Background Review Document of an *In Vitro* Approach for EPA Toxicity Labeling of Anti-Microbial Cleaning Products

Prepared for:

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List of Abbreviations

916		
917		
918	AC	Acidic (used to designate a formulation "bucket")
919	AISE	European Soap and Detergent Industry Association
920	AL	Alkaline (used to designate a formulation "bucket")
921	BCOP	Bovine Corneal Opacity and Permeability Assay
922	BRD	Background Review Document
923	CM	Cytosensor Microphysiometer
924	COLIPA	European Cosmetic, Toiletry, and Perfumery Association
925	CPSC	Consumer Products Safety Commission
926	CTFA	U.S. Cosmetics, Toiletries, and Fragrance Association
927	CV	Coefficient of Variance
928	DPIC	Drug & Poisons Information Centre
929	DMEM	Dulbecco's Modified Eagle's Medium
930	ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
931	EC/HO	European Commission/British Home Office
932	EO	EpiOcular™
933	EPA	Environmental Protection Agency
934	EU	European Union
935	FHSA	Federal Hazardous Substances Act
936	FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
937	GHS	United Nations Globally Harmonized Systems
938	ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
939	IIVS	Institute for In Vitro Sciences, Inc.
940	IRAG	Interagency Regulatory Alternatives Group
941	LVET	Low Volume Eye Test
942	MA	Microbiological Associates, Inc.
943	MMAS	Modified Maximum Average Score
944	MRD_{50}	Metabolic rate decrement of 50%
945	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
946	NICETAM	National Toxicology Program (NTP) Interagency Center for the Evaluation of
947		Alternative Toxicological Methods
948	OPP	Office of Pesticide Programs
949	P&G	The Procter & Gamble Company
950	PBS	Phosphate Buffered Saline
951	pH	An acidity/alkalinity index; the logarithm of reciprocal of the hydrogen ion
952		concentration
953	RC	Reactive chemistry, more generally referred to in this BRD as oxidizer (used to
954		designate a formulation "bucket")
955	SD	Standard Deviation
956	SEM	Standard Error of the Mean
957	SLS	Sodium Lauryl Sulfate
958	SM	Silicon Microphysiometer
959	SO	Solvent (used to designate a formulation "bucket")
960	SU	Surfactant (used to designate a formulation "bucket")
961	TSCA	Toxic Substances Control Act
962		

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963
964
965
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967 *in vitro* and/or *in vivo* data considered in this Background Review Document are
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969
970
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975 JohnsonDiversey, Inc., Sturtevant, WI USA
976 S.C. Johnson & Son, Inc., Racine, WI USA
977 The Procter and Gamble Company, Cincinnati, OH USA
978
979
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981
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983
984
985
986
987
988

989 **Preface**
990
991   On June 4, 2004, Mr. James Jones, Director, Office of Pesticide Programs, EPA
992 informed Dr. William Stokes, Director, ICCVAM that the EPA was developing, via a
993 subgroup of the Pesticide Program Dialogue Committee, a non-animal assessment
994 approach for evaluating the eye irritation potential of antimicrobial cleaning products for
995 the purpose of determining appropriate product cautionary labeling. Mr. Jones
996 requested that ICCVAM conduct a technical review of this approach when finalized.
997
998   This approach has been finalized and is presented in Figure I as a flowchart
999 which outlines how the EpiOcular (EO) Assay, Cytosensor Microphysiometer (CM)
1000 Assay and Bovine Corneal Opacity and Permeability (BCOP) Assay are to be used to
1001 determine the EPA toxicity Category (I – IV) with regards to ocular cautionary labeling
1002 for anti-microbial cleaning products.

Antimicrobial and Related Household Cleaning Chemistries
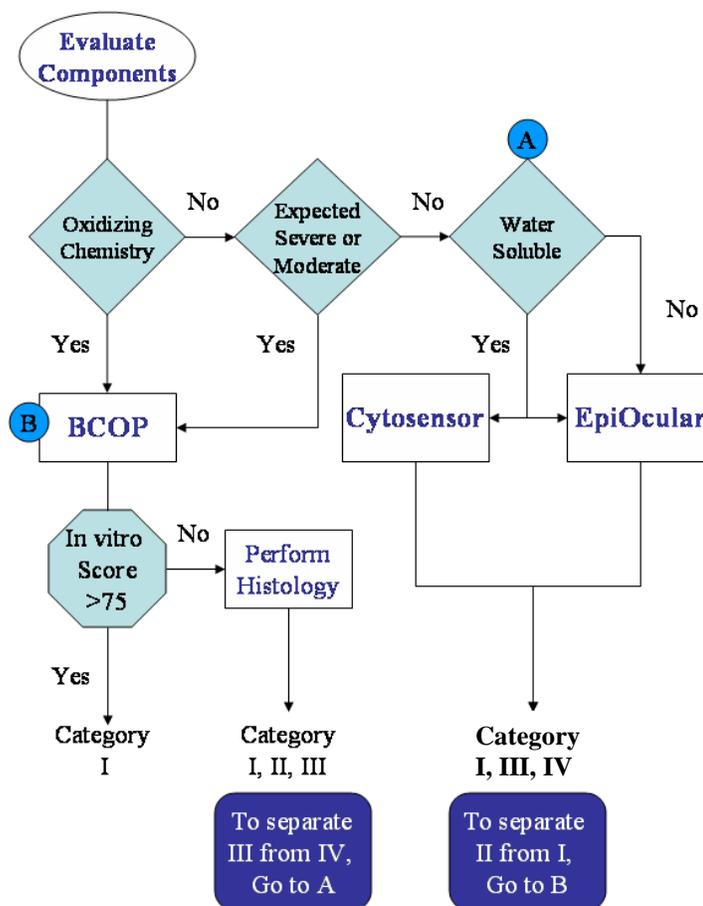
1003
1004
1005 **Figure I The proposed testing strategy for evaluating the EPA toxicity category for anti-microbial**
1006 **cleaning products.**

1007
1008   Based on the request of Mr. Jones, we now ask ICCVAM to conduct a technical
1009 review of the attached approach and supporting materials and develop an opinion on
1010 whether use of this approach will assure the EPA that, with a reasonable level of
1011 certainty, no antimicrobial product will be underlabeled.
1012

Executive Summary

1013
1014
1015   This Background Review Document (BRD) presents a description of an *in vitro*
1016 testing strategy for determining the appropriate product cautionary labeling for anti-
1017 microbial cleaning products. The strategy is flexible in that several different assays can
1018 be used either alone or combined with a second assay to obtain an EPA or GHS toxicity
1019 category. The three assays proposed are the Cytosensor Microphysiometer (CM)
1020 assay, the EpiOcular[TM] (EO) assay (MatTek Corporation, Ashland MA), and the Bovine
1021 Corneal Opacity and Permeability (BCOP) assay. A complete description of these
1022 assays and data supporting their predictive capacity and reproducibility are contained in
1023 the BRD.
1024
1025   This BRD is a joint project of seven companies – The Clorox Company, Colgate-
1026 Palmolive, The Dial Corporation, EcoLabs, JohnsonDiversey, Inc., S.C. Johnson & Son,
1027 Inc. and The Procter & Gamble Company - who manufacture anti-microbial cleaning
1028 products. Normally cleaning products are regulated by the US Consumer Product
1029 Safety Commission (CPSC), but when the product is labeled as "anti-microbial" – it is
1030 then classified as a pesticide and falls under the jurisdiction of the EPA. Registration of
1031 such products requires animal testing for several endpoints, including eye irritation, to
1032 determine the appropriate product cautionary labeling. Since many products of this type
1033 have been safely marketed (minus the anti-microbial claim) without animal testing, the
1034 companies wished to provide data supporting the position that *in vitro* test methods for
1035 eye irritation could provide adequate cautionary labeling.
1036
1037   The companies therefore provided the animal eye irritation data (using both the
1038 standard Draize test and the Low Volume Eye Test [LVET]) that were available in their
1039 files for a large set of cleaning products. At the same time data from one or more of the
1040 *in vitro* tests listed above was provided for each material, or was newly generated.
1041 These paired data sets were used to determine the predictive ability of the three *in vitro*
1042 methods. In addition, the within laboratory and between laboratory reproducibility of the
1043 *in vitro* methods was assessed.
1044
1045   As a guideline against which to asses the performance of the *in vitro* methods, an
1046 analysis of the reproducibility of the rabbit eye test was presented which shows that this
1047 *in vivo* method does not always give the same EPA toxicity category when multiple tests
1048 are run. Thus the *in vitro* methods should not be expected to provide a 100% duplication
1049 of the animal results.
1050
1051   In addition to data provided by the participating companies for the anti-microbial
1052 cleaning products, other historical studies which were conducted with similar ingredients
1053 (e.g. surfactants) or mixtures are also presented and analyzed.
1054
1055   It was found that each of the three *in vitro* tests had different areas of strength.
1056 The CM and EO assays were more sensitive and thus are useful to separate EPA
1057 category III materials from EPA category IV materials. These materials are in the milder

1058   side of the toxicity range. In contrast, the BCOP assay uses a more robust tissue and
1059   therefore is able to differentiate between EPA category I materials and EPA category II
1060   materials. These materials are in the higher side of the toxicity range. A diagram of this
1061   strategy is presented in the Preface and in Section 1. Introduction and Rationale for the
1062   Proposed Test Method.
1063
1064   The proposed *in vitro* strategy is very conservative and results in over labeling of
1065   some products, especially many EPA category IV materials which are overpredicted to
1066   be EPA category III. The participating companies are aware of these overpredictions
1067   and have accepted it as a small consequence of adopting non-animal testing strategy.
1068
1069   <u>Test Method Predictive Capacity</u>

1070   Prediction models for the three *in vitro* assays (CM, EO and BCOP) were
1071   constructed using the same approach (a graphical one). For each model all the paired
1072   *in vitro* and *in vivo* data provided were used, and the *in vitro* data were plotted against
1073   the *in vivo*-defined toxicity category (both EPA and GHS). In some cases only data from
1074   an LVET assay were available, and in other cases only data from a Draize test were
1075   available. Generally each type of data was analyzed separately, although it was
1076   concluded that the prediction models were the same regardless of the *in vivo* assay
1077   used.
1078
1079   Once the data were graphed, cut-off lines were fitted by eye to provide the "best"
1080   predictions. A description of these cut-offs then became the prediction model. The
1081   strategy in setting the cut-offs was to minimize under predictions of toxicity at the
1082   expense of over predictions. Of course, over and under predictions are somewhat
1083   arbitrary terms since we have shown earlier in this BRD (Section 4.8.1) that repeated
1084   three-rabbit eye irritation tests do not necessarily provide identical toxicity
1085   classifications. In other words, a second rabbit test may over or under predict the first
1086   test.
1087
1088   Although data from the testing of anti-microbial cleaning products (and related
1089   cleaning products) were primarily used to set the cut-offs, additional data from
1090   chemically related formulations and some pure substances (e.g. surfactants) were used
1091   to provide supporting information for our decisions.
1092
1093   Summary contingency tables showing concordance, under prediction and over
1094   prediction are presented below for each of the methods.
1095
1096   a) Cytosensor
1097
1098   The following table shows the performance of the Cytosensor in predicting the
1099   EPA toxicity category (defined by the LVET test) of 108 cleaning products.
1100   <u>There were no underpredictions of EPA toxicity categories</u>, but 89% of the
1101   Category IV materials were overpredicted as Category III or higher. However

1102   the CM was able to clearly identify some Category IV materials. Results for
1103   the prediction of GHS categories were similar.
1104

1105   The CM should be useful in clearly identifying materials as EPA Category III
1106   or Category IV, but cannot separate EPA toxicity category I from category II.
1107   Oxidizing materials, or materials not completely aqueous soluble at the
1108   highest dilution, should not be tested in the CM.
1109

LVET- Determined EPA Category	CM Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	9	0	0	9	100%	NA	0%
II	11	0	0	11	0%	100%	0%
III	40	20	0	60	33%	67%	0%
IV	4	21	3	28	11%	89%	NA
Total	64	41	3	108	30%		
Predictivity	14%	49%	100%				
Category under predicted	NA	0%	0%				
Category over predicted	86%	51%	NA				

1110
1111   b) EpiOcular[TM]
1112

1113   Animal eye irritation data from both the Draize test and the LVET were
1114   supplied paired with EO data. The following two tables show the performance
1115   of the EpiOcular[TM] assay in predicting the EPA toxicity categories defined by
1116   the by each of the *in vivo* tests. <u>There was only one underprediction for the 41
1117   total materials</u>. The EO method was able to clearly separate a few EPA
1118   category IV materials, although most Category IV materials will be
1119   overpredicted as Category III. Results for the prediction of GHS categories
1120   were similar.
1121

1122   The EO assay should be useful in clearly identifying materials as EPA
1123   Category III or Category IV, but cannot separate EPA toxicity category I from
1124   category II. Oxidizing materials should not be tested in the CM, but both water
1125   soluble and water insoluble materials can be tested.
1126

LVET- Determined EPA Category	EpiOcular Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	1	0	0	1	100%	NA	0%
II	0	0	0	0	0%	0%	0%
III	2	7	0	9	78%	22%	0%
IV	2	4	0	6	0%	100%	NA
Total	5	11	0	16	50%		
Predictivity	20%	64%	0%				
Category under predicted	NA	0%	0%				
Category over predicted	80%	36%	NA				

1127

1128

Draize- Determined EPA Category	EpiOcular Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	12	0	0	12	100%	NA	0%
II	0	1	0	1	0%	0%	100%
III	1	3	0	4	75%	25%	0%
IV	1	4	3	8	38%	63%	NA
Total	14	8	3	25	72%		
Predictivity	86%	38%	100%				
Category under predicted	NA	12%	0%				
Category over predicted	14%	50%	NA				

1129
1130   c)   BCOP
1131

1132   The vast majority of animal data used in the analysis of the BCOP assay
1133 were from the Draize test; only two tests were conducted using the LVET.
1134 Histopathological examination of the treated bovine corneas was included in
1135 the analysis in addition to the traditional *in Vitro* Score which measures the
1136 opacity and permeability of the cornea.

1137
1138 The following table shows the performance of the BCOP assay (including
1139 histopathology) in predicting EPA toxicity categories. Only 2 of 61 materials
1140 (8%) were underpredicted. All of the EPA toxicity category IV materials are
1141 overpredicted as Category III since the BCOP does not seem to be able to
1142 differentiate between materials at this lower end of the toxicity scale. The
1143 BCOP assay does differentiate between EPA Category I and II materials, so it
1144 is most useful in this higher range.

1145
1146 If the anti-microbial cleaning product is a High Solvent (>5 solvent)
1147 formulation, it should be tested in the BCOP assay using a 3 minute exposure
1148 instead of the normal 10 minute exposure.
1149

Draize- Determined EPA Category	BCOP Predicted (with histology) EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	II	III	Total			
I	23	2	0	25	92%	NA	8%
II	4	1	0	5	20%	80%	0%
III	3	2	7	12	58%	42%	0%
IV	0	1	18	19	0%	100%	NA
Total	30	6	25	61	51%		
Predictivity	77%	17%	28%				
Category under predicted	NA	33%	0%				
Category over predicted	23%	50%	72%				

1150 <u>Test Method Reliability</u>

1151

1152   This parameter was assessed by measuring the within and between laboratory
1153   reproducibility for each of the *in vitro* methods. Within assay repeatability was also
1154   assessed when the values were available. The coefficient of variation (CV) between
1155   repeat values was used as a measure of reliability.
1156
1157   Although the primary data used to calculate the CV's was from studies with anti-
1158   microbial (or similar cleaning products), the BRD also contains supporting data from
1159   other studies which used individual ingredients or mixtures (e.g. of surfactants).
1160
1161   a) Cytosensor
1162
1163   Within laboratory reproducibility was assessed from the results of two
1164   international validation studies. In the first study (EC/HO study), the mean CV
1165   for 31 chemicals (three CM runs each) was 23.9%. For the second study
1166   (Colipa eye irritation validation), one laboratory had a mean CV of 19.7% for
1167   surfactant materials and 15.4% for non-surfactant materials. A second
1168   laboratory had a mean CV of 14.3% for the surfactant materials and 10.4% for
1169   the non-surfactant materials.
1170
1171   Interlaboratory reproducibility was also assessed from data generated in the
1172   above validation studies. In the HO/EC study, four laboratories had a mean
1173   between laboratory CV of 37% for surfactant materials and 50.6% for non-
1174   surfactant materials. For the Colipa study two laboratories had a mean
1175   between laboratory CV of 23.3% for surfactant materials, 16.5% for surfactant-
1176   based formulations and mixtures, and 32.5% for non-surfactant ingredients
1177   and mixtures.
1178
1179   b) EpiOcular^{TM}
1180
1181   Within laboratory reproducibility was estimated from the repeated testing of a
1182   single material (0.3% Triton X-100) over a nine year period in two laboratories.
1183   The CV for these repeats was 20.7%.
1184
1185   Interlaboratory reproducibility was assessed from two phases of a validation
1186   study conducted by Colgate-Palmolive. Nineteen pure surfactants and
1187   mixtures were tested by four laboratories in Phase I with a mean between
1188   laboratories CV of 18.1%. Fifty-four pure surfactants and mixtures were tested
1189   by two laboratories in Phase II with a mean between laboratories CV of 11.8%.
1190   c). BCOP
1191
1192   Within run reproducibility was estimated for the BCOP assay from anti-
1193   microbial cleaning products tested for this BRD. When the overall *In Vitro*
1194   *Score* was low (≤10), the within run CV could be quite high (mean CV = 266%
1195   for opacity and 167% for permeability) because small changes in low numbers
1196   result in high CV's. However, such small differences in magnitude in opacity or
1197   permeability scores are relatively meaningless with respect to the overall

1198   range of scores that is possible. However for materials where the mean *In*
1199   *Vitro Score* was >10, the mean CV for opacity was 27.9% and for permeability
1200   was 24.1%.

1201

1202   c) BCOP

1203

1204   BCOP Intralaboratory reproducibility for the anti-microbial cleaning products
1205   was 20.3% for five materials (2 – 6 values per material). Intralaboratory CV's
1206   found by NICEATM in their BCOP Test Method Review Document ranged
1207   from 12.6% to 14.8%.

1208

1209   Interlaboratory reproducibility for the BCOP assay was assessed from three
1210   studies where the <u>median </u>CV's were: Study 1 (11-12 laboratories) 46.9%,
1211   Study 2 (5 laboratories) 30.6% and Study 3 (3 laboratories) 22.8%. The
1212   median CV is presented for these studies since the mean CV was strongly
1213   affected by large CV's for materials where the overall *In Vitro Score* was ≤10.

1214

1215 <u>Overall Testing Strategy</u>

1216

1217   A strategy is presented in this BRD where materials can be tested in one or more
1218 *in vitro* assays to reach a final EPA or GHS toxicity category. Oxidizing formulations are
1219 always tested in the BCOP assay, but other formulation types could be tested in any of
1220 the three assays, as long as their physical characteristics are compatible with that
1221 system. However a second assay may be needed since the BCOP can not identify an
1222 EPA category IV material, while the CM and EO are able to. Conversely the BCOP
1223 assay may be used differentiate between an EPA toxicity category I and II, but the CM
1224 and EO are not able to do that.

1225 **1 Introduction and Rationale for the Proposed Test Method**

1226 1.1 Introduction

1227 1.1.1 Description of framework for development of program
1228
1229   For the past twenty years, extensive research has been conducted to develop
1230 non-animal approaches for evaluating the eye irritation potential of household and
1231 commercial cleaning products. This research involved developing a detailed
1232 understanding of the mechanism by which these products induced eye injury and then
1233 developing *in vitro* and *ex vivo* assays that modeled that mechanism.
1234
1235   In the mid to late 1990's, manufacturers of household and commercial cleaning
1236 products started to conduct internal evaluations of these assays to evaluate whether
1237 they could be used to determine the appropriate ocular precautionary labeling for their
1238 specific products.
1239
1240   These internal studies were successful and for nearly a decade these non-animal
1241 methods together with a weight-of-evidence approach have been used in lieu of
1242 traditional rabbit models for the determination of ocular precautionary labeling of
1243 products.
1244
1245   For the vast majority of household and commercial cleaning products, the
1246 Consumer Products Safety Commission (CPSC) has regulatory authority for ocular
1247 cautionary labeling.   The CPSC itself actively encouraged companies to use non-
1248 animal tests. Its publication "Requirements under the Federal Toxic Substances Act:
1249 Labeling and Banning Requirements for Chemicals and Other Toxic Substances"
1250 states:
1251
1252   "The FHSA only requires that a product be labeled to reflect the toxicities it
1253   presents. It does not require anyone to perform animal tests. The Commission
1254   policy is, whenever possible, to evaluate product toxicities by using alternatives
1255   to animal testing. We encourage anyone evaluating products to determine
1256   whether they present toxicities listed in the FHSA to follow a similar policy."
1257
1258   A small percentage of household and commercial cleaning products carry the
1259 claim, "anti-microbial".   These are considered pesticidal products and regulatory
1260 authority for ocular precautionary labeling for these products rests with EPA's Office of
1261 Pesticide Programs (OPP).   <u>In contrast to regulations for non-pesticidal cleaning</u>
1262 <u>products, EPA regulations for pesticide registration require that animal tests be</u>
1263 <u>performed to determine ocular precautionary labeling.</u>
1264
1265   Since non-animal methods are predominately used today to determine the ocular
1266 precautionary labeling for the vast majority of household and commercial cleaning
1267 products, a project (which has resulted in this Background Review Document) was

1268   initiated with the goal of gaining adoption of these methods for ocular precautionary
1269   labeling decisions for a subset of specific products regulated by OPP – *i.e.,* anti-
1270   microbial cleaning products.
1271
1272   Within this document is a proposed approach and supporting materials which
1273   outline how these non-animal methods can be used to determine the EPA toxicity
1274   Category (I – IV) for ocular cautionary labeling of anti-microbial cleaning products.
1275
1276   It is now requested that ICCVAM conduct a technical review of this approach and
1277   supporting materials and develop an opinion on whether the use of this approach will
1278   assure the EPA that, with a reasonable level of certainty, antimicrobial cleaning
1279   products will not be under labeled.

