Development of a Non-Animal Testing Strategy for Ocular Hazard Labeling of Some Specific EPA-Regulated Products

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Summary

A joint effort between industry and the EPA led to the development of a three-assay in vitro testing strategy for determining hazard labeling for ocular toxicity. The EPA then instituted an 18-month pilot program whereby manufacturers were encouraged to submit data using the in vitro testing strategy to support ocular hazard labeling requirements for registration of cleaning products with antimicrobial claims. Six acceptable in vitro eye irritation studies were received during the pilot program. Based on these results and, in concert with the data generated to develop the in vitro ocular labeling strategy, the EPA has determined that the BCOP, EpiOcular™ , and Cytosensor assays, when used according to the strategy, can be employed to categorize antimicrobial cleaning products with respect to the four categories of ocular hazard categorization.

Keywords: ocular toxicity, alternatives to animal testing, labeling

1 Introduction

More than five thousand regulatory decisions are made annually by the United States (US) Environmental Protection Agency's (EPA) Office of Pesticide Programs (OPP), which ensures the safety of the approximately 1,100 active pesticide ingredients found in approximately 19,000 pesticide products used in the US. OPP's mission is to evaluate the properties of pesticide products to ensure that, if used according to the label, they will be safe for human health and the environment.

Thus, safety evaluations are required for both human and environmental health, and all available and relevant scientifically sound information is used to ensure that the best possible regulatory decisions are made.[1] Evaluations are made about the safety of "active" ingredients, which include conventional, biochemical, and antimicrobial active ingredients, as well as "inert" ingredients for both food- and non-food use pesticides. However, the data required for registration varies with the type of pesticide chemical being registered, with extensive data submission required for food use pesticides with conventional active ingredients, and lesser requirements for non-food use pesticides and active ingredients.

2 Challenge

The regulatory decisions that need to be made each year on a large number of chemicals that may cause many possible adverse outcomes require the expenditure of both resources and time. However, only finite resources and time are available to meet OPP's mission and the public's expectation for scientific soundness and timeliness. Furthermore, the science available for safety evaluation and risk assessment is increasingly complex and changing, so the process is constantly evolving to meet the risk assessment and risk management challenges that always arise. In this context, it is critical for OPP to ensure transparency and build confidence in the new, more efficient and improved tools and methods being used to evaluate safety so they can be increasingly used to inform decisions.

It is clear that while the current paradigm is time tested and provides a strong basis for credible and reliable risk management decisions, it is also time consuming and resource intensive, both in terms of dollars and animal usage. Furthermore, it is not easily adaptable when new issues arise (e.g., endocrine disruption, cumulative risk), and therefore an alternative approach is needed that provides the broad coverage of information needed to effectively evaluate human and environmental health risks and that can be readily augmented as knowledge continues to evolve. The long-term solution is not to generate more information faster, but rather to identify what specific data for which chemicals, which exposures, and which populations are essential to assess risk (Bradbury et al., 2004). In recognition of this challenge, OPP's objectives are to evaluate pesticides more efficiently and with greater accuracy to enhance its abilities to target effects of concern and to focus on the information most relevant to the assessment.[2]

Disclaimer: The views expressed in this paper are those of the authors and do not necessarily reflect the official views of the U.S. Environmental Protection Agency.

[1] http://www.epa.gov/pesticides/about/aboutus.htm

[2] http://www.epa.gov/pesticides/science/testing-assessment.html

3 Approach

The National Academy of Sciences vision for Toxicity Testing in the 21st Century (NRC, 2007), in conjunction with OECD's integrated approach to testing and assessment (IATA) (OECD, 2008), have been adopted by OPP to provide the means for moving from the current status (where there is a heavy reliance on animal studies to generate information for all possible outcomes based on traditional toxicity studies) to a future where there is less reliance on animal studies and where data generation is tailored based on an understanding of toxicity pathways.

