

Improving the Accessibility and Efficiency of the Human Cell Line Activation Test

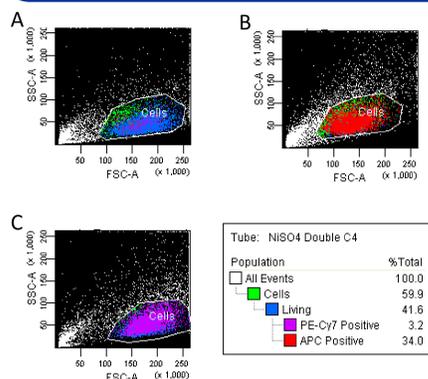
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ABSTRACT

The Human Cell Line Activation Test (h-CLAT) is an *in vitro* method for determining skin sensitization potential of test chemicals by addressing dendritic cell activation. The assay monitors expression of cell surface markers CD54 and CD86 on THP-1 cells using flow cytometry. Developed by Kao Corporation and Shiseido Company, h-CLAT has been shown to accurately identify skin sensitizers, and a draft OECD test guideline for the assay is currently under review. Based on the complexity of the sensitization pathway, h-CLAT can be used in conjunction with the Direct Peptide Reactivity Assay and KeratinoSens™ assay as part of an integrated approach to testing and assessment. Therefore, it is important to incorporate h-CLAT into standard *in vitro* sensitization testing in a variety of laboratories using varying flow cytometry platforms. We performed the assay using a BD FACSAry platform which uses different lasers and requires different fluorophores than those identified in the draft guideline. Additionally, we examined the ability to identify both surface markers simultaneously using different fluorophores (ie: PE-Cy7 labeled CD86 and APC labeled CD54). This would increase assay efficiency and permit faster data acquisition. During this project, THP-1 cells were exposed to the ten proficiency chemicals listed in the draft OECD guideline for 24 hours. Following exposure, cells were stained with PE-Cy7 labeled mouse anti-human CD86 antibody, APC labeled mouse anti-human CD54 antibody and/or APC labeled mouse anti-human CD86 antibody. Propidium iodide (PI) was used to determine viability. Samples were examined by flow cytometry (n=10,000 events) to determine the Relative Fluorescence Intensity of surface marker expression. Our results showed that we were able to correctly identify sensitizers and non-sensitizers among the proficiency chemicals. These results indicate that it may be possible to expand the h-CLAT procedures to allow different laboratories to utilize available equipment by demonstrating proficiency using the chemical set. Also, it may be possible to incorporate double staining to enhance the overall efficiency of the assay.

BACKGROUND



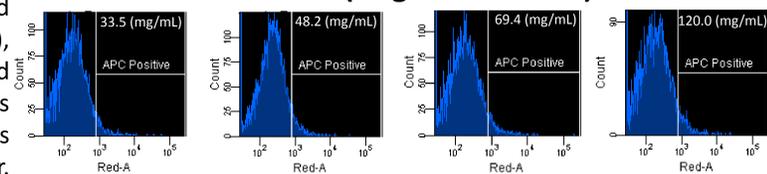
This study was conducted using three colors for staining and reading on a BD FACSAry. In order to classify a test material as a sensitizer or non-sensitizer, the test material concentrations tested and analyzed must result in a viability greater than or equal to 50%. PI was used to stain the non-living cells; therefore, allowing the living cells to be gated. These cells are indicated as blue data points in the dot plot (A). Two additional fluorophores were used, APC (B) and PE-Cy7 (C), in order to indicate the occurrence of CD54 and CD86 on the surface of the cells.

SINGLE STAINING

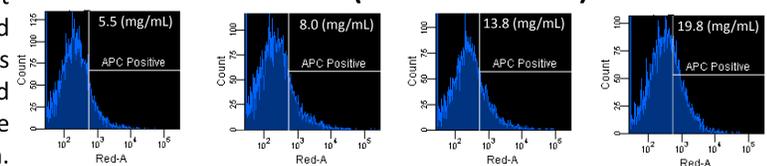
These are results for single staining of the negative control (lactic acid; LA) and the positive control (NiSO₄), both stained with APC labeled CD54 antibody. The graphs show the number of events detected using the red laser. As expected, CD54 is not detected on the lactic acid treated cells; CD54 is detected on the NiSO₄ treated cells and appears to increase with increasing concentration.

APC CD54

Lactic Acid (Negative Control)

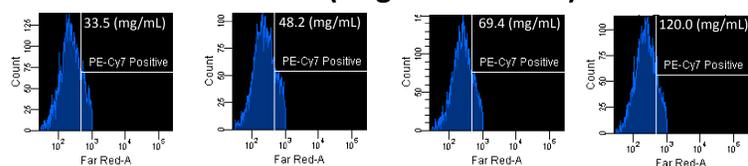


Nickel Sulfate (Positive Control)

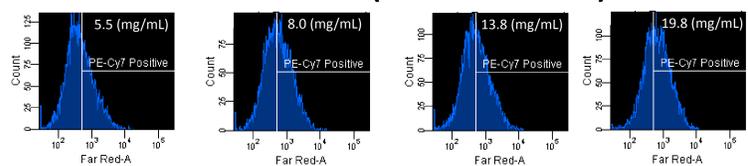


PE-Cy7 CD86

Lactic Acid (Negative Control)