1280   ## 1.1.2 Summary of Project History

1281
1282   The genesis of the herein described non-animal testing approach occurred within
1283   the Pesticide Program Dialog Committee, a Federal Advisory Committee established to
1284   advise EPA on the concerns of its many and diverse stakeholders. The concern was
1285   broached in this committee that since cleaning products had apparently been safely
1286   marketed for many years without the use of new animal tests, it seemed unreasonable
1287   to force them to be tested in animals just because of a different claim. Their thought was
1288   that as long as the non-animal methods would allow products to be adequately labeled,
1289   then those options should be available and acceptable.
1290
1291   EPA/OPP Director Jim Jones agreed with the advice of the committee to
1292   investigate the feasibility of accepting non-animal methods for the labeling of cleaning
1293   products, and began supporting efforts to develop a non-animal testing approach. The
1294   effort was taken up by two major manufacturers of anti-microbial cleaning products, the
1295   Procter & Gamble Company and S.C. Johnson & Son, Inc. A specialized *in vitro*
1296   laboratory – The Institute for *In Vitro* Sciences, Inc. (IIVS) was asked to help coordinate
1297   the program, perform any needed testing, and prepare the eventual submission.
1298
1299   Although the project was originally scheduled to be presented directly to the
1300   EPA's science advisory panel, it was later determined that the Interagency Coordinating
1301   Committee on the Validation of Alternative Methods (ICCVAM) would oversee the
1302   technical review and then present their findings and recommendations to the EPA.
1303   Therefore, this submission is being prepared according to the formatting suggested by
1304   ICCVAM.
1305
1306   To initiate the project, companies that manufacture anti-microbial cleaning
1307   products or materials with similar formulations were invited to participate and to share
1308   their animal data, *in vitro* data, and toxicological expertise. If this program is successful,
1309   there will be several advantages for a manufacturer, for example, the ability to:
1310
1311   • normalize standard practices for non-regulated product development with
1312   regulated product requirements, and

1313   • use formulation development data obtained *in vitro* to support registration and
1314   labeling
1315
1316   The following seven companies agreed to assist the project by supplying animal
1317   and/or *in vitro* data:
1318
1319   • Clorox
1320   • Colgate -Palmolive Company
1321   • The Dial Corporation
1322   • EcoLabs
1323   • JohnsonDiversey, Inc.
1324   • S.C. Johnson & Son, Inc.
1325   • The Procter & Gamble Company
1326
1327   Each company was informed that the specific data that they contributed would be
1328   coded so that it could not be linked directly to them. They were asked to supply the
1329   following type of information for each cleaning formulation that would be used in the
1330   program:
1331
1332   1) Complete data (carried out to 21 days) from individual animals used to test a
1333   substance
1334   2) Detailed description of the animal test protocol, if possible
1335   3) Characterization of the suspected chemical activity category of the
1336   formulation (see below)
1337   4) Description of the ingredients contained in the test formulation at the level of
1338   detail that would be supplied to a poison control center
1339   5) Description of the *in vitro* test used with the test substance
1340   6) Raw data from the *in vitro* test, if possible
1341
1342   A sample Excel® spread sheet was provided to each potential participant which
1343   included the input form that each submitter was asked to fill out for each animal tested
1344   with each formulation.
1345
1346   The following chemical descriptors were suggested to characterize the different
1347   types of chemically-induced mechanisms associated with ocular irritation. These were
1348   chosen based on existing information about the mechanisms of ocular irritation and the
1349   common types of formulation chemistries used in commercial and household cleaning
1350   products.
1351
1352   • Surfactants (SU) (e.*g.,* cationic, anionic, and nonionic with limited acid or alkaline
1353   activity)
1354   • Acids (AC) (*e.g.,* with pH <4, especially where reserve acidity would contribute to
1355   the irritation potential)
1356   • Alkaline (AL) products (bases) (*e.g.,* with pH >9, especially where reserve
1357   alkalinity would contribute to the irritation potential)

1358   • Solvents (SO) (where organic solvents are expected to contribute to the irritancy
1359   potential (*e.g.,* alcohols, glycol ethers, etc.))
1360   • Oxidizers (RC; reactive chemistry) (formulations containing specific reactive
1361   chemicals, *e.g.,* hypochlorite, peroxide, percarbonate, oxygen bleaches, etc.)
1362
1363   The process began by collecting data (both animal and non-animal data) from
1364   the historic records of the participating companies and combining it in a database (at
1365   IIVS) to determine the effectiveness of the methods to predict the EPA toxicity labeling
1366   categories of anti-microbial products. We compared the specific EPA categories with
1367   the *in vitro* scores to determine prediction models for each *in vitro* test which could be
1368   used to set cut offs for the various categories. Since knowing the correct EPA toxicity
1369   category for the substances was imperative, raw data for the individual test animals
1370   were absolutely required.
1371
1372   *In vivo* methods: Data from two types of rabbit tests were submitted during this
1373   project. One set was from the traditional Draize rabbit eye test, and the second was
1374   from a similar test – the Low Volume Eye Test (LVET). The LVET is also a rabbit eye
1375   test, but it differs from the traditional Draize assay in the volume tested and the location
1376   on which the material is placed on the eye. The LVET uses one-tenth the volume of the
1377   Draize test (10 µL vs. 100 µL) and places the material directly on the central surface of
1378   the cornea as opposed to instilling the material in the conjunctival sac. This volume and
1379   placement is thought to more closely mimic a typical human accidental exposure. Excel
1380   spreadsheets were created to convert raw animal data into the appropriate EPA or GHS
1381   scoring scale.
1382
1383   *In vitro* methods: Three different *in vitro* assays for eye irritation were in common
1384   use by the participating manufacturers. These were the EpiOcular (EO) assay, the
1385   Cytosensor Microphysiometer (CM) assay, and the Bovine Cornea Opacity and
1386   Permeability (BCOP) assay. The EO assay is a three-dimensional, non-keratinized,
1387   tissue constructed from human epithelial cells. It is designed to have a similar
1388   construction and histological appearance to the epithelial cell layers covering the
1389   cornea. The CM is an instrument which measures changes in the metabolism of cells.
1390   Increasing amounts of test article are exposed to the cells until the metabolic rate falls
1391   by 50% (MRD_{50}). The lower the MRD_{50} value, the higher is the potential for eye
1392   irritation. The BCOP assay uses isolated bovine corneas dissected from whole globe
1393   eyes obtained from slaughterhouses. Test substances can be placed directly on the
1394   surface of these corneas and subsequent changes in both the opacity and the barrier
1395   function of the epithelial cell layer can be measured. Additionally, histopathology can be
1396   performed on the corneas so that the induced damage can be visualized.
1397
1398   Anti-microbial cleaning products can be formulated in different ways. To prepare
1399   for the possibility that each different type of formulation might have a slightly different
1400   pattern of toxicity when used in the different *in vitro* tests, we described each product
1401   according to what was thought would be the major driver of eye irritation for that
1402   product. The descriptors chosen were acid, alkaline, oxidizer, surfactant, and solvent.
1403

1404   The first part of our study was purely retrospective. Graphical comparisons
1405   between the toxicity categories determined by the *in vivo* and the *in vitro* scores for the
1406   same test materials were made. This helped to decide if sufficient materials were
1407   available in each toxicity category to allow the determination of potential cut-off values
1408   that would ultimately define EPA classifications. Although this determination was
1409   possible in some cases, in others we found that the data were lacking to clearly indicate
1410   where the cut-off values should fall. However, probable cut-off ranges were still
1411   hypothesized based on the distribution of the data and known irritation profiles
1412   determined based on *in vivo* animal data.
1413

1414   Materials were then sought with which to generate additional *in vitro* data from
1415   the database of animal studies without paired *in vitro* data. It was hoped that these
1416   additional studies would clarify where the cut-off values should lie. Attempts were made
1417   to find materials from the toxicity categories that had low representation (for example,
1418   EPA Category II materials were significantly underrepresented), or where the cut-off
1419   values were difficult to determine. These materials were requested from the appropriate
1420   manufacturers, and if the manufacturers chose to have them tested in the *in vitro* assay
1421   that was suggested, they were instructed to code the materials before submitting them
1422   for testing. The materials were then tested under code at IIVS. If the *in vitro* test
1423   selected was the BCOP assay, the corneas were also submitted for histopathology
1424   which was conducted either by IIVS staff or by an IIVS contractor skilled in ocular
1425   histopathology. The histopathology results were then compared to the BCOP *in vitro*
1426   scores to determine if they were reflective of the *in vitro* scores, or if the toxicity
1427   category of the material should be increased. Materials were not decoded until after the
1428   final decision as to the ocular irritation potential of the substance was made.
1429

1430   These new data were then combined with the previous data to determine if they
1431   supported the initial determination of cut-offs or if they provided more information which
1432   allowed a better estimation of the cut-off.
1433

1434   After the predictive capacity of each *in vitro* test was examined, we investigated
1435   whether any of the tests could be stand-alone predictors of all of the EPA labeling
1436   categories, or whether the tests had good predictive ability only for a portion of the
1437   irritation scale. We found that the latter case was true for the data we analyzed. This led
1438   us to develop a testing strategy which utilizes the Cytosensor assay and the EpiOcular
1439   assay to identify the mild products, *e.g.,* Categories III and IV, depending on the
1440   physical state of the material. Substances which scored more irritating than a Category
1441   III were moved to the more robust BCOP assay to determine if the materials were either
1442   Category I or Category II materials.
1443

1444   This testing scheme also can begin with the BCOP assay for materials expected
1445   from their composition to be highly irritating. However, if the BCOP assay shows the
1446   substance to be of a lower (Category III) irritation potential, the substance may be
1447   retested in the Cytosensor or EpiOcular assay to determine if it is a Category III or
1448   Category IV material. This strategy is depicted in Figure 1-1.
1449
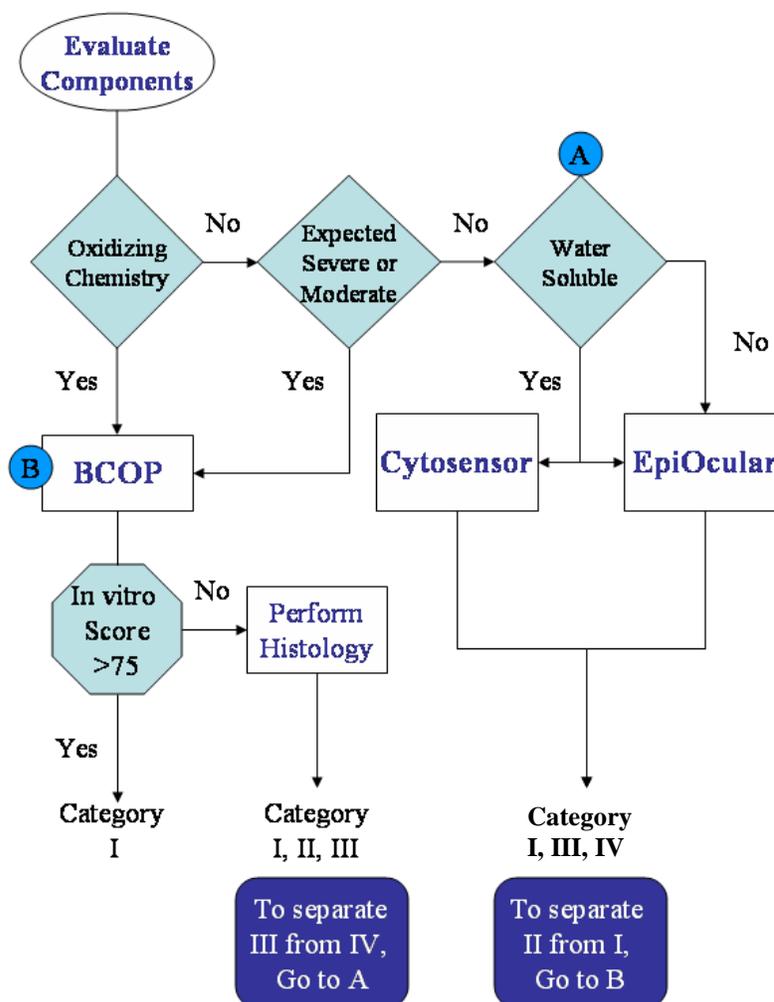
Figure 1-1 The proposed testing strategy for determining the EPA toxicity category for anti-microbial cleaning products.

We wish to make it clear that the above strategy is self-correcting if the initial estimate of irritation potential of a test substance is incorrect. If a highly irritating material is tested in the Cytosensor or EpiOcular assays, it will receive a score indicating that it is a highly irritating (category I) material. If further resolution is desired (to determine if it is actually a Category II material rather than a Category I material), the formulation can then be further tested in the BCOP assay. Similarly a mild material will be identified as a Category III material by the BCOP assay. If it is important to the company to distinguish between a Category III and IV for labeling and marketing

1466   purposes, then an additional Cytosensor or EpiOcular assay may be required to make
1467   that determination.
1468

1469   1.1.3 Confidential information

1470

1471   Manufacturers who are participating in this program by submitting data have
1472   agreed that any information that is contained in this submission is <u>non-confidential</u>.
1473   However, the submitters do desire that individual data not be linked to a specific
1474   company. Therefore, that information is not included, and the data are grouped so that
1475   no linkage can be made to the company that generated it.
1476

1477   1.2 Regulatory rationale and applicability

1478   1.2.1 Current regulatory testing requirements for which the proposed test method
1479   is applicable

1480

1481   The proposed test methods will be used to make labeling decisions for anti-
1482   microbial cleaning products as required by the EPA's Office of Pesticide Programs (EPA
1483   2003).

1484

1485   The traditional method of making the labeling decisions is based on the Draize
1486   rabbit eye irritation test (Draize, Woodard et al. 1944). In this test, a scoring scheme is
1487   applied to the eyes of albino rabbits whose eyes have been exposed to a test material
1488   by application within the conjunctival sac. The degree of irritation is classified according
1489   to the ocular irritation criteria of Kay and Calandra (1962). This process is described in
1490   Acute Eye Irritation (EPA 1998) published in August 1998. The same scoring system is
1491   also used for grading and interpretation of data using the Low Volume Eye Test (LVET)
1492   method.

1493

1494   As stated in the BRD produced by NICEATM for the BCOP assay: "The EPA
1495   ocular irritation classification regulation and testing guidelines (EPA 1998; EPA 2003)
1496   are based on the most severe response in one animal in a group of three or more
1497   animals. This classification system takes into consideration the kinds of ocular effects
1498   produced, as well as the reversibility and the severity of the effects. The EPA classifies
1499   substances into four ocular irritant categories, ranging from I to IV (Table 1-1) (EPA
1500   2003). Category I substances are defined as corrosive or severe irritants, while
1501   classification from II to IV is based on decreasing irritation severity, as well as the time
1502   required for irritation to clear. Irritation that clears in 8 to 21 days is classified as
1503   Category II, while irritation that clears within seven days is classified as Category III. For
1504   Category IV substances, irritation clears within 24 hours."

Table 1-1 *In vivo* **Ocular Irritancy Classification Systems**

Regulatory Agency (Authorizing Act)	Number of Animals	Minimum Observations Times (after treatment)	Mean Score Taken?	Positive Response	Irritant/Nonirritant Classification
EPA (FIFRA; TSCA; and The Federal Environmental Pesticide Control Act)	**At least 3***	**1 hour, 1, 2, 3, 7, 14, and 21 days**	**No**	**-Maximum score in an animal used for classification** **-Opacity or Iritis ≥ 1 or Redness or chemosis ≥ 2**	**One or more positive animals needed for classification in categories below:** **I = Corrosive, corneal involvement, or irritation persisting more than 21 days** **II = Corneal involvement or irritation clearing in 8-21 days** **III = Corneal involvement or irritation clearing in 7 days or less** **IV = Minimal effects clearing in less than 24 hours**
GHS – Irreversible Eye Effects	**3**	**1, 2, 3, days (observation until Day 21)**	**Yes**	**Mean animal values (over Days 1, 2, and 3) of:** • **Opacity ≥ 3 and/or** • **Iritis ≥ 1.5**	**1 = At least 2 positive response animals** **1 = At least 1 animal where Opacity, Chemosis, Redness, or Iritis > 0 on Day 21**
GHS – Reversible Eye Effects	**3**	**1, 2, 3 days (observation until Day 21)**	**Yes**	**Mean animal values (over Days 1, 2, and 3) of:** • **Opacity or Iritis ≥ 1 or** • **Redness or Chemosis ≥ 2**	**2A = At least 2 positive response animals and the effect fully reverses in 21 days** **2B = At least 2 positive response animals and effect fully reverses in 7 days**

Abbreviations: EPA = U.S. Environmental Protection Agency; FIFRA = Federal Insecticide, Fungicide, and Rodenticide Act; GHS = United Nations Globally Harmonized System; TSCA = Toxic Substances Control Act.

* - Only one animal is required if the result in that animal is corrosive.

Table 1-1 Cont'd

Regulatory Agency (Authorizing Act)	Number of Animals	Minimum Observations Times (after treatment)	Mean Score Taken?	Positive Response	Irritant/Nonirritant Classification

European Union (EU)	**Current Directive:** 1 if severe effects are suspected or 3 if no severe effects are suspected
	
	Prior Directive: 3 or 6 animals
		1, 2, 3 days (observation until Day 21)	**Yes**	<u>6 Animals</u>
				Mean study values (scores averaged over all animals in study over Days 1, 2, and 3) of:
				• **Opacity or Chemosis ≥ 2**
				• **Redness ≥ 2.5**
				   or
				• **Iritis ≥ 1**
				
				<u>3 Animals</u>
				Individual animal mean values (scores for each endpoint are averaged for each animal over Days 1, 2, and 3) of:
				• **Opacity or Chemosis ≥ 2**
				• **Redness ≥ 2.5**
				   or
				• **Iritis ≥ 1**
					R36 =
					• **Mean study value (when more than 3 animals are tested) where:**
					   ○ **2 ≤ Opacity < 3 or**
					   ○ **1 ≤ Iritis < 1.5 or**
					   ○ **Redness ≥ 2.5 or**
					   ○ **Chemosis ≥ 2**
					• **If 2 of 3 tested animal have individual animal mean values that falls into one of the following categories:**
					   ○ **2 ≤ Opacity < 3 or**
					   ○ **1 ≤ Iritis < 1.5 or**
					   ○ **Redness ≥ 2.5 or**
					   ○ **Chemosis ≥ 2**
					R41 =
					• **Mean study value (when more than three animals are tested) where:**
					   ○ **Opacity ≥ 3 or Iritis > 1.5**
					• **If 2 of 3 tested animals have individual animal mean values that fall into one of the following categories:**
					   ○ **Opacity ≥ 3 or Iritis = 2**
					• **At least one animal where ocular lesions are still present at the end of the observation period, typically Day 21**

1112   1.2.2 Intended regulatory use (*i.e.,* replacement) of the proposed method
1113
1114   The proposed testing scheme is designed to replace the Draize rabbit eye
1115   irritation test for the purpose of toxicity labeling of anti-microbial cleaning products
1116   (see above).

1117   1.2.3 Similarities between data obtained using this method and the current *in*
1118   *vivo* data
1119
1120   The current *in vivo* data consist of information about the cornea (area and
1121   amount of opacity), the iris (iritis) and the conjunctiva (redness and chemosis).
1122
1123   Data obtained from the proposed *in vitro* testing scheme give information
1124   about toxicity mainly to the cornea and the conjunctiva. Two of the *in vitro* ocular
1125   irritation tests proposed (EO and CM) give information about the direct toxicity of the
1126   test material to cells. This is the same type of toxicity that occurs in the outer surface
1127   of the cornea and to the conjunctiva. The third *in vitro* ocular test utilizes an excised
1128   bovine cornea, and thus the type of initial damage that is seen in this *in vitro* (or *ex*
1129   *vivo*) test is very similar to what occurs to the animal cornea during a traditional eye
1130   irritation test.

1131   1.2.4 Fit of method into the overall strategy of toxicity or safety assessment
1132
1133   The proposed *in vitro* testing strategy provides a complete tiered assessment
1134   process to determine the EPA toxicity category and product labeling for eye irritation
1135   caused by anti-microbial cleaning products.

1136   1.3 Scientific basis for the proposed test method

1137   1.3.1 Purpose and mechanistic basis of the proposed test methods
1138
1139   Data from three *in vitro* methods are used in this submission. These *in vitro*
1140   methods – the Cytosensor assay, the EpiOcular assay and/or the BCOP assay –
1141   were primarily chosen because they had been extensively used by participating
1142   companies to assess cleaning products and similar materials, and because there
1143   were *in vivo* data available which could be paired with the *in vitro* data. The
1144   mechanistic basis of each of these assays is described in detail below.

1145   1.3.1.1 Cytosensor Microphysiometer (CM) Assay
1146
1147   The Cytosensor is a machine which measures the metabolic activity of a
1148   small population of cells grown as a monolayer in a Transwell cup. The cells are
1149   exposed to increasing concentrations of a test substance, and their metabolic
1150   activity (an estimate of their viability) is measured after each exposure. As the
1151   toxicity of the test substance increases, the metabolic activity decreases until

1152   eventually the cells may be completely killed. The endpoint of the assay is the
1153 MRD_{50} (concentration of test material which reduces the metabolic rate to 50% of
1154   the control rate). The more irritating the test material, the lower the MRD_{50}.
1155

1156   1.3.1.1.1 Intended uses / purpose of the CM
1157

1158   Currently the CM is used by industry early in the new product development
1159   process to screen primarily liquid ingredients for cosmetic, personal care, and
1160   household cleaning products. This screening is then often followed by evaluations of
1161   the final formulations for final in-house safety and labeling decisions. Data from the
1162   CM may be combined with information from other *in vitro,* existing *in vivo,* and *in*
1163   *silico* assays on the formulation and/or the ingredients contained within to provide a
1164   "weight of evidence" evaluation of the formulation. Information from this assay is
1165   generally not combined with new animal data in making the final safety decision for
1166   the product.
1167

1168   At the time the CM technology was developed, a number of *in vitro* assays
1169   such as the Neutral Red Uptake assay were already proposed as potential
1170   replacements for the Draize eye irritation test. However, the great advantage of the
1171   CM, or its predecessor the silicon microphysiometer (SM), technology was that
1172   measurement could be made of the cytotoxic response of the target cells in real
1173   time, as opposed to the 2-3 days or longer time which was required of the existing
1174   cytotoxicity assays. Thus, the assay was mainly created not to reveal a completely
1175   new endpoint, but rather to provide data in a much shorter time period.
1176   Subsequently, it was realized that greater sensitivity of the CM method made it
1177   useful in identifying differences between formulations which were already
1178   determined to be very mild.

1179   1.3.1.1.2 Regulatory rationale and applicability of the CM
1180

1181   To the best of our knowledge, the CM assay is not currently included in the
1182   regulatory scheme of any country. Data are used primarily to evaluate raw materials
1183   and formulations where regulatory registration is not required. It has been reviewed
1184   informally by regulatory agencies in the US as part of the Interagency Regulatory
1185   Alternatives Group (IRAG) evaluation of alternative ocular irritation assays (Botham,
1186   Osborne et al. 1997). A BRD on the performance of the CM test method is currently
1187   being prepared for review by ECVAM as part of their ocular toxicity method
1188   validation program.

