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Successful prevalidation of the slug mucosal irritation test to assess the eye irritation potency of chemicals

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ABSTRACT

A previous 'in house' validation study showed that the SMI assay can be used as an alternative to the *in vivo* Draize eye irritation test. The aim of this multi-centre study with four participating laboratories was to assess the transferability and inter-laboratory variability of the assay using 20 reference chemicals covering the whole irritancy range. The eye irritation potency of the chemicals was assessed by measuring the amount of mucus produced during a 60-min contact period with a 1% dilution, and a second 60-min treatment with a 3.5% dilution. After each contact period the protein release from the mucosal surface was measured. Linear discriminant equations were used to convert the results into the corresponding EU eye irritation categories (NI, R36 and R41). All the non-irritants were predicted correctly by the four laboratories resulting in a 100% specificity. For the R36 compounds a correct classification rate of 89% (VITO) and 100% (SPL, JNJ and UGent) was obtained. The R41 compounds were classified correctly in 78% of the cases for VITO, 89% for SPL and JNJ and 100% for UGent. We can conclude that the SMI assay is a relevant, easily transferable and reproducible alternative to predict the eye irritation potency of chemicals.

1. Introduction

Regardless of major efforts by many organizations, validation of a complete replacement to the Draize eye irritation test was not successful up to now. On 27 April 2007, however, the Scientific Advisory Committee (ESAC) of the European Centre for the Validation of Alternatives (ECVAM) announced the validation of two *in vitro* assays (the Bovine Corneal Opacity and Permeability (BCOP) and the Isolated Chicken Eye (ICE) test) (EC, 2007). The *in vitro* tests will replace the use of animals to identify severe eye irritants, for mild eye irritants animal studies will still be required. Positive results (severe irritants) of the Isolated Rabbit Eye (IRE) test and the Hen's Egg Test on the Chorio-allantoic Membrane (HET-CAM) are also accepted by regulatory agencies in France, Germany, the Netherlands and the UK (Worth and Balls, 2002).

Full replacement of the Draize eye irritation test can only be accomplished when the full range of ocular irritation is covered. As generally recognized, it is unlikely that a single assay will replace the Draize test, rather a testing strategy will be required that combines several alternative methods (Eskes et al., 2005). However, according to Jester it is realistic that a single *in vitro* system that is based on initial depth of injury can replace the rabbit test (Jester, 2006). Several studies have shown that regardless the process that is involved, the depth of initial corneal injury is predictive of the final outcome of ocular irritation (Jester, 2006; Jester et al., 2001; Maurer et al., 2002). The Slug Mucosal Irritation (SMI) assay was initially developed at the Laboratory of Pharmaceutical Technology (Ghent University – Belgium) to predict the mucosal irritation potency of pharmaceutical formulations and ingredients. The test utilizes the terrestrial slug *Arion lusitanicus*. Slugs that are placed on an irritating substance produce mucus. Additionally, tissue damage can be induced which results in the release of proteins and enzymes from the mucosal surface. Mild irritants that have only a superficial effect will mainly result in an increased mucus production with only a limited effect on the protein and enzyme

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release. Severe irritants such as benzalkonium chloride will results in an increased mucus production and deep tissue damage that is related with an increased protein and enzyme release (Adriaens and Remon, 2007). Several studies have shown that the SMI assay is a useful tool for evaluating the local tolerance of pharmaceutical formulations and ingredients to nasal, buccal and vaginal tissue (Adriaens, 2006; Adriaens et al., 2003; Adriaens et al., 2001; Adriaens and Remon, 1999; Callens et al., 2001; Ceulemans et al., 2001; Dhondt et al., 2004; Dhondt et al., 2005; Weyenberg et al., 2004). The predictivity of the SMI assay towards eye irritation was also assessed during an 'in house' validation study. The further validation of the SMI assay was performed according to the modular approach of the ECVAM principles on test validity (Hartung et al., 2004). Module 1 (test definition) and module 2 (within-laboratory variability) were already performed in the Laboratory of Pharmaceutical Technology (Ghent University – Belgium). Twenty-eight reference chemicals that covered the whole irritancy range, representing different chemical classes were selected from the ECETOC data bank for eye irritation (ECETOC, 1998). The results of this study demonstrated that the SMI assay is a reliable and reproducible method. Of the 28 chemicals tested, 20 (71%) were classified the same during the five repeated trials with an overall correct classification rate into the three EU eye irritation categories non-irritant (NI), irritating to the eyes (R36) and risk of serious damage to the eyes (R41) of 71%. The detailed results are described in Adriaens et al. (Adriaens et al., 2005).

The aim of the current project was to assess the transferability (module 3), the inter-laboratory variability (module 4) and the predictive capacity (module 5) of the SMI assay. Twenty chemicals of which 15 were already tested during the in-house validation study were selected from the ECETOC data bank (ECETOC, 1998). The chemicals covered the whole eye irritancy range and represented different chemicals classes. The reference chemicals were tested in four laboratories under blind conditions. This study was conducted in two phases. Five reference chemicals covering the whole irritancy range were selected for the training phase. The reference chemicals of the training phase were used to demonstrate the procedure in each laboratory. Then the same chemicals were tested three times by the laboratory technicians under supervision of the trainer (three experimental runs conducted on different occasions). During the testing phase, another 15 ECETOC chemicals were tested in three experimental runs on different occasions (five chemicals tested per run) without the supervision of the trainer. The within- and the between-laboratory variability was assessed and the predictive capacity of the SMI assay was evaluated. Additionally, we investigated if the prediction model could be optimized.

2. Materials and methods

2.1. Chemicals

Twenty reference chemicals were selected from the ECETOC database (ECETOC, 1998). The suppliers and *in vivo* classifications of the chemicals are presented in Table 1. The EU classification and labelling system was applied in accordance to the Commission Directive 2004/73/EC (EU, 2004). The Globally Harmonized System (GHS) was applied according to the guidelines provided by the United Nations Globally Harmonized System (GHS) for the classification and labeling of hazardous chemicals (UN, 2003). The chemicals are also listed as Candidates for the Recommended List of Reference Chemicals for *in vitro* Ocular Test Methods for Identifying Ocular Corrosives/Severe Irritants (BRD, 2006), hence *in vitro* data from the BCOP, HET-CAM, IRE and ICE assay are available for each test chemical.

2.2. Participating laboratories and study design

The Laboratory of Pharmaceutical Technology from the University of Ghent, (UGent, Belgium) was the coordinating laboratory. The other participating laboratories were Safepharm Laboratories (SPL, Derby, United Kingdom), VITO (Mol, Belgium) and Johnson & Johnson Pharmaceutical Research and Development (J& JPRD, Beerse, Belgium). The coding of the reference chemicals was done by the Laboratory of Pharmaceutical Technology (University of Ghent, Belgium). A sealed envelope containing the MSDS and Certificate of Analysis was delivered per experimental run together with the coded compounds. When the study was finished, the sealed envelope was sent back to the lead laboratory (Laboratory of Pharmaceutical Technology, University of Ghent, Belgium).

During the training phase, the test procedure was demonstrated by the trainer (UGent staff) in each laboratory. Five blind coded reference chemicals (two NI's, two R36's and one R41 labelled com-

Table 1
In vivo Draize ocular irritancy classifications of the reference chemicals

No.	Chemical	CAS	Supplier	MMAS	EU/GHS
1	3-Methoxy-1,2-propanediol	623-39-2	Sigma–Aldrich	0	NI
2	PEG 400	25322-68-3	α Pharma	0	NI
3	Potassium tetrafluoroborate	14075-53-7	Aldrich	0	NI
4	Glycerol	56-81-5	Sigma–Aldrich	1.7	NI
5[a]	Methylcyclopentane	96-37-7	Aldrich	3.7	NI
6[a]	Tween-20	9005-64-5	α Pharma	4	NI
7	2,4,5,6-Tetraaminopyrimidine sulfate	5392-28-9	Aldrich	10.3	NI
8	Ethyl acetate	141-78-6	Aldrich	15	NI
9[a]	Ammonium nitrate	6484-52-2	Sigma–Aldrich	18.3	R36/Cat2
10	1-Octanol	111-87-5	Sigma	41	R36/Cat2
11	2-Ethyl-1-hexanol	104-76-7	Fluka	51.3	R36/Cat2
12[a]	1-Hexanol	111-27-3	Fluka	64.8	R36/Cat2
13	Acetone	67-64-1	Aldrich	65.8	R36/Cat2
14[a]	Imidazole	288-32-4	Sigma	59.3	R41/Cat1
15	Sodium oxalate	62-76-0	Fluka	61.3	R41/Cat1
16	Triton X-100 (10%)	9002-93-1	Sigma–Aldrich	68.7	R41/Cat1
17	Promethazine HCl	58-33-3	Sigma	71.7	R41/Cat1
18	Chlorexidine	55-56-1	Aldrich	82.3	R41/Cat1
19	Cetylpyridinium Br (10%)	140-72-7	Sigma	89.7	R41/Cat1
20	Benzalkonium Cl (10%)	8001-54-5	Sigma–Aldrich	108	R41/Cat1

MMAS: modified maximum average score; EU: European Union; GHS: globally harmonized system of classification and labelling of chemicals.
[a] Chemicals tested during the training phase.

pound) were tested (Table 1). Then, from day 2 to day 4, each individual laboratory performed the assay three times with the same reference chemicals under supervision of the trainer. Each laboratory received detailed Standard Operating Procedures, Excel templates and standard raw data forms to record all experimental variables and deviations from the protocol. During the testing phase, 15 chemicals were tested in three experiments (five chemicals/experiment).

