DB-ALM Protocol n° 217: Kinetic Direct Peptide Reactivity Assay (kDPRA)

Skin Sensitisation & Allergic Contact Dermatitis

The Kinetic Direct Peptide Reactivity Assay (kDPRA) is a modification of the *in chemico* method DPRA (described in Appendix I of OECD TG 442C, **DB-ALM Protocol n°154**) wherein the reaction kinetics of a test chemical towards a synthetic cysteine-containing peptide is evaluated. Kinetic rates of cysteine-peptide depletion are calculated to distinguish between two levels of skin sensitisation potency, i.e. to differentiate United Nations' Globally Harmonised System of Classification and Labelling of Chemicals (UN GHS) Cat 1A sensitisers from UN GHS Cat 1B/ not classified. In addition, kinetic rates generated with this method have a strong quantitative correlation to sensitising potency and can therefore be used in defined approaches (DA) with a quantitative data integration procedure (DIP) for skin sensitisation potency assessment.

Résumé

The basic method of the kDPRA has been described for the first time in (Natsch et al. 2007). The approach to use it for rate constant determinations was described in (Roberts and Natsch 2009) and later applied to more chemicals from specific domains (Natsch et al. 2011). Thereafter it was applied to a more diverse set of chemicals and a tentative prediction model was proposed to discriminate between GHS categories 1A and 1B (Wareing et al. 2017). Finally, a ring trial study of the kDPRA with 24 blind-coded chemicals was conducted in seven labs and an extension of the kDPRA database substances was performed to further assess the predictive capacity of the assay (Natsch et al. 2020b; Wareing et al. 2020).
Further details on the context of use and applicability domain can be found in OECD TG No. 442C "Key–Event-Based Test Guideline For In Chemico Skin Sensitisation Assays Addressing The Adverse Outcome Pathway Key Event On Covalent Binding To Proteins" (OECD 2020, under preparation) and kDPRA training video (Landsiedel et al, 2020 available at http://doi.org/10.5281/zenodo.3901109 in English language; https://www.3r-smart.de/index.php?id=13164 in German language).

Experimental Description

Endpoint and Endpoint Measurement:

REACTION KINETICS OF DIRECT PEPTIDE REACTIVITY: measured by quantifying the depletion of a synthetic heptapeptide containing cysteine followed by calculation of kinetic rate constants from these depletions.

kDPRA addresses mechanisms described under the molecular initiating event (covalent binding to proteins) of the Adverse Outcome Pathway (AOP) for skin sensitisation (OECD 2020 under preparation; OECD 2012).

Endpoint Value:

The maximum rate constant observed, log k_{max} [M^{-1} s^{-1}], is used in the kDPRA to distinguish between two levels of skin sensitisation potency, i.e. to discriminate between GHS subcategory 1A from GHS subcategory 1B/ not classified.

Log k_{max} is the maximum rate constant observed amongst all rate constants calculated from the peptide depletion at the respective reaction time points and test chemical concentrations.

Experimental System:

Synthetic heptapeptide containing cysteine (Ac-RFAACAA-COOH).

Discussion

- Management of ethical issues: The test method is an *in chemico* assay and does not require human- or animal derived material.
- Special equipment needed: The kDPRA can be performed using common lab equipment. A fluorometer capable of reading 96-well plates with an excitation filter of 390 nm and an emission filter of 480 nm is required.
- Use of materials which require special handling or restricted access: The fluorimetric dye monobromobimane is light- and temperature sensitive. Handling of monobromobimane should be performed with special care as it may interact with DNA. The synthetic peptide is temperature- and oxygen-sensitive.
- Amount of training required: The five naïve labs participating in the ring trail /validation study did not attend a hands-on training, but were able to perform the assay within short time after reading the SOP.
- Duration of the test: A kDPRA run is performed within 2 days.
- Particular technical difficulties and critical attention points: Precise pipetting and timing are crucial.
- Possible adaptations of the protocol for high-throughput testing and/or combinations with other assays: The assay is designed to test 3 chemicals in parallel in one 96-well plate. The number of assay plates conducted in parallel may be increased by using pipetting robots.
- Costs (e.g., approximate cost for testing 10 compounds): Three test chemicals can be tested in parallel on one plate. The costs associated with the testing of three test chemicals is hence comparable to 1 test chemical in the DPRA.

Status

Known Laboratory Use:

- Givaudan Schweiz AG, Switzerland
- BASF SE, Experimental Toxicology and Ecology, Germany
- L'Oréal Research & Innovation, France
- Institute for In Vitro Sciences, Inc., USA
- Procter & Gamble, USA
- Charles River Laboratories Den Bosch BV, The Netherlands
- National Institute of Public Health, Czech Republic

Participation in Evaluation Study:

The test method developers evaluated a database of rate constants for 180 chemicals (Natsch et al. 2020b). Based on these data, an optimal prediction cut-off to identify strong sensitisers (GHS 1A) according to the Globally Harmonised System classification scheme was derived. This threshold has a balanced accuracy of 85% vs. the local lymph node assay for discriminating UN GHS subcategory 1A skin sensitisers versus a combination of subcategory 1B-and-non-sensitisers according to UN GHS. At the same time, correlation analysis against LLNA EC3 values indicates that the rate constant has the strongest contribution to potency of all the parameters measured in the validated assays for skin sensitisation. The kDPRA is proposed as a stand-alone assay for identification of GHS sub-category 1A sensitisers.

