:	ChromeleonLocal	*Updated On:*	31/Aug/20 08:31:48
No. of Injections:	47	*Updated By:*	US Army

By Component Cystene

No.	Injection Name	Ret.Time min Cystene UV_VIS_1	Area mAU*min Cystene UV_VIS_1	Height mAU Cystene UV_VIS_1	Amount mM peptide Cystene UV_VIS_1	Rel.Area % Cystene UV_VIS_1	Peak Type Cystene UV_VIS_1
1	ACN blk	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	STD 7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	STD 6	10.932	1.300	11.791	0.018	100.00	M
6	STD 5	10.918	2.429	24.027	0.033	100.00	M
7	STD 4	10.922	4.731	47.919	0.066	98.47	M
8	STD 3	10.908	9.265	92.953	0.133	98.72	M
9	STD 2	10.900	17.731	173.371	0.267	97.06	M
10	STD 1	10.888	32.156	295.820	0.534	100.00	BMB*
11	ref control A	10.885	31.912	294.345	0.529	100.00	BMB*
12	ref control A	10.897	31.494	295.691	0.520	100.00	BMB*
13	ref control A	10.888	31.611	294.979	0.523	100.00	BMB*
14	ref control B	10.895	29.234	270.118	0.475	100.00	BMB*
15	ref control B	10.885	28.975	268.435	0.470	100.00	BMB*
16	ref control B	10.877	28.938	268.485	0.469	100.00	BMB*
17	ref control C	10.880	31.077	283.797	0.512	100.00	BMB*
18	Acetone	10.888	29.986	274.196	0.490	100.00	BMB*
19	DMSO	10.907	14.260	140.313	0.211	100.00	BMB*
20	Co-el PrQ	10.900	0.144	0.862	0.002	50.16	M
21	Co-el IAMMDA52	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
22	Co-el OFHMBS	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
23	Cinnamaldehyde	10.915	7.465	73.476	0.106	98.84	M
24	PrNQ	10.883	30.718	281.527	0.504	100.00	BMB*
25	IAMMDA52	10.830	0.631	5.427	0.008	100.00	M
26	OFHMBS	10.887	29.370	268.530	0.477	100.00	BMB*
27	reference control C	10.878	30.001	277.215	0.490	100.00	BMB*
28	Acetone	10.877	28.675	263.433	0.464	100.00	BMB*
29	DMSO	10.895	12.207	120.591	0.178	100.00	BMB*
30	Cinnamaldehyde	10.902	7.206	70.285	0.102	99.07	BM
31	PrNQ	10.888	30.625	278.525	0.503	100.00	BMB*
32	IAMMDA52	10.830	0.689	5.995	0.009	100.00	M
33	OFHMBS	10.887	28.790	266.009	0.466	100.00	BMB*
34	reference control C	10.888	29.435	272.405	0.479	100.00	BMB*
35	Acetone	10.880	27.745	255.756	0.448	100.00	BMB*
36	DMSO	10.900	11.037	109.559	0.160	100.00	BMB*
37	Cinnamaldehyde	10.898	6.656	64.162	0.094	97.26	M
38	PrNQ	10.878	30.105	276.888	0.492	100.00	BMB*
39	IAMMDA52	10.818	0.764	6.540	0.010	100.00	M
40	OFHMBS	10.885	28.200	261.218	0.455	98.01	M
41	reference control C	10.890	28.984	266.286	0.470	100.00	BMB*
42	Cinnamaldehyde	10.897	6.423	64.037	0.091	97.79	M
43	Reference control B	10.885	26.404	246.430	0.420	100.00	BMB*

44	Reference control B	10.875	26.526	245.157	0.423	100.00	BMB*
45	Reference control B	10.882	26.401	245.491	0.420	100.00	BMB*
46	10% ACN	10.912	0.139	1.161	0.001	100.00	M
47	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Raw Data Lysine #2

Summary

Sequence Details

Name:	DP-DPRA-HEATN_COGA_RUN2_OFHDA_PrN	*Created On:*	26/Feb/19 14:30:42
Directory:	DNK\Data	*Created By:*	US Army
Data Vault:	ChromeleonLocal	*Updated On:*	28/Aug/20 08:23:39
No. of Injections:	44	*Updated By:*	US Army

