

December 30, 2004

**Via electronic transmission: stokes@niehs.nih.gov**

Dr. William S. Stokes, Director  
NTP Interagency Center for the Evaluation of Alternative Toxicological Methods  
National Institute of Environmental Health Sciences  
P.O. Box 12233  
Research Triangle Park, NC 27709

**Re: NICEATM Federal Register Notice (69 FR 64081; November 3, 2004)  
Assessment of the Current Validation Status of In Vitro Testing Methods for  
Identifying Potential Ocular Irritants; Request for Comments**

Dear Dr. Stokes:

The following comments are submitted on behalf of the Alternatives Research & Development Foundation (ARDF), the American Anti-Vivisection Society (AAVS), the Animal Protection Institute (API), the Doris Day Animal League (DDAL), People for the Ethical Treatment of Animals (PETA), and the Physicians Committee for Responsible Medicine (PCRM)—a coalition of national scientific, animal protection, and health advocacy organizations. Our comments are being submitted in response to the aforementioned Federal Register Notice soliciting public comment on four Background Review Documents (BRDs) that assess the current validation status of the Hen's Egg Test – Chorioallantoic Membrane (HET-CAM), Bovine Corneal Opacity and Permeability (BCOP), Isolated Chicken Eye (ICE), and Isolated Rabbit Eye (IRE) test for identifying severe and corrosive eye irritants.

The parties to this submission have a number of concerns regarding both the BRDs and the composition of the expert panel that has recently been formed to review these documents; however, our comments are put forth in the spirit of support for this important effort by NICEATM and ICCVAM.

### **General Comments on the BRDs**

1. We strongly challenge the BRDs' conclusions that: "Based on the analyses conducted, optimization and validation studies using a standardized BCOP [HET-CAM/ICE/IRE] test method protocol are proposed prior to its routine use for regulatory hazard classification and labeling purposes to identify ocular corrosives and severe irritants in a regulatory tiered testing strategy (e.g., GHS [UN 2003])" (Executive Summary to BRDs; also stated in Section 12.5). This statement strongly suggests that NICEATM/ICCVAM do not consider the BCOP and the other methods to be ready yet for even routine use as 'positive screens' to identify ocular corrosives or severe irritants, and that acceptance for this purpose would be contingent upon further validation. If U.S. agencies adopt this perspective as a matter of policy, it will effectively ensure that this country remains nearly a decade behind its European counterparts in terms of acceptance of these already widely used alternative methods.

Furthermore, we object to the language contained in lines 56-60 in the Executive Summaries to the BCOP and ICE BRDs, and lines 52-54 in the Executive Summary to the IRE BRD, which states: "In this strategy, positive in vitro test results are considered in a weight-of-evidence decision as to whether to classify the substance as an ocular corrosive or severe irritant. Negative results and suspected false positive in vitro results proceed to standard in vivo testing or to validated in vitro test methods that are capable of detecting false negative corrosives and severe irritants." These statements are inserted in the BRDs in a manner to indicate this is how industry uses these methods in a decision scheme, but the information is misleading to readers of the BRDs, and at least one company has already commented that this is **not** the process it uses for the assessment of in vitro data. The statements in question describe the process used in the GHS scheme, but not all companies using these in vitro methods follow the GHS scheme by conducting in vivo testing on "negative results and suspected false positive in vitro results." And, absolutely none of them proceed to validated in vitro test methods, because there are none. The inclusion of this kind of information in the BRDs has given some readers the impression that ICCVAM and NICEATM support recommending animal eye testing of severe/corrosive materials as preferable to possible over-labeling of a material based on an in vitro result. We are strongly opposed to any recommendation for additional testing of a potential severe/corrosive material in an animal's eye.

2. The BRDs should more clearly describe the assessment of test method performance, the criteria for acceptable performance, and clearly acknowledge that the calculation of an alternative method's "accuracy" is based on a comparison with a reference test (i.e., the Draize test) that has never been properly validated itself. To this end, the well known limitations of the Draize test—including its subjectivity, reproducibility, and its over- and under-prediction rate—must be openly acknowledged **and accounted for** when attempting to calculate the accuracy of a new alternative method.

When an alternative method is being compared to rabbit in vivo data, it is being assessed on how accurately the in vitro method predicts rabbit results rather than human results. Accordingly, the statement that test method accuracy is "the proportion of correct outcomes of a test method" (Footnote 3, Executive Summary) should be deleted from the BRDs. While this statement may be part of the definition adopted by ICCVAM, it is incorrect, because it would require assuming that the rabbit eye Draize data correctly predicts human eye injury/irritation.

