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The BlueScreen HC assay to predict the genotoxic potential of fragrance materials

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Abstract

BlueScreen HC is a mammalian cell-based assay for measuring the genotoxicity and cytotoxicity of chemical compounds and mixtures. The BlueScreen HC assay has been utilized at the Research Institute for Fragrance Materials in a safety assessment program as a screening tool to prioritize fragrance materials for higher-tier testing, as supporting evidence when using a read-across approach, and as evidence to adjust the threshold of toxicological concern. Predictive values for the BlueScreen HC assay were evaluated based on the ability of the assay to predict the outcome of *in vitro* and *in vivo* mutagenicity and chromosomal damage genotoxicity assays. A set of 371 fragrance materials was assessed in the BlueScreen HC assay along with existing or newly generated *in vitro* and *in vivo* genotoxicity data. Based on a weight-of-evidence approach, the majority of materials in the data set were deemed negative and concluded not to have the potential to be genotoxic, while only a small proportion of materials were determined to show genotoxic effects in these assays. Analysis of the data set showed a combination of high positive agreement but low negative agreement between BlueScreen HC results, *in vitro* regulatory genotoxicity assays, and higher-tier test results. The BlueScreen HC assay did not generate any false negatives, thereby providing robustness when utilizing it as a high-throughput screening tool to evaluate the large inventory of fragrance materials. From the perspective of protecting public health, it is desirable to have no or minimal false negatives, as a false-negative result may incorrectly indicate the lack of a genotoxicity hazard. However, the assay did have a high percentage of false-positive results, resulting in poor positive predictivity of the *in vitro* genotoxicity test battery outcome. Overall, the assay generated 100% negative predictivity and 3.9% positive predictivity. In addition to the data set of 371 fragrance materials, 30 natural complex substances were evaluated for BlueScreen HC, Ames, and *in vitro* micronucleus assay, and a good correlation in all three assays was observed. Overall, while a positive result may have to be further investigated, these findings suggest that the BlueScreen HC assay can be a valuable screening tool to detect the genotoxic potential of fragrance materials and mixtures.

Keywords: BlueScreen; genotoxicity; fragrance materials

Introduction

In vitro genotoxicity assays based on prokaryotic and eukaryotic systems have been of great importance in evaluating the genotoxic potential of chemicals. It is well established that a single regulatory approved assay is not sufficient to evaluate the genotoxic potential of a chemical. Identification of genotoxic compounds is required by regulatory schemes globally. For example, according to the European regulation for

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the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), the approach for genotoxicity testing of chemicals is a process based on production tonnage [1]: no testing is required for chemicals produced below 1 ton per year, and test requirements increase as production levels increase (e.g. only an Ames test for the 1–10 tonnage band through two *in vivo* genotoxicity, if indicated by unfavorable results in lower-tier testing).

Since the vast majority of fragrance materials are manufactured at extremely low production levels, in most cases only minimal data (e.g. an Ames test) is typically required to assess genotoxic potential. In assuring the safety of any chemical, the absence of genotoxic potential is of paramount importance; therefore, an adequate understanding of both mutagenicity and clastogenicity is needed [2, 3], which the Ames test alone cannot provide. The Research Institute for Fragrance Materials Inc. (RIFM) has developed a stepwise process for evaluating the toxicity of low production fragrance materials for all endpoints, including genotoxicity. RIFM's evaluation process also involves the use of *in silico* tools, read-across, and the threshold of toxicological concern (TTC). The TTC is an exposure-based safety assessment tool used to evaluate the safety of chemicals. For substances with exposures below an appropriate TTC value, the probability that they would cause adverse health effects is low. For substances considered to have the potential to be DNA-reactive mutagens and/or carcinogens based on the weight of evidence (WoE), the relevant TTC value is 0.0025 μg/kg body weight (bw) per day. As described in Api *et al.* (2015), the first step involves evaluating all available data on the material. Depending on the availability of sufficient data, the evaluation process is customized for that particular material. If no data are available for a given material, then the use of *in silico* and *in vitro* tools and a read-across approach to identify close analogs with adequate safety data are considered in the second step [4].

If there is insufficient data and no read-across analog that can support the safe use of the material, a credible estimate of consumer exposure is required so that the TTC decision tree can be utilized, as the third step in the evaluation process. RIFM exposure estimates are based on the total systemic 95th percentile consumer usage levels modeled in the RIFM-Creme exposure model [5]. As summarized in Table 1, the TTC for the genotoxicity endpoint can be utilized in a safety assessment on a fragrance material that does not have a structural alert, is negative in a genotoxicity screening assay, and has been identified as having a consumer exposure below a default threshold value of 1.5 μg/person/day. This value corresponds to the threshold of regulation derived by the US Food and Drug Administration [6–8], which has been derived to protect against all types of toxicity, including carcinogenicity. On the other hand, if a material exhibits a structural alert or has positive genotoxicity screening assay data, the material is assessed using the default TTC value for potentially genotoxic materials of 0.15 μg/person/day [9]. Dewhurst and Renwick considered that to move from 0.15 to 1.5 μg/day based on an absence of alerts for genotoxic carcinogenicity was adequate, but a greater degree of proof of no DNA reactivity was necessary before moving to the Cramer class tiers. Thus, when the estimated consumer exposure of a compound without adequate safety data or compelling read-across analogy to a material regarded as safe exceeds the respective TTC value of 0.15 μg/person/day, the generation of adequate safety data through testing is required as the final step in the evaluation process [10]. Various *in silico* tools can be used to identify structural alerts such as DEREK, MultiCASE, Oncologic, TOPKAT, TIMES, OECD toolbox etc.

Table 1. Genotoxicity TTC values used in fragrance safety assessment.

No.	BlueScreen HC result	*In silico* structural alert	TTC-based exposure limit
1	Positive	Yes[a]	0.15 μg/day
2	Negative	Yes	0.15 μg/day
3	Positive	No[b]	0.15 μg/day
4	Negative	No	1.5 μg/day

aIt was considered to be "Yes" if the prediction was certain, probable, plausible, equivocal, or doubted in at least one of the endpoint results, such as "carcinogenicity," "chromosomal damage," or "mutagenicity *in vitro*."
bIt was considered to be "No" if the prediction was either impossible or improbable for all of the endpoint results, such as "carcinogenicity," "chromosomal damage," and "mutagenicity *in vitro*."

When RIFM's safety assessment process was implemented, less than 50% materials had sufficient genotoxicity data to complete an endpoint safety evaluation. The BlueScreen HC has previously been shown to be a useful tool to prioritize fragrance and flavor materials, and it can be used to fill data gaps [11]. Therefore, the BlueScreen HC was selected as a high-throughput screening (HTS) assay that could support the safety evaluation process for fragrance materials. The BlueScreen HC screening assay uses a patented *Gaussia* luciferase (GLuc) reporter system that exploits the regulation of the *GADD45a* gene, reflecting the adaptive response to genotoxic stress. This reporter system is incorporated into a genetically modified strain of cultured human lymphoblastoid TK6 cells [12–17]. Exposure to a genotoxic compound increases the expression of GLuc, which is quantified by the detection of luminescence generated from the reaction of GLuc with a coelenterazine substrate that is added to the microplate wells just before measurement [16]. BlueScreen HC is conducted both with and without metabolic activation, so it is also considered to respond to metabolites that may be responsible for causing genotoxicity.

