Phase 1 Validation of the Electrophilic Allergen Screening Assay

J Strickland[1], J Gordon[2], J Hettick[3], B Law[3], E Petersen[4], N Solomotis[5], J Truax[1], R Uhl[2], J Yourick[5], D Allen[1], N Kleinstreuer[6]

[1]ILS, RTP, NC, USA

[2]CPSC, Rockville, MD, USA

[3]CDC/NIOSH/HELD, Morgantown, WV, USA

[4]NIST, Gaithersburg, MD, USA

[5]FDA/CFSAN, Laurel, MD, USA

[6]NIH/NIEHS/DNTP/NICEATM, RTP, NC, USA

Introduction

- The adverse outcome pathway for skin sensitization has been previously defined (OECD 2012a, 2012b).
- Covalent binding of an electrophilic chemical to a nucleophilic binding site on skin protein is a known molecular initiating event in this pathway (**Figure 1**).
- The electrophilic allergen screening assay (EASA) measures this event (Chipinda et al. 2010, 2011, 2014).
  - Two probe chemicals are used as surrogates for skin proteins.
  - Electrophilic chemicals covalently bind to one or both probe chemicals.
  - Binding is measured as depletion of probe absorbance or fluorescence (Figure 2).
- NICEATM is conducting a validation study to characterize usefulness and limitations of the EASA for classifying substances as sensitizers or nonsensitizers. The study will evaluate:
  - Intra- and inter-laboratory reproducibility
  - Accuracy for the classification of sensitizers and nonsensitizers relative to murine local lymph node assay (LLNA) and human outcomes
- This poster reports the results of the Phase 1 testing of 10 coded test chemicals.

Methods

EASA Workflow

- The EASA consists of three separate tests (**Table 1**):
  - An absorbance assay using 4-nitrobenzenethiol (NBT) as the probe chemical
  - An absorbance or fluorescence assay using pyridoxylamine (PDA) as the probe chemical
    - The PDA absorbance assay is run first (sample data shown in **Figure 2**).
    - The PDA fluorescence assay is run only if a test chemical interferes with PDA absorbance.
- If a test chemical has a positive response at any time in any assay, a positive outcome is assigned without further testing.
- **Figure 3** shows the EASA workflow and decision criteria used to assign a skin sensitization classification. Confirmation tests extend the incubation time to 240 min or double the test chemical concentration.

Phase 1 Testing Protocol

- Three laboratories tested 10 coded chemicals three times each to determine proficiency and to provide a preliminary assessment of reproducibility and accuracy.
- Each test was performed with triplicate cuvettes.

Table 1 Specifications of EASA Component Assays

	NBT Absorbance Assay	PDA Absorbance Assay	PDA Fluorescence Assay
Wavelength (nm)	412	324	324 excitation 398 emission
Molar ratio of test chemical to probe	2:1	5:1	5:1
Measurement times	5, 20, 120 min	5, 20, 120 min	5, 20, 120 min
Positive control	2,4-Dinitrochlorobenzene	Glutaraldehyde	Glutaraldehyde
Negative control	Solvent[a]	Solvent	Solvent
Negative response criterion	<10% depletion of absorbance at 120 min	<10% depletion of absorbance at 120 min	<15% depletion of fluorescence at 120 min
Positive response criterion	≥30% depletion of probe absorbance	≥30% depletion of probe absorbance	≥30% depletion of probe fluorescence

[a]Solvent is acetonitrile:0.1M phosphate buffer, pH 7.4 (1:1)