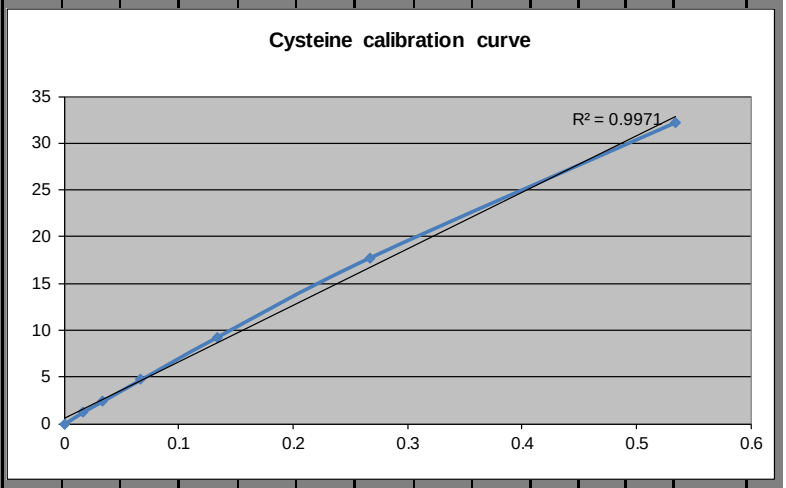
By Component Lysine

No.	Injection Name	Ret.Time min Lysine UV_VIS_1	Area mAU*min Lysine UV_VIS_1	Height mAU Lysine UV_VIS_1	Amount mM peptide Lysine UV_VIS_1	Rel.Area % Lysine UV_VIS_1	Peak Type Lysine UV_VIS_1
1	ACN blk	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	STD 7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	STD 6	8.365	1.100	12.969	0.016	100.00	BMB*
6	STD 5	8.357	2.202	25.696	0.032	100.00	BMB*
7	STD 4	8.347	4.459	50.311	0.066	100.00	BMB*
8	STD 3	8.338	9.028	96.146	0.135	100.00	BMB*
9	STD 2	8.312	17.752	172.980	0.267	100.00	BMB*
10	STD 1	8.293	35.555	297.342	0.534	99.67	BMB*
11	ref control A	8.290	33.285	281.657	0.500	100.00	BMB*
12	ref control A	8.302	33.303	283.274	0.500	100.00	BMB*
13	ref control A	8.307	33.215	282.198	0.499	99.64	BMB*
14	ref control B	8.297	33.302	281.793	0.500	100.00	BMB*
15	ref control B	8.287	33.561	282.506	0.504	100.00	BMB*
16	ref control B	8.298	33.458	281.404	0.503	100.00	BMB*
17	ref control C	8.295	33.357	283.405	0.501	100.00	BMB*
18	Co_Elut_OFHDA	10.753	0.090	0.713	0.000	26.53	M
19	Co_Elut_HEATN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
20	Co_Elut_COGA	11.393	0.081	0.323	0.000	100.00	M
21	Cinnamaldehyde	8.355	6.781	71.322	0.101	98.83	BMB*
22	HEATN	8.303	33.087	282.281	0.497	100.00	BMB*
23	COGA	8.293	32.680	279.296	0.491	100.00	BMB*
24	OFHDA	8.225	33.381	241.303	0.501	98.57	BMB*
25	PrNQ	8.290	33.543	285.097	0.504	100.00	BMB*
26	reference control C	8.287	33.288	280.429	0.500	99.73	BMB*
27	Cinnamaldehyde	8.343	6.913	72.771	0.103	97.40	BMB*
28	HEATN	8.302	33.337	281.929	0.501	100.00	BMB*
29	COGA	8.295	32.910	278.833	0.494	99.91	BMB*
30	OFHDA	8.233	32.582	240.749	0.489	98.95	BMB*
31	PrNQ	8.297	33.302	283.684	0.500	100.00	BMB*
32	reference control C	8.292	33.486	283.184	0.503	100.00	BMB*
33	Cinnamaldehyde	8.332	6.745	73.325	0.101	97.00	BMB*
34	HEATN	8.293	33.348	281.157	0.501	100.00	BMB*
35	COGA	8.287	32.640	277.651	0.490	99.32	BMB*
36	OFHDA	8.218	32.949	239.657	0.495	98.73	BMB*
37	PrNQ	8.297	33.666	282.725	0.506	100.00	BMB*
38	reference control C	8.297	33.045	283.007	0.496	99.61	BMB*
39	Cinnamaldehyde	8.345	6.964	75.421	0.104	95.26	BMB*
40	Reference control B	8.287	33.726	284.064	0.507	100.00	BMB*
41	Reference control B	8.293	33.776	285.623	0.507	99.57	BMB*
42	Reference control B	8.288	33.483	285.421	0.503	100.00	BMB*
43	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