IATA is not a new concept in that it integrates existing knowledge with information from new technologies, coupled with combined estimates of exposure in a manner that leads to better predictions of risk for regulatory endpoints. Plausible and testable hypotheses are formulated to target *in vivo* testing on chemicals and endpoints of concern. It has been used extensively to evaluate chemicals that lack data in OPP for the evaluation of pesticide inerts and in the EPA's industrial chemicals program, where (Q)SAR, read-across, and various other tools and types of information are used in a weight of evidence approach to evaluate safety.

The Adverse Outcome Pathway (AOP) concept (Ankley et al., 2010) (Fig. 1) is the key to achieving IATA, providing a framework that portrays existing knowledge about the linkage between a direct molecular initiating event and an adverse outcome at a level of biological organization relevant for risk assessment. AOPs provide the basis for using lower tier tests and non-animal models for read across, risk assessment, and the development of expert systems.

OPP has traditionally used the Draize rabbit eye test to determine ocular hazards for required hazard labeling for pesticide products (Fig. 2). Recently a testing strategy using three *in vitro* assays – the Bovine Corneal Opacity and Permeability

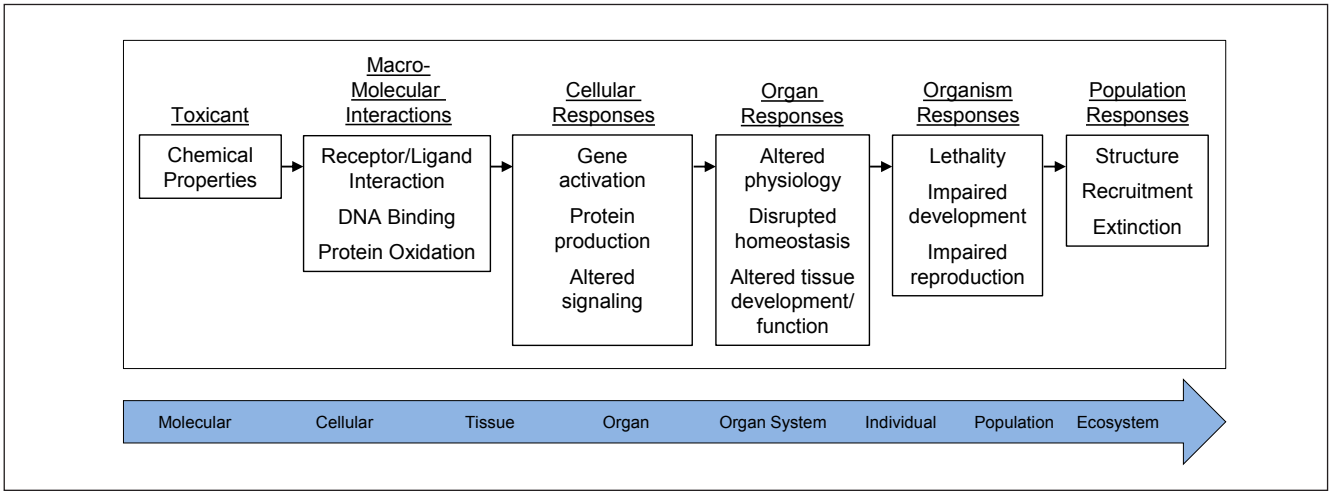

Fig. 1: Adverse Outcome Pathway

An Adverse Outcome Pathway (AOP) is a conceptual framework that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a level of biological organization relevant to risk assessment (Ankley et al., 2010).