Results from the assay can also play a key role in strengthening the confidence in using a particular chemical analog for read-across for fragrance materials. If the read-across structural analog and the target chemical respond similarly in the BlueScreen HC assay, it supports the appropriateness of the selected read-across analog. Using this approach integrates chemical and biological information to support selecting an appropriate read-across material [18]. An example of this approach is shown in the published fragrance safety assessment for 1,1-diethoxyheptane, (CAS # 688-82-4) [19]. Both target chemical and read-across chemical used to complete data gap for 1,1-diethoxyheptane produced negative results in the BlueScreen HC assay. In the RIFM evaluation process, BlueScreen HC results are also used to enable prioritization with respect to extended testing of the fragrance material inventory. Extended testing of fragrance materials includes assessing genotoxic potential using a two-test battery approach, as suggested by Pfuhler et al. [20]. This includes a test for bacterial mutagenicity (e.g. the Ames test) and one for chromosomal damage and aneugenicity potential (e.g. the micronucleus test), and this battery is considered to fully

address the genotoxicity potential of fragrance materials, although additional tests may be considered when necessary/available. Fragrance materials with negative BlueScreen HC results are primarily considered to be a lower priority, requiring case-by-case evaluation for higher-tier testing. Materials that tested positive in the BlueScreen HC assay are given a higher priority for higher-tier testing. The current analysis was conducted to further strengthen the abovementioned uses and to establish the predictivity of the BlueScreen HC assay in the context of a standard genotoxicity testing battery.

Materials and methods

Selection of materials

Approximately 2800 fragrance materials make up the RIFM inventory. A total of 1419 fragrance materials were tested in the BlueScreen HC assay, and 371 of these were considered in this analysis. The selection for analysis was based on the availability of higher-tier test data, including both the bacterial reverse mutation test and the *in vitro* mammalian cell micronucleus test, conducted in compliance with GLP regulations and in accordance with OECD guidelines (OECD 471 and OECD 487, respectively). Subsequently, 26 of the 371 materials were also assessed in the *in vivo* micronucleus test (OECD 474) and/or a 3D skin-based micronucleus assay. Most of the samples were supplied by fragrance manufacturers that are members of RIFM in a quality representative of what is sold in the global fragrance market. However, some materials were also purchased from external suppliers.

BlueScreen HC assay

The BlueScreen HC was conducted according to manufacturer instructions (Gentronix Ltd). A dilution series of eight concentrations for each test material was generated in black 96-well microplates with an optically clear base. Up to four compounds were tested per microplate, and a known genotoxic compound (4-nitroquinoline 1-oxide; 4-NQO) was included on each microplate as a positive control. Each dilution of the test material was tested on human lymphoblastoid TK6 cells. The microplates were covered with a breathable membrane (Breathe-Easy; Diversified Biotech, Boston, MA, USA) and incubated at 37°C with 5% CO_2 and 95% humidity for 48 h. The microplates were analyzed using a microplate reader [Tecan Infinite F500 plate reader (Tecan UK Ltd, Reading, UK)], which provides measurements of fluorescence and flash luminescence for cells and solutions in each microplate well. Following a recent protocol enhancement, cytotoxicity was measured by lysis of the cells with 10 ml of 4% v/v Triton X solution in D-PBS and the addition of a fluorescent DNA binding stain (thiazole orange), followed by an assessment of the resulting fluorescence [16]. This technique for the estimation of relative cell density is a replacement of the previous optical absorbance measure [16]. Fluorescence is proportional to cell proliferation, which would be lowered by toxic analytes and luminescence, as a measure of the GADD45a gene expression, the intensity of luminescence is proportional to the activity of the cell's DNA repair system, which is triggered after genotoxic damage. Luminescence was normalized to the fluorescence signal to correct for variation in cell number caused by cytotoxicity. Raw luminescence and fluorescence data collected from the assay plates were automatically saved to an MS Excel template. This template automatically analyzed the data, giving a semiquantitative assessment of cytotoxicity and genotoxicity, and summarized it in both tabulated and graphical form. The overall assay outcome presented for a compound is the average of the duplicate test series performed for the compound. All the BlueScreen tests discussed in this manuscript were conducted at a contract research organization, Gentronix LLC (UK).

Further evaluation by regulatory approved assays

All the fragrance materials were also assessed for mutagenicity and the induction of chromosomal damage to ascertain full coverage of genotoxicity potential.

Mutagenicity assays

All 371 materials in the data set were evaluated for their mutagenic potential in a standard bacterial mutagenicity assay (Ames, OECD 471). A subset of materials was also evaluated in a human cell line-based mutagenicity assay, such as the mouse lymphoma assay (MLA)/hypoxanthine phosphoribosyltransferase (HPRT) assay (OECD 476), *in vivo* genotoxicity testing such as the comet assay (OECD 489), or a skin tissue-based assay, such as the 3D skin comet assay. These assays can be used in a WoE approach to conclude on the genotoxic potential of a material. Standard assays were conducted in compliance with OECD test guidelines, where available. The other assays were performed following accepted protocols [21].

The Phenion Full-Thickness Skin Model provided by Henkel (Germany) was used for the 3D skin comet assay tests. The similarity in histological and physiological parameters of the model with human skin makes the model well suited for genotoxicity testing. This 3D skin model has been successfully validated and was shown to detect the DNA damage of test substances acting via different mechanisms [22, 23]. The assay design was partially based on the standards recommended by international expert groups for *in vitro* and *in vivo* comet procedures for single-cell preparation and analysis of the nuclei [24, 25]. The SCCS has recommended using both the RS comet and RSMN assays as a follow-up for suspected misleading positive results from the standard *in vitro* test battery, based on the outcome on the case studies for three hair dyes [26]. Additionally, the *in vivo* comet assay has been shown to efficiently detect *in vivo* and *in vitro* mutagens [27], and hence it is considered an appropriate for follow-up testing of mutagenic substances in the *in vitro* battery.

Chromosomal damage assays

All 371 materials in the data set were evaluated for their potential to induce chromosomal damage by utilizing a standard *in vitro* chromosomal aberration assay (OECD 473), *in vitro* micronucleus assay (OECD 487), *in vivo* micronucleus assay (OECD 474), or skin tissue-based assays such as the RSMN assay. All assays were conducted following OECD test guidelines except the RSMN assay which followed accepted protocols [28, 29].

The RSMN assay uses reconstructed skin (EpiDerm™, MatTek Corporation, Ashland, MA, USA) and is especially relevant for chemicals for which human dermal exposure is expected [22, 28–34]. The EpiDerm model is a multilayered, differentiated tissue consisting of basal, spinous, granular, and cornified layers resembling the normal human epidermis [28].

This system has been successfully validated and demonstrated to be sensitive to the genotoxic activity of a large variety of chemicals [35].

Data analysis

In order to evaluate the predictive power of the BlueScreen HC, predictivity and agreement of results were calculated by comparing to the results from *in vitro* and *in vivo* mutagenicity as well as chromosomal damage studies. Both positive and negative predictivity and agreement results were considered for completeness of the data analysis for prospective and retrospective analysis.