1189   1.3.1.1.3 Scientific basis for the CM test
1190

1191   Topical applications of chemicals can kill cells in several ways; among these
1192   are lysis of membranes, denaturation of proteins, saponification of lipids, and
1193   alkylation or other covalent interactions with macromolecules. The first three modes
1194   of action kill or damage very rapidly while the last may act rapidly but the evidence

1195 of the action may take some time to be manifested (Maurer, Parker et al. 2002).
1196 Certain chemical classes are associated with these modes of action. Surfactants are
1197 primarily associated with membrane lysis although cationic surfactants may also act
1198 to precipitate proteins and other macromolecules. Organic solvents can act to
1199 delipidize and thus lyse membranes as well as denature (coagulate or precipitate)
1200 proteins. Acids tend to coagulate or precipitate proteins. Alkalis saponify lipids and
1201 denature proteins in a way that tends to allow them to penetrate into the cornea.
1202 Bleaches, peroxides, alkylators (*e.g.,* mustards) bind to macromolecules (especially
1203 DNA) leading to cell death.
1204
1205   Damage to the eye is a function of the inherent cytotoxicity potential of the
1206 chemical or mixture, the effective concentration impacting the tissues and the
1207 residence time at that concentration on or in the tissues. The effective exposure is a
1208 combination of concentration and time of exposure (Figure 1-2). For example, a
1209 neat organic solvent may have a high cytotoxic potential but if it rapidly evaporates,
1210 the effective residence time will be less. Putting a large volume into a closed sac
1211 (*e.g.,* lower conjunctival sac of the rabbit eye) will produce a very different effective
1212 exposure than a smaller amount placed (or accidentally splashed) onto the open
1213 surface of the cornea. Another solvent may have a longer residence time but have
1214 its cytotoxic potential rapidly reduced by dilution with tears. In this case, the irritation
1215 potential in a species with a low propensity to tear could show much more irritation
1216 than in a species with a high propensity to tear. The effective exposure to solids
1217 (powders) in the eye is a particular challenge. Powders placed into the conjunctival
1218 sac may have a residence time that ranges from minutes to a full day (and longer in
1219 some older studies) (Prinsen 2006). Traditional studies of eye irritation potential do
1220 not measure or control the effective exposure within or among studies. Thus, efforts
1221 to model exposure in alternative test systems are based on best estimates and
1222 approximations.
1223
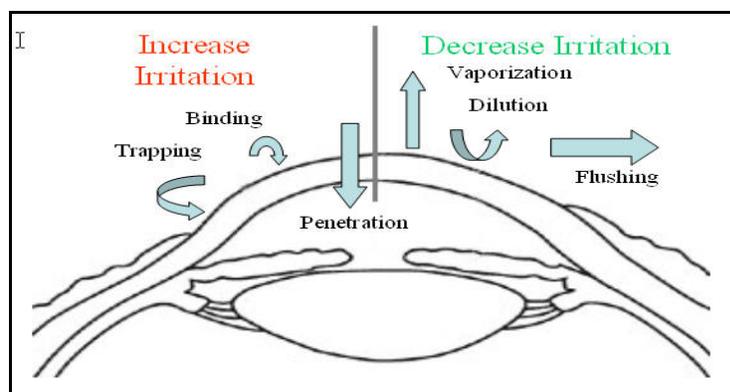
1224
1225   **Figure 1-2 Factors that impact exposure to the eye**
1226
1227   Mechanistically, this cytotoxicity assay is intended to model the action of the
1228 surfactant on the cell membranes of the corneal and conjunctival epithelium where
1229 the test article would reside in an *in vivo* exposure. The potency of the surfactant (or
1230 surfactant formulation) *in vivo* is related to the area and number of cell layers that
1231 can be lysed during the effective exposure period. More potent (and/or more

1232   substantive) surfactants will be more effective at a given concentration and
1233   exposure period. Potency can be a function of concentration (*e.g.,* in a formulation)
1234   or chemical structure. Thus, a lower concentration of a more potent surfactant or
1235   more concentrated formulation would be required to lyse the membranes, and thus
1236   kill a given fraction of the cells in the epithelia (both corneal and conjunctival).
1237   Expressed another way, a given concentration of a more potent test material should
1238   lyse more cells (*i.e.,* greater depth of penetration and injury). Initial depth of injury
1239   has been shown by Maurer, Jester, and collaborators (Jester, Petroll et al. 1998;
1240   Jester, Li et al. 2001; Maurer, Parker et al. 2002) to relate directly to the degree and
1241   duration of ocular injury (Figure 1-3). Their work has shown the relationship between
1242   cell initial killing and the resulting irritation. In the cytotoxicity assays with monolayer
1243   cells, a similar relationship between potency and effective concentration is expected
1244   for killing 50% of the target cell population (Harbell, Koontz et al. 1997).
1245
1246   Non   Slight   Mild   Moderate Severe Irritation
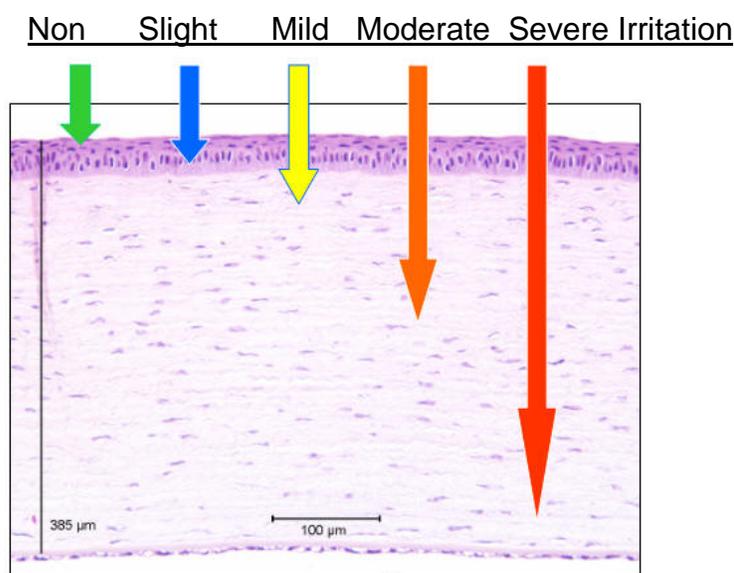
1247
1248   **Figure 1-3 Summary of the Depth of Injury Model**
1249
1250   The CM estimates the metabolic rate (glucose utilization rate) of a population
1251   of cells by measuring the rate of excretion of acid by-products and resulting
1252   decrease in pH of the surrounding medium in an enclosed chamber. The rate of
1253   change in pH per unit time becomes the metabolic rate of the population. The basal
1254   metabolic rate and the ratio of glycolytic to aerobic metabolism (Krebs Cycle) may
1255   be different for different cell types. However, for the population of any one cell type,
1256   the ratio remains similar if the cells are handled in a consistent fashion. If a test
1257   material causes cytotoxicity to this population of cells it is assumed that the
1258   metabolic rate will fall. However, the metabolic rate may not fall immediately after
1259   exposure of the cells to a dilute concentration of toxicant. Populations of cells in
1260   culture are reported to metabolize glucose at only a fraction of their maximal
1261   metabolic rate (McConnell, Owicki et al. 1992). Thus, an up regulation of glucose
1262   metabolism can occur if the cells need energy to maintain their integrity in the face
1263   of a mild biochemical insult. For example, exposure to a subcytotoxic concentration
1264   of surfactant can increase membrane leakage (to ions and water). This in turn can

1265   lead to an increase in the activity of ATP-dependent ion pumps and increased
1266   glucose metabolism. Thus early points in a killing curve can show increases in
1267   metabolic rate of 2- to 3-fold, but this metabolic rate then soon falls below 100% as
1268   higher concentrations of test material overwhelm the homeostatic controls within the
1269   cells (Figure 1-4).
1270
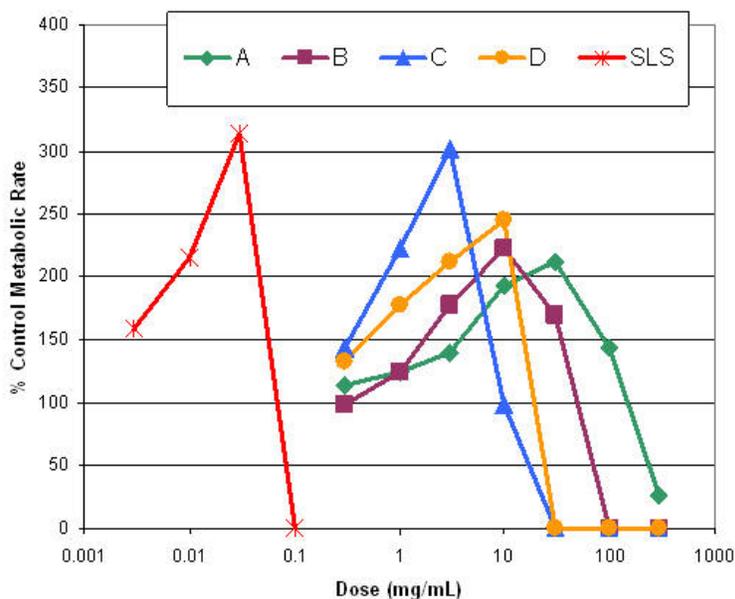
1271
1272   **Figure 1-4 Example of the metabolic rate data as a function of surfactant type and**
1273   **concentration**
1274

1275   Although the metabolic rate is the physical parameter which is measured
1276   during the CM assay, the magnitude of metabolic rate itself is not directly related to
1277   eye irritation potential. Rather, the reduction of the metabolic rate to 50% of its basal
1278   rate is the parameter used to measure the impact of the test article on the test
1279   system (L929 cells in almost all cases). The CM assay exposes a population of cells
1280   to increasing concentrations of the test article (diluted in medium). The exposure
1281   follows a three step process where the first step is the exposure to the diluted test
1282   article, the second is the test article rinse-out and the third is the measurement of
1283   the metabolic activity. This means that the impact of the exposure is measured
1284   immediately and then a subsequent exposure is performed until the highest testable
1285   concentration has been used or the population of cells is severely damaged and the
1286   metabolic rate has declined to effectively zero. From the concentration response
1287   curve, the concentration that leads to a 50% decline in the metabolic rate of the
1288   population (the MRD_{50}) is calculated from the curve. The MRD_{50} values are used to
1289   compare test materials and provide a measure of ocular irritancy potential. By
1290   current convention, the units of the MRD_{50} are mg/mL.
1291

1292   For ease in understanding the mechanistic basis of the CM assay, a table
1293   (Table 1-2) has been compiled describing the events that are commonly considered
1294   to occur during eye irritation. Those events that are modeled (or are closely related)
1295   by the CM assay are indicated by a Y (yes) indication. It can be seen that the CM

1296 assay most closely models some of the initial stages of interaction of an eye irritant
1297 with the cornea. The more distal occurrences in eye irritation such as gross tissue
1298 changes in the corneal stroma, and the recovery from the lesions, are not directly
1299 modeled. However, if the hypothesis of Jester, Mauer, and others that initial area
1300 and depth of injury is predictive of time to, and extent of, recovery, then the
1301 measurements made by the CM may have a relationship to recovery as well.
1302
1303 **Table 1-2 Summary of events involved in chemical-induced eye irritation *in vivo*. Text in**
1304 **italics represents irreversible responses.**

Events involved in chemical-induced eye irritation	Modeled by the CM assay?
Chemical interaction with tear film (Klyce and Beuerman 1988; Hackett and McDonald 1994)	N
Chemical binding to the conjunctival epithelium (Hogan and Zimmerman 1962; Hackett and McDonald 1994)	Y
Adhesion molecules compromised (Farquhar and Palade 1963; Van Meer, van Hof et al. 1992; Katahira, Sugiyama et al. 1997)	N
Corneal epithelium damage (Dua, Gomes et al. 1994)	Y
• Inhibition of receptor-mediated membrane transport (Dearman, Cumberbatch et al. 2003)	Y
• Compromise of cell membrane integrity of upper corneal epithelium (Dua, Gomes et al. 1994; Hackett and McDonald 1994; Maurer and Parker 1996)	Y
• Cell membrane lysis of all corneal epithelium layers (Hackett and McDonald 1994)	Y
Hydration of corneal stroma (Hackett and McDonald 1994)	N
Cross-linking of proteins in corneal stroma (Butler and Hammond 1980; Eurell, Sinn et al. 1991; Chan and Hayes 1994)	N
Erosion of corneal stroma (Baldwin, McDonald et al. 1973; Hackett and McDonald 1994; Maurer and Parker 1996)	N
Cell damage to corneal epithelium and limbus (Jacobs and Martens 1990; Wilhelmus 2001)	Partially
Dilation and increased lymphatic leakage from scleral vasculature (Hackett and McDonald 1994)	N
Stimulation of nerve endings, i.e., enhanced blinking, tearing (Chan and Hayes 1994)	N
Erosion of nerve endings in cornea and sclera (Butler and Hammond 1980; Klyce and Beuerman 1988; Araki, Ohahsi et al. 1994)	N
Duration of response, *i.e.,* length of time cell responses deteriorate. Duration of response covers the effects of reactive chemicals which can cause coagulation, saponification, that are effects which develop and increase over time. (Hubert 1992; Maurer and Parker 1996)	N
Recovery from response, *i.e.,* length of time for cell responses to return to control levels (Hubert 1992)	N

1305   1.3.1.2 EpiOcular

1306

1307   The *in vitro* method using the EpiOcular tissue model was developed as a
1308   replacement for the Draize eye irritation test (Draize, Woodard et al. 1944; Draize,
1309   Woodward et al. 1944). The Draize scoring system is heavily weighted towards
1310   corneal damage (80 out of a total of 110 total points) because irreversible damage
1311   to the cornea can lead to blindness. Since damage to the cornea is so important
1312   both in the Draize scoring scale and to human health, the cornea (specifically its
1313   outer surface, the epithelium) is the tissue that is modeled by the EpiOcular tissue
1314   model. The EpiOcular protocol models very closely the Low Volume Eye Test
1315   (LVET) (Griffith, Nixon et al. 1980) where test materials are applied directly to the
1316   surface of the cornea.

1317

1318   The topical application method described in this BRD utilizes a commercially
1319   available three-dimensional tissue construct called EpiOcular (Model OCL-200,
1320   MatTek Corporation, Ashland, MA) (herein referred to as the EpiOcular tissue
1321   model). The EpiOcular tissue model consists of normal, human-derived epidermal
1322   keratinocytes that have been cultured to form a stratified, squamous epithelium
1323   similar to that found in the human cornea (Figure 1-5). In this model, keratinocytes
1324   progressively flatten as the apical surface of the tissue is approached and
1325   differentiate to form a multi-layered structure that closely resembles the corneal
1326   epithelium *in vivo*. *In vivo*-like growth characteristics are reproduced and include
1327   mitotically and metabolically active cells that produce pro-inflammatory growth
1328   factors and cytokines important in ocular irritation and inflammation (Thakur, Clegg
1329   et al. 1997). Test materials can be applied directly to the surface of the tissue
1330   construct to approximate exposure conditions *in vivo*. Damage to the tissue, as
1331   reflected by cell cytotoxicity, can be quantified via the chemical reduction of 3-(4,5-
1332   dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and related to a test
1333   material's potential for ocular irritation. The current submission describes the
1334   relationship between *in vitro* cytotoxicity (time-to-toxicity) and *in vivo* ocular irritation.

1335

1336

a) EpiOcular: Magnification 360x   b) Rabbit Cornea: Magnification 360X

c) Human Cornea
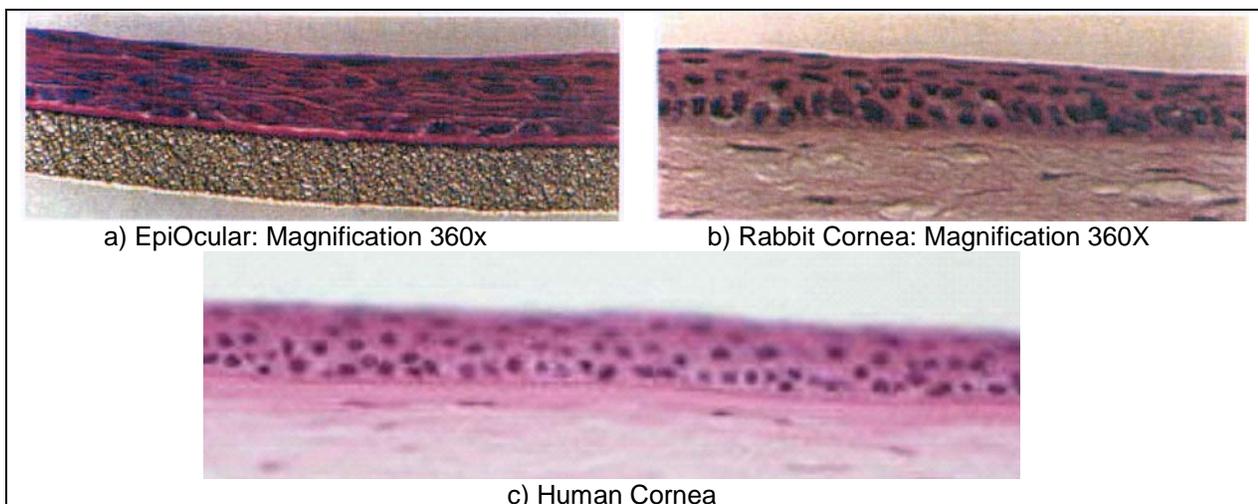
1337
1338 **Figure 1-5 Photomicrographs of a) the EpiOcular model showing the stratification and lack of**
1339 **surface keratinization (photo from MatTek Corporation, Ashland, MA), b) the cornea of a rabbit**
1340 **eye (photo courtesy of MatTek Corporation, Ashland, MA), and c) a human cornea.**
1341

1342 1.3.1.2.1 Intended uses / purpose of the EpiOcular assay

1343

1344   Very similar to what was described earlier for the CM (Section 1.3.1.1.1), the
1345 EpiOcular assay is used by industry early in the new product development process
1346 to screen solid or liquid ingredients for cosmetic, personal care, and household
1347 cleaning products, as well as assessment of irritation potential of final formulations.
1348 One advantage that this method has in comparison to the CM test method is that
1349 common product formulations like gels, pastes, creams, and powders are
1350 completely compatible with the EpiOcular tissue. Toxicity screening activity is then
1351 often followed by further EpiOcular evaluations of the final formulations for final in-
1352 house safety decisions. Data from the EpiOcular assay may be combined with
1353 information from other *in vitro* or *in silico* assays to provide a "weight of evidence"
1354 evaluation of the formulation. Information from this assay is generally not combined
1355 with new animal data in making the final safety decision for the product.

1356 1.3.1.2.2 Regulatory rationale and applicability of the EpiOcular test method

1357

1358   To the best of our knowledge, the EpiOcular test method is not currently
1359 included in the regulatory scheme of any country. Data are used primarily to
1360 evaluate raw materials and formulations where regulatory registration is not
1361 required. It is in the process of being reviewed by ECVAM as part of their ocular
1362 toxicity method validation program.

1363

1364   1.3.1.2.3 Scientific basis for the EpiOcular test method

1365

1366   As described above, the EpiOcular test method is an attempt to model early
1367   changes that occur in the cornea after exposure to a potential eye irritant. The
1368   model, as shown in Figure 1-5, closely resembles the non-keratinized squamous
1369   epithelium of the mammalian cornea. Because this model is maintained at the
1370   air:medium interface, the apical surface is accessible for direct application of test
1371   material as might occur during a traditional Draize or LVET rabbit eye test or an
1372   accidental human exposure.

1373

1374   Since the damage induced by eye irritants is generally progressive from the
1375   corneal epithelium through the stroma and potentially to the endothelium, the
1376   EpiOcular assay is able to provide information on the first stages of this progression.
1377   As an irritant kills cells as it moves through the corneal epithelium, the cytotoxic
1378   progress can be estimated by measuring the loss of MTT reducing activity in the
1379   EpiOcular tissue using standardized methods. Although the model only represents
1380   the corneal epithelium, (very mild responses would also be reflective of some
1381   conjunctival irritation), it can be used to estimate deeper damage into the stroma
1382   because of the time-to–toxicity measurements (ET_{50}'s) that are made. The quicker a
1383   material kills 50% of the cells in the model the more likely it is to progress to deeper
1384   layers of the cornea.

1385

1386   It should be clear from this discussion that the EpiOcular assay is most
1387   valuable in addressing the milder end of the irritation scale. Very mild materials may
1388   take up to 4 hours to kill 50% of the cells. Thus it is relatively easy to differentiate
1389   between the degrees of mildness of two closely related mild substances. However, if
1390   extremely irritating materials are used with the EpiOcular assay, the rather thin layer
1391   of cells comprising the model is killed quite rapidly (on the order of seconds for
1392   extremely toxic materials). When materials act this rapidly, it is extremely difficult to
1393   differentiate one very toxic material from another which is only slightly less toxic.
1394   Thus the EpiOcular assay has been used most successfully with materials which
1395   exist in the lower range of irritancy potential. That fact is borne out by the data in this
1396   BRD which show that the EpiOcular assay can be used to identify and differentiate
1397   EPA Category III from Category IV materials, while the BCOP assay cannot.
1398   Conversely the EpiOcular assay does not seem to be able to differentiate EPA
1399   Category II materials from EPA Category I materials as easily as the BCOP assay.

1400   For ease in understanding the mechanistic basis of the EO assay, a table
1401   (Table 1-3) has been compiled describing the events that are commonly considered to
1402   occur during eye irritation. Those events that are modeled (or are closely related) by
1403   the EpiOcular assay are indicated by a Y (yes) indication.

1404

1405   It can be seen that the EpiOcular assay most closely models some of the
1406   initial stages of interaction of an eye irritant with the cornea. The more distal
1407   occurrences in eye irritation such as gross tissue changes in the corneal stroma,
1408   and the recovery from the lesions, are not directly modeled. However, if the

1409 hypothesis of Jester, Mauer, and others that initial area and depth of injury is
1410 predictive of time to, and extent of, recovery, then the measurements made by the
1411 EpiOcular assay may have a relationship to recovery as well.
1412
1413 **Table 1-3 Summary of events involved in chemical-induced eye irritation *in vivo*. Text in**
1414 **italics represents irreversible responses.**

Events involved in chemical-induced eye irritation	Modeled by the EpiOcular assay?
Chemical interaction with tear film (Klyce and Beuerman 1988; Hackett and McDonald 1994)	N
Chemical binding to the conjunctival epithelium (Hogan and Zimmerman 1962; Hackett and McDonald 1994)	Y
Adhesion molecules compromised (Farquhar and Palade 1963; Van Meer, van Hof et al. 1992; Katahira, Sugiyama et al. 1997)	Y
Corneal epithelium damage (Dua, Gomes et al. 1994)	Y
• Inhibition of receptor-mediated membrane transport (Dearman, Cumberbatch et al. 2003)	Y
• Compromise of cell membrane integrity of upper corneal epithelium (Dua, Gomes et al. 1994; Hackett and McDonald 1994; Maurer and Parker 1996)	Y
• Cell membrane lysis of all corneal epithelium layers (Hackett and McDonald 1994)	Y
Hydration of corneal stroma (Hackett and McDonald 1994)	N
Cross-linking of proteins in corneal stroma (Butler and Hammond 1980; Eurell, Sinn et al. 1991; Chan and Hayes 1994)	N
Erosion of corneal stroma (Baldwin, McDonald et al. 1973; Hackett and McDonald 1994; Maurer and Parker 1996)	N
Cell damage to corneal epithelium and limbus (Jacobs and Martens 1990; Wilhelmus 2001)	Partially
Dilation and increased lymphatic leakage from scleral vasculature (Hackett and McDonald 1994)	N
Stimulation of nerve endings, i.e., enhanced blinking, tearing (Chan and Hayes 1994)	N
Erosion of nerve endings in cornea and sclera (Butler and Hammond 1980; Klyce and Beuerman 1988; Araki, Ohahsi et al. 1994)	N
Duration of response, *i.e.,* length of time cell responses deteriorate. Duration of response covers the effects of reactive chemicals which can cause coagulation, saponification, that are effects which develop and increase over time. (Hubert 1992; Maurer and Parker 1996)	N
Recovery from response, *i.e.,* length of time for cell responses to return to control levels (Hubert 1992)	N

1415   1.3.1.3 BCOP

1416

1417   The test system (target tissue) for the BCOP assay is the isolated bovine
1418   cornea obtained as a by-product from freshly slaughtered animals (Figure 1-6). The
1419   procedures for preparing and handling the test system were developed by
1420   Gautheron *et al.* (1992). The assay measures two important components that are
1421   predictive of eye irritation; corneal opacity and permeability (Sina 1994). When
1422   necessary, the depth and degree of injury may be assessed by histological
1423   evaluation.

1424
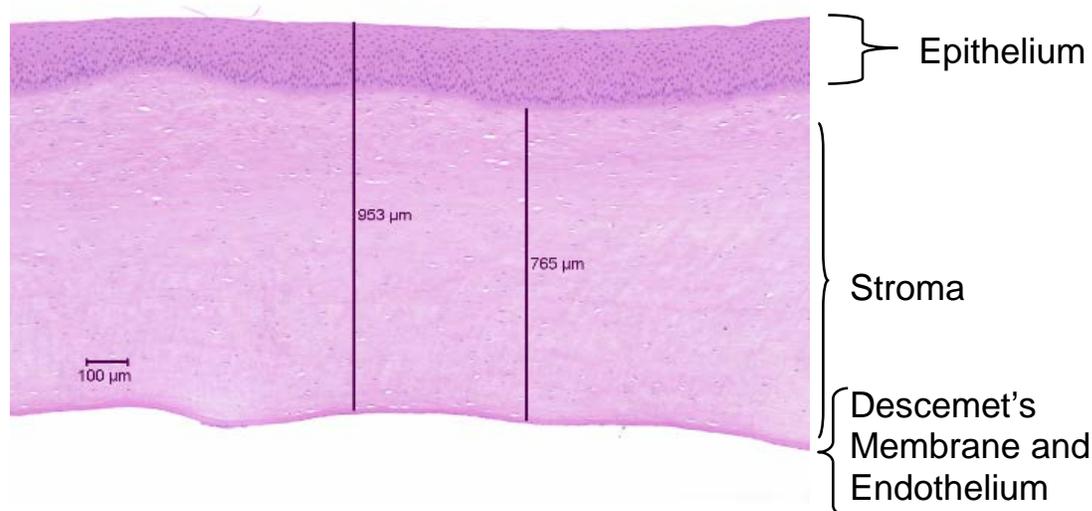
1425   **Figure 1-6 A cross-section of a typical bovine cornea as used in the BCOP assay. (H&E stain)**

1426

1427   Since the apical surface of the bovine cornea is easily accessible in the organ
1428   culture chamber in which the cornea is held, liquid test substances can be easily
1429   applied and tested neat unless information about exact in-use (diluted) conditions
1430   are desired. Solid test substances are usually tested as a 20% slurry in sterile
1431   deionized water. Changes in opacity, permeability to fluorescein, and tissue
1432   architecture (depth of injury) are measured and used to assess the relative potential
1433   for ocular irritancy of the test substances.

