The molecular initiating event in the adverse outcome pathway for skin sensitization is the covalent binding of the sensitizer to skin proteins, and a first method to address this key event was adopted as OECD TG 442C in 2015. This method, the direct peptide reactivity assay (DPRA), uses two synthetic peptides (one containing a cysteine, one containing a lysine residue) that are incubated with a single concentration of the test substance. After 24 hours incubation, the concentrations of remaining, non-depleted peptide are determined using HPLC.

All currently adopted non-animal OECD TGs to assess skin sensitization, including the DPRA, provide information on the classification of potency into UN GHS sub-categories 1A and 1B remains challenging. The kinetic direct peptide reactivity assay (kDPRA) is a modification of the DPRA wherein the reaction kinetics of a test substance towards a synthetic cysteine-containing peptide are evaluated. For this purpose, several concentrations of the test substance are incubated with the synthetic peptide for several incubation times. The reaction is stopped by addition of monobromobimane, which forms a fluorescent complex with the free cysteine of the model peptide. The relative remaining non-depleted amount of peptide is determined. Kinetic rate constants are derived from the depletion vs concentration and time matrix and used to distinguish between UN GHS sub-category 1A sensitizers and test substances in sub-category 1B/not classified test substances. In this study, we present a ring trial of the kDPRA with 24 blind-coded test substances in seven laboratories. The intra- and inter-laboratory reproducibility were 96% and 88%, respectively (both for differentiating GHS Cat 1A sensitizers from GHS Cat 1B/not classified). Following an independent peer review, the kDPRA was considered to be acceptable for the identification of GHS Cat 1A skin sensitizers. Besides GHS Cat 1A identification, the kDPRA can be used as part of a defined approach(es) with a quantitative data integration procedure for skin sensitization potency assessment. For this aim, next to reproducibility of classification, the quantitative reproducibility and variability of the rate constants were quantified in this study.

1 Introduction

The mechanism underlying skin sensitization is complex, but it is well understood and described as an adverse outcome pathway by OECD (OECD, 2014). During the last decade, significant progress has been made in the field of non-animal tests, and several test methods to address skin sensitization have meanwhile been adopted as OECD test guidelines (TGs). To date however, the regulatory accepted test methods were validated to address skin sensitization hazard but not sensitizer potency, although potency information is essential for risk assessment purposes.
skin sensitizing hazard but cannot be used as a stand-alone test to address skin sensitization (OECD, 2020) or sufficiently address skin sensitizing potency (Wareing et al., 2017). A score-based approach for identification of GHS 1A chemicals has been proposed, however, reproducibility of the underlying scores derived from quantitative data in the DPRA and the lCLAT assay has not yet been documented (Nukada et al., 2013).

The kinetic direct peptide reactivity assay (kDPRA) is a modification of the OECD-adopted in chemico DPRA (described in Appendix I of OECD TG 442C, (OECD, 2020)). The kDPRA uses the cysteine-containing test peptide (Ac-RFAACAA-COOH; Cys), also used in the DPRA, while it does not use a lysine-containing peptide. The final concentration of the Cys peptide (0.5 mM) and the reaction medium (25% acetonitrile in phosphate buffer) are identical in the kDPRA and in the DPRA. While the DPRA measures only at one concentration of the test substance (5 mM in the Cys peptide reaction mixture) and at one, not exactly defined, time point (≥ 24 h), the kDPRA performs parallel reactions at five test substance concentrations (5, 2.5, 1.25, 0.625 and 0.3125 mM) and at six defined time points (10, 30, 90, 210 and 1440 min) at 25 ±2.5°C. The residual concentration in % relative to the VC of the Cys peptide after the respective reaction time is measured by stopping the reaction by the addition of monobromobimane (mBrB). Highly reactive and non-fluorescent mBrB rapidly reacts with unbound cysteine moieties of the model peptide to form a fluorescent complex. The residual concentration in % relative to the VC of the Cys thus can be determined, and the depletion vs time and concentration matrix is used to calculate rate constants.

It has been shown that kDPRA differentiates GHS Cat 1A sensitizers from GHS 1B/not classified substances with a balanced accuracy of 85% (based on 180 test substances), with a sensitivity of 84% (38/45), and a specificity of 86% (116/135) relative to LLNA results (Natsch et al., 2020). In addition, the prediction of human skin sensitization for 123 test substances that fall within the kDPRA’s applicability domain has a balanced accuracy of 76%, a sensitivity of 64% (21/33), and a specificity of 89% (80/90) (Natsch et al., 2020). On the basis of the overall data available (n = 180), the kDPRA’s applicability domain was shown to include a variety of organic functional groups, the full range of skin sensitization potencies (as determined in in vivo studies), and diverse physicochemical properties.

After setting up a standard operating procedure (SOP) to conduct the kDPRA and to perform rate constant calculations, the primary goal of this study was to assess the transferability of the method (using six test substances) to five naive labs and then assess intra- and inter-laboratory reproducibility of the method using 24 blind-coded substances. The reproducibility of the assay based on the log-transformed rate constants as well as the classification reproducibility to differentiate Cat 1A sensitizers from Cat 1B sensitizers/not classified substances according to UN GHS were evaluated.

