Validation of the Electrophilic Allergen Screening Assay (EASA) to Detect Substances that Impact the Initial Key Event in the Adverse Outcome Pathway for Skin Sensitization

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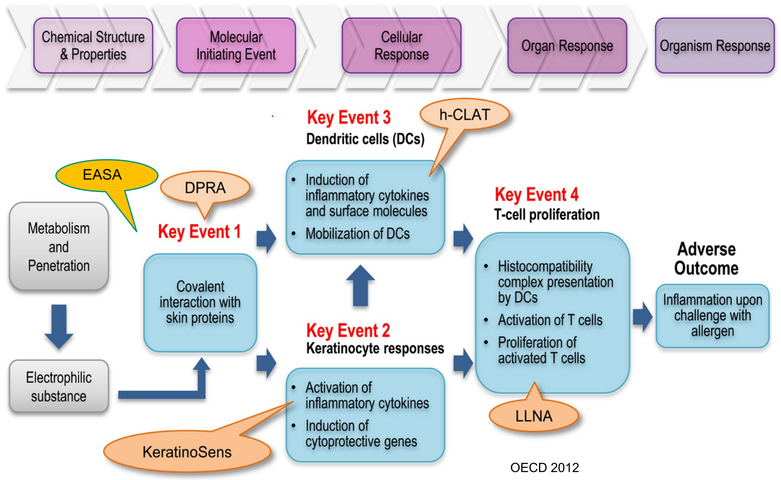
Introduction

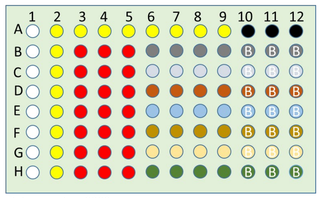
- Chemical binding to skin proteins is the initial key event in the adverse outcome pathway for skin sensitization (**Figure 1**). The electrophilic allergen screening assay (EASA) was originally developed by the National Institute of Occupational Safety and Health as a cuvette-based assay to identify substances that have the potential to cause allergic contact dermatitis (Chipinda et al. 2011, 2014).
- The EASA evaluates a substance's ability to bind nitrobenzenthiol (NBT) or pyridoxylamine (PDA) probes used as surrogates for thiol- or amine-containing skin proteins (**Table 1**). Skin sensitizers bind with amino acids containing thiol or amine groups to form haptens, which initiates skin sensitization (**Figure 1**).
- Probe depletion is measured by absorbance (NBT) or fluorescence (PDA) spectroscopy. A test substance is positive when it meets the positive depletion criterion for either NBT or PDA and is negative when the depletion fails to meet the positive criterion for both tests (**Figure 3**).
- The EASA was subsequently modified by the U.S. Consumer Product Safety Commission (CPSC) and the National Institute of Standards and Technology (NIST) into a higher-throughput assay using a 96-well format through a measurement science approach (**Figure 2,** Petersen et al. 2022).
- Four laboratories participated in a validation study of the EASA:
  - U.S. Food and Drug Administration Center for Devices and Radiological Health (FDA/CDRH)
  - Defense Centers for Public Health - Aberdeen (DoD)
  - Burleson Research Technologies, Inc. (BRT)
  - CPSC/NIST (lead laboratory)
- The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) assembled a validation management team to oversee the study.
- The validation study tested 20 reference chemicals from the Direct Peptide and Amino Acid Derivative Reactivity Assay (DPRA/ADRA) Performance Standards (OECD 2019), 12 of which are tested three times for the assessment of within-laboratory reproducibility (**Table 2**). The performance of the EASA was determined by comparison with local lymph node assay outcomes noted in the performance standards document (OECD 2019).

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



OECD 2012

Figure 2. EASA Plate Layout Map for NBT and PDA Assays



TCs are added starting in columns 6 – 12 in rows B – H, horizontally.
○ - NC/PC Blank wells (40 µl ACN + 160 µl SS without Probe)
● - PC (40 µl PC in ACN + 160 µl SS with Probe)
● - NC (ACN) (40 µl ACN + 160 µl SS with Probe)
● - TC (40 µl TC in ACN + 160 µl SS with Probe)
● - TC Blanks (40 µl TC in ACN + 160 µl SS without Probe)
● - Not used– no additions

NC = negative control, PC = positive control, ACN = acetonitrile, TC = test chemical, SS = solvent system

Petersen et al. 2022

Table 1. Characteristics of EASA Component Assays

	NBT Absorbance Assay	PDA Fluorescence Assay
Wavelength (nm)	412	324 excitation 398 emission
Measurement times	5, 20, 35, **50 min***	5, 20, 35, **50 min**
Positive control	Benzyl bromide	Glutaraldehyde
Negative control	Solvent without probe	Solvent without probe
Negative response criterion	No statistically significant depletion of probe based on protocol parameters	
Positive response criterion	Statistically significant depletion of probe based on protocol parameters	

*The 50 min time point is used to determine a final positive or negative response.

