A skin sensitizer refers to a substance leading to an allergic reaction after repeated skin contact. The prediction of skin sensitization is recognized as an important endpoint in toxicology, required by regulatory agencies for hazard identification, as well as used for risk assessment. A battery of in vitro tests have been developed based on the current knowledge of the key biological events leading to skin sensitization. The h-CLAT assesses the activation of monocytes and dendritic cells by determining the expression of cell surface markers, CD69 and CD45, in a human monocyte/macrophage cell line (THP-1). Although the h-CLAT has been found to be applicable to chemicals with diverse functional groups, its predictive performance is limited to substances that are soluble in the assay solvents (DMSO and Sal) specified in the validated method. Therefore, we aimed to determine the compatibility of various solvents with the h-CLAT and to evaluate its predictive capacity. The h-CLAT was performed as previously described (OECD TG 442E, 2019). In addition to the standard solvents, Acetone (AC), Acetonitrile (ACN), Ethanol (EtOH), Methanol (MeOH), and Isopropanol (iPrO) were evaluated. All the solvents were determined to be non-Sensitizers and non-Cytotoxic. Ten test substances were selected for assessment of their sensitization potential: the profency chemical lactic acid (LA) and benzyl salicylate (BS), a known ‘difficult to work’ chemical in h-CLAT, LA was found to be soluble in all the test solvents while BS was found to be insoluble in all the solvents except for Saline. LA was correctly predicted as a non-sensitizer when prepared in EtOH, AC, and MeOH, however, it was mispredicted in ACN and iPrO. BS was classified as a non-Sensitizer in DMSO, EtOH, MeOH, while it was classified as a Sensitizer in AC, ACN and iPrO. BS was regarded as a Sensitizer based on visual data, however, results from human experiments suggest this is a false positive. Based on the LA results, EtOH, ACN, and MeOH appear to be alternative solvents for the h-CLAT. Furthermore, iPrO and MeOH, classified consistently in line with human data which is the most relevant dataset to compare our results. Our data indicate that the selected solvent has a profound effect on the results of the h-CLAT. The inclusion of DMSO and MeOH as primary solvents may improve and extend the applicability domain of the h-CLAT.

Introduction
Poorly soluble test substances are difficult to assess via flow cytometry, may produce inconclusive results and miss classification of the sensitization potential. Our goal was to determine if other solvents besides DMSO or Saline can be used, and thereby expand the applicability of the h-CLAT. The five solvents selected for testing were anticipated to be non-cytotoxic on their use in other applications. Our test substances, Benzyl Salicylate (BS) and Lactic acid (LA), were chosen based on their reported sensitization potential and solubility. BS is used as a fragrance ingredient which is poorly soluble in water with a log Kow (octanol-water partition coefficient) of 4.3. This is higher than the 3.5 threshold applicable to the h-CLAT. BS is soluble in alcohol and this better solubility was achieved using some of our test solvents. BS is a predicted sensitizer based on its use in vitro and in vivo trials. However, in human clinical trials, BS has been determined as a non-sensitizer. The positive results are likely a false-positive due to challenges with preclinical test approaches. Therefore, BS was considered as non-sensitizer for the current study. LA is a non-sensitizer included as a proficiency chemc in h-CLAT, and soluble in most polar solvents; LA can react with alcohols in an esterification reaction, which may affect the h-CLAT results, and thus, was included as a reference test substance.

Materials & Methods

Figure 2. Methods for the h-CLAT definitive assay. The h-CLAT was performed according to the OECD TG 442E, 2019. The test substances were diluted in the selected solvents and then a total of 8 serial dilutions were prepared. A dose range finding assay was performed to establish the dose used in the definitive assay (not shown). THP-1 cells were cultured in a 24-well plate at a final density of 1x10^5 cells/well for 96 h. After dosing, the cells were washed with PBS buffer for 3 times. A blocking solution was added to the cells and they were incubated at 4°C for 15 minutes. The cells were then plated on a 96 well plate and added with 3 different FITC-conjugated antibodies (CD69, CD45, and isotype control). After a 30 minutes incubation the cells were rinsing and then the surface marker expression was determined by the Mass cytometer.

Table 2. Relative Fluorescence Intensity (RFI) and cell viability results of the selected solvents in the h-CLAT. The solvents were tested at a single final concentration of 0.2%. The presented values are the average of two independent trials. In order for a solvent to be used in the h-CLAT (the following three criteria must be met: a) the RFI for CD69 must be less than 200%; b) the RFI for CD45 must be less than 150%; and c) cell-viability must be greater than 90%). The selected solvents were considered usable in the h-CLAT since they meet the three criteria.

Figure 3. Expression of CD69 and CD45 for Benzyl Salicylate and Lactic acid in the test solvents. Two independent definitive trials were performed (shown as purple and red bars, respectively). For a test substance to be determined as a sensitizer the CD45 RFI must be higher than 200% and/or the CD69 RFI must be higher than 150%, and the results must be unequivocal in two independent trials. The black trend line in the graphs indicates the threshold for a test substance to be considered a sensitizer.

Conclusions & Future Directions

- Ethanol and Methanol allowed to correctly classify the skin sensitization potential of the test substances in the h-CLAT.
- Isopropanol and Acetone lead to an incorrect sensitization prediction of the test substances in the h-CLAT.
- Acetone correctly predicted Lactic acid as a non-sensitizer while it mis-predicted Benzyl Salicylate as a sensitizer.
- Overall, our results indicate that Ethanol and Methanol are promising solvents to be used in the h-CLAT. Further testing is recommended to corroborate the results in a larger set.
- The methodology presented may useful for the screening of novel solvents to be used on the h-CLAT.

References