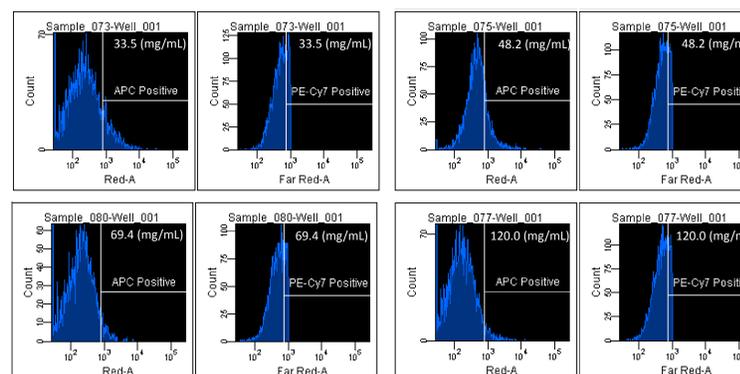
Nickel Sulfate (Positive Control)



These results show the staining for CD86 using PE-Cy7 labeled antibody. The negative control shows few positive events, while the positive control shows increasing amounts of positive events as the NiSO₄ concentrations increase. The standard assay is typically done with one fluorophore, but for purposes of double staining we tested samples with both APC and PE-Cy7 labeled antibodies.

DOUBLE STAINING

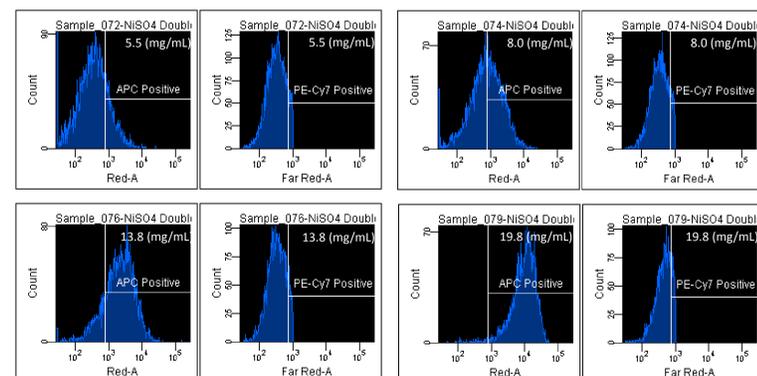
APC CD54 and PE-Cy7 CD86 Lactic Acid (Negative Control)



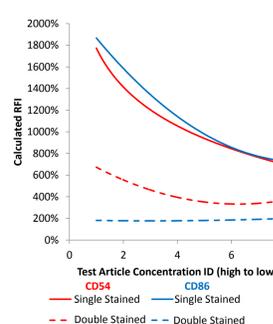
The graphs represent double staining using APC labeled CD54 antibody and PE-Cy7 labeled CD86 antibody. The results for lactic acid are consistent with what was seen for the single staining of lactic acid. There are not significant APC and PE-Cy7 positive events detected for the negative control treated samples.

Nickel Sulfate (Positive Control)

The graphs represent the double staining for the positive control, again using APC labeled CD54 antibody and PE-Cy7 labeled CD86 antibody. From the graphs shown, there is a response for APC positive events, which would lead to the conclusion that NiSO₄ is a sensitizer. Contrary to expectations, a positive response from the PE-Cy7 labeled antibody is not observed.



COMPARISON OF STAINING TECHNIQUES



The single staining and double staining techniques were both performed for the ten proficiency chemicals listed in the draft OECD Guidelines. This graph represents results from the single and double staining assays performed using the validation chemical, imidazole. According to the draft guideline and our single staining assay results, imidazole is a skin sensitizer (values over 200% and 150% are considered positive for CD54 and CD86, respectively). The double staining assay results confirm the designation as a sensitizer; however, relative to the single staining results, the observed expression of CD54 (indicated in red) and CD86 (indicated in blue) is reduced.

CONCLUSION

We sought to show that it is possible to adapt h-CLAT to use equipment other than what was used for the development and validation of the assay. Our study has shown that simple modifications, such as the fluorophores used for staining can be done and the assay can still yield results conclusive with the original validation. Also, the h-CLAT protocol uses single staining with one fluorophore, but there are two cell surface markers monitored in the assay. This means that there needs to be two samples of cells treated with each test article concentration as one sample will need to be exposed to antibody for CD54 and one sample will need to be exposed to antibody for CD86. As we were testing our modifications to the assay, we began testing the possibility to double stain. This allowed us to use one sample of cells treated with the test article for each test article concentration. The cells were stained with two different fluorophores, allowing for simultaneous detection. Our results have shown that the assay is transferable to another flow cytometry platform with minor adaptations to the procedure. The biggest obstacle we found using a different platform is that the concentrations of antibody mentioned in the protocol did not work for our system and we needed to test different concentrations of antibodies to find what allowed us to detect increases in CD54 and CD86. With the double staining, there were mixed results when testing the set of ten validation chemicals. There were instances where double staining produced results comparable to the single staining, and there were other instances where the double staining led to false positive or negative results. Some of the false results may have been associated with spillover among the fluorophores. Future work needs to focus on testing different fluorophores and concentrations of fluorophores to find a better set that works for double staining on our instrument.