# Appendix 11
# The detail of the revision of the SOP in the Phase I-C

In the Phase I-C, cross-contaminations by volatile compound are observed. Cross-contamination could affect the gene expressions of other compounds in the same plate, leading to either overestimate or underestimate. Therefore, in order to avoid cross-contamination, revision of the SOP was needed. This report describes the detail of the revision of the SOP in the Phase I-C.

a. Verification of potential cross-contamination effects of volatile test chemical

Figure 1 depicts the dose-dependent effects of MHC (as obtained by lead laboratory) on the expression of the four marker genes and on cell viability. The fold induction of all marker genes exceeded the respective cut-off values, and fold inductions of both the *GCLM* and *DNAJB4* were especially high at a low MHC concentration (*GCLM*: 18.4-fold at 0.2% w/v and *DNAJB4*: 6.7-fold at 0.2% w/v, respectively). On the other hand, the EC (also known as the estimated concentration resulting into a respective cut-off value in the expression of a marker gene) values for the *ATF3* and *IL-8* were around 1% w/v, and as such, cross-contaminated MHC is unlikely to be able to affect any of these marker genes as the concentration of cross-contaminated MHC is unlikely to reach 1% w/v. Therefore, it was considered that the cross-contaminated MHC would mainly affect the *GCLM* and *DNAJB4* expression; in fact, the extrapolated EC value for *GCLM* and *DNAJB4* were 0.0056% and 0.059% w/v, respectively. Figure 2 presents the Imax values of lactic acid for the four marker genes after three repetitions in the three participating laboratories. It is clearly shown that there are two outliers with regard to the *GCLM* and *DNAJB4* expression at the 3rd experiment undertaken by both KOSÉ and FDSC. Moreover, lactic acid at these two experiments was added in the same plate as MHC. These results suggest that a cross-contamination with MHC might affect the co-examined test chemicals. In addition, cross-contamination could either overestimate or underestimate the other chemicals' effects. In the first case, when a chemical is placed in the same plate as MHC, its effect on the expression of *GCLM* and *DNAJB4* may be significantly overestimated as a result of a cross-contamination with MHC. Furthermore, when the vehicle controls are placed in the same plate with MHC, their *GCLM* and *DNAJB4* expressions might appear increased; as a result, the gene expression of the test chemicals may be underestimated due to the fact that the gene expression of the latter is calculated and expressed by relative quantitation.

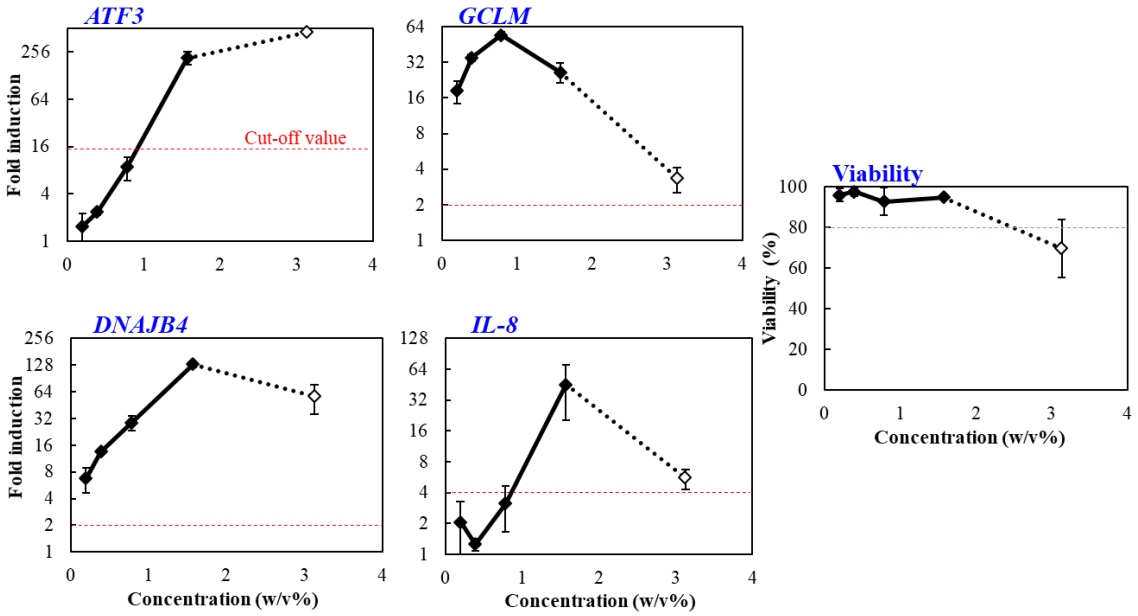


Figure 1. Dose-dependency of the effects of methyl heptine carbonate on the expression of the four marker genes and on cell viability, as assessed by the lead laboratory. Red dashed lines indicate the respective cut-off values of each of the marker genes, while the green dashed line represents the acceptable cell viability criterion.

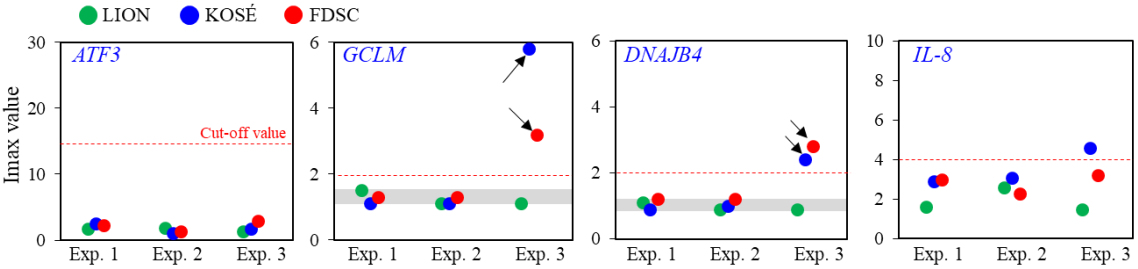


Figure 2. Effects of the exposure to lactic acid (with Imax values) on the expression of the four marker genes, after three repetitions at the three participating laboratories. Red dashed lines indicate the respective cut-off values of each of the marker genes. Black arrows indicate potential outliers. Gray areas represent the variation of the Imax value across three repetitions in the three participating laboratories, except for outliers.