Figure 1 **Adverse Outcome Pathway for Skin Sensitization**

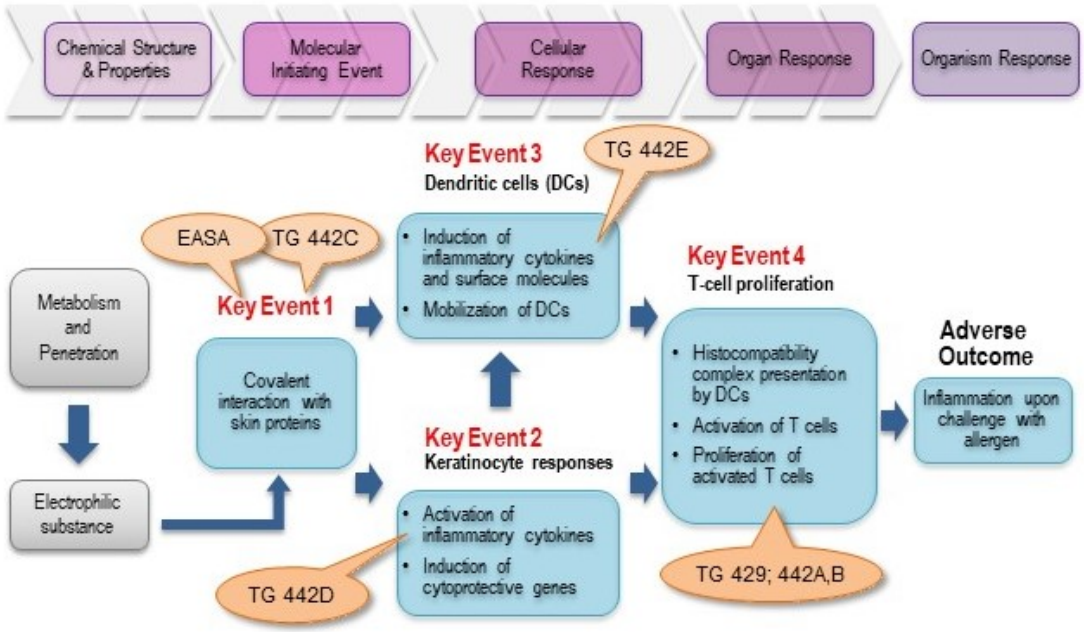


Figure 2 **Sample Data: PDA Absorbance Test**

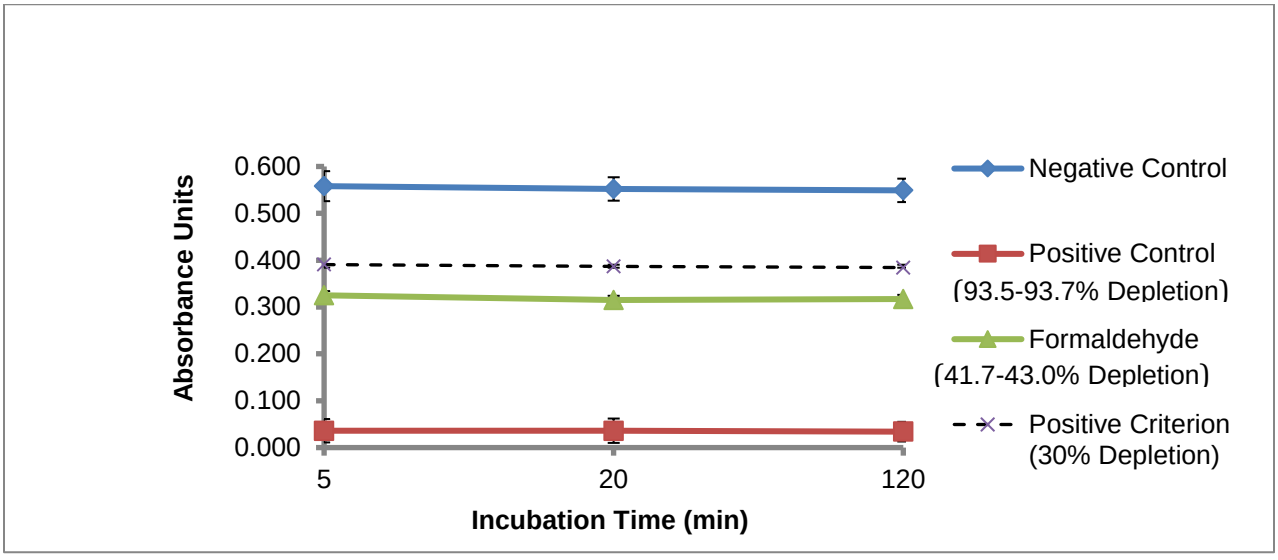
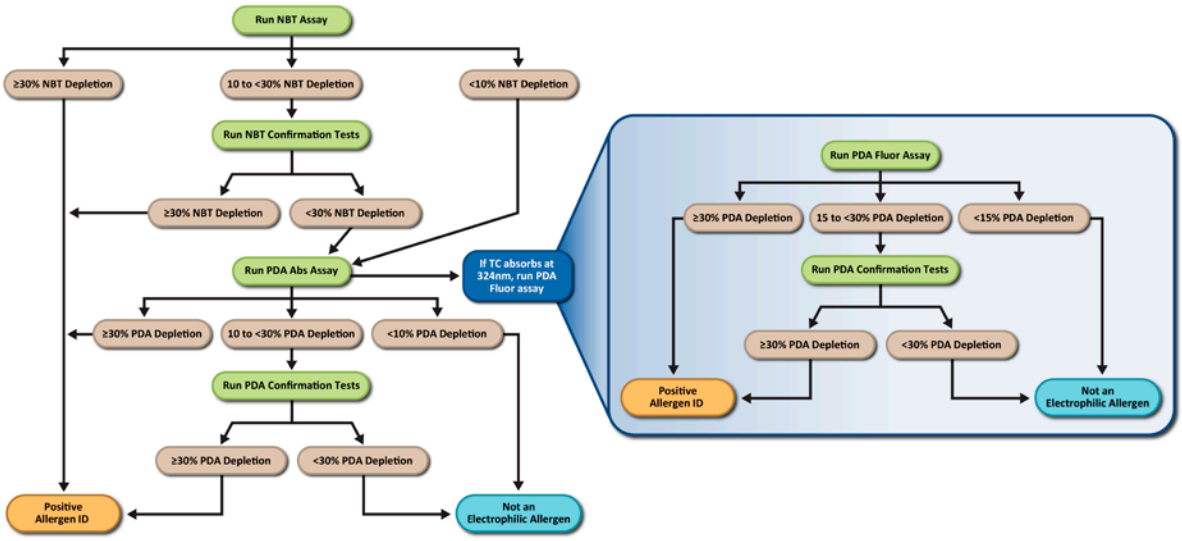


Figure 3 EASA Workflow and Decision Criteria



Results

Reproducibility of Triplicate Cuvette Measurements

- **Table 2** shows the coefficient of variation (CV) ranges for the triplicate cuvettes tested in each run for the negative and positive controls.
- For the 10 test chemicals:
  - Average CVs for the PDA absorbance assay (5 min time point) are shown in **Figure 4.**
  - In the NBT absorbance assay (5 min time point; data not shown), which was negative for all chemicals, average CVs ranged from 2% to 7%.
  - In the PDA fluorescence assay (5 min time point; data not shown), average CVs for the five chemicals tested ranged from 2 to 11%.

Reproducibility of Sensitizer vs. Nonsensitizer Classifications

- Reproducibility results for the test chemicals are shown in **Tables 3-5** for each independent test in the individual assays.
- Within each laboratory, the sensitizer or nonsensitizer classification results of the three runs were concordant for all 10 of the test chemicals.
- Classification results among all three laboratories were concordant for 9 of 10 test chemicals.

- The classification results between the FDA/CFSAN and CPSC/NIST laboratories were concordant for all 10 of the test chemicals.

Classification Accuracy

- **Table 6** shows classification accuracy results for all three laboratories relative to LLNA outcomes for all 10 chemicals and to human outcomes for nine chemicals.
- FDA/CFSAN and CPSC/NIST results correctly predicted LLNA outcomes for 7 of 10 test chemicals.
- NIOSH results correctly predicted LLNA outcomes for 6 of 10 test chemicals.
- FDA/CFSAN and CPSC/NIST correctly predicted human outcomes for 6 of 9 test chemicals.
- NIOSH correctly predicted human outcomes for 5 of 9 test chemicals.
- All misclassifications were false negatives.
  - 4-Phenylenediamine is a pre-hapten and would not be expected to be active in the EASA.
  - Poor solubility of squaric acid may have impacted its activity in the EASA assays.
  - 2,3-Butanedione is a weak sensitizer in the LLNA (EC3 = 11%); no human data could be located.
  - Sulfanilamide is positive in humans and negative in the LLNA. It was negative in the EASA.

