Attachment 4c

Test report

Further evaluation of the intralaboratory reproducibility of the KeratinoSens assay to detect skin sensitizers

Study period:	**January 17 – March 4, 2011**
Study laboratory:	**Givaudan *in vitro* toxicology laboratory**
Report date:	**17. March 2011, sent to ECVAM 24. March 2011**
Updated version:	**Revised 31. March 2011 after un-blinding of chemicals by ECVAM**
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1) Goal of the study

The prediction model of the KeratinoSens assay requires that at least 2 of 3 independent repetitions give a statistically significant gene induction above the threshold in order to rate chemicals positive. 28 chemicals had been evaluated twice in the same laboratory with this prediction model before. However, to explore intralaboratory reproducibility, it is desirable to have for a certain number of chemicals three full experiments, i.e. 3 times 3 repetitions. Therefore, ECVAM suggested to generate more intralaboratory reproducibility data prior to submission of the KeratinoSens assay to peer review for assessment of reliability and validity.

2) Test chemicals

ECVAM provided eight test chemicals as blind coded items. Givaudan selected 6 further chemicals which were not in our previous publication and not used in the ring study. Table 1 lists the available information on these chemicals.

Table 1: Test chemical specifications

Test chemicals	**Abbrevi-ation**	**Solvent**	**CAS-Nr.**	**MW**	**Sensitization potential**	**LLNA EC3 (%)**
Beryllium sulphate *	GMP	Water	7787-56-6	177.1	Sensitizer, positive in LLNA and GPMT	Not reported
Nickel chloride *	GFN	Water	7718-54-9	129.6	Sensitizer, false-negative in LLNA	n.a.
Chloramine T *	GCS	DMSO	149358-73-6	227.6	Strong Sensitizer	0.4
Chlorpromazine hydrochloride *	GWA	DMSO	69-09-0	355.3	Sensitizer, Photosensitizer	EC3 not reported, < 10%
R(+) Limonene *	GER	DMSO	5989-27-5	136.2	Probably sensitizer, clearly sensitizer after air oxidation	31 / <10 / 38 / 63 / 22
Methyl metacrylate *	GBT	DMSO	80-62-6	100.1	Very weak sensitizer in LLNA, positive in guinea pig tests	90
4-amino benzoic acid *	GLD	DMSO	150-13-0	137.1	Non-sensitizer	>10
2,4-Dinitrobenzene-sulfonic acid, Na salt	DNBS	Water	885-62-1	248.2	Moderate sensitizer	2
Streptomycin sulfate	Strep	Water	3810-74-0	1457.4	Non-sensitizer in LLNA, positive in Guinea pig	NC
Xylene *	GQZ	DMSO	1330-20-7	106.2	Non-sensitizer, very weak in LLNA, considered false-positive in ICCVAM performance standards	95.8
2-Ethylhexyl acrylate	2-EHA	DMSO	103-11-7	184.3	Weak	10
2-Phenylpropionaldehyde	2-PPA	DMSO	93-53-8	134.2	moderate	6.3
4-Amino-m-cresol	4-AmC	DMSO	2835-99-6	123.1	Moderate	1.5
Clofibrate (Ethyl (2-(4-chlorophenoxy)-2-methylpropanoate)	Clofi	DMSO	637-07-0	242.7	Non-sensitizer	NC

* Chemicals received from ECVAM as blind-coded items

3) Study setup

The 14 chemicals in Table 1 were tested in two master-plates, which were set-up according to the standard plate layout of the SOP. All the chemical solutions and master plates were prepared freshly for the different runs. Streptomycin was tested up to a maximal concentration of 274 µM due to the high molecular weight. Each master plate was run in three runs (each in triplicate) and the complete procedure was then repeated three times. These, in total nine, runs were all performed on different test days with different passages of the test cells. For all procedures, there was strict adherence to the SOP, and the method is therefore not further described here.

4) Test results

4.1. Maximal gene induction

The maximal gene induction (I_{max}) shows the dynamic range of gene induction by the different chemicals. As the prediction model rates any chemical positive with an I_{max} , which is statistically significant above solvent control and above the threshold of 1.5, the absolute value of the I_{max} is not very important for the prediction, but this parameter is illustrative to compare the reproducibility of gene induction over the experiments. These data are thus shown in Table 2.

The full dose response curves for each run and for the average of each experiment (hence 12 dose-response curves) are given in the Appendix and the separate file (Attachment4c_second intralab study_appendix-dose-response_new.pdf) for each chemical.

Table 2. Maximal gene induction (fold over solvent control) in the full dose response.