2 Materials and methods

2.1 Test substance selection

Test substances for the ring trial for evaluation of transferability and reproducibility were selected based upon their published characterization for potency in mice and humans (Baskett et al., 2014; ICCVAM, 2011).

Transfer phase

During the transfer phase, 6 DPRA-positive sensitisers were tested twice: 2,4-dichloronitrobenzene (CAS RN 97-00-7), oxazolone (CAS RN 15646-46-5), formaldehyde (CAS RN 50-00-0), ethylene glycol dimethacrylate (CAS RN 97-90-5), benzylidene acetone (CAS RN 122-57-6), and 2,3-butanedione (CAS RN 431-03-8). While ethylene glycol dimethacrylate was the positive control originally reported for the kDPRA (Wareing et al., 2017), the remaining 5 are proficiency chemicals for the DPRA listed in OECD TG 442C (OECD, 2020). The transfer phase test substances were purchased from Sigma Aldrich and distributed by BASF non-blinded.

Inter- and intra-laboratory trial (reproducibility assessment)

The 24 test substances used during the blind-coded testing included two correct negatives and 21 correct positives in the Cys-only DPRA according to literature data (ICCVAM, 2011). The test set intentionally also included one sensitiser known to be negative/minimally reactive in the DPRA (i.e., dihydrocoumarin). The 24 test substances are listed in Table 1 including their protein reactivity rank and concentration matrix is used to calculate rate constants.

It has been shown that kDPRA differentiates GHS Cat 1A sensitizers from GHS 1B/not classified substances with a balanced accuracy of 85% (based on 180 test substances), with a sensitivity of 84% (38/45), and a specificity of 86% (116/135) relative to LLNA results (Natsch et al., 2020). In addition, the prediction of human skin sensitization for 123 test substances that fall within the kDPRA’s applicability domain has a balanced accuracy of 76%, a sensitivity of 64% (21/33), and a specificity of 89% (80/90) (Natsch et al., 2020). On the basis of the overall data available (n = 180), the kDPRA’s applicability domain was shown to include a variety of organic functional groups, the full range of skin sensitization potencies (as determined in in vivo studies), and diverse physicochemical properties.

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2.2 Participating laboratories

This ring trial validation study was conducted by a total of 7 laboratories (in alphabetical order): BASF SE Experimental Toxicology and Ecology (Germany), Charles River Laboratories Den Bosch BV (The Netherlands), Givaudan Schweiz AG (Switzerland), Institute for In Vitro Sciences, Inc. (USA), L’Oréal Research & Innovation (France), National Institute of Public Health (Czech Republic), Procter & Gamble (USA). The lead labs, BASF SE
Experimental Toxicology and Ecology (Germany) and Givaudan Schweiz AG (Switzerland), were responsible for protocol authorship, organization of test-substance selection and procurement, and statistical evaluations. The other 5 laboratories were naïve to the kDPRA (thereof 2 were also naïve to the standard DPRA according to OECD TG 442C, (OECD, 2020)).

### 2.3 Kinetic direct peptide reactivity assay (kDPRA)

**Procedure**

Test substances were dissolved in acetonitrile (ACN) or in pH 7.5 phosphate buffer, if not soluble in ACN, to yield stock solutions of 20 mM. Thereafter, dilution series of 20, 10, 5, 2.5 and 1.25 mM were prepared.
The kDPRA consisted of the following steps: In case of ACN-soluble substances, 120 µL of 0.667 mM Cys-peptide solution in pH 7.5 phosphate buffer was added to each well of a black 96-well plate. Next, 40 µL of the respective substance solution was added to each well. This yielded 0.5 mM peptide concentration and substance concentrations of 5, 2.5, 1.25, 0.625 and 0.3125 mM (fifinal ratios of peptide: test substance = 1:10, 1:5, 2:5, 4:5, 8:5). All substances were tested in triplicate within the same run.

In case of pH 7.5 phosphate-buffer-soluble substances, 80 µL of 1.0 mM Cys-peptide solution in phosphate buffer (pH 7.5) was added to each well of a black 96-well plate. Next, 40 µL ACN was added and, finally, 40 µL of the respective substance solution. This yielded the same composition of samples as for the ACN-soluble substances described above.

Each 96-well plate comprised control samples as follows: 12 wells of a negative control (NC) containing the peptide and vehicle; 12 wells of blank control (BC) containing pH 7.5 phosphate buffer (without peptide) and the vehicle; 1 sample per concentration of the positive control (PC) cinnamic aldehyde, and 1 sample per substance and concentration of a substance control (SC) containing the respective test substance and the buffer but no peptide. The SC served for identification of interference of the test substance with the fluorescence measurement and as a background measurement.

The plates were sealed with impermeable foil directly after application of the substance, shaken for 5 min on a plate shaker and thereafter incubated in the dark at 25°C ±2.5°C. The substances were incubated for 10, 30, 90, 150, 210 and 1440 min. After the respective reaction time, each test run was stopped by the addition of 40 µL of 3 mM mBrB solution (diluted in acetonitrile). Highly reactive non-fluorescent mBrB rapidly reacts with unbound cysteine moieties of the model peptide to form a fluorescent complex. The higher the intensity in fluorescence, the more cysteine moieties remained unbound after the respective reaction time and the less peptide-reactive is the test substance. The substance solutions containing mBrB were then further incubated for 5 min in the dark on a plate shaker. Then fluorescence was detected using an excitation filter of 390 nm and an emission filter of 480 nm.