Table 2 CV Ranges for Controls

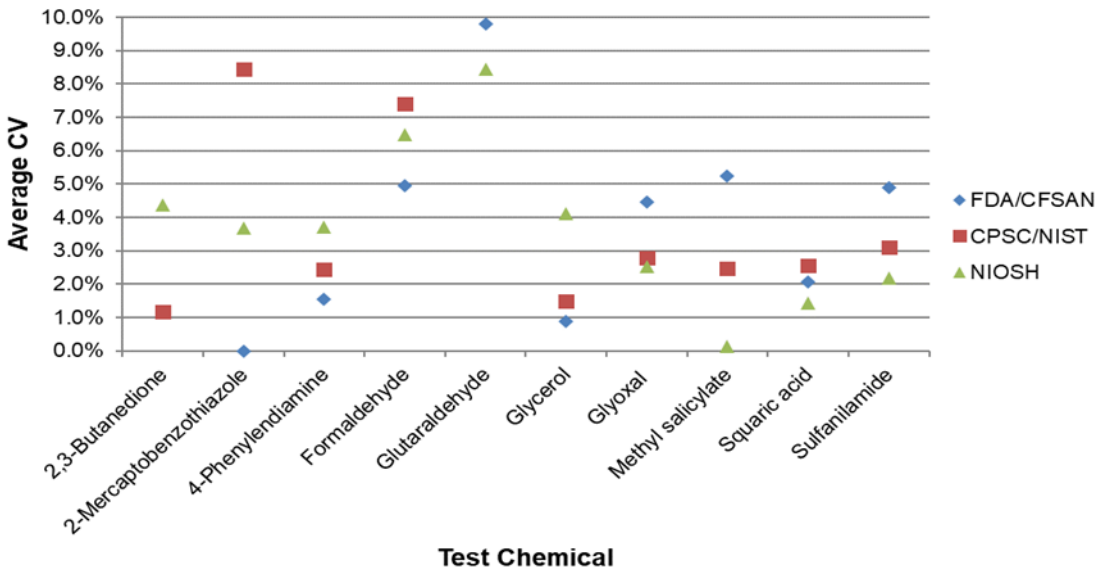
Test[a]	FDA/CFSAN	CPSC/NIST	NIOSH
NBT Abs NC	0.0 - 1.9% (n = 13)	1.7 – 4.0% (n = 8)	0.4 - 6.8% (n = 7)
PDA Abs NC	0.5 - 2.7% (n = 12)	0.6 - 4.9% (n = 7)	0.9 - 6.6% (n = 12)
PDA Fluor NC	6.8 - 14.3% (n = 5)	3.0 - 10.2% (n = 3)	1.0 - 5.5% (n = 5)
NBT Abs PC	1.0 – 6.0% (n = 13)	1.5 - 4.6% (n = 8)	1.9 - 8.9% (n = 7)
PDA Abs PC	3.4 - 13.1% (n = 12)	-143.9 – 110.3%[b] (n = 7)	1.6 - 68.8% (n = 12)
PDA Fluor PC	2.1 – 15.3% (n = 5)	-217.3 - 22.5% (n = 3)	1.0 – 30.1% (n = 5)

Abs = absorbance; Fluor = fluorescence; NC = negative control; PC = positive control.

[a] Negative control assays are run for 120 min, positive control assays are run until the result is positive.

[b] As the mean absorbance values approach zero or become negative with strong depletion of the probe, the CV cannot be determined accurately.

Figure 4 Average CV at 5 Min in PDA Absorbance Assay



A CV for glutaraldehyde could not be accurately determined in the CPSC/NIST laboratory due to negative absorbance values with greater than 100% depletion.

