Electrophilic Allergen Screen Assay Protocol

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

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List of Abbreviations and Acronyms

ACN	Acetonitrile
NBT	4-Nitrobenzenethiol
PB	0.1M Phosphate Buffer, pH 7.4
PDA	Pyridoxylamine
SS	Solvent System of 50% ACN/PB
TC	Test Chemical
Ttc	Time to Completion as defined as loss of 80% of PDA or NBT absorbance or fluorescence

1. Introduction and Rationale

Several validated assays have been adopted by Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for regulatory safety assessment of allergic contact dermatitis (ACD) hazards. These include the murine local lymph node assay (LLNA) and modified LLNAs that reduce the number of animals required and/or eliminate the need for radiolabeled tracers. With the goal of reducing or eliminating the need for use of animals for identification of ACD hazards NICEATM/ICCVAM and their European and Japanese counterparts (European Centre for the Validation of Alternative Methods, and Japanese Center for the Validation of Alternative Methods) are evaluating the applicability of *in vitro* and *in chemico* methods that may be incorporated in an integrated decision making process. The *in chemico* direct peptide reactivity assay (DPRA) under evaluation assesses binding of chemical allergens to nucleophilic sites on model peptides. The DPRA involves incubation of chemical allergens with the model peptides for 24 hrs, followed by measurement of loss of unbound peptide using a high performance liquid chromatograph coupled to an ultraviolet detector (HPLC-UV). Test chemicals producing a loss ≥ 10% of unbound peptide are considered allergens (Gerberick *et al*. (2004) *Tox Sci*. 81, 332-343). Sensitivity, specificity and accuracy based on 81 chemicals (vs. LLNA classification) were reported to be 88%, 90% and 89%, respectively (Gerberick *et al.* (2007) Tox Sci. 97(2), 417-427). Although, the DPRA is promising as a screening ACD hazard test several drawbacks have been reported (Natsch and Gfeller (2008) *Toxicol Sci.* 106(2):464-478). In particular, Natsch and Gfeller noted loss of unreacted peptide due to oxidation and precipitation (caused by the high test chemical concentrations employed), potential co-elution of test chemical and peptide and use of equilibrium conditions vs. kinetic-type endpoint assessment. Their solution to the identified drawbacks of the DPRA was to replace the UV detector with a mass spectrometer (HPLC-MS) that would allow identification and measurement of adducts formed as well as oxidation products. This approach, however, greatly increases the cost and expertise required.

A critical step in the development of chemical-induced allergic sensitization is the covalent binding of the chemical allergen (or its metabolite) to an endogenous protein(s). The DPRA

assay uses model peptides with known nucleophilic centers as surrogates for proteins. We further suggest that the relative binding of a chemical allergen is not dependent on the protein/peptide nature of the probe, but rather follows the HSAB (hard/soft (Lewis) acid/base) concept. The HSAB concept with respect to electrophilic allergens implies that soft electrophiles react faster and form stronger bonds with nucleophiles of comparable softness, whereas hard electrophiles react faster and form stronger bonds with nucleophiles of comparable hardness. (For a review of the HSAB concept as applied to electrophilic/nucleophilic covalent interactions see Lopachin *et al*. (2012); *Chem Res Toxicol,* 20;25(2):239-251.) Under the HSAB concept, appropriate non-protein/peptide probes can be employed to evaluate chemical allergen protein binding potentials. The use of chemical probes vs. peptide probes for allergen screening include (1) ability to directly monitor the chemical reaction kinetically or via an endpoint assay, (2) lower concentrations of test chemical required resulting in decreased solubility and precipitation problems, (3) shorter assay times and (4) significantly lower cost.

The present submission details an *in chemico* low molecular weight chemical probe assay for the identification of electrophilic ACD hazards. Two probes are employed; 4-Nitrobenzenethiol (NBT) is the "soft" nucleophile and pyridoxylamine (PDA) is the "hard" nucleophile employed in this *in chemico* assay. NBT and PDA effectively replace the cysteine (thiol) and lysine (ε-amine) containing model peptides in the DPRA, respectively. These probes are very water soluble; have high absorbance coefficients (and fluorescence) and are readily commercially available with nominal cost. Covalent binding of an electrophilic allergen to the amine or thiol on these probes produces a shift in the absorbance and/or fluorescence (for PDA) of these probes that are independent of the species bound. The shift (loss) of absorbance/fluorescence is directly related to the allergen's chemical reactivity and can be directly monitored continuously or through end-point measures. This eliminates the requirement for physical separation of free from bound probe (i.e. need for HPLC).