3. There are at least two methods and/or test batteries for assessing severe/corrosive ocular irritants that should be considered for validation at this time for use in the GHS tiered scheme. We recommend that the following methods, and possibly others, be considered for ICCVAM review at this time without requiring additional optimization or validation studies:

A. The BCOP assay: For certain classes of materials, the BCOP assay has shown acceptable performance for assessing severe/corrosive materials, and data from this test method are accepted for this purpose by regulatory authorities in some European countries. Therefore, we would have expected that the BCOP BRD would have included a comprehensive review of this method vis-a-vis the chemical and product classes for which its

historical performance has been acceptable, and were alarmed to read in S.C. Johnson's comments that data sets submitted by the company were apparently not included in NICEATM's assessment of the BCOP. If this is indeed the case, the BCOP BRD should be revised to give proper consideration of **all** available data.

Additional information that should be considered in a retrospective evaluation of the BCOP assay was only briefly mentioned. Lines 187-192 of the Executive Summary state that "Since the current in vitro categorization scheme described in this BRD was developed based on correlation to maximum average scores (MAS) obtained in the Draize rabbit eye test, it may be possible to increase the accuracy of the BCOP test method for detecting GHS Category 1 (UN 2003) and EPA Category I (EPA 1996) substances by further optimizing the decision criteria for these classification systems." Revision of the in vitro classification scheme(s), and the use of this in vitro data for correlation to MAS and other in vivo scores would provide information on how much the BCOP could be optimized by using the correct in vivo score(s) and in vitro classification schemes for test method analysis. Additional optimization and validation studies are not required to perform this analysis, and it would be useful to have this information included in a revised BCOP BRD.

B. A test battery: The BRD's evaluate each of the four ocular test methods as a stand-alone test, and the rationale for this approach is unclear as some tests would clearly be of more use as part of a test battery. Several of the methods being reviewed have been in use for 20 years or more, and have been optimized and evaluated in more than one validation study. Thus, there is no reason to subject these methods to additional studies. All of the methods reviewed in the BRDs have shown an ability to identify severe/corrosive eye irritants. Acknowledging that each method alone has different weaknesses and strengths, we recommend that the complementary strengths of several of these assays be used to identify a test battery for assessing severe/corrosive ocular irritants. There may be sufficient experience by industry users of these methods to identify an appropriate test battery scheme, or sufficient data in the BRDs to identify a feasible approach for use at this time. The test battery will then serve as the "in vitro assay" to identify severe and corrosive eye irritants in the GHS tiered scheme. [An example of this is described on page xxxv, lines 61-63, of the HET-CAM BRD where industry as part of a tiered test scheme used HET-CAM to identify severe/corrosive eye irritants and then used a second in vitro method to detect possible false negative severe/corrosive materials.]

If it is determined, after an exhaustive analysis, that a proposed test battery requires additional validation, ICCVAM should revise its guidelines for validation of a test battery. It is currently stated that each test in a test battery should be individually validated. This is not a workable approach, and is doomed to failure. A test battery that uses several in vitro methods to make one determination of a material's degree of irritancy should be "validated" as a unit—by evaluation of the test battery results, rather than by validating the individual test methods. We recommend that ICCVAM review and amend the validation guideline regarding this issue.

4. After providing its assessment of the in vitro methods in Section 12 of the BRDs, NICEATM presented several arguments that have historically been used as barriers to the validation and

regulatory acceptance of alternative methods to the Draize test: 1) the inability of the alternative method to assess reversibility of adverse ocular effects, and 2) the lack of data on all three ocular tissues when using the alternative model. These are issues that have sufficiently been addressed in the literature, but this information was omitted from the discussion in these BRDs. The information on page 5 of the comments submitted by S.C. Johnson (their comment for page 1-15 of the BCOP BRD) also adequately addresses these issues. Reversibility of the adverse effects cannot be directly measured in the in vitro models; however, it has been demonstrated by Jester, et al. (1998) and Maurer, et al. (2002) that the degree of acute corneal injury typically correlates with the extent and duration of the injury. The panel of ophthalmology experts assembled to consult with the ILSI Animal Alternatives Task Force concluded that consumer products do not typically penetrate the cornea and cause deeper damage without significant corneal effects (personal communication, Sherry Ward, September 1996). Even for a human cell-based corneal epithelial model, the in vitro endpoint was found to correlate reasonably well with the in vivo “days to clear” score (Kruszewski, et al., 1997). John Ubels and others have suggested that endothelial damage could be used in a corneal organ culture model such as the BCOP assay as an indicator of reversible damage. The Draize test was developed many decades ago, and scientific advances **have** occurred since that time. Therefore, we should not let our ability to progress on to new methods be hindered by inflexible thinking. We can evaluate in vitro test method data in a way that can predict severe/irreversible effects.