Participation in Validation Study:

The kDPRA protocol was used in a ring trial/validation study for assessment of its transferability and within- and between-laboratory reproducibility. This study was initiated by Givaudan and BASF and its study validation plan was shared and reviewed by EURL ECVAM prior to initiation of the study. The two test developers Givaudan and BASF and 5 further naïve labs participated in this validation study. The study was reviewed by an *ad hoc* validation panel with members nominated by EURL ECVAM, JaCVAM and ICCVAM-NICETAM (Natsch et al. 2020b). The panel concluded that "the data provided by the test method developers is sufficient and adequately supports the scientific validity of the kDPRA for the identification of UN GHS Subcategory 1A."

Regulatory Accepted:

The test method was adopted as OECD Test Guideline No. 442C "Key–Event -Based Test Guideline For In Chemico Skin Sensitisation Assays Addressing The Adverse Outcome Pathway Key Event On Covalent Binding To Proteins" (OECD, 2020 under preparation). TG 442C describes *in chemico* procedures addressing the molecular initiating event in the skin sensitization adverse outcome pathway. In addition to the methods proposed for supporting the discrimination between skin sensitisers and non-sensitisers, the kDPRA differentiates UN GHS subcategory 1A skin sensitisers versus a combination of subcategory 1B-and-non-sensitisers according to UN GHS (UN 2019).

Proprietary Issues

None.

Health and Safety Issues

General Precautions

General safety instructions should be followed at all times. Appropriate personal safety equipment should be worn in accordance with local requirements. Unknown chemicals should generally be considered as potential sensitizing agents and toxic and must be handled with maximum care.
Unknown chemicals as well as monobromobimane should be handled in a chemical safety hood, exclusively.

MSDS Information

Current version of the MSDS for the following components should be derived before handling from the supplier of the components:

- Monobromobimane
- Cinnamic Aldehyde
- Acetonitrile (ACN)
- Sodium Phosphate, Monobasic Monohydrate
- Sodium Phosphate, Dibasic Heptahydrate

Abbreviations and Definitions

ACN	Acetonitrile
AOP	Adverse Outcome Pathway
BC	Blank Control
CV	Coefficient of Variance
Cys	Cysteine
DPRA	Direct Peptide Reactivity Assay
DA	Defined Approaches
DIP	Data Integration Procedure
EURL ECVAM	European Union Reference Laboratory for alternatives to animal testing
JaCVAM	Japanese Center for the Validation of Alternative Methods
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
k	kinetic rate constant
kDPRA	Kinetic Direct Peptide Reactivity Assay
mBrB	Monobromobimane
(M)SDS	(Material) Safety Data Sheet
MW	Molecular Weight
NICETAM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organisation for Economic Co-operation and Development

PC	Positive Control
SC	Substance Control (=test chemical control)
SD	Standard Deviation
SOP	Standard Operating Procedure
UN GHS	United Nations' Globally Harmonised System of Classification and Labelling of Chemicals
VC	Vehicle Control

Last update: 17Jun 2020

PROCEDURE DETAILS, Latest Version: 18 May 2020

Kinetic Peptide Reactivity Assay (kDPRA)

DB-ALM Protocol n° 217

kDPRA is used to identify strong sensitisers (GHS 1A) according to the Globally Harmonised System classification scheme. The protocol is based on the Standard Operating Procedure (SOP) used in the ring trial/validation study on the Kinetic Peptide Reactivity Assay (kDPRA). A validated data evaluation template (kDPRA_Evaluation_Template_t6.xltx) is available as supplementary material for download.

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Materials and Preparations

CELL OR EXPERIMENTAL SYSTEM

The kDPRA is an *in chemico* assay. A nucleophile-containing peptide (cysteine peptide – Ac-RFAACAA-COOH) is used to screen for skin sensitisation potency by measuring peptide depletions following different incubation times and different concentrations of allergens and by calculating kinetic rate constants from these depletions.

EQUIPMENT

Apparatus	**Suggested type (or equivalent)**
Analytical balance:	capable of accurately weighing up to 30 g with 0.1 mg readability
Dispensing pipets capable of delivering 40 and 120 µL:	Eppendorf Xplorer, 8-channel, 15-300 µL
Dispensing pipets capable of delivering up to 600 µL:	Eppendorf Xplorer, 8-channel, 50-1200 µL or Eppendorf Xplorer, 12-channel, 50-1200 µL (optional)
Dispensing pipets capable of delivering up to 1000 µL	Eppendorf Research Adjustable Pipets
Incubator:	Midi 40 Incubator, Thermo Scientific; for incubation at 25°C ± 2.5°C
pH meter with electrode and calibration buffers:	capable of reading +/- 0.01 pH units
Photometer for fluorescence measurement:	TriStar² Multimode Reader LB 942, Berthold Technologies, excitation filter F390, emission filter F480
Plate shaker:	Thermo-Orb-Shaker DTS-4, elmi Ltd., min. 200 RPM

Consumables:	**Suggested type (or equivalent)**
Volumetric flask:	certified with defined volume, e.g. 5 mL / 37256 or 10 mL / 37267, Brand
Pipets for higher volumes:	serological pipettes, e.g. 10, 25, 50mL, TPP
Deepwell plates (for preparing the test chemical dilutions):	U96 PP 2 mL Deepwell Natural, 278752, Thermo Scientific
Assay plates:	OptiPlate™ 96F, Black, 96 well, Pinch bar design, Polystyrene, 6005270, Perkin Elmer or equivalent
Gas-tight plate sealers:	Adhesive foil for MicroWell™ plates, Nunc™, 732-2610, Thermo Fischer