Chemical allergens are often referred to as haptens (or incomplete allergens) as they must bind covalently to a protein to be allergenic. Chemical allergens that require metabolic or (air/chemical) oxidative modification of electrophilic species are preferred to as prohaptens. The DPRA and the proposed NBT/PDA assays, at present, will not identify prohaptens. In addition,

allergens that react with proteins through co-ordinate covalent bonding such as the metals will be false negatives in the NBT/PDA assay. Therefore, the NBT/PDA proposed test method is for screening of test chemicals for ACD hazard identification. It may be possible to incorporate metabolic activation steps into the NBT/PDA assay, but at present, chemicals that are negative in the NBT/PDA assay would need to be tested with the LLNA (or alternate validated ACD hazard identification assay). In addition, chemical oxidants are expected to produce disulfide formation (dimer) of NBT producing a chemical shift similar to that observed with covalent binding to the thiol.

The NBT assay has been published (Chipinda et al. (2010) *Chem Res Toxicol*. 17;23(5):918-925) and the PDA assay method manuscript is in preparation. In addition to our laboratory the assays have been performed in Dr. Reuben Simoyi's laboratory at Portland State University, Department of Chemistry. Additional peer review of the NBT/PDA has not been conducted.

2. Test Method Protocol Components

2.1 Overview: The NBT/PDA method measures loss of specific absorbance and/or fluorescence of NBT and PDA with covalent binding by electrophilic haptens (chemical allergens) at 25°C. The reaction solvent system is 50% acetonitrile/0.1M phosphate buffer, pH 7.4. Haptens are added at 2:1 or 5:1 molar ratios to NBT or PDA, respectively to start the reaction. We recommend either continuous monitoring or taking absorbance/fluorescence readings at 0, 3, 20 and 120 min. Controls containing only NBT or PDA in the solvent system should be run concurrently. Time to completion (Ttc) has been defined as time to 80% loss of NBT or PDA absorbance/fluorescence. Reaction to extremely weak haptens will not be complete within the 120 min monitoring period. Ttcs up to 244 min have been observed. In addition, a small percent of the positive hapten reactions proceed only to 50-60% completion (independent of hapten:probe ratio employed).

2.2 Chemicals (All were purchased from Sigma-Aldrich, St. Louis, Mo.)

Phosphate buffer (PB), 0.1 M, pH 7.4

Acetonitrile (ACN)

Acetone

Pyridoxylamine (PDA) (CAS# 524-36-7),

4-Nitrobenzenethiol (NBT) (CAS# 1849-36-1),

Test chemicals (TC)

2.3 Equipment and Supplies

Spectrophotometer with temperature control

Spectrofluorometer with temperature control

Glass or Quartz Cuvettes (preferably matched), 1 cm path length, with Teflon lids; *Note: Methods detailed herein use a 0.5 mL total assay volume for absorbance readings. Volumes can be adjusted to accommodate cuvette/spectrophotometer requirements. Disposable cuvettes (methacrylate) have not been tested in this assay and may not be suitable for use with the SS.*

Pipettes able to handle volumes from 0.05 to 5 mL. (Positive displacement pipettes are preferred for handling organic solvents)

2.4 Dose-Selection: Test chemicals are assayed at 0.2 mM final concentration for the NBT photometric and PDA fluorescent assays, and 0.5 mM final concentration for the PDA photometric assay. This provides reaction molar ratios of 2:1 and 5:1 test chemical to NBT or PDA, respectively. These concentrations were chosen to allow for all but extremely weak electrophilic reactions run to completion within 2 hrs and to decrease potential interferences caused by competing reactions (ex. Hydrolysis of water unstable test compounds).

2.5 *Endpoints Measured*: Loss of NBT absorbance at λ=412 nm; of PDA absorbance at λ=324 nm or PDA fluorescence at λ=324ex/398em.

2.6 *Duration of Exposure*: Reactions are run for up to 2 hrs. Further monitoring may be required for very weak electrophiles where NBT or PDA depletion is between 10-30%.

2.7 *Known Limits of Use*: This assay will only detect electrophilic allergens. Metals and pro/prehaptens will be negative in this assay. Test chemicals have been found that absorb at 324 nm prohibiting use of the PDA spectrophometric protocol. The PDA fluorometric assay should be used in such cases.

2.8 *Nature of the Response Assessed*: Loss of unbound (free) probe due to covalent binding to electrophilic allergen.