Fluorescence intensities were normalized relative to the substance without the peptide (SC) as well as the phosphate buffer and acetonitrile (BC; background fluorescence). Relative peptide depletion was expressed as percent decrease in relation to the mean of the NC wells. Further, a pair-wise comparison of each substance concentration group with the VC was performed using the Welch t-test (two-sided) for the hypothesis of equal means.

Data evaluation
Depletion values were further evaluated if the criteria for positivity was reached at the highest concentration of one reaction time point (13.89% Cys-peptide depletion, based on the Cys-only prediction model described in OECD TG 442C (OECD, 2020)) and if the depletion was statistically significantly different (p < 0.05) from NC.

For each incubation time, the remaining (non-reacted) amount of Cys-peptide was determined and its natural logarithm plotted against the respective substance concentration. For each time point for which the regression line gave a correlation > 0.9, the calculated slope was divided by the incubation time to determine the second order reaction rate constant k in (min⁻¹ mM⁻¹) (Roberts and Natsch, 2009). This value was transformed to the rate constant in (s⁻¹ M⁻¹) and the logarithm was taken. The maximum value observed at any time point was taken as the log kmax and used for the further evaluation.

Acceptance criteria
The results of a 96-well test plate were considered valid if the following conditions were met:
- Positive control (PC): The log k of the PC at 90 min is within the following range: -1.75 M⁻¹s⁻¹ to -1.40 M⁻¹s⁻¹. If no log k is obtained at 90 min, the value at 150 min is used instead and lies in the following range: -1.90 M⁻¹s⁻¹ to -1.45 M⁻¹s⁻¹
- Vehicle control (VC): The coefficient of variation of the 12 VC values of a plate is < 12.5% for at least 5 of the 6 time points. If one or more of these criteria were not met, a repetition of the run was considered. Further, the runs for substances were repeated if non-linear behavior of results was obtained in order to exclude data bias due to artifacts, e.g., pipetting errors.

Prediction model
The maximum rate constant observed, log kmax, is used in the kDPRA to distinguish between two levels of skin sensitization potency, i.e., to discriminate GHS subcategory 1A from GHS subcategory 1B/not classified. The prediction model was developed from a dataset on 180 substances with LLNA reference data (Natsch et al., 2020):

| Reaction rate | kDPRA prediction |
|---------------|------------------|
| log kmax ≥ -2.0 | GHS subcategory 1A |
| log kmax < -2.0 | GHS subcategory 1B or not classified |

2.4 Transfer phase
For the transfer phase, the naïve laboratories received the protocol, an evaluation sheet and run validity criteria, and a preliminary proficiency range based on previous results of the lead labs with the same test substances. Two telephone conferences were held to clarify questions related to the conduct of the assay, but no hands-on training was conducted.

2.5 Blind-coded testing
In total, 24 different test substances were assessed under GLP-like conditions during the blind-coded testing in seven different laboratories. Test-substance procurement, blind-coding and distribution were conducted by an external service (BioTeSys GmbH, Esslingen, Germany). All seven participating labs tested all 24 test substances in one repetition (inter-laboratory reproducibility). Further, out of the 24 test substances, a random subset of 12 test substances was tested in two additional repetitions (with a different code for each run) in three or four labs (additional intra-laboratory reproducibility). Thus, for 12 test substances the intra-laboratory comparison was conducted in 3 labs and for the re-
Cys-peptide (in % relative to the VC) is plotted against test substance concentration.

3.1 Transferability
The five naïve labs tested six (non-coded) known sensitizers and the positive control (PC) cinnamic aldehyde to establish the assay within their labs and to familiarize themselves with the study protocol. Overall, the results reported were very similar to those obtained by the two lead labs (Fig. 2) with the exception of formaldehyde, which was much less reactive in all three repetitions performed at Lab E.

The kDPRA was easily transferable to five naïve labs without hands-on training, and the SOP was found sufficiently detailed to perform the test in all participating laboratories. There were no remaining 12 test substances in 4 labs (in total 12 x 3 + 12 x 4 = 84 intra-laboratory comparisons). This was fully randomized, so no laboratory received the same test substances for intra-laboratory reproducibility. The codes Lab1 - Lab8 are thus attributed to different labs for each chemical, Lab 5 - Lab 8 always referring to the labs performing intra-laboratory testing for a particular chemical.

3 Results and discussion

To illustrate a typical result of the kDPRA assay, the depletion matrices for DNCB determined in the seven participating laboratories during the transfer phase are shown in Figure 1. For each exposure time point, the natural logarithm of the remaining

![Fig. 1: Depletion matrices for DNCB in one run from all seven laboratories](image)
The natural logarithm of the non-depleted peptide concentrations is plotted vs the concentration of the test substance at each time point for laboratories A-G.

Cys-peptide (in % relative to the VC) is plotted against test substance concentration.

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significant technical obstacles specific to the method. Figure 2 indicates that the six test substances first tested in the two lead laboratories resulted in very similar log \( k_{\text{max}} \) values when tested in the five naïve labs. Moreover, the standard deviation (SD) from the four experiments in the two lead labs is similar to the SD in the eleven experiments in the five naïve labs, and thus the variability was not significantly increased by moving from the lead labs to the naïve labs.

