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 predictive value 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
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 vitro 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.

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 GADD45α 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

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

alt 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.”

alt 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.”
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 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% CO2 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 487), 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:

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

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

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

\[
\text{Predictivity (Negative)} = \frac{\text{True Negative}}{\text{True Negative} + \text{False Negative}}
\]

**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       | 3/77                  | 3.9%                 |

*Final 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, 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 negative in BlueScreen HC.

**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 vitro* 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 vitro 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 doses 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 [38]. 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 vitro 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) |
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 \textit{in vitro} and \textit{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.

\begin{table}
\centering
\caption{Summary of genotoxicity data set for natural extracts.}
\begin{tabular}{|l|l|l|l|l|}
\hline
Principal NCS name & CAS Number & BlueScreen HC & Bacterial reverse mutation test result (OECD 471) & \textit{In vitro} micronucleus test result (OECD 487) \\
\hline
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 \\
Cabrauva 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 (\textit{Santalam spicata}) & 8024-35-9 & Negative & Negative & Negative \\
Sandalwood Oil E.I. Type (\textit{Santalum album}) & 8006-87-9 & Negative & Negative & Negative \\
Chamomile Oil Blue Egyptian (\textit{Matricaria chamomilla}) & 8002-66-2 & Negative & Negative & Negative \\
Chamomile Oil Roman (\textit{Anthemis nobilis}, syn. \textit{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 \\
\hline
\end{tabular}
\end{table}

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 \textit{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.

\section*{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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