In order to clarify the cross-contamination effect of MHC on the positive/negative judgment undertaken by Phase I-C, a three-step verification of the potential cross-contamination effect was performed. The first step was by checking if any of the test chemicals or the vehicle control were placed on the same plate as MHC.

The second step was to establish that the effect of MHC on the Imax value was verified by checking if the Imax value fell within the variation range produced by the three repetitions in each of the three participating laboratories. Regarding the potential overestimation (when a test chemical, called "chemical X," is put along with MHC on the same plate), three types of relationships were assumed between the variation range of the Imax value and that of the chemical X: (i) that the Imax of the cross-contaminated chemical X lies above the variation of Imax, (ii) that the Imax of the cross-contaminated chemical X lies within the variation of Imax, and (iii) that the Imax of the cross-contaminated chemical X lies below the variation of Imax (see Figure 3). When the Imax value for the *GCLM* or *DNAJB4* expression as a result of the exposure to a cross-contaminated chemical X is above the variation of Imax, the overestimation is likely caused. However, when the Imax value is within or below that same variation, the overestimation is unlikely to be caused. Likewise, in the case of an underestimation (where the vehicle control is placed on the same plate as MHC, and a chemical Y is exposed along with the same vehicle and tested at the same experiment), if the Imax value of the *GCLM* or *DNAJB4* expression for the chemical Y lies above or within the variation of Imax, then an underestimation is unlikely to be caused. On the other hand, when the Imax value lies below the Imax variation, then an underestimation is likely caused (see Figure 4).

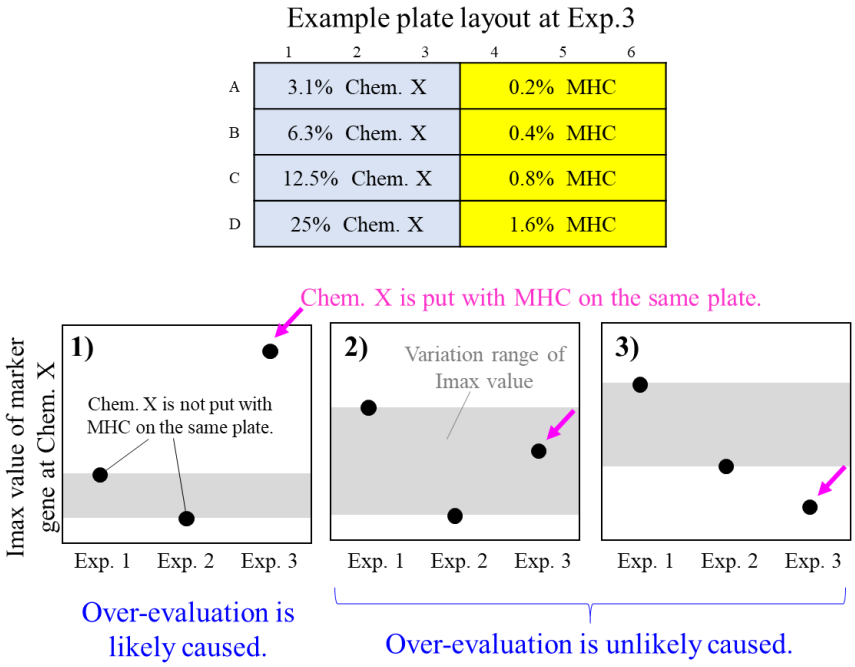


Figure 3. Assumed relationships between the variation range of the Imax value and the Imax value of the chemical X; the latter is placed on the same plate as methyl heptine carbonate (MHC) at the 3rd experiment, but not at the 1st and 2nd experiment.

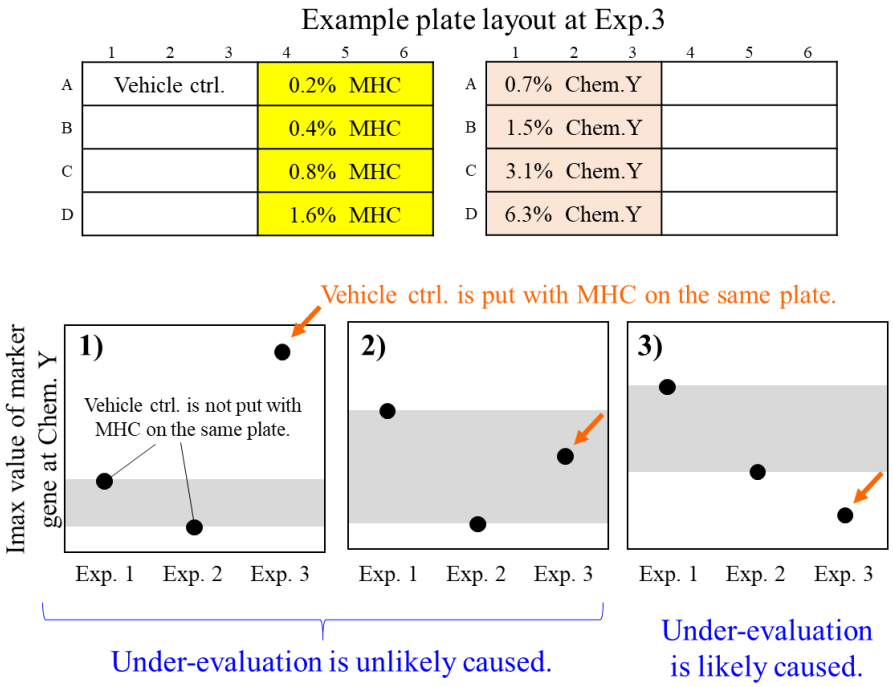


Figure 4. Assumed relationships between the variation range of the Imax value and the Imax value of chemical Y. The vehicle control is placed on the same plate as methyl heptine carbonate (MHC) at the 3rd experiment, but do not at the 1st and 2nd experiment.