### 3.2 Blind-coded testing

Once the laboratories had successfully tested the six substances of the transfer phase, they progressed to testing the 24 blind-coded substances. After the test of the blind-coded substances was completed at the seven participating laboratories, all spreadsheets containing the blind-coded data were collected by the lead labs and provided to BioTeSys as an external and independent data repository site before the code for unblinding was provided by the latter. Analysis and biostatistics on the decoded data were then conducted at Givaudan.

**Reproducibility of positive control (PC)**

For the PC cinnamic aldehyde, the log \( k_{\text{max}} \) values and the rate constants at a fixed time (90 min and 150 min) were reported for each experiment (summarized in Tab. 2).

### Tab. 2: Reproducibility of positive control in the blind-coded phase: log \( k_{\text{max}}, \log k_{90 \text{ min}} \) and log \( k_{150 \text{ min}} \) values (M\(^{-1}\)s\(^{-1}\))

|                | \( \log k_{\text{max}} \) (M\(^{-1}\)s\(^{-1}\)) | \( \log k_{90 \text{ min}} \) (M\(^{-1}\)s\(^{-1}\)) | \( \log k_{150 \text{ min}} \) (M\(^{-1}\)s\(^{-1}\)) |
|----------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
|                | AVG   | SD   | Min  | Max  | AVG   | SD   | Min  | Max  | AVG   | SD   | Min  | Max  |
| **All labs**   | -1.35 | 0.13  | -1.51 | -1.15 | -1.58 | 0.04  | -1.64 | -1.53 | -1.66 | 0.04  | -1.75 | -1.62 |
| **Lab A**      | -1.37 | 0.16  | -1.59 | -0.87 | -1.53 | 0.07  | -1.66 | -1.43 | -1.62 | 0.05  | -1.71 | -1.50 |
| **Lab B**      | -1.44 | 0.14  | -1.63 | -1.16 | -1.60 | 0.04  | -1.68 | -1.53 | -1.67 | 0.03  | -1.71 | -1.57 |
| **Lab C**      | -1.32 | 0.19  | -1.59 | -0.99 | -1.54 | 0.07  | -1.66 | -1.37 | -1.63 | 0.06  | -1.74 | -1.51 |
| **Lab D**      | -1.46 | 0.23  | -1.88 | -0.81 | -1.60 | 0.08  | -1.75 | -1.48 | -1.75 | 0.07  | -1.88 | -1.62 |
| **Lab E**      | -1.15 | 0.23  | -1.52 | -0.73 | -1.62 | 0.08  | -1.75 | -1.48 | -1.65 | 0.06  | -1.73 | -1.53 |
| **Lab F**      | -1.51 | 0.18  | -1.70 | -1.09 | -1.64 | 0.07  | -1.75 | -1.52 | -1.69 | 0.04  | -1.77 | -1.65 |
| **Lab G**      | -1.22 | 0.15  | -1.54 | -1.01 | -1.56 | 0.04  | -1.64 | -1.47 | -1.64 | 0.09  | -1.71 | -1.26 |
| **All labs**   | -1.35 | 0.22  | -1.76 | -0.94 | -1.60 | 0.08  | -1.73 | -1.38 | -1.68 | 0.09  | -1.92 | -1.45 |

*Shown for comparison; \(^{a}\) The \( k_{90 \text{ min}} \) value is the rate used to decide on acceptability of an experiment. In case no rate is calculated at 90 min (reaction not linear or not statistically significant), then \( k_{150 \text{ min}} \) can be considered. Labs had to report the 150 min value instead of the 90 min value in only 4 of 148 runs during the blind-coded testing. AVG, average; SD, standard deviation; Min, minimum; Max, maximum.*
Intra-laboratory reproducibility

The average log $k_{\text{max}}$ of all valid runs during the blind-coded testing phase was -1.35, and this value is identical to the value obtained as average value of all labs in the transfer phase. Thus, very comparable results were obtained in both phases of this study. The average log $k_{\text{max}}$ value varied between -1.15 and -1.51 for the seven labs. The SD for intra-laboratory reproducibility of the log $k_{\text{max}}$ was between 0.14 and 0.23, similar to the average SD obtained for all test substances in the intra-laboratory reproducibility (0.158; see subsection on intra-laboratory reproducibility).

The inter-laboratory variability of the PC was even lower for the rate constants derived at 90 and 150 min, which are used as acceptance criteria, and log $k_{90 \text{ min}}$ varied between -1.53 and -1.64 for the seven labs (Tab. 2), while the intra-laboratory SD was between 0.04 and 0.08. The overall SD for all runs was 0.04. Log $k_{150 \text{ min}}$ varied between -1.62 and -1.75 for the seven labs (Tab. 2), while the intra-laboratory SD was between 0.03 and 0.09. The overall SD for all runs was 0.04.

Intra-laboratory reproducibility

The average and the SDs of log $k_{\text{max}}$ are shown in Figure 3 for test substances tested in four labs and in Figure 4 for test substances tested in three labs.