The following equations were used to evaluate the comparisons:

$$\text{Positive agreement} = \frac{\text{TruePositive}}{\text{True Positive} + \text{False Negative}}$$

$$\text{Negative agreement} = \frac{\text{True Negative}}{\text{True Negative} + \text{False Positive}}$$

$$\text{Predictivity (Positive)} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Positive}}$$

$$\text{Predictivity (Negative)} = \frac{\text{True Negative}}{\text{True Negaive} + \text{False Negative}}$$

Definitions

1. True Positive: Materials positive in the BlueScreen HC assay concluded to be positive according to results from regulatory approved assays as well as other assays considered in the WoE
2. False Negative: Materials negative in the BlueScreen HC assay but concluded to be positive according to results from regulatory approved assays
3. True negative: Materials negative in the BlueScreen HC concluded to be negative according to results from regulatory approved assays
4. False Positive: Materials positive in the BlueScreen HC but concluded to be negative according to results from regulatory approved assays

In accordance with regulatory practice, the outcome from guideline-compliant *in vivo* studies, if available, superseded the *in vitro* findings in defining these positives and negatives.

Results

Availability of genotoxicity data for the fragrance materials evaluated

Considering the 1419 fragrance materials tested in BlueScreen HC, 371 have genotoxicity testing data. Of these, 371 materials (100%) have Ames data, 371 (100%) have *in vitro* micronucleus data, 18 (4.85%) have *in vitro* chromosome aberration data, 29 (7%) have MLA or HPRT assay data, 4 (1.1%) have *in vitro* 3D skin comet data, 13 (3.5%) have *in vitro* 3D skin MNT data, and 28 (7.5%) have *in vivo* genotoxicity data.

Predictivity

Predictivity was determined by evaluating BlueScreen HC data and comparing it to standard assays as described earlier. Positive predictive value is the probability that a positive BlueScreen HC result truly is positive, and negative predictive value is the probability that a negative BlueScreen HC result truly is negative. Positive/negative predictive value provides a measure of the confidence that one can have in a positive or negative result being an accurate predictor of the genotoxicity or lack thereof of the test material.

Positive predictivity

A total of 371 fragrance materials were evaluated in this analysis, and as shown in Table 2, 77 (20.8%) generated positive results in the BlueScreen HC. When considering the additional *in vitro* data for these 77 fragrance materials, 9 (11.7%) have positive results in the Ames test, and 18 (23.3%) have positive results in the *in vitro* micronucleus test. Furthermore, 2 out of 7 (28.6 %) were also positive in the *in vitro* mammalian cell mutagenicity study, and 7 out of 17 (41.1 %) were also positive in the *in vitro* chromosomal aberration study. In totality, when considering all available *in vitro* genotoxicity data, 19 (24.6%) of the 77 materials with positive BlueScreen HC results also generated positive results in one or more *in vitro* study. Additionally, 14/77 materials were also tested *in vivo* (including *in vivo* comet assay, dominant lethal assay, *in vivo* micronucleus assay, and *in vivo* chromosomal aberration studies). Only 1 (7.14%) out of 14 were positive *in vivo*. When considering all available *in vitro* and *in vivo* data, 3 of 77 materials were concluded to be positive for genotoxicity (conclusion considering the WoE of data available), equivalent to an overall positive predictive value of 3.9% for the whole test battery.

Negative predictivity

Of the 371 fragrance materials evaluated in this analysis, 294 (79.2%) generated negative results in the BlueScreen HC. When considering the additional available *in vitro* data for these 294 fragrance materials, 292 (99.3%) and 289 (98.2%) produced negative results in the Ames test and *in vitro* micronucleus test, respectively. Additionally, 19 out of 22 (86.3 %) were negative in the *in vitro* mammalian cell mutagenicity study, and 8 out of 11 (72.2 %) were negative

Table 2. Positive predictivity.

	Number of positives/ BlueScreen HC positive	Positive predictivity
Ames	9/77	11.7%
In vitro MNT	18/77	23.3%
In vitro mammalian cell mutagenicity	2/7	28.6%
In vitro chromosomal aberration	7/17	41.1%
In vitro genotoxicity battery	19/77	24.6%
In vivo	1/14	7.14%
Final genotoxic potential conclusion[a]	3/77	3.9%

aFinal conclusion is based on currently available genotoxicity test battery from regulatory approved assays and WoE/expert judgment.

in the *in vitro* chromosomal aberration study. When considering all available *in vitro* genotoxicity data, 286 (97.3%) of the 294 materials with negative BlueScreen HC results also generated negative results in one or more *in vitro* studies. Out of 294 materials, *in vivo* studies were also available on 19 of them (which includes *in vivo* comet assay, dominant lethal assay, *in vivo* micronucleus assay, as well as *in vivo* chromosomal aberration study; not conducted as a part of this project), and 19 (100%) out of 19 were negative in these *in vivo* studies. When considering the WoE of all available *in vitro* and *in vivo* data for both mutagenicity and clastogenicity, 294 of 294 materials were concluded to be negative for genotoxicity based on a battery of assays giving a negative predictive value of 100% for BlueScreen HC (Table 3).

Agreement

Agreement was determined by evaluating available data for standard assays as described earlier and comparing it to BlueScreen HC data. The positive agreement value is the probability that a material that is truly positive will also result in positive in the BlueScreen HC test. The negative agreement value is the probability that a material that is truly negative will also result in negative in the BlueScreen HC test.

Positive agreement

Of the 371 fragrance materials evaluated in this analysis, three materials (0.8%) were concluded to be positive based on the results from regulatory approved assays and WoE/expert judgment. As shown in Table 4, in a retrospective analysis of the *in vitro* data for these 371 fragrance materials, 7 out of 9 (77.8%) materials that were positive in Ames were also positive in BlueScreen HC, and 13 out of 18 (72.2%) materials that were positive in the *in vitro* MNT were also positive in BlueScreen HC. Additionally, 1 out of 4 (25 %) materials that was positive in an *in vitro* mammalian cell mutagenicity assay was also positive in BlueScreen HC, and 4 out of 7 (57.1 %) materials that were positive in the *in vitro* chromosomal aberration assay were also positive in BlueScreen HC. A total of 16 materials were determined to have genotoxic potential when considering all available *in vitro* genotoxicity studies (including bacterial and mammalian cell mutagenicity assay, *in vitro* micronucleus assay, and *in vitro* chromosomal assay), and 8 out of 16 (50 %) were also positive in BlueScreen HC. Three materials that were also tested in *in vivo* studies (including *in vivo* comet assay, dominant lethal assay, *in vivo* micronucleus, as well as *in vivo* chromosomal aberration assay) were concluded to be positive for genotoxicity, and all also concluded to be positive in BlueScreen HC, giving a positive agreement value of 100%.