1434   1.3.1.3.1 Intended uses / purpose of the BCOP assay

1435

1436   Very similar to what was described earlier for the CM assay (Section
1437   1.3.1.1.1) and the EpiOcular assay (Section 1.3.1.2.1), the BCOP assay is used by
1438   industry early in the product development process to screen solid or liquid
1439   ingredients for cosmetic, personal care, and household cleaning products, as well
1440   as final formulations. One advantage that this method has in comparison to the CM
1441   and EpiOcular test methods is that actual ocular tissue is used in the assay, and, if
1442   desired, damage to the cornea can be visualized by conducting histopathological
1443   analysis after test article treatment. Often final in-house safety decisions are made
1444   based on results from the BCOP assay. Data from the BCOP assay may be
1445   combined with information from other *in vitro* or in silico assays to provide a "weight

1446 of evidence" evaluation of the formulation. Information from this assay is generally
1447 not combined with new animal data in making the final safety decision for the
1448 product.
1449

1450 1.3.1.3.2 Regulatory rationale and applicability of the BCOP test method

1451

1452   To the best of our knowledge, the BCOP test method is not currently included
1453 in the regulatory scheme of any country. However, data from the assay that
1454 indicates severe irritation has been accepted by regulators from several European
1455 Union countries in lieu of animal tests. The test has been reviewed by ICCVAM in
1456 their evaluation of the "Current Status of *In vitro* Test Methods for identifying Ocular
1457 Corrosives and Severe Irritants." The BRD for the BCOP that was constructed for
1458 this effort is appended to this report. We have also quoted freely from this NICEATM
1459 report in the preparation of the BCOP portion of this current BRD. The final
1460 conclusion of ICCVAM concerning the BCOP assay was that there are sufficient
1461 data to support the use of the BCOP test method, in appropriate circumstances and
1462 with certain limitations, as a screening test to identify substances as ocular
1463 corrosives and severe irritants (*i.e.,* EPA Category I, UN GHS Category 1, EU R41)
1464 in a tiered-testing strategy, as part of a weight-of-evidence approach.
1465

1466   Within industry, many toxicologists use results from the BCOP assay (with or
1467 without histopathology analysis) to make final safety and labeling decisions for
1468 products which do not have formal regulatory registration requirements.
1469

1470 1.3.1.3.3 Scientific basis for the BCOP method

1471

1472   The following discussion of the scientific basis for the BCOP assay is quoted
1473 from the NICEATM BRD "Current Status of *In vitro* Test Methods for Identifying
1474 Ocular Corrosives and Severe Irritants: Bovine Corneal Opacity and Permeability
1475 Test Method."

1476

1477   "The BCOP is an organotypic model (*i.e.,* isolated whole organ, or
1478 component thereof) that provides short-term maintenance of normal physiological
1479 and biochemical function of the cornea in an isolated system (Chamberlain, Gad et
1480 al. 1997). As noted above, the BCOP was developed as an alternative eye irritation
1481 test method in order to obviate the need for laboratory animals as the source for test
1482 eyes.

1483

1484   The most commonly used endpoints evaluated in the BCOP assay to
1485 measure the extent of damage to the cornea following exposure to a chemical
1486 substance are corneal opacity and permeability. Opacity is quantitatively measured
1487 by the amount of light transmission through the cornea, and permeability is
1488 quantitatively measured as the amount of the small molecule, sodium fluorescein,
1489 that penetrates all corneal cell layers. Irritant-induced opacity in the cornea indicates

1490   denaturation/precipitation of proteins in the epithelial or stromal layers and/or
1491   swelling, vacuolization, or damage to the cells in the stromal layer (Millichamp
1492   1999). Development of opacity in the cornea, which is normally a transparent tissue,
1493   is a significant adverse effect of some irritants that can lead to vision loss. Increased
1494   corneal permeability results from damage to the corneal epithelium, which normally
1495   serves as a barrier function. In addition, histopathological evaluation of the treated
1496   cornea provides useful descriptive information of corneal damage (Curren, Evans et
1497   al. 2000; Cooper, Earl et al. 2001).
1498
1499   Histopathology or confocal microscopy would allow for a more accurate
1500   assessment of the extent of corneal injury. Maurer *et al.* (2002) proposed that the
1501   extent of ocular injury, as measured by confocal microscopy, has the greatest
1502   impact on the outcome of such an injury. Live/dead cell staining methods evaluated
1503   with confocal microscopy have also been used to determine the extent or depth of
1504   corneal injury (Maurer, Li et al. 1997) and in an *ex vivo* corneal button assay (Jester,
1505   Li et al. 2001). These studies prompted the authors to suggest that the extent of
1506   corneal injury could be used as the basis for developing alternative methods to
1507   predict the level of damage produced by ocular irritants." Thus, the BCOP offers the
1508   possibility of using depth-of-injury analysis through histopathology to predict the
1509   potential outcome of eye injury produced by ocular irritants.
1510
1511   For ease in understanding the mechanistic basis of the BCOP assay, a table
1512   (Table 1-4) has been compiled describing the events that are commonly considered
1513   to occur during eye irritation. Those events that are modeled (or are closely related)
1514   by the BCOP assay are indicated by a Y (yes) indication.
1515
1516   It can be seen that the BCOP assay closely models not only most of the initial
1517   stages of interaction of an eye irritant with the cornea, but also some of the more
1518   distal occurrences in eye irritation such as gross tissue changes in the corneal
1519   stroma. However, the short time period that the cornea can be kept in organ culture
1520   limits the amount of recovery, if any, which may occur. Again, if the hypothesis of
1521   Jester, Mauer, and others that initial area and depth of injury is predictive of time to,
1522   and extent of recovery, then the measurements made by the BCOP assay may
1523   have a relationship to recovery as well.
1524
1525
1526

1527   **Table 1-4 Summary of events involved in chemical-induced eye irritation *in vivo*. Text in**
1528   **italics represents irreversible responses.**

Events involved in chemical-induced eye irritation	Modeled by the BCOP assay?
Chemical interaction with tear film (Klyce and Beuerman 1988; Hackett and McDonald 1994)	N
Chemical binding to the conjunctival epithelium (Hogan and Zimmerman 1962; Hackett and McDonald 1994)	Y
Adhesion molecules compromised (Farquhar and Palade 1963; Van Meer, van Hof et al. 1992; Katahira, Sugiyama et al. 1997)	Y
Corneal epithelium damage (Dua, Gomes et al. 1994)	Y
• Inhibition of receptor-mediated membrane transport (Dearman, Cumberbatch et al. 2003)	Y
• Compromise of cell membrane integrity of upper corneal epithelium (Dua, Gomes et al. 1994; Hackett and McDonald 1994; Maurer and Parker 1996)	Y
• Cell membrane lysis of all corneal epithelium layers (Hackett and McDonald 1994)	Y
Hydration of corneal stroma (Hackett and McDonald 1994)	Y
Cross-linking of proteins in corneal stroma (Butler and Hammond 1980; Eurell, Sinn et al. 1991; Chan and Hayes 1994)	Y
Erosion of corneal stroma (Baldwin, McDonald et al. 1973; Hackett and McDonald 1994; Maurer and Parker 1996)	Y
Cell damage to corneal epithelium and limbus (Jacobs and Martens 1990; Wilhelmus 2001)	Y
Dilation and increased lymphatic leakage from scleral vasculature (Hackett and McDonald 1994)	N
Stimulation of nerve endings, i.e., enhanced blinking, tearing (Chan and Hayes 1994)	N
Erosion of nerve endings in cornea and sclera (Butler and Hammond 1980; Klyce and Beuerman 1988; Araki, Ohahsi et al. 1994)	N
Duration of response, *i.e.,* length of time cell responses deteriorate. Duration of response covers the effects of reactive chemicals which can cause coagulation, saponification, that are effects which develop and increase over time. (Hubert 1992; Maurer and Parker 1996)	Partially
Recovery from response, *i.e.,* length of time for cell responses to return to control levels (Hubert 1992)	N

1529
1530

2 Test Method Components

2.1 Overview of the proposed testing approach

A general review of how this project was structured and how the testing approach was determined has been presented in Section 1 – Introduction and Rationale. The testing approach itself is presented in Figure 1-1 and relies on using one of three *in vitro* assays potentially supplemented with a second *in vitro* assay to further refine the appropriate labeling category.

Anti-microbial cleaning products can be formulated in different ways. Although to begin this study we characterized the formulations into several different classes, i.e. acids, bases, surfactants, solvents, and oxidizing chemistries, we found that most of these classes reacted similarly in the in vitro assays. We eventually concluded that only those materials with oxidizing chemistry and those with a high solvent concentration (>5%) should be treated somewhat differently from the others. It is also useful to determine the water solubility of the formulation since only fully water soluble materials can be tested in the Cytosensor Microphysiometer.

The proposed testing strategy (see Figure 1-1) begins by evaluating the components of the formulation. If the formulation is characterized as having oxidizing chemistry, then the first step is to test it using the BCOP assay. This is done because the oxidizers seem to be overpredicted in the other assay systems (see Section 6 – Test Method Predictive Capacity). Any of the other types of formulations may also be tested in the BCOP assay, although we suggest that formulations thought to be mild or non-irritating (e.g. EPA labeling categories III or IV) be tested first in either the Cytosensor or EpiOcular assays. This is suggested since the latter two assays are better able to identify EPA IV materials than the BCOP assay (see Section 6 – Test Method Predictive Capacity). Conversely, if the formulation is thought to be a strong eye irritant, (e.g. EPA I or II) it is suggested that it first be tested in the BCOP assay. If the formulation is characterized as a high solvent (>5%) product, the BCOP assay should be conducted with a 3 minute exposure rather than the traditional ten minute exposure. This is because our studies showed that some high solvent materials were overclassified by the BCOP if the longer exposure was used (see discussion in Section 6.3.2.2.3).

Table 2-1 describes the BCOP assay *in vitro* score cut-off values for the EPA category designations. If the testing results in a BCOP *in vitro* score that is ≥75 it is given a Category I designation. If testing results in a score ≥25, it is initially given a Category II designation, but histopatholgy of the corneas is conducted to verify the designation (see Section 6.3.3). Similarly, a material scoring <25 (Category III) should have histopathology performed to verify its designation, or it could be retested in the Cytosensor or EpiOcular assays to determine whether it was actually a Category IV rather than a Category III.

1576   **Table 2-1 BCOP *in vitro* score and EPA category designation**

BCOP *In vitro* Score	EPA Category
in vitro score ≥ 75	Category I
75 > *in vitro* score ≥ 25	Category II (Histopathology should be performed)
in vitro score < 25	Assume Category III (Histopathology should be performed) or retest in Cytosensor or EpiOcular to determine if Category III or IV

1577
1578   When conducting the BCOP assay the following conclusions from Section 6
1579   should be considered:
1580
1581   1)   **In general, when testing anti-microbial cleaning product**
1582   **formulations, the BCOP assay should be conducted with a ten**
1583   **minute exposure.**
1584
1585   2)   **If the anti-microbial cleaning product contains a solvent at the level**
1586   **of 5% or greater, it should be tested with a three minute exposure.**
1587
1588   3)   **All anti-microbial cleaning products having an *In Vitro* Score ≥75**
1589   **should be classified as an EPA Category I or a GHS Category 1. <u>No</u>**
1590   **<u>histopathology needs to be conducted.</u>**
1591
1592   4)   **Anti-microbial cleaning products having an *In Vitro* Score <75 and ≥**
1593   **25 are given a preliminary classification of EPA Category II or GHS**
1594   **Category 2A. They should be further assessed with a**
1595   **histopathological evaluation and given the final categorization of**
1596   **whichever determination (*In Vitro* Score or histological evaluation)**
1597   **is more severe.**
1598
1599   5)   **Anti-microbial cleaning products having an *In Vitro* Score <25 are**
1600   **given a preliminary classification of EPA Category III or GHS**
1601   **Category 2B. They should be further assessed with a**
1602   **histopathological evaluation and given the final categorization of**
1603   **whichever determination (in vitro score or histological evaluation)**
1604   **is more severe.**
1605
1606   6)   **(Optional) To determine if an anti-microbial cleaning product which**
1607   **was categorized as either EPA III or GHS 2B is actually an EPA IV**
1608   **or a GHS NI, it should be further tested in either the Cytosensor or**
1609   **EpiOcular assays.**
1610
1611
1612

1613   For materials not characterized as having oxidizing chemistry and not
1614   suspected to be a severe irritant, either the Cytosensor or EpiOcular test is chosen.
1615   Liquids and aqueous soluble materials can be tested with the Cytosensor. Granular,
1616   non-aqueous soluble materials and liquid, aqueous soluble materials can be tested
1617   in the EpiOcular assay. The choice, other than considering the water solubility
1618   requirement of the Cytosensor, would be based solely on the experience of the user
1619   with one method or the other. However, as discussed in Section 2.2.1, it is likely that
1620   in a few years the Cytosensor assay may no longer be available since its
1621   manufacturer is no longer supporting the instrument. At that time the EpiOcular
1622   assay (or a similar three-dimensional tissue model) will be the only *in vitro* model
1623   available to identify EPA Category IV materials – unless another assay is found in
1624   the meantime that can be shown to reliably identify the extremely mild materials.
1625

1626   Using cut-off values for either the Cytosensor or EpiOcular assays that are
1627   described later in this submission, a decision can be made whether the material is a
1628   Category IV, III, or I. Both of these tests were designed to evaluate mild materials
1629   and although both can identify severe materials, they do not have the ability to
1630   discriminate between Category I and Category II materials. If there is a desire to
1631   differentiate between Category I and II materials the BCOP assay must be used.
1632

1633   When conducting the Cytosensor assay the following conclusions from
1634   Section 6 should be considered:
1635

1636   **1) Anti-microbial cleaning products having an oxidizing chemistry**
1637   **should not be tested with the Cytosensor assay.**
1638

1639   **2) Only fully water soluble anti-microbial cleaning products can be**
1640   **tested with the Cytosensor assay.**
1641

1642   **3) If the anti-microbial cleaning product has an MRD_{50} score of <2**
1643   **mg/ml, it is classified as EPA Category I or GHS Category 1.**
1644

1645   **4) If the anti-microbial cleaning product has an MRD_{50} score of ≥2**
1646   **mg/ml, but < 80 mg/ml, it is classified as EPA Category III. If the anti-**
1647   **microbial cleaning product has an MRD_{50} score of ≥2 mg/ml, but <10**
1648   **mg/ml, it is classified as GHS Category 2B.**
1649

1650   **5) If the anti-microbial cleaning product has an MRD_{50} score of ≥80**
1651   **mg/ml, it is classified as EPA Category IV. If the anti-microbial**
1652   **cleaning product has an MRD_{50} score of ≥10 mg/ml, it is classified**
1653   **GHS Category NI.**
1654

1655   **6) (Optional) To determine if an anti-microbial cleaning product which**
1656   **was categorized as either EPA I or GHS 1 is actually an EPA II or a**
1657   **GHS 2A, it should be further tested in the BCOP assay.**

1658
1659
1660   When conducting the EpiOcular assay the following conclusions from Section
1661   6 should be considered:
1662
1663   **1) Anti-microbial cleaning products having an oxidizing chemistry**
1664   **should not be tested with the EpiOcular assay.**
1665
1666   **2) Both water soluble and water insoluble anti-microbial cleaning**
1667   **products can be tested with the EpiOcular assay.**
1668
1669   **3) If the anti-microbial cleaning product has an ET$_{50}$ score of <4**
1670   **minutes, it is classified as EPA Category I or GHS Category 1.**
1671
1672   **4) If the anti-microbial cleaning product has an ET$_{50}$ score of ≥4**
1673   **minutes, but <70 minutes, it is classified as EPA Category III or GHS**
1674   **Category 2B.**
1675
1676   **5) If the anti-microbial cleaning product has an ET$_{50}$ score of ≥70**
1677   **minutes, it is classified as EPA Category IV or GHS Category NI.**
1678
1679   **6) (Optional) To determine if an anti-microbial cleaning product which**
1680   **was categorized as either EPA I or GHS 1 is actually an EPA II or a**
1681   **GHS 2A, it should be further tested in the BCOP assay.**
1682
1683   The above strategy - which provides the option for using several different *in vitro*
1684   methods – was devised because we found that no single *in vitro* test was able
1685   adequately cover the entire range of irritation that is covered by the EPA labeling
1686   categories I – IV. The BCOP is a more robust tissue and is able to differentiate the
1687   more aggressive materials from each other, while the CM and EO are more
1688   sensitive methods and thus better able to resolve differences between milder
1689   materials. **Note that if the original decision that the test material falls in the**
1690   **severe range or in the mild range proves to be false when the material is**
1691   **actually tested, the strategy still works; the testing may just take longer**
1692   **because a second assay may have to be used.** If a mild material is mistakenly
1693   put into the BCOP it will be identified as a Category III (remember: the BCOP cannot
1694   differentiate a IV from a III, and in such a case the more conservative category must
1695   be given). To determine if this material is a IV, a second assay in Cytosensor or
1696   EpiOcular would have to be conducted.
1697
1698   Similarly if a severe material is tested in the EpiOcular or Cytosensor assays it
1699   will be identified as a Category I. If it is necessary to find out if it's actually a
1700   Category II, it must be retested in the BCOP. <u>The strategy is self-correcting so there</u>
1701   <u>is no worry about initially choosing an incorrect test method using this approach</u>.
1702

1703   ## 2.2 Detailed description and rationale for each assay

1704
1705   The methodologies utilized in the proposed *in vitro* strategy for toxicity
1706   classification of anti-microbial cleaning products are the Cytosensor assay, the
1707   EpiOcular assay, and the BCOP assay. The methodology used for each of these
1708   assays is described below.

1709   ### 2.2.1 Overview of how the CM test method is conducted

1710
1711   The CM uses a low volume flow-through chamber and a light-addressable
1712   potentiometer to measure the metabolic rate of a cell population. Metabolic rate is
1713   determined indirectly by the number of protons excreted into the low buffer medium
1714   (change in pH) per unit time. The light-addressable potentiometer forms the bottom
1715   of the flow-through chamber and serves as a very sensitive and stable pH meter.
1716   While medium is flowing through the chamber, the pH is stable and governed by the
1717   medium. When the flow of medium is stopped, the pH begins to drop in a linear
1718   fashion over time. The actual change in pH during this measurement is generally
1719   less than 0.2 pH units.
1720
1721   Data contained in this BRD were generated with two different instruments.
1722   One was the predecessor instrument to the current CM, the Silicon
1723   Microphysiometer (SM). In the SM (Figure 2-1) target cells were grown on a glass
1724   coverslip and the coverslip was inverted over the top of the sensor chip to form a
1725   flow-through chamber (Figure 2-2). A minority of data was generated with the SM
1726   protocol. The majority of the data in this BRD were generated with the Cytosensor.
1727
1728   The positive control currently used for CM studies at IIVS is SLS (using a
1729   stock concentration of 100 mg/mL in water). The current (as of 4/28/08) acceptable
1730 MRD_{50} is 79.8 µg/mL± 11.3 µg/mL.
1731
1732   To conduct the Cytosensor protocol as used for the majority of studies
1733   reported in this BRD (see Annex A1), cells are grown on a Transwell membrane
1734   (discussed below). The whole Transwell is placed into the sensor chamber and a
1735   plunger (with a spacer) pressed down on the membrane to seal it. The sensor
1736   chamber is composed of the light-addressable potentiometer sensor (sensor chip)
1737   on the bottom and ports for the medium (inlet and outlet). There is a small medium-
1738   filled space between the sensor chip and the bottom of the Transwell. The cells are
1739   attached to the top of the membrane so that the acid metabolites must pass through
1740   the membrane pores to reach the space in the lower part of the chamber. The
1741   medium is passed over the cells on the upper side of the membrane. Figure 2-3
1742   shows the operating components of the instrument and Figure 2-4 shows the low
1743   volume sensor chamber (Transwell configuration). Based on the comparison of data
1744   generated in both the SM and CM, Procter & Gamble established a conversion
1745   algorithm so that all results generated initially from the SM could be compared to the
1746   results generated with the CM (details provided in section 2.2.1.1).
1747

1748
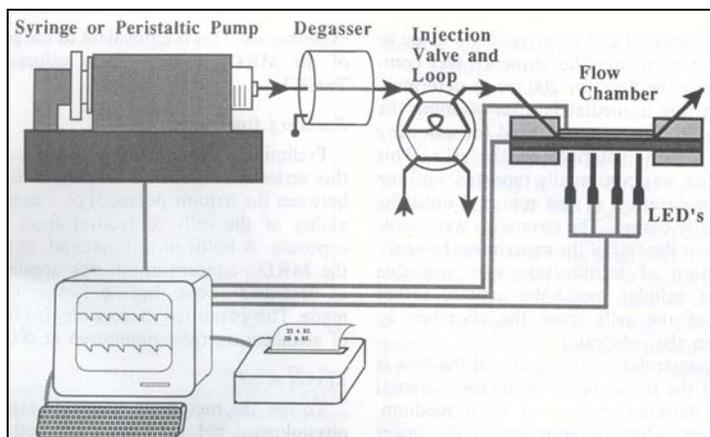
1749
1750   **Figure 2-1 Diagram of the operating components of the silicon microphysiometer (Bruner,**
1751   **Miller et al. 1991)**
1752
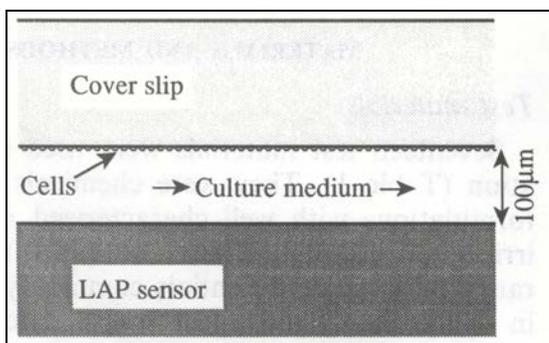
1753
1754   **Figure 2-2 The original silicon microphysiometer sensor chamber with the coverslip in place**
1755   **(Bruner, Miller et al. 1991)**
1756
1757
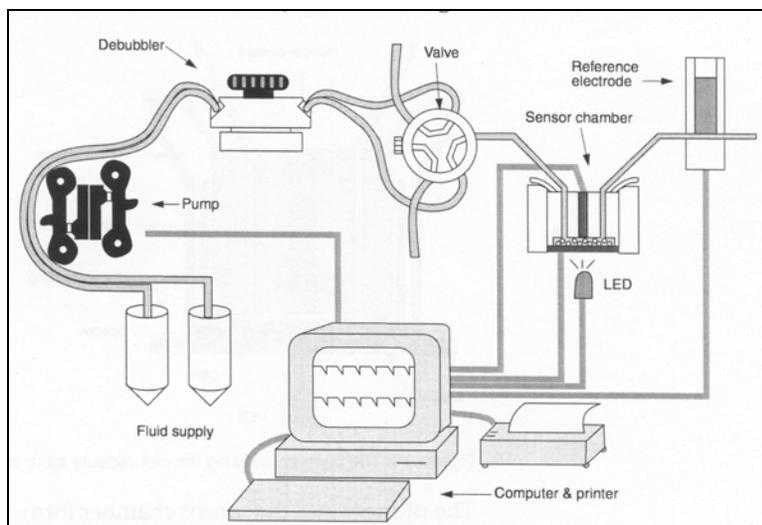
1758
1759   **Figure 2-3 Diagram of the operating components of the Cytosensor (Cytosensor Manual)**
1760
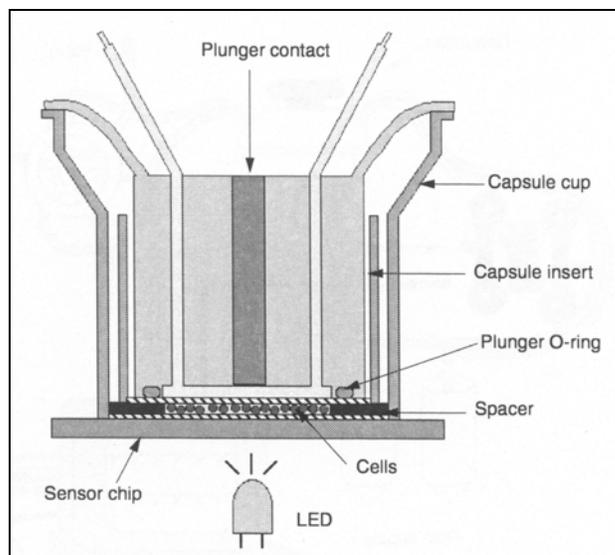
1761
1762 **Figure 2-4 The Cytosensor chamber with the Transwell in place (Cytosensor Manual)**
1763

1764   Originally, the silicon microphysiometer (coverslip chamber) used a 15-
1765 minute exposure, rinse, and read cycle. The cells were exposed to each
1766 concentration in two phases. In the first phase, the diluted test article was pumped
1767 (1.67 µL/sec) through the chamber for 120 seconds and then the flow halted for 200
1768 seconds (total of 320 seconds of exposure). The chamber was then rinsed with
1769 fresh medium at the same rate for 380 seconds. The flow was then stopped for 200
1770 seconds while the acidification rate was measured. This exposure protocol was
1771 used primarily on normal human epidermal keratinocytes (Bruner, Miller et al. 1991).
1772 Most of the studies in this BRD used L929 cells as the test system. The exposure
1773 protocol was altered so that the cells were exposed to the test article for a total of
1774 500 seconds (300 seconds of flow and 200 seconds with the flow off), rinsed for 400
1775 seconds, and the metabolic rate determined for 169 seconds. Flow was restarted
1776 with medium before the next dose was introduced. Because the valves were turned
1777 manually, the total cycle time was 1100 seconds.
1778

1779   In contrast, the Cytosensor (both the commercial instrument and the silicon
1780 microphysiometer with "Cytosensor-like" chambers used a 20-minute (1200-second)
1781 exposure, rinse, and read cycle. This is still the current protocol. The cells are
1782 exposed 810 seconds (100 µL per minute for one minute and 20 µL per minute for
1783 12.5 minutes). The rinse cycle lasts for 6 minutes and the flow is 100 µL per minute.
1784 Finally, the flow is stopped for 25 seconds and the change in pH is measured. For
1785 the purposes of the BRD, this will be the standard Transwell protocol (for either the
1786 converted silicon microphysiometer or the Cytosensor).
1787

1788   The bulk of the available data come from the Transwell protocol using the
1789 810-second exposure. The Transwell was introduced by Molecular Devices, Inc. to
1790 allow more efficient introduction of the test system to the sensor chambers
1791 (including non-adherent cells in a gelatin matrix). However, this change limited the
1792 cell density and types of cells that could be used. The Transwells have 3 micron
1793 pores that allow efficient communication between the upper surface of the

1794   membrane (with the cells) and the lower surface that faces the sensor itself.
1795   Confluent cell layers would interfere with this communication and so the cell density
1796   was reduced to a standard 6×10^5 cells per well (seeded the day before use). The
1797   Transwell uses a polycarbonate filter membrane that is less prone to interaction with
1798   test materials than other types of membranes but does not allow the human
1799   keratinocytes to attach. Thus, the L929 cells were selected because they would
1800   readily attach and were easy to grow in continuous culture. With the change to L929
1801   cells, the SM exposure protocol was changed to 500 seconds. This is the protocol
1802   that was used for most of the SM studies in this BRD. This is also the same protocol
1803   that was used in the IIVS positive control database before a switch was made to the
1804   CM.