MEDIA, REAGENTS, SERA, OTHERS

Chemicals and special materials:	Suggested type (or equivalent)
Cysteine peptide (store deep-frozen, under inert gas) Ac-RFAACAA-COOH, MW = 751.9 g/mol; ≥ 95% purity	commercially available from e.g.: GenScript USA Inc., Piscataway, NJ 08854, USA JPT Peptide Technologies GmbH, Berlin, Germany RS Synthesis, Louisville KY, USA or Bachem AG, 4416 Bubendorf, Switzerland
Monobromobimane (mBrB) (store deep-frozen)	B4380, Sigma Aldrich or ENZ-52501-0000 Enzo Life Sciences
Sodium phosphate, monobasic monohydrate (NaH_2PO_4 H_2O, FW = 138.0 g/mol); CAS-No 10049-21-5	ACS Reagent Grade, Sigma Aldrich S9638
Sodium phosphate, dibasic heptahydrate (Na_2HPO_4 $7H_2O$, FW = 268.0 g/mol); CAS-No 7782-85-6	ACS Reagent Grade, Sigma Aldrich S9390
Cinnamic aldehyde CAS No. 104-55-2	W228613, Sigma Aldrich
Acetonitrile:	e.g. CHROMASOLV® gradient grade, for HPLC, ≥99.9%, 34851-1L, Sigma Aldrich
De-ionized water:	HPLC Grade or Millipore Milli-Q grade

PREPARATIONS

Media and Endpoint Assay Solutions

100 mM sodium phosphate, monobasic	Using a 1 L volumetric flask, dissolve 13.8 g of sodium phosphate monobasic monohydrate in purified water and dilute to final volume of 1 L. Store refrigerated up to 4 weeks.
100 mM sodium phosphate, dibasic	Using a 1 L volumetric flask, dissolve 26.8 g of sodium phosphate dibasic heptahydrate in purified water and dilute to final volume of 1 L. Store refrigerated up to 4 weeks.
100 mM phosphate buffer, pH 7.5	Combine 18 mL of 100 mM sodium phosphate monobasic with 82 mL of 100 mM sodium phosphate dibasic. Mix well and measure pH using a calibrated pH meter. Adjust pH to 7.50 +/- 0.05 with either the monobasic (to acidify) or dibasic (basify) solution. Store refrigerated up to 4 weeks.
3 mM monobromobimane (3 mM mBrB)	Using a 10 mL volumetric flask, dissolve 8.1 mg of mBrB in acetonitrile and dilute to final volume of 10 mL. Prepare fresh at least for each test day. When not in use, store refrigerated and in the dark.

Test chemicals pre-work preparation

Solubility assessment

Prior to the assay the solubility of the test chemical at a concentration of 20 mM should be tested. A suitable non-reactive, water-miscible solvent should dissolve the test chemical completely (no visible precipitation or cloudiness of the test chemical preparation). The preferred solvent is acetonitrile.
When a substance is not soluble in acetonitrile, solubilisation in pH 7.5 phosphate buffer is tried. Alternatively de-ionized water may be used for solubility assessment if the buffer is not at hand. (*Note: substances, which are soluble in water will be prepared and diluted in pH 7.5 phosphate buffer*).
Further vehicles have not been tested yet but may be used if demonstrated that the vehicle does not interfere with the assay, e.g. all controls should be prepared using the same vehicle.

Note: All further pre-work preparations listed below must be prepared on the day of the assay fresh before use.

Preparation of 20 mM stock solutions

Calculate the target weight of test chemical needed to prepare 5 mL of a 20 mM stock solution, considering molecular weight and purity of the test chemical, if purity is available. If no purity is available, assume 100% purity.

$$5\ mL * \frac{1L}{1000} mL * 20 \frac{mmol}{L} * MW\ \frac{mg}{mmol} * \frac{100}{\%purity} = \frac{MW}{\%purity} x\ 10 = Target\ weight\ [mg]$$

Based on the amount of stock solution needed, weigh an appropriate amount of substance into a suitable vessel. Add the appropriate amount of vehicle using a pipette. The appropriate amount of vehicle is calculated based on the actual weight of substance in the vial. *Note: the volume of the weighed substance is subtracted (density is considered to be 1).*

$$\frac{Actual\ weigh\ \ [mg]}{Target\ weigh\ \ [mg]} * 1000\ \mu L - absolute\ value\ of\ actual\ weight\ [\mu L] = V\ (Solvent)\ [\mu L]$$

Example:

To prepare a 20 mM stock solution of a test chemical with molecular weight of 300 g/mol, 30 mg of substance is required for a volume of 5 mL.

31.4 mg were weighed into a vessel.
The volume to be added is:
(31.4 mg x 5000 µL / 30 mg) – 31.4 µL = 5201.9 µL

Dissolve the substance in the solvent using a vortex. If not soluble, use ultra-sonication for several minutes.

Note: For test chemicals that are expensive or short in supply it is possible to prepare smaller volumes of solutions (i.e. 2 mL), as long as the final concentration remains to be 20 mM.

Positive Control

Cinnamic aldehyde is soluble in acetonitrile. It is used as the PC and is included on every test plate. A 20 mM stock solution is prepared in the same way as the test chemicals described above.

