



# Direct Reading Alternative *In Chemico* Method for Screening Dermal Sensitizers (Electrophilic Allergen Screening Assay)

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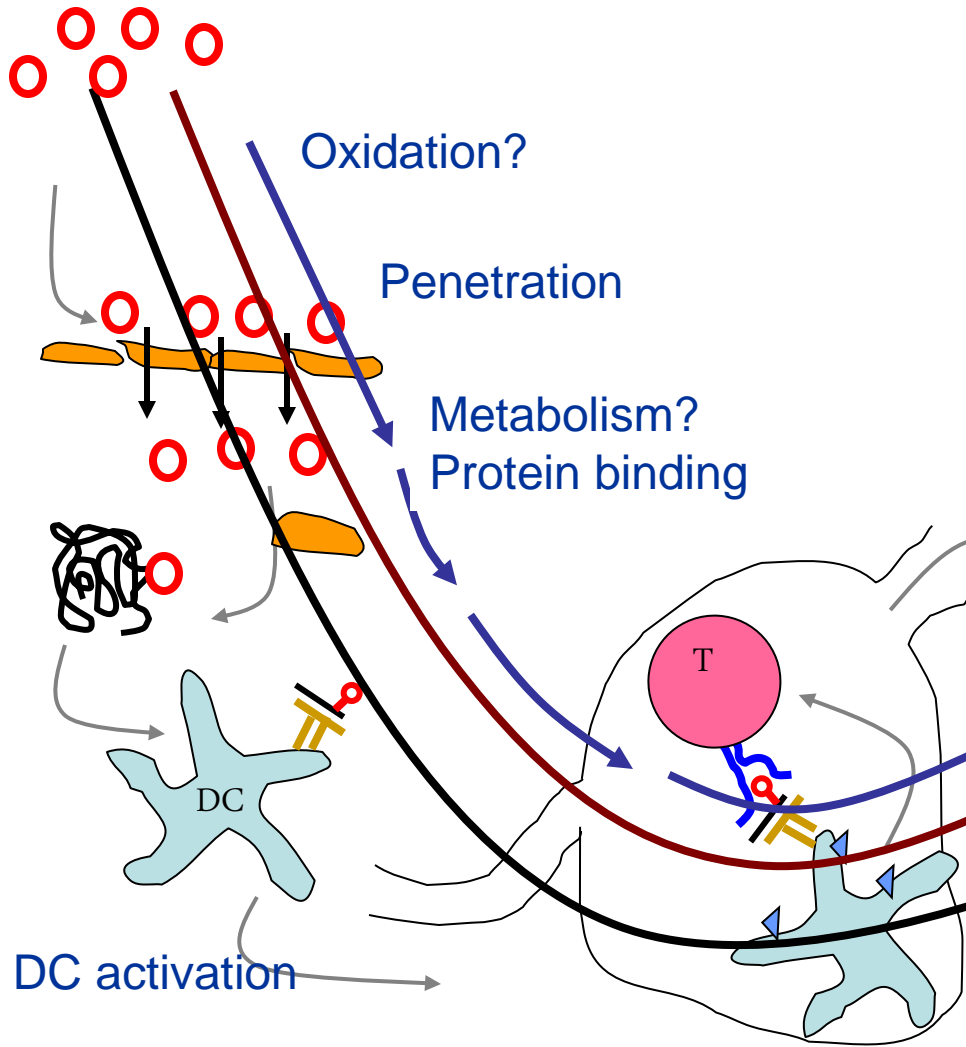
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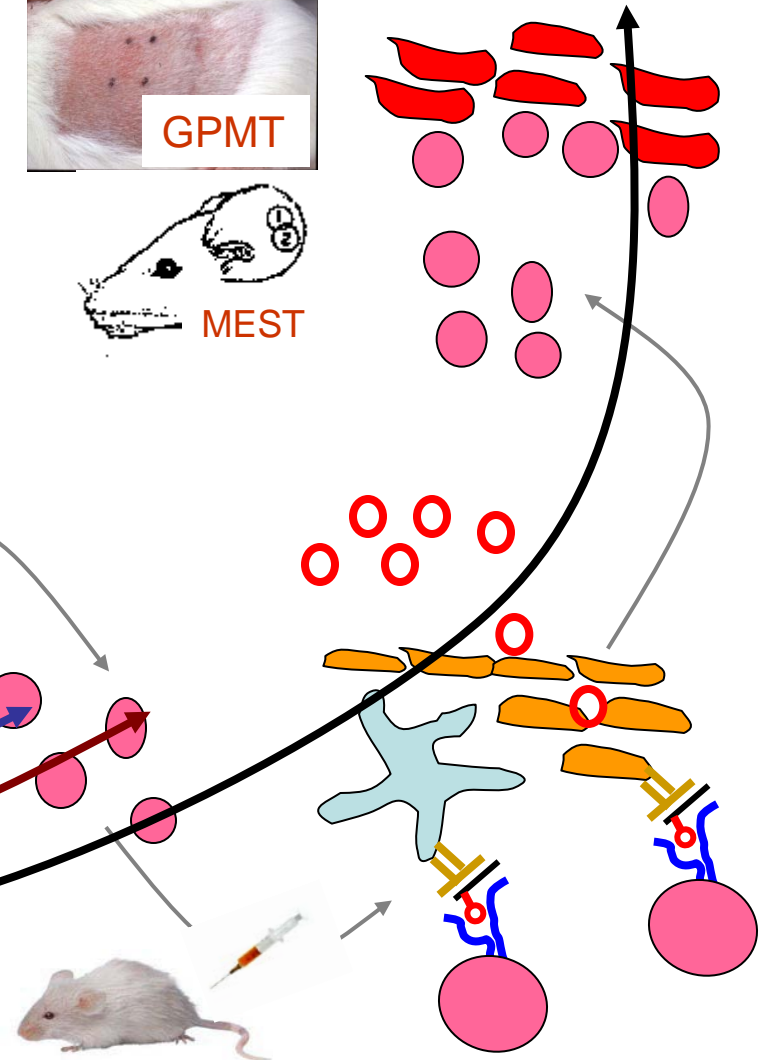
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# Current Models – Animal vs Non-Animal

