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Prevalidation of the "EpiDerm™ Phototoxicity Test"

FINAL REPORT

(Phases I, II,III)

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CONTENT

1 ABSTRACT

ECVAM's tiered prevalidation procedure is designed to assess if a method adequately fulfils the criteria to be accepted in a validation study. It comprises three consecutive phases (protocol refinement (phase I), protocol transfer (phase II) and protocol performance (phase III)) employing one laboratory in the beginning and ending up with three to four laboratories. For the ECVAM project "Evaluation of the prevalidation process" five *in vitro* tests were selected to undergo the prevalidation procedure. Objectives of the project were on the one hand evaluation of the applicability of the theoretical concept of prevalidation and on the other hand further development and evaluation of the five tests selected.

The EpiDerm™ Phototoxicity Test was chosen for prevalidation since production of the full human *in vitro* skin model Skin² was stopped by the manufacturer in October 1996. ZEBET had developed a test protocol of a Skin² Phototoxicity Test and after refinement of the method ZEBET obtained promising results with this test in the blind trial of an EU/COLIPA validation project of "In Vitro Photoirritation" tests.

The need for the EpiDerm™ Phototoxicity Test is based on the role the test is expected to play in a tiered *in vitro* phototoxicity testing strategy: The EU/COLIPA Validation study has shown that the phototoxic potential of chemicals can be correctly predicted in a well validated *in vitro* photocytotoxicity assay, the 3T3-NRU-PT. Since the phototoxic potential of a chemical may not be relevant if it is topically applied to the skin in a formulation there is a need for an adjunct test, which may allow for assessment of safe usage concentrations on a dose per area basis. Reconstituted skin models and epidermis models have shown to be able to predict both, photoirritant as well as photoprotective properties of chemicals. In addition, in contrast to the 3T3 NRU-PT test which is a cellular cytotoxicity assay, skin models can handle formulations (e.g. emulsions, suspensions). Thus, in an *in vitro* testing strategy for phototoxicity, there is a need for *in vitro* tests, which allow to assess phototoxic potency and safety of formulations. In addition, a phototoxicity test using human skin models may be useful for risk benefit analysis of dermal pharmaceuticals.

The mechanistic basis of the EpiDerm™ Phototoxicity Test is strong, since skin is the target organ of phototoxicity. By using an appropriate test design (chemical dose-response in the presence and absence of a non-cytotoxic UVA-vis irradiation) only chemical induced phototoxic reactions will be detected. The only limitations are the facts that the epidermal model has no fibroblasts and that the endpoint is reduction of viability instead of measuring mediators of inflammation. Since all mechanisms of phototoxicity will finally lead to cell damage assessment of the inflammation response may not be important. Furthermore, the correlation between the cytotoxicity endpoint (MTT-measurement) and release of the inflammatory cytokine IL-1α is well established.

In the prevalidation study of the EpiDerm™ Phototoxicity Test, ZEBET (Berlin, D) acted as laboratory 1, Beiersdorf AG (Hamburg, D) as laboratory 2 and Procter & Gamble (Cincinnati, USA) as laboratory 3. The time frame for conducting the study was March-July 1997 for Phase I, August-September 1997 for Phase II and October-December 1997 for Phase III. Independent biostatistical analysis was performed from February 1998 to May 1998.

In Phase I ZEBET drafted a standard operating procedure (SOP) and a project plan for conducting the study. ZEBET's major task in phase I was to adopt the existing Skin² methodology to the specific needs of the epidermal model EpiDerm™. A refinement of the prediction model used for Skin² did not seem necessary but a confirmation that the prediction model of the Skin² Phototoxicity Test could also be applied

to the EpiDerm™ phototoxicity test. Therefore, phase I was focused on thorough determination of the UV sensitivity of EpiDerm™ and on improving chemical exposure conditions. UVA sensitivity experiments with EpiDerm™ revealed 6 J/cm² as the highest non-cytotoxic UVA dose. Exposure to test materials for 3, 6 and 21 hrs before UVA irradiation revealed 21 hrs as the optimum exposure time. Established phototoxins, e.g. chlorpromazine (CPZ), promethazine, 5-methoxypsoralene (5-MOP), 8-methoxypsoralene (8-MOP) and Bergamot oil, were used to improve the SOP. They were correctly identified as phototoxins. The lowest phototoxic concentration was 0.1% for 5-MOP, whereas 8-MOP showed phototoxic activity up to 0.0000001%. CPZ, which is soluble in water was tested in H_20, in a water-in-oil emulsion as well as suspended in oil, revealed its lowest phototoxic concentrations at 0.002% (H_20), 0.2% (H_20/oil) and 1.0% (oil). 4 UV filter chemicals which are non-phototoxic *in vivo* were correctly classified negative in the EpiDerm™ test. However, musk ambrette and 6-methylcoumarine, which are photoallergens *in vivo* and show a low acute phototoxic potential, were classified negative in the EpiDerm™ assay. Anthracene, which is phototoxic *in vivo*, showed phototoxic activity in 5 of 9 tests. These data confirmed the promising results obtained with the Skin² phototoxicity test, since neither the test design nor the prediction model had to be changed for applying the SOP to the EpiDerm™ Phototoxicity Test. It was, therefore, decided to proceed to phase II.

At the begin of phase II, at a meeting of the three laboratories in Berlin it was agreed to slightly amended the application technique, taking into account experience from phase I. a training session was held for laboratory II, which had no experience with skin models. Although it was the main task in phase II to produce sufficient data to asses the reproducibility of the EpiDerm™ Phototoxicity Test, it was decided to establish the applicability of a new patch application technique by testing 4 chemicals with and without patch application in all three laboratories. The result confirmed that the patch technique is useful for chemicals applied in oil, but not for chemicals solved in water. Since reproducibility of the data within and among the three laboratories was excellent in phase II, it was decided to proceed to phase III.

Before phase III was started, ZEBET drafted the final reversion of the SOP. For the blind trial 16 test chemicals (8 phototoxins and 8 non-phototoxins) were selected by ZEBET, BDF and P&G. Out of this list BIBRA selected 10 chemicals (5 pt and 5 non-pt) which were coded and distributed to the laboratories of ZEBET, BDF and P&G. Each of the 10 chemicals was tested twice independently in each laboratory and the data were submitted to the Humboldt University (Berlin) for biometrical analysis in December 1997. The statistical analysis confirmed the expected high predictivity and robustness of the test: Only one positive chemical (Tetracycline, free base) was not detected as a phototoxin in two laboratories and none of the negative chemicals was overpredicted as a phototoxin. In addition, the historical prediction model derived from the Skin² test was confirmed to be the best choice for the EpiDerm™ Phototoxicity Test.

The promising results of the prevalidation study of the EpiDerm™ Phototoxicity Test will be presented to the COLIPA Task Force "In Vitro Photoirritation" in September of 1998. It is expected that the role of the assay as an adjunct in an *in vitro* phototoxicity test strategy will be defined in this meeting. In conclusion, the prevalidation exercise suggested by the ECVAM prevalidation task force proved successful for the EpiDerm™ Phototoxicity Test in particular when the general concept is handled flexible.

2 INTRODUCTION

2.1 Background of the project

Formal validation studies can be compromised by methods which have not sufficiently undergone a process of optimisation and transfer to other laboratories. The ECVAM Task Force "Prevalidation" has, therefore, proposed a tiered prevalidation procedure (**1**), designed to allow assessment if a method adequately fulfils the criteria defined for inclusion in a validation study. It comprises three consecutive phases (protocol refinement, protocol transfer, and protocol performance) employing one laboratory in the beginning and ending up with three to four laboratories. For the ECVAM project „*Evaluation of the prevalidation process"* five in vitro tests were selected to undergo the procedure of prevalidation. Objectives of the project were on the one hand the evaluation of the applicability of the theoretical concept of prevalidation and on the other hand a further development and evaluation of the five test candidates selected. The EpiDerm Phototoxicity Test was selected since due to the discontinuation of the production of Skin^{2TM} (Advanced Tissue Sciences, USA) a promising test protocol was existing, worth to be adopted to an available Skin model (EpiDerm^{TM}).

2.2 Need for the EpiDerm™ Phototoxicity Test

It has been shown in a joint EU/COLIPA validation project (**2, 3**), that the phototoxic potential of chemicals can be correctly predicted by using cell culture monolayers in a specially designed cytotoxicity assay, the 3T3-NRU-phototoxicity test. Since the phototoxic potential of a chemical predicted using a cellular system may not be relevant when topically applied to the skin at low concentrations (e.g. in a formulation) there is a need for adjunct tests, which allow for the assessment of safe usage concentrations on a dose per area basis before testing them in humans. Reconstituted skin models and epidermis models have shown to be able to predict both, photoirritancy (**4, 5, 6, 7**), as well as the photoprotective action of sunscreens (**5**). In addition, skin models can handle formulations (e.g. emulsions, suspensions) which the 3T3 test cannot handle. Thus, in a testing strategy which is based purely on *in vitro* tests, there is a need to combine the basic 3T3 NRU PT with other *in vitro* tests, which may allow to assess safety or phototoxic potency of formulations. In addition, a phototoxicity test involving a human skin model may be useful for risk benefit analysis of dermal pharmaceuticals.

2.3 Basis of the Test

The scientific basis of the EpiDerm™ Phototoxicity Test is strong, as skin is the target organ of photoxicity. By using an appropriate test design only chemically induced phototoxic reactions will be detected. The only limitations to this statement are the facts that the epidermal model has no fibroblasts and that the endpoint is viability reduction instead of inflammatory mediators. Since all known mechanisms of photoxicity finally lead to a cell damage the latter is not an important limitation. Furthermore, the correlation between the cytotoxicity endpoint MTT and release of the inflammatory cytokine IL-1α is well established.

2.4 Status of the test previous to prevalidation

In 1993/94 an *in vitro* phototoxicity test with the human skin model Skin^2 (Advanced Tissue Sciences, USA) was developed by ZEBET and Advanced Tissue Sciences (**6**). This test showed promising results in phase II of the EU/COLIPA joint project "In vitro Photoirritation" when 30 chemicals were tested under blind conditions. After stop of the production of Skin^2 in October 1996, ZEBET adopted the Skin² methodology to the human in vitro model of reconstituted human epidermis EpiDerm^{TM} (MatTek, Ashland, USA) and established a small data base (**8**). Data showed that neither the Skin² protocol nor the prediction model needed to be changed significantly.