Table 3 NBT Absorbance Results

Table 3a FDA/CFSAN

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	<10%	<10%	<10%
2-Mercaptobenzothiazole	10-<30%[a]	<10%	<10%
4-Phenylenediamine	10-<30%	<10%	<10%
Formaldehyde	<10%	<10%	<10%
Glutaraldehyde	<10%	<10%	<10%
Glycerol	<10%	<10%	<10%
Glyoxal	<10%	<10%	<10%
Methyl salicylate	<10%	<10%	<10%
Squaric acid	<10%	<10%	<10%
Sulfanilamide	10-<30%	<10%	<10%

Table 3b CPSC/NIST

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	<10%	<10%	<10%
2-Mercaptobenzothiazole	<10%	<10%	<10%
4-Phenylenediamine	<10%	<10%	<10%
Formaldehyde	<10%	<10%	<10%
Glutaraldehyde	<10%	<10%	<10%
Glycerol	<10%	<10%	<10%
Glyoxal	<10%	<10%	<10%
Methyl salicylate	<10%	<10%	<10%
Squaric acid	<10%	<10%	<10%
Sulfanilamide	<10%	<10%	<10%

Table 3c NIOSH

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	<10%	<10%	10-<30%
2-Mercaptobenzothiazole	<10%	<10%	<10%
4-Phenylenediamine	10-<30%	10-<30%	10-<30%
Formaldehyde	10-<30%	<10%	10-<30%
Glutaraldehyde	<10%	<10%	<10%
Glycerol	10-<30%	<10%	<10%
Glyoxal	<10%	<10%	10-<30%
Methyl salicylate	<10%	<10%	<10%
Squaric acid	<10%	<10%	<10%
Sulfanilamide	<10%	<10%	<10%

[a] 100% of the FDA/CFSAN (3/3) and NIOSH (8/8) intermediate runs were confirmed as negative in the NBT absorbance confirmation test at the 2x concentration.

Table 4 PDA Absorbance Results

Table 4a FDA/CFSAN

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	<10%	<10%	<10%
2-Mercaptobenzothiazole	Int	Int	Int
4-Phenylenediamine	Int	Int	Int
Formaldehyde	≥30%	≥30%	≥30%
Glutaraldehyde	≥30%	≥30%	≥30%
Glycerol	<10%	<10%	<10%
Glyoxal	≥30%	≥30%	≥30%
Methyl salicylate	Int	Int	Int
Squaric acid	<10%	<10%	<10%
Sulfanilamide	<10%	<10%	<10%

Table 4b CPSC/NIST

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	<10%	<10%	<10%
2-Mercaptobenzothiazole	Int	Int	Int
4-Phenylenediamine	Int	Int	Int
Formaldehyde	≥30%	≥30%	≥30%
Glutaraldehyde	≥30%	≥30%	≥30%
Glycerol	<10%	<10%	<10%
Glyoxal	≥30%	≥30%	≥30%
Methyl salicylate	Int	Int	Int
Squaric acid	<10%	<10%	<10%
Sulfanilamide	<10%	<10%	<10%

Table 4c NIOSH

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	<10%	<10%	<10%
2-Mercaptobenzothiazole	Int	Int	Int
4-Phenylenediamine	Int	Int	Int
Formaldehyde	≥30%	≥30%	≥30%
Glutaraldehyde	≥30%	≥30%	≥30%
Glycerol	<10%	Int	<10%
Glyoxal	<10%	<10%	<10%
Methyl salicylate	Int	Int	Int
Squaric acid	<10%	<10%	<10%
Sulfanilamide	<10%	Int	<10%

Int = interference (less than minus 10% depletion)

Table 5 PDA Fluorescence Results

Table 5a FDA/CFSAN

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	-	-	-
2-Mercaptobenzothiazole	≥30%	≥30%	≥30%
4-Phenylenediamine	<15%	<15%	<15%
Formaldehyde	-	-	-
Glutaraldehyde	-	-	-
Glycerol	-	-	-
Glyoxal	-	-	-
Methyl salicylate	>15%<30%[a]	<15%	>15%<30%
Squaric acid	-	-	-
Sulfanilamide	-	-	-

