

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

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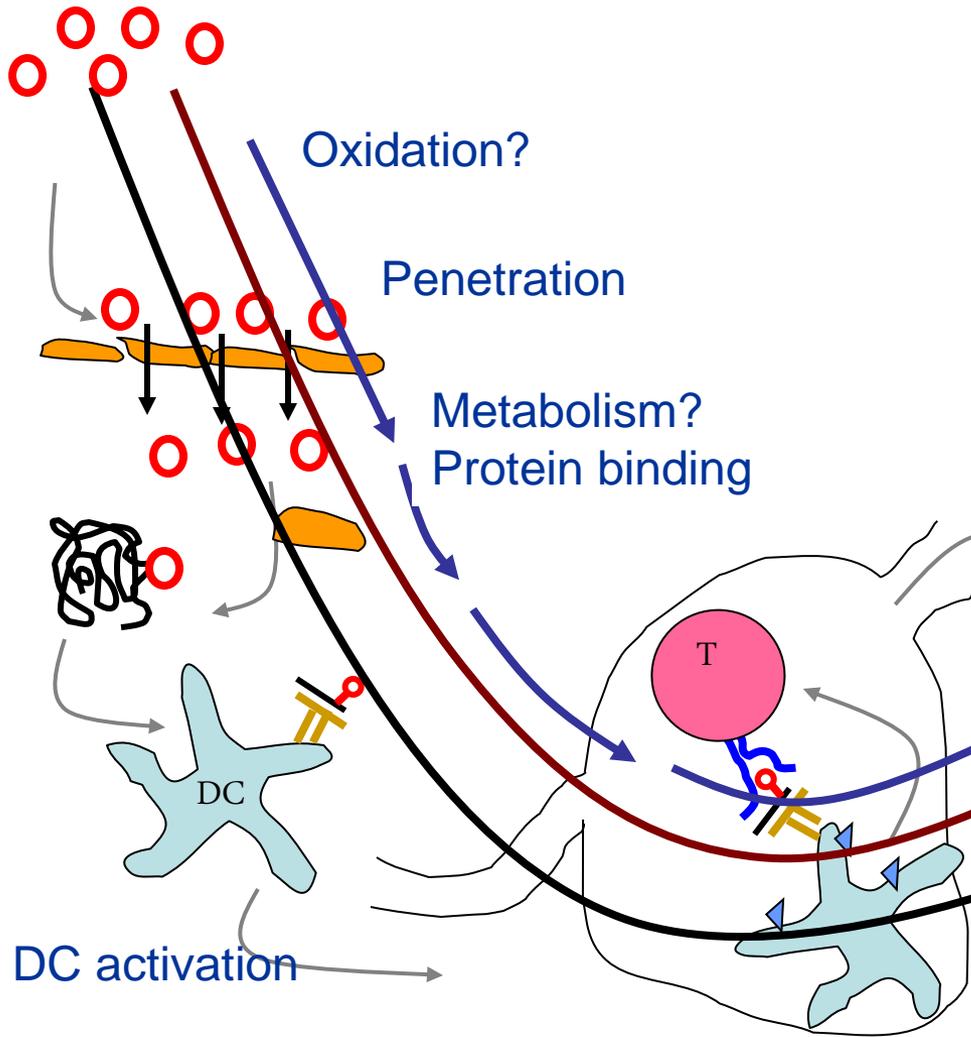
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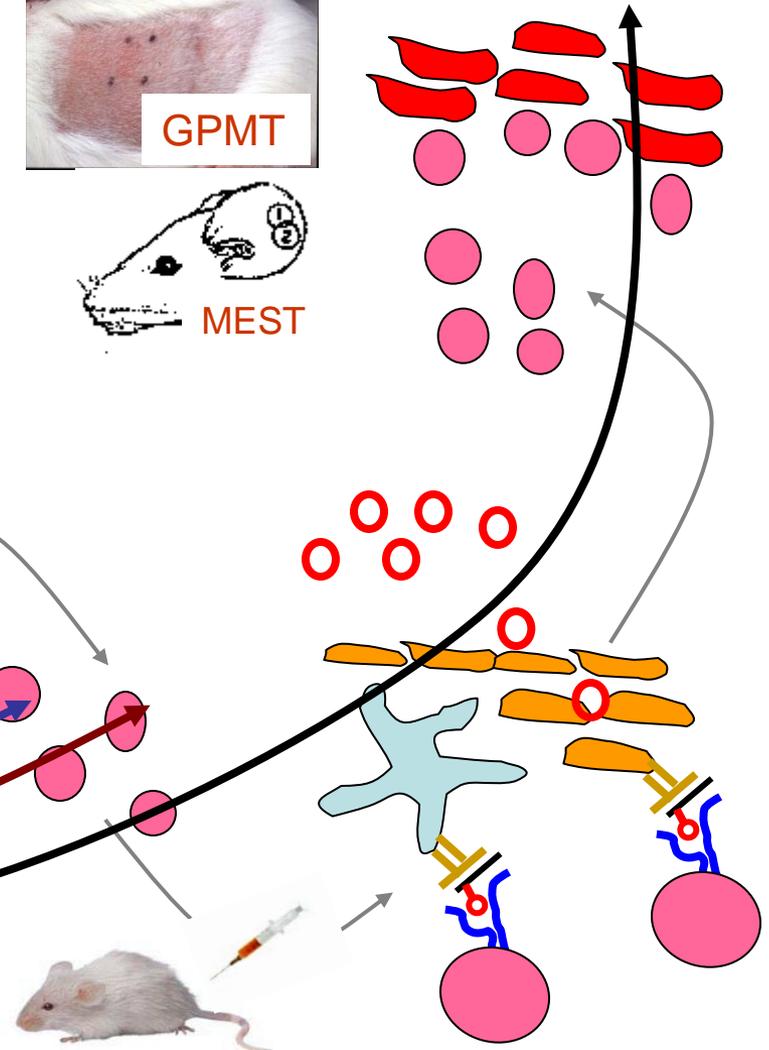
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Current Models – Animal vs Non-Animal