	Experiment 1				Experiment 2				Experiment 3			
Compound	**rep1**	**rep2**	**rep3**	**Avg**	**rep1**	**rep2**	**rep3**	**Avg**	**rep1**	**rep2**	**rep3**	**Avg**
GMP	5.48[1)]	8.69	5.33	**6.50**	5.65	3.89	3.78	**4.44**	6.49	6.60	5.24	**6.11**
GFN	1.80	2.15	4.96	**2.97**	1.59 [2)]	1.78	2.29	**1.89**	3.98	2.71	3.40	**3.36**
GCS	55.18	3.49	15.14	**24.60**	12.49	5.37	28.34	**15.40**	41.02	37.04	51.23	**43.10**
GWA	0.91	1.31	0.89	**1.04**	1.47	1.04	1.23	**1.24**	1.25	1.11	1.03	**1.13**
GER	1.33	1.52	1.51	**1.45**	1.56	1.46	1.14	**1.39**	1.49	1.56	1.28	**1.44**
GBT	2.62	2.39	2.57	**2.53**	1.55	2.59	2.15	**2.10**	2.43	2.27	1.85	**2.18**
GLD	1.33	1.37	1.30	**1.33**	1.14	1.46	1.48	**1.36**	1.57	1.37	1.29	**1.41**
DNBS	21.85	21.52	26.04	**23.14**	22.33	18.70	24.67	**21.90**	53.09	23.04	34.71	**36.95**
Strep	1.06	1.13	1.11	**1.10**	1.12	1.20	1.02	**1.11**	1.34	1.37	1.45	**1.38**
GQZ	1.08	1.14	1.17	**1.13**	1.05	1.02	1.14	**1.07**	1.18	1.05	1.27	**1.17**
2-EHA	3.89	1.52	3.69	**3.03**	6.64	6.42	5.64	**6.23**	6.39	5.41	5.40	**5.73**
2-PPA	3.04	8.69	2.64	**4.79**	18.47	2.29	10.82	**10.53**	4.40	4.65	3.82	**4.29**
4-AmC	15.31	15.62	8.87	**13.27**	21.39	14.47	36.51	**24.12**	29.11	7.44	27.78	**21.45**
Clofi	1.15	1.10	1.08	**1.11**	1.08	1.10	1.13	**1.10**	1.11	0.97	1.42	**1.16**

[1)] Shown are for each run (rep1 to 3) the average values from the three replicate plates. For each of the experiments the average of the three runs is also given in the bold columns.
[2)] Induction above 1.5 but not statistically significant in this run

4.2. EC1.5 concentration values for gene induction

For each run, the calculated EC1.5 concentration value, extrapolated from the data point above and below the 1.5-fold induction threshold was calculated. These data are shown in Table 3. EC 1.5 values are only shown for those runs, for which the induction above the threshold is statistically significant.
By comparing the bold columns, it becomes obvious that for most chemicals the EC 1.5 values obtained in the three experiments are quite close to each other, and vary less than a factor of two. The variability was

further compared by calculating the geometric standard deviation[1] (see Table 3b). For most chemicals these standard deviations within the experiments (which indicate the factor of variation), were below 1.41 and on the average for all experiments they are at 1.22. In the last column of Table 3b also the geometric standard deviations over the geometric means of each experiment are given. This is the true measure of the intralaboratory variability of the EC1.5 value in full experiments. For most chemicals these were below 1.2, which indicates that the 95% confidence interval is less than ½ well down and up in the dilution series.

Table 3. EC1.5 values (concentration in µM for 1.5 fold gene induction)

	Experiment 1				Experiment 2				Experiment 3			
Compound	**Rep1**	**Rep2**	**rep3**	**Avg**	**rep1**	**rep2**	**rep3**	**Avg**	**rep1**	**rep2**	**rep3**	**Avg**
GMP	13.7	5.2	10.1	**9.0**	17.7	20.0	40.9	**24.4**	13.0	11.2	14.2	**12.7**
GFN	413.0	332.8	265.9	**331.9**	Non sig. [1)]	419.1	375.2	**396.5**	293.9	333.2	309.7	**311.9**
GCS	252.0	221.5	250.6	**240.9**	251.8	262.1	253.9	**255.9**	194.7	250.6	250.7	**230.4**
GWA	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**
GER	n.i.	118.8	61.1	**85.2**	221.3	n.i.	n.i.	**n.i.**	n.i.	55.8	n.i.	**n.i.**
GBT	352.1	310.4	241.7	**297.8**	866.0	895.9	484.5	**721.7**	270.3	548.8	470.4	**411.7**
GLD	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**	505.5	n.i.	n.i.	**n.i.**
DNBS	94.1	89.0	67.9	**82.9**	99.4	79.4	88.7	**88.8**	67.6	84.2	59.3	**69.6**
Strep	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**
GQZ	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**
2-EHA	33.5	60.4	33.4	**40.7**	53.5	32.7	32.4	**38.4**	31.9	32.6	32.1	**32.2**
2-PPA	39.0	48.5	37.4	**41.3**	46.0	40.6	63.3	**49.1**	52.2	43.5	41.9	**45.7**
4-AmC	11.7	8.1	8.6	**9.3**	8.0	11.6	13.3	**10.7**	9.6	7.8	12.1	**9.7**
Clofi	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**	n.i.	n.i.	n.i.	**n.i.**

[1)] Induction above 1.5 but not statistically significant in this run
n.i. indicates run with no statistically significant induction above 1.5-fold.