Negative agreement

Of the 371 fragrance materials evaluated in this analysis, 368 materials (99.2%) were concluded to be negative based on the results from regulatory approved assays and WoE assessment on the material. As shown in Table 5, a retrospective analysis of the *in vitro* data for these 371 fragrance materials resulted in 292 out of 362 (80.7%) materials negative in Ames that were also negative in BlueScreen HC and 289 out of 353 (81.9%) materials negative in *in vitro* MNT that were also negative in BlueScreen HC. Additionally, 20 out of 25 (80%) materials that were negative in an *in vitro* mammalian cell mutagenicity assay were also negative in BlueScreen HC, and 7 out of 10 (70%) materials that were negative in the *in vitro* chromosomal aberration assay were also negative in BlueScreen HC. When considering all available *in vitro* genotoxicity data, 286 out of 352 (81.3%) materials that were negative in all the *in vitro* genotoxicity studies were also

Table 3. Negative predictivity.

	Number of negatives/ BlueScreen HC negative	Negative predictivity
Ames	292/294	99.3%
In vitro MNT	289/294	98.2%
In vitro mammalian cell mutagenicity	19/22	86.3%
In vitro chromosomal aberration	8/11	72.7%
In vitro genotoxicity battery	286/294	97.3%
In vivo	19/19	100%
Final genotoxic potential conclusion	294/294	100%

Table 4. Positive agreement.

	BlueScreen HC positive/number of positives	Positive agreement
Ames	7/9	77.8%
In vitro MNT	13/18	72.2%
In vitro mammalian cell mutagenicity	1/4	25%
In vitro chromosomal aberration	4/7	57.1%
In vitro genotoxicity battery	8/16	50%
Final genotoxic potential conclusion based on *in vivo* studies	3/3	100%

Table 5. Negative agreement.

	BlueScreen HC negative/number of negatives	Negative agreement
Ames	292/362	80.7%
In vitro MNT	289/353	81.9%
In vitro mammalian cell mutagenicity	20/25	80%
In vitro chromosomal aberration	7/10	70%
In vitro genotoxicity battery	286/352	81.3%
Final genotoxic potential conclusion	294/368	79.9%

negative in BlueScreen HC. Furthermore, 294 out of 368 materials concluded to be negative in various *in vitro* and *in vivo* studies for genotoxicity were also concluded to be negative in BlueScreen HC, giving a negative agreement value of 79.9%.

Discussion

A total of 1419 fragrance materials were tested in the BlueScreen HC as part of a testing prioritization process. Results from the BlueScreen HC testing enabled RIFM to prioritize fragrance materials for higher-tier mutagenicity and clastogenicity testing in order to conclude on the genotoxic potential of each fragrance material. A total of 371 materials were selected for the current analysis, based on the availability of data from a complete genotoxicity test battery consisting of regulatory approved assays for mutagenicity and clastogenicity. It should be noted that the majority of materials in the data set were deemed negative based on a WoE approach, and only a small proportion was deemed true positive on the basis of *in vivo* data. Based on the analysis of the data set, overall positive predictivity was determined to be 3.9%, whereas negative predictivity was 100%. For agreement analysis, the positive agreement was determined to be 100%, while negative agreement was 79.9%. One of the limitations of the current research is the availability of materials with positive results, to calculate agreement analysis. From the 371 fragrance materials only 19 generated positive results in the *in vitro* genotoxicity battery and only 3 of these showed positive results in the full genotoxicity potential conclusion, based on *in vivo* studies. Overall, the results showed a combination of high positive agreement and negative predictivity. However, considering that only a limited number of materials were available which showed positive results for the final genotoxic potential determination, additional materials showing positive results would be ideal for a more robust analysis of the positive agreement. Since the BlueScreen HC assay did not generate any false-negative results, its inclusion in the RIFM assessment paradigm strengthens the clearance of a material as nongenotoxic and provides a robust, HTS method to evaluate the large inventory of fragrance materials. From the perspective of protecting public health, it is desirable to have no or minimal false negatives, as a false-negative result may incorrectly indicate a lack of hazard. However, the assay did have a high percentage of false-positive results, resulting in poor positive predictivity of *in vitro* genotoxicity test battery outcome. In this analysis, a WoE approach was applied when concluding on the overall genotoxic potential for each material. There are several examples where a fragrance material produced a negative result in the BlueScreen HC but generated a positive result in higher-tiered or regulatory approved assay. This is not unexpected and is the case with the entire battery of *in vitro* genotoxicity assays. Detailed examination and expert assessment of all available data, taking into account the quality of the study, the robustness of the protocol and quality of the test material, and historical control data, allowed decisions to be made on the validity of the data. As such, some of the supposed guideline-compliant *in vitro* studies were determined to have little or no biological relevance, and therefore the results were considered to not impact the overall conclusion on a material's true genotoxic potential. These WoE determinations affected predictive and agreement analysis in the current evaluation, as discussed below.

Bacterial mutagenicity

Two materials were positive in the Ames assay but negative in BlueScreen HC (anisyl formate, CAS: 122-91-8; and 1-(3,5,6-trimethyl-3-cyclohexen-1-yl)ethan-1-one, CAS: 68480-14-8). Anisyl formate was found positive only in *Salmonella typhimurium* strain TA 100 without metabolic activation, but it did not induce a higher number of revertant colonies when tested in TA 100 in the presence of metabolic activation and when tested in the other four bacterial tester strains either with or without metabolic activation. The material was negative in a mammalian cell line mutagenicity study (MLA/HPRT) [36] and also negative in an *in vitro* micronucleus assay [37]. The isolated positive Ames result was determined to be biologically nonrelevant for various reasons. Anisyl formate may produce a false-positive response in the Ames assay as a result of the formation of oxidative free radicals or weak electrophiles [38]. Enzymatic ester hydrolysis of anisyl formate yields anisyl alcohol and formic acid, which both have negative results in Ames mutagenicity studies [39, 40]. In conclusion, while anisyl formate generated an isolated positive result in an assay without metabolic activation, it did not exert genotoxic effects in metabolically competent mammalian cells. At a European Centre for the Validation of Alternative Methods (ECVAM) workshop, it was concluded that a single Ames positive result together with at least two negative responses in *in vitro* mammalian genotoxicity tests, covering two different endpoints, is unlikely to be genotoxic *in vivo* [41, 42]. This conclusion is applicable to anisyl formate, given the negative Ames test on its hydrolysis products and the negative results in the HPRT and *in vitro* micronucleus assays. Taken together, the WoE for anisyl formate indicates that it does not have genotoxic potential *in vivo*.