2.9 *Appropriate vehicle, positive and negative controls*: Solvent System (SS) = 50:50 acetonitrile:0.1M phosphate buffer, pH 7.4. Acetone can be substituted for acetonitrile if required for test chemical solubilization. A NBT or PDA only negative control should be run concurrently. A positive control electrophile has not been established. Suggested positive controls are given in 2.16 Component Integrity.

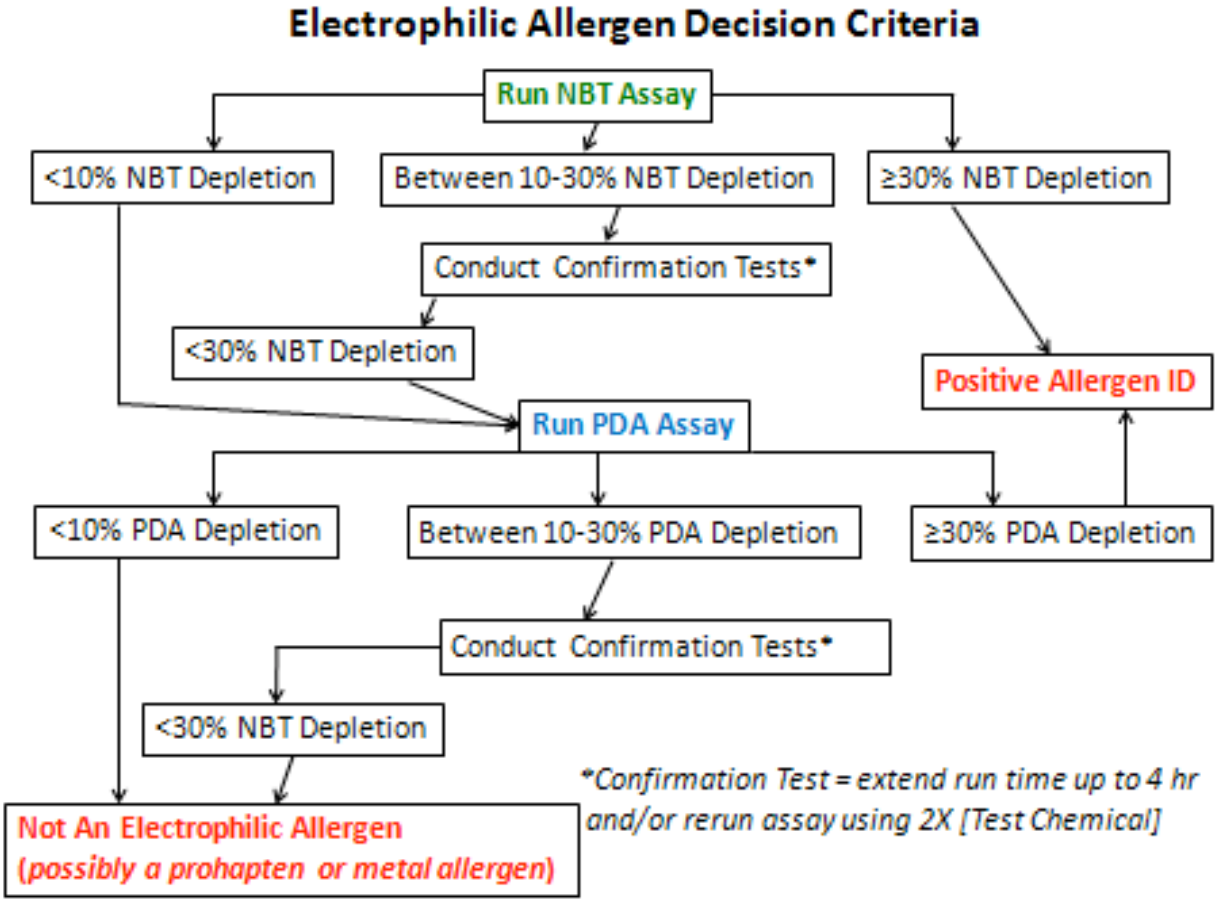
2.10 *Acceptable range of vehicle and controls*: The vehicle does not have an absorbance or fluorescence at test wavelengths. Substrate (NBT or PDA) negative control absorbance or fluorescence should not change > 10% over the reaction period. NBT and PDA are stable at 25°C in the solvent system. Absorbance/fluorescence drift may differ from instrument to instrument.

2.11 *Data Collected*: Absorbance or Fluorescence values at 0, 3, 20 and 120 min should be recorded. Continuous data logging may be used if available with instrument software. Additional absorbance or fluorescence measures beyond 120 min may be needed for confirmation that a chemical is electrophilic.