2.3. Test procedure

The slugs (*Arion lusitanicus*) were bred in the laboratory in an acclimatized room (18–20 °C). Slugs weighing between 3 and 6 g were isolated from the cultures two days before the start of any experiment. The body wall was inspected carefully for evidence of macroscopic injuries. Only slugs with clear tubercles and with a foot surface that showed no evidence of injuries were used for testing purposes. Two days before the start of the study, the slugs were placed in a plastic box lined with paper towel moistened with PBS and were kept at 18–20 °C. The body wall of the slugs was wetted daily with 300 μl PBS using a micropipette.

The eye irritation potency of the reference compounds, negative and positive controls was evaluated by placing five slugs per treatment group during 60 min (contact period) on a membrane filter (cellulose acetate 0.45 μm, 90 mm diameter, Sartorius AG, Goettingen, Germany) moistened with 2 ml of a 1% w/v dilution of the test item. The mucus produced during this 60-min contact period (MPCP1) was measured by weighing the Petri dishes with the test item before and after the 60-min contact period and was expressed as % of the initial body weight. Two hours later the slugs were placed on a 3.5% w/v dilution of the test item. After each 60-min contact period the slugs were transferred to a fresh Petri dish and 1 ml PBS was added. After 60 min the PBS was collected with a micropipette and analyzed for the presence of proteins and lactate dehydrogenase (LDH, EC 1.1.1.27). After the first sample the slugs were transferred to a fresh Petri dish with 1 ml PBS for a 60-min rest period.

Phosphate buffered saline (PBS, pH 7.4) was used as negative control, benzalkonium chloride (BAC) was used as positive control. The positive control and the reference compounds were diluted with PBS.

2.4. Protein determination

The total protein concentration present in the PBS samples was determined with a NanoOrange® protein quantitation kit (Invitrogen™, Merelbeke, Belgium). The NanoOrange® reagent allows accurate detection of proteins in solutions at concentrations between 10 ng and 10 μg/ml (Harvey et al., 2001). Bovine serum albumin was used as a standard. Fluorescence was measured on a fluorometer (Wallac 1420 multilabel counter, PerkinElmer, Turku, Finland) using excitation/emission wavelengths of 485/590 nm. The protein concentration is expressed in μg/ml per g body weight.

2.5. Lactate dehydrogenase determination

The lactate dehydrogenase activity (LDH, EC 1.1.1.27) was measured with an enzyme kit (LDH/HBDH 2.8, ABX diagnostics, Montpellier, France). The enzyme activity measurements were conducted on a Cobas Plus analyser (ABX, Brussels, Belgium) at 37 °C. The enzyme activity is expressed as IU/l per g body weight.

2.6. Data analysis

A classification prediction model was available from the in-house validation study. Two parameters (mucus production and tissue damage, assessed by the protein and LDH release from the mucosal surface) were used in a stepwise strategy to classify the compounds into three eye irritation categories (Fig. 1). For the first endpoint (MPCP1), the mean amount of mucus produced for each treatment group ($n = 5$) during the 1st 60-min contact period was calculated. The tissue damage score combines the protein release after the 1st contact period (P1) and the LDH and protein release after the second contact period (P2 and LDH2). For each slug the protein release in μg/ml per g body weight of sample 1 and sample 2 and the LDH activity in IU/L per gram body weight was calculated. These values were then combined in a tissue damage score, then the mean tissue damage score was calculated for each treatment group ($n = 5$). The reproducibility of the original prediction model was assessed by evaluating the within- and between-laboratory variability of the classification results. These results will be described briefly.

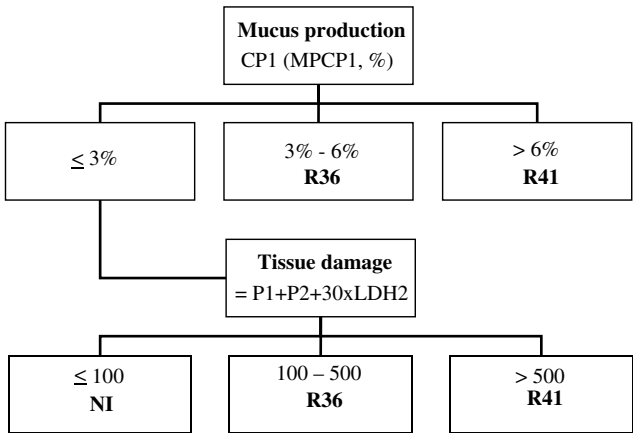

Fig. 1. Classification prediction model of the SMI assay.

The results of this multicenter study were used to see if the prediction model could be optimized. Linear discriminant function analysis (DA) was applied to determine which variables discriminate best between the three EU eye irritation categories. The individual data ($n = 760$) of this prevalidation study were used to build the model (all individual measurements except the data of the demonstration of the procedure by the trainer). DA determines functions of the variables (linear combinations) that separate the groups (EU categories) as much as possible. DA assumes that within the groups the data are multivariate normally distributed and the populations within-group covariance matrix is the same for all groups.

The quality of the model was assessed by the Wilks λ parameter ($\lambda = 0$: perfect discrimination; $\lambda = 1$: no discrimination). Once the DA model was finalized, the within- and between-laboratory variability of the variables that were used in the final DA model was assessed. Therefore, a one-way ANOVA was performed to investigate the effect of the 'run' (within-laboratory) or the 'laboratory' (between-laboratory) on the different endpoints of the SMI assay for each chemical and the negative and positive controls. In case of a significant overall group difference a Scheffé post hoc test was performed. A significance level of 5% was chosen. Because the variance increased with the mean for all endpoints, the data were log-transformed to meet the homogeneity of variances assumption, this was tested with the Levene's test. The normality of the residuals was assessed with a Kolmogorov–Smirnov test. For all the statistical analyses, the computer program SPSS (version 15.0; SPSS, Chicago, IL) was used.

Finally, the predictivity of the DA model was assessed. The classification functions were used to calculate the classification scores and a case is classified to the group (EU category) for which it has

the highest classification score. To evaluate the classification rule the true class is compared with the predicted class and the proportion of misclassified observations is computed. This will, however, result in an underestimation of the misclassification error since all the observations were used to develop the classification rule. A better estimation of this error can be obtained with the leave-one-out method (cross validation). Using this method the classification rule for each observation *i* is obtained by omitting observation *i* from the training set. The validity of the SMI assay (predictive capacity) was assessed by comparing the predicted classes (based on the mean data, $n = 5$ per test substance) with the EU label of the reference chemicals in terms of the Cooper statistics (Cooper et al., 1979). Therefore, 2×2 contingency tables were constructed [non-irritants (NI) versus irritants (R36 and R41)] and the sensitivity, specificity, negative and positive predictive value were calculated. The *sensitivity* is defined as the percentage of *in vivo* irritant chemicals, which the alternative model predicts to be irritant. The *specificity* is the percentage of *in vivo* non-irritant chemicals, which the alternative method predicts to be non-irritant. The *concordance* is the percentage of chemicals, which the prediction model classifies correctly. The *negative predictive value* is the percentage of chemicals that is predicted non-irritant by the alternative method and that give negative results *in vivo*. The *positive predictive value* is the percentage of chemicals that is predicted irritant and that are irritant *in vivo*.