Table 5b CPSC/NIST

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	-	-	-
2-Mercaptobenzothiazole	≥30%	≥30%	≥30%
4-Phenylenediamine	<15%	<15%	<15%
Formaldehyde	-	-	-
Glutaraldehyde	-	-	-
Glycerol	-	-	-
Glyoxal	-	-	-
Methyl salicylate	<15%	<15%	<15%
Squaric acid	-	-	-
Sulfanilamide	-	-	-

Table 3c NIOSH

Test Compound	Run 1	Run 2	Run 3
2,3-Butanedione	-	-	-
2-Mercaptobenzothiazole	≥30%	≥30%	≥30%
4-Phenylenediamine	<15%	<15%	<15%
Formaldehyde	-	-	-
Glutaraldehyde	-	-	-
Glycerol	-	<15%	-
Glyoxal	-	-	-
Methyl salicylate	<15%	<15%	<15%
Squaric acid	-	-	-
Sulfanilamide	-	<15%	-

[a] 100% (2/2) of the PDA fluorescence intermediate runs results were confirmed negative in the confirmation tests at the 1x concentration.

Table 6 Classification Results

Test Compound	FDA/ CFSAN	CPSC/ NIST	NIOSH	LLNA	Human
2,3-Butanedione	NS	NS	NS	S	-
Formaldehyde	S	S	S	S	S
Glycerol	NS	NS	NS	NS	NS
Glyoxal	S	S	NS	S	S
Glutaraldehyde	S	S	S	S	S
2-Mercaptobenzothiazole	S	S	S	S	S
Methyl salicylate	NS	NS	NS	NS	NS
4-Phenylenediamine	NS	NS	NS	S	S
Squaric acid	NS	NS	NS	S	S
Sulfanilamide	NS	NS	NS	NS	S

NS = nonsensitizer; S = sensitizer.
Black text = concordant with LLNA and human results.
Blue text = false negative with respect to LLNA and human results (no human data for 2,3-butanedione.)
Green text = concordant with LLNA result; false negative with respect to human data.
Red text = discordant with the other two laboratories.

Discussion and Conclusion

- The CVs of triplicate cuvette measurements for positive and negative controls and test chemicals showed that the laboratories were consistent in performing assay procedures.
- Intralaboratory reproducibility (100% concordance) and interlaboratory reproducibility (90% concordance) were very good.
- Accuracy ranged from 60-70% in predicting LLNA outcomes (n=10) and 56-67% in predicting human outcomes (n=9).
- All misclassifications were false negatives. Of the four misclassified chemicals, one was a pre-hapten, one was a weak sensitizer in LLNA with no human data, one had solubility issues, and one was negative in the LLNA but positive in human studies.

- The validation management team has concluded that the reproducibility and accuracy for this small number of chemicals (n=10) support further evaluation of the EASA. Phase 2 of the study will begin after a 96-well format is developed to increase throughput and accessibility of the assay.

References

Chipinda et al. 2010. Chem Res Toxicology. 23:918-925.

Chipinda et al. 2011. Journal of Allergy. 2011:1-11 (Review article).

Chipinda et al. 2014. Toxicology. 315:102-109.

LoPachin et al. 2012. Chem Res Toxicology. 25:239-251.

OECD. 2012a. ENV/JM/MONO(2012)10/PART1.

OECD. 2012b. ENV/JM/MONO(2012)10/PART2.

Acknowledgements

We thank Catherine Sprankle, ILS, for editing the poster text. The Intramural Research Program of the National Institute of Environmental Health Sciences (NIEHS) supported this poster. Technical support was provided by ILS under NIEHS contract HHSN273201500010C.

The views expressed above do not necessarily represent the official positions of any federal agency. Since the poster was written as part of the official duties of the authors, it can be freely copied.

Subscribe to the NICEATM News Email List

To get announcements of NICEATM activities, visit the NIH mailing list page for NICEATM News at https://list.nih.gov/cgi-bin/wa.exe?SUBED1=niceatm-l&A=1 and click “Subscribe”