Table 3 b. Geometric standard deviation of the EC1.5 values for the positive chemicals

	Experiment 1	Experiment 2	Experiment 3	Between experiments
GMP	1.65 [1]	1.57	1.13	1.66 [2]
GFN	1.25	1.08	1.07	1.13
GCS	1.08	1.02	1.16	1.05
GBT	1.21	1.41	1.45	1.56
DNBS	1.19	1.12	1.19	1.13
2-EHA	1.41	1.33	1.01	1.13
2-PPA	1.15	1.26	1.12	1.09
4-AmC	1.22	1.30	1.24	1.07
Average	**1.27**	**1.26**	**1.17**	**1.23**

[1] For each Experiment the geometric standard deviation of the three repetitions was calculated
[2] Indicates the geometric standard deviation over the geometric means of each experiment

[1] Based on Log2-transformed values, the logarithmic standard deviations were calculated. These values were then retransformed calculating the exponential function with base, thereby rendering the geometrical standard deviation, which corresponds to a factor. (Numerical example: If the standard deviation of the Log2 transformed values is 0.5, the geometric standard deviation is 1.414 or the square root of 2. The 95.4% confidence interval of the Log2 transformed values then becomes ± 1 (i.e. twice the standard deviation) and the geometric (or re-transformed) 95.4% confidence interval is confined by a factor of 2. Thus in this specific case, the 95.4% confidence interval is covered by the concentration range one well in the microtiter plate up and down of the geometric mean.)

4.3. IC50 values for cytotoxicity

Table 4 lists all the cytotoxicity values. Given are extrapolated values for 50% reduction in cellular viability as determined by the MTT assay. With the exception of the compound GMP, the IC50 values obtained in the three experiments are nicely reproducible. Compound GMP has very flat dose-response curves for cytotoxicity, and this leads also to higher variability of the IC50 values. This result will be further discussed below. The variability was further compared by calculating the geometric standard deviation (see Table 4b). For most chemicals these standard deviations within the experiments (which indicate the factor of variation), were below 1.41 and on the average for all experiments, excluding the variable chemical GMP, they are at 1.21. In the last column of Table 4b also the geometric standard deviations over the geometric means of each experiment are given. This is the true measure of the intralaboratory variability of the IC50 value in full experiments. For most chemicals these were below 1.2, which indicates that the 95% confidence interval is less than ½ well down and up in the dilution series.

Table 4. IC50 values (concentration in µM for 50% reduction in viability)

	Experiment 1				Experiment 2				Experiment 3			
Compound	**Rep1**	**rep2**	**rep3**	**Avg**	**Rep1**	**rep2**	**rep3**	**Avg**	**rep1**	**rep2**	**rep3**	**Avg**
GMP	17.6	102.1	6.4	**22.6**	45.8	65.7	322.2	**98.9**	51.1	23.0	29.3	**32.5**
GFN	807.9	745.1	870.1	**806.0**	878.6	845.2	876.1	**866.5**	874.3	905.5	899.2	**892.9**
GCS	745.6	725.5	788.3	**752.7**	803.6	734.7	759.6	**765.4**	773.3	818.7	774.6	**788.6**
GWA	9.8	10.0	7.4	**8.9**	13.2	9.7	10.6	**11.1**	10.3	10.9	9.7	**10.3**
GER	96.8	394.2	96.0	**154.2**	391.0	192.1	95.0	**192.5**	90.6	102.4	99.2	**97.3**
GBT	>2000	>2000	>2000	**>2000**	>2000	>2000	>2000	**>2000**	>2000	>2000	>2000	**>2000**
GLD	>2000	>2000	>2000	**>2000**	>2000	>2000	>2000	**>2000**	>2000	>2000	>2000	**>2000**
DNBS	633.1	882.0	739.6	**744.7**	972.0	730.9	785.6	**823.3**	657.1	680.9	758.8	**697.6**
Strep	>274	>274	>274	**>274**	>274	>274	>274	**>274**	>274	>274	>274	**>274**
GQZ	1294.8	>2000	1254.0	**1274.2**	1427.9	1771.4	772.3	**1250.1**	1449.7	1517.3	1283.4	**1413.3**
2-EHA	62.7	92.1	94.1	**81.6**	132.1	95.8	89.8	**104.4**	47.7	75.8	78.0	**65.6**
2-PPA	101.3	126.6	103.8	**110.0**	112.6	103.3	113.3	**109.6**	97.9	104.1	106.3	**102.7**
4-AmC	56.7	86.9	49.8	**62.6**	99.1	54.9	88.5	**78.4**	51.0	51.6	69.3	**56.7**
Clofi	195.3	248.1	269.1	**235.4**	209.2	224.5	201.4	**211.5**	196.8	199.2	195.9	**197.3**

Table 4 b. Geometric standard deviations of the IC50 values for the cytotoxic chemicals

	Experiment 1	Experiment 2	Experiment 3	Between experiments
GMP	4.05[1]	2.82	1.51	2.16[2]
GFN	1.08	1.02	1.02	1.05
GCS	1.04	1.05	1.03	1.02
GWA	1.18	1.17	1.06	1.12
GER	2.25	2.03	1.07	1.42
DNBS	1.18	1.16	1.08	1.09
GQZ	1.02	1.54	1.09	1.07
2-EHA	1.26	1.23	1.32	1.26
2-PPA	1.13	1.05	1.04	1.04
4-AmC	1.34	1.37	1.19	1.18
Clofi	1.18	1.06	1.01	1.09
Average	**1.52**	**1.41**	**1.13**	**1.23**

[1] For each Experiment the geometric standard deviation of the three repetitions was calculated
[2] Indicates the geometric standard deviation of the geometric mean of each experiment

4.4. Positive / negative rating of chemicals

Based on the induction above the 1.5-fold threshold at non-cytotoxic concentrations, the individual runs were then evaluated for positive or negative ratings according the prediction model, and Table 5 lists this rating for each run and the overall rating for each experiment. Table 5b then lists the final conclusion from each experiment. This final conclusion was always the same for 12 of the 14 chemicals. For two chemicals discordant results were obtained in one experiment. These will be discussed in the discussion section.