In-vitro, In-Chemico, In-Silico



In-Vivo



Protein Reactivity

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

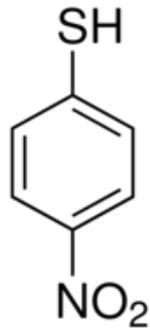
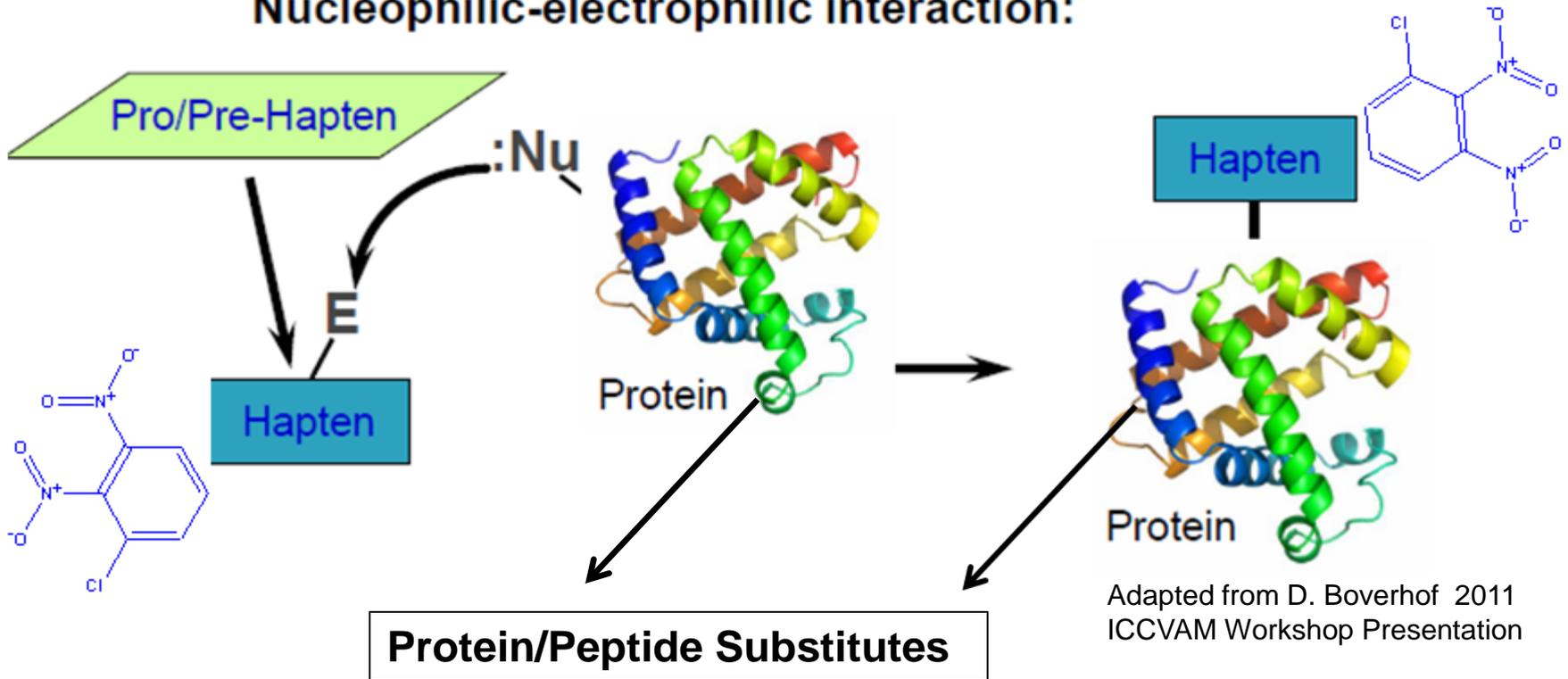
-Relevant Bonds:

covalent bonds (200-420 kJ/mol)

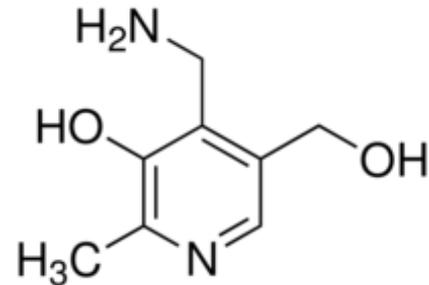
co-ordinate bonding (M^{n+} allergy)

- 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:



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 ≈ 1.1 at 412 nm
- 0.1 mM PDA and should be should ≈ 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

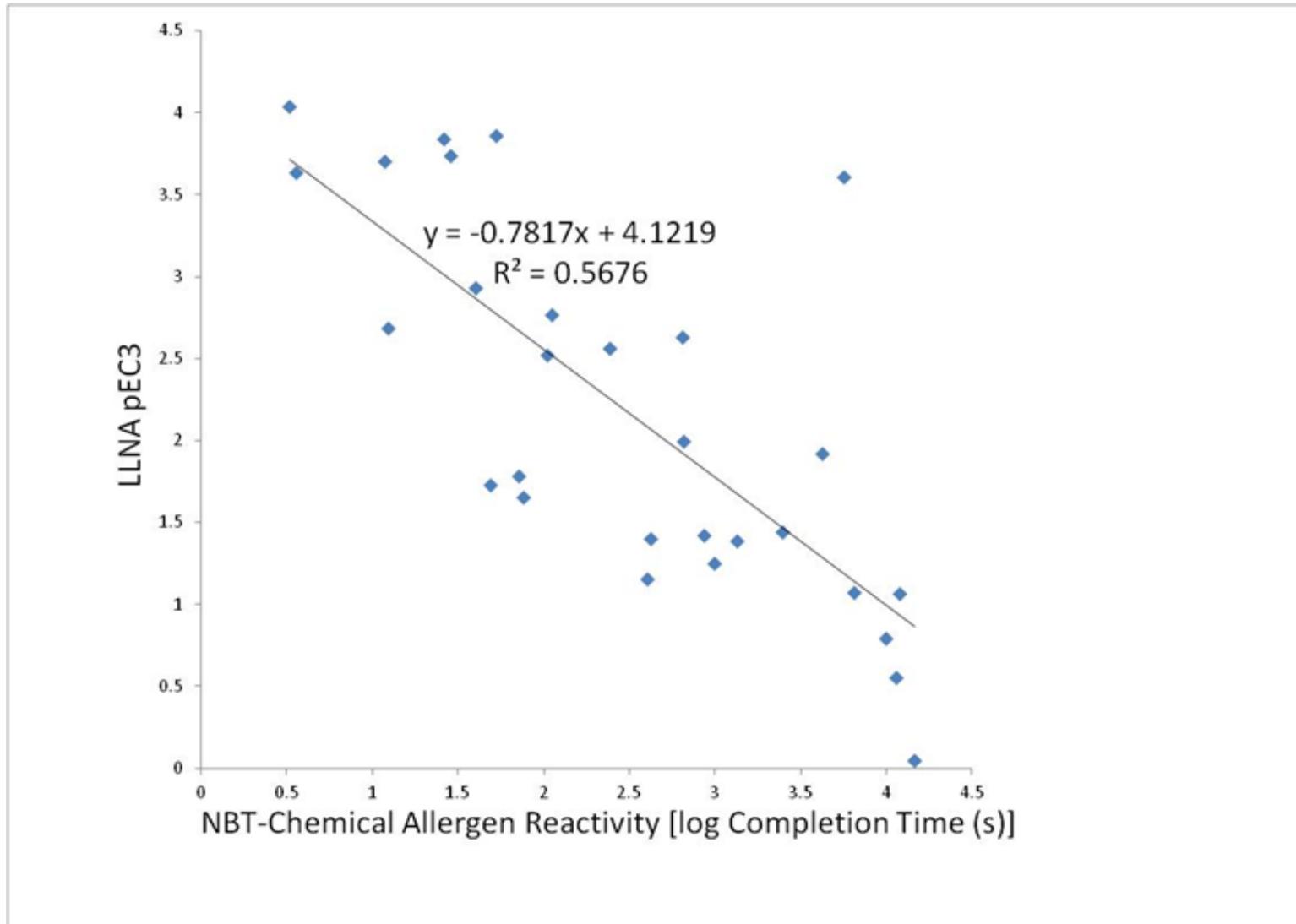
+ = positive
 - = negative
 0 = not tested