3. Results

3.1. Evaluation of the original prediction model

The classifications based on the original prediction model of the SMI assay will be described briefly. The predictions were based on the mean data shown between brackets in Tables 8 and 10 (LDH release data not shown). Seven of the 20 chemicals were under or overpredicted by at least one laboratory (Table 2). Generally, 14 of the 20 compounds (70%) were always predicted the same by the four laboratories. When considering the three eye irritancy categories, a correct classification rate of 87% was obtained for SPL, 85% for UGent, 83% for JNJ and 80% for VITO. The percentage of correct predictions was the highest for the NI's and varied between 88% and 100%, the R41 chemicals were correctly predicted in 78–100% of the cases whereas the prediction of the R36 chemicals was lower (60–67%). The overprediction of the R36 compounds was always caused by the tissue damage score and more specifically by the LDH release. The score combines the LDH release (multiplied by 30) and the protein release. Some compounds (1-hexanol and 1-octanol) induced sometimes high LDH release (varying between 12.3 and 18.3 IU/l g) resulting in a tissue damage score ranging from 535 to 791. The cut-off value between the R36 and R41 category for the tissue damage score is 500. Since the prediction of the R36 category was unsatisfactory, linear discriminant analysis (LDA) was used to determine if the prediction of this category could be optimized without affecting the predictivity of the other categories.

Table 3
Standardized discriminant functions

Predictor	DF 1	DF 2
Log(MPCP1 + 10)	0.645	0.567
Log(P1 + 1)	0.043	0.445
Log(P2 + 1)	0.620	−0.841

DF: discriminant function.

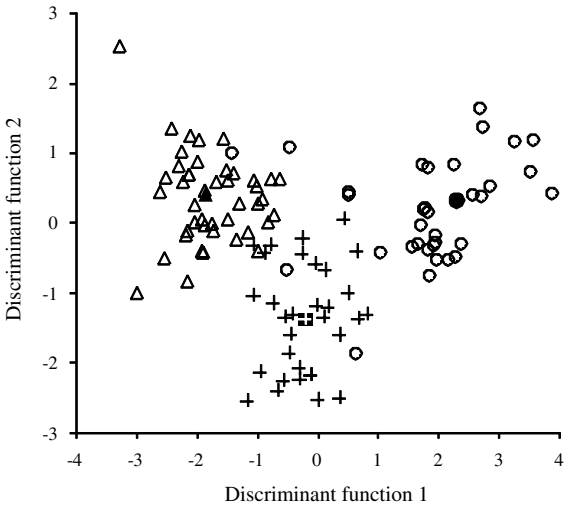

Fig. 2. Canonical discriminant functions plot for the reference chemicals (training phase and testing phase). NI (Δ), R36 (+) and R41 (○) compounds; group centroids of the NI's (▲), R36 (■) and R41 (●) compounds.

3.2. Optimization of the original prediction model

The individual data of the prevalidation phase were used for the improvement of the prediction model. The data were log-transformed to obtain a more homogeneous variance covariance matrix. A preliminary stepwise linear discriminant analysis demonstrated that the mucus production during the first contact period and the protein release after the first and the second contact period were the best variables to discriminate between the three EU eye irritation categories. The LDH release after the second contact period did not result in a better prediction and was, therefore, excluded from the model (results not shown).

In the final LDA model, two discriminant functions were included. The first discriminant function (DF1) accounted for 87% of the total among-groups variance (Wilk's $\lambda = 0.150$, $P < 0.001$). The mucus produced during the first contact period (Log(MPCP1 + 10))

Table 2
Reference chemicals that were under or overpredicted by the SMI assay according to the original prediction model: results of the training and testing phase

No.	Chemical	EU	Classifications based on SMI assay (number)		
			Underpredicted	Correct	Overpredicted
7	Tetraaminopyr.sulf salt	NI	–	1	3 (VITO, JNJ, Ugent)
9	Ammonium nitrate[a]	R36	1 (SPL)	9	–
10	1-Octanol	R36	–	3	1 (VITO)
12	1-Hexanol[a]	R36	–	5	5 (all labs)
13	Aceton	R36	4 (all labs)	–	–
14	Imidazole[a]	R41	2 (VITO, JNJ)	8	–
19	Cetylpyridinium Br	R41	2 (SPL, VITO)	2	–

[a] Compounds tested during the training phase.

and the protein release after the second contact period (Log(P2 + 1)) contributed most to the first discriminant function (DF1) as indicated by the standardized discriminant functions (Table 3). The group centroids demonstrated that DF1 separates best between the NI's and R41 compounds (Fig. 2). The second discriminant function (DF2) accounted for the remaining 13% of the variation (Wilk's $\lambda = 0.666$, $P < 0.001$). The protein release after the second contact period (Log(P2+1)) contributed most to the second discriminant function. DF 2 separates the R36 compounds from the NI's and R41 compounds (Fig. 2). The classification functions are shown in Table 4, a case is classified into the group for which it has the highest classification score. A misclassification rate of 11.6% was obtained for the individual data. The misclassification rate of the cross validation was almost comparable (12%), indicating that the classes can be predicted quit well by the computed linear discriminant functions.

3.3. Reproducibility of the controls

An excellent within-laboratory reproducibility for the negative and positive controls was observed at SPL (Tables 5 and 6), for none of the endpoints significant differences were observed between the repeated runs. At VITO and UGent, significant differences between the runs were only observed for the mucus produced by the negative control slugs. At JNJ, a significant lower protein release (P2) was observed during the second run for the negative control. This difference was, however, biologically not relevant. For the positive controls, a statistical difference between the runs was observed for the mucus production and the protein release after the first contact period.

The variation observed between the laboratories was also acceptable (Table 7). The mucus production was statistically comparable between SPL, JNJ and UGent, lower values were reported by VITO for both the negative and positive controls. Generally, the protein release observed at UGent was higher and this was more pronounced after the second contact period (P2). These differences, however, did not affect the classifications, the negative controls were always predicted NI whereas the positive controls were always predicted R41 by all the laboratories.

3.4. Transferability: training phase

Each laboratory tested five reference chemicals three times to assess the transferability of the assay. Generally, the protein release after the first contact period showed the best within- and between-laboratory reproducibility (Tables 8 and 9). The mucus production and the protein release after the second contact period showed more variation within and between the laboratories. A significant within-laboratory variability was reported for methylcyclopentane at VITO (1 endpoint) and at JNJ (two endpoints), and the protein release after the second contact period was significantly different between the laboratories. These differences did, however, not result in a different classification. For Tween-20 an excellent within- and between-laboratory reproducibility was obtained. For ammonium nitrate no differences were observed between the repeated runs, but the mucus production measured at VITO was significantly lower when compared to JNJ and UGent. This chemical was predicted NI by VITO during the last run, whereas the other laboratories predicted ammonium nitrate always correctly as R36. The repeated runs for 1-hexanol were statistically comparable within the laboratories, but a difference was observed between the laboratories for the mucus production and the protein release (P2) without affecting the classification. For

Table 4
Fisher's linear classification function coefficients

EU class	Discriminant equation
NI	$100.0 * \text{Log}(\text{MPCP1+10}) - 3.2 * \text{Log}(\text{P1+1}) + 3.1 * \text{Log}(\text{P2+1}) - 51.9$
R36	$100.6 * \text{Log}(\text{MPCP1+10}) - 4.8 * \text{Log}(\text{P1+1}) + 9.7 * \text{Log}(\text{P2+1}) - 60.9$
R41	$125.8 * \text{Log}(\text{MPCP1+10}) - 2.9 * \text{Log}(\text{P1+1}) + 10.1 * \text{Log}(\text{P2+1}) - 94.9$