Vehicle Control(s)

Vehicle control (VC): peptide (dissolved in buffer) and vehicle without test chemical. The peptide-depletion of substance-incubated samples is calculated in relation to the respective VC.

Background measurement control (BC): vehicle and buffer without test chemical or peptide. Serves as a background measurement for the VC.

Substance control (=test chemical control,SC): Respective test-chemical concentration in the vehicle and buffer without peptide. For identification of interference of the test chemical with the fluorescence measurement and as a background measurement.

Preparation of peptide stock solution

Note: Do not prepare until ready to use!

Cysteine peptide Ac-RFAACAA-COOH, 0.667 mM (0.501 mg/mL):

Calculate the amount of peptide stock solution needed for the run (calculation of all amounts needed for 1 – 3 test chemicals using the standard layout is included in the excel evaluation sheet). All samples in a run should use the identical peptide stock solution.

Based on the amount of peptide stock needed, weigh an appropriate amount of peptide into a glass beaker. Add the appropriate amount of pH 7.5 phosphate buffer to make a 0.667 mM solution of Cysteine peptide. The appropriate amount of buffer is calculated based on the actual weight of peptide in the vial using the equation:

$$\mathbf{mL\ pH\ 7.5\ Buffer} = \frac{\mathbf{mg\ Peptide}}{\mathbf{0.501\ mg/mL}}$$

For example, to prepare ca. 50 mL of peptide stock solution, weigh > 25.05 mg of cysteine peptide (note the exact weight, for example 25.5 mg) and add the required amount of buffer (in this case: 25.5 mg/0.501 mg/mL = 50.898 mL). Smaller or larger quantities may be prepared as appropriate. For these calculations, the purity of the peptide is assumed to be 100%.

The peptide is dissolved using a vortex or magnetic stirrer. Only use gentle agitation however, to prevent peptide oxidation. Ultra-sonication may also be used. It is then recommended to add ice to the ultra-sonic bath in order to prevent heating of the peptide stock solution. Short ultra-sonication is also recommended at the end of the preparation process in order to degas the peptide stock solution.

Method

TEST SUBSTANCE EXPOSURE PROCEDURES

The assay is performed in a 96-well plate format. The plate layout is designed to test 3 test chemicals in parallel. For each reaction time point one plate is prepared, hence a routine assay will generally consist of 6 plates.

It is recommended that only one type of vehicle is used per run. When more than one vehicle is used per run it is recommended that each vehicle is processed on separate test plates. The positive control is always dissolved in acetonitrile.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	VC (Peptide + Vehicle)											
B	BC (Buffer + Vehicle)											
C	Substance A 0.31 mM + Peptide 0.5 mM			Substance B 0.31 mM + Peptide 0.5 mM			Substance C 0.31 mM + Peptide 0.5 mM			SC Subst. A - 0.31 mM + Buffer	SC Subst. B - 0.31 mM + Buffer	SC Subst. C - 0.31 mM + Buffer
D	Substance A 0.63 mM + Peptide 0.5 mM			Substance B 0.63 mM + Peptide 0.5 mM			Substance C 0.63 mM + Peptide 0.5 mM			SC Subst. A - 0.63 mM + Buffer	SC Subst. B - 0.63 mM + Buffer	SC Subst. C - 0.63 mM + Buffer
E	Substance A 1.25 mM + Peptide 0.5 mM			Substance B 1.25 mM + Peptide 0.5 mM			Substance C 1.25 mM + Peptide 0.5 mM			SC Subst. A - 1.25 mM + Buffer	SC Subst. B - 1.25 mM + Buffer	SC Subst. C - 1.25 mM + Buffer
F	Substance A 2.5 mM + Peptide 0.5 mM			Substance B 2.5 mM + Peptide 0.5 mM			Substance C 2.5 mM + Peptide 0.5 mM			SC Subst. A - 2.5 mM + Buffer	SC Subst. B - 2.5 mM + Buffer	SC Subst. C - 2.5 mM + Buffer
G	Substance A 5 mM + Peptide 0.5 mM			Substance B 5 mM + Peptide 0.5 mM			Substance C 5 mM + Peptide 0.5 mM			SC Subst. A - 5 mM + Buffer	SC Subst. B - 5 mM + Buffer	SC Subst. C - 5 mM + Buffer
H	0.31 mM PC + Peptide 0.5 mM	0.63 mM PC + Peptide 0.5 mM	1.25 mM PC + Peptide 0.5 mM	2.5 mM PC + Peptide 0.5 mM	5 mM PC + Peptide 0.5 mM			SC PC 0.31 mM + Buffer	SC PC 0.63 mM + Buffer	SC PC 1.25 mM + Buffer	SC PC 25 mM + Buffer	SC PC 5 mM + Buffer

Each test-chemical concentration is tested in triplicates. The plate contains further 12 samples of the vehicle control (VC), blank control (BC) as well as one sample per concentration of the positive control (PC) and one sample per concentration for each test chemical control (SC).
Serial dilutions of the test chemical and PC are prepared in 96-well plate referred to as the **application plate**. Further, a 96-well assay plate for each exposure time is prepared, referred to as the **assay plates**, by adding the relevant reagents (i.e., peptide stock solution, vehicle and buffer solution). The plate layout is identical for both application and assay plates.
Details on the preparation of application and assay plates are provided below.