1805   2.2.1.1 Development of Conversion Algorithm between SM and CM

1806   At the time that the SM was replaced with the CM by Molecular Devices, Inc.,
1807   The Procter & Gamble Company sponsored a study to compare data obtained with
1808   the SM (coverslip protocol) for a set of 11 surfactant-containing materials with data
1809   obtained for the same materials with the CM (Transwell protocol). The studies were
1810   carried out concurrently at a single laboratory (Microbiological Associates, Inc.). The
1811   testing protocol utilized a preliminary trial followed by at least three definitive trials.
1812   Data produced by the SM and CM are shown in Tables 2-2 & 2-3, respectively. It
1813   can be seen that the overall mean CV for each of the two methods is very similar
1814   (22.8% for the SM; 21.8% for the CM).

1815   Following data collection from both instruments, the data were compared and
1816   the following equation was derived to translate SM coverslip data to CM Transwell
1817   data:
1818
1819 $\text{Log}_{10}(\text{Cytosensor MRD}_{50}) = 0.135 + 0.7753 \times \text{Log}_{10}(\text{Silicon Microphysiometer MRD}_{50})$.
1820
1821   A graph depicting the relationship between the SM and CM is given in Figure
1822   2-5. The current standard Cytosensor protocol is attached in Annex A1.
1823

1824   **Table 2-2 Silicon Microphysiometer data for 11 surfactant-containing materials from P&G**

Substance	Prelim*	Trial 1	Trial 2	Trial 3	Trial 4	Mean MRD$_{50}$ (mg/mL)	SD	CV (%)
#1	21.368	18.116	25.510	20.408		21.345	3.785	17.7
#2	+	0.083	0.085	0.082		0.083	0.001	1.7
#3	+	0.291	0.266	0.263		0.273	0.015	5.5
#4	+	0.247	0.153	0.435	0.298	0.283	0.117	41.5
#5	+	13.643	13.004	9.434		12.027	2.268	18.9
#6	+	0.042	0.027	0.026		0.032	0.009	28.2
#7	0.161	0.093	0.139	0.198		0.143	0.053	36.8
#8	0.714	2.020	1.239	1.595		1.618	0.391	24.2
#9	0.094	0.043	0.032	0.039		0.038	0.006	14.7
#10	0.020	0.045	0.038	0.026		0.036	0.010	26.9
#11	+	0.081	0.094	0.152		0.109	0.038	34.5
Mean								**22.8**
Median								**24.2**

1825   * Not included in the mean calculation
1826   + Value not determined during assay
1827
1828   **Table 2-3 Cytosensor Microphysiometer data for 11 surfactant-containing materials from P&G**

Substance	Prelim*	Trial 1	Trial 2	Trial 3	Trial 4	Mean MRD$_{50}$ (mg/mL)	SD	CV (%)
#1	90.909	56.497	48.544	62.500		55.847	7.001	12.5
#2	0.223	0.254	0.424	0.283		0.320	0.091	28.4
#3	0.758	0.794	0.552	0.820		0.722	0.147	20.4
#4	0.452	0.442	0.412	0.431		0.428	0.016	3.7
#5	19.120	9.091	11.429	5.319		8.613	3.083	35.8
#6	0.067	0.074	0.052	0.075		0.067	0.013	19.2
#7	0.251	0.177	0.288	0.267		0.244	0.059	24.3
#8	2.288	2.110	2.016	2.457		2.194	0.232	10.6
#9	3.497	1.475	4.367	3.802		3.215	1.533	47.7
#10	0.282	+	0.139	0.151	0.165	0.152	0.013	8.5
#11	0.251	0.268	0.159	0.281		0.236	0.067	28.4
Mean								**21.8**
Median								**20.4**

1829   * Not included in the mean calculation
1830   + Value not determined during assay
1831

1832
1833

Comparison of SM and CM
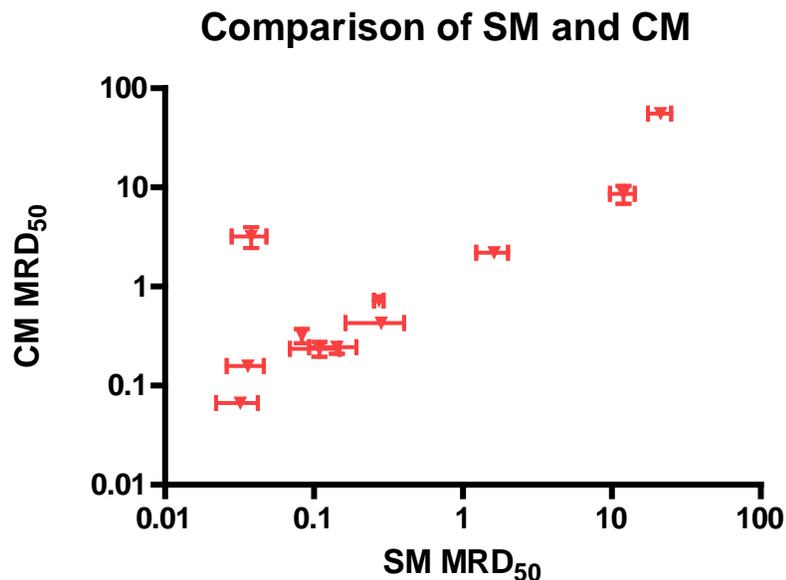
1834
1835 **Figure 2-5 A comparison of data obtained from 11 surfactant-containing products with SM and**
1836 **CM.**
1837

1838   A more complete description of the Cytosensor is given in a Background
1839 Review Document recently prepared under contract to ECVAM. Because this BRD
1840 is still in the review process it could not be directly appended to this document, but it
1841 is quoted from extensively in this BRD. It will be referred to repeatedly in this
1842 submission where more detail is required.
1843

1844 2.2.2 Overview of how the EpiOcular test method is conducted

1845 2.2.2.1 Preparation of the EpiOcular tissue (Description provided by the
1846 manufacturer, MatTek Corporation, Ashland, MA)

1847

1848   The EpiOcular model is prepared using proprietary manufacturing
1849 techniques in which normal human neonatal foreskin keratinocytes, derived from a
1850 single donor, are grown under standardized conditions to produce a highly uniform,
1851 reproducible cornea-like tissue. The keratinocytes are expanded in monolayer
1852 culture and harvested using trypsinization according to standard techniques
1853 described in literature available from Cascade Biologics, Inc. (Portland, OR), the
1854 commercial vendor from which the keratinocytes are currently obtained. Single cell
1855 suspensions of keratinocytes are aliquoted into 10-mm ID Millicell® PCF cell
1856 culture inserts (Millipore Corporation, Bedford, MA); polycarbonate Nunc[TM] cell
1857 culture inserts (Nalge Nunc International, Rochester, NY) also serve as suitable
1858 substrates. The inserts are placed in a 37ºC, 5% CO_2 incubator and cultured at
1859 the air liquid interface, *i.e.,* only the basal side of the cell culture inserts is exposed

1860   to the medium (see Figure 2-6).   The culture medium is Dulbecco's Modified
1861   Eagle's Medium (DMEM) to which a proprietary mixture of nutrients, growth
1862   factors, and hormones has been added; all media are serum free.   After
1863   approximately one week of culture, the cell culture inserts containing the stratified
1864   tissue are placed atop DMEM-enriched agarose gel in a 24-well tissue culture
1865   plate. This 24-well plate is hermetically sealed ("packaged") and shipped for
1866   commercial sale or stored at 4°C for 24-72 hours prior to its use for testing.   For
1867   commercial purposes, these packaged tissues are shipped every Monday on wet
1868   ice (c.a. 4ºC) via overnight express delivery.
1869
1870
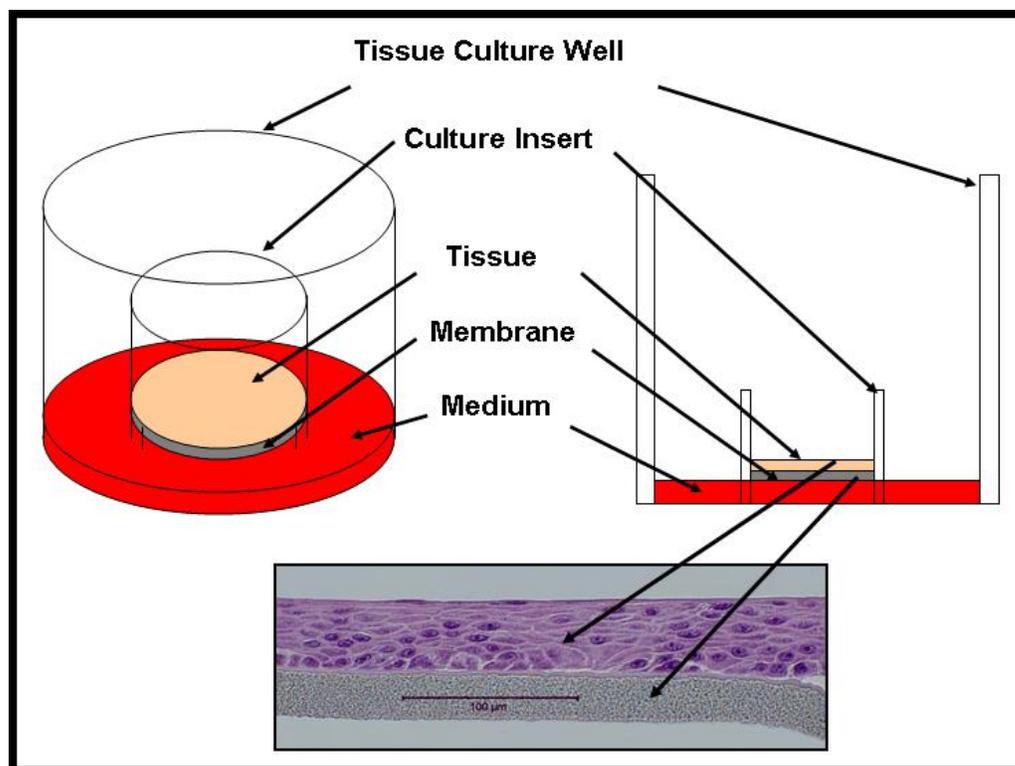
1871   **Figure 2-6 Diagrammatic representation of EpiOcular tissue growing in a milliicell chamber**
1872   **placed within a well of a 24-well plate. A photomicrograph of a cross section through the**
1873   **tissue and underlying membrane is included.**

1874   2.2.2.2 Test methodology
1875
1876   The protocol used for the majority of EO studies in this BRD can be found in
1877   Annex A3. On arrival at the laboratory, EpiOcular tissues are examined for obvious
1878   defects and may be rejected based on blistering, excess fluid on the tissue
1879   (evidence of an incomplete barrier), air bubbles below the tissue insert, etc. Tissues
1880   can be used within 48 hours of receipt. Prior to test article dosing, tissues are
1881   transferred (using sterile technique) to 6-well plates that contain fresh assay
1882   medium. The tissues are incubated at standard conditions (5% CO_2, 37°C, 95%
1883   humidity) for at least 1 hour before use.
1884

1885   EpiOcular tissues which are not used immediately should be equilibrated by
1886   placement into a 5% CO_2 environment and stored at 4°C. Experience indicates that
1887   repeated equilibration at 5% CO_2, 37°C, 95% humidity (*i.e.,* tissue culture incubator)
1888   can produce variability in tissue performance. Prior to dosing with test materials or
1889   controls, the tissues are re-fed with fresh, prewarmed assay medium and generally
1890   dosed within 30 minutes of refeeding.
1891
1892   The positive control currently used for EO studies at IIVS is 0.3% TRITON[®]
1893   X-100 in water. The current (as of 4/28/08) acceptable ET_{50} is 27.3 min ± 5.0 min..
1894
1895   Dosing of aqueous or semi-viscous test materials is performed with a positive
1896   displacement pipette. Solid materials are "sprinkled" onto the surface of the tissue. A
1897   dosing device (*e.g.,* the flat end of a sterile push pin) can be used to ensure that the
1898   test material covers the complete tissue surface. After application of the test
1899   material, the tissues are incubated at standard conditions for various amounts of
1900   time estimated to cover the time at which the test material causes 50% toxicity to
1901   the tissues. Exposure times generally range from 1 minute to 24 hours. Figure 2-7
1902   presents diagrammatically the procedures used in the EpiOcular assay.
1903
1904   At the end of the incubation period the tissues are removed from the
1905   incubator, and the test material is removed from the tissue surface using phosphate
1906   buffered saline (PBS). The PBS is sprayed against the Millicell® wall to create a
1907   gentle vortex which aids in test material removal. The tissues are then "soaked" in
1908   medium at room temperature to ensure a more complete removal of any remaining
1909   test material. Following the soak process, the tissues are rinsed again with PBS
1910   prior to the MTT reduction step. Complete test material removal is necessary to
1911   prevent prolonged exposure and an erroneous estimate of toxicity. Individual tissues
1912   are placed into wells containing unreduced *3-(4,5-dimethylthiazol-2-yl)-2,5-*
1913   *diphenyltetrazolium bromide* (MTT) solution. The tissues are incubated at standard
1914   conditions for 3 hours. Viable tissue reduces the colorless MTT solution to a dark
1915   blue or purple color.
1916
1917   Following exposure to MTT, the tissues are removed and placed into
1918   isopropanol for 2 hours at room temperature to extract the reduced MTT. Extracted
1919   MTT is thoroughly mixed and transferred to a 96-well plate. The amount of
1920   MTT/ethanol in each well is then quantified using a microplate reader. Raw OD_{550}
1921   values are used to calculate the final ET_{50} values which are reported in minutes.
1922
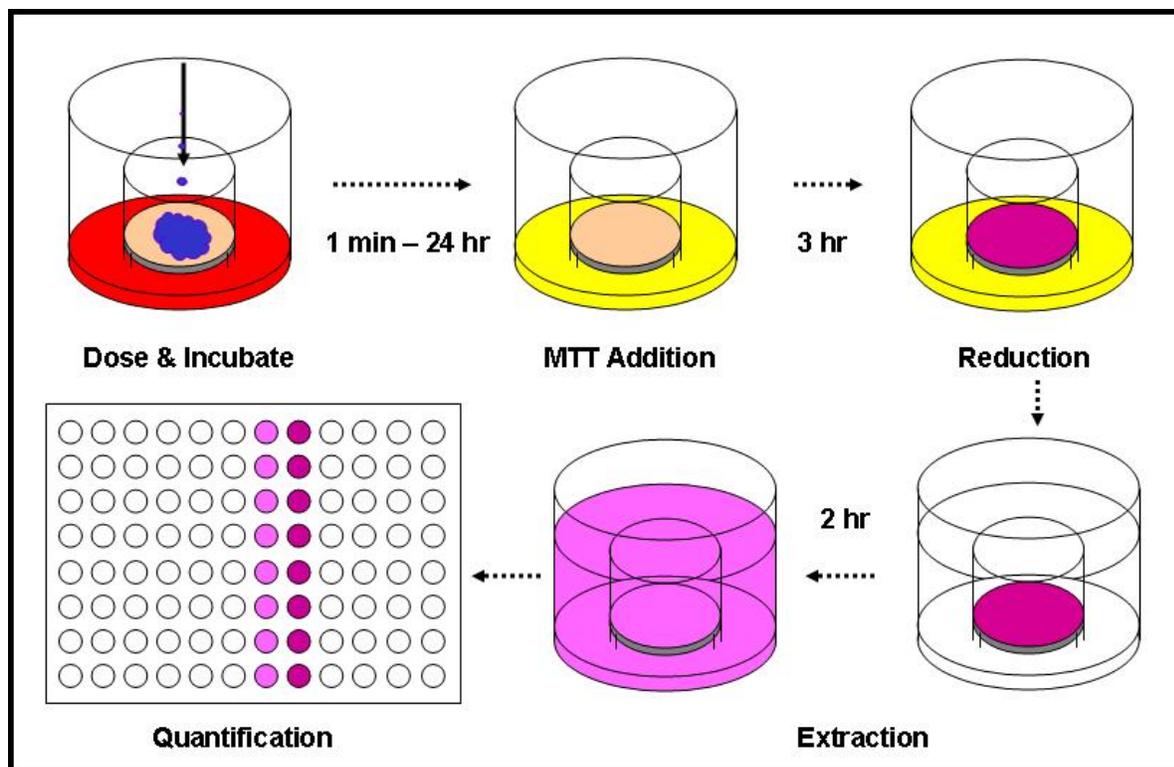
1924 Figure 2-7 Diagrammatic representation of the testing procedure using EpiOcular tissue.
1925 Incubation is carried out at 37°C, and test material is thoroughly removed before the addition
1926 of MTT.
1927

1928   One technical detail of the assay that can cause serious underestimation of
1929 toxicity, and therefore must be carefully controlled, is the possible reduction of MTT
1930 by a test material which itself has reducing properties (Liebsch, Traue et al. 2000). If
1931 a test material has reducing properties <u>and it binds to the tissue or underlying</u>
1932 <u>membrane</u> such that it is not removed during the washing step, then it may reduce
1933 the MTT solution resulting in a masking of toxicity to the EpiOcular tissue. This
1934 would result in an underprediction of the toxicity category for the test material. This
1935 situation can be addressed by screening all test materials for the presence of
1936 reducing activity by incubating them directly in MTT solution. If they have reducing
1937 properties they will turn the solution purple (see top middle photograph in Figure 2-
1938 8). If direct reduction is observed, its actual effect on the assay can be determined
1939 by conducting a sham exposure on EpiOcular tissue that has been freeze–killed. If
1940 no MTT reduction is seen, then no test material remained on the tissue or
1941 membrane after the wash step and the reducing properties of the test material are
1942 not of a concern. However, if reduction has occurred the amount can be calculated
1943 and that value can be subtracted from the MTT reduction at the identical time point
1944 in the full assay so that the true viability of the tissue can be determined.
1945

1946   Other aspects of the assay that can be visualized are shown in Figure 2-8.
1947 For example, the photograph in the top left illustrates the results of testing a material
1948 with hydroscopic properties. Almost all the medium has been absorbed by the test

1949 material likely causing toxicity to the EpiOcular tissue which might not occur in an *in*
1950 *vivo* situation. Similarly artifactual results can occur unless the presence of air
1951 bubbles under the membrane is carefully monitored (Figure 2-8 top right
1952 photograph). Large air bubbles can significantly block the passage of MTT into the
1953 tissue.
1954
1955   The lower row of photographs in Figure 2-8 demonstrate that the viability of
1956 the tissue can be visualized at the conclusion of the MTT exposure step, and
1957 therefore these recorded observations of toxicity can be compared to subsequent
1958 viability values calculated from the absorbance values.
1959
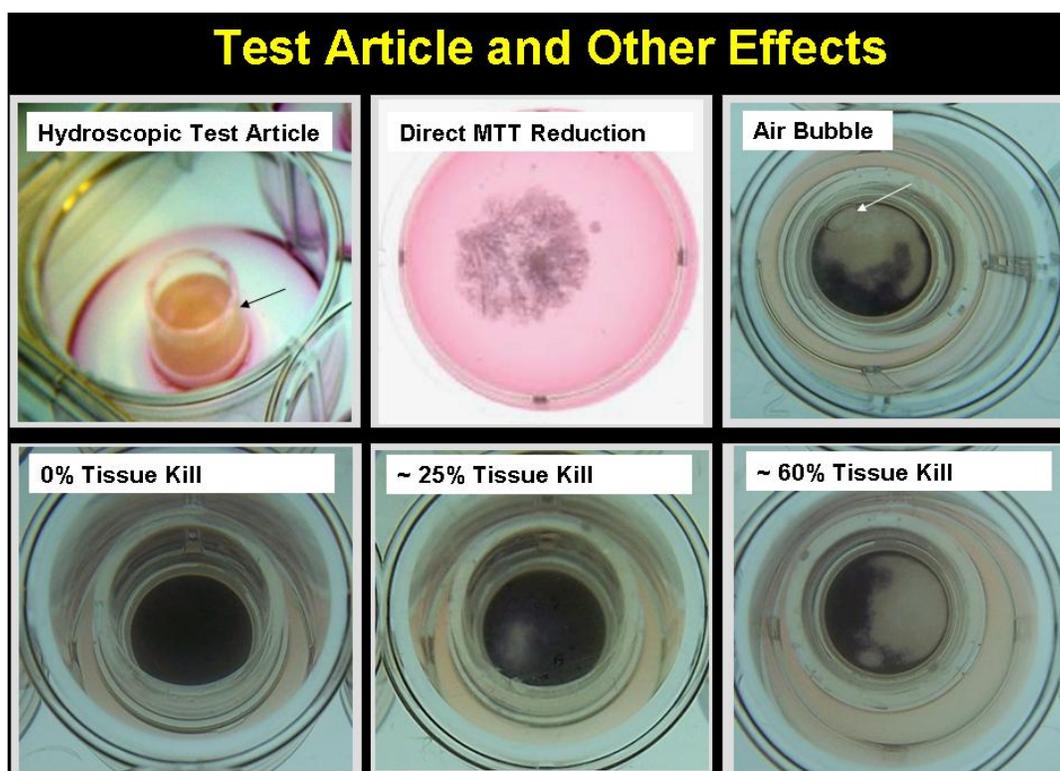
1960
1961   **Figure 2-8 Photographs of various aspects of the EpiOcular assay.**
1962

1963   A more complete description of the EpiOcular assay is given in a Background
1964 Review Document recently prepared for submission to ECVAM. Although the
1965 EpiOcular BRD focuses on a prediction model different from that proposed in this
1966 BRD, the treatment protocol is essentially identical, with the exception that the
1967 ECVAM BRD protocol uses a dilution of the test article before application. The
1968 ECVAM BRD will be referred to repeatedly in this submission where more detail is
1969 required.
1970

1971 2.2.3 Overview of how the BCOP test method is conducted
1972
1973   The overview of the BCOP test method procedures given below is taken
1974 directly from the NICEATM BRD "Current Status of *In vitro* Test Methods for