For non-reactive test substances (log $k_{\text{max}} < -3.46$ corresponding to Cys-depletion of $< 13.89\%$ at 5 mM after 24 h), a default value of -3.5 was assigned to allow plotting the results. Abbreviated test substance names (see Tab. 1) and the number attributed to the test laboratory for testing that particular test substance are indicated on the x-axis. The solid red line indicates the cut-off log $k_{\text{max}} = -2.0$. 

**Fig. 3:** Intra-laboratory testing: Variability expressed as average values and SD in repeated intra-laboratory testing (3 times each) in four labs

**Fig. 4:** Intra-laboratory testing: Variability expressed as average values and SD in repeated intra-laboratory testing (3 times each) in three labs

For non-reactive test substances (log $k_{\text{max}} < -3.46$, corresponding to Cys-depletion of $< 13.89\%$ at 5 mM after 24 h) a default value of -3.5 was assigned to allow plotting the results. Abbreviated test substance names (see Tab. 1) and the number attributed to the test laboratory for testing that particular test substance are indicated on the x-axis. The solid red line indicates the cut-off log $k_{\text{max}} = -2.0$. 

The average and the SDs of log $k_{\text{max}}$ are shown in Figure 3 for test substances tested in four labs and in Figure 4 for test substances tested in three labs.
Coumarin, which are also non-Cys-reactive in DPRA (Natsch et al., 2013). In addition, phenylbenzoate and bourgeonal were reproducibly non-reactive in kDPRA despite the fact that Cys-depletion had been reported in the DPRA (Bauch et al., 2011 and unpublished data). This low intra-laboratory variability of the rate constants and quantification of this variability should be considered an important aspect for uncertainty analysis when these data are later used in defined approaches for potency assessment on a continuous scale. For most validated in vitro assays, only reproducibility of hazard classification has been fully documented.

For most test substances, the intra-laboratory variability was low and the SDs between individual runs were very small. For those test substances with very low SD (below 0.3 on the logarithmic scale corresponding to a two-fold difference in the kinetic rate), the values reported by the different laboratories were also very close to each other. The average SD of the 24 test substances in intra-laboratory testing was 0.158, and the average was < 0.1 for 10 test substances, with a further five test substances being non-reactive in all laboratories. These included the two non-sensitizers (4-methoxy-acetophenone and chlorobenzene) and 3,4-dihydrocoumarin, which are also non-Cys-reactive in DPRA (Natsch et al., 2013). In addition, phenylbenzoate and bourgeonal were reproducibly non-reactive in kDPRA despite the fact that Cys-depletion had been reported in the DPRA (Bauch et al., 2011 and unpublished data). This low intra-laboratory variability of the rate constants and quantification of this variability should be considered an important aspect for uncertainty analysis when these data are later used in defined approaches for potency assessment on a continuous scale. For most validated in vitro assays, only reproducibility of hazard classification has been fully document-

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Tab. 3: Intra-laboratory reproducibility of the classification of test substances

| Substance                        | Lab 5 rep1 | Lab 5 rep2 | Lab 5 rep3 | Lab 6 rep1 | Lab 6 rep2 | Lab 6 rep3 | Lab 7 rep1 | Lab 7 rep2 | Lab 7 rep3 | Lab 8 rep1 | Lab 8 rep2 | Lab 8 rep3 | Lab 5 AVG | Lab 6 AVG | Lab 7 AVG | Lab 8 AVG | Labs with consistent repetitions |
|----------------------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|----------------------------------|
| (Chloro)methylisothiazolinone    | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 4 of 4                            |
| Glyoxal                          | 1B         | 1B         | 1B         | 1A         | 1A         | 1B         | 1B         | 1A         | 1A         | 1A         | 1B         | 1A         | 1A         | 1B         | 1A         | 3 of 4                            |
| Methylisothiazolinone            | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 4 of 4                            |
| Methyl-2-octynoate               | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 3 of 3                            |
| 4-Phenylenediamine               | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 3 of 3                             |
| Tetrachlorosalicylanilide        | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 3 of 3                             |
| Isoeugenol                       | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 3 of 3                             |
| Bourgeonal                       | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | 3 of 3                             |
| Carvone                          | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 3 of 4                             |
| Dihydropyocumarin                | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | 3 of 3                             |
| Hydroxycitronellial              | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | 1B         | 1B         | 1B         | 1B         | 1B         | 4 of 4                             |
| Imidazolidinyl urea              | 1A         | 1A         | 1B         | n-r        | 1A         | 1A         | 1A         | 1A         | 1A         | 1B         | 1B         | 1A         | 1A         | 1A         | 4 of 4                             |
| Methylhexanediolide              | 1B         | 1B         | 1B         | n-r        | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 3 of 3                             |
| Phenylbenzoate                   | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | 4 of 4                             |
| Phenylpropanaldehyde             | 1B         | 1B         | n-r        | 1B         | 1A         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 2 of 3                             |
| Tetramethylthiuram disulfide     | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 4 of 4                             |
| Benzisothiazolinone              | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 4 of 4                             |
| Benzyldiene acetone              | 1A         | 1B         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 2 of 3                             |
| δ-Damascone                      | 1A         | 1A         | 1A         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 1B         | 4 of 4                             |
| Diethylmaleate                   | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 3 of 3                             |
| trans-2-Hexenal                  | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 1A         | 4 of 4                             |
| 4-Methoxyacetophenone            | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | 4 of 4                             |
| Chlorobenzene                    | 1B         | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | n-r        | 3 of 3                             |
| **Total**                        |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            | 81 of 84                           |

*a Abbreviations: n-r, non-reactive; b AVG indicates the rating of the test substance by the average log kmax determined from all repetitions in a particular lab, this value is used for inter-laboratory reproducibility of class prediction below.*
ing test substances like CAR and MHD may have higher variation, as variation over a prolonged incubation time may be cumulative. Test substances triggering peptide oxidation like PPA are known to be subject to higher variability. For PPA, this had already been observed in the DPRA pre-validation study (reported in Dimitrov et al., 2016). Finally, pre-haptens that spontaneously oxidize, like IE, may be more variable, as autoxidation is known to be a self-catalyzed process and hence prone to more stochastic effects.