Preparation of the application and assay plates

It is recommended to first prepare the assay plates. Then the test-chemical dilutions on the application plate should be prepared and immediately applied to the prepared assay plates in order to avoid evaporation of the solvent. A quick but precise working technique is required.

When the assay is performed by two technicians it is advisable that one technician prepares the peptide stock solution and the assay plates as described above, while the other technician prepares the application plate. The plates may be covered with the gas-tight foil until needed.

Preparation of the test-chemical dilutions (application plate)

Note: Do not prepare until ready to use or cover plate thoroughly!

The test-chemical and PC-dilutions are prepared by serial dilution in 96-well plates. Using a 2 mL deep-well plate, an **application plate** is prepared as follows (volume is sufficient to apply 6 plates):

	1	2	3	4	5	6	7	8	9	10	11	12
A	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle
B	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle	vehicle
C	1.25mM subst. A	1.25mM subst. A	1.25mM subst. A	1.25mM subst. B	1.25mM subst. B	1.25mM subst. B	1.25mM subst. C	1.25mM subst. C	1.25mM subst. C	1.25mM subst. A	1.25mM subst. B	1.25mM subst. C
D	2.5mM subst. A	2.5mM subst. A	2.5mM subst. A	2.5mM subst. B	2.5mM subst. B	2.5mM subst. B	2.5mM subst. C	2.5mM subst. C	2.5mM subst. C	2.5mM subst. A	2.5mM subst. B	2.5mM subst. C
E	5mM subst. A	5mM subst. A	5mM subst. A	5mM subst. B	5mM subst. B	5mM subst. B	5mM subst. C	5mM subst. C	5mM subst. C	5mM subst. A	5mM subst. B	5mM subst. C
F	10mM subst. A	10mM subst. A	10mM subst. A	10mM subst. B	10mM subst. B	10mM subst. B	10mM subst. C	10mM subst. C	10mM subst. C	10mM subst. A	10mM subst. B	10mM subst. C
G	20mM subst. A	20mM subst. A	20mM subst. A	20mM subst. B	20mM subst. B	20mM subst. B	20mM subst. C	20mM subst. C	20mM subst. C	20mM subst. A	20mM subst. B	20mM subst. C
H	1.25 mM PC	2.5 mM PC	5 mM PC	10 mM PC	20 mM PC			1.25 mM PC	2.5 mM PC	5 mM PC	10 mM PC	20 mM PC

- 300 µL of the respective vehicle is pipetted into each well of row A and B
- 300 µL of the respective vehicle is pipetted to each used well of rows C to F
- 600 µL of the respective 20 mM test chemical stock solution is pipetted to each used well of row G
- Serial dilution: Using a multi-channel pipette, 300 µL are pipetted from row G to row F, mixed thoroughly (e.g. using the P/M-function (pipetting + mix) of the Eppendorf Explorer pipette) and 300 µL are then pipetted from row F to row E, mixed thoroughly and so on until all concentrations are prepared.

  It is recommended that this procedure is followed for each test chemical separately, using 3 tips of the multi-channel pipette, in order to carefully observe thorough mixing and accurate pipetting without air bubbles. However, when feeling confident with the procedure, more than one or even all test chemicals could be pipetted simultaneously (a 12-channel pipette is required then).

- Prepare the PC dilution series in wells H1 to H5:

  add 600 µL of the vehicle to wells H1 – H4 and 1200 µL of the 20 mM stock preparation to H5. Transfer and mix 600 µL as described above.

  Transfer 300 µL of the readily prepared dilution series from wells H1 – H5 to wells H8 – H12 using a multi-channel pipette.

Note: Other ways of pipetting than described are acceptable if the quality criteria and time schedule of the assay are met for all steps.

Preparation of the assay plates

Using the black 96 - well plates for fluorescence measurements, the **assay plates** are prepared according to the following plate layout for each reaction time point (e.g. 6x). Each sample has a final total volume of 160 µL.

	1	2	3	4	5	6	7	8	9	10	11	12
A	VC (Peptide + Vehicle)											
B	BC (Buffer + Vehicle)											
C	Substance A 0.31 mM + Peptide 0.5 mM			Substance B 0.31 mM + Peptide 0.5 mM			Substance C 0.31 mM + Peptide 0.5 mM			SC Subst. A - 0.31 mM + Buffer	SC Subst. B - 0.31 mM + Buffer	SC Subst. C - 0.31 mM + Buffer
D	Substance A 0.63 mM + Peptide 0.5 mM			Substance B 0.63 mM + Peptide 0.5 mM			Substance C 0.63 mM + Peptide 0.5 mM			SC Subst. A - 0.63 mM + Buffer	SC Subst. B - 0.63 mM + Buffer	SC Subst. C - 0.63 mM + Buffer
E	Substance A 1.25 mM + Peptide 0.5 mM			Substance B 1.25 mM + Peptide 0.5 mM			Substance C 1.25 mM + Peptide 0.5 mM			SC Subst. A - 1.25 mM + Buffer	SC Subst. B - 1.25 mM + Buffer	SC Subst. C - 1.25 mM + Buffer
F	Substance A 2.5 mM + Peptide 0.5 mM			Substance B 2.5 mM + Peptide 0.5 mM			Substance C 2.5 mM + Peptide 0.5 mM			SC Subst. A - 2.5 mM + Buffer	SC Subst. B - 2.5 mM + Buffer	SC Subst. C - 2.5 mM + Buffer
G	Substance A 5 mM + Peptide 0.5 mM			Substance B 5 mM + Peptide 0.5 mM			Substance C 5 mM + Peptide 0.5 mM			SC Subst. A - 5 mM + Buffer	SC Subst. B - 5 mM + Buffer	SC Subst. C - 5 mM + Buffer
H	0.31 mM PC + Peptide 0.5 mM	0.63 mM PC + Peptide 0.5 mM	1.25 mM PC + Peptide 0.5 mM	2.5 mM PC + Peptide 0.5 mM	5 mM PC + Peptide 0.5 mM			SC PC 0.31 mM + Buffer	SC PC 0.63 mM + Buffer	SC PC 1.25 mM + Buffer	SC PC 25 mM + Buffer	SC PC 5 mM + Buffer