Table 5
Within-laboratory variability for the negative controls

No.	Lab	Run	MPCP1	P1	P2	Class
NC	SPL	1	0.95 ± 0.04 (−1.0)	0.92 ± 0.20 (7)	0.84 ± 0.27 (6)^{A}	NI
		2	0.99 ± 0.08 (−0.2)	1.10 ± 0.20 (12)	1.04 ± 0.10 (10)^{A}	NI
		3	0.99 ± 0.04 (−0.2)	0.94 ± 0.46 (8)	0.86 ± 0.23 (6)^{A}	NI
		4	1.01 ± 0.04 (0.1)	1.33 ± 0.15 (21)	1.03 ± 0.33 (10)^{A}	NI
		5	1.00 ± 0.03 (0.0)	1.28 ± 0.29 (18)	1.36 ± 0.12 (22)^{A}	NI
		6	0.99 ± 0.02 (−0.2)	1.27 ± 0.50 (18)	1.28 ± 0.29 (18)^{A}	NI
Effect run		*P*	0.581	0.225	0.009	
NC	VITO	1	0.94 ± 0.04 (−1.3)^{A}	1.14 ± 0.40 (13)^{A}	0.94 ± 0.30 (8)	NI
		2	1.01 ± 0.02 (0.2)^{AB}	1.19 ± 0.39 (15)^{A}	1.03 ± 0.63 (10)	NI
		3	0.95 ± 0.06 (−1.2)^{AB}	0.12 ± 0.28 (0)^{A}	0.90 ± 0.53 (7)	NI
		4	0.94 ± 0.05 (−1.2)^{A}	0.47 ± 0.68 (2)^{A}	0.95 ± 0.56 (8)	NI
		5	1.04 ± 0.03 (1.1)^{AB}	1.10 ± 0.36 (12)^{A}	0.63 ± 0.33 (3)	NI
		6	1.02 ± 0.03 (0.6)^{B}	1.18 ± 0.65 (14)^{A}	1.13 ± 0.17 (13)	NI
Effect run		*P*	0.001	0.005	0.621	
NC	JNJ	1	1.04 ± 0.06 (0.9)	0.97 ± 0.33 (8)	0.87 ± 0.15 (6)^{B}	NI
		2	1.00 ± 0.02 (0.1)	1.23 ± 0.52 (16)	0.28 ± 0.19 (1)^{A}	NI
		3	1.00 ± 0.05 (0.1)	1.11 ± 0.16 (12)	0.73 ± 0.20 (4)^{AB}	NI
		4	1.02 ± 0.06 (0.4)	0.89 ± 0.82 (7)	0.63 ± 0.12 (3)^{AB}	NI
		5	1.06 ± 0.01 (1.5)	0.82 ± 0.58 (6)	1.08 ± 0.36 (11)^{B}	NI
		6	1.02 ± 0.03 (0.5)	0.99 ± 0.20 (9)	1.01 ± 0.24 (9)^{B}	NI
Effect run		*P*	0.303	0.816	< 0.001	
NC	UGent	1	1.06 ± 0.03 (1.4)^{B}	1.22 ± 0.23 (16)	1.31 ± 0.31 (19)	NI
		2	1.01 ± 0.00 (0.2)^{A}	1.37 ± 0.24 (22)	1.50 ± 0.40 (30)	NI
		3	1.03 ± 0.03 (0.7)^{AB}	1.50 ± 0.10 (31)	1.58 ± 0.20 (37)	NI
Effect run		*P*	0.024	0.117	0.414	

Values represent the mean ± SD of the log-transformed data ($n = 5$), the back transformations are shown in parentheses. In case of a significant difference between the runs (effect run: ($P < 0.05$, one-way ANOVA)), mean values with a different superscript (A and B) are significantly different from each other (Scheffé post hoc test).

Table 6
Within-laboratory variability for the positive controls

No.	Lab	Run	MPCP1	P1	P2	Class
PC	SPL	1	1.50 ± 0.09 (21.4)	2.20 ± 0.39 (159)	2.36 ± 0.10 (227)^{A}	R41
		2	1.45 ± 0.10 (18.0)	2.16 ± 0.39 (142)	2.26 ± 0.18 (180)^{A}	R41
		3	1.36 ± 0.08 (12.9)	1.89 ± 0.38 (77)	2.52 ± 0.08 (332)^{A}	R41
		4	1.45 ± 0.04 (18.2)	2.00 ± 0.33 (100)	2.49 ± 0.22 (305)^{A}	R41
		5	1.46 ± 0.05 (18.8)	1.72 ± 0.24 (51)	2.55 ± 0.11 (356)^{A}	R41
		6	1.34 ± 0.15 (11.7)	1.79 ± 0.30 (60)	2.43 ± 0.09 (266)^{A}	R41
Effect run		*P*	0.075	0.185	0.023	
PC	VITO	1	1.46 ± 0.01 (19.0)	2.31 ± 0.23 (202) ^{A}	2.47 ± 0.12 (295)	R41
		2	1.43 ± 0.02 (16.9)	2.33 ± 0.38 (212)^{A}	2.41 ± 0.16 (257)	R41
		3	1.38 ± 0.16 (14.1)	1.89 ± 0.38 (77)^{A}	2.29 ± 0.46 (194)	R41
		4	1.39 ± 0.08 (14.7)	2.12 ± 0.23 (131)^{A}	2.30 ± 0.16 (201)	R41
		5	1.33 ± 0.20 (11.4)	1.45 ± 0.70 (27)^{A}	2.55 ± 0.13 (353)	R41
		6	1.31 ± 0.07 (10.5)	2.05 ± 0.33 (112)^{A}	2.60 ± 0.14 (394)	R41
Effect run		*P*	0.308	0.023	0.229	
PC	JNJ	1	1.36 ± 0.06 (13.2)^{A}	1.50 ± 0.13 (31)^{A}	2.44 ± 0.14 (272)	R41
		2	1.50 ± 0.04 (21.6)^{AB}	2.20 ± 0.32 (156)^{B}	2.45 ± 0.10 (279)	R41
		3	1.38 ± 0.03 (14.1)^{A}	1.74 ± 0.19 (55)^{AB}	2.43 ± 0.07 (269)	R41
		4	1.45 ± 0.07 (18.1)^{AB}	1.68 ± 0.46 (47)^{AB}	2.36 ± 0.04 (229)	R41
		5	1.53 ± 0.06 (23.9)^{B}	2.30 ± 0.16 (197)^{B}	2.44 ± 0.10 (272)	R41
		6	1.41 ± 0.08 (15.9)^{AB}	1.78 ± 0.29 (59)^{AB}	2.46 ± 0.05 (286)	R41
Effect run		*P*	0.001	0.001	0.607	
PC	UGent	1	1.49 ± 0.04 (20.7)	2.30 ± 0.21 (201)	2.65 ± 0.08 (446)	R41
		2	1.55 ± 0.06 (25.5)	2.44 ± 0.11 (277)	2.72 ± 0.02 (528)	R41
		3	1.49 ± 0.06 (20.8)	2.14 ± 0.30 (138)	2.72 ± 0.19 (529)	R41
Effect run		*P*	0.125	0.138	0.553	

Values represent the mean ± SD of the log-transformed data ($n = 5$), the back transformations are shown in parentheses. In case of a significant difference between the runs (effect run: $P < 0.05$, one-way ANOVA), mean values with a different superscript (A and B) are significantly different from each other (Scheffé post hoc test).

Table 7
Between-laboratory variability for the negative and positive controls

No.	Lab	MPCP1	P1	P2
NC	SPL	0.99 ± 0.05 (−0.2)^{AB}	1.14 ± 0.34 (13)^{AB}	1.07 ± 0.30 (11)^{B}
	VITO	0.98 ± 0.06 (−0.4)^{A}	0.87 ± 0.61 (6)^{A}	0.93 ± 0.44 (8)^{AB}
	JNJ	1.02 ± 0.04 (0.6)^{B}	1.00 ± 0.47 (9)^{B}	0.77 ± 0.34 (5)^{A}
	UGent	1.03 ± 0.03 (0.7)^{B}	1.36 ± 0.22 (22)^{B}	1.46 ± 0.32 (28)^{C}
Effect Lab	*P*	0.001	0.006	< 0.001
PC	SPL	1.43 ± 0.10 (16.6)^{AB}	1.96 ± 0.36 (90)^{AB}	2.43 ± 0.16 (270)^{A}
	VITO	1.39 ± 0.12 (14.3)^{A}	2.02 ± 0.48 (105)^{AB}	2.44 ± 0.24 (273)^{A}
	JNJ	1.44 ± 0.08 (17.5)^{AB}	1.87 ± 0.39 (72)^{A}	2.43 ± 0.09 (267)^{A}
	UGent	1.51 ± 0.06 (22.3)^{B}	2.30 ± 0.24 (197)^{B}	2.70 ± 0.12 (499)^{B}
Effect Lab	*P*	0.001	0.008	< 0.001

Values represent the mean ± SD of the log-transformed data (SPL, VITO and JNJ $n = 30$; UGent $n = 15$), the back transformations are shown in parentheses. In case of a significant difference between the laboratories (effect lab: $P < 0.05$, one-way ANOVA), mean values with a different superscript (A and B) are significantly different from each other (Scheffé post hoc test).

the severe eye irritant imidazole a significant lower mucus production was observed during the first run at JNJ but it did not affect the classification. Imidazole was predicted NI by VITO during the third run, the values for the three endpoints were lower in comparison to the other runs. The differences were, however, not significant because of the high within-laboratory variability.