Toxicity Category	Signal Word	Statements
I	DANGER	Corrosive.* Causes irreversible eye damage. Do not get in eyes or on clothing. Wear (specify appropriate protective eyewear such as goggles, face shield, or safety glasses). Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum, using tobacco or using the toilet. Remove and wash contaminated clothing before reuse.
II	WARNING	Causes substantial but temporary eye injury. Do not get in eyes or on clothing. Wear (specify appropriate protective eyewear such as goggles, face shield, or safety glasses). Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum, using tobacco or using the toilet. Remove and wash contaminated clothing before reuse.
III	CAUTION	Causes moderate eye irritation. Avoid contact with eyes or clothing. Wear (specify protective eyewear, if appropriate). Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum, using tobacco or using the toilet.
IV	CAUTION (optional)	No statements are required. However, the registrant may choose to use category III labeling.

Fig. 2: Hazard classification categories for primary eye irritation

Test (BCOP), the EpiOcular™ model (EO), and the Cytosensor Microphysiometer assay (CM) – was developed and evaluated as an alternative for ocular hazard labeling of antimicrobial products with cleaning claims in the Antimicrobial Cleaning Products Pilot Program (AMCP).[3]

An *in vitro* AMCP approach was suggested by OPP's Pesticide Program Dialog Committee (PPDC) because many companies marketing cleaning products did not conduct animal testing for eye irritation but rather used an *in vitro* testing strategy. However, if the cleaning product is claimed to have "antimicrobial" properties, even if it is identical to a previously marketed product that was not required to be reviewed by EPA, it now becomes subject to EPA's pesticide registration jurisdiction. Under EPA regulations, animal testing is now required. Both EPA and the industry want a predictive and conservative *in vitro* testing strategy that uses as much existing data as possible, that builds upon human experience whenever possible, and that does not require additional animal testing. The AMCP manufacturers participating in the pilot program are listed in Table 1. They agreed to share their data from non-animal *in vitro* ocular studies as a basis for comparison with historical *in vivo* animal data. A database was created to allow OPP to differentiate between the four irritation hazard categories used by the Agency by gaining experience with these non-animal test methods to assess eye irritation and comparing data generated from them to data generated from conventional ocular toxicity tests building on the IATA philosophy.

Tab. 1: Participants in the Antimicrobial Cleaning Products Pilot Program

Clorox
Colgate Palmolive
Dial
EcoLabs
JohnsonDiversey
Proctor and Gamble
SC Johnson
The Accord Group
The Institute for In Vitro Sciences

The PPDC established an Alternative Test Working Group (ATWG) to help design, evaluate, and refine the process. The first step suggested by the ATWG was to clearly define the context and purpose of the pilot progam, and it was decided to limit it to the use of non-animal hazard evaluation of antimicrobial cleaning products to define ocular hazard labeling.

The next step was to build on what is understood about the common modes of action leading to eye injury, including membrane lysis, protein coagulation and denaturation, saponification, alkylation, and oxidative damage to macromolecules. The ATWG built on this body of knowledge to define an AOP that, coupled with the physical and chemical properties of the active ingredient and the formulation and linkages to Draize Test results from related compounds, provided a predictive basis for defining labeling categories. Based on this experience, it was concluded that one *in vitro* assay is not sufficient for eye irritation labeling purposes, and a "bottom up/top down" testing strategy was proposed (Scott et al., 2010). It also was noted that the depth of injury is predictive of the degree and duration of injury (Maurer et al., 2002), and this depth of injury model provides a first approximation to an AOP. As shown in Figure 3, there is a continuity of sensitivity for the types of damage caused by different categories of chemicals, as well as for the types of damage that can be measured by the different types of *in vitro* tests.