1975   Identifying Ocular Corrosives and Severe Irritants: Bovine Corneal Opacity and
1976   Permeability Test Method."
1977
1978   "The basic procedures used to assess the effects of a test substance on an
1979   isolated bovine cornea were first reported by Gautheron *et al.* (1992). As described
1980   by Sina and Gautheron (1994, 1998), the BCOP assay uses isolated corneas from
1981   the eyes of freshly slaughtered cattle. Corneas free of defects are dissected with a 2
1982   to 3 mm rim of sclera remaining to assist in subsequent handling, with care taken to
1983   avoid damage to the corneal epithelium and endothelium. Isolated corneas are
1984   mounted in specially designed corneal holders that consist of anterior and posterior
1985   compartments, which interface with the epithelial and endothelial sides of the
1986   cornea, respectively (Figure 2-9 – upper left). Both chambers are filled with medium
1987   and the device is then incubated at 32 ± 1°C for one hour to allow the corneas to
1988   equilibrate with the medium and to resume normal metabolic activity. Following the
1989   equilibration period, fresh medium is added to both chambers, and a baseline
1990   opacity measurement is performed. Corneal opacity is measured quantitatively as
1991   the amount of light transmission through the cornea (Figure 2-9 – upper right).
1992
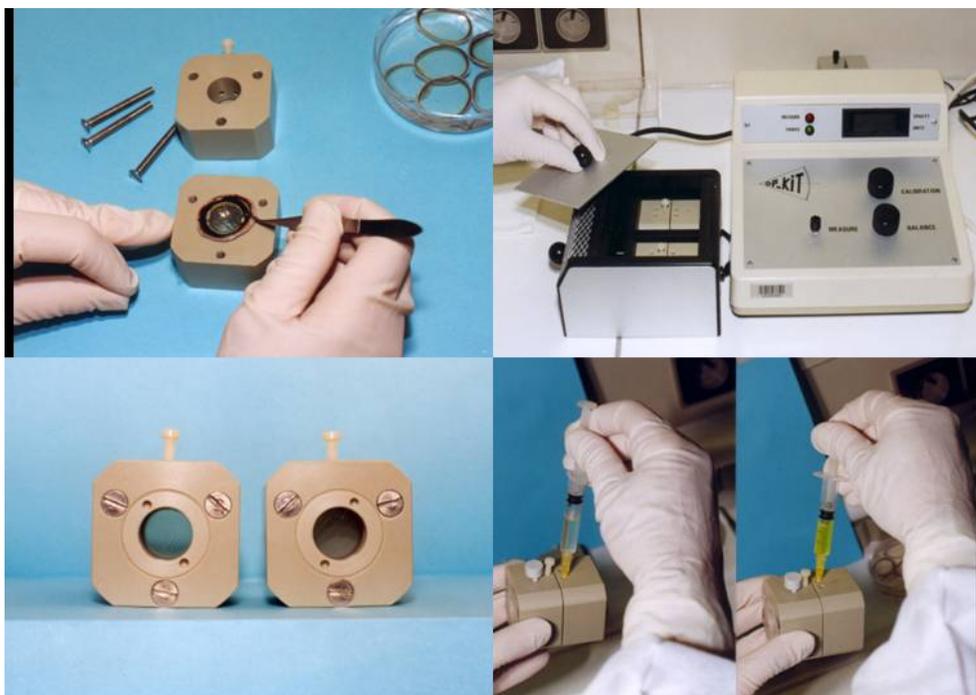
1993
1994
1995   **Figure 2-9 Photographs of various procedures occurring in the BCOP protocol. Upper left –**
1996   **Placing an excised cornea on the corneal holder. Upper right – Using the opacitometer to**
1997   **measure the opacity of a bovine cornea contained in a corneal holder. Bottom left – Visual**
1998   **comparison of the transparency of an untreated cornea on the left and a cornea treated with**
1999   **an irritating material on the right. Lower right – removing fluorescein solution from the**
2000   **posterior chamber prior to measuring its optical density in a spectrophotometer.**
2001
2002   Two treatment protocols are used, one for liquids and surfactants, and one
2003   for solids. The protocol used by IIVS for the majority of the studies in this BRD is

2004   given in Annex A4. Test substances are applied to the epithelial surface of the
2005   cornea by addition to the anterior chamber of the corneal holder.
2006
2007   The positive controls currently used for BCOP studies at IIVS are ethanol
2008   (neat) for the liquids protocol, and imidazole (200 mg/mL in complete MEM without
2009   phenol red) for the solids protocol. The current (as of 4/28/08) acceptable *In Vitro*
2010   Scores are 51.9 ± 6.2 for ethanol and 100.0 ± 15.9 for imidazole..
2011
2012   Liquids are tested undiluted; pure surfactants are generally tested at a
2013   concentration of 10% in saline or deionized water. Corneas are incubated
2014   horizontally for 10 ± 1 minutes at 32 ± 1 °C. The test substance is removed from the
2015   anterior compartment and the epithelial surface is washed at least three times. After
2016   refilling both chambers with fresh medium, a second opacity measurement is taken
2017   and the corneas are incubated again at 32 ± 1 °C for two hours prior to taking a final
2018   opacity measurement.
2019
2020   Solids are tested as solutions or suspensions at 20% concentration in saline
2021   or deionized water. Corneas are incubated horizontally for four hours at 32 ± 1°C.
2022   The test substance is removed from the compartment and the epithelial surface is
2023   washed at least three times with medium or until the corneal surface is free of visible
2024   particles. Fresh medium is added to both chambers and an opacity measurement is
2025   taken without further incubation.
2026
2027   Immediately after completing the final opacity measurements, corneal
2028   permeability is determined quantitatively by evaluating changes in the barrier
2029   properties of the epithelium to sodium fluorescein. To the anterior compartment of
2030   the corneal holder, 1 mL of sodium fluorescein (0.4% for liquids and surfactants,
2031   0.5% for solids) is added. The corneas are incubated horizontally for 90 minutes at
2032   32 ± 1°C. The amount of dye that penetrates the cornea is determined by measuring
2033   the OD of the medium in the posterior chamber (Figure 2-9 – lower right) with a
2034   microplate reader or UV/VIS spectrophotometer set at 490 nm.
2035
2036   A mean corrected opacity value (± standard deviation [SD]) and a mean
2037   corrected permeability value (OD units ± SD) are calculated for each treatment
2038   group. Most BCOP studies calculate an *In vitro* Score for irritancy that combines
2039   both values using the following empirically derived formula (Sina, Galer et al. 1995):
2040   *In vitro* Score = opacity value + 15 x OD_{490} value.
2041
2042   Generally, a substance producing an *In Vitro* Score from 0 to 25 is
2043   considered a mild irritant, from 25.1 to 75 (to 55 in early studies with pharmaceutical
2044   intermediates) a moderate irritant, and from 75.1 and above a severe irritant. A few
2045   laboratories do not calculate an *In Vitro* Score, but evaluate the opacity and
2046   permeability values independently. Also, some companies, such as S.C. Johnson &
2047   Son, Inc., do not use the classification system described above to assign an ocular
2048   irritancy classification, but instead compare BCOP data for newly tested substances
2049   to benchmark materials, relying on a system of comparative toxicity instead of cutoff

2050   scores (Cuellar N and Swanson J, personal communication). In some cases, S.C.
2051   Johnson could also use a combination of classification scheme, control scores,
2052   histology, and knowledge about the chemistry of the formula to evaluate the test
2053   substance appropriately (Cuellar, N, personal communication).
2054

2055   These procedures were initially developed to assess the ocular irritation
2056   potential of pharmaceutical manufacturing intermediates and raw materials
2057   (Gautheron, Giroux et al. 1994; Sina 1994). However, as the BCOP test method
2058   gained more widespread use, the protocol has been modified by different
2059   investigators interested in using the assay to evaluate the ocular irritancy potential
2060   of other types of materials, including surfactant-based personal care cleaning
2061   formulations (Gettings, Lordo et al. 1996), home care products (Casterton, Potts et
2062   al. 1996), alkaline liquid laundry detergents (Cater, Nusair et al. 2002),
2063   oxidizing/reactive cleaning products (Swanson, White et al. 2003) and
2064   petrochemical products (Bailey, Freeman et al. 2004). As a result of the different
2065   testing needs of different investigators, additional endpoints have been used, such
2066   as assessment of corneal hydration (Ubels 1998; Cooper, Earl et al. 2001; Jones,
2067   Budynsky et al. 2001), and histological assessment of morphological alterations in
2068   the cornea (Curren, Evans et al. 2000; Swanson and Harbell 2000; Cater, Raabe et
2069   al. 2001; Cooper, Earl et al. 2001; Jones, Budynsky et al. 2001; Burdick, Merrill et
2070   al. 2002).
2071

2072   If a histological evaluation of the cornea is performed, the cornea is fixed in
2073   an appropriate fixative (*e.g.,* 10% neutral buffered formalin) after completing the
2074   corneal permeability steps of the assay. The cornea is fixed at room temperature for
2075   at least 24 hours before processing. After embedding the corneas, they are
2076   sectioned and stained with an appropriate stain such as hematoxylin and eosin.
2077   Corneal sections are examined for lesions in the epithelium, stroma, and
2078   endothelium. Sections from treated corneas are compared to those from concurrent
2079   negative and positive control corneas (Evans 1998; Curren, Evans et al. 2000)).
2080

2081
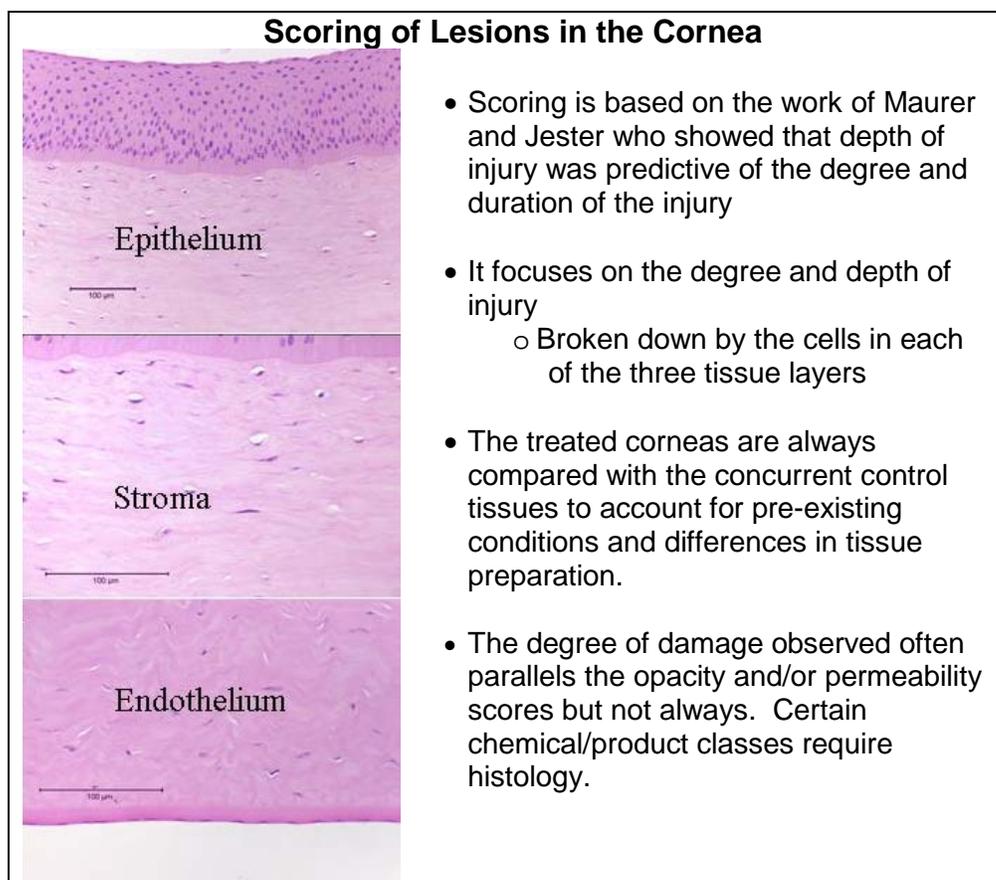
Figure 2-10 Histological evaluation of corneas

2082
2083
2084   Other common modifications to the basic BCOP protocol include use of
2085 variable test substance exposure times and post-exposure periods that are specific
2086 to certain types of substances or products. For example, shorter exposure times are
2087 sometimes used for volatile organic solvents (Harbell J, personal communication;
2088 (Cuellar, Lloyd et al. 2003; Cuellar, Lloyd et al. 2004), longer exposure times are
2089 used for diluted materials or for increased sensitivity in the mild range of irritancy
2090 (Gettings, Lordo et al. 1996; Bruner, Carr et al. 1998; Cater, Nusair et al. 2002;
2091 Cater, Mun et al. 2003), and longer post-exposure expression periods are
2092 sometimes used to test substances with a potentially delayed onset of irritancy
2093 (Rees, Swanson et al. 2001; Cuellar, Lloyd et al. 2003; Gran, Swanson et al. 2003;
2094 Cuellar, Lloyd et al. 2004)."
2095
2096   A more complete description of the BCOP assay is given in a Background
2097 Review Document prepared by NICEATM and amended by a Peer Review Panel.
2098 The BRD is attached as an annex to this submission and will be referred to
2099 repeatedly in this submission where more detail is required.
2100

2101   ## 2.3 Use of histology in conjunction with the BCOP assay

2102

2103   Histological evaluation of bovine corneas has been conducted at IIVS for
2104   approximately 8 years. During this time we have developed standard practice for the
2105   evaluations which have been consolidated into a guidebook (Annex G). The
2106   guidebook describes the process of evaluation and also contains a set of
2107   photomicrographs illustrating the various lesions that are found in treated corneas.
2108   This guidebook can be found in Annex G. A recent meeting (June 2008) of experts
2109   in ocular histopathology examined this document and will continue to work together
2110   to create a final consensus guidebook for the field. Figure 2-11 gives examples of
2111   epithelial damage, upper stroma damage, and lower stroma/endothelial damage.
2112

(a) Epithelial damage with squamous layer coagulation and cytoplasmic and nuclear vacuolization in the wing and basal layers (20X).

(b) Severe collagen matrix vacuolization of the upper stroma. Note also the destruction of the upper keratocytes.

(c) Damage to the endothelial cell layer, cytoplasmic, and deep stromal collagen matrix vacuolization (severe).
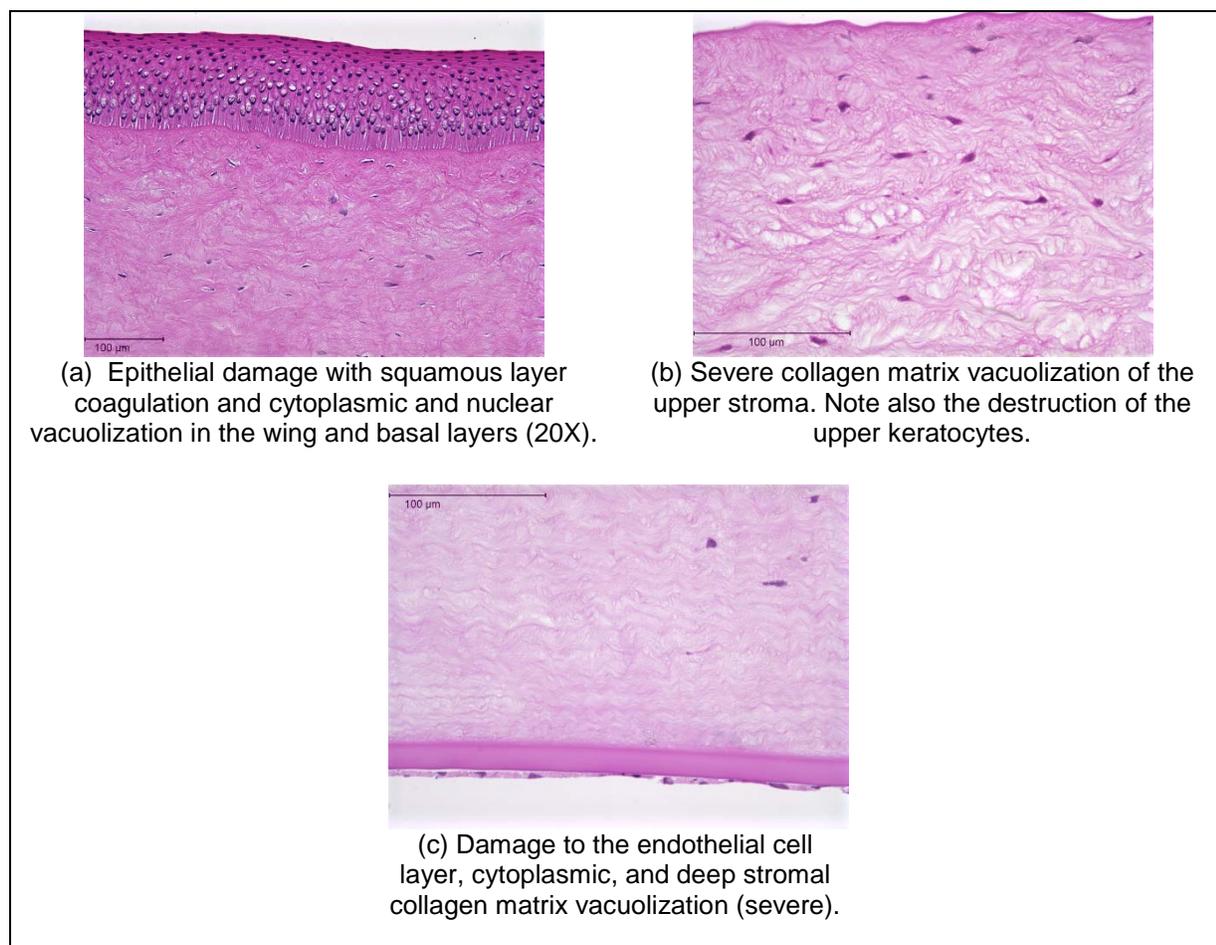
2113   **Figure 2-11 Corneal damage after exposure to test article in the BCOP assay.**

2114 **3 Substances Used For Validation of the Proposed Testing**
2115 **Approach**

2116 3.1 Rationale for the products selected, including rationale for
2117 solicitation of additional test materials to fill in gaps
2118
2119   The goal of this BRD is to present evidence that an *in vitro* testing strategy
2120 can provide for adequate protective labeling of a well-defined product category –
2121 anti-microbial cleaning products. Therefore, only this class of products (or products
2122 which have similar formulations) were used to determine the relationship between
2123 the results of the *in vitro* tests and the results from historical *in vivo* testing (Draize
2124 or LVET eye irritation test), *i.e.,* the relevance of the test. To do this, the
2125 manufacturers who participated in this program chose to submit data on products for
2126 which *in vitro* and *in vivo* data existed and in many cases also for products for which
2127 *in vitro* data only was available.
2128
2129   When considering the reproducibility of the assays; however, it seemed
2130 reasonable to utilize as much information as was available even though this
2131 information was derived from a wide range of products and ingredients. Thus, we
2132 incorporated reproducibility information for the three individual assays that was
2133 available in previously written BRD's even though some of these data were derived
2134 from products which did not fall into the anti-microbial cleaning product category.

2135 3.2 Rationale for dividing substances into "buckets"
2136
2137   Anti-microbial cleaning products can be formulated with various types of
2138 chemistries. Some products – generally containing solvents or surfactants - clean by
2139 causing physical changes to the soil which allows the soil to be more easily
2140 removed from the surface. Other products clean by causing chemical changes to
2141 the soil. This can be accomplished by using strongly alkaline or acidic formulations,
2142 or by using extremely reactive formulations containing such ingredients as bleach,
2143 peroxides, or percarbonates.
2144
2145   Because there very likely could be different modes of action whereby these
2146 products could cause eye irritation, we thought it prudent at the beginning of the
2147 study to classify each anti-microbial cleaning product into one (or more) of five
2148 subcategories – solvents, oxidizers, acids, bases, or surfactants – depending on the
2149 specific formulation. In many cases a product might also be assigned to a second or
2150 third subcategory if more than one mode of action was suspected. We thought it
2151 possible that certain types of products might have to be handled differently as they
2152 progressed through an *in vitro* testing strategy.
2153
2154   The following chemical descriptors were used to characterize the different
2155 types of chemically-induced mechanisms associated with ocular irritation. These

2156   were chosen based on existing information about the mechanisms of ocular irritation
2157   and the common types of formulation chemistries used in commercial and
2158   household cleaning products. The primary (and additional) categories were
2159   assigned by the company toxicologist(s) whose product was being evaluated in this
2160   program.
2161
2162   • Surfactants (SU) (*e.g.,* cationic, anionic, and nonionic with limited acid or
2163   alkaline activity)
2164   • Acids (AC) (*e.g.,* with pH <4, especially where reserve acidity would
2165   contribute to the irritation potential)
2166   • Alkaline (AL) products (bases) (*e.g.,* with pH >9, especially where reserve
2167   alkalinity would contribute to the irritation potential)
2168   • Solvents (SO) (where organic solvents are expected to contribute to the
2169   irritancy potential (*e.g.,* alcohols, glycol ethers, etc.))
2170   • Oxidizers (RC; Reactive chemistry) (formulations containing specific reactive
2171   chemicals, *e.g.,* hypochlorite, peroxide, percarbonate, oxygen bleaches, etc.)
2172
2173   As the results of our *in vitro/in vivo* comparisons became available we
2174   planned to look at each subcategory of cleaning products separately to see if they
2175   were possibly responsible for a greater number of overpredictions or
2176   underpredictions than the other subcategories. If not, then there would be no reason
2177   to treat individual subcategories in a special way, and all of anti-microbial cleaning
2178   products could progress through exactly the same *in vitro* testing scheme.
2179
2180   At the end of the study we concluded that only two types of chemical
2181   formulations should be assigned a special testing program. We recommend that
2182   Oxidizers, because they were often overpredicted by the CM and EO assays,
2183   should be tested only with the BCOP assay. We also recommend that formulations
2184   with "high solvent" concentrations (>5%) – if they are tested in the BCOP assay –
2185   should be tested with a three minute exposure time rather than the normal ten
2186   minute exposure time.

2187   3.3 Rationale for number of substances included in the study
2188
2189   The number of substances included in this study was determined only by the
2190   number of formulations for which paired *in vivo* and *in vitro* data existed. After
2191   evaluating these data and constructing preliminary prediction models, we tested the
2192   prediction models by *in vitro* testing of either existing products or product
2193   reformulations which had previously been tested *in vivo* but not *in vitro.* There was
2194   no statistical basis for the number of substances; the number was only limited by
2195   availability of previously animal tested products which were relevant for this
2196   initiative. No new animal testing was done for the purposes of this project.
2197

2198   ## 3.4 Chemicals or products evaluated
2199
2200   The anti-microbial cleaning products were broken down into six
2201   subcategories depending on the composition of their formulation: solvents,
2202   oxidizers, surfactants, acids, bases, or other. Table 3-1 gives the distribution of
2203   each subcategory of chemicals based on the *in vitro* assay system.
2204
2205   **Table 3-1 Descriptive subcategory of products tested in the individual assays. Final graphs**
2206   **may contain fewer materials as final applicability domains were determined.**

Paired *In vitro* & *In vivo* Data Sets			
Subcategory of	Number of substances tested per assay		
cleaning products	Cytosensor	EpiOcular	BCOP	Total
Solvents	18	10	12	39
Oxidizers	0	13	16	33
Surfactants	82	17	18	114
Acids	1	2	7	10
Bases	4	11	14	29
Other	-	2	1	3
Total	105	55	68	228

2207   ## 3.5 Coding procedures
2208
2209   The individual manufacturers who participated in this study stated that the *in*
2210   *vivo* testing was generally done by providing the testing laboratory a product coded
2211   by a system that they had developed in house. Often these products were
2212   accompanied by an MSDS that would have described in general terms their
2213   chemical composition.
2214
2215   The same type of coding was used for materials that had undergone *in vitro*
2216   testing before the start of this project. Products which underwent *in vitro* testing in
2217   the course of this project were coded by the manufacturer before shipping to IIVS.
2218   The materials were accompanied with MSDS's contained in sealed envelopes. In
2219   case of emergency the envelopes could be opened to obtain safety information. In
2220   all cases, the envelopes were not opened and the products decoded until after the
2221   *in vitro* testing. In the case of the BCOP assay, some products were decoded after
2222   the primary assay, but the identity of the materials was withheld from the individuals
2223   responsible for histopathological evaluation of the samples until after the evaluations
2224   were completed.