3.5. Between-laboratory variability

Table 10 shows the between laboratory reproducibility of the 15 eye reference chemicals that were tested during the testing phase. The highest between-laboratory reproducibility was again observed for the protein release after the first contact period, only for 4 of the 15 chemicals significant difference between the laboratories were reported. The mucus production was statistically different between the laboratories for 6 of the 15 chemicals. The protein release after the second contact period showed the highest between laboratory variability, a significant difference between the laboratories was observed for 9 of the 15 chemicals. The statistical differences between the laboratories that were observed for the endpoints resulted only in two cases in a different classification. For Triton X-100, the higher mucus production that was observed at SPL, VITO and UGent resulted in a R41 classification whereas this chemical was classified R36 by JNJ. Cetylpyridinium bromide showed the highest discrepancy between the predicted eye irritation categories, significant differences between the laboratories were observed for the three endpoints. At VITO, this compound induced only a minimal response resulting in an NI classification. At SPL the protein release after the second contact period was significantly higher in comparison with VITO and the compound was predicted R36. For JNJ and UGent a significant increase was observed for all the endpoints resulting in an R41 classification.

3.6. Predictive capacity

The number of correct, under and overpredictions based on the optimized prediction model of the SMI assay is shown in Table 11. For SPL, VITO and JNJ the results of the training phase (3 runs) were

Table 8
Within-laboratory variability for the different endpoints of the SMI assay

No.	Lab	Run	MPCP1	P1	P2	Class
5	SPL	1	0.98 ± 0.06 (−0.4)	0.74 ± 0.43 (4)	1.07 ± 0.19 (11)	NI
		2	0.99 ± 0.06 (−0.2)	1.07 ± 0.12 (11)	1.08 ± 0.08 (11)	NI
		3	1.03 ± 0.06 (0.7)	0.92 ± 0.49 (7)	0.90 ± 0.34 (7)	NI
Effect run		*P*	0.459	0.410	0.404	
5	VITO	1	0.99 ± 0.06 (−0.2)	1.53 ± 0.40 (33)^{B}	0.93 ± 0.42 (8)	NI
		2	0.99 ± 0.05 (−0.2)	1.06 ± 0.62 (11)^{AB}	1.16 ± 0.61 (14)	NI
		3	1.02 ± 0.03 (0.5)	0.74 ± 0.21 (4)^{A}	1.17 ± 0.41 (14)	NI
Effect run		*P*	0.538	0.043	0.683	
5	JNJ	1	1.01 ± 0.03 (0.1)^{A}	0.68 ± 0.31 (4)	0.76 ± 0.20 (5)^{B}	NI
		2	1.06 ± 0.03 (1.5)^{B}	1.02 ± 0.68 (9)	0.78 ± 0.30 (5)^{B}	NI
		3	1.04 ± 0.02 (1.0)^{AB}	0.98 ± 0.37 (9)	0.14 ± 0.27 (0)^{A}	NI
Effect run		*P*	0.020	0.487	0.003	
6	SPL	1	0.94 ± 0.05 (−1.3)	1.15 ± 0.21 (13)	1.18 ± 0.28 (14)	NI
		2	0.96 ± 0.09 (−0.9)	1.27 ± 0.11 (18)	1.20 ± 0.15 (15)	NI
		3	0.91 ± 0.20 (−1.9)	0.83 ± 0.52 (6)	1.11 ± 0.25 (12)	NI
Effect run		*P*	0.824	0.136	0.801	
6	VITO	1	0.79 ± 0.27 (−3.8)	1.31 ± 0.32 (19)	1.25 ± 0.20 (17)	NI
		2	0.89 ± 0.06 (−2.2)	1.20 ± 0.09 (15)	1.38 ± 0.22 (23)	NI
		3	0.95 ± 0.07 (−1.2)	1.21 ± 0.25 (15)	1.33 ± 0.44 (20)	NI
Effect run		*P*	0.357	0.740	0.813	
6	JNJ	1	0.98 ± 0.07 (−0.4)	1.15 ± 0.20 (13)	1.22 ± 0.15 (16)	NI
		2	1.00 ± 0.03 (0.0)	1.17 ± 0.26 (14)	0.89 ± 0.52 (7)	NI
		3	0.99 ± 0.02 (−0.3)	1.23 ± 0.42 (16)	0.98 ± 0.24 (8)	NI
Effect run		*P*	0.799	0.921	0.311	
9	SPL	1	1.10 ± 0.05 (2.5)	1.30 ± 0.31 (19)	1.76 ± 0.22 (56)	R36
		2	1.18 ± 0.10 (5.1)	1.39 ± 0.55 (23)	1.99 ± 0.20 (96)	R36
		3	1.10 ± 0.10 (2.5)	1.15 ± 0.41 (13)	2.03 ± 0.15 (107)	R36
Effect run		*P*	0.251	0.688	0.082	
	VITO	1	1.01 ± 0.06 (0.2)	1.12 ± 0.26 (12)	1.98 ± 0.39 (95)	R36
		2	1.11 ± 0.08 (2.9)	0.74 ± 0.70 (5)	1.88 ± 0.30 (75)	R36
		3	1.05 ± 0.03 (1.2)	1.03 ± 0.51 (10)	1.46 ± 0.65 (28)	NI
Effect run		*P*	0.071	0.510	0.222	
	JNJ	1	1.14 ± 0.03 (3.9)	0.94 ± 0.21 (8)	1.61 ± 0.56 (40)	R36
		2	1.11 ± 0.08 (2.9)	1.30 ± 0.18 (19)	1.85 ± 0.23 (70)	R36
		3	1.19 ± 0.06 (5.7)	1.35 ± 0.58 (21)	1.80 ± 0.13 (63)	R36
Effect run		*P*	0.125	0.203	0.560	
12	SPL	1	0.94 ± 0.04 (−1.3)	1.06 ± 0.29 (10)	2.14 ± 0.20 (136)	R36
		2	0.98 ± 0.06 (−0.5)	0.44 ± 0.30 (2)	1.84 ± 0.31 (68)	R36
		3	1.00 ± 0.03 (−0.1)	1.05 ± 0.76 (10)	2.03 ± 0.16 (107)	R36
Effect run		*P*	0.162	0.124	0.147	
12	VITO	1	0.91 ± 0.07 (−1.9)^{A}	0.68 ± 0.45 (4)	1.96 ± 0.30 (91)	R36
		2	1.00 ± 0.03 (0.0)^{A}	1.00 ± 0.62 (9)	2.12 ± 0.26 (130)	R36
		3	0.97 ± 0.05 (−0.6)^{A}	0.87 ± 0.15 (6)	2.07 ± 0.26 (116)	R36
Effect run		*P*	0.047	0.546	0.670	
12	JNJ	1	1.03 ± 0.02 (0.7)	0.61 ± 0.18 (3)	2.22 ± 0.19 (164)	R36
		2	1.02 ± 0.03 (0.6)	0.84 ± 0.40 (6)	2.15 ± 0.15 (139)	R36
		3	1.04 ± 0.06 (0.9)	0.67 ± 0.14 (4)	2.11 ± 0.21 (128)	R36
Effect run		*P*	0.842	0.391	0.658	
14	SPL	1	1.23 ± 0.06 (7.1)^{A}	2.32 ± 0.14 (206)	2.49 ± 0.22 (311)	R41
		2	1.29 ± 0.03 (9.5)^{A}	1.92 ± 0.39 (82)	2.22 ± 0.28 (167)	R41
		3	1.22 ± 0.02 (6.8)^{A}	1.98 ± 0.13 (94)	2.33 ± 0.09 (214)	R41
Effect run		*P*	0.037	0.060	0.170	
14	VITO	1	1.24 ± 0.06 (7.6)	2.19 ± 0.13 (154)	2.34 ± 0.35 (218)	R41
		2	1.26 ± 0.02 (8.3)	2.23 ± 0.15 (168)	2.30 ± 0.36 (199)	R41
		3	1.15 ± 0.11 (4.1)	1.74 ± 0.53 (54)	1.40 ± 1.28 (24)	NI
Effect run		*P*	0.075	0.068	0.144	
14	JNJ	1	1.17 ± 0.06 (4.9)^{A}	1.92 ± 0.31 (81)	2.22 ± 0.41 (166)	R41
		2	1.28 ± 0.04 (9.0)^{B}	2.05 ± 0.17 (112)	2.28 ± 0.16 (190)	R41
		3	1.27 ± 0.04 (8.7)^{B}	2.01 ± 0.05 (101)	2.39 ± 0.21 (243)	R41
Effect run		*P*	0.007	0.581	0.659	