As far as the third step is concerned, the effect of MHC on the final judgment (P versus N) is confirmed by checking the Imax values of the *ATF3* and *IL-8* expressions as the cross-contamination by MHC is unlikely to affect these marker genes. At the example result described in Figure 5, the overestimation as a result of a cross-contamination by MHC is likely evident on the *GCLM* and *DNAJB4* expression in the 3rd experiment, while the Imax values exceed the cut-off ones. In our case, EpiSensA will judge a test chemical as "positive" when its Imax values exceed the respective cut-off values for at least one out of four marker genes. Therefore, when the Imax values of the *ATF3* and *IL-8* expression as a result of an exposure to chemical X are basically lower than the respective cut-off values, the final judgment at the 3rd experiment is dependent on the *GCLM* and *DNAJB4* expressions. Consequently, a cross-contamination with MHC at the 3rd experiment might affect the positive judgment. On the other hand, if the Imax values of *ATF3* expression for chemical X exceed the cut-off value, than the positive judgment at the 3rd experiment is not dependent on *GCLM* and *DNAJB4* (Figure 6). Likewise, at the example result presented in Figure 7, the underestimations are likely to be caused for the

effects on the *GCLM* and *DNAJB4* expression in the 3rd experiment, and the Imax values would not exceed the cut-off ones. Moreover, the Imax values for the *ATF3* and *IL-8* expression as a result of an exposure to chemical Y are basically lower than the respective cut-off values; as a result, a negative judgment based on the findings of the 3rd experiment is dependent on the *GCLM* and *DNAJB4* expressions. Therefore, the cross-contamination with MHC at the 3rd experiment may affect the negative judgment. On the other hand, if the Imax values for the *IL-8* expression after an exposure to chemical Y exceed the cut-off ones, then a positive judgment based on the findings of the 3rd experiment is not dependent upon the expressions of *GCLM* and *DNAJB4* (Figure 8).

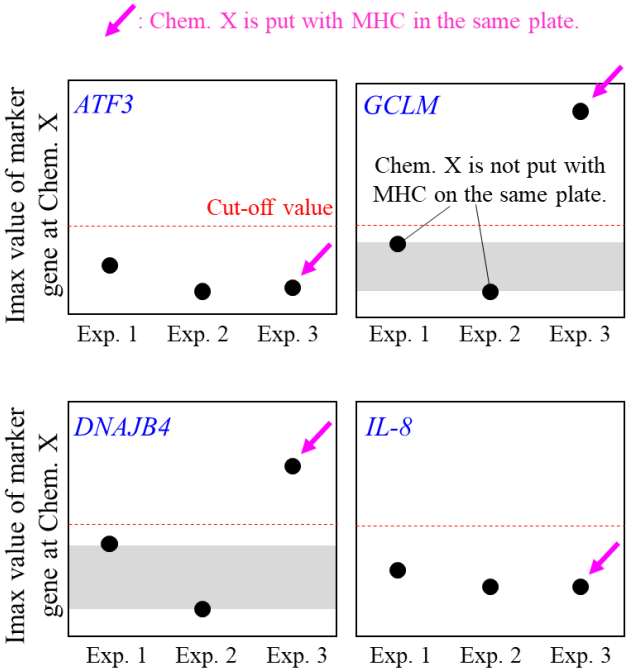


Figure 5. Example results for chemical X which is theoretically affected by a cross-contamination with methyl heptine carbonate (MHC) at the 3rd experiment. As a result, the cross-contamination may affect the positive judgment at the 3rd experiment.

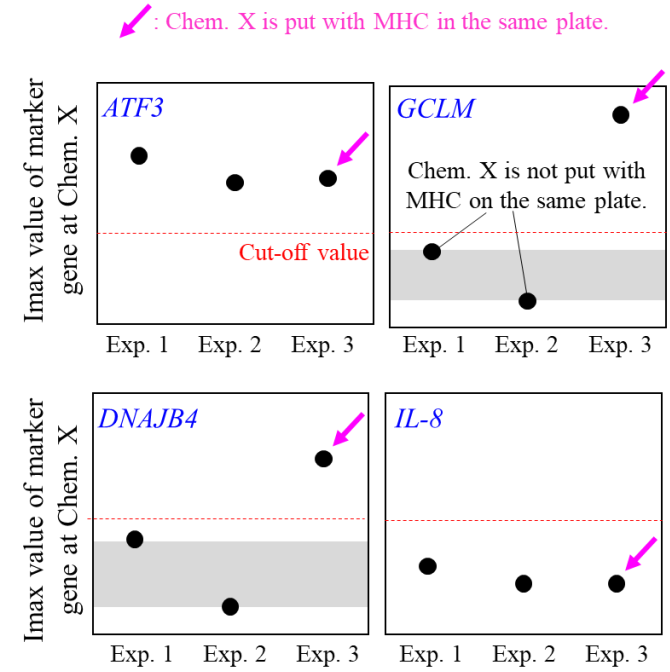


Figure 6. Example results for chemical X which is theoretically affected by a cross-contamination with methyl heptine carbonate (MHC) at the 3rd experiment. However, the cross-contamination will not affect the positive judgment at the 3rd experiment.