2.5 Work programme

The full work programme for laboratories 1, 2, and 3 proposed at the beginning of the project is given in detail in the project plan (see **ANNEX 1**). Briefly, apart from managing the project, the following main tasks had to be fulfilled by Laboratory 1:

Phase I

- elaboration of a project plan for the whole study
- elaboration of a draft standard operating procedure (SOP)
- definition and characterisation of a positive and negative control
- definition of criteria for assay quality assurance
- optimisation of the test protocol
- elaboration of a GLP compliant documentation of assay data and assay conditions
- verification of the proposed prediction model

Phase II

- provide draft SOP to Laboratory 2 and give support in establishing the test in this laboratory
- test several chemicals several times to allow assessment intralaboratory reproducibility and interlaboratory comparability
- refine SOP and draft a final SOP to be used in Phase III
- refine prediction model, if necessary
- select test chemicals for Phase III

Phase III

- contract independent biostatistician for analysis of Phase III data
- give support in establishing the test in Laboratory 3
- provide draft final SOP to Laboratory 2 and Laboratory 3
- agree on final SOP with Laboratory 2 and Laboratory 3
- test 10 chemicals (5 PT and 5 NPT) twice independently under blind conditions and submit data to the biostatistician

- approve statistical report in co-operation with Laboratory 2 and Laboratory 3

3 PHASE I

3.1 elaboration of a project plan

A project plan giving sufficient details to allow acquisition of partner laboratories was elaborated by ZEBET at the end of Phase I (April 1997). Based on this plan Procter & Gamble, (P&G), USA and Beiersdorf AG, (BDF), Hamburg, D agreed to participate in the project. Since this plan had to be approved by the P&G and BDF, it was updated in May 1997 with regard to timing details. This version of the plan is enclosed with this report as **ANNEX I**.

3.2 elaboration of a draft standard operating procedure (SOP)

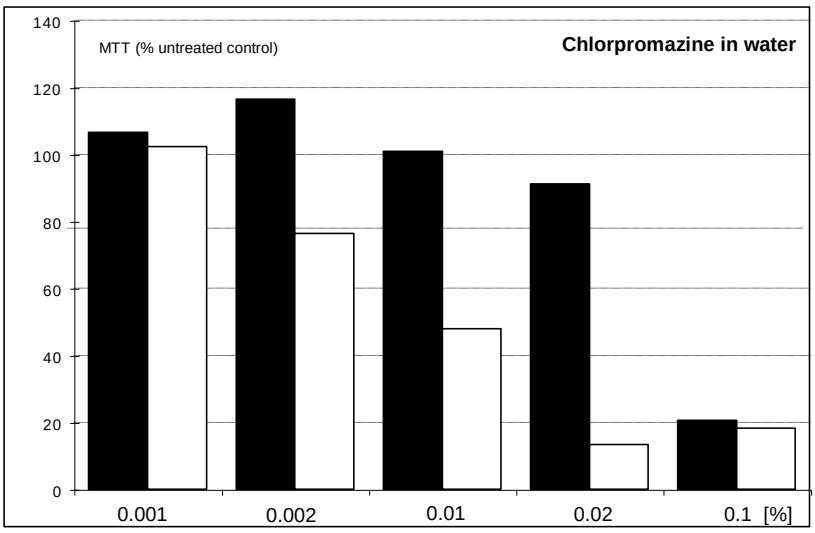
A standard operating procedure (SOP) was drafted by ZEBET and distributed to putative participants end of May 1997. This first version of the SOP is enclosed with this report as **ANNEX 2**. Briefly, the procedure consisted of steps shown in **Figure 1** (page 8).

3.3 definition and characterisation of a positive and negative reference chemical

Chlorpromazine (CPZ) is widely used as a positive reference chemical in phototoxicity tests in vivo and in vitro, and was therefore established as positive reference chemical in the EpiDerm™ Phototoxicity Test.

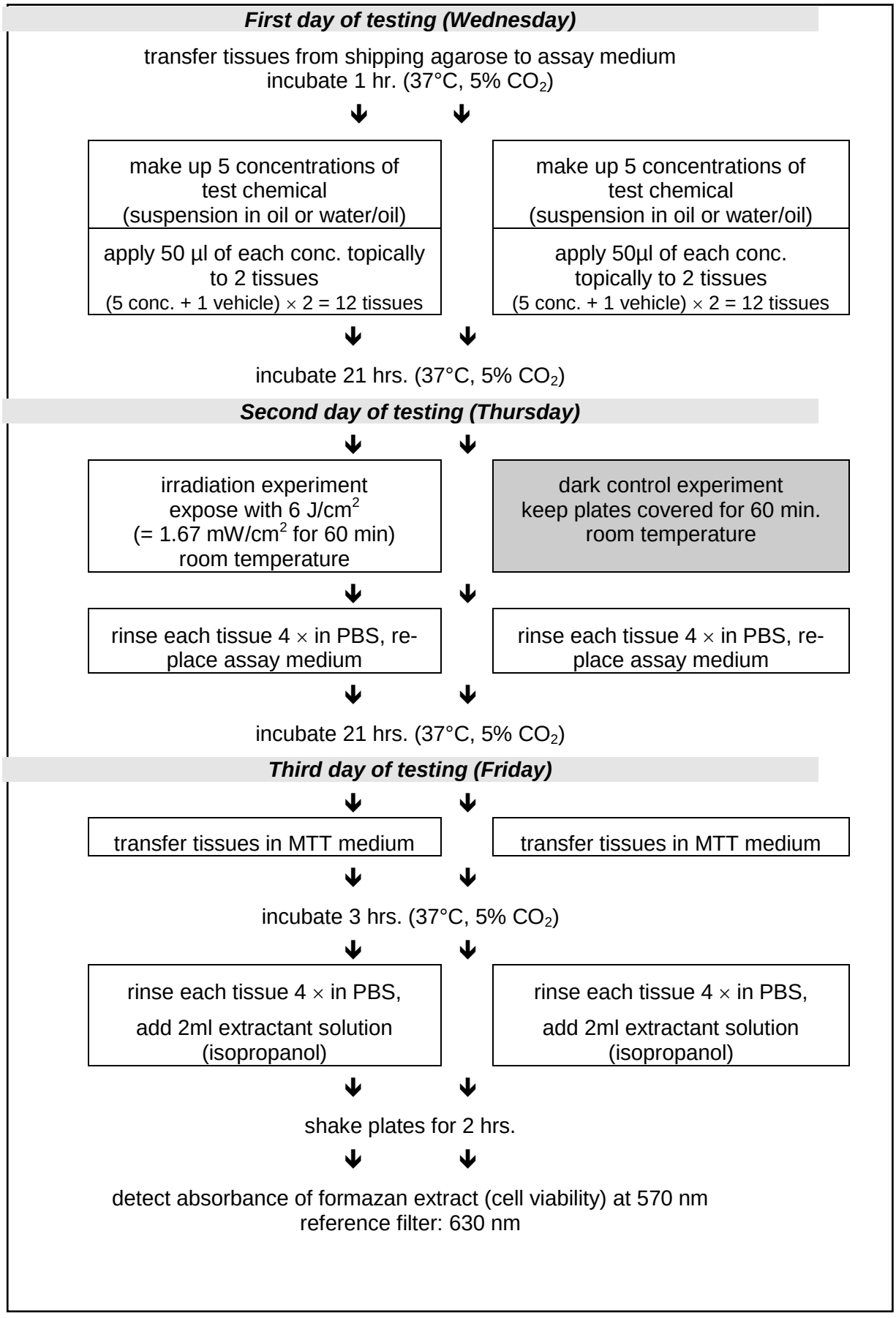
Fifty microliters of CPZ, solved in H_20 were tested 3 times independently at five concentrations in the range between 0.001% to 0.1%. A significant (>30%), dose dependent viability reduction of the irradiated tissues compared to the non-irradiated tissues was observed between 0.002% and 0.02% CPZ (**Figure 2**). At the highest test concentration of 0.1% CPZ was cytotoxic in both, the (+UVA) and the (-UVA) tissues. Since the effect of CPZ was reproducible in independent tests (see **Table 1**) and, moreover, the range of phototoxic concentrations of CPZ was in agreement with results formerly obtained with Skin², these tests were regarded a sufficient establishment of a positive control.

Figure 2 **Positive Reference Chemical: Chlorpromazine**



(example: 1 of 3 experiments)

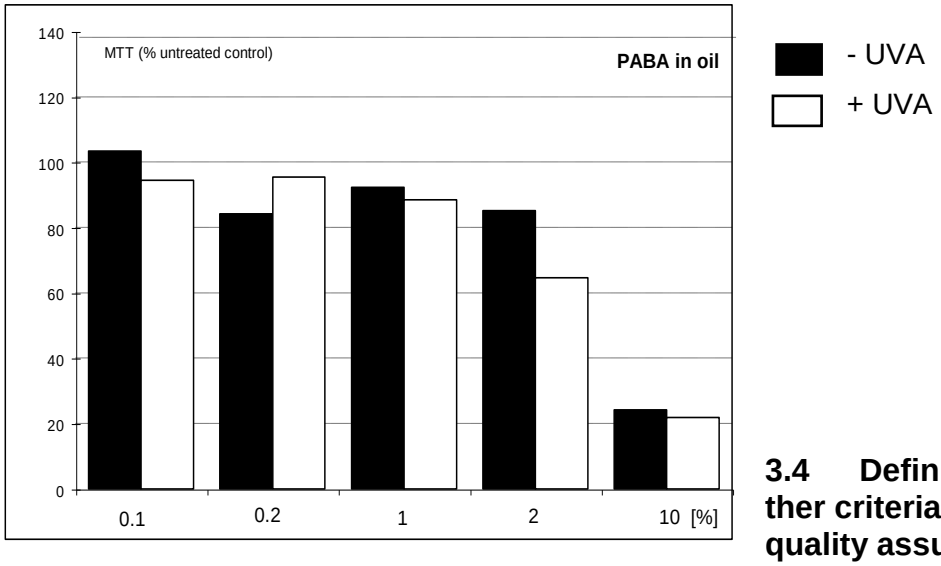
Figure 1 Design of the EpiDerm™ Phototoxicity test at the end of Phase I



Para-amino-benzoic acid (PABA), an in vivo non-phototoxic UV absorbing chemical was selected to act as negative reference chemical, because it can be tested up to cytotoxic concentrations. Fifty microliters of PABA, solved in oil were tested 3 times independently at five concentrations in the range between 0.1% to 10%. No significant (>30%) viability reduction of the irradiated tissues compared to the non-irradiated tissues was observed (**Figure 2**). At the highest test concentration of 10% PABA was cytotoxic in both, the (+UVA) and the (-UVA) tissues.