Table 5. Positive / negative rating of the chemicals in the individual runs

	Experiment 1				Experiment 2				Experiment 3			
Compound	**rep1**	**rep2**	**rep3**	**Avg**	**Rep1**	**rep2**	**rep3**	**Avg**	**rep1**	**rep2**	**rep3**	**Avg**
GMP	Cytotox[1)]	1	cytotox	1 of 3	cytotox	cytotox	1	1 of 3	1	cytotox	cytotox	1 of 3
GFN	1	1	1	3 of 3	0	1	1	2 of 3	1	1	1	3 of 3
GCS	1	1	1	3 of 3	1	1	1	3 of 3	1	1	1	3 of 3
GWA	0	0	0	0 of 3	0	0	0	0 of 3	0	0	0	0 of 3
GER	0	1	1	2 of 3	1	0	0	1 of 3	0	1	0	1 of 3
GBT	1	1	1	3 of 3	1	1	1	3 of 3	1	1	1	3 of 3
GLD	0	0	0	0 of 3	0	0	0	0 of 3	1	0	0	1 of 3
DNBS	1	1	1	3 of 3	1	1	1	3 of 3	1	1	1	3 of 3
Strep	0	0	0	0 of 3	0	0	0	0 of 3	0	0	0	0 of 3
GQZ	0	0	0	0 of 3	0	0	0	0 of 3	0	0	0	0 of 3
2-EHA	cytotox	1	1	2 of 3	1	1	1	3 of 3	cytotox	cytotox	cytotox	cytotox
2-PPA	1	1	1	3 of 3	1	1	1	3 of 3	1	1	1	3 of 3
4-AmC	1	1	1	3 of 3	1	1	1	3 of 3	1	1	1	3 of 3
Clofi	0	0	0	0 of 3	0	0	0	0 of 3	0	0	0	0 of 3

[1)] '0' indicates negative, '1' indicates positive, 'cytotox' indicates statistically significant induction above threshold of 1.5, but only at < 70% cell viability

Table 5b. Overall assessment of the chemicals in the three experiments

Test chemicals	Abreviation	Experiment 1	Experiment 2	Experiment 3
Beryllium sulphate	GMP	Negative (*)	Negative (*)	Negative (*)
Nickel chloride	GFN	Positive	Positive	Positive
Chloramine T	GCS	Positive	Positive	Positive
Chlorpromazine hydrochloride	GWA	Negative	Negative	Negative
R(+) Limonene	GER	Positive	Negative	Negative
Methyl metacrylate	GBT	Positive	Positive	Positive
4-amino benzoic acid	GLD	Negative	Negative	Negative
2,4-Dinitrobenzene-sulfonic acid	DNBS	Positive	Positive	Positive
Streptomycin sulfate	Strep	Negative	Negative	Negative
Xylene	GQZ	Negative	Negative	Negative
2-Ethylhexyl acrylate	2-EHA	Positive	Positive	Negative (*)
2-Phenylpropionaldehyde	2-PPA	Positive	Positive	Positive
4-Amino-m-cresol	4-AmC	Positive	Positive	Positive
Clofibrate (Ethyl (2-(4-chloro-phenoxy)-2-methylpropanoate)	Clofi	Negative	Negative	Negative

(*) negative ratings due to cytotoxicity at inducing concentrations

4.5. Gene induction by the positive control cinnamic aldehyde and variability in the solvent control wells.

Each test plate contained a five-point dose-response of the positive control cinnamic aldehyde. These results are listed in Table 6. This Table also includes the extrapolated EC1.5 values, and it indicates whether the acceptance criteria were fulfilled. Figure 1 summarizes all the dose-response curves for cinnamic aldehyde, and, for comparison purposes, the results of the lead lab from the ring study. Cinnamic aldehyde induced the luciferase gene in all runs and the EC1.5 value was between 18 and 24 µM in all experiments. The acceptance criteria were fulfilled for all runs with the exception of the EC1.5 in Experiment 2, plate 2, rep 2 being at 30.08 instead of a maximal of 30 and the induction at 64 µM in Exp. 3, plate 1, rep 3 being at 1.99 instead of a minimum of 2. Since these values were so close to the target values and the other two criteria were fulfilled in these runs, these two runs were still accepted.

Table 6b lists the variability of the solvent control values and indicates fulfillment of the acceptance criterion of this parameter. All runs were below the target of maximal 20% variability, with an average of 10.1% and individual runs varying between 6.4 and 15.2% of variation in the solvent controls.