In-vitro, In-Chemico, In-Silico



In-Vivo



# Protein Reactivity

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- Protein binding is an initial step in allergic skin sensitization.

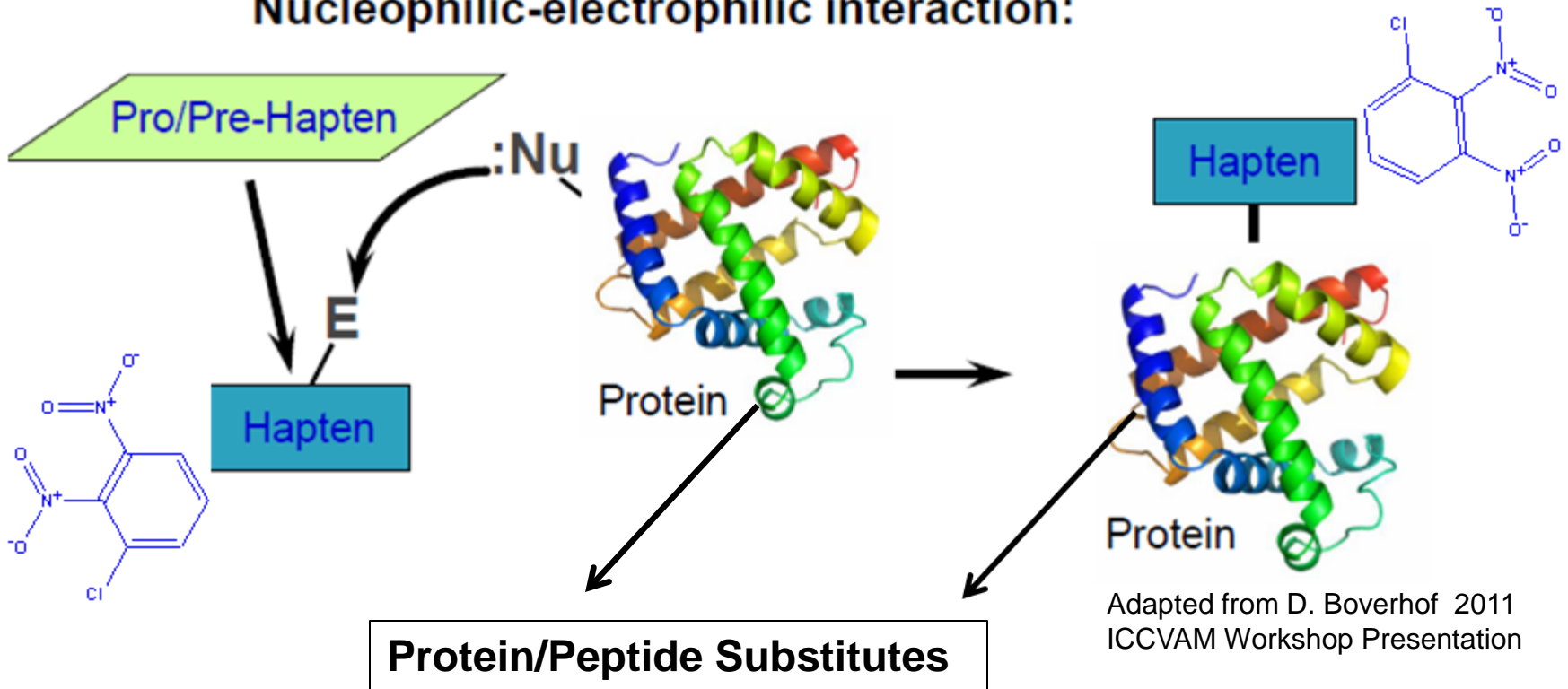
## -Relevant Bonds:

covalent bonds (200-420 kJ/mol)

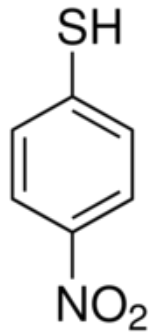
co-ordinate bonding ( $M^{n+}$  allergy)

- Electrophilic allergens can bind to soft (thiol) and/or hard (amine) protein nucleophiles – HSAB concept applies.
- Binding (depletion) assays involving model peptides, GSH and cysteine have been reported.
- Limitations - Measurement of rapid reactions, probe oxidation, solubility incompatibilities between E and Nu.

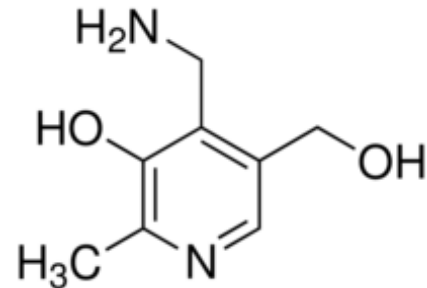
## Nucleophilic-electrophilic interaction:



Adapted from D. Boverhof 2011  
ICCVAM Workshop Presentation



**4-Nitrobenzenethiol (NBT)**



**Pyridoxylamine (PDA)**

# End Point Assay Methodology

## **Soft Electrophilic Allergen 4-Nitrobenzenethiol (NBT) Assay:**

1. Solvent System (SS) = 50:50 acetonitrile:0.1M phosphate buffer, pH 7.4
2. Mix NBT + test chemical in SS to have a final concentration of 0.1 mM NBT  $\pm$  0.2 mM test chemical. Measure loss of absorbance at 412 nm at 25°C for up to 2 hr.

## **Hard Electrophilic Allergen Pyridoxylamine (PDA) Absorbance Assay:**

1. Solvent System (SS) = 50:50 acetonitrile:0.1M phosphate buffer, pH 7.4
2. Mix PDA + test chemical in SS to have a final concentration of 0.1 mM PDA  $\pm$  0.5mM test chemical. Measure loss of absorbance at 324 nm at 25°C for up to 2 hr.

## **Hard Electrophilic Allergen Pyridoxylamine (PDA) Fluorescence Assay:**

1. Solvent System (SS) = 50:50 acetonitrile:0.1M phosphate buffer, pH 7.4
2. Mix PDA + test chemical in SS to have a final concentration of 0.02 mM PDA  $\pm$  0.1 mM test chemical. Measure loss of fluorescence at  $\lambda_{\text{ex}} = 324 \text{ nm} / \lambda_{\text{em}} = 398 \text{ nm}$  at 25°C for up to 2 hr.