Since this result could be reproduced in independent tests (see **Table 2**) the establishment of a negative reference chemical was regarded successful.

Figure 3 **Negative Reference Chemical: PABA**



3.4 Definition of further criteria for assay quality assurance

3.4.1 UVA sensitivity of EpiDerm™ (model: EPI-200)

Since the test is based on application of the test chemical plus a non-cytotoxic UVA dose the sensitivity of EpiDerm™ tissues to an increasing dose of UVA-vis irradiation was determined in four experiments. Using the solar simulator SOL-500 (Dr. Hönle, Planegg, D) and a H1-filter a series of UVA doses ranging from 3 J/cm² to 21 J/cm² was applied to three tissues each and compared with tissues kept in the dark (**Figure 4a** and **4b**).

Figure 4 a **EpiDerm™ UVA-Sensitivity (4 independent experiments)**

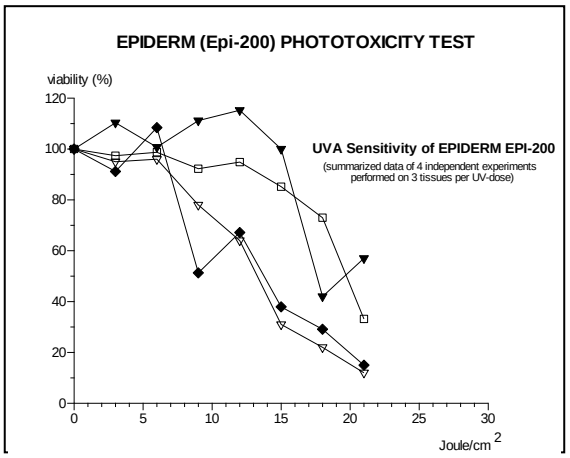
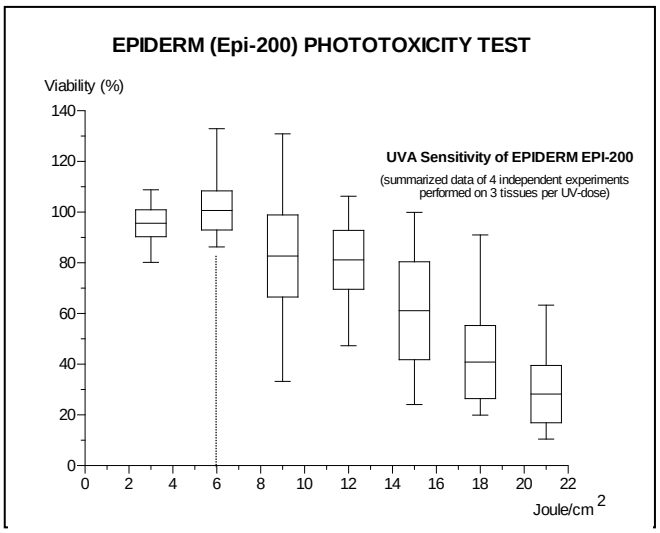


Figure 4 b **EpiDerm™ UVA-Sensitivity (box plot of four experiments)**

median ---
95% c.i. □
min ⊥
max T



According to **Figure 4a** the dose of **6 J/cm²** was non-cytotoxic in all single experiments. This dose was therefore selected as fixed dose to be used in the EpiDerm™ Phototoxicity Test. This dose is sufficient to sensitisize phototoxic chemicals (**2**) (**3**).

Confidence intervals in **Figure 4b** show that at a dose of 6 J/cm² UVA a maximum viability reduction of 10% (95% confidence interval) to 20% (99% c.i.) may be expected.

- Therefore, in the SOP a maximum difference of 20% viability between non-irradiated and irradiated vehicle controls was defined as assay acceptance criterion.

3.4.2 Viability of untreated vehicle controls

Whereas the positive control (**3.3**) is used to assure that the sensitivity of the tissues is within the historical acceptance range, viability of the untreated negative controls should be used as assay acceptance criterion as well: the **absolute OD** of negative control (NC) tissues in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after the shipping procedure and under specific conditions of the assay.

Based on first data obtained at ZEBET with untreated tissues and keeping in mind that ODs to some extend depend on specialities of plate readers used, in the first draft SOP the following acceptance criterion was specified for the negative control:

- "Absolute optical density of vehicle control tissues should be not less then 0.8"

3.5 optimisation of the test protocol: chemical exposure time

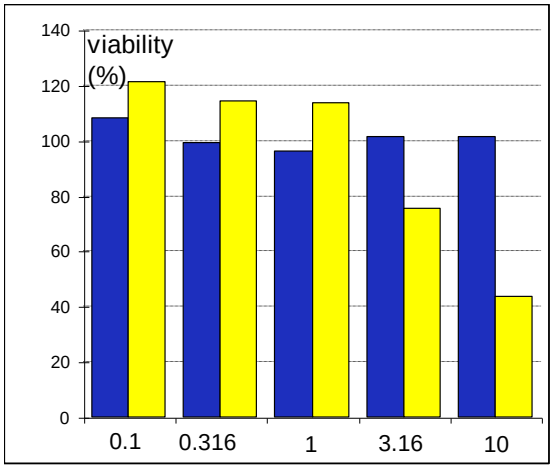
At the beginning of phase I the test design of the Skin² methodology (**4, 6**) was used with only adapting the UVA-dose (**Figure 1**). Since the time period of chemical exposure previous to UVA-vis irradiation may significantly have influence on the sensitivity of the test, two phototoxins, Bergamot oil and Anthracene were tested at three different exposure times (3 hrs, 6hrs and 21 hrs) previous to UVA-vis irradiation with 6 J/cm² (**Figure 5**).

Data shown in **Figure 5** reveal 21 hrs exposure to be the most sensitive, and therefore, most appropriate test design. Bergamot oil was positive at 10% after 3 hrs exposure, at 3,16% 6 hrs exposure and at 1% after 21 hrs exposure. Anthracene was

positive at 3.16% after 3 hrs or 6 hrs exposure respectively and at 1% after 21 hrs exposure.

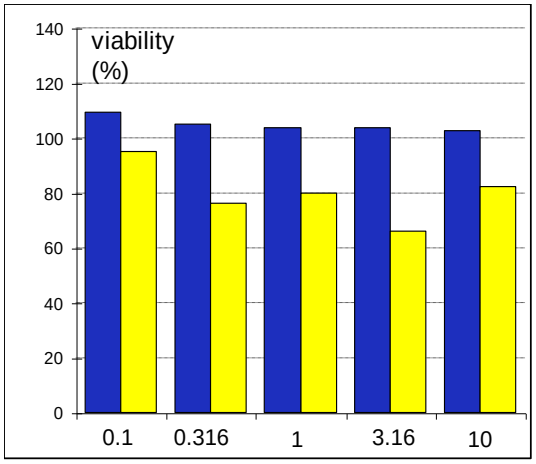
- Conclusion: The optimum exposure time regime (21 hrs incubation previous to irradiation) determined for Skin² (6) was confirmed for EpiDerm™.

Figure 5 Protocol optimisation: Influence of chemical exposure time



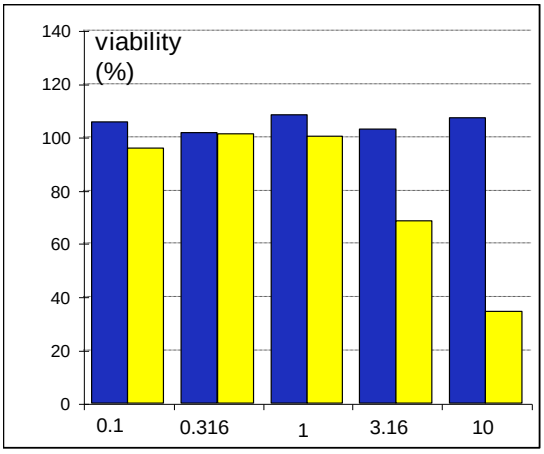
Bergamot oil

3 hours application

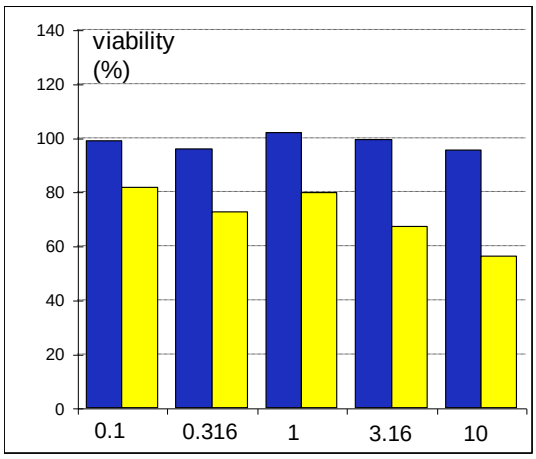


Anthracene

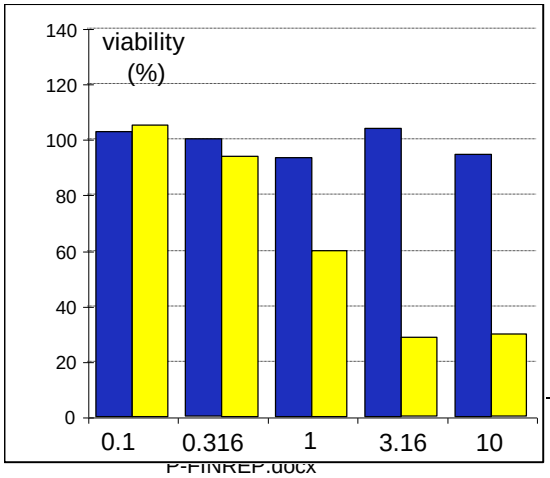
3 hours application

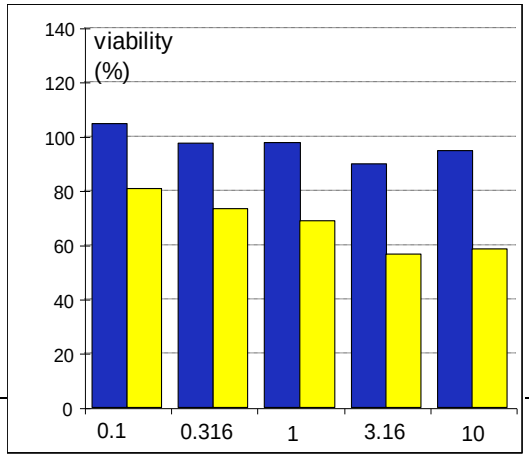


6 hours application



6 hours application





21 hours application

21 hours application

3.6 Verification of the prediction model (PM) by testing chemicals

The PM developed for the Skin² Phototoxicity Test (**6**) was based on investigating viability differences in pairs of untreated tissues according to which any difference exceeding 30% is a significant effect at the 99% confidence level. Consequently, the PM defined a difference in tissue viability of >30% obtained +UVA and -UVA at identical concentrations of the test chemical as significant positive phototoxic reaction. In phase I of the prevalidation study the Skin² PM was applied to the EpiDerm™ Phototoxicity Test and had to be verified.