Values represent the mean ± SD of the log-transformed data ($n = 5$), the back transformations are shown in parentheses. In case of a significant difference between the runs (effect run: $P < 0.05$, one-way ANOVA), mean values with a different superscript (A and B) are significantly different from each other (Scheffé post hoc test).

included. Generally, 16 of the 20 compounds (80%) were always predicted correctly by the different laboratories. A correct classification rate into the three eye irritation categories of 100% was obtained for UGent, 96.7% for SPL and JNJ and 90% for VITO. All the NI's were correctly predicted by the four laboratories, the R36 compounds were correctly predicted in 88.9% to 100% of the cases and the R41 compounds in 77.8–100% of the cases. The number of chemicals that were misclassified during at least one experiment

Table 9
Between-laboratory variability for the different endpoints of the SMI assay: training phase

No.	Lab	MPCP1	P1	P2	Class
5	SPL	1.00 ± 0.06 (0.0)	0.91 ± 0.38 (7)	1.02 ± 0.23 (9)^{AB}	
	VITO	1.00 ± 0.05 (0.0)	1.11 ± 0.53 (12)	1.09 ± 0.46 (11)^{BC}	
	JNJ	1.04 ± 0.03 (0.9)	0.89 ± 0.47 (7)	0.56 ± 0.39 (3)^{A}	
	UGent	1.02 ± 0.02 (0.4)	1.47 ± 0.13 (28)	1.50 ± 0.17 (30)^{C}	
Main effect lab	*P*	0.137	0.060	< 0.001	
6	SPL	0.94 ± 0.12 (−1.3)^{A}	1.08 ± 0.36 (11)	1.16 ± 0.22 (14)^{A}	
	VITO	0.88 ± 0.17 (−2.5)^{A}	1.24 ± 0.23 (16)	1.32 ± 0.29 (20)^{A}	
	JNJ	0.99 ± 0.04 (−0.2)^{A}	1.18 ± 0.29 (14)	1.03 ± 0.35 (10)^{A}	
	UGent	1.02 ± 0.05 (0.5)^{A}	1.16 ± 0.34 (14)	1.41 ± 0.36 (24)^{A}	
Main effect lab	*P*	0.036	0.549	0.027	
9	SPL	1.12 ± 0.09 (3.3)^{AB}	1.28 ± 0.42 (18)	1.92 ± 0.21 (83)	
	VITO	1.06 ± 0.07 (1.4)^{A}	0.96 ± 0.51 (8)	1.78 ± 0.49 (59)	
	JNJ	1.15 ± 0.07 (4.1)^{B}	1.19 ± 0.39 (15)	1.76 ± 0.35 (56)	
	UGent	1.14 ± 0.03 (3.8)^{AB}	1.21 ± 0.51 (15)	2.07 ± 0.36 (116)	
Main effect lab	*P*	0.009	0.270	0.280	
12	SPL	0.97 ± 0.05 (−0.6)^{A}	0.85 ± 0.55 (6)	2.00 ± 0.25 (100)^{A}	
	VITO	0.96 ± 0.06 (−0.9)^{A}	0.85 ± 0.44 (6)	2.05 ± 0.26 (111)^{AB}	
	JNJ	1.03 ± 0.04 (0.7)^{B}	0.71 ± 0.27 (4)	2.16 ± 0.18 (143)^{AB}	
	UGent	1.05 ± 0.02 (1.2)^{B}	0.84 ± 0.16 (6)	2.35 ± 0.08 (224)^{B}	
Main effect lab	*P*	< 0.001	0.763	0.019	
14	SPL	1.25 ± 0.05 (7.8)	2.07 ± 0.29 (117)	2.35 ± 0.23 (223)	
	VITO	1.22 ± 0.09 (6.5)	2.05 ± 0.38 (112)	2.01 ± 0.86 (102)	
	JNJ	1.24 ± 0.07 (7.5)	1.99 ± 0.20 (97)	2.30 ± 0.27 (197)	
	UGent	1.24 ± 0.07 (7.2)	2.36 ± 0.17 (226)	2.50 ± 0.20 (314)	
Main effect lab	*P*	0.616	0.135	0.187	

For each chemical the effect of the laboratory was investigated, values represent the mean ± SD of the log-transformed data (SPL, VITO and JNJ $n = 15$; UGent $n = 5$), the back transformations are shown in parentheses. In case of a significant difference between the laboratories (main effect lab: $P < 0.05$, one-way ANOVA), mean values with a different superscript (A and B) are significantly different from each other (Scheffé post hoc test).

was the highest for VITO, with three false negatives: ammonium nitrate, imidazole and cetylpyridinium bromide. JNJ underpredicted the eye irritation potency of triton X-100. At SPL the irritation potency of cetylpyridinium bromide was underpredicted. The sensitivity, specificity, negative and positive predictive value were calculated based on 2×2 contingency tables (non-irritants (NI) versus irritants (R36 and R41)). At SPL, JNJ and UGent a sensitivity and specificity of 100% was observed. A 100% specificity with a 83% sensitivity was reported for VITO.

4. Discussion

A previous in-house validation study showed that the Slug Mucosal irritation assay was a reliable method that can accurately predict the eye irritation potency of chemicals into three irritation categories corresponding with the EU label. (Adriaens et al., 2005; Adriaens and Remon, 2002; Dhondt et al., 2006). During this prevalidation study, 20 blind coded reference compounds were evaluated for their eye irritating properties in four laboratories to assess the transferability and between-laboratory variability of the assay. An empirically derived prediction model (PM), developed during the in house validation study, was used to predict the eye irritation potency of the compounds. This original PM combined four endpoints: the mucus produced during the first 60-min contact period (1), the protein release after the first (2) and the second 60-min contact period (3) and the LDH release after the second contact period (4) with the reference chemical. During the training and testing phase 14 of the 20 compounds (70%) were always predicted the same by the four laboratories. For the NI and R41 category a correct classification rate of 78–100% was obtained. The prediction of the R36 category was, however, unsatisfactory, only 60 to 67% of the chemicals were predicted correctly.

Based on the data of this prevalidation study an improved prediction model was developed using stepwise linear discriminant analysis (DA). Since the LDH release did not result in a better prediction only the first three endpoints were included in the final model that was based on two discriminant functions. The first function (DF1) was mainly influenced by the mucus production and the protein after the second contact period and discriminated best between the NI's and the R41 compounds. The second function (DF2) separated the R36 category from the NI and R41 category and this function was mainly influenced by the protein release after the second contact period. Compounds that score low on the three endpoints will most probably be predicted as NI's. Compounds that score low to intermediate for the mucus production and for the protein release after the first contact period and that result in an increased protein release after the second contact period will most probably be predicted as R36 whereas compounds that induce a high mucus production and an increased protein release after the first and the second contact period will most probably be predicted as a R41 compound.

This optimized prediction model resulted in an improvement of the transferability, the inter-laboratory variability and the predictive capacity of the SMI assay. A training of four days with a demonstration of the procedure by the trainer on the first day, seemed to be sufficient to transfer the SMI assay to an unexperienced laboratory. An excellent within- and between-laboratory reproducibility was observed for the negative and positive controls that were always predicted NI and R41 by all the laboratories, respectively. At SPL and JNJ the five compounds that were tested three times during the training phase were always predicted correctly. At VITO, ammonium nitrate and imidazole were predicted NI during the third run where a high intra-assay variability was observed for the protein release after the second contact period. An increased protein release after the second contact period is decisive for an R36 or R41 prediction. Three of the five slugs treated with ammonium nitrate showed low protein release levels and for two of the five slugs treated with imidazole no protein release (0 μg/ml g) was measured after the second contact period resulting

Table 10
Between-laboratory variability for the different endpoints of the SMI assay