Table 6. Dose-response results for cinnamic aldehyde

		Fold-induction					EC 1.5 (conc µM)	EC1.5 (between 7 µM band 30 µM)	Induction at 64 µM (between 2 and 8 fold)
	Conc.	**4 µM**	**8 µM**	**16 µM**	**32 µM**	**64 µM**			
Experiment 1, plate 1	rep1a	1.13	1.32	1.38	1.81	2.61	20.51	TRUE	TRUE
Experiment 1, plate 1	rep2a	1.18	1.22	1.33	1.75	2.31	22.38	TRUE	TRUE
Experiment 1, plate 1	rep3a	1.17	1.36	1.57	2.11	3.39	13.35	TRUE	TRUE
	Average	**1.16**	**1.30**	**1.43**	**1.89**	**2.77**	**18.75**		
Experiment 1, plate 2	rep1b	1.20	1.11	1.36	1.64	2.72	23.85	TRUE	TRUE
Experiment 1, plate 2	rep2b	1.17	1.32	1.38	1.66	2.65	22.86	TRUE	TRUE
Experiment 1, plate 2	rep3b	1.33	1.19	1.51	2.05	3.78	15.83	TRUE	TRUE
	Average	**1.24**	**1.21**	**1.41**	**1.78**	**3.05**	**20.85**		
Experiment 2, plate 1	rep1a	1.12	1.26	1.43	1.60	2.65	22.16	TRUE	TRUE
Experiment 2, plate 1	rep2a	1.01	1.23	1.42	1.71	2.55	20.24	TRUE	TRUE
Experiment 2, plate 1	rep3a	1.10	1.35	1.53	1.72	2.83	14.56	TRUE	TRUE
	Average	**1.07**	**1.28**	**1.46**	**1.68**	**2.68**	**18.98**		
Experiment 2, plate 2	rep1b	1.13	1.22	1.41	1.65	2.59	21.92	TRUE	TRUE
Experiment 2, plate 2	rep2b	1.10	1.21	1.50	1.57	2.69	30.08	FALSE	TRUE
Experiment 2, plate 2	rep3b	0.98	1.26	1.30	1.98	2.95	20.70	TRUE	TRUE
	Average	**1.07**	**1.23**	**1.40**	**1.73**	**2.74**	**24.23**		
Experiment 3, plate 1	rep1a	1.17	1.41	1.54	2.10	3.17	13.42	TRUE	TRUE
Experiment 3, plate 1	rep2a	1.08	1.27	1.44	1.65	2.39	20.58	TRUE	TRUE
Experiment 3, plate 1	rep3a	1.06	1.09	1.27	1.73	1.99	23.98	TRUE	FALSE
	Average	**1.10**	**1.26**	**1.42**	**1.83**	**2.52**	**19.33**		
Experiment 3, plate 2	rep1b	1.17	1.28	1.56	2.17	3.05	14.31	TRUE	TRUE
Experiment 3, plate 2	rep2b	1.03	1.24	1.29	1.70	2.81	24.19	TRUE	TRUE
Experiment 3, plate 2	rep3b	0.78	1.29	1.25	1.54	2.31	29.74	TRUE	TRUE
	Average	**0.99**	**1.27**	**1.37**	**1.80**	**2.72**	**22.75**		