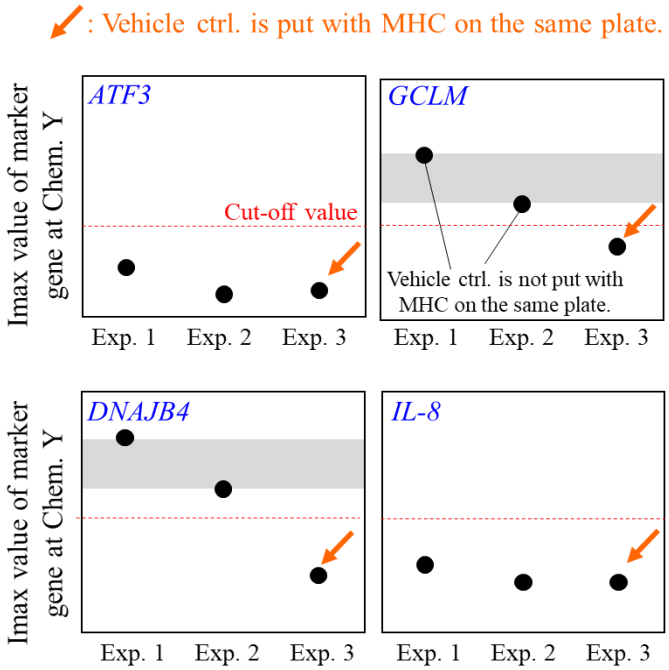


Figure 7. Example results for chemical Y. The vehicle control is placed on the same plate as methyl heptine carbonate (MHC) at the 3rd experiment. Chemical Y's effects are affected by that same vehicle. As a result, the cross-contamination may affect the negative judgment at the 3rd experiment.

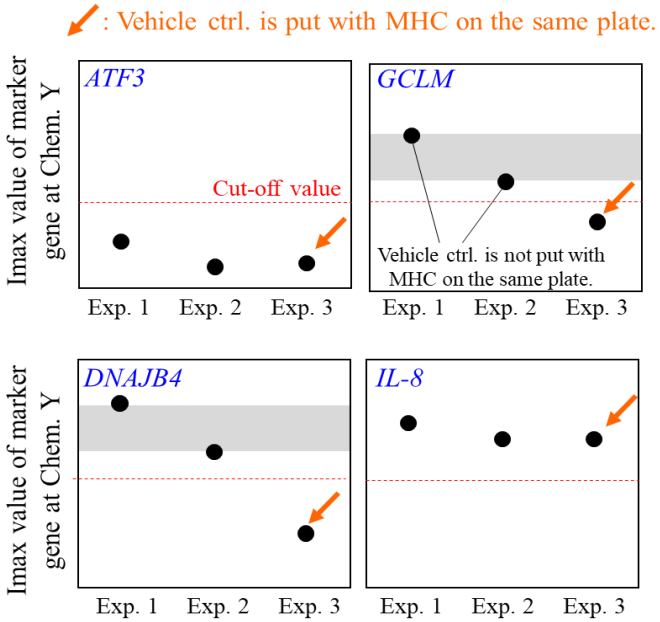


Figure 8. Example results for chemical Y. The vehicle control is placed on the same plate as methyl heptine carbonate (MHC) at the 3rd experiment. Chemical Y's effects are affected by that same vehicle. However, the cross-contamination will not affect the positive judgment at the 3rd experiment.

Figure 9 presents the Imax values for the four marker genes, after three repetitions in the three participating laboratories when lactic acid was tested. Regarding the first step, no test chemical or corresponding vehicle control was put on the same plate as MHC at LION. On the other hand, the test chemical was put with MHC on the same plate at the 3rd experiment conducted by KOSÉ and FDSC. As far as the second step is concerned, both of these Imax values for the *GCLM* and *DNAJB4* expressions fell outside of the variation, and thus the overestimation was likely caused by a cross-contamination. Finally, for the third step, the Imax value for the *IL-8* expression at the 3rd experiment undertaken by KOSÉ exceeded the cut-off value, and as a result, the cross-contamination did not affect to the final judgment. However, both the Imax values for the *ATF3* and *IL-8* expressions were lower than the cut-off ones at the 3rd experiment of FDSC, thus a cross-contamination has likely affected the positive judgment.

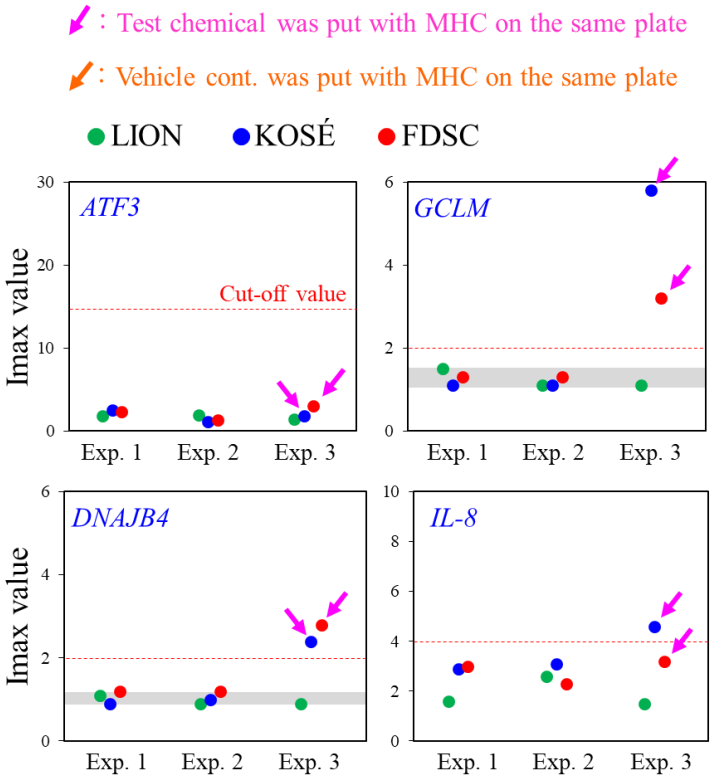


Figure 9. Imax values of the four marker genes as obtained from three repetitions in the three participating laboratories, when lactic acid was tested. Gray areas represent the variation of the Imax value among three repetitions in the three participating laboratories, except for the results that are likely to be affected by a cross-contamination with MHC.