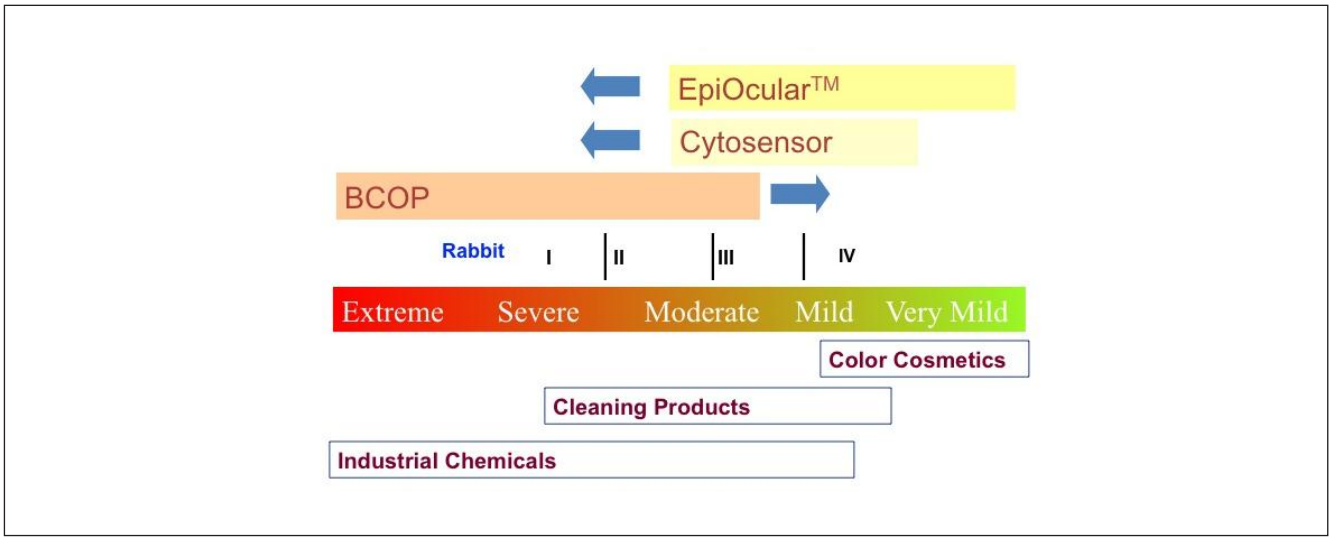

Fig. 3: A generalized representation of the continuum of sensitivity for eye irritation*
*Courtesy of the Institute for In Vitro Sciences, Inc.

[3] http://www.epa.gov/oppad001/eye-irritation.pdf

The third step in the ATWG process was to collect data, beginning with an analysis of existing historical data for the various chemicals to be analyzed and then collecting additional *in vitro* testing data where needed, in this case using the BCOP, CM, and EpiOcular™ assays. Chemistries were considered important in thinking about how to stage the tests in terms of an efficient testing process. Choice of starting point did not affect the outcome of the effort, just the efficiency of the process. The products were first divided into formulations based on oxidizing chemistries and those not based on oxidizing chemistries, and these classes were further divided into subclasses including: surfactants, acids (pH less than 4), alkalines (pH greater than 9), solvents, high solvents (greater than 5% solvent), and oxidizing chemistries (e.g., hypochlorite, peroxide, percarbonate, oxygen bleaches, etc.).

The EpiOcular™ test is a human epithelial construct of roughly 6-8 cell layers thickness, and the chemicals to be tested are applied by topical application – similar to *in vivo* exposures. The endpoint measured is the exposure time required to reach a 50% reduction in viability, or ET_{50}, which is dependent on both the cytotoxic potential of the test substance and its rate of penetration.

The Cytosensor assay provides real time measures of cellular metabolism via small changes in the metabolic rate of L929 cells (i.e., the dose calculated to reduce the population metabolic rate as measured by the secretion of hydrogen ions from glucose metabolism to 50% of the initial rate [MRD_{50}]). The Cytosensor assay employs a single cell layer and is a dilution-based assay with a wide dynamic range, but it is applicable only to those water soluble chemicals in the mild to moderate ocular toxicity categories. It provides an *in vitro* model of damage to the epithelium of the cornea.[4]

The BCOP uses corneal explants taken from cows sacrificed for food production. The test chemical is applied topically, and direct measures are then taken of opacity and epithelial integrity. An *in vitro* score is calculated based on opacity and permeability, and additional histopathology may be conducted if warranted. Because it employs ocular explants, the BCOP provides a model of all corneal cell layers – epithelium, stroma, and endothelium.[5]

As shown in Figure 4, the three assays can be mapped to the AOP based on the depth of injury model. The results of the appropriate assay or combination of assays then can be used to more completely evaluate the ocular toxicity of the chemicals tested (Redden et al., 2009).