The Draize test evaluates damage from a test material to the cornea, conjunctiva, and iris of the rabbit eye, and corneal effects account for 73% of the Draize score. Three of the models evaluated in the BRDs are corneal models, and one conjunctival. Therefore, the in vitro models cannot literally replace the Draize test, but again they don't need to do this to perform well. Lovell (1996) analyzed Draize tissue scores using Principal Component Analysis, and showed that the Draize tissue scores are related to one another. In support of Lovell's conclusion, the individual Draize tissue scores for cornea, conjunctiva, and iris for 25 surfactant formulations all correlated well with the in vitro test results from the Gillette HCE-T TEP assay (personal communication, Sherry Ward).

5. We remain concerned that confirmatory animal testing will still be required for ocular irritants and non-irritants by use of the GHS tiered scheme. It was stated that the GHS tiered scheme would soon be in global use. We would like ICCVAM to determine the feasibility of working with the international regulatory community to revise the GHS scheme for ocular irritants to eliminate confirmatory animal testing of materials that were negative for Category 1 or 2 classifications.

One way to achieve this would be to validate (using existing data) one of the human cell-based methods that has shown excellent performance for classifying materials in the low range of irritancy (non- and mild irritants). We recommend that existing data from human cell models (including, but not limited to: EpiOcular, EpiDerm, and HCE-T) be reviewed as soon as possible by ICCVAM for applicability as a validated in vitro model(s) to use in the GHS tiered scheme for classifying non-irritants and irritants.

6. There are animal welfare concerns with all of the proposed methods, in that all four of the proposed methods use animal tissues: animal eyes (BCOP, ICE, and IRE), and animal embryos (HET-CAM). Not enough focus has been given to available non-animal methodologies. The specific animal welfare concerns related to each method are addressed in the method-specific comments provided below.

### **General Comments on NICEATM Processes**

1. The selection process for any ICCVAM expert panel should be open and transparent, but has instead been secretive with respect to the ocular expert panel. The panel members and their affiliations were not made public until December 27—just two weeks prior to the panel meeting. Stakeholders in the process had no opportunity to make comments on panel member selection, including potential conflicts of interest.
2. The purpose of the ocular expert panel and the weight of their recommendations in the final decision-making process are unclear, especially given the fact that NICEATM appears to have taken over some of the panel's duties and decisions. Instead of presenting panel members with the data and allowing them to make recommendations on the validation status and protocol for each of the four methods, NICEATM took the liberty of making these recommendations itself in the BRDs. The information as presented in the BRDs is "leading" the expert panel to NICEATM's/ICCVAM's desired outcome. NICEATM decided what subset of the submitted data to be considered for the data analysis used to make recommendations, interpreted the results, made comments on the performance of each method, and then went so far as to state that additional optimization and validation studies are needed for each method. The expert panel should have decided whether to make these recommendations, and should now be made aware of all other options, such as possible retrospective validation of one of these methods and/or a test battery. Now that the expert panel has been subjected to this "group think," it will be more difficult for them to explore alternative approaches. A cynical person might think that the opinions of the expert panel are not really of interest to NICEATM/ICCVAM.
3. NICEATM requested in a Federal Register notice that individuals and companies submit existing in vivo Draize data, in vitro test data, and corresponding test material information for their use in developing the list of test materials to be used in future ocular test method optimization and validation studies. Two comments: 1) There is still a great deal of ocular test data that industry has not submitted, and NICEATM should explore additional options for obtaining that data (regulatory agency submissions, contacting the companies directly, etc.); 2) ICCVAM should be more proactive in obtaining this kind of information to support the development and validation of other types of in vitro test methods.
4. NICEATM should be as helpful to industry-sponsored studies as they have been to this EPA-requested analysis of the four ocular methods. As one example, NICEATM could have requested existing Draize data (as described in #3 above) to support several previous industry-sponsored validation studies of in vitro ocular methods.