Figure 10 presents the Imax values of the four marker genes as obtained from three repetitions in the three participating laboratories, when *p*-phenylene diamine was tested. Regarding the first step, no test chemical or corresponding vehicle control was put with MHC on the same plate by LION. On the other hand, the test chemical was put with MHC on the same plate at the 2$^{nd}$ experiment conducted by KOSÉ. In addition, the vehicle control was put with MHC on the same plate at the 2$^{nd}$ experiment conducted by FDSC. As a second step, the Imax values for the *GCLM* and *DNAJB4* expressions at the 2$^{nd}$ experiment of KOSÉ fell inside the variation, thus suggesting that an overestimation was unlikely. On the other hand, the Imax value at the 2$^{nd}$ experiment conducted by FDSC fell outside the variation, thus suggesting that an underestimation was likely. However, at the third step, the Imax value for the *ATF3* expression at the 2$^{nd}$ experiment of FDSC exceeded the cut-off value, and as a result, there was no effect on the final judgment due to cross-contamination.

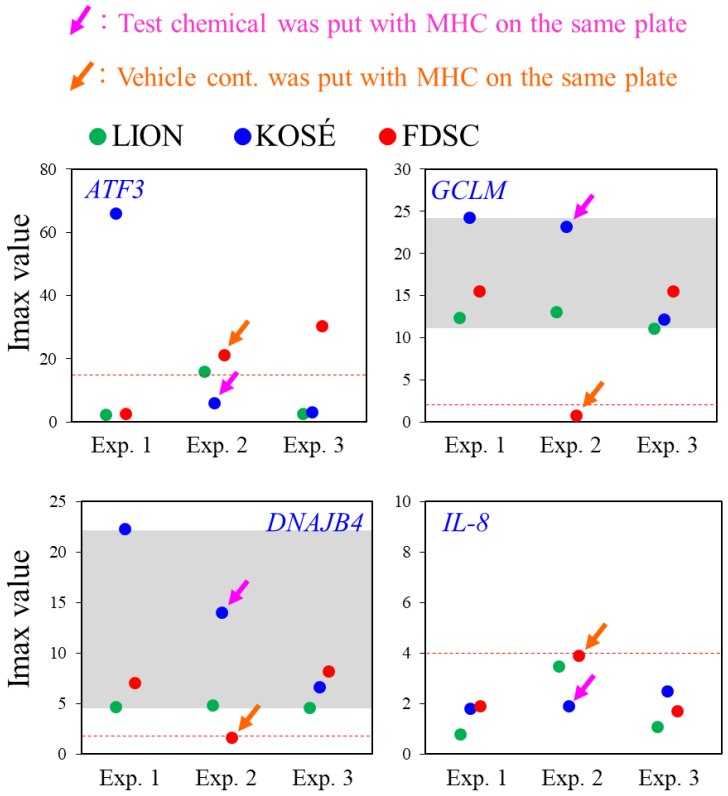


Figure 10. Imax values of the four marker genes as obtained from three repetitions in the three participating laboratories, when *p*-phenylene diamine was tested. Gray areas represent the variation of the Imax value among three repetitions in the three participating laboratories, except for the results that are likely to be affected by a cross-contamination with MHC.

Figure 11 presents the Imax values of the four marker genes as obtained from three repetitions in the three participating laboratories, when MHC was tested. For MHC itself, the vehicle control was put on the same plate with MHC at the 2$^{nd}$ experiment conducted by KOSÉ, but not at any sets assessed by LION or FDSC. In addition, the Imax value for the *DNAJB4* expression at the 2$^{nd}$ experiment of KOSÉ fell outside the variation, thus suggesting that an underestimation was likely. However, the Imax values for the *ATF3* and *IL-8* expressions exceeded the respective cut-off ones, and as a result there was no effect on the final judgment caused by cross-contamination.