# System Checks etc.

- 0.1 mM NBT and should be should  $\approx 1.1$  at 412 nm
- 0.1 mM PDA and should be should  $\approx 0.78$  at 324 nm
- Monitor rate of absorbance/fluorescence loss

# Allergens Tested in the Electrophilic Allergen Screening Assay

	<i>Positive Allergen Test</i>	NBT	PDA
1	benzyl bromide	+	+
2	nitrobenzyl bromide	+	+
3	methyl methane sulfonate	+	+
4	diethylthiocarbamoyl chloride	+	+
5	formaldehyde	+	+
6	acetic anhydride	+	+
7	benzoquinone	+	+
8	hydroxyethylacrylate	+	+
9	methyl methacrylate	+	-
10	ethylacrylate	+	-
11	2,4-dihydroxychalcone	+	-
12	tetraethylthiuram disulfide	+	-
13	aminophenyl disulfide	+	-
14	phenylacetaldehyde	-	+
15	glyoxal	-	+
16	maleic anhydride	-	+
17	propyl gallate	-	+
18	palmitoyl chloride	-	+
19	glutaraldehyde	-	+
20	O-phthalaldehyde	-	+
21	phthalic anhydride	-	+
22	trimellitic anhydride	-	+
23	methyl pyruvate	-	+
24	cyanuric chloride	-	+
25	2,3-butanedione	-	+
26	linalool aldehyde	-	+
27	lauryl gallate	-	+
28	oxalic acid	-	+
29	hexylcinnamaldehyde	-	+
30	phenyl cinnamaldehyde	-	+
31	hydroquinone	-	+

	<i>Positive Allergen Test</i>	NBT	PD A
32	propiolactone	+	0
33	methyl isothiazolinone	+	0
34	isothiazolinone	+	0
35	Phenylmethanesulfonyl chloride	-	0
36	2,4-toluene diisocyanate	+	0
37	4-hexen-3-one	+	0
38	diphenylcyclopropenone	+	0
39	3,4-dihydroxy-3-cyclobuta-1,2-dione	+	0
40	ethoxyloxazolone	+	0
41	oxazolone	+	0
42	benzisothiazolinone	+	0
43	carvone	+	0
44	nonanoyl chloride	+	0
45	1-phenyl-2-methylbutane-1,3-dione	+	0
46	citral	+	0
47	2,4-hexadienal	+	0
48	dinitrochlorobenzene	+	0
49	kathon	+	0
50	2,5-dichlorobenzoquinone	+	0
51	2-chlorobenzoquinone	+	0
52	2,6-dichlorobenzoquinone	+	0
53	2-methylbenzoquinone	+	0
54	2,5-dimethylbenzoquinone	+	0
55	2-tert-butylbenzoquinone	+	0

+ = positive  
 - = negative  
 0 = not tested

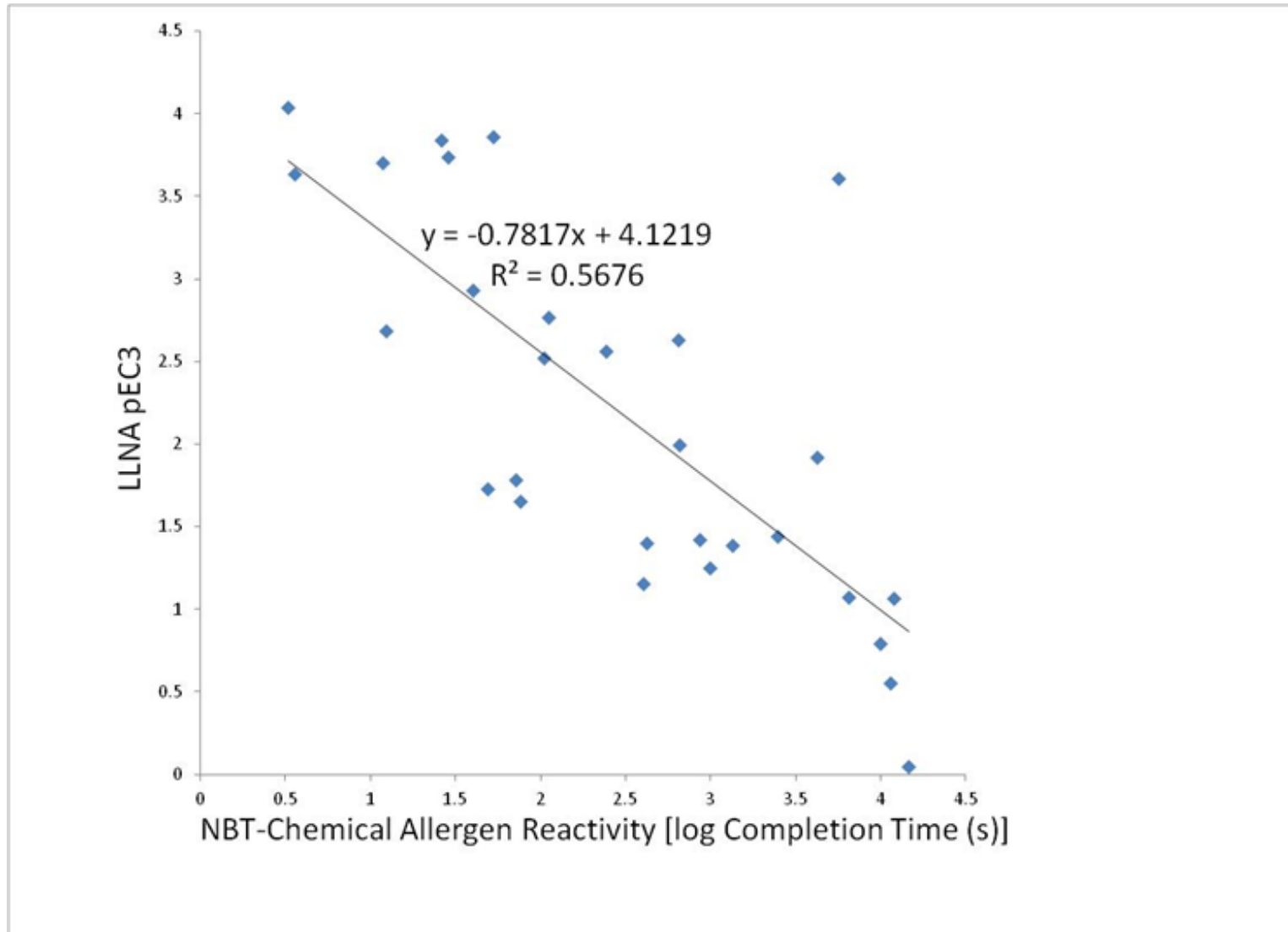
# Allergens Tested in the Electrophilic Allergen Screening Assay (continued)