The final step in the process used to evaluate the three tests for use in a test battery for ocular hazard labeling was to analyze the data via graphical presentations of the *in vivo*/*in vitro* data sets where possible. The findings were that the BCOP can be used to differentiate Category I and II materials, but it is sometimes overpredictive for Category I, because approximately 12% of the time it yielded Category I results where the Draize test results in Category II damage.

It was further concluded that the EpiOcular™ test could be used to differentiate Category III materials from Category IV materials, as could the Cytosensor assay. (Redden et al., 2009). However, a recent European Centre for the Validation of Alternative Testing (ECVAM) evaluation of the Cytosensor Micro-

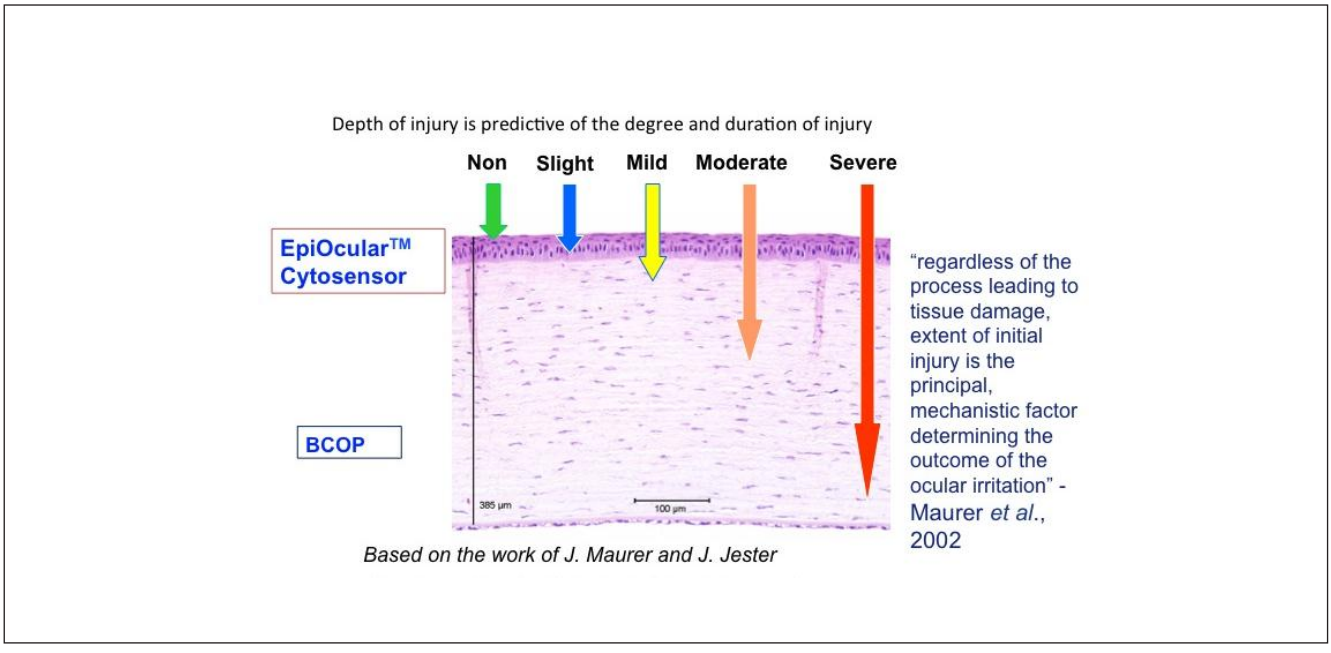

Fig. 4: A representation of the depth of injury model for ocular irritation*

*Courtesy of the Institute for In Vitro Sciences, Inc.