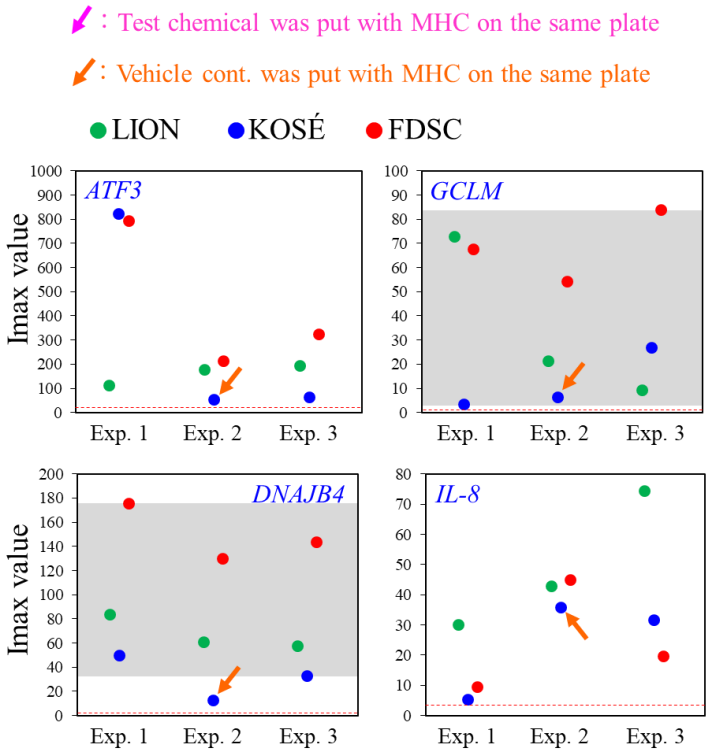


Figure 11. Imax values of the four marker genes as obtained from three repetitions in the three participating laboratories, when methyl heptine carbonate (MHC) was tested. Gray areas represent the variation of the Imax value among three repetitions in the three participating laboratories, except for the results that are likely to be affected by a cross-contamination with MHC.

With regard to the results of abietic acid presented in Figure 12, test chemicals were put on the same plate with MHC at the 1st and 2nd experiment conducted by FDSC. Furthermore, both the Imax values for the *GCLM* and *DNAJB4* expressions at the 1st and 2nd experiment conducted by FDSC fell outside the variation, and consequently the overestimation was considered as likely. However, the Imax values for the *ATF3* and *IL-8* expressions exceeded the cut-off ones, and as a result there was no evidence of an effect on final judgment exerted by cross-contamination.

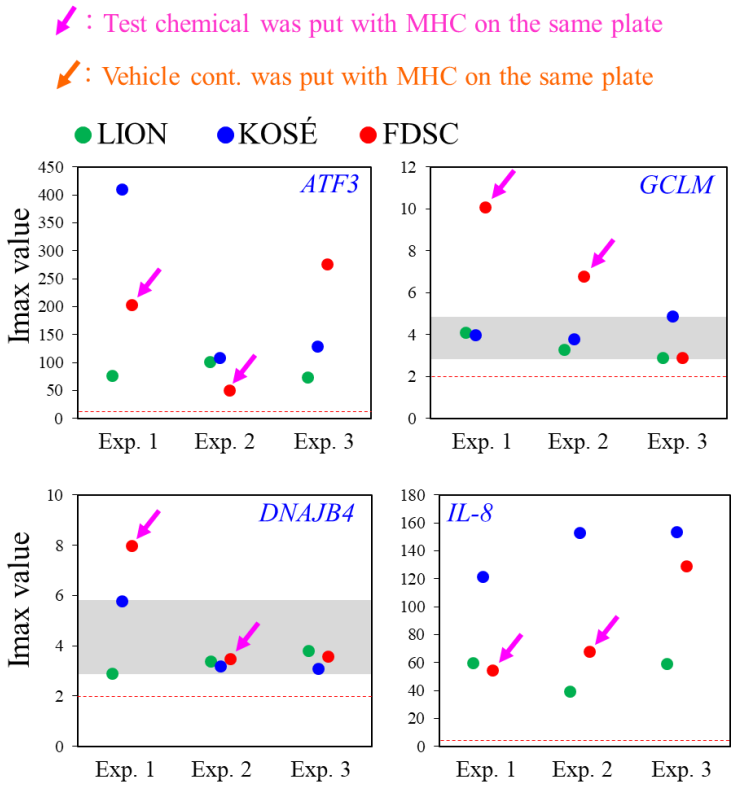


Figure 12. Imax values of the four marker genes as obtained from three repetitions in the three participating laboratories, when abietic acid was tested. Gray areas represent the variation of the Imax value among three repetitions in the three participating laboratories, except for the results that are likely to be affected by a cross-contamination with MHC.

Regarding the results of farnesol presented in Figure 13, test chemicals were put on the same plate with MHC at the 1st experiment conducted by KOSÉ and FDSC. Furthermore, both the Imax values for the *GCLM* and *DNAJB4* expressions fell outside of the variation, so an overestimation was likely caused. However, the Imax values for the *ATF3* and *IL-8* expressions exceeded the cut-off ones, and as a result there was no effect on the final judgment due to cross-contamination.

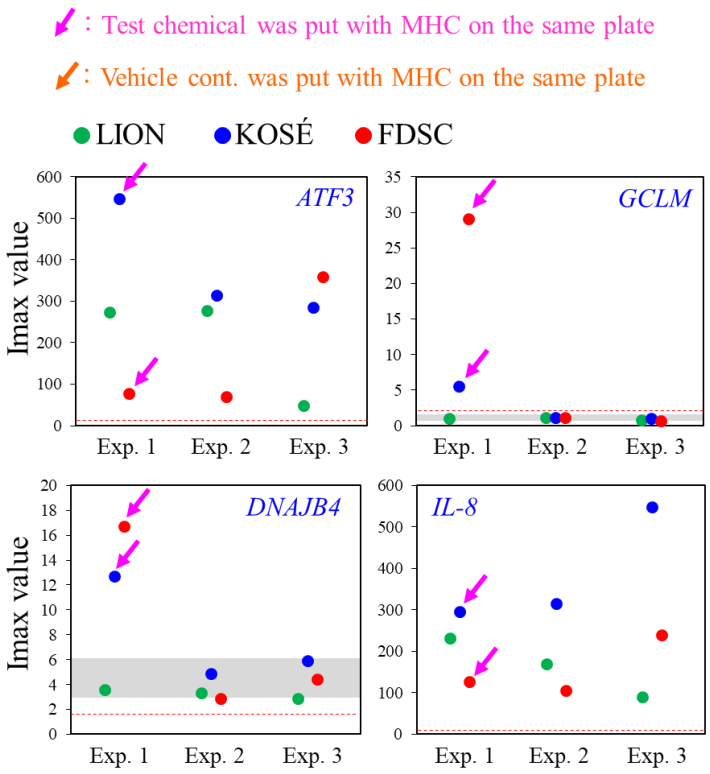


Figure 13. Imax values of the four marker genes as obtained from three repetitions in the three participating laboratories, when farnesol was tested. Gray areas represent the variation of the Imax value among three repetitions in the three participating laboratories, except for the results that are likely to be affected by a cross-contamination with MHC.