In the Ames assay, 1-(3,5,6-trimethyl-3-cyclohexen-1-yl) ethan-1-one generated positive results only in *Escherichia coli* strain WP2uvrA without metabolic activation; however, this result has questionable biological relevance. While dose-dependent increases of >2-fold were observed in both the initial as well as the confirmatory studies in the same experiment, no positive effects were observed in a third confirmatory study conducted using similar concentrations in two strains, *E. coli* WP2uvrA or TA102, which are both included in the testing protocol to identify certain oxidizing mutagens or cross-linking agents and hydrazines, and both have an AT base pair at the primary reversion site [43]. Additionally, data on many other unsaturated cyclohexyl ketones are also negative in all the strains tested in the Ames assay [44]. The positive outcome in the initial Ames study on 1-(3,5,6-trimethyl-3-cyclohexen-1-yl)ethan-1-one may be due to keto-enol tautomerism and a potential propensity of the enolic hydroxyl group to participate in reactive oxygen species (ROS) radical formation, in conjunction with insufficient phase II detoxification in the *in vitro* testing conditions [45]. However, in the *in vivo* conditions, the compound may undergo cytochrome P450 oxidation to form an epoxide that may be hydrolyzed, and the resultant dihydrodiol may be conjugated and excreted [46, 47]. Alternatively, carbonyl reduction may occur, and the cyclic alcohol may be conjugated and excreted. Since the material tested negative in a further confirmatory Ames test, in a mammalian cell line mutagenicity study (HPRT), and in an *in vitro* micronucleus assay [48, 49], the weak initial Ames positive result is

considered of no biological relevance, and this material is concluded unlikely to have genotoxic potential *in vivo*.

In viro mammalian cell gene mutation test (HPRT)/ MLA

Isobutyric acid (CAS: 79-31-2) was positive in the L5178 TK +/– cell MLA only in the presence of metabolic activation at the time performed [50]. This positive response would not be considered to be a relevant positive result according to the current OECD guideline because the effect was observed at cytotoxic concentrations [51] only. Additionally, isobutyric acid was confirmed to be negative in an Ames test and in an *in vitro* MNT with and without metabolic activation [51]. A negative *in vivo* micronucleus test result on isobutyl alcohol also adds to the WoE, discounting the effects observed in the mouse lymphoma study only at does exceeding toxicity limits [51].

In vitro mammalian cell micronucleus test (MNT)

In current dataset, five materials were positive in the *in vitro* MNT and negative in BlueScreen HC: 4-thujanol (CAS: 546-79-2), isobornyl methyl ether (CAS:5331-32-8), 5-methylquinoxaline (CAS: 13708-12-8), 3-methyl-5-phenylpent-2-enenitrile (CAS: 93893-89-1), and 1,5-dimethylbicyclo[3.2.1]octan-8-one-oxime (CAS: 75147-23-8).

4-Thujanol was positive in the 4-h time point *in vitro* micronucleus study in the absence of metabolic activation [52]. Since 4-thujanol was negative in an *in vivo* micronucleus study, the positive *in vitro* finding may be attributed to the lack of competing conjugation metabolism leading to efficient detoxification of the substance by phase II enzymes [53]. *In vivo*, 4-thujanol is expected to be eliminated after glucuronidation [38, 54]. Additional WoE for the lack of a genotoxic potential of 4-thujanol is provided by a negative 3D skin micronucleus assay [53].

Isobornyl methyl ether was positive in an *in vitro* micronucleus study in the presence of metabolic activation at the 4-h time point [55]. A confirmatory assay resulted in increases only at the highest dose level, but a dose response was not observed [55]. *In vivo*, isobornyl methyl ether is expected to be eliminated after glucuronidation, a key detoxifying step absent *in vitro* [54] (FEMA GRAS assessment). Furthermore, the increases observed in the first *in vitro* micronucleus study were within the historical control range of the test lab, albeit outside the 95% percentile confidence level. Hence, the increases observed in the first test are considered to be of little biological relevance. Additional WoE for the lack of genotoxic potential of isobornyl methyl ether is provided by a negative 3D skin micronucleus study [56], and the 3D skin models utilized have been shown to be capable of glucuronidation [57].

5-Methylquinoxaline was found to be positive without metabolic activation in the 24-h treatment of the *in vitro* micronucleus study, but negative in the 4-h treatment [58]. It has been proposed that glutathione depletion in the 24-h treatment may be responsible for the apparent positive result [38]. Additionally, in contrast to the *in vitro* positive response, two *in vivo* micronucleus assays were negative [59]. This further supports the WoE evaluation that 5-methylquinoxaline can be considered devoid of genotoxic activity *in vivo*.

Positive results were also produced in *in vitro* micronucleus assays by 3-methyl-5-phenylpent-2-enenitrile at the 4-h time point with metabolic activation and 1,5-dimethylbicyclo[3.2.1]octan-8-one-oxime at both 3- and 24- h time points with and without metabolic activation. Both these materials also tested negative in *in vivo* micronucleus tests [60–63]. Again, the WoE supports the conclusion that these fragrance materials are not genotoxic.

Chromosomal aberration

Four materials, α-ionone, methyl acetoacetate, cinnamyl nitrile, and methyl anthranilate, all generated positive results in chromosomal aberration assays in either Chinese hamster lung, ovary, or fibroblast cells, as shown in Table 6 but were negative in the BlueScreen HC assay. It is known that all of the above cell lines have a compromised p53 gene and are p53-deficient, which may lead to false-positive outcomes [64, 65]. Accordingly, the four materials tested negative in p53-proficient cell lines, such as human lymphocytes or the TK6 cell line (also used in BlueScreen HC). For example, α-ionone was negative in an *in vitro* micronucleus study conducted in human peripheral lymphocytes and also in an *in vivo* mouse micronucleus study [66]. Both methyl acetoacetate and cinnamyl nitrile also showed negative *in vitro* micronucleus results in human lymphocytes [67] and Chinese hamster V79 cells [68], respectively. Methyl anthranilate was positive in an *in vitro* chromosomal aberration study using a Chinese hamster fibroblast cell line (B 241); however, a 2-year *in vivo* carcinogenicity study in mice did not show any carcinogenic effects of methyl anthranilate [69]. These findings support the WoE conclusion that these materials lack genotoxic potential *in vivo*.

The BlueScreen HC may be considered a conservative screening assay, in view of the fact that a high false-positive rate but no false negatives were identified in our data set. Only false negatives have been discussed here considering the fact that the observed false positives in the BlueScreen HC assay, in connection with compellingly negative results in guideline-compliant and other *in vitro* studies, may be reconciled with various causative factors. Amongst those may be physicochemical parameters like pH value, osmolarity, solubility, and/or ionic imbalance, but also effects like enzyme inhibition, imbalance of DNA precursors, energy depletion, production of active oxygen species, lipid peroxidation, sulfhydryl depletion, nuclease release from lysosomes, inhibition of protein synthesis, protein denaturation, or other unknown mechanisms as described in Kirkland et al. [38] In general, such factors are of relevance for all *in vitro* mammalian cell line studies, including the BlueScreen HC assay.

The overall outcome of the BlueScreen HC assay, however, is reassuring, indicating that no true genotoxic materials were missed by screening the RIFM material library with this HTS.

Table 6. Fragrance materials positive in *in vitro* chromosomal aberration study and negative in BlueScreen HC assay.

CAS#	Name	Positive test conditions
127-41-3	α-Ionone	CHL cells (+/– S9)
105-45-3	Methyl acetoacetate	CHL cells (+ S9)
1885-38-7	Cinnamyl nitrile	V79 cells (+/– S9)
134-20-3	Methyl anthranilate	Chinese hamster fibroblast cells (+/– S9)

Table 7. Summary of genotoxicity data set for natural extracts.