2225 **4 *In vivo* Reference data used for the assessment of**
2226 **accuracy**

2227 ## 4.1 Protocols used to generate the *in vivo* data

2228 ### 4.1.1 Draize rabbit eye irritation protocol
2229

2230   The test method currently utilized for the majority of eye irritation tests
2231 conducted today, and also for the majority of *in vivo* eye irritation data presented in
2232 this BRD, is the Draize rabbit eye test. A good description of the Draize test is
2233 presented in the NICEATM BRD for the BCOP assay and is quoted directly below:
2234

2235   "The methodology, originally described by Draize *et al.* (1944), involves
2236   instillation of 0.1 mL of the test substance (*e.g.,* liquids, solutions, and
2237   ointments) into the conjunctival sac of an albino rabbit eye. In this test
2238   method, one eye is treated while the other eye serves as the untreated
2239   control. The eye is examined at selected time intervals after exposure
2240   and any injuries to the cornea, conjunctiva, and the iris are scored.
2241   Scoring is subjective and based on a discrete, arbitrary scale
2242   (*reference omitted*) for grading the severity of ocular lesions. The
2243   scores for the observed ocular injuries range from 1 to 2 for iris effects,
2244   from 1 to 3 for conjunctival redness and discharge, and from 1 to 4 for
2245   corneal effects and conjunctival chemosis. A score of zero is assigned
2246   when the eye is normal and no adverse effects are observed. In the
2247   original protocol, the eyes were observed up to 4 days after application
2248   of the test substance. However, in current practice these time points
2249   vary according to the degree of irritation, the clearing time, and testing
2250   requirements imposed by the various regulatory agencies.

2251   The original Draize protocol describes a scoring system in which each
2252   ocular parameter is graded on a continuous numerical scale. The
2253   scores may be weighted (see Table 4-1); however, most classification
2254   systems today do not use a weighting factor. The weighting of the score
2255   by Draize *et al.* (1944) is biased more heavily for corneal injury, since
2256   injury to the cornea has the greatest probability of producing irreparable
2257   eye damage. To illustrate, each ocular parameter shown in (Table 4-1)
2258   is evaluated for each rabbit. The product of the opacity and area scores
2259   is obtained, then multiplied by a weighting factor of 5; the maximum
2260   corneal score is 80. The iris score is multiplied by a weighting factor of
2261   5; the maximum score is 10. The scores for the three conjunctival
2262   parameters are added together and then the total is multiplied by a
2263   weighting factor of 2; the maximum score is 20. The overall score for
2264   each rabbit is calculated by adding the values for each parameter; the
2265   maximum total score is 110."
2266

2267 **Table 4-1 Scale of weighted scores for grading the severity of ocular lesions (Draize, Woodard**
2268 **et al. 1944).**
2269

I. Cornea	
A. Opacity-Degree of density (area which is most dense is taken for reading)	
Scattered or diffuse area-details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
B. Area of cornea involved	
One quarter (or less), but not zero	1
Greater than one qu arter, but less than one -half	2
Greater than one-half, but less than three quarters	3
Greater than three quarters up to whole area	4
Score equals A x B x 5 Total maximum = 80	
II. Iris	
A. Values	
Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage; gross destruction (any one or all of these)	2
Score equals A x 5 Total possible maximum = 10	
III. Conjunctiva	
A. Redness (refers to palpebral conjunctiva only)	
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. Chemosis	
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C. Discharge	
Any amount differ ent from normal (does not include small amount observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3
Score equals (A + B + C) x 2 Total maximum = 20	

2270   Although the above paragraph refers to the calculation of a numerical score to
2271 characterize eye irritation potential, the approach taken in this BRD is to translate
2272 individual tissue scores observed into toxicity categories, *e.g.,* the EPA toxicity
2273 categories or the GHS categories, which are described later (Sections 4.3 and 4.4).

2274   A more detailed description of the Draize eye irritation method for observing
2275 and scoring tissue lesions, test guidelines for various international regulatory
2276 agencies, and other details of the test are given in the NICEATM BRD on the BCOP
2277 assay. In some cases a modified Draize procedure which utilized a 30 µl dose of

2278   test material to the conjunctival sac was used (See section 4.2 Original reference
2279   data).
2280
2281   Despite the common use of the Draize eye irritation test it is not without its
2282   serious detractors (Daston and Freeberg 1991; Prinsen 2006).

2283   4.1.2 LVET rabbit eye irritation protocol
2284
2285   The traditional Draize methodology described above has often been criticized
2286   for being very overpredictive of human response (Walker 1985). For example, 1) the
2287   amount of material (100 µL) dosed into the eye is more than the human eye, or even
2288   the rabbit eye can retain, 2) dosing in the conjunctival sac of the rabbit allows for
2289   much greater exposure to the test material than would the typical accidental
2290   exposure scenario to the human eye which would be a splash to the surface of the
2291   cornea, and 3) direct comparison of the human and rabbit ocular response to
2292   several types of cleaning products (Freeberg, Nixon et al. 1986; Roggeband, York et
2293   al. 2000) indicates that the rabbit response with the Draize protocol is much greater
2294   than that seen in the human.
2295
2296   In response to these concerns, a modification of the Draize eye irritation test
2297   – the Low Volume Eye Test (LVET) (Griffith, Nixon et al. 1980) – was developed
2298   and has been well characterized over a number of years. The essential difference is
2299   in dosing of the animals. In the LVET, a 10 µl dose is placed in the center of the
2300   cornea, in contrast to the traditional Draize methodology in which 100 µL is placed
2301   into the conjunctival sac. The LVET dosing regimen was to more closely model
2302   expected human exposure with a volume small enough that it could be retained in
2303   the eye. Scoring of the LVET is conducted identically to that of the Draize test
2304   according to the scale presented in Table 4.1.

2305   The approach taken in this BRD is to translate the individual tissue scores
2306   observed into toxicity categories, *e.g.,* the EPA toxicity categories or the GHS
2307   categories, which are described later (Sections 4.3 and 4.4).

2308   4.1.3 Comparison of Draize and LVET
2309
2310   It has been well reported that results obtained with the Draize eye irritation
2311   protocol (Draize, Woodard et al. 1944) do not reflect the eye irritation toxicity for
2312   humans. This was shown by the early work of Beckley (Beckley 1965; Beckley
2313   1969). The rabbit Draize test grossly overpredicted the effects that you would see in
2314   the human eye (Lambert, Chambers et al. 1993).
2315
2316   The dose volume is one of the most influential factors that contribute to
2317   overprediction of the human response to detergent and cleaning products by the
2318   rabbit Draize test. The volume that is instilled into the lower conjunctival sac is
2319   100µL, which exceeds the volume capacity of the rabbit eye lower conjunctival sac
2320   that can maximally hold ~80µL without blinking (Swanston 1985). The blink reflex is

2321   also an important point. In the human, the spontaneous blink rate is about 12-20 per
2322   minute (Bell, Emslie-Smith et al. 1976; Karson, Berman et al. 1981) and serves to
2323   refresh the tear film at each blink. This is much more frequent than the spontaneous
2324   blink rate of about 3 blinks per hour in the rabbit (Mann and Pullinger 1942). Besides
2325   this spontaneous blinking, there is forced blinking in man in response to threat or
2326   injury. The blink reflex is a natural and involuntary response to a foreign material
2327   contacting the surface of the eye. Since the blink reflex is poorly developed in
2328   rabbits and highly developed in man, it is reasonable to take the blink reflex into
2329   account when considering the volume of a material that can contact the human eye.
2330   A volume of 100µL is approximately 10 times the normal volume of liquid (~10µL)
2331   residing in the human eye after blinking (Ehlers 1976; Swanston 1985). Equally
2332   important is that a volume of 100µL greatly exceeds (>10 times) the volume that
2333   directly covers the eye, *i.e.,* the tear volume of both the rabbit and the human eye (~
2334   7µL) (Mishima, Gasset et al. 1966; Chrai, Patton et al. 1973). Taking into account
2335   the anatomical facts, it is clear that the 10µL volume is more than the volume that
2336   can be in direct contact with either the rabbit or the human eye, *i.e.,* more than the
2337   tear volume.
2338
2339   The rabbit low volume eye test (LVET) addressed issues associated with the
2340   gross over-dosing and the animal welfare concerns of the Draize method (Griffith,
2341   Nixon et al. 1980). Correlation of recovery in the LVET with recovery in human
2342   accidents (Freeberg, Griffith et al. 1984; Freeberg, Hooker et al. 1986), and
2343   controlled comparative studies with 100µL and 10µL of detergent based products
2344   (Freeberg, Nixon et al. 1986), have shown that the LVET method is a better
2345   predictor than the Draize test, yet the LVET still overpredicts the human recovery
2346   time. Tables 4-2 and 4-3 summerize the results of the Freeberg *et al.* 1986 study
2347   where both rabbits and human volunteers (who were fully informed and participated
2348   in an Institutional Human Subjects Review Board-approved study) were exposed to
2349   identical concentrations of four representative household cleaning products. Table
2350   4-2 shows that days-to-clear in the human were better predicted by the rabbit LVET
2351   assay than by the rabbit Draize assay, <u>although the rabbit LVET assay still
2352   overpredicted the effects of both the human 100 µL or 10µL exposure</u>. Table 4-3
2353   extends this finding to the traditional Draize scoring scale. Again it can be seen that
2354   the rabbit LVET protocol predicts the human eye score better than the rabbit Draize
2355   protocol and <u>that the rabbit LVET protocol still overpredicts the effects of both the
2356   human 100 µL and 10 µL exposure</u>. Another example comes from Ghassemi *et al.*
2357   1993 who compared the response of humans and rabbits to a liquid household
2358   cleaner (Table 4-4). By enumerating the number of eyes affected at the corneal,
2359   conjunctival or iridial level (or days-to-clear), it was again found that the rabbit LVET
2360   protocol overestimated the human reponse for all parameters with the exception of
2361   conjunctival involvement where it was equivalent.
2362
2363

2364 **Table 4-2 Mean time to clear after direct instillation of household cleaning products to both**
2365 **rabbits and humans. Compiled from Freeberg *et al.* 1986.**

Product	Draize Protocol		LVET Protocol	
	Rabbit	**Human**	**Rabbit**	**Human**
Liquid fabric softener (100%)	3.5 days	12.5 hours	1.1 days	13.2 hours
Liquid shampoo (20%)	2.6 days	7.9 hours	1.4 days	7.5 hours
Liquid hand soap (10%)	2.7 days	9.1 hours	1.8 days	10.5 hours
Liquid laundry detergent (4%)	3.1 days	19.8 hours	1.7 days	4.8 hours

2366
2367
2368 **Table 4-3 Rabbit and human eye responses after exposure to either 100 μL (Draize protocol)**
2369 **or 10 μL (LVET protocol). All scoring done by the traditional Draize scoring scale. Compiled**
2370 **from Freeberg *et al.* (1986)**

Reading time (hours)	Draize Protocol		LVET Protocol	
	Mean rabbit score	**Mean human score**	**Mean rabbit score**	**Mean human score**
Liquid Fabric softener (100%)				
1	4.3	0.8	4.8	1.8
24	6.5	-[a]	0.3	-
48	3.0	-	0.0	-
72	0.8	-	-	-
Liquid Shampoo (20%)				
1	11.1	4.0	6.0	2.0
24	7.0	-	0.8	-
48	4.3	-	0.0	-
72	0.9	-	-	-
Liquid hand soap (10%)				
1	8.0	3.0	4.0	2.5
24	13.9	-	1.8	-
48	4.3	-	0.3	-
72	0.3	-	0.3	-
Liquid laundry detergent (4%)				
1	8.3	4.0	4.5	2.3
24	13.3	-	1.8	0.0
48	9.0	-	0.5	-
72	1.4	-	0.0	-

2371 [a]not scored

2372
2373 **Table 4-4 Rabbit and human eye responses after exposure to either 100 μL (Draize protocol)**
2374 **or 10 μL (LVET protocol) for the liquid household cleaner. All scoring done by the tradititial**
2375 **Draize scoring scale. Compiled from Ghassemi *et al.* (1993)**

Dosing Procedure	Number of Eyes Affected			Max. Time to Clear
	Cornea	**Iris**	**Conjunctiva**	
Rabbit LVET	3/3	2/3	3/3	7 days
Human LVET	0/10	0/10	10/10	2 days
Human Draize	0/10	0/10	10/10	< 3 days

2376

2377   In addition, comparisons can be made between predictions made by either
2378 the rabbit LVET or Draize test and human experience from accidental exposure
2379 (Freeburg et al 1986b). Table 4-5 shows that mean Time-to-Clear in days for these
2380 household cleaning products is always shorter in the human accidental exposure
2381 data than was predicted by either the Draize of LVET information. Additional

2382   information exists on the overprediction of the LVET protocol (Bruner and Kohrman
2383   1993; Cormier, Hunter et al. 1995), including an additional study directly comparing
2384   effects of low volumes of undiluted detergent and cleaning products in humans and
2385   rabbits (Ghassemi, Sauers et al. 1993; Roggeband, York et al. 2000).
2386
2387
2388   **Table 4-5 Average Time-to-Clear (days) for ocular effects following accidental exposure in**
2389   **humans and in rabbit eye irritation tests (LVET and Draize test) to household and cleaning**
2390   **products (Freeberg, Hooker et al. 1986).**

Product[a]	Average Time-to-Clear (Days)		
	Human Data	LVET	Draize
Liquid Laundry Product #1	1.92	26.6	35
Liquid Dishwashing Product #1	0.77	8.2	25.7
Dry Dishwashing Product #1	0.59	4.6	18.3
Liquid Dishwashing Product #2	0.43	7.7	11.7
Liquid Household Cleaning Product #1	0.38	-	11.1
Liquid Dishwashing Product #3	0.3	3.9	22.2
Liquid Household Cleaning Product #2	0.23	4	15.2
Dry Household Cleaning Product #1	0.19	1.3	29.2
Dry Dishwashing Product #1	0.08	2.1	13.8
Dry Dishwashing Product #2	0.06	2.9	15.1

2391   [a]Laundry Products: additives, main wash detergents, fabric softeners; Dishwashing products:
2392   automatic and hand detergents; Household Cleaning Products: hard surface cleaners, non-
2393   abrasive cleaners

2394   4.2 Original reference data

2395
2396   Supporting animal data for the comparisons made in this BRD came from
2397   three basic methodologies: 1) the traditional Draize protocol utilizing 100 µL (or 100
2398   mg) dose of test article into the conjunctival sac, 2) a modified Draize protocol which
2399   involved dosing with 30 µL (or 30 mg) of material into the conjunctival sac, and 3)
2400   the LVET which involves dosing with 10 µL directly onto the surface of the cornea.
2401
2402   In one case, animal data came from the EPA guideline for assessing
2403   aerosols. In this protocol, the animal eye was held open while a 1 second spray of
2404   the test article was directed onto the cornea. This one data point was then paired
2405   with data from a specially designed BCOP study in which the bovine cornea was
2406   exposed to a similar 1 second spray of the test material. Other aspects of the BCOP
2407   protocol remained the same.
2408
2409   Some of the animal data from the 30 µL Draize protocol could not be used for
2410   the comparisons found in this BRD. If the final classifications were less than an EPA
2411   Category I or less than a GHS Category 1, the data were not used since it could be
2412   assumed that a higher dose of test material might have resulted in a higher
2413   categorization. On the other hand, if the resulting score was an EPA Category I or a
2414   GHS Category 1, the data were used since the assumption was that a higher dose
2415   would not have resulted in a lower score. Seven materials are included in this BRD
2416   which had the 30 µL protocol and resulted in an EPA Category of 1, while only six

2417   materials could be included in the GHS analysis since one of the seven materials
2418   had a GHS Category of 2A.
2419
2420   The actual animal data were supplied to IIVS in one of two ways; either as
2421   copies of the final reports from the organization that conducted the animal studies or
2422   as Excel$^{©}$ spreadsheets which contained the full tissue scores that had been
2423   entered by the staff of the submitter. The Excel$^{©}$ spreadsheets which were
2424   submitted are contained in Annex C. For reasons of confidentiality, copies of final
2425   reports that were submitted as the primary source for the animal scores are not
2426   included in this BRD; only spreadsheets containing the data transcribed by IIVS
2427   employees are appended. However, some of the final reports are available for
2428   inspection by NICEATM or EPA staff upon request.
2429

2430   ## 4.3 Description of EPA toxicity categories

2431
2432   The EPA uses four toxicity categories which determine the labeling
2433   information for the product. Table 4-6 lists the four categories along with the ocular
2434   endpoints for determining the toxicity category.
2435
2436   **Table 4-6 EPA Eye irritation toxicity categories (EPA 2003)**

EPA Category	Draize Eye Test Scoring
Category I	- Corrosive, corneal involvement or irritation (iris or cornea score ≥ 1 or redness or chemosis ≥ 2) persisting more than 21 days or Corneal effects that are not expected to reverse by 21 days
Category II	- Corneal involvement or irritation clearing in 8-21 days
Category III	- Corneal involvement or irritation clearing in 7 days or less
Category IV	- Minimal or no effects clearing in less than 24 hours*

2437   * Based on positive scores for conjunctival irritation ≥ 2
2438
2439   The eye irritation toxicity indicator is based on the outcome of the Draize eye
2440   test. In this BRD we have also classified the toxicity on the basis of the LVET. At
2441   least three animals are tested per chemical (a one-animal screen protocol is
2442   permitted to determine if the chemical is a severe irritant). The most severe
2443   response of the animals is used to calculate the EPA toxicity category. A single
2444   animal with a Category I response would lead to a Category I classification
2445   regardless of the outcome of the other animals. The criteria used to determine if a
2446   given animal result could be used for the analyses in this BRD are the same as
2447   were used by NICEATM in their BRD on the BCOP test and are quoted below:
2448
2449   ▪ "At least three rabbits were tested in the study, unless a severe
2450   effect (*e.g.*, corrosion of the cornea) was noted in a single rabbit.
2451   In such cases, substance classification could proceed based on
2452   the effects observed in less than three rabbits.
2453   ▪ A volume of 0.1 mL or 0.1 g was tested in each rabbit. A study in
2454   which a lower quantity was applied to the eye was accepted for

2455 substance classification, provided that a severe effect (*e.g.,*
2456 corrosion of the cornea, lesion persistence) was observed in a
2457 rabbit.
2458 ▪ Observations of the eye must have been made, at minimum, at
2459 24-, 48-, and 72-hours following test substance application, if no
2460 severe effect was observed.
2461 ▪ Observations of the eye must have been made until reversibility
2462 was assessed, typically meaning that all endpoint scores were
2463 cleared. Results from a study terminated early were not used,
2464 unless the reason for the early termination was documented."

2465 ## 4.4 Description of GHS toxicity categories
2466
2467 The GHS (UN 2003) classification system for eye irritation is also utilized in
2468 this BRD because of the likelihood that EPA labeling decisions will eventually be
2469 made on the basis of this system. The classification system was applied to animal
2470 data in this BRD in an identical fashion to that used by NICEATM in their BRD on
2471 the BCOP assay. This methodology is described below in an extract from their BRD.
2472
2473 "The classification of substances using the GHS classification system (UN
2474 2003) was conducted sequentially. Initially, each rabbit tested was classified
2475 into one of four categories (Category 1, Category 2A, Category 2B, and
2476 nonirritant) based on the criteria outlined in Table 4-7. The criteria provided
2477 in this table are identical to those described in the GHS classification and
2478 labeling manual (UN 2003). Once all rabbits were categorized, the
2479 substance classification was determined based on the proportion of rabbits
2480 with a single irritancy category."

2481
2482 **Table 4-7 Criteria for Classification of rabbits according to the GHS classification system**

GHS Category	Rabbit Category Necessary for Classification
Category 1	Group A: • Effects in the cornea, iris, or conjunctiva that were not expected to reverse or did not fully reverse[1] within the observation period of 21 days, or • A corneal opacity score of 4 at any time during the test Group B: Rabbit with mean scores (averaging of the scores on day 1, 2, and 3) for opacity ≥3 and/or iritis ≥1.5
Category 2A	Rabbit with mean scores (rabbit values are averaged across observation days 1, 2, and 3) for one or more of the following: • 1 ≤ Iritis < 1.5 • 1 ≤ Corneal opacity < 3 • Redness ≥ 2 • Chemosis ≥ 2

	and the effects fully reverse within 21 days
Category 2B	Rabbit with mean scores (rabbit values are averaged across observation days 1, 2, and 3) for one or more of the following: • 1 ≤ Iritis < 1.5 • 1 ≤ Corneal opacity < 3 • Redness ≥ 2 • Chemosis ≥ 2 and the effects fully reverse within 7 days
Nonirritant	Rabbit mean scores fall below threshold values for Category 1, 2A, and 2B

2483   [1]Full reversal of the effects was defined as corneal, iritis, redness, and chemosis = 0.

2484   After each rabbit was categorized, the ocular irritancy potential of the
2485   substance was determined. As shown in Table 4-8, substance classification
2486   depended on the proportion of rabbits that produced the same response. As noted
2487   above, if a substance was tested in more than three rabbits, decision criteria were
2488   expanded. Generally, the proportionality needed for classification was maintained
2489   (*e.g.,* 1 out of 3 or 2 out 6 rabbits were required for classification for most
2490   categories). However, in some cases, additional classification rules were
2491   necessary to include the available data. These additional rules are distinguished
2492   by italicized text in Table 4-8.
2493
2494   If an unequivocal substance classification could not be made due to the
2495   response pattern of the tested rabbits for a substance (*e.g.,* one rabbit classified as
2496   Category 1, Group B; two rabbits classified as Category 2B; three rabbits classified
2497   as nonirritant), the data were not used in the analysis.
2498
2499   **Table 4-8 Criteria for Classification of Substance According to the GHS Classification System**
2500   **(Modified from UN 2003)**

GHS Category	Criteria Necessary for Substance Classification
Category 1	1. At least 1 of 3 rabbits or 2 of 6 rabbits classified as Category 1, Group A *2. One of six rabbits classified as Category 1, Group A and at least 1 of 6 rabbits classified as Category 1, Group B* 3. At least 2 of 3 rabbits or 4 of 6 rabbits classified as Category 1, Group B
Category 2A	1. At least 2 of 3 rabbits or 4 of 6 rabbits classified as Category 2A *2. One of 3 (2 of 6) rabbits classified as Category 2A and 1 of 3 (2 of 6) rabbits classified as Category 2B*
Category 2B	1. At least 2 of 3 rabbits or 4 of 6 rabbits classified as Category 2B
Nonirritant	1. At least 2 of 3 rabbits or 4 of 6 rabbits classified as nonirritant

2501   Italicized text indicates rules that were developed to include additional data.

2502 ## 4.5 Transformation of original data to toxicity categories
2503
2504 To transform the original data – existing either as a submitted spreadsheet or
2505 as an original report from the laboratory conducting the Draize eye irritation test –
2506 individual eye scores were entered into Excel® spreadsheets designed to
2507 categorize the scores according to the above listed criteria. Example spreadsheets
2508 can be found in Annex C. The spreadsheet used to determine EPA toxicity
2509 categories was designed at IIVS, and the spreadsheet used to determine GHS
2510 toxicity classifications was designed at ECVAM and supplied to IIVS.
2511
2512 As part of our additional analysis of the EPA and GHS classifications,
2513 information from six rabbit Draize tests was entered into a secondary spreadsheet
2514 which calculated GHS and EPA categories for each of the 20 distinct sets of 3 rabbit
2515 combinations as described in Section 4.8.

2516 ## 4.6 Quality of *in vivo* data
2517
2518 It is the generally stated goal of most validation authorities that data
2519 submitted in support of a validation effort should be conducted to comply with GLP
2520 guidelines (ICCVAM 1997; Hartung, Bremer et al. 2004). The GLP-compliance
2521 status of the majority of the animal studies in this BRD is not known since that
2522 information was not supplied by the sponsors. However, for a minority of the data
2523 the actual study reports were available, and it could be determined from these
2524 reports whether or not the studies were GLP-compliant. In cases where the studies
2525 were determined to be GLP-compliant this fact was noted in the spreadsheets.