In summary, the cross-contamination has likely affected the final judgment of only the lactic acid when tested by FDSC as part of its 3rd experiment.

b. How to avoid the cross-contamination effect

The lead laboratory has examined whether cross-contamination can be avoided by separating the liquid test chemicals from others. MHC was retested by the lead laboratory based on the plate design of FDSC for its 3rd experiment, in the presence and absence of MHC. In Figure 14, lactic acid was placed in the same plate as MHC, and it was able to reproduce the Imax value that was obtained by FDSC at the 3rd experiment. In addition, lactic acid demonstrated comparable Imax values at the 1st and 2nd experiment of FDSC, in the absence of MHC. These results verified that cross-contamination could be avoided

by separating the plates and allowing for MHC to be tested in an isolated manner.

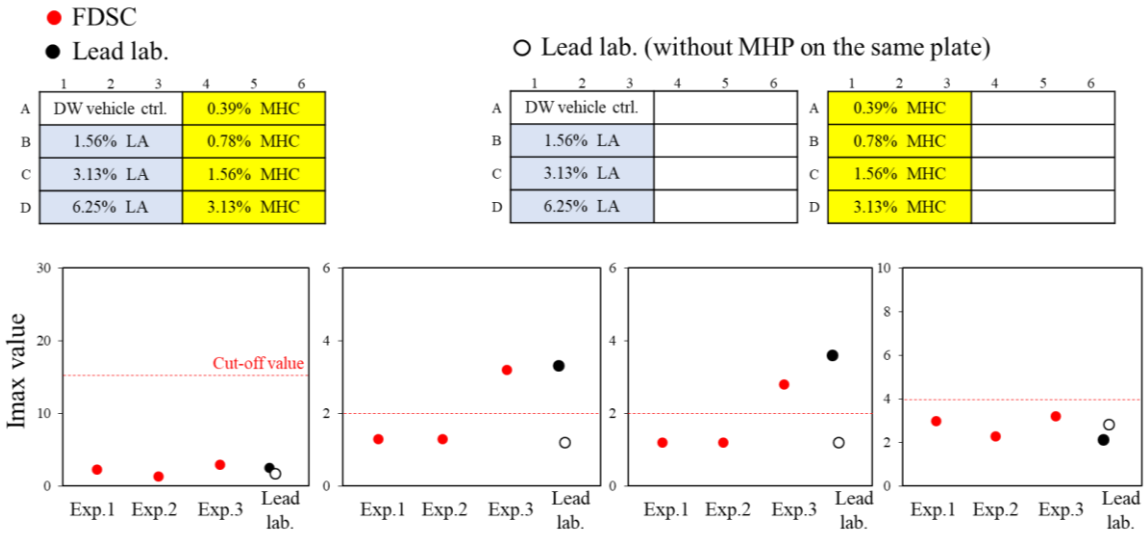


Figure 14. The results of the retest performed by the lead laboratory, based on the plate design of FDSC for the undertaking of the 3rd experiment in the presence and absence of MHC on the same plate. The results were compared to those obtained by FDSC.

c. Cross-contamination effects of volatile test chemicals in Phases I-A and I-B

The cross-contamination effects may occur when liquid and EpiSensA-positive chemicals are tested. So far, we have come across three liquid and EpiSensA-positive test chemicals (namely the glyoxal 40% solution, diethyl phthalate, and ethyl acrylate) in Phases I-A and I-B. Glyoxal has a lower boiling point (50°C) than diethyl phthalate (294°C) or ethyl acrylate (99.5°C), and as a result, a comparatively higher volatility of this compound is assumed. Figure 15 presents the dose-responses of the four marker genes for these three test chemicals and for MHC. In addition, Table 1 presents the respective EC values for the induction of the four marker genes as well as the minimum EC value (as defined by the lowest EC value of the four marker genes). The minimum EC values of glyoxal, diethyl phthalate and ethyl acrylate are much higher than that of MHC, suggesting that any cross-contamination effects on these chemicals might not be important.