5. Two comments on test material selection for future validation studies: 1) Ophthalmologists and cell biologists should be consulted to identify what chemical classes and product types may be incompatible with specific in vitro/organ culture models. This would prevent data from incompatible materials (as far as prospectively can be determined) from unknowingly skewing the performance evaluation results for classes of materials that are compatible with that particular assay; 2) The manufacturing history should be traced as much as possible on test materials that are to be included in future validation studies. Triton X-100, for example, is commonly used in studies to evaluate the performance of in vitro ocular methods. When the manufacturing history of Triton X-100 was evaluated, it was found that since the development of the in vivo Draize data that ownership of this detergent had changed and the location for manufacturing the material had changed two times. Thus, there is a very high degree of uncertainty as to whether the Triton X-100 that is available on the market today is of the same purity and composition as what was tested in rabbit eyes many years ago. Therefore, it is probably irrelevant to compare test results from new assays to the historical Draize data for this substance.
6. ICCVAM's guidelines for validation of new methods state that a new test method should cover the range of expected in vivo toxicity and also be compatible with all of the chemical classes and product types typically tested (this concept is also mentioned in the BRDs). Neither of these concepts is typically feasible for an in vitro test method, and there is no justification for this guideline, because an in vitro assay can be validated for any subset of test materials or range of in vivo irritancy as long as that is clearly defined in the purpose stated for what the method has been validated. ICCVAM itself has deviated from this guideline, because the four ocular methods are being reviewed for only the upper range of irritancy (severe/corrosive), and all of the methods appear to have some chemical classes or product types for which they will be incompatible. Therefore, we recommend that ICCVAM delete or revise this guideline, so as to make a workable process more obvious to future test method developers.

### **Specific Comments on Individual BRDs**

#### **Hen's Egg Test – Chorioallantoic Membrane (HET-CAM)**

The HET-CAM method was developed as a model for the conjunctival ocular surface prior to the development of human cell models and refinements to the BCOP assay and other organ culture models. Corneal effects comprise 73% of the Draize score, and conjunctival effects only 18%. Furthermore, the test is based on using an egg membrane rather than ocular cells, which raises some doubt as to the biological relevance of the model and the end points evaluated. However, performance data for the HET-CAM method (Table 12-2) was as good as, or better than, the other methods being evaluated. We think the HET-CAM method would be most useful as part of a test battery to provide data that will complement one of the other methods. The historical HET-CAM data had very few false-negative results. Therefore, this method might be paired with the ICE method, which was found to produce few false-positives, as a test battery for use in the GHS tiered scheme as the "in vitro method" to identify severe/corrosive materials. The complementary strengths of these two methods are apparent in the table of comparative performance data shown on page 12-4 of the HET-CAM BRD. It is recommended that the ocular

panel and the ICCVAM evaluate the possibility of validating this or a similar test battery approach.

*Animal Welfare Considerations:* The recommended protocol for the HET-CAM assay requires the use of fertilized hen's eggs after 9 days of incubation (on day 10). The BRD states that some regulations consider a bird a protected animal at times greater than half of the gestation period, or at 10.5 days (page 2-6, HET-CAM BRD). Using the eggs at 10 days would likely result in some of them being used at >10.5 days. Therefore, the egg incubation time should be decreased by 1-2 days. It is stated that the CAM membrane is formed by day 6 of gestation (page 2-5, HET-CAM BRD). If the HET-CAM test is selected for further use, then we recommend that the revised protocol call for incubation of the eggs for only 7 (or 8) days and testing on day 8 (or 9). This revision takes into consideration the fact that the earlier the eggs are used the less likely a chick embryo is to experience pain and suffering. Additionally, the references to "personal communication" with European researchers are insufficient proof that the embryo is unlikely to experience pain prior to day 10, or that the CAM is not sensitive to pain (page 10-2, HET-CAM BRD). More specific information on these points should be obtained from avian biologists and/or veterinarians.

*Recommendations:* It is recommended that no additional investment be made in refining or validating the HET-CAM method as a stand-alone test, but that it be considered for use as part of a test battery. Because of the large amount of data on this method, retrospective validation of a test battery using existing data should be considered. If it is determined that a test battery needs additional validation, then the HET-CAM assay should only be refined and validated as a component of that test battery (see comment #2B).