3.6.1 Positive chemicals: Chorpromazine (CPZ) and Promethazine (PMZ)

Table 1 shows that for the phototoxins CPZ and PMZ the Skin² PM gave correct predictions. Lowest concentrations at which a positive effect (>30% difference) was determined are marked with a shadow. Furthermore, if the two water soluble chemicals were tested in a water-in-oil emulsion (exp. 3 & 6) the lowest phototoxic concentrations were shifted ~ 1:100. If CPZ was tested in oil the threshold was shifted ~1:500 (0.002% CPZ ⇨ 1% CPZ).

- Besides a verification of the PM (each classification was correct), data indicate that the assay is able to handle different "formulations" of the same chemical.

Table 1 Tests of *in vivo* phototoxic chemicals CPZ and PMZ

experim. no	chemical	vehicle	test conc. (%)	EpiDerm™ viabilty -UVA (%)	EpiDerm™ viabilty +UVA (%)	pred.
1	Chlorpromazine (1. experiment)	**H_20**	0,001	90	71	
			0,002	89	58	pt
			0,01	84	27	
			0,02	83	16	
			0,1	15	15	
2	Chlorpromazine (replicate)	**H_20**	0,001	107	102	
			0,002	116	76	pt
			0,01	101	48	
			0,02	91	13	
			0,1	21	18	
3	Chlorpromazine	**H_20/oil**	0,1	91	54	
			0,2	87	30	pt
			1	10	10	
			2	11	9	
			10	12	19	
4	Chlorpromazine	**oil**	0,01	98	91	
			0,02	98	102	
			0,1	91	95	
			0,2	91	85	
			1	91	24	pt
5	Promethazine	**H_20**	0.003	98	91	
			0,01	98	58	pt
			0,3	88	16	
			0,1	9	7	
			0,3	7	6	
6	Promethazine	**H_20/oil**	0,1	118	96	
			0,2	40	11	
			1	57	20	pt
			2	47	29	

			10	16	16	

3.6.2 Positive chemicals: 5-Methoxypsoralene (5-MOP) and 8-MOP

Both psoralens 5-MOP and 8-MOP are in vivo phototoxic, and, by applying the Skin² PM, were correctly predicted to be phototoxic with the EpiDerm™ Phototoxicity Test (**Table 2**). For 5-MOP the lowest phototoxic concentration was determined to be 0.01-0.02% (exp. 1& 2) revealing a negative experiment, in which 0.01% was the highest concentration tested. In contrast, 8-MOP was tested at much lower concentrations and gave a positive result even in the lowest concentrations tested (exp.4: 0.00001%, exp.5: 0.000001%). It is worth to note, that in vivo 8-MOP is known to be the more potent phototoxin than 5-MOP.

- Besides a verification of the PM, data indicate that the assay may have a capacity to rank chemicals that act as topical dermal phototoxins according to their "potency".

Table 2 **Comparison of the "relative phototoxic potency" of two *in vivo* phototoxic psoralens 5-Methoxypsoralen (5-MOP) and 8-MOP**

experim. No.	chemical	vehicle	test-conc. (%)	EpiDerm™ viabilty -UVA (%)	EpiDerm™ viabilty +UVA (%)	pred.
1	5-MOP	OIL	0,01	91	33	pt
			0,02	84	35	
			0,1	86	37	
			0,2	90	34	
			1	95	43	
2	5-MOP	OIL	0,001	97	103	
			0,002	100	83	
			0,01	99	100	
			0,02	94	59	pt
			0,1	91	38	
3	5-MOP	OIL	0,00001	121	134	
			0,00002	114	114	
			0,0001	106	105	
			0,0002	106	130	
			0,001	99	106	
4	8-MOP	OIL	0,00001	91	33	pt
			0,00002	84	35	
			0,0001	86	37	
			0,0002	90	34	
			0,001	95	43	
5	8-MOP	OIL	0.000001	104	47	pt
			0.000002	106	39	
			0,00001	103	41	
			0,00002	99	42	
			0,0001	102	46	

The lowest concentration of a test chemical classified phototoxic is marked with a grey shadow.

3.6.3 Negative chemicals: UV-filters

Since false positive predictions never occurred in the Skin² Phototoxicity Test only four in vivo non phototoxic UV filter chemicals were tested in the EpiDerm™ Phototoxicity Test to confirm this experience (**Table 3**): Para-amino-benzoic-acid (PABA), Benzophenone-3 (Colipa No. S38), Methoxy-cinnamate (Colipa No. S28), and Mexoryl SX (Colipa No. S71).

- The UV filters PABA, Benzophenone-3, Methoxy-cinnamate, and Mexoryl SX were correctly classified non phototoxic. PABA was the only UV filter which showed a slight cytotoxicity at 2% and a strong cytotoxicity at 10%. In addition, PABA showed a tendency to be more toxic +UVA at the highest test concentration. It is worth to note that of the three "modern" UV filters (S38, S28, and S71) the sulfonic acid Mexoryl SX (S71) showed a positive phototoxic potential in the monolayer cell culture photocytotoxicity test 3T3 NRU PT at high concentrations (10), which was not confirmed in the EpiDerm™ Phototoxicity Test.

Table 3 Tests of *in vivo* non phototoxic UV-filter chemicals with the EpiDerm™ Phototoxicity Test

chemical name (COLIPA No.)	vehicle	test-conc. (%)	EpiDerm™ viabilty -UVA (%)	EpiDerm™ viabilty +UVA (%)	viability difference ± UVA (%)
Para-aminobenzoic acid (1st experiment)	oil	0,1	103	94	
		0,2	84	96	
		1	92	89	< 30
		2	85	65	
		10	24	22	
Para-aminobenzoic acid (2nd experiment)	oil	0,1	104	118	
		0,2	89	103	
		1	95	105	< 30
		2	70	74	
		10	41	12	
Benzophenone-3 COLIPA S38	oil	0,1	103	111	
		0,3	100	117	
		1	96	114	< 30
		3	109	119	
		10	117	124	
Methoxy cinnamate COLIPA S28	oil	0,1	100	100	
		0,3	101	105	
		1	100	105	< 30
		3	102	104	
		10	96	98	
Mexoryl SX COLIPA S71	oil	0,1	101	105	
		0,3	99	104	
		1	96	99	< 30
		3	95	91	
		10	81	69	

3.6.4 Photoallergens

Two chemicals, which are in vivo photoallergenes (6-methylcoumarin and musk ambrette), which also show a weak acute phototoxicity were tested in the EpiDerm™ Phototoxicity Test.

- Both chemicals were predicted to be non phototoxic with the EpiDerm™ Phototoxicity Test. A possible explanation for 6-methylcoumarin is that the chemical may need a certain amount of UVB for sensitisation (11). In the 3T3 NRU-PT 6-MC was one of the chemicals that was not uniquely classified positive by all laboratories, whereas musk ambrette was classified positive in all laboratories.

Table 4 **Tests *in vivo* photoallergens 6-Methylcoumarin and Musk ambrette**

chemical name	vehicle	test-conc. (%)	EpiDerm™ viabilty -UVA (%)	EpiDerm™ viabilty +UVA (%)	viability difference ± UVA (%)
6-Methylcoumarin	oil	0,1	95	121	
		0,2	98	109	
		1	85	111	< 30
		2	80	61	
		10	7	10	
Musk ambrette	oil	0,1	109	112	
		0,3	98	112	
		1	108	110	< 30
		3	102	109	
		10	110	109	

3.7 elaboration of GLP compliant documentation of data and test conditions

For data recording of and for calculations necessary for *in vitro* prediction of phototoxicity an MS EXCEL Spreadsheet was developed at ZEBET.

Apart from electronically data recording, for documentation of experimental conditions a "method documentation sheet" (MDS) was elaborated, which allowed quality assurance of experimental circumstances of each experiment 'in the spirit of GLP' (see **ANNEX 2 and 3**).

3.8 Conclusions

Since data obtained in phase I of the prevalidation study with the EpiDerm™ Phototoxicity Test confirmed the promising results obtained with the SKIN^{2}™ Phototoxicity Test within the EU/COLIPA project "In Vitro Photoirritation" neither the basic test design nor the prediction model needed to be changed. The only change necessary was the adoption of the UVA dose to the sensitivity of the in vitro model EpiDerm™.

Therefore, phase I was regarded to have been completed successfully and it was decided to proceed to phase II of the prevalidation study.

4 PHASE II

The main target of phase II in a prevalidation study is to ensure a successful protocol transfer from laboratory 1 to laboratory 2 and to establish interlaboratory data allowing for assessment of reproducibility. Procter & Gamble (P&G), USA acted as laboratory 2.

To ensure a successful protocol transfer before proceeding into the experimental stage of phase II a meeting was held at ZEBET in Berlin on 3-5 September 1997. The meeting comprised two parts, a practical training in the laboratory and a discussion of the methodology. To ensure an optimum of agreement laboratory 3, Beiersdorf AG (BDF) participated in the discussion part of the meeting.

4.1 Protocol transfer

4.1.1 Practical Training

Two tests were performed by P&G at the laboratory of ZEBET in Berlin, one using an in vivo non-phototoxic chemical (Benzophenone-3) and one using the phototoxin Demecloclycine which had not been tested so far with the EpiDerm™ Phototoxicity Test. Both tests gave correct predictions.