For better overview: The blue script refers to content of application plate, which is only added to the plate at application (not at time of plate preparation).

- **VC:** 120 µL of 0.667 mM peptide stock solution
  + 40 µL of the vehicle
- **BC:** 120 µL of phosphate buffer, pH=7.5
  + 40 µL of the vehicle
- **SC:** 120 µL of phosphate buffer, pH=7.5
  + 40 µL of the respective test-chemical/PC concentration
- **PC:** 120 µL of 0.667 mM peptide stock solution
  + 40 µL of the respective PC concentration
- **Test-chemical samples:** 120 µL of 0.667 mM peptide stock solution
  + 40 µL of the respective test-chemical concentration

The 6 assay plates are simultaneously prepared using a multi-channel pipette (dispense function):

- Pipette 120 µL of phosphate buffer (marked by dotted background in the plate layout)
  - to wells B1 - B12 (using 6 tips)
  - to wells C10 – G10 to C12 – G12 (using 5 tips)
  - to wells H8 – H12 (using 5 tips)
- Pipette 120 µL of the 0.667 mM peptide stock solution
  - to all wells of row A (using 6 tips)
  - to C1 – H1 to C5 – H5 of each test-chemical plate (using 6 tips)
  - to wells C6 – G6 to C9 – G9 of each test-chemical plate (using 5 tips)

Preparation of the application and assay plates: alternative procedure for phosphate buffer-soluble chemicals

Preparation of peptide stock solution:

The peptide stock solution is prepared in the same way as described above but as a 1 mM concentration:

Cysteine peptide Ac-RFAACAA-COOH, 1 mM = 0.752 mg/mL

Preparation of the test-chemical dilutions (Application plate)

In case phosphate buffer soluble chemicals are tested, the **application plate** is prepared as described above for row A, B and H, containing either ACN or the PC preparations in ACN.
In rows C – G the (water-soluble) chemicals can be added and diluted in phosphate buffer (same layout as for chemicals dissolved in ACN).

Preparation of assay plates

If phosphate buffer-soluble chemicals are tested, the **assay plates** are prepared as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	VC (Peptide + ACN)											
B	BC (Buffer + ACN)											
C	Substance A 0.31 mM + Peptide 0.5 mM			Substance B 0.31 mM + Peptide 0.5 mM			Substance C 0.31 mM + Peptide 0.5 mM			SC Subst. A - 0.31 mM + Buffer	SC Subst. B - 0.31 mM + Buffer	SC Subst. C - 0.31 mM + Buffer
D	Substance A 0.63 mM + Peptide 0.5 mM			Substance B 0.63 mM + Peptide 0.5 mM			Substance C 0.63 mM + Peptide 0.5 mM			SC Subst. A - 0.63 mM + Buffer	SC Subst. B - 0.63 mM + Buffer	SC Subst. C - 0.63 mM + Buffer
E	Substance A 1.25 mM + Peptide 0.5 mM			Substance B 1.25 mM + Peptide 0.5 mM			Substance C 1.25 mM + Peptide 0.5 mM			SC Subst. A - 1.25 mM + Buffer	SC Subst. B - 1.25 mM + Buffer	SC Subst. C - 1.25 mM + Buffer
F	Substance A 2.5 mM + Peptide 0.5 mM			Substance B 2.5 mM + Peptide 0.5 mM			Substance C 2.5 mM + Peptide 0.5 mM			SC Subst. A - 2.5 mM + Buffer	SC Subst. B - 2.5 mM + Buffer	SC Subst. C - 2.5 mM + Buffer
G	Substance A 5 mM + Peptide 0.5 mM			Substance B 5 mM + Peptide 0.5 mM			Substance C 5 mM + Peptide 0.5 mM			SC Subst. A - 5 mM + Buffer	SC Subst. B - 5 mM + Buffer	SC Subst. C - 5 mM + Buffer
H	0.31 mM PC + Peptide 0.5 mM	0.63 mM PC + Peptide 0.5 mM	1.25 mM PC + Peptide 0.5 mM	2.5 mM PC + Peptide 0.5 mM	5 mM PC + Peptide 0.5 mM			SC PC 0.31 mM + Buffer	SC PC 0.63 mM + Buffer	SC PC 1.25 mM + Buffer	SC PC 25 mM + Buffer	SC PC 5 mM + Buffer

For better overview: The blue script refers to content of application plate, which is only added to the plate at application (not at time of plate preparation).