2.12 *Type of Data Storage Media*: Not specified

2.13 *Measures of Variability*: Variability between replicates (n=3 to 5) for reaction rate completion times averaged < 10% (range 1.5 to 15.3% cv) for all electrophilic allergens and control NBT absorbance measured to date. Other measures of variability have not been assessed.

2.14 *Decision Criteria*: Schematic below is an outline of the criteria for identification of electrophilic contact allergen hazards. Identification of the allergen is based on loss of the free nucleophilic probe (NBT and PDA) absorbance/fluorescence upon complexation with an electrophilic allergen. It is suggested that the NBT assay be conducted first and the PDA conducted NBT-negative test chemicals. If the test chemical causes a loss of NBT absorbance (at 414 nm) ≥30% within 2 hr the test chemical is classified as a contact allergen. NBT-negative test chemical is one in which <10% loss of NBT absorbance over 2 hr at 25°C is observed or < 30% loss of NBT absorbance of confirmatory test. The confirmatory tests consist of extending assay time to up to 4 hr (to observe progressive loss of NBT absorbance with time to > 30%) and/or rerun the test chemical increasing the concentration to 4:1 test chemical:NBT (mole:mole). NBT-negative test chemicals will be assayed against PDA and if ≥ 30% loss of PDA absorbance or fluorescence is observed within 2 hr the test chemical is classified as a contact allergen. If <10% PDA loss is observed or <30% following confirmation test is observed the test chemical is classified as not an electrophilic contact allergen. Negative test chemicals may be able to produce allergy indirectly (prohaptens activated to an electrophilic species through metabolism or chemical/air oxidation) or a metal allergen and thus, should be further evaluated by other assays.



2.*15 Acceptance Criteria*: Acceptance or rejection of a test is based on evaluation of positive and negative control results. Control PDA and NBT absorbance or fluorescence readings during the 2 hr reaction period should be within 10% of the initial reading (0 time point). Excessive increase in readings over the incubation period may indicate improperly capped cuvettes/tubes. A reference standard should be run with each working solution (daily) of PDA and NBT. The reference standard should be an electrophilic chemical for which the laboratory has established historical data. Suggested reference standards for the NBT reaction are 4-hexen-3-one (80% depletion of NBT absorbance reported to be 16.6 ± 1.3 min) or propiolactone (80% depletion of NBT absorbance reported to be 4.1 ± 0.1 min) and for the PDA reaction are benzyl bromide (80% depletion of PDA absorbance reported to be 5.3 ± 0.4 min) or methanesulfonyl chloride

(80% depletion of PDA fluorescence reported to be 31.7 ± 0.6 min). Depletion by incubation with the reference standard ≥80% of the free NBT or PDA should be observed by the end of the 2 hr. reaction period.

2.16 *Component integrity*: NBT and PDA are available from commercial laboratory chemical suppliers. Thiols, including NBT, are subject to air oxidation. It is suggested that 0.1 mM NBT absorbance at 412 nm be within 15% of 1.1 with a 1 cm pathlength. The absorbance of 0.1 mM PDA at 324 nM is 0.78 with a 1 cm pathlength. All assays should be run in capped glass or quartz cuvettes or test tubes to prevent volatilization losses of solvent system components.

2.17 *Replicates*: Three to 5 replicates per test chemical reaction to PDA and NBT were run during the development of this method. Preliminary assessment from 1 laboratory suggest n=3 is sufficient.

3. **Basis for Selection of Test System**

The NBT/PDA assay was developed as a fast, inexpensive preliminary screening assay to be used in conjunction with animal based assays. Based on 67 test chemicals tested, to date, sensitivity and positive predictability was 100%. All electrophilic allergens tested, to date, have caused ≥30% NBT or PDA depletion within 2 hr, however; confirmation test are included due to the limited number of very weak electrophilic allergens within that group. Chemicals that test negative in this assay would need to be further screened for allergenic potential.

4. Detailed Assay Protocols

4.1 ***NBT Assay*** *(Note: The volumes stated are for use with a semi-micro cuvette. Volumes can be adjusted accordingly for use with standard cuvettes requiring larger volumes).*

1) Turn on the spectrophotometer, set the wavelength to 414 nm. Allow the instrument to warm up/stabilize according to manufacturer's guidelines.

2) Dissolve 1.55 mg of NBT (MW 155.17 g/mol) in 10 mL PB (0.1 M, pH 7.4) to make a 1 mM stock solution. Sonicate for a few minutes to aid dissolution. (stable stored up to at least 1 week at -20°C).

3) Mix 5 mL of PB with 5 mL of acetonitrile to make 50:50 solvent system (SS). Volumes can be adjusted for number of test to be run.