It is also recommended that NICEATM revise the HET-CAM BRD to reflect the corrections [and possibly missing data] submitted by ZEBET.

### **Bovine Corneal Opacity and Permeability (BCOP)**

The BCOP assay has been used successfully for many years by industry to make product development and safety decisions. There is a great amount of data available for analysis for retrospective validation of this method. NICEATM selected a subset of the data for a limited analysis, and concluded that the method is not ready for validation at this time. The BCOP is perhaps the most "optimized" and "validated" in vitro model that we have at this time. If it is not possible to validate this method for only a limited range of irritancy (severe/corrosive irritants), and certain classes of chemicals, even when negatives will still be re-evaluated in an in vivo test, then what is the point of doing additional studies on this method. If ICCVAM cannot validate the BCOP method for at least certain classes of materials at this time for limited use in the GHS tiered scheme, then we question their intent to ever validate an alternative method to the Draize test.

One of the limitations identified from the analysis of the BCOP data (and a reason stated for the need for additional studies) was "the limited database of substances for which the BCOP test method has been evaluated..." (lines 192-195, Executive Summary, BCOP BRD). This statement is then contradicted by the data that shows the BCOP assay has been used to test a

large variety of chemical classes and product types. The statement in the Executive Summary is further contradicted by the following statements made later in the same BRD (Section 12.1.1, BCOP BRD): “A broad range of chemical and product classes that have included both solid and liquid substances have been tested using BCOP (see Section 3.0)” and “When compared to the other three *in vitro* test methods proposed for identifying GHS-defined ocular corrosives and severe irritants (i.e., HET-CAM, ICE, and IRE), the BCOP test method has the largest dataset, with data from a variety of chemical and product classes.” Furthermore, due to limited availability of historical Draize data of sufficient quality for use in a validation study, it is not clear that the recommendation to perform “additional comparative studies to increase the numbers of substances tested per chemical class...” (Section 12.1.1, lines 156-157, BCOP BRD) will not be the basis for using live rabbits to develop new Draize data for use in the proposed studies.

It has been known for at least a decade that the porcine cornea is the preferred animal corneal model for the evaluation of toxic effects to the human eye. The bovine corneal epithelium is many cell layers thicker than the human (or rabbit) cornea, and can therefore be expected to be less sensitive than the human or rabbit eye to topical exposures to chemical eye irritants, which is probably the basis for the large false negative rate with the current BCOP assay. It is a great concern and wonderment that regulators, industry, and contract testing labs have not jumped at the opportunity to significantly improve the performance of the “BCOP” assay with the use of porcine cornea. If availability of well-maintained and functional porcine cornea is a concern, Dr. Ubels could put NICEATM/ICCVAM in contact with a supplier.

*Animal Welfare Considerations:* The use of bovine eye tissue requires using the tissue of an animal killed for slaughter. This potentially increases the economic viability and justification of such slaughter. The animal protection community does not endorse the use of animal tissues for testing purposes, but does find it preferable to the use of live rabbits in the Draize test.

*Recommendations:* It is recommended that ICCVAM consider validating the BCOP method at this time using the existing data for use in the GHS tiered scheme as the *in vitro* method for the assessment of severe/corrosive ocular irritants. European regulatory experience with this use of the BCOP assay can be cited as the basis of this recommendation, and the accepted protocol could be classified as temporary and subject to ongoing refinement.

We also recommend that NICEATM/ICCVAM provide financial support and appropriate project management for the continued refinement of the BCOP assay, as recommended by Dr. Ubels in his posted public comment. When the BCOP assay is further refined using the proposed new holder, a different species of cornea, and possibly additional endpoints, it may perform better at identifying severe/corrosive materials, and may also show acceptable performance for assessing other degrees of eye irritation (irritant and non-irritant) as part of the GHS tiered scheme, and would therefore eliminate the need for confirmatory animal testing of any material for eye irritation potential.

We also recommend that the ocular expert panel and NICEATM/ICCVAM carefully review the comments and data on the BCOP assay submitted by IIVS and S.C. Johnson, and revise the BCOP BRD and their recommendations as needed.



### **Isolated Chicken Eye (ICE)**

The use of chicken eyes as the tissue for developing an alternative assay is appealing for a number of reasons – availability, ease of handling, a tissue-appropriate model, etc. However, the use of an intact animal eye for the evaluation of chemical toxicity effects to the cornea is a step backwards compared to the use of a corneal organ culture model. Except for the cornea, the eye globe is a vascularized tissue that deteriorates rapidly after death of the animal and excision of the tissue. The mediators released from adjacent tissues can adversely affect the integrity of the cornea under these conditions. It is therefore preferable to remove the avascular cornea and culture it separately for use in assays that require evaluating toxic effects on the corneal integrity.