Allergens Tested in the Electrophilic Allergen Screening Assay (continued)

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

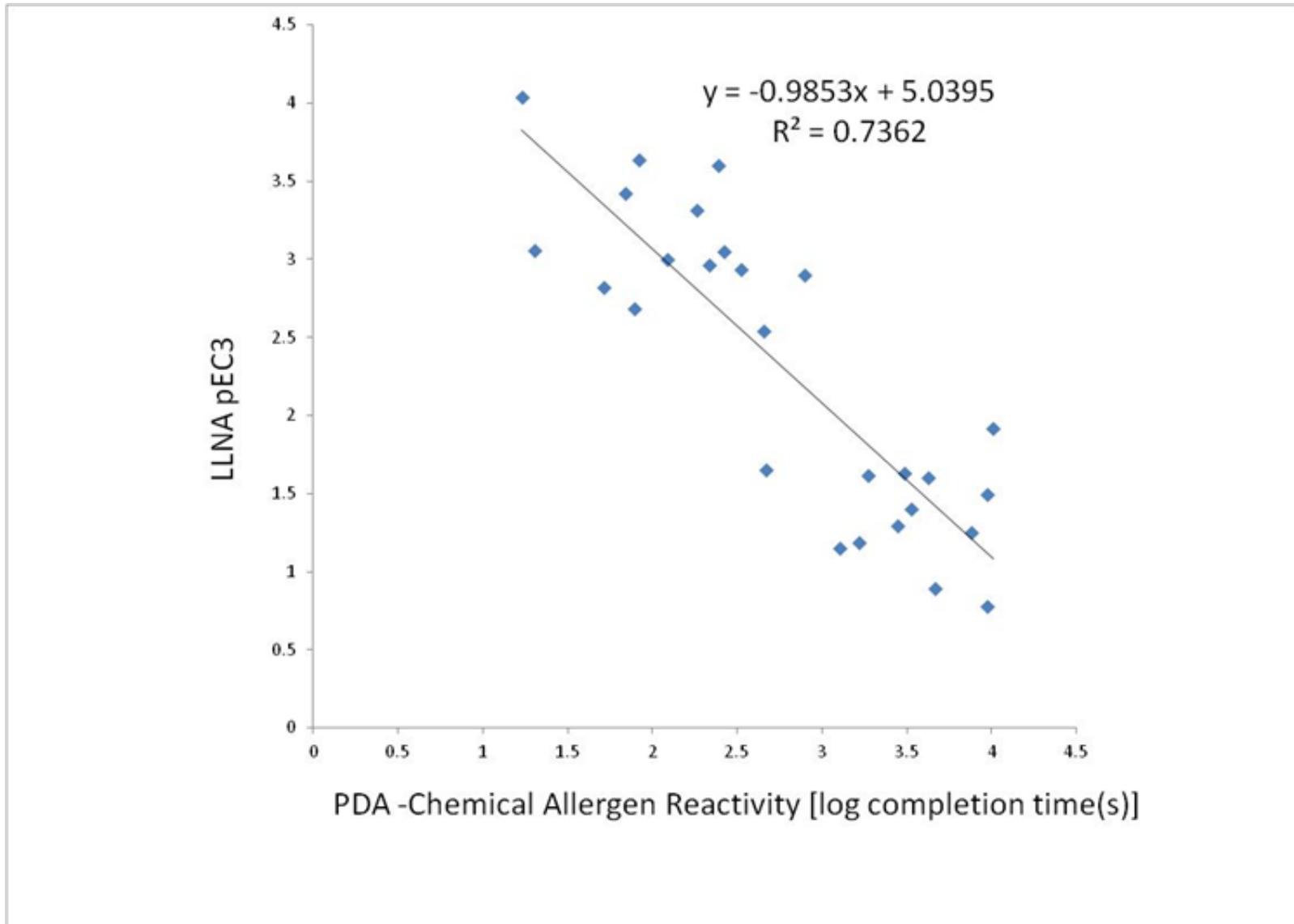
	<i>Non-Allergens Tested</i>	NBT	PDA
1	dichlorofluoronitrobenzene	-	-
2	sulfanilamide	-	-
3	benzaldehyde	-	-
4	dinitrophenol	-	-
5	chlorobenzene	-	-
6	sodium lauryl sulphate	-	-
7	benzyl cinnamate	-	-
8	acetonitrile	-	-
9	glycerol	-	-
10	acetone	-	-
11	methyl salicylate	-	-