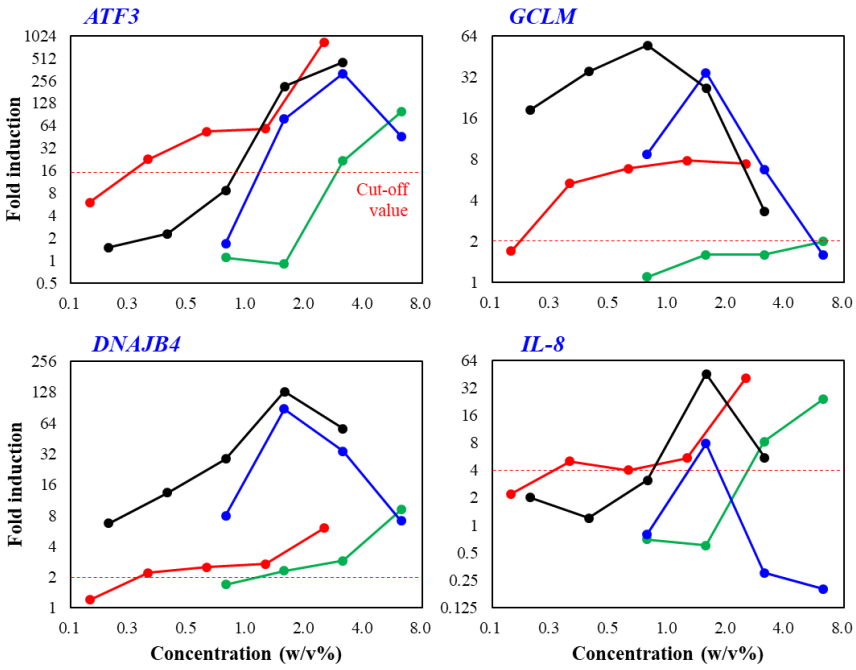


Figure 15. Dose-response graphs of the fold induction of the four marker genes when exposed to liquid test chemicals during Phases I-A and I-B, and when methyl heptine carbonate (MHC) was tested. Red line: glyoxal; green line: diethyl phthalate; blue line: ethyl acrylate; black line; MHC. Red dashed lines indicate respective cut-off values.

Table 1. The EC values of the induction effects on the four marker genes and the minimum EC value for 4 test chemicals. EC values were interpolated from the dose-response curve. Parenthesized EC values were extrapolated by using the fold inductions at the lowest two tested concentrations.

| | *ATF3* | *GCLM* | *DNAJB4* | *IL-8* | Min. |
|---|---|---|---|---|---|
| Glyoxal | 0.24 | 0.17 | 0.28 | 0.26 | 0.17 |
| Diethyl phthalate | 2.6 | 6.3 | 1.2 | 2.3 | 1.2 |
| Ethyl acrylate | 0.92 | (0.65) | (0.74) | 1.1 | (0.65) |
| Methyl heptine carbonate | 0.81 | (0.0056) | (0.059) | 0.80 | (0.0056) |

In the following, the verification of glyoxal was demonstrated as an example, because glyoxal has the lowest minimum EC value and the lowest boiling point amongst the three chemicals. The EC values ranged from 0.17% to 0.28%, and were comparable (Table 1). This means that if glyoxal was able to affect the other test chemicals by cross-

contamination, then the Imax values of all marker genes for these chemicals might have been misestimated. From this point of view, when the Imax values of all genes fell outside the variation, an over/underestimation was likely caused by cross-contamination. Figure 16 presents the results of the effects of sodium lauryl sulfate as a representative chemical for Phase I-A. The test chemical was put on the same plate as glyoxal in the 3rd experiment conducted by LION. However, the Imax values on all marker genes fell inside the variation, and as such, a misestimation is unlikely. Therefore, there was no effect on the final judgment of sodium lauryl sulfate caused by a possible cross-contamination with glyoxal. Moreover, the cross-contamination effects of glyoxal were not confirmed at other Phase I-A chemicals either (data not shown). Based on these results, glyoxal did not cause the cross-contamination effect. Furthermore, other test chemicals with a higher EC value than glyoxal (diethyl phthalate and ethyl acrylate) did not cause the cross-contamination effect either (data not shown). In conclusion, cross-contamination was unlikely to cause any obvious effect on the results of Phases I-A and I-B.

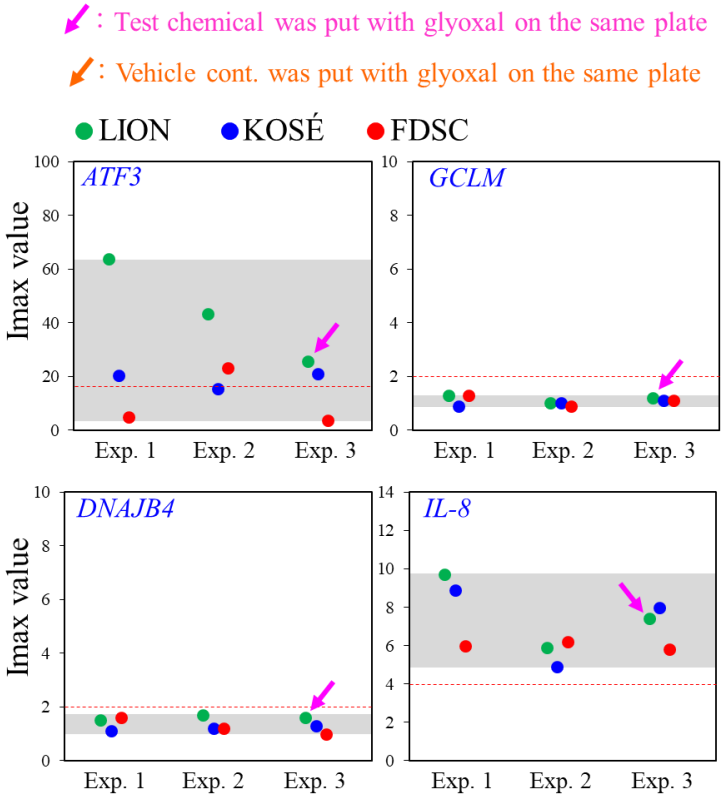


Figure 16. Imax values of the four marker genes as obtained from three repetitions in the three participating laboratories, when sodium lauryl sulfate was tested. Gray areas represent the variation of the Imax value among three repetitions in the three participating laboratories except for the results that are likely to be affected by a cross-contamination with glyoxal.

d. Revision of the SOP

The following revision was proposed, discussed and approved at a web meeting of the VMT (that took place on the 23rd of September 2020) after the end of the Phase I-C study; this modification was, thus, included in the SOP version 2.4.

- A cautionary note regarding the liquid chemical exposure was added. In order to avoid cross-contamination by volatile compounds, the tissue units that are used for liquid test chemicals should be kept separated from other test chemicals and controls (e.g., positive controls and vehicle controls) into individual 24-well plates.