Principal NCS name	CAS Number	BlueScreen HC	Bacterial reverse mutation test result (OECD 471)	*In vitro* micronucleus test result (OECD 487)
Lavender Oil	8000-28-0	Negative	Negative	Negative
Citronella Oil Java Type	8000-29-1	Negative	Negative	Negative
Rosemary Oil	8000-25-7	Negative	Negative	Negative
Eucalyptus Oil Citriodora	85203-56-1	Negative	Negative	Negative
Fir Needle Oil Siberian	8021-29-2	Negative	Negative	Negative
Geranium Oil African	8000-46-2	Negative	Negative	Negative
Petitgrain Oil Paraguay	8014-17-3	Negative	Negative	Negative
Rose Oil Bulgarian	8007-01-0	Negative	Negative	Negative
Amyris Oil	8015-65-4	Negative	Negative	Negative
Cananga Oil	68606-83-7	Negative	Negative	Negative
Guaiacwood Oil	8016-23-7	Negative	Negative	Negative
Star Anise Oil	68952-43-2	Negative	Negative	Negative
Clary Sage Oil	8016-63-5	Negative	Negative	Negative
Bay Oil W.I.	8006-78-8	Negative	Negative	Negative
Wormwood Oil American	8008-93-3	Negative	Negative	Negative
Bois de Rose Oil (Rosewood)	8015-77-8	Negative	Negative	Negative
Cabreuva Oil	68188-03-4	Negative	Negative	Negative
Eucalyptus Dives Oil	8000-48-4; 90028-48-1	Negative	Negative	Negative
Buchu Crenulata Leaf Oil	92346-85-5	Negative	Negative	Negative
Celery Seed Oil Indian	8015-90-5	Negative	Negative	Negative
Sandalwood Oil Australian Type (*Santalum spicata*)	8024-35-9	Negative	Negative	Negative
Sandalwood Oil E.I. Type (*Santalum album*)	8006-87-9	Negative	Negative	Negative
Chamomile Oil Blue Egyptian (*Matricaria chamomilla*)	8002-66-2	Negative	Negative	Negative
Chamomile Oil Roman (*Anthemis nobilis*, syn. *Chamaemelum nobile*) (English chemotype)	8015-92-7	Negative	Negative	Negative
Ylang Oil I	8006-81-3; 83683-30-3	Negative	Negative	Negative
Ylang Oil III	8006-81-3; 83683-30-3	Negative	Negative	Negative
Buchu Oil Crenulata	92346-85-5	Negative	Negative	Negative
Chamomile Oil Roman Italian	8015-92-7	Negative	Negative	Negative
Coriander Herb Oil	8008-52-4	Negative	Negative	Negative
Pimento Leaf Oil	8006-77-7	Negative	Negative	Negative

According to this data set, the BlueScreen HC assay has a high sensitivity (100%) for identifying a true genotoxic material in the fragrance material domain, as confirmed by guideline-compliant, regulatory approved assays. Following expert judgment analysis, all three materials that were concluded to have true positive results in *in vitro* and *in vivo* testing systems were also positive in the BlueScreen HC assay. In contrast, for identifying nongenotoxic fragrance materials, the BlueScreen HC assay is considered to have only a modest specificity (79.9%), indicating that the assay is good at identifying materials that actually have genotoxic potential but may be considered conservative in that it also has a high rate of false positives. It is acknowledged that the total number of true genotoxic agents in the data set was low and that the data set was imbalanced in terms of true BlueScreen positives, which were underrepresented. Both factors may have had an impact on the overall numbers but were a result of the "real-world" testing scenario reported here.

In addition to the test set of 371 defined fragrance materials, RIFM has also tested about 30 naturally derived fragrance materials (Table 7) (which contain multiple chemical constituents) in three different assays (BlueScreen HC, Ames, and *in vitro* micronucleus assays) and found a good agreement of the respective outcomes. Although more work is required on the assessment of naturals, the data to date support the conclusion that the BlueScreen HC assay also may be useful as a high-throughput screen for natural complexes. There are greater than 800 natural complexes currently listed in the RIFM Database, of which more than 80% lack genotoxicity data.

Conclusions

The relevance and ability to use the BlueScreen HC assay as a screening and prioritization tool in the safety assessment of fragrance materials was assessed using an extensive

and representative set of 371 fragrance materials tested in the BlueScreen HC assay along with *in vitro* and *in vivo* genotoxicity data. The majority of materials in the data set were deemed negative and concluded not to have the potential to be genotoxic based on a weight-of-evidence approach, while only a small proportion of materials were determined to be true positives. Analysis of the data set showed a combination of high positive agreement but low negative agreement between BlueScreen HC results, *in vitro* regulatory genotoxicity assays and higher-tier test results. The BlueScreen HC assay did not generate any false negatives, thereby providing robustness when utilizing it as an HTS tool to evaluate the large inventory of fragrance materials. The BlueScreen HC assay can, therefore, be considered a key screening tool in identifying genotoxic materials in the fragrance material domain. Since it generates higher false positives, it may be considered a conservative screening approach. It is important to note that in the data set analyzed, BlueScreen HC is a sensitive predictor of the outcome of the *in vitro* two-test battery, and analysis of the few *in vitro* positives it missed revealed that these substances were not considered to have biologically relevant genotoxic properties *in vivo*. The evaluation of some naturally derived fragrance materials provided a similar finding, suggesting the potential to consider the BlueScreen HC a valuable screening tool to evaluate the genotoxic potential of fragrance materials derived from natural sources

Supplementary Data

Supplementary data is available at *Mutagenesis* online.

Conflict of Interest Statement

None declared.