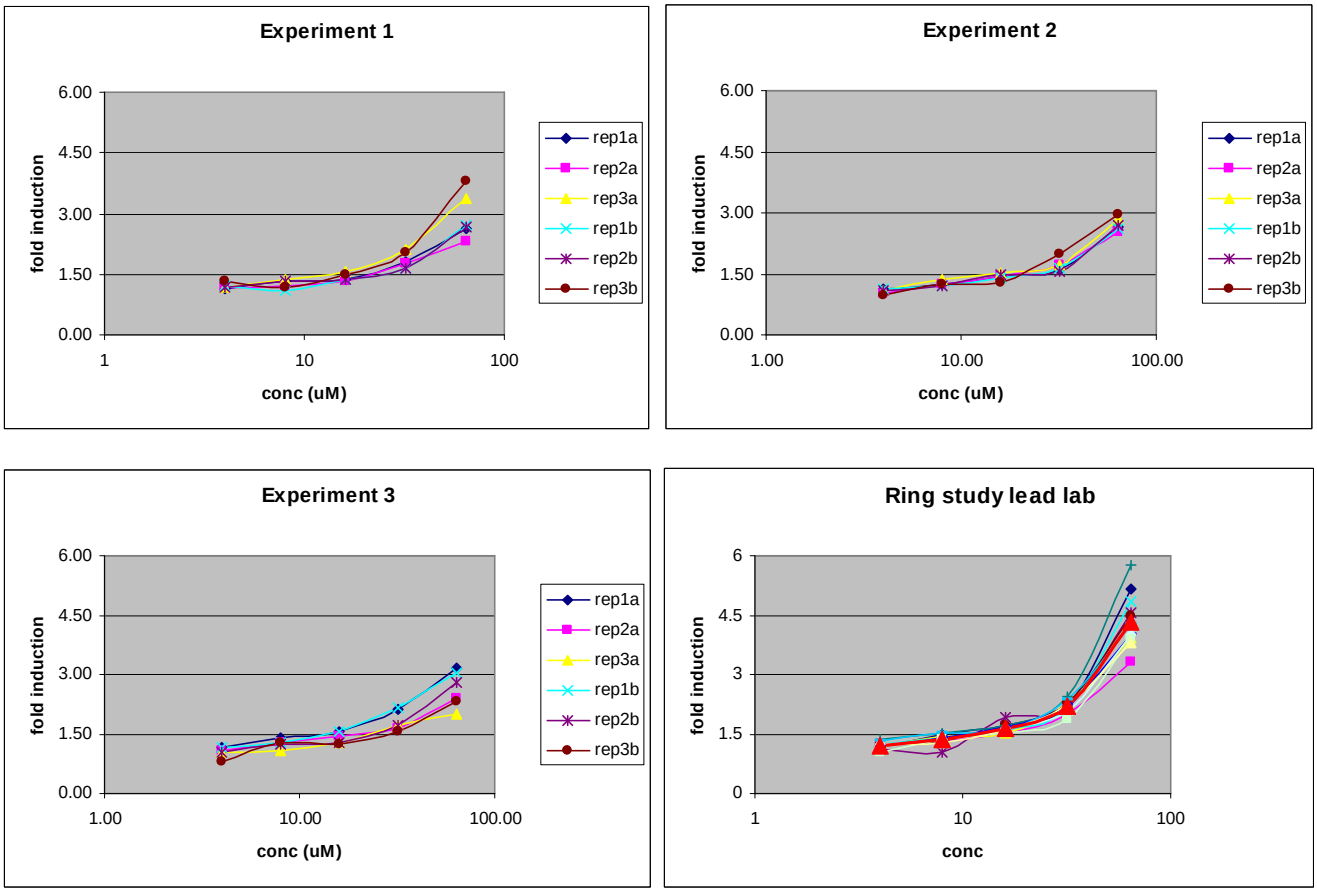

Figure 1: The dose-response curves for the six runs (2plates, 3 reps) in each of the three experiments. As comparison the results of the same lab in the ring study are shown

Table 6b. Variability of the solvent control values in each run

	% standard deviation blanks	
Experiment 1, plate 1	6.44	**ACCEPTED**
Experiment 1, plate 1	9.96	**ACCEPTED**
Experiment 1, plate 1	6.83	**ACCEPTED**
Experiment 1, plate 2	11.66	**ACCEPTED**
Experiment 1, plate 2	11.34	**ACCEPTED**
Experiment 1, plate 2	10.94	**ACCEPTED**
Experiment 2, plate 1	10.96	**ACCEPTED**
Experiment 2, plate 1	11.63	**ACCEPTED**
Experiment 2, plate 1	15.26	**ACCEPTED**
Experiment 2, plate 2	6.69	**ACCEPTED**
Experiment 2, plate 2	8.20	**ACCEPTED**
Experiment 2, plate 2	11.25	**ACCEPTED**
Experiment 3, plate 1	10.51	**ACCEPTED**
Experiment 3, plate 1	9.92	**ACCEPTED**
Experiment 3, plate 1	12.02	**ACCEPTED**
Experiment 3, plate 2	13.18	**ACCEPTED**
Experiment 3, plate 2	7.84	**ACCEPTED**
Experiment 3, plate 2	7.19	**ACCEPTED**

5) Non-qualified runs

At the beginning of the testing phase an initial run was performed, which was contaminated by bacterial growth. The source was a contaminated flask of culture medium[2]. The variability in the control wells was > 50% and the bacterial contaminations was obvious from change in media pH and microscopic observation. This run was discarded, but thereafter no further runs had to be discarded. The two cases with borderline values of the positive control cinnamic aldehyde could be accepted, as the values were so close to acceptance criteria, and the other acceptance criteria data indicated these were clearly valid runs (see above).

6) Discussion

6.1. Chemicals with clear-cut result in the intra-laboratory repeatability

The chemicals

- GFN **Nickel chloride**
- GCS **Chloramine T**
- GBT **Methyl metacrylate**
- DNBS **2,4-Dinitrobenzene-sulfonic acid**
- 2-PPA **2-Phenylpropionaldehyde**
- 4-AmC **4-Amino-m-cresol**

are clearly rated positive and are predicted as sensitizers by all the three experiments.

The chemicals

- GWA **Chlorpromazine hydrochloride**
- GLD **4-amino benzoic acid**
- Strep **Streptomycin sulfate**
- GQZ **Xylene**
- Clofi **Clofibrate**

would be rated as negative and thus non-sensitizers taking the evidence from KeratinoSens only.

6.2. Chemicals with some ambiguity

R(+) Limonene (GER)
Limonene was positive in one experiment, with two runs giving statistically significant gene induction just above the threshold. It was positive in one run of the other experiments, hence it is rated once positive and twice negative. However looking at the average I_{max}, which was 1.44, 1.45 and 1.39 in the three experiments, and also considering the dose response curves, it becomes clear that overall this test item gave consistent responses, but since gene induction is so close to the threshold of the prediction model, a variation in the yes/no rating is obtained.