	<i>False Negative Allergen Test</i>	<b>NBT</b>	<b>PDA</b>	<b>Prohaptan</b>
<b>1</b>	<b>aniline</b>	-	-	+
<b>2</b>	<b>2-mercaptobenzothiazole</b>	-	-	+
<b>3</b>	<b>cinnamic alcohol</b>	-	-	+
<b>4</b>	<b>4-nitrobenzene-1,2-diamine</b>	-	-	+
<b>5</b>	<b>imidazolidinylurea</b>	-	-	+
<b>6</b>	<b>4-phenylenediamine</b>	-	-	+
<b>7</b>	<b>eugenol</b>	-	-	+
<b>8</b>	<b>isoeugenol</b>	-	-	+
<b>9</b>	<b>dihydroeugenol</b>	-	-	+
<b>10</b>	<b>limonene</b>	-	-	+
<b>11</b>	<b>thioglycerol</b>	-	-	+ (?)
<b>12</b>	<b>benzyl salicylate</b>	-	-	+ (?)
<b>13</b>	<b>nickel chloride</b>	-	-	(metal)

	<i>Non-Allergens Tested</i>	<b>NBT</b>	<b>PDA</b>
<b>1</b>	<b>dichlorofluoronitrobenzene</b>	-	-
<b>2</b>	<b>sulfanilamide</b>	-	-
<b>3</b>	<b>benzaldehyde</b>	-	-
<b>4</b>	<b>dinitrophenol</b>	-	-
<b>5</b>	<b>chlorobenzene</b>	-	-
<b>6</b>	<b>sodium lauryl sulphate</b>	-	-
<b>7</b>	<b>benzyl cinnamate</b>	-	-
<b>8</b>	<b>acetonitrile</b>	-	-
<b>9</b>	<b>glycerol</b>	-	-
<b>10</b>	<b>acetone</b>	-	-
<b>11</b>	<b>methyl salicylate</b>	-	-