[4] http://www.iivs.org/scientific-services/laboratory-services/ocular-irritation/cytosensor/

[5] http://www.iivs.org/scientific-services/laboratory-services/ocular-irritation/bcop/

physiometer supports its use to classify both Category I (for all water-soluble chemicals) and Category IV ocular irritants (for water-soluble surfactants and surfactant-based products). Following the ECVAM evaluation, the OTWG concurred with this decision (Redden et al., 2009).

4 Proposed process for classification

Figure 5 outlines ATWG's classification process for using the three *in vitro* assays for ocular hazard labeling (Redden et al., 2009; Bradbury et al., 2004; Scott et al., 2010). The first step is to evaluate the product's components to determine if it is based on oxidizing chemistry. If yes, test using the BCOP assay. If not, is the chemistry expected to cause severe or moderate irritation? If so, test with the BCOP assay, and if not, is the chemistry expected to be water soluble? If yes, test with either the Cytosensor assay or EpiOcular™ assay, and if not, test with the EpiOcular™ assay.

The next steps are dictated by the results of the assays. If the results of the BCOP assay give a score of greater than 75, label the material as a Category I irritant. If less than 75, perform a histological evaluation to classify as a Category I, II, or III, as appropriate. If it is desired to separate Category III from IV substances at this point, evaluate whether the material is water-soluble and then test with either the Cytosensor or EpiOcular™ assay, as appropriate. For materials that are not based on oxidizing chemistries and that are not expected to result in severe or moderate irritation, the results of the Cytosensor and EpiOcular™ assays can be used to categorize substances into Categories I, III, or IV. To separate Category I from Category II substances, conduct a BCOP assay.

5 Results

The US EPA instituted an 18-month pilot program whereby manufacturers were encouraged to submit AMCP products for registration using the proposed *in vitro* testing strategy for ocular irritation in place of animal testing. Six acceptable *in vitro* eye irritation studies were received, as shown in Table 2.

Based on the evaluation phase and the pilot, the EPA concluded that the ocular hazard labeling can be conducted accurately for antimicrobial cleaning products using the BCOP, the Cyto-