2-ethyl-hexyl-acrylate

This chemical gave significant gene induction in all runs, with nicely reproducible EC1.5 values. However, the IC50 values were lower in one run in Experiment 1 and in all runs in Experiment 3, and in these runs the EC1.5-determining concentration fell in the cytotoxic range. Based on this finding it had to be rated as negative in the 3rd experiment.

[2] Note: The KeratinoSens assay and all the culture steps are always performed without the addition of antibacterial / antimycotic antibiotics to the cell culture medium, such as Penicillin / Streptomycin, as Penicillin itself is a weak skin sensitizer and could interfere with the assay

Looking at the dose-response, it is a borderline case: The gene induction at the non-cytotoxic concentrations in the four negative runs is just below the threshold in the last viable concentration. Ethyl-hexyl acrylate is a reactive molecule with surfactant properties, and thus may affect cell viability by both reactive and narcotic action. The result for such molecules may be confounding in few cases if the narcotic /cytotoxic action of the molecule becomes dominant. A similar effect was observed before for hexyl-cinnamic aldehyde (see publication of the ring-study for discussion).

***GMP* - Beryllium sulphate**

The chemical GMP is consistently rated negative due to cytotoxic action at the inducing concentration in two of the three runs in each experiment. It should be noted that this chemical has a particular effect on the cellular viability. While for most chemicals viability drops from 100% to 0% within 2-3 wells of the dilution series (see separate Appendix for dose-response curves), GMP has a flat dose-response over the full tested range of concentrations (See Figure 2 and Appendix).

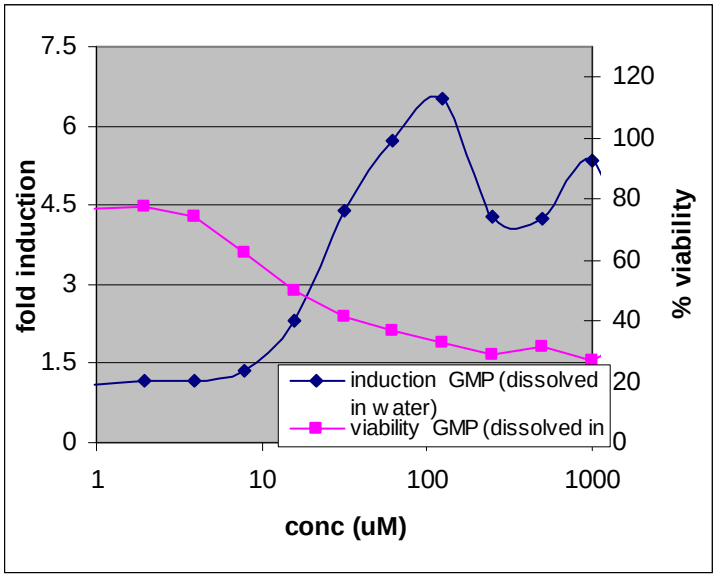

Figure 2: Dose response in Experiment 1 for the test item GMP

6.3. Discussion of the prediction of the sensitization potential

For the following chemicals, the prediction of the weight-of-evidence *in vivo* sensitization potential appears correct.

- GFN **Nickel chloride**
- GCS **Chloramine T**
- GBT **Methyl metacrylate**
- DNBS **2,4-Dinitrobenzene-sulfonic acid**
- 2-PPA **2-Phenylpropionaldehyde**
- 4-AmC **4-Amino-m-cresol**
- GLD **4-amino benzoic acid**
- GQZ **Xylene**
- Clofi **Clofibrate**
- 2-EHA **2-ethyl-hexyl-acrylate**

For **Streptomycin sulfate,** the LLNA result is predicted correctly, but it does differ from the guinea pig and human result. Note: Streptomycin had in this study only been tested up to 274 µM (4% stock solution)

due to its high molecular weight. It was later also repeated up to 2000 µM and remained negative even at higher test concentrations.

***GMP* - Beryllium sulphate** is not correctly predicted. This test item did induce significant and reproducible luciferase activity, but (probably due to the very flat cytotoxicity dose-response curves) it was positive only at cytotoxic levels in most runs, and is thus rated negative.

R(+) Limonene (*GER)*

R- Limonene was rated negative in the current study with the exception of one experiment. The results indicate it is a borderline chemical.

For Limonene, the sensitization potential of the parent compound is disputed and the LLNA EC3 varies significantly between studies (Table 1). Forced oxidation of limonene clearly enhances its sensitization potential, and oxidized limonene is widely described as a skin sensitizer (Karlberg et al., 1992; Karlberg and Dooms-Goossens, 1997; Matura et al., 2002). We had tested this test item before in an assay with a forced-oxidation step: Limonene was stirred as a thin layer in an atmosphere of pure oxygen for 28 days. At repeated intervals, a sample was taken and investigated in the KeratinoSens. In parallel, the sample was analyzed by GC-MS. Figure 3 gives a summary of the results, clearly indicating that Limonene and a related prehapten become positive in the KeratinoSens if an oxidation step is included. The increasing I_{max} is paralleled by a decrease of the parent compound detected in GC-MS.