4) Make 1 mM solutions of the test chemicals (TC) in SS (or acetone + PB (1:1) for chemicals that will not dissolve in ACN). Maintain at 25°C. NOTE: TC that are water labile should be made up in dry acetone or ACN (for example diisocyanates or organic acid anhydrides).

5) Dilute the stock NBT 1:10 immediately prior to use (i.e. 1 mL NBT stock + 9 mL SS). Maintain working solutions at 25°C.

6) Blank the spectrophotometer to SS (at λ = 412 nm).

7) To 100 µL of TC add 400 µL dilute NBT solution (from step #5). This will give a molar ratio of TC:NBT of 2:1. Mix well directly in the cuvette or in a test tube and transferring immediately to the cuvette. Immediately read the absorbance.

8) A control consisting of 100 µL of SS + 400 µL NBT solution (step #5) should be run concurrently (if possible).

9) Read and record the absorbance at 0, 3, 20 and 120 min of control and TC. (Or if instrument allows for continuous monitoring additional data points can be collected).

10) Percent Absorbance loss = 100 X Control absorbance (#8) minus TC absorbance (# 7)/Control absorbance.

4.2 ***PDA Absorbance Assay –*** *Not all TC can be assayed by the PDA Fluorescent Assay. The PDA Absorbance Assay is optional if TC interference is not observed.*

1) Dissolve 2.41 mg of PDA (MW 241.11 g/mol) in 10 mL PB to make a 1 mM stock solution (stable for 1 month at room temperature).

2) Mix 5 mL of PB with 5 mL of acetonitrile to make 50:50 solvent system (SS). Volumes can be adjusted for number of test to be run.

3) Make 1 mM solutions of the test chemicals (TC) in SS (or acetone + PB for chemicals that will not dissolve in the SS). NOTE: TC that are water labile should be made up in dry acetone or ACN (for example diisocyanates or organic acid anhydrides).

4) Blank the spectrophotometer to SS (at λ = 324 nm).

5) Mix 250 µL SS + 250 µL TC (0.5 mM). If the absorbance at 324 nm is greater than 0.08 it is suggested that the fluorescence method should be employed. Otherwise proceed to the next step.

6) Dilute the stock PDA 1:5 immediately prior to use (i.e. 1 mL PDA stock + 4 mL SS).

7) To 250 µL of TC Add 250 µL of dilute PDA (from step #6). Mix well directly in the cuvette or in test tube and immediately place into the cuvette to start the reaction and read the absorbance.

8) A control consisting of 250 µL of dilute PDA + 250 µL of SS should be run (concurrently if possible).

9) Read and record absorbance of control and TC at 0, 3, 20 and 120 min. (Or if instrument allows for continuous monitoring additional data points can be collected.)

10) Percent Absorbance loss = 100 X Control absorbance (#8) minus TC absorbance (# 7)/Control absorbance.

4.3 ***PDA Fluorescence Assay***

1) Dissolve 2.41 mg of PDA (MW 241.11 g/mol) in 100 mL PB to make a 1 mM stock solution.

2) Mix 5 mL of PB with 5 mL of acetonitrile to make 50:50 solvent system (SS).

3) Make 1 mM solutions of the test chemicals (TC) in SS (and acetone + PB for chemicals that will not dissolve in ACN). NOTE: TC that are water labile should be made up in dry acetone or ACN (for example diisocyanates or organic acid anhydrides).

4) Set excitation wavelength to 324 nm and emission wavelength to 398 nm. Set Excitation slit width = 10. Set Emission slit width = 10. Blank the instrument with

3000 µL of SS. Diluting 60 µL of PDA into 2,940 µL should give intensity of ≥ 700. Slit widths should be adjusted to provide optimal fluorescence (this will be instrument dependent and will vary with age of the light source). The fluorescence measured is for 0.02 mM PDA.

5) Add 0.6 mL of stock PDA to 26.4 mL SS to provide the working dilute PDA solution. Total volumes can be adjusted for number of assays required.

6) To 300 µL of TC add 2700 µL of dilute PDA (from Step #5), mix well in the cuvette or in a test tube and add to the cuvette to start the reaction. Immediately, read the fluorescence.

7) A control with 300 µL SS + 2700 µL dilute PDA should be run (concurrently if possible).

8) Read and record the fluorescence of control and TC at 0, 3, 20 and 120 min (or by continuous monitoring if time drive is available).

9) Percent Fluorescence loss = 100 X Control fluorescence (#7) minus
TC absorbance (# 8)/Control absorbance.