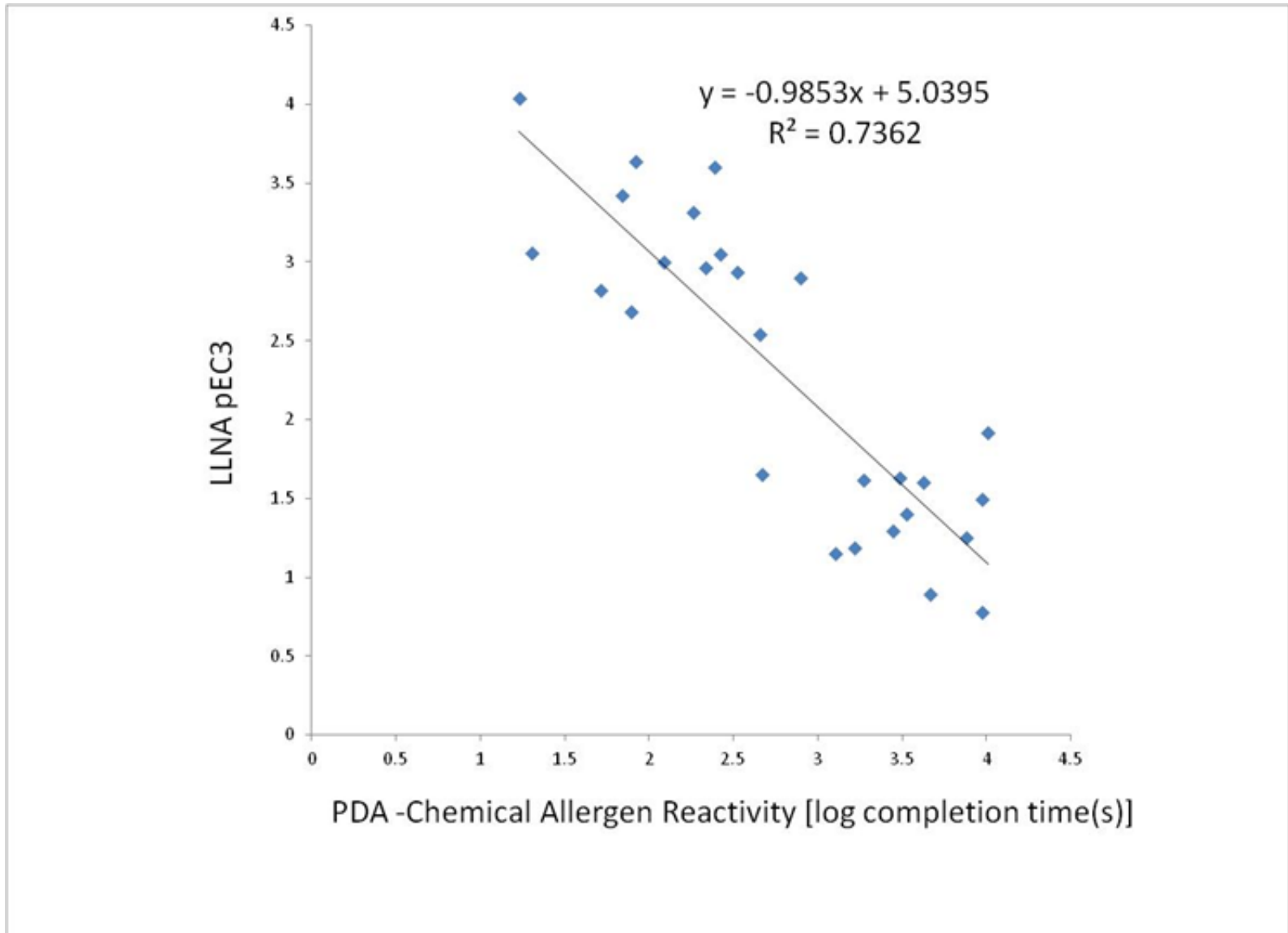


# NBT Reactivity vs. LLNA pEC3

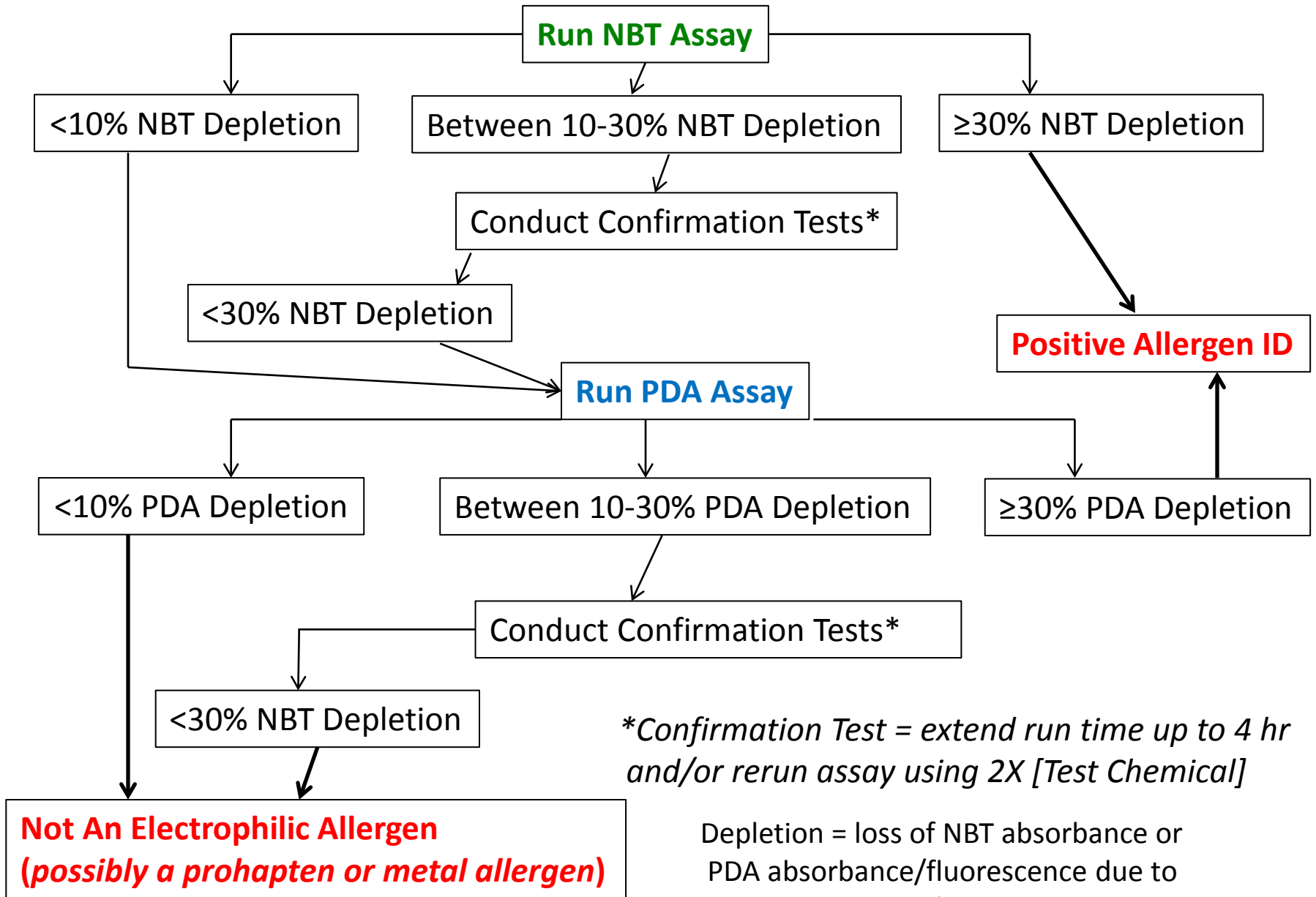


Time to completion = 80% decrease in absorbance

# PDA Reactivity vs. LLNA pEC3



# Electrophilic Allergen Decision Criteria



# Assay Performance to date

Chemical Class	Predicted Classification based on Reactivity to NBT/PDA			
		Non-sensitizer	Sensitizer	Total
	Non-sensitizer	11	0	11
	Sensitizer	13	55	68
	Total	24	55	79

- Sensitivity =  $(55/68) \times 100 = 80\%$
- Specificity =  $(11/11) \times 100 = 100\%$
- Positive Predictivity =  $(55/55) \times 100 = 100\%$
- Negative Predictivity =  $(11/24) \times 100 = 46\%$
- Accuracy =  $(66/79) \times 100 = 84\%$

# False Negatives

- Allergens that do not react to either NBT or PDA may be classified as non-electrophilic species.
- They are most likely prohaptens requiring metabolic or chemical oxidation to reactive species, or are metals.
- These would also be expected to be negative in the DPRA.

# Summary and Conclusion

## PDA/NBT Reactivity vs Peptide Reactivity and LLNA

	PDA/NBT	DPRA	LLNA
<b>Conc Range</b>	0.1-0.5 mM	5-25 mM	3 orders of magnitude
<b>Potency Ranking</b>	+	+/-	+
<b>Assay Time</b>	≤ 2 h	>24 h	6 d
<b>Cost</b>	+	++	+++
<b>Prohaptens and Non Electrophile ID</b>	-	-	+