44	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

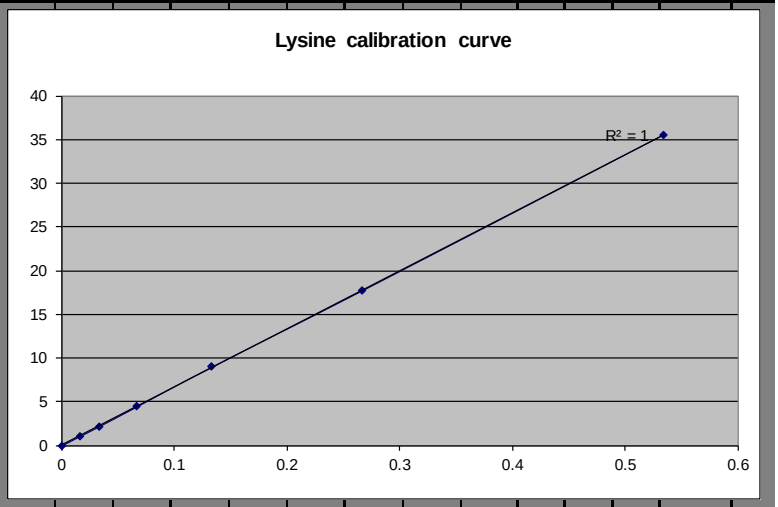
Calculations Cysteine #2

Vehicle	Code	Conc/ replicate	Cysteine: Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Precipitates	Re-solub. Or Centrif.	Absorbs at 220 nm	Retent. Time similar to pept.	Co-elution
	STANDARD																			
		STD1	32	0.5																
		STD2	18	0.3																
		STD3	9.3	0.1																
		STD4	4.7	0.1																
		STD5	2.4	0																
		STD6	1.3	0																
		Dil Buff	0	0																
	REF CTRL A																			
		r1	31.912	0.519047132																
		r2	31	0.512118757						**0.52**	**0.00**	**0.01**								
		r3	32	0.514058039																
	REF CTRL B																			
		r1	29.234	0.474659126																
		r2	28.975	0.470366186																
		r3	28.938	0.469752909			**27.746**	**1.4314**	**0.0516**	**0.45**	**0.02**	**0.05**								
		r4	26.404	0.427751713																
		r5	26.526	0.42977387																
		r6	26.401	0.427701987																
Acetonitrile	**REF CTRL C**																			
		r1	30.001	0.487372196																
		r2	29	0.477990713			**29.473**	**0.5096**	**0.0173**	**0.4786**	**0.0084**	**0.0176**								
		r3	29	0.470515362																
Acetonitrile	**POSITIVE CONTROL**																			
	Cinnamic Aldehyde	r1	7.206	0.109543751	0.755507804	0.755507804														
		r2	7	0.100427469	0.77416874	0.77416874	**6.7617**	**0.4021**	**0.0595**	**0.1022**	**0.0067**	**0.0652**	**0.7706**	**0.0136**	**0.0177**					
		r3	6	0.09656548	0.782074191	0.782074191														
Acetonitrile	**CHEMICAL 1**																			
	PrNQ 1	r1	30.718	0.499256512	-0.042230265	0														
		r2	31	0.497715032	-0.03907487	0	**30.483**	**0.3304**	**0.0108**	**0.4954**	**0.0055**	**0.0111**	**0**	**0**	######	Y/N	Y/N	Y/N	Y/N	Y/N
		r3	30	0.489096002	-0.021431803	0														