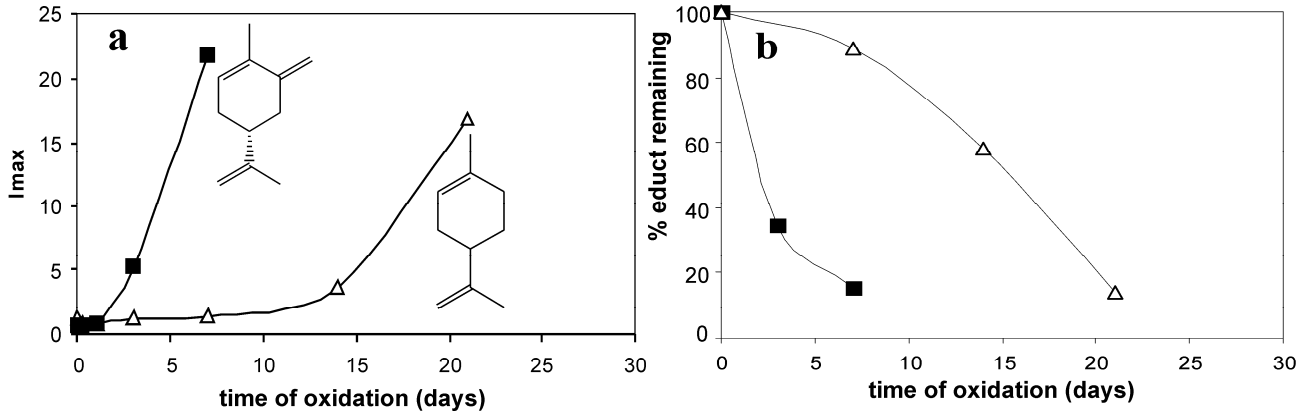

Figure 3. Induction of ARE-dependent luciferase activity by Limonene and a related pro/prehapten (Nilsson et al., 2005) oxidized for increasing time ranges. Shown is (a) the I_{max} value as a function of time, indicating at each time point the maximal fold-induction of luciferase in a dose-response curve between 1 and 2000 µM and (b) the analytical result showing % of the non-oxidized compound remaining in the O_2-exposed sample. Closed squares, conjugated diene; open triangles, limonene.

Chlorpromazine hydrochloride is known both as sensitizer (positive in guinea pig tests and the LLNA), but it is particularly known as a photo-sensitizer.

The direct acting sensitization potential was not apparent in the KeratinoSens assay in this study.

We had tested this test item before in an assay with a photo-activation step. Results are shown in Figure 4. If cells were irradiated with daylight UVA-light for 1 h after substance addition, Chlorpromazine was positive in the KeratinoSens assay, but not if cells were kept in darkness in a parallel treatment. The result of this dark treatment is very similar as the result of the blind coded item tested in the current study (IC50 for cytotoxicity of 7.8 µM, current study geometric mean of IC50 of 10.1 µM, no gene induction in both studies.).

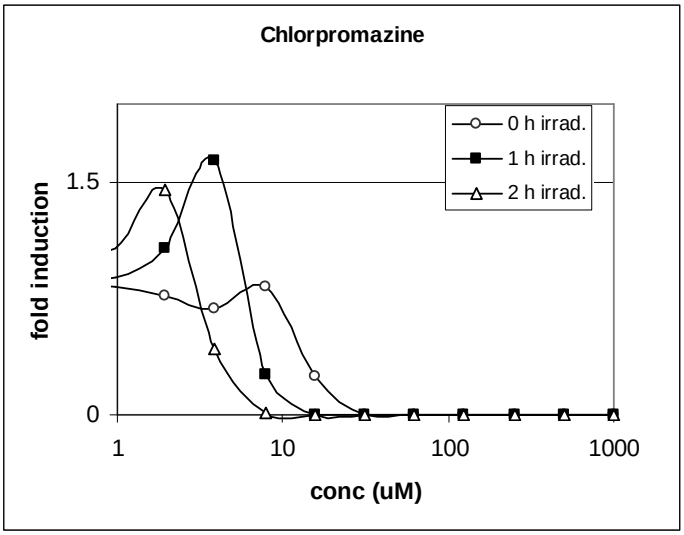

Figure 4. Historical data for Chlorpromazine tested according to the SOP, but including an irradiation step. (Irradiation for 1 -2 h by Philips daylight lamp filtered for UVB).

Karlberg, A. T.Dooms-Goossens, A., 1997. Contact allergy to oxidized d-limonene among dermatitis patients. Contact Dermatitis 36, 201-206.

Karlberg, A. T., Magnusson, K.Nilsson, U., 1992. Air oxidation of d-limonene (the citrus solvent) creates potent allergens. Contact Dermatitis 26, 332-40.

Matura, M., Goossens, A., Bordalo, O., Garcia-Bravo, B., Magnusson, K., Wrangsjö, K.Karlberg, A. T., 2002. Oxidized citrus oil (R-limonene): A frequent skin sensitizer in Europe. Journal of the American Academy of Dermatology 47, 709-714.

Nilsson, A. M., Bergström, M. A., Luthman, K., Nilsson, J. L. G.Karlberg, A. T., 2005. A conjugated diene identified as a prohapten: Contact allergenic activity and chemical reactivity of proposed epoxide metabolites. Chemical Research in Toxicology 18, 308-316.