*Animal Welfare Considerations:* The use of chicken eyes requires using the tissue of an animal killed for slaughter. This potentially increases the economic viability and justification of such slaughter. The animal protection community does not endorse the use of animal tissues for testing purposes, but does find it preferable to the use of live rabbits in the Draize test.

*Recommendations:* Due to the complementary predictive nature of the two assays (using non-identical test material data sets, but this is all we have at this time), we recommend the ICE and HET-CAM assay data be retrospectively evaluated for potential use as a test battery to serve as the “in vitro” assay to identify severe/corrosive materials in the GHS tiered scheme. This retrospective analysis should include optimization of the decision criteria for the in vitro methods, which was noted by NICEATM as a possible way to enhance the predictive power of the in vitro assays. Additional comparisons and statistical analysis of certain data subsets could identify optimized performance parameters of these assays for use in a test battery or sequential test scheme without the need for conducting new studies.

### **Isolated Rabbit Eye (IRE)**

The use of rabbit eyes as the tissue for developing an alternative assay is appealing for a number of reasons—availability, ease of handling, a tissue-appropriate model, prior experience/comfort in using rabbits in the Draize test, etc. Again, the use of an intact eye is not the preferred method for evaluating corneal effects, for reasons cited above (ICE).

Of the four assays evaluated, only the IRE and the HET-CAM had very low false negative rates, an extremely important performance measure for an assay assigned to identify severe/corrosive eye irritants. However, the overall performance of the IRE assay, as reported by NICEATM in Section 12 of the BRD, was not as good as the performance of the HET-CAM assay, and the number of materials evaluated with the IRE assay is more limited. Therefore, even if used as only part of a test battery, the HET-CAM would be preferred over the IRE.

*Animal Welfare Considerations:* The use of rabbit eyes requires using the tissue of an animal killed for slaughter. This potentially increases the economic viability and justification of such slaughter. Furthermore, rabbits are slaughtered at a lesser rate than chickens and cows, and the use of their eyes could lead to their slaughter solely for procuring the eye tissue. The animal protection community does not endorse the use of animal tissues for testing purposes. Besides

the biological inappropriateness of using an intact rabbit eye for testing, we find that replacing the Draize test with an assay that requires using an intact rabbit eye in its place defeats the purpose of the initiative to replace the Draize test.

*Recommendations:* It is recommended that the IRE assay not be further assessed at this time, since other assays with greater databases and comparable or better performance are available.

### **Summary**

We have proposed a number of recommendations regarding the alternative test methods proposed to assess severe/corrosive ocular irritants for use in the GHS tiered scheme, and the BRDs that describe these test methods. All four of the alternative ocular methods reviewed in the BRDs use animal tissues, and, therefore, we strongly encourage additional resources be used to support the development and validation of more technologically sophisticated and effective non-animal methods. At this time, however, we are most interested in seeing that one of these methods (and/or a test battery) is rapidly recommended for validation for this very specific application in assessing severe/corrosive ocular irritants without requiring additional studies.

The parties to this submission are strongly committed to advancing the development, validation, and regulatory acceptance of test methods that will reduce, and ultimately replace, the use of animals in toxicity testing. To support that goal, the undersigned representatives of PCRM, PETA, DDAL, ARDF, API and the AAVS respectfully submit these recommendations, and request that you review them and take appropriate action to see that they are considered during the formal review process of the four alternative ocular test methods.

Sincerely,



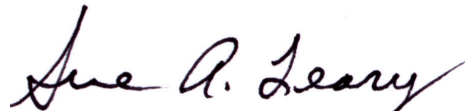
Sherry L. Ward  
Associate Director for Toxicology & Research  
Physicians Committee for Responsible Medicine



Troy Seidle  
Director of Science Policy  
People for the Ethical Treatment of Animals



Sara Amundson  
Deputy & Legislative Director  
Doris Day Animal League



Sue A. Leary  
President  
Alternatives Research & Development Foundation



Michelle Thew  
Chief Executive  
Animal Protection Institute



Tina Nelson  
Executive Director  
American Anti-Vivisection Society

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