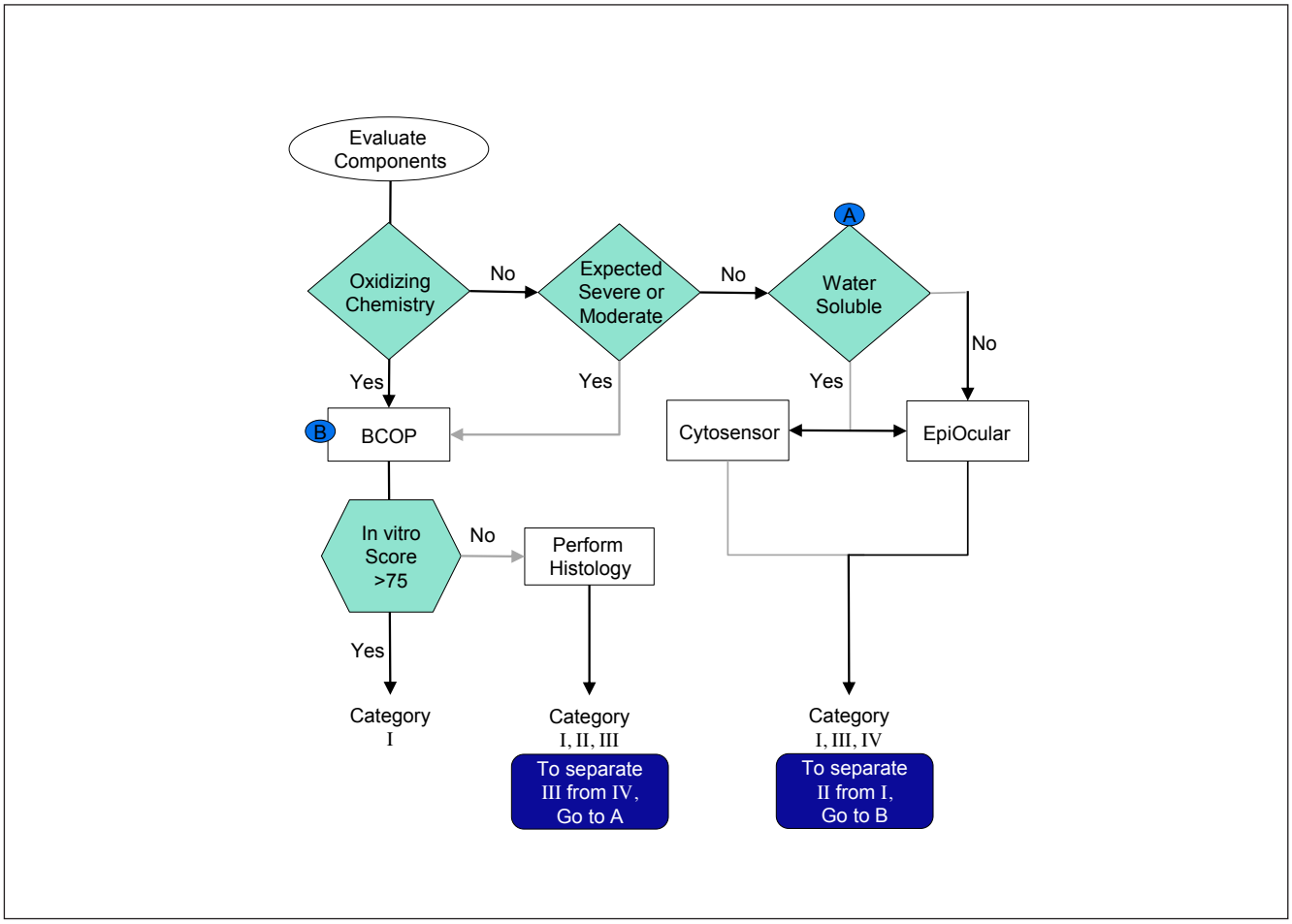

Fig. 5: Proposed process for classification

Tab. 2: Acceptable AMCP Pilot Studies submitted to EPA over the 18-month pilot period

Number Studies/Type of Assay	Chemical
2/BCOP with optional histology	beta-Alanine, N-acetyl-N-butyl-, ethyl ester
	L-Lactic acid
2/Cytosensor	Alkyl* dimethyl benzyl ammonium chloride *(60%C14, 30%C16, 5%C18, 5%C12)
	Alkyl* dimethyl ethylbenzyl ammonium chloride *(68%C12, 32%C14)
2/Epiocular™	Alkyl* dimethyl benzyl ammonium chloride *(60%C14, 30%C16, 5%C18, 5%C12)\
	Alkyl* dimethyl ethylbenzyl ammonium chloride *(68%C12, 32%C14)

sensor, and the EpiOcular™ assays, as shown in Figure 5. These *in vitro* assays were selected to address the modes of action, and they complement each other to cover the range of irritancy potential. The testing strategy is consistent with current practice and with the proposed ECVAM strategy developed with input from ICCVAM representatives.

The key reasons for success of the program were:

– It used an IATA approach.
– It only covered a limited applicability domain (i.e., Antimicrobial Cleaning Products).
– Cooperation among companies provided a larger test material set.
– There was a continual interactive dialog with regulators and company representatives throughout the process.
– Animal test variability was highlighted.
– The purpose of the test battery was for labeling purposes only.
– The approach was very conservative (i.e., only a few false negatives).

References

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