No.	Lab	MPCP1	P1	P2	Class
1	SPL	1.06 ± 0.03 (1.6)	1.11 ± 0.09 (12)	1.26 ± 0.27 (17)^{AB}	NI
	VITO	1.03 ± 0.09 (0.8)	1.27 ± 0.28 (18)	1.12 ± 0.31 (12)^{A}	NI
	JNJ	1.03 ± 0.04 (0.8)	1.16 ± 0.35 (14)	0.94 ± 0.26 (8)^{A}	NI
	UGent	1.07 ± 0.04 (1.8)	1.54 ± 0.21 (34)	1.69 ± 0.31 (48)^{B}	NI
Effect Lab	*P*	0.646	0.064	0.005	
2	SPL	1.01 ± 0.08 (0.3)	0.96 ± 0.27 (8)	1.19 ± 0.23 (14)^{AB}	NI
	VITO	1.04 ± 0.05 (1.0)	0.86 ± 0.50 (6)	1.01 ± 0.54 (9)^{A}	NI
	JNJ	1.04 ± 0.11 (1.0)	0.97 ± 0.65 (8)	0.66 ± 0.25 (4)^{A}	NI
	UGent	1.02 ± 0.03 (0.5)	1.38 ± 0.13 (23)	1.60 ± 0.13 (38)^{B}	NI
Effect Lab	*P*	0.882	0.274	0.003	
3	SPL	1.08 ± 0.05 (2.1)	1.14 ± 0.43 (13)	1.12 ± 0.22 (12)	NI
	VITO	1.08 ± 0.04 (2.1)	1.37 ± 0.31 (22)	1.01 ± 0.30 (9)	NI
	JNJ	1.07 ± 0.03 (1.8)	0.99 ± 0.26 (9)	0.82 ± 0.27 (6)	NI
	UGent	1.09 ± 0.02 (2.4)	1.33 ± 0.24 (20)	1.29 ± 0.25 (18)	NI
Effect Lab	*P*	0.869	0.249	0.067	
4	SPL	1.03 ± 0.05 (0.8)	1.05 ± 0.19 (10)	1.26 ± 0.15 (17)	NI
	VITO	1.08 ± 0.04 (1.9)	1.00 ± 0.17 (9)	1.10 ± 0.24 (12)	NI
	JNJ	1.07 ± 0.07 (1.8)	1.15 ± 0.07 (13)	1.09 ± 0.17 (11)	NI
	UGent	1.08 ± 0.04 (2.0)	1.44 ± 0.46 (27)	1.34 ± 0.09 (21)	NI
Effect Lab	*P*	0.398	0.069	0.083	
7	SPL	1.08 ± 0.05 (1.9)^{A}	1.33 ± 0.39 (20)	1.39 ± 0.26 (24)	NI
	VITO	1.13 ± 0.04 (3.5)^{AB}	0.76 ± 0.67 (5)	1.25 ± 0.31 (17)	NI
	JNJ	1.17 ± 0.02 (4.9)^{B}	0.83 ± 0.95 (6)	1.26 ± 0.41 (17)	NI
	UGent	1.14 ± 0.02 (3.7)^{AB}	1.10 ± 0.38 (12)	1.28 ± 0.25 (18)	NI
Effect Lab	*P*	0.005	0.496	0.895	
8	SPL	0.95 ± 0.06 (−1.0)	1.16 ± 0.10 (14)^{AB}	1.31 ± 0.12 (19)^{AB}	NI
	VITO	0.96 ± 0.22 (−0.9)	0.98 ± 0.09 (9)^{A}	0.87 ± 0.25 (6)^{A}	NI
	JNJ	0.99 ± 0.05 (−0.1)	1.32 ± 0.25 (20)^{AB}	1.00 ± 0.14 (9)^{A}	NI
	UGent	1.07 ± 0.03 (1.6)	1.42 ± 0.26 (25)^{B}	1.53 ± 0.45 (33)^{B}	NI
Effect Lab	*P*	0.449	0.013	0.007	
10	SPL	1.00 ± 0.04 (−0.1)^{A}	0.92 ± 0.30 (7)	2.14 ± 0.29 (138)^{AB}	R36
	VITO	1.11 ± 0.07 (2.8)^{B}	1.48 ± 0.50 (29)	2.28 ± 0.08 (192)^{B}	R36
	JNJ	1.01 ± 0.04 (0.2)^{A}	1.19 ± 0.78 (15)	1.81 ± 0.26 (63)^{A}	R36
	UGent	1.11 ± 0.02 (2.9)^{B}	1.65 ± 0.36 (44)	2.34 ± 0.24 (220)^{B}	R36
Effect Lab	*P*	0.001	0.169	0.009	
11	SPL	1.03 ± 0.07 (0.8)	1.84 ± 0.31 (68)^{B}	2.36 ± 0.28 (228)	R36
	VITO	1.01 ± 0.02 (0.2)	1.30 ± 0.16 (19)^{A}	1.94 ± 0.23 (87)	R36
	JNJ	1.08 ± 0.02 (2.0)	1.81 ± 0.30 (63)^{B}	2.04 ± 0.16 (108)	R36
	UGent	1.04 ± 0.03 (1.1)	1.58 ± 0.12 (37)^{AB}	2.16 ± 0.24 (143)	R36
Effect Lab	*P*	0.102	0.009	0.063	
13	SPL	1.06 ± 0.06 (1.5)	1.01 ± 0.18 (9)	1.53 ± 0.18 (33)	R36
	VITO	1.01 ± 0.05 (0.3)	0.87 ± 0.35 (6)	1.63 ± 0.24 (41)	R36
	JNJ	1.05 ± 0.09 (1.2)	1.38 ± 0.39 (23)	1.62 ± 0.18 (41)	R36
	UGent	1.06 ± 0.03 (1.6)	0.74 ± 0.54 (5)	1.81 ± 0.24 (64)	R36
Effect Lab	*P*	0.519	0.095	0.225	
15	SPL	1.34 ± 0.07 (11.9)	1.88 ± 0.17 (75)	2.06 ± 0.12 (114)^{A}	R41
	VITO	1.28 ± 0.07 (9.2)	1.54 ± 0.39 (33)	2.30 ± 0.10 (200)^{B}	R41
	JNJ	1.35 ± 0.11 (12.2)	1.72 ± 0.41 (52)	1.99 ± 0.12 (97)^{A}	R41
	UGent	1.26 ± 0.09 (8.1)	1.88 ± 0.48 (75)	2.46 ± 0.06 (288)^{B}	R41
Effect Lab	*P*	0.319	0.457	< 0.001	
16	SPL	1.28 ± 0.09 (9.0)^{B}	1.48 ± 0.41 (29)	2.22 ± 0.25 (166)^{B}	R41
	VITO	1.21 ± 0.03 (6.3)^{AB}	1.54 ± 0.36 (33)	1.76 ± 0.29 (57)^{A}	R41
	JNJ	1.10 ± 0.06 (2.5)^{A}	1.09 ± 0.58 (11)	2.31 ± 0.09 (204)^{B}	R36
	UGent	1.30 ± 0.09 (10.1)^{B}	1.75 ± 0.40 (55)	2.49 ± 0.12 (309)^{B}	R41
Effect Lab	*P*	0.001	0.175	< 0.001	
17	SPL	1.48 ± 0.04 (20.2)	2.18 ± 0.26 (151)	2.38 ± 0.21 (241)^{BC}	R41
	VITO	1.44 ± 0.12 (17.7)	2.19 ± 0.26 (155)	2.20 ± 0.08 (158)^{AB}	R41
	JNJ	1.47 ± 0.06 (19.4)	2.03 ± 0.36 (107)	2.08 ± 0.11 (119)^{A}	R41
	UGent	1.46 ± 0.06 (18.6)	2.41 ± 0.24 (257)	2.62 ± 0.07 (414)^{C}	R41
Effect Lab	*P*	0.892	0.253	< 0.001	
18	SPL	1.29 ± 0.06 (9.7)^{AB}	1.67 ± 0.35 (46)^{A}	2.45 ± 0.29 (282)	R41
	VITO	1.26 ± 0.07 (8.3)^{A}	1.50 ± 0.41 (30)^{A}	2.40 ± 0.11 (249)	R41
	JNJ	1.39 ± 0.04 (14.3)^{B}	1.97 ± 0.20 (92)^{AB}	2.41 ± 0.16 (256)	R41
	UGent	1.39 ± 0.05 (14.4)^{B}	2.27 ± 0.19 (187)^{B}	2.48 ± 0.27 (298)	R41
Effect Lab	*P*	0.006	0.005	0.939	
19	SPL	1.08 ± 0.05 (2.0)^{A}	1.01 ± 0.20 (9)^{A}	1.68 ± 0.34 (47)^{B}	R36
	VITO	1.09 ± 0.02 (2.3)^{A}	1.28 ± 0.45 (18)^{A}	1.08 ± 0.37 (11)^{A}	NI
	JNJ	1.22 ± 0.10 (6.6)^{B}	1.45 ± 0.29 (27)^{AB}	1.75 ± 0.26 (55)^{B}	R41
	UGent	1.37 ± 0.05 (13.6)^{C}	2.01 ± 0.17 (101) ^{B}	2.19 ± 0.23 (154)^{B}	R41
Effect Lab	*P*	< 0.001	0.001	< 0.001	

(*continued on next page*)

Table 10 (*continued*)

No.	Lab	MPCP1	P1	P2	Class
20	SPL	1.33 ± 0.06 (11.5)^{A}	1.65 ± 0.29 (44)	2.44 ± 0.12 (273)^{A}	R41
	VITO	1.39 ± 0.06 (14.4)^{AB}	1.81 ± 0.31 (63)	2.33 ± 0.10 (215)^{A}	R41
	JNJ	1.52 ± 0.09 (23.0)^{B}	2.08 ± 0.43 (118)	2.43 ± 0.13 (269)^{A}	R41
	UGent	1.49 ± 0.06 (20.8)^{B}	2.14 ± 0.30 (138)	2.72 ± 0.19 (529)^{B}	R41
Effect Lab	*P*	0.002	0.113	0.002	

Values represent the mean ± SD of the log-transformed data ($n = 5$), the back transformations are shown in parentheses. In case of a significant difference between the laboratories (effect lab: $P < 0.05$, one-way ANOVA), mean values with a different superscript (A and B) are significantly different from each other (Scheffé post hoc test).