Table 5 Protocol transfer: Results of the training of P&G held in Berlin

chemical name (COLIPA No.)	vehicle	test-conc. (%)	EpiDerm™ viabilty -UVA (%)	EpiDerm™ viabilty +UVA (%)	EpiDerm predict. pt / npt
Demeclocycline	oil	0,01	97	64	
		0,0316	94	62	
		0,1	91	13	**pt**
		0,316	91	20	
		1,0	65	42	
Benzophenone-3	oil	0,1	113	101	
		0,316	107	105	
		1,0	108	100	**npt**
		3,16	108	95	
		10,0	116	118	

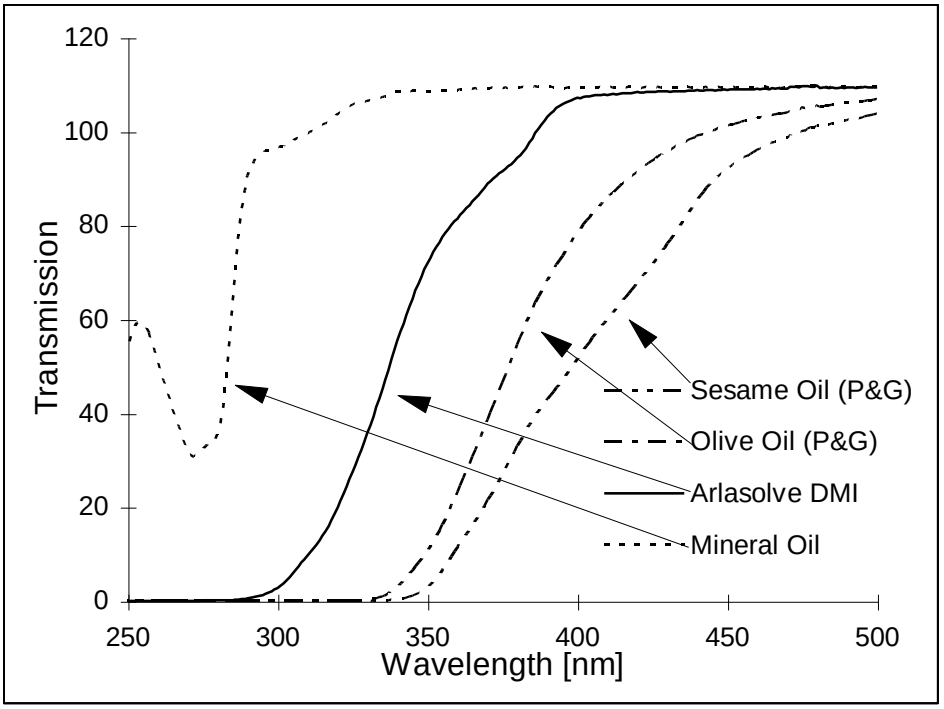
4.1.2 Refinements of the SOP to be used for phase II

At the meeting P&G, BDF and ZEBET the following points were discussed to ensure refinement of the SOP wherever regarded necessary.

4.1.2.1 Solvents/vehicle:

During phase I of the prevalidation study ZEBET had used sesame oil as vehicle for chemicals which could not be sufficiently solved in H_20. Beiersdorf (laboratory 3), although not jet involved in the project, had tested the absorption / transmission spectra of several oils and an alternative solvent dimethylisosorbide (DMI) to allow evaluation of the optimum solvent for water-insoluble test chemicals. The absorption spectra shown in **Figure 6** were discussed at the meeting: For DMI there was no experience of the dermal toxicity. Mineral oil (less UV absorbing) might be an alternative, but the properties as a solvent were not sufficiently known. The UVA absorption of olive oil was similar to sesame oil.

Figure 6 Absorption / transmission spectra of 3 oils and DMI



- Although mineral oil might have been the 'first choice vehicle' for future tests, it was decided to stay with **sesame oil** as it was used by ZEBET in phase I and phototoxins solved or suspended in sesame oil were correctly detected. This suggests that, if there is no excess of vehicle present on the stratum corneum the UVA absorption may not be a serious problem.

4.1.2.2 Patch technique

In phase I chemicals were topically applied dissolved or suspended without using a patch. To achieve the highest possible similarity between the application technique used in the EpiDerm™ Phototoxicity Test and techniques used in vivo in dermal phototoirritation tests it was decided to use a patch technique in phases II and III of the prevalidation study. Furthermore, difficulties in sufficiently spreading solutions or suspensions of test chemicals in oil should be avoided by using a patch application technique.

During the meeting several filter papers and a blotting paper were tested for their capacity to completely soak the application volume of 50 µL. **The maximum application volume revealed to be 20 µL if pads were used**.

It was finally decided to use **Finn chamber paper pads of ∅ 8 mm**, (Purchase # D 9503, HERMAL, Scholzstr. 3, D-21465 Reinbeck, Germany), which are frequently used in human patch tests all over the world and for which a sufficient purity of the cellulose could be assumed. It was decided that the pads should be used sterilised (autoclave) in the EpiDerm™ Phototoxicity Test.

Since the introduction of a new application technique was not backed by any data from phase I, it was decided that the testing programme of phase II had to be changed in the following way: The number of test chemicals should not be reduced, but at the cost of several repeat tests the laboratories should test in an extra study 2 water-soluble and 2 oil-soluble chemicals in parallel with and without using the Finn chamber paper pads. In addition, it was agreed that BDF (which according to the

concept of prevalidation would join the study not before coming into force of phase III) will participate in this extra study on the usability of a patch technique.

4.1.3 Selection of test chemicals for Phase II

At the meeting at ZEBET, out of a list of candidate chemicals provided by Laboratory 2 (P&G), the following chemicals were chosen to be used for establishing intra- and interlaboratory reproducibility.

(1) 5-methoxypsoralene (5-MOP)
(2) Promethazine (PMZ)
(3) Bithionol
(4) Chlorpromazine (CPZ)
(5) Bergamot oil

4.2 Results obtained in Phase II

4.2.1 Establishment of the test

To establish the EpiDerm™ Phototoxicity Test, five tests were performed at P&G using the phototoxic chemicals CPZ, 5-MOP and PMZ (**Table 6**). The chemicals were classified correctly in each single test, although data obtained with CPZ either applied with or without pad are indicating the patch technique is influencing the test sensitivity.

Table 6 **Tests to establish the assay in Laboratory 2: Procter & Gamble**

Chemical	Concentration (%)	without UVA (% viability)	with UVA (% viability)
Chlorpromazine (H_2O) (**with** pad)	0.002	115	78
	0.01	108	50
	0.02	137	10
	0.1	11	9
	0.2	11	9
Chlorpromazine (H_2O) (**without** pad)	0.001	102	107
	0.002	62	60
	0.01	48	91
	0.02	101	30
	0.1	15	13
5-MOP (oil) (**with** pad)	0.1	104	47
	0.316	94	45
	1	98	52
	3.16	93	51
	10	103	53
Promethazine (H_2O) (**without** pad)	0.003	102	100
	0.01	87	69
	0.03	102	43
	0.1	15	11
	0.316	13	10
Promethazine (H_2O) (**without** pad)	0.003	104	74
	0.01	110	63
	0.03	106	56
	0.1	96	18
	0.316	12	13

The lowest concentration of a test chemical classified phototoxic is marked with a black shadow.

4.2.2 Reproducibility (P&G, ZEBET)

Results of repeated tests of Bithionol, Chlorpromazine and Bergamot oil obtained at ZEBET and P&G are compiled in **Table 7**. The test programme was reduced to allow for additional testing with and without patch application. Again, the lowest concentration of a test chemical classified phototoxic is marked with a black shadow.

Table 7 Intra- and interlaboratory reproducibility

Chemical	Conc. (%)	Procter & Gable		ZEBET	
		without UVA (% viability)	with UVA (% viability)	without UVA (% viability)	with UVA (% viability)
Bithionol (oil) (with pad)	0.003	81	98	115	94
	0.01	103	102	111	88
	0.03	105	92	114	86
	0.1	90	85	99	42
	0.316	46	32	22	19
Bithionol (oil) (with pad)	0.003	104	82	103	100
	0.01	104	81	103	96
	0.03	103	96	110	92
	0.1	79	47	85	53
	0.316	16	24	60	54
Chlorpromazine (H_2O) (without pad)	0.001	97	98	95	90
	0.002	96	95	104	81
	0.01	42	39	91	17
	0.02	27	26	87	9
	0.1	8	8	8	6
Chlorpromazine (H_2O) (with pad)	0.002	59	76	84	72
	0.01	53	46	71	71
	0.02	126	18	57	17
	0.1	51	10	74	7
	0.2	20	15	8	7
Chlorpromazine (H_2O) (with pad)	0.001	72	68		
	0.002	103	68	98	111
	0.01	85	25	87	14
	0.02	84	21	99	16
	0.1	12	12	77	11
	0.2			8	8
Bergamot oil (oil) (without pad)	0.1	106	108	95	103
	0.316	92	75	92	98
	1	77	96	98	53
	3.16	113	79	98	31
	10	126	56	98	28
Bergamot oil (oil) (with pad)	0.1	85	43	81	118
	0.316	89	64	103	76
	1	66	33	93	48
	3.16	58	28	107	33
	10	89	27	56	22

- For reasons unknown, CPZ and Bithionol revealed a false negative prediction in one of the two test runs at P&G, so that 5 pairs of tests were concordant in both laboratories and two were discordant.
- Data of CPZ (dissolved in H_20) applied with patch technique revealed an increase in the variability of tissue viabilities in both laboratories, indicating the technique is

not suitable for chemicals applied in water. Since tissue viability was even reduced in "no-effect" concentrations, and pads seemed to "dry" on the tissue surface over night a mechanical damage of the cells when removing the pads could not be excluded.

- Of the **12/14** tests, in which the phototoxins were correctly classified positive at P&G and ZEBET, the lowest concentrations of test chemicals classified as phototoxic were in **five cases identical**, in **four cases differed by a factor of 2-3** and only in **one case differed by one log** (factor 10).
- Although introduction of the patch technique had caused problems when applied with H_20 as solvent, it can be stated that with exception of 2/14 tests both laboratories obtained identical classifications and results obtained in a single test by were confirmed by second experiment.

4.2.3 Assessment of the new patch technique (P&G, BDF, ZEBET)

Data shown in **4.2.1** and **4.2.2.** suggested the patch technique not applicable to chemicals applied as solutions in water. In addition BDF had made the same experience of increasing variations of tissue viability when pads with H_20 were used (data not shown). Therefore, it was decided in a conference call between the three laboratories that all laboratories test two water-soluble chemicals at 50 µL without using a pad (Chlorpromazine and Promethazine) and two chemicals dissolved / suspended in sesame oil at 20 µL (Bithionol and 5-MOP) with using a pad (**Table 8**).