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References

1. Cihák R. REACH - an overview. *Interdiscip Toxicol* 2009;2:42–4.
2. Kirkland D, Aardema M, Henderson L et al. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. *Mutat Res* 2005;584:1–256.
3. Kirkland D. Improvements in the reliability of in vitro genotoxicity testing. *Expert Opin Drug Metab Toxicol* 2011;7:1513–20.
4. Api AM, Belsito D, Bruze M et al. Criteria for the Research Institute for Fragrance Materials, Inc. (RIFM) safety evaluation process for fragrance ingredients. *Food Chem Toxicol* 2015;82:S1–19.
5. Safford B, Api AM, Barratt C et al. Use of an aggregate exposure model to estimate consumer exposure to fragrance ingredients in personal care and cosmetic products. *Regul Toxicol Pharmacol* 2015;72:673–82.
6. Rulis AM. *De Minimis and the Threshold of Regulation*. Chelsea, MI: Lewis Publishers, 1986.
7. Rulis AM. *Establishing a Threshold of Concern*. New York, NY: Plenum Press, 1989.
8. Rulis AM. *Threshold of Regulation: Options for Handling Minimal Risk Situations*. Washington, DC: American Chemical Society, 1992.
9. Kroes R, Renwick AG, Cheeseman M et al.; European Branch of the International Life Sciences Institute. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. *Food Chem Toxicol* 2004;42:65–83.
10. Dewhurst I, Renwick AG. Evaluation of the threshold of toxicological concern (TTC)—challenges and approaches. *Regul Toxicol Pharmacol* 2013;65:168–77.
11. Etter S, Birrell L, Cahill P et al. The 'BlueScreen HC' assay as a decision making test in the genotoxicity assessment of flavour and fragrance materials. *Toxicol In Vitro* 2015;29:1425–35.
12. Billinton N, Hastwell PW, Beerens D et al. Interlaboratory assessment of the GreenScreen HC GADD45a-GFP genotoxicity screening assay: an enabling study for independent validation as an alternative method. *Mutat Res* 2008;653:23–33.
13. Birrell L, Cahill P, Hughes C et al. GADD45a-GFP GreenScreen HC assay results for the ECVAM recommended lists of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests. *Mutat Res* 2010;695:87–95.
14. Hastwell PW, Chai LL, Roberts KJ et al. High-specificity and high-sensitivity genotoxicity assessment in a human cell line: validation of the GreenScreen HC GADD45a-GFP genotoxicity assay. *Mutat Res* 2006;607:160–75.
15. Hastwell PW, Webster TW, Tate M et al. Analysis of 75 marketed pharmaceuticals using the GADD45a-GFP 'GreenScreen HC' genotoxicity assay. *Mutagenesis* 2009;24:455–63.
16. Hughes C, Rabinowitz A, Tate M et al. Development of a high-throughput *Gaussia* luciferase reporter assay for the activation of the GADD45a gene by mutagens, promutagens, clastogens, and aneugens. *J Biomol Screen* 2012;17:1302–15.
17. Knight AW, Little S, Houck K et al. Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast chemicals. *Regul Toxicol Pharmacol* 2009;55:188–99.
18. Low Y, Sedykh A, Fourches D et al. Integrative chemical-biological read-across approach for chemical hazard classification. *Chem Res Toxicol* 2013;26:1199–208.
19. Api AM, Belsito D, Botelho D et al. RIFM fragrance ingredient safety assessment, 1,1-diethoxyheptane, CAS Registry Number 688-82-4. *Food Chem Toxicol* 2018;122:S558–65.
20. Pfuhler S, Albertini S, Fautz R et al.; Gesellschaft fuer Umwelt-Mutationsforschung. Genetic toxicity assessment: employing the best science for human safety evaluation part IV: recommendation of a working group of the Gesellschaft fuer Umwelt-Mutationsforschung (GUM) for a simple and straightforward approach to genotoxicity testing. *Toxicol Sci* 2007;97:237–40.
21. Reus AA, Reisinger K, Downs TR et al. Comet assay in reconstructed 3D human epidermal skin models—investigation of intra- and inter-laboratory reproducibility with coded chemicals. *Mutagenesis* 2013;28:709–20.
22. Pfuhler S, Van Benthem J, Curren R et al. Use of in vitro 3D tissue models in genotoxicity testing: strategic fit, validation status and way forward. Report of the working group from the 7(th) International Workshop on Genotoxicity Testing (IWGT). *Mutat Res* 2020;850–851:503135.
23. Pfuhler S, Downs TR, Hewitt NJ et al. Validation of the 3D reconstructed human skin micronucleus (RSMN) assay: an animal-free alternative for following-up positive results from standard in vitro genotoxicity assays. *Mutagenesis* 2021;36:1–17.
24. Hartmann A, Agurell E, Beevers C et al.; 4th International Comet Assay Workshop. Recommendations for conducting the in vivo alkaline Comet assay. 4th International Comet Assay Workshop. *Mutagenesis* 2003;18:45–51.
25. Tice RR, Agurell E, Anderson D et al. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 2000;35:206–21.