- **VC:** 80 µL of 1 mM peptide stock solution + 40 µL phosphate buffer, pH=7.5 + 40 µL ACN
- **BC:** 80 µL of phosphate buffer, pH=7.5 + 40 µL phosphate buffer, pH=7.5 + 40 µL ACN
- **SC of PC:** 80 µL of phosphate buffer, pH=7.5 + 40 µL phosphate buffer, pH=7.5 + 40 µL of the PC concentration (dissolved/diluted in ACN)
- **SC of water-soluble test chemical:** 80 µL of phosphate buffer, pH=7.5 + 40 µL ACN + 40 µL of the respective test-chemical concentration
- **PC:** 80 µL of 1 mM peptide stock solution + 40 µL phosphate buffer, pH=7.5 + 40 µL of the PC concentration (dissolved in ACN)

- **Test-chemical samples:** 80 µL of 1 mM peptide stock solution + 40 µL ACN + 40 µL of the respective test-chemical concentration

- Pipette **80 µL of phosphate buffer** (marked by dotted background in the plate layout)
  - to wells B1 - B12 (using 6 tips)
  - to wells C10 – G10 to C12 – G12 (using 5 tips)
  - to wells H8 – H12 (using 5 tips)

- Pipette **80 µL of the 1mM** peptide stock solution
  - to all wells of row A (using 6 tips)
  - to C1 – H1 to C5 – H5 of each test-chemical plate (using 6 tips)
  - to wells C6 – G6 to C9 – G9 of each test-chemical plate (using 5 tips)

- Add **40 µL of ACN** to all wells C1 – G12
  (= wells in range C1 – G12 where chemicals dissolved in buffer are tested)

- Add **40 µL of phosphate buffer**, pH=7.5 to
  - wells A1 - A12
  - wells B1 - B12
  - wells H1 – H5
  - wells H8 – H12
    (= wells in range C1 – G12 where chemicals dissolved in ACN are tested)

- Pipette 40 µL of each well from the application plate to the respective well of the assay plate (plate layout is identical).

Note: Final level of acetonitrile in all wells is 40 µL, peptide concentration is 0.5 mM and phosphate buffer is 120 µL, identical to the test setup when only testing chemicals dissolved in acetonitrile.

ENDPOINT MEASUREMENT

The application is performed using a multi-channel pipette as follows:

- Pipette 40 µL of each well from the **application plate** to the respective well of the **assay plate** (plate layout is identical)

When the application is completed a timer is started (counting down the time interval of the first measurement) and the exact application time is recorded. All plates are covered with the gas-tight adhesive foil and shaken at min. 200 rpm for 5 minutes. Then the assay plates are placed into the incubator at 25 °C.

Incubation time = time interval from application of test chemical and PC dilutions to addition of mBrB.

Standard incubation times are as follows:

- 10 min ± 30 sec
- 30 min ± 3 min
- 90 min ± 5 min
- 150 min ± 10 min
- 210 min ± 10 min
- 1440 min ± 15 min

As the incubation times are relevant for calculation of the kinetic rate constants, it is crucial to meet the times given above. Any deviation from the given time spans should be recorded and the exact time should be used for calculation.

Incubation times may be adopted in order to investigate relevant time points for a given test chemical. For fast reacting test chemicals, it may be more suitable to test additional shorter incubation times and omit longer incubation times. However, 1440 min should always be tested as the highest test chemical concentration at this time point corresponds to cysteine-sample determined in the DPRA.

Fluorescence measurement

The 3 mM MBrB solution should be prepared fresh for at least each test day. When not in use it is stored in the dark in the refrigerator (only taken out for the time of use). The room should be darkened as much as reasonably possible. The mBrB solution is transferred to a tip tub.

At the end of the incubation time the adhesive foil is carefully removed from the test plate. 40 µL of 3 mM mBrB are added to each treated well of the plate using a multi-channel pipette.

Note: The mBrB solution should be added by rapid dispensing speed, which helps to immediately mix the solution well with the test sample. This helps to reduce data variability.

Carefully seal the plates again with the adhesive foil. Shake the plates at min. 200 rpm for 5 minutes. Measure fluorescence using an excitation filter of 390 nm and an emission filter of 480 nm.

ACCEPTANCE CRITERIA

Validity criteria for a test run:

- **PC**: the log k_{max} of the PC at 90 min should be within the following range: -1.75 $M^{-1}s^{-1}$ to -1.40 $M^{-1}s^{-1}$

  If no log k_{max} is obtained at 90 min, the value at 150 min can be taken into account and should lie in the following range: -1.90 $M^{-1}s^{-1}$ to -1.45 $M^{-1}s^{-1}$

- **VC**: The coefficient of variation of the 12 VC values of a plate should be <12.5% for 5 of the 6 time points.

Further acceptance criteria / special cases:

Historic control data should be established and the fluorescence readout of the VC and BC should be comparable to historic control data.

Furthermore, there may be special cases, where the automatic calculations of the evaluation template could be misleading. This can be due to

- Outliers / pipetting errors
- Intrinsic non-linear behavior of a test chemical
- Fluorescence interference
- High variability in control wells
- Highly reactive chemicals

These special cases should be separated by considering repetitions. How these cases are recognized, when a repetition is needed and which log k_{max} is finally reported is given in **Annex I - Special cases, outliers and non-linear reaction kinetics** which is available as Supplementary Material for download.

Data Analysis

Preparation of the Excel evaluation sheet

For each test run a separate Excel-evaluation spreadsheet is used. A data evaluation template is provided as Supplementary Material for download. A maximum of 3 test chemicals can be evaluated per test run/excel spreadsheet. When more than 3 test chemicals are run in parallel, several excel-spreadsheets may be prepared.