Figure 3. EASA Workflow and Decision Criteria

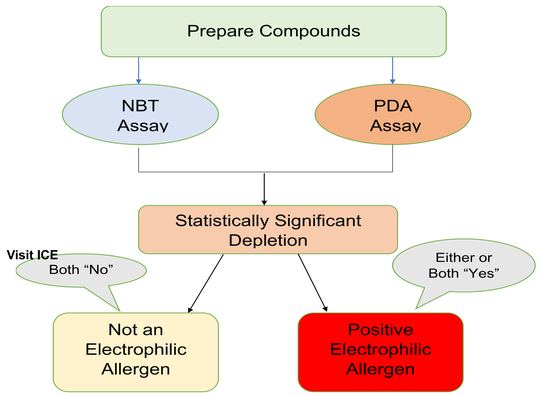


Table 2. EASA NBT/PDA and Final Outcomes by Laboratory Compared to LLNA and DPRA/ADRA

Test Chemical	EASA Outcomes – NBT/PDA/Final[1]				LLNA Outcomes	DPRA/ADRA Outcomes[3]
	Lab 1	Lab 2	Lab 3	Lab 4		
Lauryl gallate	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	**Pos**	**Pos**/**Pos**
Chloramine T trihydrate	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	**Pos**	**Pos**/**Pos**
Metol (4-methyl amino phenol)	Pos/Inc[2]/**Pos**	Pos/Neg/**Pos**	Pos/Inc/**Pos**	Pos/Inc/**Pos**	**Pos**	**Pos**/**Pos**
2-Mercaptobenzothiazole	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	**Pos**	**Pos**/**Pos**
Benzyl salicylate	Neg/Pos/**Pos**	Neg/Pos/**Pos**	Neg/Pos/**Pos**	Neg/Pos/**Pos**	**Pos**	**Pos**-**Neg**/**Pos**
Cinnamaldehyde	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	**Pos**	**Pos**/**Pos**
Imidazolidinyl urea	Neg/Pos/**Pos**	Neg/Pos/**Pos**	Neg/Pos/**Pos**	Neg/Pos/**Pos**	**Pos**	**Pos**/**Pos**
Ethyl acrylate	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	**Pos**	**Pos**/**Pos**
Salicylic acid	Neg/Inc[3]/**Inc**	Neg/Neg/**Neg**	Neg/Inc/**Inc**	Neg/Inc/**Inc**	**Neg**	**Pos**-**Neg**/**Neg**
Benzyl alcohol	Pos/Neg/**Pos**	Pos/Neg/**Pos**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	**Neg**	**Pos**-**Neg**/**Neg**
Glycerol	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	**Neg**	**Neg**/**Neg**
Isopropanol	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	**Neg**	**Neg**/**Neg**
Benzoquinone	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	**Pos**	**Pos**/**Pos**
Dihydroeugenol	Neg/Neg/**Neg**	Neg/Pos/**Pos**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	**Pos**	**Pos**-**Neg**/**Pos**-**Neg**
Palmitoyl chloride	Pos/Inc/**Pos**	Pos/Pos/**Pos**	Pos/Neg/**Pos**	Pos/Pos/**Pos**	**Pos**	**Pos**/**Pos**
Farnesol	Pos/Pos/**Pos**	Neg/Pos/**Pos**	Pos/Pos/**Pos**	Pos/Pos/**Pos**	**Pos**	**Pos**/**Pos**
Dimethyl isophthalate	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	**Neg**	**Neg**/**Neg**
Methyl salicylate	Neg/Pos/**Pos**	Neg/Pos/**Pos**	Neg/Pos/**Pos**	Neg/Pos/**Pos**	**Neg**	**Pos**-**Neg**/**Neg**
4-Aminobenzoic acid	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	Neg/Neg/**Neg**	**Neg**	**Neg**/**Neg**
Benzyl cinnamate	Pos/Pos/**Pos**	Neg/Pos/**Pos**	Neg/Pos/**Pos**	Neg/Pos/**Pos**	**Pos**	**Neg**/**Neg**

[1] EASA NBT and PDA outcomes were determined as described in **Figure 3**. The final EASA call is bolded, **RED** indicates a positive call, **BLUE** indicates a negative call, and **BLACK** indicates an inconclusive call.
[2] Inc = Inconclusive; the substance tested negative at concentrations lower than that specified in the protocol.
[3] OECD 2019.

Table 3. EASA Performance by Laboratory

Lab #	Balanced Accuracy	Sensitivity	Specificity	Within Lab Reproducibility	Between Lab Reproducibility
1	76%	85%	67%	94%	96%
2	82%	92%	71%	100%	
3	84%	85%	83%	97%	
4	84%	85%	83%	94%	

Balanced Accuracy: the average of sensitivity and specificity.
Sensitivity: proportion of all positive chemicals correctly classified.
Specificity: proportion of all negative chemicals correctly classified.

Conclusions

- This preliminary assessment yielded performance statistics for the EASA (**Table 3**) that meet the acceptance criteria established by the Organisation for Economic Co-operation and Development (OECD) for similar assays (OECD 2019). This suggests that the EASA may be useful for identifying potential skin sensitizers.
- Development of the validation report is underway. The report will undergo peer review upon acceptance by the validation management team. Results will also be reported in the peer-reviewed literature.
- This method may be proposed to OECD as an addition to OECD Test Guideline 442C.

Acknowledgments

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