Intra-laboratory data for prediction of UN GHS sensitizer classes according to the prediction cut-off log $k_{\text{max}}$ -2 are provid-
These data demonstrate the inter-laboratory variability of the 7 laboratories for all 24 test substances. The inter-laboratory reproducibility was high for most test substances with an average SD for inter-laboratory comparison of 0.244, which was slightly higher than in the intra-laboratory comparisons and which corresponds to a variation around the geometric mean of 1.75 fold. Again, quantification of the uncertainty of the rate constant determinations on a continuous scale is an important attribute for uncertainty analysis of subsequent models and risk assessments relying on log \( k_{\text{max}} \) values. In general, similar test substances that had higher variability in intra-laboratory testing also exhibited higher inter-laboratory variability (GLY, CAR, MHD, PPA), indicating that this is an intrinsic property of the test substances and not due to experimental variability.

There is one significant outlier in the whole dataset: Lab E re-handed in Table 3. In summary, using the kDPRA to differentiate GHS Cat 1A vs GHS Cat 1B/not classified, attribution to GHS Cat 1A was consistent in 81 of 84 instances, hence intra-laboratory reproducibility with the classification prediction model for identifying 1A test substances was 96%. Variable predictions were mostly observed for test substances with a log \( k_{\text{max}} \) very close to the classification cut-off.

Based on data in Figures 3 and 4 and in Table 3, the kDPRA has proven to be very reproducible in intra-laboratory testing, both when predicting binary classification as well as when considering the numerical log \( k_{\text{max}} \) values on a continuous scale.

**Inter-laboratory reproducibility**

Log \( k_{\text{max}} \) values obtained during the inter-laboratory reproducibility assessment are summarized in Table 4 and Figure 5.

| Substance                        | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 | Lab 7 | Lab 8 | AVG  | SD  |
|----------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|------|-----|
| (Chloro)methylisothiazolinone    | 0.58  | 0.64  | 0.57  | 0.60  | 0.61  | 0.61  | 0.56  | 0.60  | 0.029|
| Glyoxal                          | -1.57 | -2.23 | -1.25 | -2.51 | -1.64 | -2.37 | -1.65 | -1.89 | 0.478|
| Methylisothiazolinone            | -0.26 | -0.15 | -0.19 | -0.51 | -0.17 | -0.13 | -0.21 | -0.23 | 0.130|
| Methyl-2-octynoate               | -1.28 | -1.60 | -1.71 | -1.22 | -1.47 | -1.73 | -1.61 | -1.52 | 0.202|
| 4-phenylenediamine               | -1.50 | -1.17 | -0.86 | -1.47 | -1.07 | -1.02 | -1.05 | -1.16 | 0.238|
| Tetrachlorsalicylanilide         | -0.55 | -0.35 | -0.44 | -0.47 | -0.46 | -0.36 | -0.57 | -0.46 | 0.086|
| Isoeugenol                       | -1.07 | -1.22 | -1.23 | -1.16 | -1.36 | -1.12 | -1.31 | -1.21 | 0.103|
| Bourgeonal                       | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r  |
| Carvone                          | -2.16 | n-r   | -3.37 | -3.30 | -2.83 | -2.26 | -2.56 | -2.75 | 0.512|
| Dihydrocoumarin                  | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r  |
| Hydroxycitronellial              | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r  |
| Imidazolidinyl urea              | -1.02 | -0.73 | -1.41 | -0.76 | n-r   | -0.94 | -0.95 | -1.30 | 0.245|
| Methylhexaneidione               | -2.44 | -3.61 | -1.22 | -2.12 | -2.54 | -2.43 | -3.39 | -2.54 | 0.795|
| Perillaaldehyde                  | -3.44 | -3.19 | -2.56 | -2.44 | -2.54 | -3.25 | -3.04 | -2.92 | 0.401|
| Phenyl benzoate                  | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r  |
| Phenylpropionaldehyde            | -3.17 | -3.10 | -2.37 | -2.90 | -2.87 | -2.29 | -2.64 | -2.76 | 0.343|
| Tetramethylduriam disulfide      | 0.75  | 0.74  | 0.73  | 0.71  | 0.77  | 0.74  | 0.73  | 0.74  | 0.020|
| Benzisothiazolinone              | -0.07 | 0.18  | -0.21 | -0.20 | -0.26 | 0.06  | -0.17 | -0.10 | 0.161|
| Benzylidene acetone              | -1.94 | -1.98 | -1.78 | -1.82 | -2.08 | -1.91 | -1.72 | -1.89 | 0.125|
| δ-Damascone                      | -2.06 | -2.34 | -2.17 | -1.90 | -2.38 | -2.26 | -2.09 | -2.17 | 0.169|
| Diethylmaleate                   | -1.38 | -1.25 | -1.32 | -1.38 | -1.21 | -1.08 | -1.20 | -1.26 | 0.109|
| trans-2-Hexenal                  | -0.38 | -0.37 | -0.34 | -0.41 | -0.43 | -0.57 | -0.59 | -0.44 | 0.100|
| 4-Methoxy-acetophenone           | n-r   | -2.91 | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r  |
| Chlorobenzene                    | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r   | n-r  |