Table 8 Assessment of the new patch technique

Chemical	Concentration (%)	P&G		BDF		ZEBET	
		without UVA (% viability)	with UVA (% viability)	without UVA (% viability)	with UVA (% viability)	without UVA (% viability)	with UVA (% viability)
Chlorpromazine (H_20) (without pad)	0.001	89	91	88	94	98	99
	0.00316	113	78	89	86	97	51
	0.01	97	39	91	63	93	20
	0.0316	89	17	81	19	70	8
	0.1	16	15	12	13	7	8
Promethazine (H_20) (without pad)	0.00316	102	96	86	57	98	91
	0.01	96	96	20	12	98	58
	0.0316	41	37	10	10	88	16
	0.1	9	9	12	13	9	7
	0.316	8	7	15	21	7	6
Bithionol (oil) (with pad)	0.00316	107	104	109	98	97	83
	0.01	116	95	103	84	100	88
	0.0316	114	83	82	44	95	89
	0.1	111	70	22	21	83	82
	0.316	64	19	15	17	69	28
5-MOP (oil) (with pad)	0.001			107	89		
	0.00316			104	73		
	0.01			94	88		
	0.0316			101	83		
	0.1	95	48	100	60	103	60
	0.316	87	46			102	52
	1	88	44			96	62
	3.16	91	49			94	57
	10	93	56			98	68

The lowest concentration of a test chemical classified phototoxic is marked with a black shadow.

Tests, in which PMZ was classified false negative are marked with a grey shadow.

Description and interpretation of the data shown on **Table 8**

- Data obtained with CPZ and PMZ (applied as aqueous solutions without pad) match historical data revealing dose responses in all three laboratories without significant variability of the viabilities obtained at "no-effect" concentrations.
- The applicability of the patch technique when oil is used as vehicle was confirmed, as data of Bithionol and 5-MOP did not show variances of tissue viability at the "no-effect-concentrations".
- The predictions for CPZ, Bithionol and 5-MOP were concordant in all three laboratories and the lowest concentrations at which a phototoxic effect was observed was identical for 5-MOP and similar for CPZ and Bithionol.
- The discordant classification of PMZ may have the following reasons:

(1) PMZ is not easy to detect, as the non irradiated tissues shows a steep dose response (viability decreasing from ~100% to ~10% within less than one log). Thus, only at 1-2 test concentrations +UVA dependent increase in toxicity can be observed.

(2) Unfortunately, test chemicals used in phase II were purchased individually by the laboratories. A post-hoc analysis of the sources for PMZ revealed ZEBET had used PMZ from Sigma (#P-4651, Lot# 29F0572) taken from EU/COLIPA Phase I (1992), P&G had used newly purchased PMZ from Sigma (#P-4651, Lot#17H0826), and BDF had used PMZ from Aldrich (#28.411-4, Lot# 01517/076) taken from the ECVAM/COLIPA Special Study (1997).

4.3 Confirmation of the prediction model (PM)

The prediction model (PM) used in the EpiDerm™ Phototoxicity Test is identical with the PM used in the Skin² Phototoxicity Test. In contrast to many other in vitro tests, in which the prediction of an in vivo effect is based on an algorithm to transform the endpoint measures into an in vivo prediction (e.g. cytotoxicity into eye irritation potential) no mathematical algorithm is necessary for the in vivo prediction in the present test:

In the EpiDerm™ Phototoxicity Test the viability of tissues treated identically with certain concentrations of the test chemical in the presence and absence of a non-cytotoxic UVA irradiation is compared. Since the absence of cytotoxic activity of the UVA light is controlled in each experiment, any **significant** reduction of viability observed in UVA irradiated tissues treated with identical chemical concentrations is indicating a positive phototoxic reaction.

Thus, the PM is based on definition of the magnitude of viability reduction representing a significant effect. Usually the significance would be determined by comparing the irradiated and the non-irradiated tissues with a statistical test (e.g. Student's t-Test) but this is not possible, as 2 irradiated and 2 non-irradiated tissues cannot compared statistically.

Therefore, the historical data base of the difference occurring between two untreated EpiDerm™ tissues was used to calculate a mean difference in tissue viabilities and a confidence interval for this difference, characteristically for the model EpiDerm™. Any difference exceeding the 95% confidence interval has therefore to be regarded as a significant effect which is induced by the combined action of the chemical plus light.

Table 9 shows the compiled percent viability differences of tissue couples treated identically only with H_20 derived from 55 EpiDerm™ tests (phototoxicity as well as skin corrosivity). For the calculation, even significant outliers were not excluded. The

mean difference revealed **10.56% ± 10.16% (s.d.)** and the upper end of the 95% confidence interval was determined to be **30.4%.** Therefore, the PM used in the Skin² Phototoxicity Test was confirmed to be valid in the EpiDerm™ Phototoxicity Test as well: **Any difference between the irradiated and non-irradiated tissues exceeding 30% is predicting a phototoxic effect**.

Table 9 **Historical data base of differences in %viability between EpiDerm™ tissue couples treated with H_20**

no. of experiment	viability difference (%)	no. of experiment	viability difference (%)	no. of experiment	viability difference (%)	no. of experiment	viability difference (%)	no. of experiment	viability difference (%)
1	2.97	12	12.18	23	5.00	34	2.40	45	7.05
2	0.25	13	9.82	24	6.56	35	5.19	46	8.31
3	27.29	14	13.83	25	7.04	36	1.32	47	10.69
4	8.24	15	24.86	26	10.11	37	9.74	48	5.77
5	0.10	16	47.88	27	15.00	38	5.72	49	6.58
6	15.60	17	12.36	28	2.50	39	2.08	50	2.30
7	6.23	18	24.78	29	12.99	40	5.56	51	15.00
8	5.06	19	1.40	30	1.53	41	7.13	52	2.02
9	8.24	20	6.78	31	3.00	42	2.84	53	15.75
10	21.77	21	25.32	32	2.97	43	4.48	54	25.74
11	12.36	22	47.88	33	10.41	44	6.23	55	16.71

4.4 Conclusion

Since Intra- and interlaboratory reproducibility of the data obtained with the EpiDerm™ Phototoxicity Test in phase II were sufficient in all three laboratories, it was decided to proceed to phase III of the prevalidation study.

5 PHASE III

For ZEBET, the work programme of phase III consisted of the following tasks

(1) contracting an independent biostatistician for analysis of phase III data
(2) supporting the test establishment in laboratory 3
(3) providing a draft final SOP and an agreed final SOP to be used in the blind trial
(4) providing a list of candidates of test chemicals, of which BIBRA had to select, code and distribute 10 chemicals for the blind trial
(5) testing of 10 chemicals (5 PT and 5 NPT) twice independently under blind conditions and submitting the data to the biostatistician
(6) approving statistical report in co-operation with laboratory 2 and laboratory 3

5.1 Independent biostatistical analysis

For biostatistical analysis and assessment of the performance of the refined method (test protocol and PM) in phase III an independent biostatistician, Dr. H.G. Holzhütter, was contracted. Dr. Holzhütter is chairman of the ECVAM Task Force "Biostatistics", which has proposed guidelines for the use of statistical procedures in validation studies:

PD Dr. H.G. Holzhütter
Humboldt-University Berlin, Medical School (Charité)
Institute of Biochemistry, Monbijoustr. 2A
D-10117 Berlin, Germany
Phone: +49-30-2802-6391
Fax: +49 30 2802 6615

The work contracted comprised (1) analysis of the intra- and interlaboratory reproducibility (2) analysis of the predictive value achieved with applying the SOP PM (3) analysis of any possible refinements of the PM or suggestion of an alternative PM, if necessary.

5.2 Transfer of the assay to laboratory 3

Since laboratory 3 (BDF, Beiersdorf AG, Germany) had already participated in experiments which became necessary in phase II (**4.2.3**), the number of tests performed at BDF to establish the EpiDerm™ Phototoxicity Test had to be reduced.

Beiersdorf started in August 1997with testing the UVA sensitivity of EpiDerm™ according to the SOP (using the H1 filter, but unintentionally irradiating without the shield of the polystyrene plate lid of the 6-well plates). In addition, the UVB sensitivity of EpiDerm™ was tested with inclusion of more UVB by using the H2 filter (**Figure 7a** and **7b**). Data of the two UV-sensitivity curves suggest that the filter effect of the plate lid is negligible, when using the H1 filter (**Figure 7a**), as the sensitivity curve matches the curves determined at ZEBET during phase I. **Figure 7b** shows, that the UV sensitivity of the tissues drastically increases when the H2 filter is used and irradiation is performed without plate lid. Thus, it should be kept in mind for future developments of a modification of the EpiDerm™ Phototoxicity Test, which makes use of UVB irradiation, that further investigations will be necessary to determine optimum conditions of UVB irradiation.

Chlorpromazine (CPZ), PABA and Benzophenone-3 were tested at BDF to establish the test. The two non phototoxic chemicals Benzophenone-3 and PABA were predicted correctly, as phototoxins at concentrations obtained also at ZEBET and at

P&G, whereas Chlorpromazine was classified in one test correct positive and in one test false negative. Although the reason of the one false negative classification of CPZ could not be identified the establishment of the EpiDerm™ Phototoxicity Test was regarded to successful.