Table 11
Predictive capacity of the optimized prediction model of the SMI assay for the different laboratories

	EU class (Draize)				EU class (Draize)			
Predicted	NI	R36	R41	Predicted	I	NI	Concordance	100
SPL								
NI	12 (100%)	0	0	I	18	0	Sensitivity	100
R36	0	9 (100%)	1	NI	0	12	Specificity	100
R41	0	0	8 (89%)				PPV	100
							NPV	100
VITO								
Predicted	NI	R36	R41	Predicted	I	NI	Concordance	90
NI	12 (100%)	1	2	I	15	0	Sensitivity	83
R36	0	8 (89%)	0	NI	3	12	Specificity	100
R41	0	0	7 (78%)				PPV	100
							NPV	80
JNJ								
Predicted	NI	R36	R41	Predicted	I	NI	Concordance	100
NI	12 (100%)	0	0	I	18	0	Sensitivity	100
R36	0	9 (100%)	1	NI	0	12	Specificity	100
R41	0	0	8 (89%)				PPV	100
							NPV	100
UGent								
Predicted	NI	R36	R41	Predicted	I	NI	Concordance	100
NI	8 (100%)	0	0	I	12	0	Sensitivity	100
R36	0	5 (100%)	0	NI	0	8	Specificity	100
R41	0	0	7 (100%)				PPV	100
							NPV	100

in an underprediction of the irritation potency of these compounds. A pipetting error may be the reason for this very low protein concentration, because severe irritants can sometimes result in viscous PBS samples and the sample needed for the protein determinations is only 3 μl, resulting in a sampling error.

A good inter-laboratory reproducibility was obtained, 80% of the reference chemicals (16/20) were classified the same by the optimized prediction model against 70% for the original prediction model. The predictivity of the optimized predition model was excellent, especially the predictivity of the R36 compounds was improved, 89–100% of the R36 compounds were predicted correctly by the improved PM whereas for the original PM only 60–67% of the R36 compounds were predicted correctly. Furthermore a 100% specificity was observed in all the laboratories. The NI compounds all resulted in a minimal mucus production and a low protein release after the 1st and the 2nd 60-min contact period with the reference compounds. There was one exception, tetraaminopyrimidine sulphate resulted in a slightly increased mucus production indicating that this compound causes slight irritation, but since the protein release was not affected the compound was predicted NI. Tetraaminopyrimidine sulphate and ethyl acetate were the only NI labelled compounds that induced some corneal opacity in the Draize test one day after instillation, this effect disappeared by the second day (ECETOC, 1998). Tetraaminopyrimidine sulphate was a mild irritant according to the BCOP assay and ethyl acetate was predicted irritant by the BCOP, the Het-CAM, the ICE and the IRE test (ICCVAM Background Review Documents). For some of the NI labelled compounds human data are available. An aqueous solution of PEG 400 has been used to flush the surface of the human eye for decontamination after accidents with phenol, a 1:1 solution causes only slight burning sensations and no injury and a 1:2 solution is completely non-irritating (Grant, 1974). A repeated application of 100% glycerine in human eyes has shown that to the surface of the eye causes extensive changes in the appearance of the endothelium, but most of these changes disappear within 90 min after exposure is ended (Grant, 1986).

In general, the R36 labelled compounds induced a low to intermediate mucus production, a low to intermediate protein release after the first contact period and an increased protein release after the second contact period. Overall the R36 labelled compounds caused also irritation in the BCOP, the HET-CAM, the IRE and ICE test (ICCVAM BRD's). For several R36 labelled compounds human data are available and they generally show that these compounds may cause irritation to the eyes. Ammonium nitrate is irritating to the eyes and mucous membranes (Rao, 2005). 1-Octanol has caused transient injury of the corneal epithelium that recovered

within 48 h (Grant, 1986). There is evidence that 2-ethyl-1-hexanol can produce eye irritation in some persons and result in eye damage 24 h after instillation (Chemwatch, 2006). Recovery of corneal burns in workmen caused by 1-hexanol was complete within 48 h in four instances (Grant, 1986). Acetone exposure (1660 ppm) of 15 min causes eye irritation (ECB, IUCLID dataset).

Overall, the R41 labelled compounds caused a high mucus production and an increased protein release after the first and the second 60-min contact period except for cetylpyridinium bromide that was one time underpredicted as R36 (SPL) and was one time a false negative (VITO), whereas the compound was predicted correctly by JNJ and UGent. The mucus production and protein release induced by cetylpyridinium bromide was significantly lower at SPL and VITO when compared to UGent. For the preparation of the 1% and 3.5% w/v dilution of cetylpyridinium bromide, the compound needed to be stirred followed by immediate distribution over the membrane filter to obtain a homogeneous distribution of cetylpyridinium bromide over the filter, this may be the reason for the observed discrepancies. The irritation potency of Triton X-100 was one time underpredicted by JNJ, this compound induced a low mucus production and a low protein release after the first second 60-min contact period resulting in a R36 prediction. This compound was predicted correctly by the three other laboratories. Five of the seven R41 chemicals were classified as irritants by the BCOP, the IRE and ICE test (ICCVAM BRD's). Sodium oxalate was a false negative in the IRE and ICE assay and caused no to mild irritation in the BCOP assay. Sodium oxalate was clearly a severe irritant in the SMI assay as was observed by the increased mucus production and protein release. Human data are available for promethazine hydrochloride, chlorhexidine and benzalkonium chloride showing that these compounds are all severe irritants to the human eye. Several reports have demonstrated that accidental corneal exposure to Hibiclens (4% chlorhexidine gluconate) may lead to varying degrees of corneal epithelial defects, ranging from transient epithelial damage to endothelial destruction and permanent corneal opacification (Phinney et al., 1988; Hamed et al., 1987). A 0.1% benzalkonium chloride instilled into the eye produced burning and stinging reactions whereas a 0.02% solution seems without irritating effect, except for a few unpleasant reactions that have been reported (BIBRA, 1989). Benzalkonium chloride is toxic to the endothelium of human eyes when used intraocular (Eleftheriadis et al., 2002) and prolonged topical use of medications that contain benzalkonium chloride has also been assumed to induce endothelium degeneration requiring corneal transplantation (Lemp and Zimmerman, 1988).

The results of this prevalidation study with 20 eye reference chemicals showed that the SMI assay is a reproducible and relevant test system. The prediction model could be improved and combines three endpoints instead of four for the original prediction model. The eye irritation potency of chemicals can be predicted based on the mucus produced during the first 60-min contact period and the protein release after the first and second 60-min contact period. The more time consuming LDH measurements are no longer necessary and no sophisticated apparatus is needed. The assay was successfully transferred to three unexperienced laboratories as was shown by the high between-laboratory reproducibility, during the training and testing phase 80% of the chemicals were always predicted the same by the four laboratories. The overall correct classification rate (three EU categories) was 90% for VITO, 97% for SPL and JNJ and 100% for UGent. The NI's were most likely to be correctly classified (100% for all laboratories). The R36 compounds were correctly classified in 100% of the cases for SPL, JNJ and UGent, and 89% for VITO. The R41 compounds were classified correctly in 78% of the cases for VITO, 89% for SPL and JNJ and 100% for UGent. The SMI assay is successful in classifying compounds into three categories, this is an advantage over several other alternative assays that are only capable of discriminating between non-irritant chemicals and severely irritating chemicals.

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