Abbreviations: AVG, indicates the rating of the test substance by the average log \( k_{\text{max}} \) determined from all repetitions in all labs; n-r, non-reactive; SD, standard deviation

These data demonstrate the inter-laboratory variability of the 7 laboratories for all 24 test substances. The inter-laboratory reproducibility was high for most test substances with an average SD for inter-laboratory comparison of 0.244, which was slightly higher than in the intra-laboratory comparisons and which corresponds to a variation around the geometric mean of 1.75 fold. Again, quantification of the uncertainty of the rate constant determinations on a continuous scale is an important attribute for uncertainty analysis of subsequent models and risk assessments relying on log \( k_{\text{max}} \) values. In general, similar test substances that had higher variability in intra-laboratory testing also exhibited higher inter-laboratory variability (GLY, CAR, MHD, PPA), indicating that this is an intrinsic property of the test substances and not due to experimental variability.

There is one significant outlier in the whole dataset: Lab E re-
it was -1.80 ±0.12 in the transfer phase, hence a very similar result and similar variability was observed in both open and blinded testing.

When applying the prediction cut-off (log $k_{\text{max}}$ = -2.0) to differentiate GHS Cat 1A vs GHS Cat 1B/not classified, a consistent result was obtained for 22 test substances (when testing each test substance once, Tab. 5) and for 20 test substances (when testing each test substance three times, Tab. 6). This resulted in an inter-laboratory reproducibility for identifying 1A test substances of 92% (for laboratories testing the test substance once) and 83% (laboratories testing the test substances three times), respectively, with an average for the two indepen-

**Tab. 5: Inter-laboratory reproducibility for GHS category determination based on the cut-off log $k_{\text{max}}$ of -2.0**

Data for the laboratories testing the test substances once.

| Substance                                  | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Consistent 1A vs. 1B/NC? |
|--------------------------------------------|-------|-------|-------|-------|--------------------------|
| (Chloro)methylisothiazolinone              | 1A    | 1A    | 1A    | 1A    | YES                      |
| Glyoxal                                    | 1A    | 1B    | 1A    | 1A    | NO                       |
| Methylisothiazolinone                      | 1A    | 1A    | 1A    | 1A    | YES                      |
| Methyl-2-octynoate                         | 1A    | 1A    | 1A    | 1A    | YES                      |
| 4-phenylenediamine                         | 1A    | 1A    | 1A    | 1A    | YES                      |
| Tetrachlorosalicylanilide                  | 1A    | 1A    | 1A    | 1A    | YES                      |
| Isoeugenol                                 | 1A    | 1A    | 1A    | 1A    | YES                      |
| Bourgeonal                                 | n-r   | n-r   | n-r   | n-r   | YES                      |
| Carvone                                    | 1B    | n-r   | 1B    | YES   |
| Dihydrocoumarin                            | n-r   | n-r   | n-r   | n-r   | YES                      |
| Hydroxycitronnellal                        | n-r   | n-r   | n-r   | YES   |
| Imidazolidinyl urea                        | 1A    | 1A    | 1A    | 1A    | YES                      |
| Methylenehexanone                          | 1B    | 1B    | 1A    | 1B    | NO                       |
| Perillaldehyde                             | 1B    | 1B    | 1B    | 1B    | YES                      |
| Phenylnbenzoate                            | n-r   | n-r   | n-r   | YES   |
| Phenylpropionaldehyde                      | 1B    | 1B    | 1B    | 1B    | YES                      |
| Tetramethyliuram disulfide                 | 1A    | 1A    | 1A    | 1A    | YES                      |
| Benzisothiazolinone                        | 1A    | 1A    | 1A    | 1A    | YES                      |
| Benzylidene acetone                        | 1A    | 1A    | 1A    | 1A    | YES                      |
| δ-Damascone                                | 1B    | 1B    | 1B    | 1B    | YES                      |
| Diethylmaleate                             | 1A    | 1A    | 1A    | 1A    | YES                      |
| trans-2-Hexenal                            | 1A    | 1A    | 1A    | 1A    | YES                      |
| 4-Methoxy-acetophenone                     | n-r   | 1B    | n-r   | YES   |
| Chlorobenzene                              | n-r   | n-r   | n-r   | n-r   | YES                      |
| **n consistent**                           | **22**          |          |          |          |                          |
| **Reproducibility (%)**                    | **(22/24) 92%** |          |          |          |                          |

ported very low / no reactivity (in repeated intra-laboratory testing) for IU; thus, for an unknown reason, this lab obtained different results for test substances related to formaldehyde. This outlier is highly consistent for IU and formaldehyde in that particular lab, and it appears to be linked to the chemistry. Formaldehyde does form a reversible peptide-adduct (data not shown), and for unknown reasons the reaction must have been reversed prior to reaction or during the reaction with mBrB in that particular laboratory.