NBT Reactivity vs. LLNA pEC3

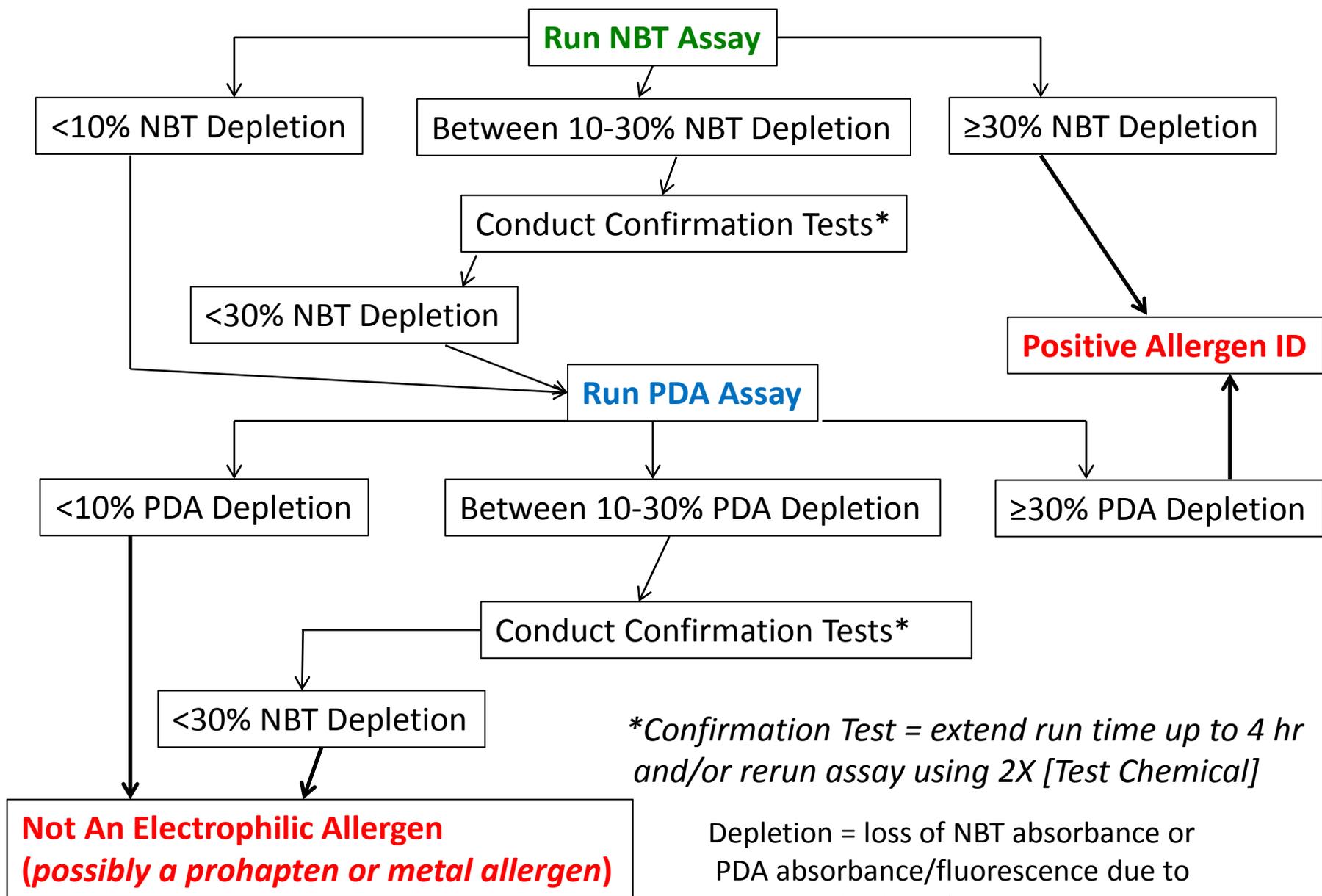


Time to completion = 80% decrease in absorbance

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