Figure 7a EpiDerm™ UV sensitivity using the H1 Filter (Dr. Hönle)

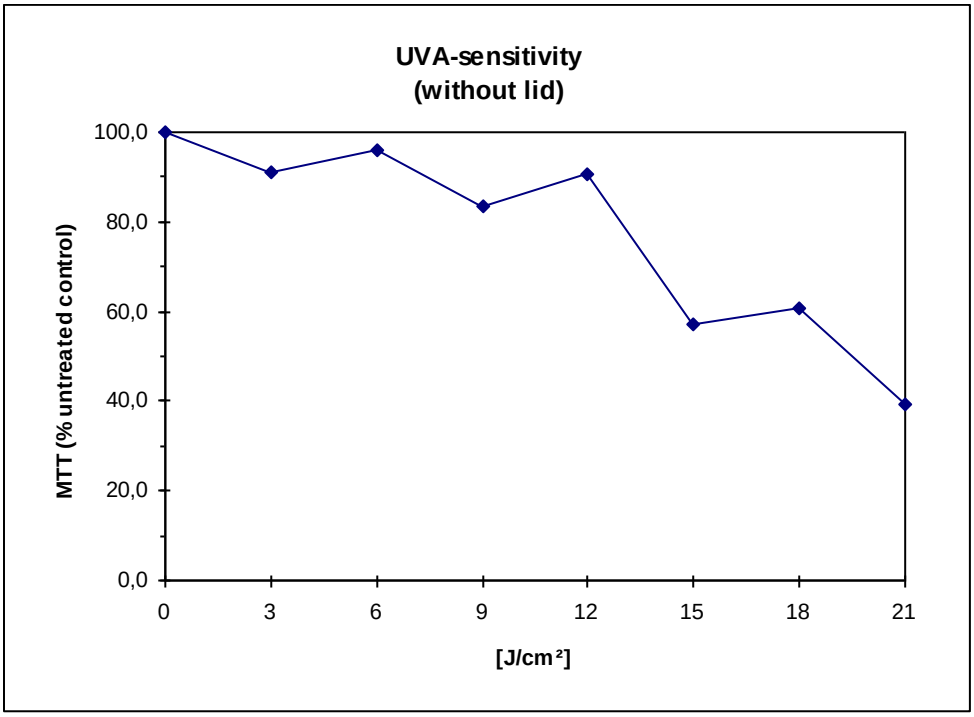
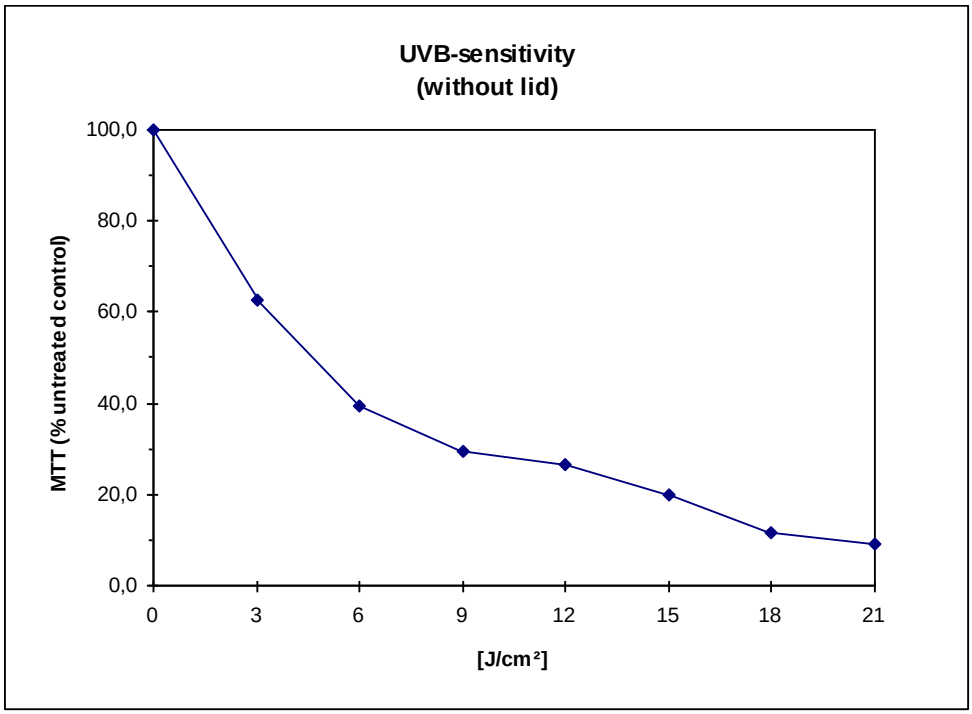


Figure 7b EpiDerm™ UV sensitivity using the H2 Filter (Dr. Hönle)



5.3 Selection of test chemicals

Since selection of phototoxic chemicals sufficiently backed by high quality in vivo data is the most crucial point in prevalidation studies and validation studies, it was decided that a list of candidate test chemicals was selected first by the participating laboratories and that out of this list an independent body (BIBRA, UK) would select a smaller set of chemicals to be tested under blind conditions. This procedure was used, since P&G, BDF and ZEBET are engaged in the EU/COLIPA joint initiative "In Vitro Photoirritation" and had knowledge about the suitability of chemicals.

In the meeting of P&G, BDF and ZEBET held in Berlin on 4-5 September 1997 participants agreed in a list of 16 chemicals (8 photoirritants, 8 non photoirritants) shown in **Table 10**

Table 10: Preselection of test chemical candidates for Phase III

8 in vivo positive chemicals
(5 to be selected by BIBRA)

chemical	CAS No.	suppl.	catalog no.	solvent
Chlorpromazine	69-09-0	Sigma	C 8138	H_20
Demeclocycline	64-73-3	Sigma	D 6140	Oil
Acridine hydrochloride	17784-47-3	Sigma	A 2145	Oil
Anthracene	120-12-7	Sigma	A 8637	Oil
8-methoxypsoralene	298-81-78	Sigma	M 3501	Oil
Bergamot oil	8007-75-8	Sigma	B 4383	Oil
Neutral red	553-24-2	Sigma	N 7005	H_20
Tetracycline free base	60-54-8	Sigma	T 3258	Oil

8 in vivo negative chemicals
(5 to be selected by BIBRA)

chemical	CAS No.	suppl.	catalog no.	solvent
Penicillin G	69-57-8	Sigma	PEN-NA	H_20
Sodium lauryl sulfate	151-21-3	Sigma	L 5750	H_20
L-Histidine	71-00-1	Sigma	H 8000	H_20
Benzophenone-3 (COLIPA S 38)	131-57-7	provided by ZEBET		Oil
Octyl salicylate (COLIPA S 13)	118-60-5	provided by ZEBET		Oil
3-(4´-Sulfobenzylidene camphor (COLIPA S 59)	56039-58-8	provided by ZEBET		Oil
4-Methylbenzylidene camphor (COLIPA S 60)	36861-47-9	provided by ZEBET		Oil
Octyl methoxycinnamate (COLIPA S28)	5466-77-3	provided by ZEBET		Oil

Out of this choice BIBRA Toxicology International (Carshalton, UK) selected 5 photoirritants and 5 non photoirritants and distributed these 10 chemicals coded to the laboratories, P&G, BDF and ZEBET for the blind trial of phase III. The final selection of BIBRA was released after all data had been submitted to the biostatistician by end of December 1997. This selection is given in **Table 11**.

Since the UV filter chemicals could not easily be purchased, ZEBET provided BIBRA with samples of all pre selected UV filter chemicals (S13, S28, S59 and S60), out of which S13, S28 and S60 were finally distributed by BIBRA (see **Table 11**).

Table 11: Final Selection of Test chemicals performed by BIBRA and code numbers of the test chemicals under which they were tested in the laboratories

TEST CHEMICAL	CAS. - No.	BDF	P&G	ZEBET
Chlorpromazine	69-09-0	4007	4030	4022
Acridine hydrochloride	17784-47-3	4003	4014	4035
Bergamot oil	8007-75-8	4038	4032	4023
Neutral red	553-24-2	4039	4031	4017
Tetracycline free base	60-54-8	4028	4019	4009
Penicillin G	69-57-8	4033	4018	4013
Lauryl sulfate sodium	151-21-3	4012	4002	4025
Octyl salicylate (S13)	118-60-5	4040	4024	4020
4-Methylbenzylidene camphor (S60)	36861-47-9	4010	4011	4016
Octyl methoxycinnamate (S28)	5466-77-3	4029	4021	4001

5.4 Final refinement of the SOP

After phase II of the prevalidation study was completed, ZEBET circulated an update of the SOP. Then, in a phone conference held on 1 October 1997, P&G, BDF and ZEBET agreed on the final SOP to be used in phase III. Apart from minor changes in details and wording, compared to the 1st draft SOP, the main amendments of the final SOP comprised:

(1) optionally, UV irradiation can be performed in 24 well plates on 0.3 mL medium instead of 6-well plates on 0.9 mL medium.

Explanation: this modification allowed a higher per week through put of test chemicals as the area under the solar simulator is the limiting factor.

This change was allowed, since 0.3 mL medium is sufficient for supply of the tissues during 60 minutes irradiation.

(2) wherever possible, chemicals shall be applied as solutions, either in oil or in H20. If chemicals cannot be dissolved either in H_20 or in sesame oil, they shall be applied as suspensions in oil.

(3) chemicals dissolved in H_20 are applied at 50 µL without using a pad.

(4) chemicals dissolved (or suspended) in oil are applied at 20 µL using a pad (Finn chamber disk, 8 mm Ø).

(5) reading of optical densities of formazan extracts is done with 570 nm (or equivalently 540 nm) without using a reference filter, since the reference recommended in the SOP and in the open literature (630 nm) is reducing the absorption signal (see formazan absorption spectrum given in the final SOP (Annex 3).

(6) a simplified Methods Documentation Sheet (MDS) is used

(7) an improved MS Excel data spreadsheet (P-SPREAD.XLS) is used

The final version of the SOP is enclosed in **ANNEX 3**

5.5 Results of the blind trial

Each of the 10 chemicals was tested twice independently in each laboratory and data were submitted to the independent biostatistician, Dr. H.-G. Holzhütter (Humboldt University, Charité, Berlin, Germany) for biometrical analysis end of December 1997. Test data were analysed during February 1998 and the analysis was approved in June 1998 after corrections which were necessary due to the fact that one laboratory had unintentionally submitted the results of one test twice saved as tests of two different chemical codes.

Since the complete report of the biostatistical analysis is enclosed with this report as **ANNEX 4** the following text is a only a brief summary of the outcome of this analysis. In addition, we have added an analysis of negative control values.

5.5.1 Reproducibility

The Biostatistical Analysis (**Annex 4**) revealed no significant differences between single assays performed in the same laboratory as well as no significant differences were found between laboratories (see Figure 2 **Annex 4**).

In addition to this biostatistical evaluation, ZEBET has compiled a table showing the lowest concentrations, at which the three laboratories detected a phototoxic effect (**Table 12**). With exception of Tetracycline free base, which was classified false in two laboratories, the lowest test concentrations at which the other four phototoxins were classified positive were in nice agreement between the laboratories, differing by a maximum of one log.

5.5.2 Predictivity

The statistical analysis confirmed the expected high predictivity of the test: Only one positive chemical (Tetracycline, free base) was not detected as a phototoxin in two of the three laboratories and all of the in vivo non phototoxic chemicals were correctly predicted as non phototoxic. It is worth to note, that the UV filter chemicals S13, S28 and S60 are poorly water soluble and could be tested up to 10% in sesame oil in EpiDerm™ Phototoxicity Test, whereas they could be tested only at very low concentrations in the in vitro 3T3 NRU (**10**).