Benzylidene acetone was tested both in the transfer phase and in the blind-coded phase. The log $k_{\text{max}}$ value from the seven labs in the blind-coded phase was -1.89 ±0.13, while it was -1.80 ±0.12 in the transfer phase, hence a very similar result and similar variability was observed in both open and blinded testing.

When applying the prediction cut-off (log $k_{\text{max}}$ = -2.0) to differentiate GHS Cat 1A vs GHS Cat 1B/not classified, a consistent result was obtained for 22 test substances (when testing each test substance once, Tab. 5) and for 20 test substances (when testing each test substance three times, Tab. 6). This resulted in an inter-laboratory reproducibility for identifying 1A test substances of 92% (for laboratories testing the test substance once) and 83% (laboratories testing the test substances three times), respectively, with an average for the two indepen-
The kDPRA proved to be transferable to laboratories without hands-on training, and highly reproducible results for the positive control were obtained. The within-laboratory reproducibility of the kDPRA for assigning GHS Cat 1A was 96%, and the between-laboratory reproducibility for 24 test substances was 88%.

The average SD of the logarithmic rate in intra-laboratory testing was 0.158, which corresponds to a variation around the geometric mean of $k_{\text{max}}$ of 1.44-fold, while the average SD for inter-laboratory comparison was 0.244, which corresponds to a variation of 1.75-fold. This quantification of variability on a continuous

dent evaluations of 88%.

When evaluating these values against other published validation studies where each test substance was typically tested three times, this analysis is a bit more stringent as 50% of the comparisons are made in 4 labs, and the random chance of congruent results falls from 25% to 13% with testing in 4 labs instead of 3 labs (i.e., the more labs, the higher the chance of producing one deviating result).

### 4 Conclusion

| Substance                        | Lab 5 | Lab 6 | Lab 7 | Lab 8 | Consistent 1A vs. 1B/NC? |
|----------------------------------|-------|-------|-------|-------|--------------------------|
| (Chloro)methylisothiazolinone    | 1A    | 1A    | 1A    | 1A    | YES                      |
| Glyoxal                          | 1B    | 1A    | 1B    | 1A    | NO                       |
| Methylisothiazolinone            | 1A    | 1A    | 1A    | 1A    | YES                      |
| Methyl-2-octynoate               | 1A    | 1A    | 1A    | 1A    | YES                      |
| 4-phenylemediamine               | 1A    | 1A    | 1A    | 1A    | YES                      |
| Tetrachloralsalicynilide         | 1A    | 1A    | 1A    | 1A    | YES                      |
| Ioseugenol                      | 1A    | 1A    | 1A    | 1A    | YES                      |
| Bourgeonal                      | n-r   | n-r   | n-r   |       | YES                      |
| Carvone                          | 1B    | 1B    | 1B    | 1B    | YES                      |
| Dihydrocoumarin                 | n-r   | n-r   | n-r   |       | YES                      |
| Hydroxyctronellal               | n-r   | 1B    | n-r   | 1B    | YES                      |
| Imidazolidinyl urea             | 1A    | 1B    | 1A    | 1A    | NO                       |
| Methylhexanediane               | 1B    | 1B    | 1B    | 1B    | YES                      |
| Perillaaldehyde                 | 1B    | 1B    |       |       | YES                      |
| Phenyl benzoate                 | n-r   | n-r   | n-r   | n-r   | YES                      |
| Phenylpropionaldehyde           | 1B    | 1B    |       |       | YES                      |
| Tetramethyldiuram disulfide      | 1A    | 1A    | 1A    | 1A    | YES                      |
| Benzosiothiazolinone            | 1A    | 1A    | 1A    | 1A    | YES                      |
| Benzylidene acetone             | 1B    | 1A    | 1A    | 1A    | NO                       |
| δ-Damascone                     | 1A    | 1B    | 1B    | 1B    | NO                       |
| Diethylmaleate                  | 1A    | 1A    | 1A    | 1A    | YES                      |
| trans-2-Hexenal                 | 1A    | 1A    | 1A    | 1A    | YES                      |
| 4-Methoxy-acetophenone          | n-r   | n-r   | n-r   | n-r   | YES                      |
| Chlorobenzene                   | n-r   | n-r   | n-r   | n-r   | YES                      |
| n consistent                    |       |       |       |       | 20                       |
| Reproducibility (%)             |       |       |       |       | (20/24) 83%              |

The average log $k_{\text{max}}$ from three intra-laboratory experiments was taken to make the prediction.
scale will be beneficial for uncertainty analysis in risk assessment.

Following an independent peer review, the kDPRA validation study was considered to demonstrate that the method is acceptable for the predictive identification of skin sensitization potency categories. The kDPRA has been added to the OECD Work Plan for the Test Guidelines Program for inclusion as an additional method in OECD TG 442C as project 4.317. Details and the draft updated OECD TG 442C will be discussed with the OECD expert group on skin sensitization during 2020.

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Conflict of interest
The authors of this publication are employees of the participating companies or organizations. The contribution to this publication was part of their work and is paid by their salaries.

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