26. SCCS. *Scientific Committee on Consumer Safety Opinion on Basic Brown 17 (B007), 24 March 2014, SCCS/1531/14, Revision of 18 June 2014*. 2014.
27. Kirkland D, Levy DD, LeBaron MJ et al. A comparison of transgenic rodent mutation and in vivo comet assay responses for 91 chemicals. *Mutat Res Genet Toxicol Environ Mutagen* 2019;839:21–35.
28. Curren RD, Mun GC, Gibson DP et al. Development of a method for assessing micronucleus induction in a 3D human skin model (EpiDerm). *Mutat Res* 2006;607:192–204.
29. Dahl EL, Curren R, Barnett BC et al. The reconstructed skin micronucleus assay (RSMN) in EpiDerm™: detailed protocol and harmonized scoring atlas. *Mutat Res* 2011;720:42–52.
30. Aardema MJ, Barnett BC, Khambatta Z et al. International prevalidation studies of the EpiDerm 3D human reconstructed skin micronucleus (RSMN) assay: transferability and reproducibility. *Mutat Res* 2010;701:123–31.
31. Pfuhler S, Kirst A, Aardema M et al. A tiered approach to the use of alternatives to animal testing for the safety assessment of cosmetics: genotoxicity. A COLIPA analysis. *Regul Toxicol Pharmacol* 2010;57:315–24.
32. Mun GC, Aardema MJ, Hu T et al. Further development of the EpiDerm 3D reconstructed human skin micronucleus (RSMN) assay. *Mutat Res* 2009;673:92–9.
33. Hu T, Bailey RE, Morrall SW et al. Dermal penetration and metabolism of p-aminophenol and p-phenylenediamine: application of the EpiDerm human reconstructed epidermis model. *Toxicol Lett* 2009;188:119–29.
34. Hu T, Kaluzhny Y, Mun GC et al. Intralaboratory and interlaboratory evaluation of the EpiDerm 3D human reconstructed skin micronucleus (RSMN) assay. *Mutat Res* 2009;673:100–8.
35. Novotna B, Pelclova D, Rossnerova A et al. The genotoxic effects in the leukocytes of workers handling nanocomposite materials. *Mutagenesis* 2020;35:331–40.
36. RIFM (Research Institute for Fragrance Materials) I. *Anisyl Formate: In Vitro L5178Y Gene Mutation Assay at the HPRT Locus, in RIFM Report Number 69972*. Woodcliff Lake, NJ: RIFM, 2016.
37. RIFM (Research Institute for Fragrance Materials) I. *Anisyl Formate (CAS No. 122-91-8): In Vitro Micronucleus Assay in Human Peripheral Blood Lymphocytes, in RIFM Study Number 70051*. Woodcliff Lake, NJ: RIFM, 2016.
38. Kirkland D, Pfuhler S, Tweats D et al. How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop. *Mutat Res* 2007;628:31–55.
39. ECHA. *Anisyl Alcohol Registration Dossier*. 2003. https://echa.europa.eu/de/registration-dossier/-/registered-dossier/16802/7/7/2 (December 2021, date last accessed).
40. ECHA. *Formic Acid Registration Dossier.* 1992. https://echa.europa.eu/lv/registration-dossier/-/registered-dossier/15127/7/7/2 (December 2021, date last accessed).
41. ECVAM. *EURL ECVAM Progress Report on the Development, Validation and Regulatory Acceptance of Alternative Methods (2010-2013)*. 2013. https://ec.europa.eu/environment/chemicals/lab_animals/pdf/EURL_ECVAM_progress_report_cosmetics_2013.pdf (December 2021, date last accessed).
42. Kirkland D, Zeiger E, Madia F et al. Can in vitro mammalian cell genotoxicity test results be used to complement positive results in the Ames test and help predict carcinogenic or in vivo genotoxic activity? I. Reports of individual databases presented at an EURL ECVAM Workshop. *Mutat Res Genet Toxicol Environ Mutagen* 2014;775–776:5–68.
43. RIFM (Research Institute for Fragrance Materials) I. *1-(3,5,6-trimethyl-3-cyclohexen-1-yl)ethan-1-one (CAS # 68480-14-8): Bacterial Reverse Mutation Assay: Plate Incorporation Method with a Confirmatory Assay in RIFM Report Number 72590*. Woodcliff Lake, NJ: RIFM, 2017.
44. Belsito D, Bickers D, Bruze M et al. A toxicological and dermatological assessment of alkyl cyclic ketones when used as fragrance ingredients. RIFM Expert Panel. *Food Chem Toxicol* 2013;62:S1–44.
45. Li D, Fedeles BI, Singh V et al. Tautomerism provides a molecular explanation for the mutagenic properties of the anti-HIV nucleoside 5-aza-5,6-dihydro-2'-deoxycytidine. *Proc Natl Acad Sci USA* 2014;111:E3252–9.
46. Chiappe C, De Rubertis A, Amato G et al. Stereochemistry of the biotransformation of 1-hexene and 2-methyl-1-hexene with rat liver microsomes and purified P450s of rats and humans. *Chem Res Toxicol* 1998;11:1487–93.
47. Nelson SD, Gordon WP. Mammalian drug metabolism. *J Nat Prod* 1983;46:71–8.
48. RIFM (Research Institute for Fragrance Materials) I. *1-(3,5,6-trimethyl-3-cyclohexen-1-yl)ethan-1-one (CAS # 68480-14-8): In Vitro L5178Y Gene Mutation Assay at the HPRT Locus, in RIFM Report Number 72599*. Woodcliff Lake, NJ: RIFM, 2017.
49. RIFM (Research Institute for Fragrance Materials) I. *1-(3,5,6-trimethyl-3-cyclohexen-1-yl)ethan-1-one (CAS # 68480-14-8): In Vitro Micronucleus Assay in Human Peripheral Blood Lymphocytes in RIFM Report Number 69878*. Woodcliff Lake, NJ: RIFM, 2016.
50. Heck JD, Vollmuth TA, Cifone MA et al. An evaluation of food flavoring ingredients in a genetic toxicity screening battery. *Toxicologist* 1989;9:257.
51. ECHA. Isobutyric Acid Registration Dossier. 2011. https://echa.europa.eu/lv/registration-dossier/-/registered-dossier/14591/7/7/2 (December 2021, date last accessed).
52. RIFM (Research Institute for Fragrance Materials) I. *4-Thujanol (CAS# 546-79-2): Micronucleus Test in Human Lymphocytes In Vitro, in RIFM Report Number 72343*. Woodcliff Lake, NJ: RIFM, 2017.
53. RIFM (Research Institute for Fragrance Materials) I. *4-Thujanol (CAS# 546-79-2): In Vivo Mammalian Erythrocyte Micronucleus Assay with Flow Cytometry Analysis in Peripheral Blood Reticulocytes and Mammalian Alkaline Comet Assay in Mice, in RIFM Report Number 76271*. Woodcliff Lake, NJ: RIFM, 2019.
54. Adams TB, Hallagan JB, Putnam JM et al. The FEMA GRAS assessment of alicyclic substances used as flavour ingredients. *Food Chem Toxicol* 1996;34:763–828.
55. RIFM (Research Institute for Fragrance Materials) I. *Isobornyl Methyl Ether (CAS #5331-32-8): In Vitro Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL) in RIFM Report Number 72542*. Woodcliff Lake, NJ: RIFM, 2017.
56. RIFM (Research Institute for Fragrance Materials) I. *Isobornyl Methyl Ether (CAS# 5331-32-8): In Vitro Micronucleus Test Using Reconstructed Skin Micronucleus (RSMN) Assay in EpiDermTM, in RIFM Report Number 72543*. Woodcliff Lake, NJ: RIFM, 2017.
57. Hewitt NJ, Edwards RJ, Fritsche E et al. Use of human in vitro skin models for accurate and ethical risk assessment: metabolic considerations. *Toxicol Sci* 2013;133:209–17.
58. RIFM (Research Institute for Fragrance Materials) I. *5-Methylquinoxaline (CAS# 13708-12-8): In Vitro Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL), in Unpublished Report from RIFM*. Woodcliff Lake, NJ: RIFM, 2016.
59. EFSA Panel on Food Contact Materials, E., Flavourings and Processing Aids (CEF). Scientific opinion on Flavouring Group Evaluation 17, Revision 2 (FEG.17Rev2): pyrazine derivatives from chemical group 24. *EFSA J* 2011;9:66.
60. RIFM (Research Institute for Fragrance Materials) I. *In Vitro Micronucleus Test in Chinese Hamster V79 Cells with 3-methyl-5-phenylpent-2-enenitrile, in RIFM Report Number 54292*. Woodcliff lake, NJ: RIFM, 2007.
61. RIFM (Research Institute for Fragrance Materials) I. *Micronucleus Assay in Bone Marrow Cells of the Mouse with 3-methyl-5-phenylpent-2-enenitrile, in RIFM Report Number 54627*. Woodcliff Lake, NJ: RIFM, 2008.
62. RIFM (Research Institute for Fragrance Materials) I. *1,5-Dimethylbicyclo[3.2.1]octan-8-one-oxime (CAS# 75147-23-8): In Vitro Micronucleus Assay in Human Peripheral Blood*

Lymphocytes, in RIFM Report Number 69943. Woodcliff Lake, NJ: RIFM: 2016.
63. RIFM (Research Institute for Fragrance Materials) I. *1,5-Dimethylbicyclo[3.2.1]octan-8-one-oxime (CAS# 75147-23-8): In Vivo Mammalian Erythrocyte Micronucleus Assay in Mice with Flow Cytometry Analysis, in RIFM Reference Number 69963*. Woodcliff Lake, NJ: RIFM, 2016.
64. Fowler P, Smith K, Young J et al. Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. I. Choice of cell type. *Mutat Res* 2012;742:11–25.
65. Chaung W, Mi LJ, Boorstein RJ. The p53 status of Chinese hamster V79 cells frequently used for studies on DNA damage and DNA repair. *Nucleic Acids Res* 1997;25:992–4.
66. ECHA. *α-Ionone Registration Dossier*. 2014. https://echa.europa.eu/lv/registration-dossier/-/registered-dossier/18612/7/7/3 (December 2021, date last accessed).
67. RIFM (Research Institute for Fragrance Materials) I. *Methyl AcetoAcetate (CAS #105-45-3): In Vitro Micronucleus Assay in Human Peripheral Blood Lymphocytes, in RIFM Reference Number 68081*. Woodcliff Lake, NJ: RIFM, 2014.
68. Bhatia SP, Politano VT, Api AM. Evaluation of genotoxicity of nitrile fragrance ingredients using in vitro and in vivo assays. *Food Chem Toxicol* 2013;59:784–92.
69. Stoner GD, Shimkin MB, Kniazeff AJ et al. Test for carcinogenicity of food additives and chemotherapeutic agents by the pulmonary tumor response in strain A mice. *Cancer Res* 1973;33:3069–85.