Calculations Lysine #2

Vehicle	Code	Conc/ replicate	Lysine: Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area mean	Peak Area SD	Peak Area CV	Peptide Conc mean	Peptide Conc SD	Peptide Conc CV	Peptide Depl. mean	Peptide Depl. SD	Peptide Depl. CV	Precipitates	Re-solub. Or Centrif.	Absorbs at 220 nm	Retent. Time similar to pept.	Co-elution
	STANDARD																			
		STD1	36	0.5																
		STD2	18	0.3																
		STD3	9	0.1																
		STD4	4.5	0.1																
		STD5	2.2	0																
		STD6	1.1	0																
		Dil Buff	0	0																
	REF CTRL A																			
		r1	33.285	0.499759578						0.4995	0.0007	0.0014								
		r2	33.303	0.500029968																
		r3	33.215	0.49870806																
	REF CTRL B																			
		r1	33.302	0.500014946																
		r2	33.561	0.503905562																
		r3	33.458	0.502358329			**33.551**	**0.177**	**0**	**0.5038**	**0.0027**	**0.0053**								
		r4	33.726	0.506384139																
		r5	33.776	0.507135224																
		r6	33.483	0.502733871																
Acetonitrile	**REF CTRL C**																			
		r1	33.357	0.500841139																
		r2	33.288	0.499804643			**33.377**	**0.1005**	**0.003**	**0.5011**	**0.0015**	**0.003**								
		r3	33.486	0.502778936																
Acetonitrile	**POSITIVE CONTROL**																			
	Cinnamic Aldehyde	r1	6.781	0.101624907	0.796836145	0.796836145														
		r2	6.913	0.103607769	0.792881325	0.792881325	**6.886**	**0.0944**	**0.0137**	**0.1032**	**0.0014**	**0.0137**	**0.7937**	**0.0028**	**0.0036**					
		r3	6.964	0.104373875	0.791353327	0.791353327														
Acetonitrile	**CHEMICAL 1**																			
	PrNQ 1	r1	33.543	0.503635172	-0.004973485	0														
		r2	33.302	0.500014946	0.002247056	0.002247056	**33.504**	**0.1852**	**0.0055**	**0.503**	**0.0028**	**0.0055**	**0.0007**	**0.0013**	**1.7321**	N	Y/N	Y	N	N
		r3	33.666	0.505482839	-0.008658657	0														



QC for Cysteine and Lysine #2

Vehicle	Code	Conc/ replicate	Mean Depletion	Reactivity Class (CYS + LYS)	Reactivity Class (CYS only)	Criterion	Criterion met?	
	STANDARD							
		STD1				Lysine: r2 > 0,99	Lys R2	0.999980117 **YES**
		STD2					Lys Intercept	0.015785278
		STD3				Cysteine: r2 > 0,99	Lys Slope	66.57043951
		STD4						
		STD5					Cys R2	0.997097033 **YES**
		STD6					Cys Intercept	0.597048683
		Dil Buff					Cys Slope	60.33161425
	REF CTRL A							
		r1				Range Mean Conc Cys [0.45 to 0.55]		**YES**
		r2						
		r3				Range Mean Conc Lys [0.45 to 0.55]		**YES**
	REF CTRL B							
		r1						
		r2				Peak Area CV ctrl B < 15% - Cys		**YES**
		r3						
		r4				Peak Area CV ctrl B < 15% - Lys		**YES**
		r5						
		r6						
Acetonitrile	**REF CTRL C**					Peak Area CV ctrl C < 15% - Cys		**YES**
		r1				Peak Area CV ctrl C < 15% - Lys		**YES**
		r2				Range Mean Conc Cys [0.45 to 0.55]		**YES**
		r3				Range Mean Conc Lys [0.45 to 0.55]		**YES**
Acetonitrile	**POSITIVE CONTROL**							
	Cinnamic Aldehyde	r1				60,8 < Mean % Depl Cys < 96,6		**YES**
		r2				40,2< Mean % Depl Lys < 69,4		**Not Met**
		r3				SD % Depl Cys < 14,9		**YES**
						SD % Depl Lys < 11,6		**YES**
Acetonitrile	**CHEMICAL 1**							
	PrNQ 1	r1	0	MINIMAL	MINIMAL	SD % Depl Cys < 14,9		**YES**
		r2				SD % Depl Lys < 11,6		**YES**
		r3						

Cinnamaldehyde is referred to as cinnamic aldehyde in this table. The cinnamaldehyde depletion was outside the specified preferred range for the lysine assay. It was depleted more than the preferred range, so is considered acceptable. Additionally, lysine is not required for a sensitization call.