Worksheet "Run specifications":
Add "run identification", "date of test performance" and the required test-chemical/PC information (yellow marked areas).
The reaction time points (incubation times) may be adjusted here, if required.

The **threshold for positivity** is defined here, but should not be changed. A value of 13.89% (at the highest test chemical concentration tested, i.e. 5 mM final test chemical concentration) is used as this value is the threshold for positivity in the "cysteine 1:10 prediction model" of the DPRA.

Worksheet "Application plate":
No input required. May be printed for orientation of pipetting procedures in the lab.

Worksheet "Assay plate layout":
No input required. May be printed for orientation of pipetting procedures in the lab. Also contains calculations of the required amounts of reagents.

Worksheets "t1" to "t5" or "t6":
For each reaction time point a separate worksheet is available. Copy-paste the raw data from the fluorescence measurement into the respective cells at the appropriate time point. The following calculations are performed automatically:

1. Correction for unspecific fluorescence signal:
    - **BC**: The mean (and SD) of the 12 BC samples is calculated
    - **VC**: The mean (and SD) of the 12 VC samples is calculated and the mean BC value is subtracted (resulting in the corrected VC)
    - **Test chemical/PC samples**: for each sample the respective value of the SC is subtracted from the sample value (resulting in the corrected test chemical or PC value)
2. Calculation of relative peptide-depletion [%]:
    - For each sample of test chemical and PC:

$$\textbf{Relative peptide depletion}\ [\%] = \left(1 - \frac{\textbf{corrected sample value}}{\textbf{corrected mean VC}}\right) * 100\%$$

    - For the test chemical the mean and SD of the three replicates is calculated.
3. Statistics
    - Statistical evaluation is performed using the Excel-function „T.TEST" as follows:
      A pair-wise comparison of each concentration group (test chemical) with the vehicle control is performed using the Welch t-test (two-sided) for the hypothesis of equal means (Welch 1947).

Worksheet “summary”:
No input required.
On this sheet depletion values < 1% are set to “1%” and depletion values >99% are set to “99%”.

Worksheet “Evaluation (Substance X)”:
For each test chemical and for the PC a separate worksheet is available.
Test substance information as well as all mean corrected depletion values are transferred automatically.
The following actions/calculations are performed automatically:

- Depletion values are transferred from the summary page only, if the criteria for positivity is reached at the highest concentration and if statistically significant ($p < 0.05$): If this is the case for any reaction time, all values of this reaction time are transferred.
- If a value was transferred, the natural logarithm is taken from 100 minus mean corr. depletion value. If 95% depletion is present at a certain concentration of a time point, no calculations are performed for higher concentrations of that time point.
- From these, the slope and correlation over all concentrations is calculated for each time point.
  This slope corresponds to the observed reaction kinetic constant ($k_{observed}$, [mM^{-1}], pseudo-first order rate constants)
- From the $k_{observed}$ value of each time point, the reaction kinetic constant per M and second is calculated for each time point as follows:

$$\mathbf{k = k_{observed}*1000/(60*time\ point[min])\ (k\ [M^{-1}\ s^{-1}])}$$

- For each time point with a correlation >0.90 the decimal logarithm of k_t ($\log k_t$) is calculated.
- The $\log k_{max}$ and the respective time point are selected automatically.

Prediction Model

The kDPRA uses kinetic rates of peptide cysteine-depletion ($\log k_{max}$) to distinguish between two levels of skin sensitization potency, i.e. to differentiate UN GHS Cat 1A sensitizers from UN GHS Cat 1B/ not classified.

Reaction rate	**kDPRA Prediction**
nonreactive or $\log k_{max} < -2.0$	CLP/GHS sub-category 1B / no category Not categorised as UN GHS subcategory Cat1A (non-subcategory 1A)
$\log k_{max} \geq -2.0$	CLP/GHS sub-category 1A

Limitations

The kDPRA only measures reactivity with the Cys-peptide. This may limit applicability / predictivity as some sensitizers exclusively react with lysine residues, such as some acyl-halides, phenol-esters or aldehydes. However, only few Cat 1A sensitizers are known with this reactivity pattern (Natsch et al. 2020a).
Pre-haptens activated to strong haptens may also be underestimated in the kDPRA– the time needed for them to oxidize will reduce the apparent kinetic rate of the reaction with the test peptide. Thus, chemicals which are spontaneously, but not instantly, transformed to very reactive species may lead to some underestimation of the sensitization potential if the lag-period for oxidation is in the range of hours. In the future it may be tested whether a pre-incubation period prior to adding the peptide could correct for this limitation (Natsch et al. 2020a). In vitro investigations using compounds requiring molecular transformation to attain a sensitizing potential have shown that most pre-haptens can readily be detected in the DPRA (Patlewicz et al. 2016; Urbisch et al. 2016).

Moreover, many pro-haptens are also activated by non-enzymatic oxidation (and therefore are both pre- and pro-haptens). As discussed widely before, the DPRA does not contain a metabolic system (Urbisch et al. 2016) and thus prediction of strict pro-haptens requiring metabolic activation (i.e. not acting as direct haptens nor pre-haptens) in theory is a significant limitation for the (k)DPRA. For more discussion see also the validation report (Wareing et al. 2020) and Natsch et al. 2020a.

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