The sensitivity obtained in single laboratories ranged from 80-100% and the specificity was 100% in all laboratories.

5.5.3 Refinement of the PM and search for alternative prediction models

The historical prediction model derived from the Skin² test (**4.3**) was confirmed to be valid also for the EpiDerm™ Phototoxicity Test. Moreover, there was no increase in false classifications if the cut-off of 30% proposed in the SOP for this PM was shifted in simulation experiments between 20% and 40% (**Annex 4**). Thus, the PM is extremely robust.

Two alternative PMs originally developed for the cellular 3T3 NRU phototoxicity test (**9**),(**10**) were also applied to the data set of the prevalidation study phase III (**Annex 4**, Table 3 and Table 4). Whereas the PIF-PM revealed a lower sensitivity of the EpiDerm™ Phototoxicity Test, the MPE-PM revealed exactly the same classifications as obtained with the SOP-PM. Therefore, we propose the simple SOP-PM based on historical data of Skin² and EpiDerm™ to be used in future use of the EpiDerm™ Phototoxicity Test.

Table 12 Lowest phototoxic concentrations of in vivo phototoxic test chemicals obtained in the three laboratories in the blind trial

Chemical	Run	UVA	Viability (% of untreated control) at conc. [%]									Lab
			0.001	0.003	0.01	0.03	0.10	0.32	1.00	3.16	10	
Tetracycline	1	plus					98	107	109	83	59	**ZEB**
free base		minus					101	103	102	100	98	
	2	plus					73	64	104	98	93	
vehicle: oil		minus					98	99	98	100	109	
Tetracycline	1	plus					88	93	83	92	83	**BDF**
free base		minus					107	105	101	98	108	
	2	plus					96	100	97	94	87	
vehicle: oil		minus					89	91	90	88	94	
Tetracycline	1	plus					96	97	89	94	93	**P&G**
free base		minus					106	99	100	97	103	
	2	plus					111	111	113	105	134	
vehicle: oil		minus					109	92	114	113	123	
Neutral Red	1	plus			53	56	53	19	19			**ZEB**
		minus			93	89	74	23	15			
vehicle: water	2	plus			12	12	12	9	11			
		minus			98	97	95	37	13			
Neutral Red	1	plus			28	22	19	11	17			**BDF**
		minus			97	81	77	34	18			
vehicle: water	2	plus	33	25	37	38	30					
		minus	97	99	91	81	84					
Neutral Red	1	plus			41	41	36	22	14			**P&G**
		minus			94	94	85	77	16			
vehicle: water	2	plus	79	55	61	44	48					
		minus	104	103	123	116	113					
Chlorpromazine	1	plus			55	18	12	12	15			**ZEB**
hydrochloride		minus			91	63	20	12	10			
	2	plus			30	9	9	10	13			
vehicle: water		minus			96	83	12	11	13			
Chlorpromazine	1	plus			35	19	15	19	25			**BDF**
hydrochloride		minus			80	63	14	15	18			
	2	plus	86	58	26	11	9					
vehicle: water		minus	89	94	67	36	12					
Chlorpromazine	1	plus			44	12	10	12	17			**P&G**
hydrochloride		minus			89	43	7	11	12			
	2	plus	93	71	56	31	16					
vehicle: water		minus	89	93	97	57	18					
Bergamot oil	1	plus					100	75	76	41	30	**ZEB**
		minus					102	108	100	99	110	
vehicle: oil	2	plus					86	95	62	36	30	
		minus					99	102	98	101	108	
Bergamot oil	1	plus					100	101	62	42	41	**BDF**
		minus					105	108	105	102	100	
vehicle: oil	2	plus					105	104	102	55	32	
		minus					93	100	95	97	107	
Bergamot oil	1	plus					92	97	99	59	44	**P&G**
		minus					89	88	95	87	94	
vehicle: oil	2	plus					104	106	95	56	40	
		minus					98	103	99	93	96	
Acridine	1	plus					36	21	9	10	8	**ZEB**
hydrochloride		minus					99	98	100	98	68	
	2	plus					28	18	12	14	12	
*vehicle: **oil***		minus					101	104	104	106	58	
Acridine	1	plus			52	36	34	18	44			**BDF**
hydrochloride		minus			100	103	86	52	35			
	2	plus		65	30	21	15	13				
*vehicle: **water***		minus		108	100	105	102	48				
Acridine	1	plus			36	21	20	10	10			**P&G**
hydrochloride		minus			100	97	96	40	12			
	2	plus	99	40	24	19	15					
*vehicle: **water***		minus	96	107	106	95	108					

5.5.4 Verification of the acceptance criteria for the negative control

In addition to investigations reported by the biostatistician, it seemed necessary after completion of phase III of the prevalidation study to compile negative control values in order to check whether this test acceptance criterion was confirmed by a larger amount of data.

The first SOP defined the minimum tissue viability of untreated vehicle controls: "Absolute optical density of vehicle control tissues should not be less then **0.8**". After completion of phase III of the prevalidation study ZEBET had performed 73 tests with oil as vehicle, 20 tests with water as vehicle (solvent) four tests with other vehicles. The absolute ODs (MTT 570) are compiled below in **Table 13**.

Table 13 shows that neither the type of vehicle (H_20 / oil) nor the application technique (with / without pad) had any effect on the mean ODs (viability) obtained with vehicle control tissues. Furthermore, a slight inhibiting effect of UVA irradiation on tissue viability of ~10% can be seen, which in addition, slightly increased the variability (**Figures 8a** and **8b** and **Table 12**: confidence intervals UVA+).

Thus, the test acceptance criterion for the negative control (tissue viability must reveal an OD of at least 0.8 of the first SOP was confirmed by the full set of data. This holds true for the negative controls of laboratories BDF and P&G.

Table 13: Negative control (acceptance criteria)

	UVA+	UVA-	UVA+	UVA-	UVA+	UVA-	UVA+	UVA-
	oil		water		water/oil		DMSO	
mean	0.966	1.052	0.911	0.991	0.967	1.058	0.998	1.125
± stdev	0.2136	0.0766	0.1651	0.1029	0.0905	0.0181		
95% confid. interval	0.55 - 1.38	0.90 - 1.20	0.59 - 1.31	0.79 - 1.19				
n tissues	73	73	20	20	3	3	1	1

= values cannot be calculated

Figure 8a: compiled negative controls (oil) (UVA+)

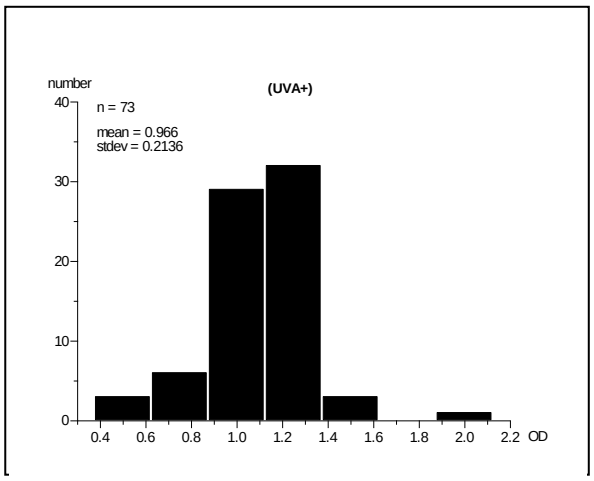
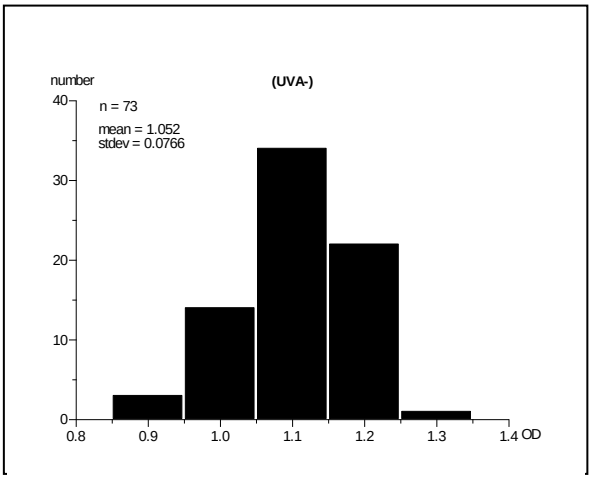


Figure 8b: compiled negative controls (oil) (UVA-)



6 CONCLUSION

- With regard to the "Evaluation of the Prevalidation Process" the present study clearly confirmed the necessity and usefulness of the prevalidation process. The test protocol underwent several refinements during the three consecutive stages contributing to the robustness of the test.
- It is strongly recommended that the general concept of prevalidation must be handled flexibly. In the present study, at the beginning of phase II it was suggested to change the application technique by using paper pads as done in vivo. Since there was no experience at all, Beiersdorf (laboratory 3) decided to participate in phase II already to help to establish a data base allowing to asses the applicability of the new technique. Thus, the concept of phase II was slightly changed: additional tests for verification of the refined application technique were performed on the costs of a large amount of repeat testing.
- For future studies it is recommended to use the final SOP of phase III of the prevalidation study and to apply the historical PM, which is now experimentally confirmed by testing 10 chemicals twice independently in three laboratories. The alternative MPE-PM (based on the biometrical analysis) which is more complicated may be applied as well but the MPE-PM has more advantage in tests revealing a dose response in any case.
- The fact that lowest phototoxic concentrations of phototoxins showed an acceptable comparability between the laboratories is indicating that the EpiDerm™ Phototoxicity Test may not only used for testing chemicals or formulations which cannot be easily handled by the *in vitro* 3T3 NRU PT, but also for assessment of topical concentrations at which a phototoxin shows a relevant phototoxicity (potency). This application of the EpiDerm™ Phototoxicity Test needs to be confirmed by a separate study using selected test chemicals for which in vivo potency tests (performed with a standard protocol) exist.
- The promising results of the prevalidation study of the EpiDerm™ Phototoxicity Test will be presented to the COLIPA Task Force "In Vitro Photoirritation" on 29 September 1998. It is expected that the role of the assay as an adjunct in a test strategy will be defined in this meeting. Summarising, it can be stated that the prevalidation exercise with the EpiDerm™ Phototoxicity Test proved the applicability and usefulness of the prevalidation process, if the general concept is handled flexible.

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