2526 ## 4.7 Human toxicity information on cleaning products
2527
2528 We have no human toxicity information for any of the specific materials that
2529 are used as references for the *in vitro* results in this BRD. However, data do exist in
2530 the literature for certain types of cleaning products. Although it is not routine,
2531 ethically designed human studies have been conducted on such products. In
2532 addition, human accidental exposure data have been collected for some household
2533 cleaning products and this information compared with data from the Draize eye
2534 irritation test, the LVET, and human clinical studies. Several of these studies have
2535 already been discussed in detail in Section 4.1.2 of this BRD.
2536
2537 Essentially, the data indicate that the results of both the Draize test and the
2538 LVET overpredict the amount of damage that would occur in the human eye;
2539 however, the Draize test overpredicts by a greater amount.
2540
2541

2542 4.7.1 Clinical Studies by Beckley *et al.* (1965) on a light duty liquid detergent
2543
2544   Beckley *et al.* (1965) compared a light duty liquid detergent (Table 4-9) on the
2545 eyes of rabbits, dogs, monkeys and humans (Beckley 1965).
2546
2547 **Table 4-9 Composition of the light duty liquid detergent from the Beckley 1965 study (Beckley**
2548 **1965)**
2549

Test Product	Ingredients	Level in Product (%)	Concentration Tested
Light Duty Liquid Detergent (pH 6.3)	Alkylbenzene sulphonate Conventional organic foam builder and solubilizer	38%	Various amounts, up to and including undiluted material
	Ethyl alcohol	12%	
	Water	50%	

2550
2551   The laboratory animal studies showed clear differences between species with
2552 the most sensitive being the rabbit, followed by the dog, and finally by the monkey.
2553 All of the animals whose eyes were not flushed showed some corneal involvement.
2554 Extracted results from the manuscript are shown in Table 4-10.
2555
2556 **Table 4-10 Mean Draize scores for individual ocular tissues of six rabbits, six dogs and four**
2557 **monkeys (unflushed) or three animals each (flushed) after instillation of 100 µL of a Light Duty**
2558 **Liquid Detergent (Beckley 1965)**
2559

Evaluation Time	Ocular Tissue	Eyes Unflushed			Eyes Flushed		
		Rabbit	Dog	Monkey	Rabbit	Dog	Monkey
1h	Cornea	33.3	40.0	20.0	15.0	40.0	0
	Iris	10.0	5.0	2.5	10.0	0	0
	Conjunctiva	12.0	4.0	1.0	10.0	0	0
1 day	Cornea	33.3	45.0	10.0	13.3	20.0	0
	Iris	10.0	5.0	0	10.0	0	0
	Conjunctiva	12.0	4.0	0	10.0	0	0
3 days	Cornea	21.7	30.0	0	5.0	20.0	0
	Iris	10.0	5.0	0	8.3	0	0
	Conjunctiva	9.3	0	0	7.3	0	0
7 days	Cornea	6.7	0	0	1.7	0	0
	Iris	8.3	0	0	3.3	0	0
	Conjunctiva	6.7	0	0	2.7	0	0

2560
2561
2562   In contrast to the animal results, three different studies using <u>human</u>
2563 <u>volunteers showed much milder reactions and no corneal involvement.</u>
2564

2565   • <u>Study A</u>: This study began with the instillation (100 µL) of increasing
2566   concentrations of the Light Duty Liquid Detergent into the lower conjunctival sac
2567   without rinsing. After it was determined that all of the diluted solutions were
2568   tolerated, undiluted solution was then instilled (100 µL) into the eyes of 15
2569   volunteers <u>for seven consecutive days</u>. Ten of the subjects had no eye damage;
2570   five had began to develop conjuctivis which disappeared when dosing was
2571   stopped. <u>There were no instances of corneal or iridial involvement</u>.
2572

2573   • <u>Study B</u>: This study was an extended dosing study, again using fifteen subjects.
2574   It began with 100 µL instillations of increasing concentrations alternating daily
2575   between the left and right eye until the undiluted solution was used. Since
2576   100 µL flooded the eye, the dosage was held in place for two minutes with a
2577   gauze pad. After removing the pad the eyes were rinsed. After the 20th day <u>100</u>
2578   <u>µL of the undiluted solution was instilled into the same conjunctival sac for 8</u>
2579   <u>days</u>. A few subjects developed a conjunctival erythema. <u>There were no</u>
2580   <u>instances of corneal or iridial involvement throughout the entire study</u>.
2581

2582   • <u>Study C</u>: This study involved instilling three drops of undiluted solution into each
2583   eye for three days. "None of the subjects developed a chronic conjunctivitis, and
2584   in no case was there involvement of the iris or cornea."
2585

2586   The conclusion from this study is that humans are not only much less
2587   sensitive to this type of cleaning product than the rabbit, but also less sensitive than
2588   the dog and monkey.
2589

2590   4.7.2 Clinical Studies by Beckley *et al.* (1969) on a soap suspension and a
2591   liquid household cleaner
2592

2593   Beckley *et al.* (1969) also compared the effects of a 5% soap solution and an
2594   undiluted all-purpose liquid household cleaner on the eyes of rabbits, monkeys, and
2595   man. The composition of the all-purpose household cleaner is provided in Table 4-
2596   11.
2597

2598   **Table 4-11 Composition of the test materials from the Beckley 1969 study (Beckley 1969)**

Test Product	Ingredients	Level in Product (%)	Concentration Tested
Soap suspension	Soap	N/A	5%
Liquid Household Cleaner (pH 10.4)	Alkylbenzene sulphonate	5	Undiluted
	Ammonium cumene sulphonate	4	
	Builder containing 3% sodium carbonate and 1% tetrapotassium pyrophosphate	13	
	Miscellaneous ingredients including 0.7% ammonia, 0.6% soap and 0.4% perfume	2	
	Water	Up to 100	

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- 5% Soap solution – Rabbits and monkeys had some corneal involvement lasting up to 72 hours for some of the animals. Both species had conjunctivitis up to 48 hours. The humans had some initial epithelial loss that was not observable at 6 hours. Conjunctivitis was seen in the humans at six hours but further measurements were not made.

2606
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2610

- Liquid Household Cleaner – Rabbits corneal stippling up through 7 days and conjunctivitis through 3 days. Monkeys had corneal stippling through seven days and conjunctivitis up to 24 hours. Humans had corneal stippling only through 6 hours and conjunctivitis through 3 days.

2611
2612
2613

The conclusion from this study was that humans had a slight corneal response to both 5% soap and the Liquid Household Cleaner, but it cleared by six hours. The laboratory animals, in contrast, had more severe responses.

2614
2615

4.7.3 Clinical Studies by Ghassemi, *et al.* (1997) on a liquid household cleaner

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2620

Ghassemi, *et al.* carried out direct installation studies in human volunteers with a liquid household cleaner of low pH. Table of 4-12 gives the composition of the cleaner.

2621 **Table 4-12 Liquid Household Cleaner composition used in the Ghassemi *et al.* (1997) study**

Test Material	Ingredients	Ingredient Concentration	Concentration Tested
Liquid Household Cleaner (pH 3)	Nonionic surfactant: • alcohol ethoxylate	2%	Undiluted
	Amphoteric surfactant: • betaine • Na H$_2$ citrate • Cumene sulphonate	2% 3% 3%	
	Solvent: • butoxypropoxypropanol/ dipropylene glycol • monobutyl ether • Water	8% to 100%	

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Undiluted Liquid Household Cleaner was instilled into one eye of ten human volunteers using either the Draize methodology (100 μL instillations) or the LVET methodlogy (10 μL onto the cornea). Rabbits were also dosed with the cleaner using the LVET method. There was no corneal or iridial involvement in the humans with either dosing procedure, but there was initial conjunctivitis which cleared by 48 hours after the 10 μL exposure and 70 hours after the 100 μL exposure. In contrast, the three rabbits had both corneal (3/3) and iridial (2/3) involvement, along with conjunctivitis which did not resolve until seven days.

2631   The conclusion from this study is that human eyes are not significantly
2632   affected by this Liquid Household Cleaner (even with the 100 μL dosing volume),
2633   but rabbits have significant ocular responses to even the LVET procedure.

2634   4.7.4 Clinical studies of liquid detergent products by Roggeband, *et al.* (2000)
2635
2636   Roggeband *et al.* conducted human clinical studies on two representative,
2637   surfactant-based cleaning products which are described in Table 4-13.
2638
2639   **Table 4-13 Composition of the test materials from the Roggeband, *et al.* (2000) study**

Test Product	Ingredients	Level in Product (%)	Concentration Tested
Concentrated Laundry Liquid	Soap	15	Undiluted
	Nonionic surfactant	27	
	Anionic surfactant	12	
	Water	Up to 100	
Concentrated Dishwasher Liquid	Non-ionic surfactant	4	Undiluted
	Anionic surfactant	38	
	Water	Up to 100	

2640
2641   Initial studies with the two test materials focused on finding dosing volumes of
2642   the two concentrated products that were just below the doses causing some corneal
2643   erosion. These doses were 3 μL for the Concentrated Laundry Liquid and 1 μL for
2644   the Concentrated Dishwashing Liquid.
2645
2646   Subsequently 10 human volunteers and six rabbits were exposed to identical
2647   doses of the Concentrated Laundry Liquid (3 μL) and the Concentrated
2648   Dishwashing Liquid (1 μL). Table 4-14 shows the results with the laundry liquid. At 1
2649   hour in the human there were corneal effects in two volunteers, but there were no
2650   corneal lesions at 24 hours. There were also conjunctival effects at 1 hour, but
2651   these resolved in all but two volunteers at 24 hours. In the rabbit; however, there
2652   were corneal effects in 5 of the 6 rabbits at 24 hours, and rather strong conjunctival
2653   effects in all rabbits at 24 hours.
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2671   **Table 4-14 Ocular responses of humans and rabbits to identical volumes (3 μL) of**
2672   **Concentrated Laundry Liquid. Modified from Roggeband, *et al* (2000).**

	Human				Rabbit				
	1 Hr		24 Hr			1 Hr		24 Hr	
Volunteer	Cornea[a]	Conjunctiva[b]	Cornea	Conjunctiva	Animal	Cornea	Conjunctiva	Cornea	Conjunctiva
A	0	1/1	0	0/0	A	0/0	1/1/0	1/2	2/1/1
B	0	1/0	0	0/0	B	0/0	1/1/0	1/2	2/1/1
C	0	1/0	0	0/0	C	0/0	1/1/0	0/0	2/1/1
D	1/2	1/0	0	1/0	D	0/0	1/1/0	1/4	2/1/0
E	1/1	1/0	0	0/0	E	0/0	1/1/0	1/3	2/1/1
F	0	1/0	0	1/0	F	0/0	1/1/0	1/4	2/1/1
G	0	1/0	0	0/0					
H	0	0/0	0	0/0					
I	0	1/0	0	0/0					
J	0	1/0	0	0/0					

2673   [a]Corneal score expressed as opacity score/area
2674   [b]Conjunctival score expressed as erythema score/edema score in humans and
2675   erythema/edema/discharge in rabbits.
2676

2677   The results with the dishwashing liquid are shown in Table 4-15. One of the
2678   human subjects had corneal involvement at 1 hour but this resolved by 24 hours.
2679   Three of the ten volunteers had a slight conjunctivial response at 1 hour, but all had
2680   resolved at 24 hours. In contrast 5 of the six rabbits had corneal opacities at 24
2681   hours and all of the rabbits had conjunctival involvement at both 1 hour and 24
2682   hours.
2683

2684   **Table 4-15 Ocular responses of humans and rabbits to identical volumes (1 μL) of**
2685   **Concentrated Dishwshing Liquid. Modified from Roggeband, *et a* (2000).**

	Human				Rabbit				
	1 Hr		24 Hr			1 Hr		24 Hr	
Volunteer	Cornea[a]	Conjunctiva[b]	Cornea	Conjunctiva	Animal	Cornea	Conjunctiva	Cornea	Conjunctiva
A	0	0/0	0	0/0	A	0/0	1/1/0	0/0	1/1/1
B	0	0/0	0	0/0	B	0/0	2/1/0	1/2	2/1/0
C	0	0/0	0	0/0	C	0/0	1/1/0	1/1	2/1/0
D	1/1	1/0	0	0/0	D	0/0	1/1/0	1/1	2/1/0
E	0	0/0	0	0/0	E	0/0	1/1/0	1/2	2/1/0
F	0	0/0	0	0/0	F	0/0	1/1/0	1/2	2/1/0
G	0	1/0	0	0/0					
H	0	0/0	0	0/0					
I	0	1/0	0	0/0					
J	0	0/0	0	0/0					

2686   [a]Corneal score expressed as opacity score/area
2687   [b]Conjunctival score expressed as erythema score/edema score in humans and
2688   erythema/edema/discharge in rabbits.
2689

2690   The conclusions of this study were that concentrated surfactant cleaning
2691   products are capable of causing ocular effects in both the human and the rabbit.
2692   However, the effects in the rabbit after an identical dose to that applied to the
2693   human volunteers were more severe and resolved much later (some between 72 hr
2694   and seven days).

2695   ## 4.8 Accuracy and reliability of the LVET and Draize tests
2696

2697   A significant problem in analyzing how well any *in vitro* test predicts the
2698   outcome of an *in vivo* test is that a single value (without any estimate of error) is
2699   generally associated with the animal score for a test material, and this single value
2700   is treated as a "gold standard". In reality, there is no single eye irritation value that
2701   characterizes a test material; the value that is obtained will generally vary each time
2702   the material is tested. Thus, it is extremely unlikely that an *in vitro* score and an *in*
2703   *vivo* score will match exactly, no matter how perfectly the *in vitro* test is performed.
2704   This fact is often overlooked in most validation studies. Generally the animal score
2705   is treated as a single fixed value (since the animal test is generally conducted only
2706   once), and the *in vitro* test is then assessed for its "accuracy" based on how well its
2707   data match that of the animal test. Only a few studies, *e.g.,* the CTFA Phase III eye
2708   irritation evaluation study (Gettings, Lordo et al. 1996), have taken the animal test
2709   variability into account. The CTFA study used bootstrap resampling to estimate with-
2710   in group variability for each test material so that Draize scores could be represented
2711   more realistically with their variability (see, for example, Figure 4-1).
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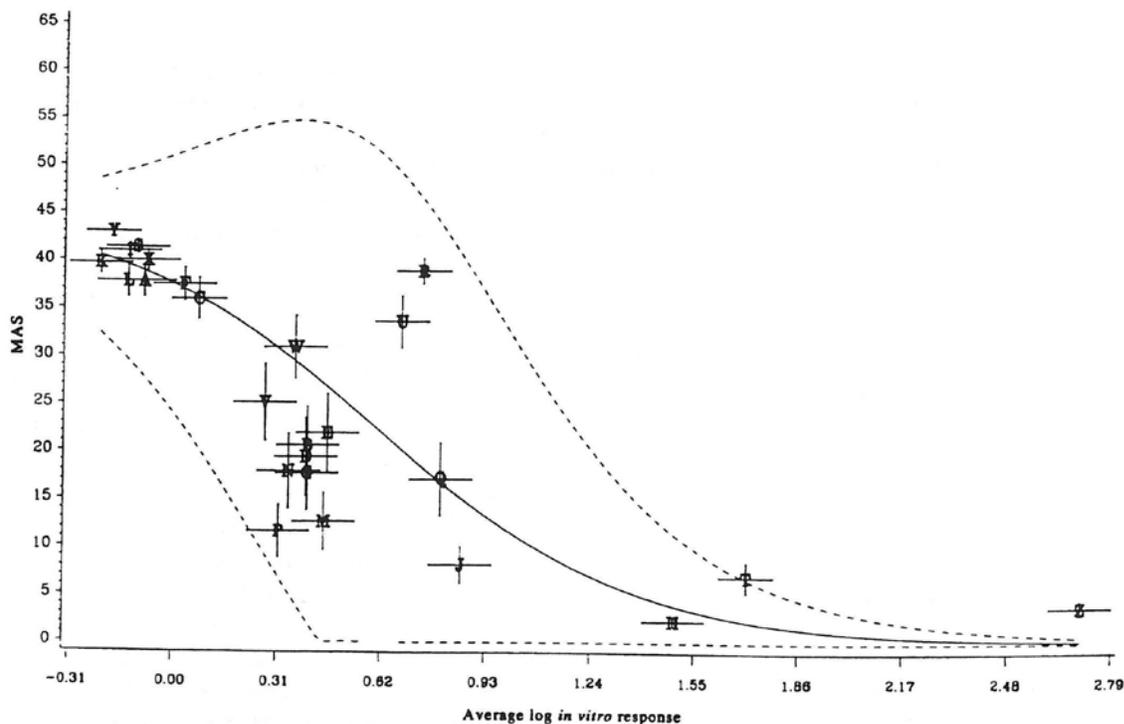
2715
2716 **Figure 4-1. Performance of the Silicon Microphysiometer in predicting the Draize MAS score**
2717 **for test materials from the CTFA Phase III study of surfactant-based formulations (Gettings,**
2718 **Lordo et al. 1996). The variability associated with both the animal test and the *in vitro* test is**
2719 **shown on the graph.**
2720

2721   4.8.1 Analysis of six rabbit tests in combinations of three
2722
2723   As mentioned above, one reason that Draize MAS scores are usually treated
2724   as unvarying values is that both ethical and financial considerations generally
2725   demand that a rabbit eye test only be conducted a single time. Thus for many
2726   materials there is no information about what score might occur in a repeat test, and
2727   without the results of multiple tests it is difficult to address variability.
2728
2729   However, there is one approach which can supply some quantitative insight
2730   into this problem. Because over the years the Draize test protocol has evolved from
2731   a six rabbit test to a three rabbit test, there is one way of estimating variability for
2732   materials which were tested with the six rabbit protocol. It is possible to analyze the
2733   ocular response of the six rabbits by placing them into smaller groups. For example,
2734   the results for each of the six individual rabbits can be recombined into multiple
2735   unique groups of three rabbits (matching the number of rabbits used in today's
2736   standard protocol). In fact, all rabbits (designated A – F in the following example) in
2737   a six rabbit test can be recombined into <u>20</u> unique three rabbit groups, e.g. ABC,
2738   ABD, ABE, ABF, etc. This is an approach already used by others in studies to
2739   determine the necessary sample size for a rabbit ocular irritation test (DeSousa,
2740   Rouse et al. 1984). Each three rabbit group can then be given a hazard
2741   classification according to the published guidelines from specific regulatory bodies.
2742   The number of subgroups in each hazard classification can then be viewed as a
2743   measure of the variability of the test. If all 20 subgroups are classified as R36, for
2744   example, then the R36 classification for that material can be considered not very
2745   variable. However, if 10 subgroups are rated as No Label and the other 10 are rated
2746   as R41, then the results for that material would be considered quite variable. In
2747   essence the above results mean that if the material were tested in multiple three
2748   rabbit tests, half of the tests would rate it as a very severe R41 material, and the
2749   other half of the tests would rate it as a mild No Label material. Therefore, an *in*
2750   *vitro* test of the same material should not necessarily be expected to always make a
2751   prediction of R41, which would be the <u>overall</u> prediction of the six rabbit test.
2752
2753   To demonstrate the level of Draize test variability which occurs in the real
2754   world, we have examined the animal data from the CTFA Phase III study. This study
2755   had arguably one of the best controlled animal studies because it was conducted
2756   under GLP's and utilized a randomized block design (3 males and 3 females) with
2757   each animal's dosing initiated on a separate day.
2758
2759   Table 4.16 shows for the CTFA Phase III study the number of three rabbit
2760   subgroups which fall into each of the hazard categories for the three regulatory
2761   classification schemes (GHS, EU, and EPA). Data which support these
2762   classifications can be found in spreadsheets contained in Annex C; CTFA Animal
2763   Data) It can be seen that in some cases all of the three rabbit subgroups give the
2764   same hazard classification as the six rabbit study, e.g. the EU classification for HZB,
2765   HZC and HZD is No Label, and each of the 20 three rabbit subgroups for each test
2766   material is also No Label. However, for those same three test materials classified by

2767   GHS criteria there is considerable difference between the subgroups and the
2768   original six rabbit study. For example, HZC is No Label by the six rabbit test, but
2769   only half (10) of the three rabbit groups are No Label; seven are 2B and 3 are
2770   category 1. This means if the test were repeated 20 times using the current three
2771   rabbit protocol there would be an equal chance of having a higher than No Label
2772   score (10 out of 20 times) as there would be of having the No Label score (10 out of
2773   20 times). Similar results can be seen for many of the materials in this study.
2774
2775   Even more dramatic examples can be found in the CTFA Phase III study.
2776   HZE, for example, is classified R41 by the six rabbit test, but only 10 of the
2777   subgroups have R41 classifications, the other 10 are No Label! Thus if the three
2778   rabbit test were run only once, there would be a 50% chance of having the lowest
2779   classification (No Label) and an equal chance of having the highest label (R41).
2780   HZP is another interesting example. Although it has a 6-rabbit GHS classification of
2781   No Label, 6 out of 20 tests (30% of the time) give a Category 1 result – three
2782   categories higher than that determined by the 6 rabbit test! Other interesting
2783   examples are highlighted in bold in the table.

2784 **Table 4-16 Recombination of each 6 rabbit test result into 20 three rabbit test subgroups. Each**
2785 **subgroup was classified separately according to the rules for each of the three classification**
2786 **systems, and the number of subgroups falling into each hazard category is indicated. Numbers**
2787 **in bold, shaded areas represent results from test materials where the subgroups differed in their**
2788 **hazard classification from the overall six rabbit classification. Data from the CTFA Phase III**
2789 **study. N = 25 materials.**

		6 animal study score			GHS Counts				EU Counts			EPA Counts			
		GHS	EU	EPA	1	2A	2B	NL	R41	R36	NL	I	II	III	IV
Shampoo 7	HZA	1	R41	1	16	4	0	0	16	3	1	16	4	0	0
Liquid Soap 1	HZB*	NL	NL	3	0	0	4	16	0	0	20	0	0	20	0
Shampoo 1	HZC*	NL	NL	3	0	0	10	10	0	0	20	0	0	20	0
Shampoo 5	HZD*	NL	NL	3	0	0	0	20	0	0	20	0	0	20	0
Gel Cleaner	HZE	NL	R41	1	10	0	0	10	10	0	10	10	0	10	0
Baby Shampoo 2	HZF	1	R41	1	16	4	0	0	16	3	1	16	4	0	0
Shampoo 8	HZG*	NL	NL	3	0	0	0	20	0	0	20	0	0	20	0
Eye Makeup re.	HZH	NL	NL	4	0	0	0	20	0	0	20	0	0	0	20
Skin Cleaner	HZI	1	R41	1	19	1	0	0	19	1	0	19	1	0	0
Mild Shampoo	HZJ	NL	NL	4	0	0	0	20	0	0	20	0	0	0	20
Bubble bath	HZK	1	R41	1	20	0	0	0	20	0	0	20	0	0	0
Foam Bath	HZL	1	R41	1	19	0	1	0	19	0	1	19	0	1	0
Shampoo 3	HZM*	NL	NL	3	0	0	0	20	0	0	20	0	0	10	10
Shampoo 6	HZN*	NL	NL	3	0	0	0	20	0	0	20	0	0	20	0
Baby Shampoo 1	HZP	NL	NL	3	0	0	0	20	0	0	20	0	0	19	1
Cleaning Gel	HZQ	NL	NL	3	0	0	0	20	0	0	20	0	0	20	0
Facial Cleaning Foam	HZR*	NL	R41	1	10	0	3	7	10	0	10	10	0	10	0
Shower Gel	HZS	1	R41	1	19	1	0	0	19	1	0	19	1	0	0
Polishing Scrub	HZT	NL	NL	4	0	0	0	20	0	0	20	0	0	0	20
Hand Soap	HZU*	NL	NL	3	0	0	4	16	0	0	20	0	0	20	0
Shampoo 4	HZV*	NL	NL	3	0	0	0	20	0	0	20	0	0	20	0
Liquid Soap 2	HZW*	2B	NL	3	0	0	16	4	0	0	20	0	0	20	0
Shampoo 2	HZX	1	R41	1	19	1	0	0	19	0	1	16	4	0	0
Shampoo AntiD	HZY	1	R41	1	16	4	0	0	16	4	0	16	4	0	0
Facial Cleaner	HZZ	NL	NL	4	0	0	0	20	0	0	20	0	0	0	20

2790 * tested at 25% (w/v) in vivo and in vitro (starting material)

2791

2792 **The main conclusion from studying this example is that neither a Draize**
2793 **MAS score nor a Draize-defined EPA toxicity classification is an unvarying**
2794 **physical constant for the test material. Therefore, an *in vitro* test should not**
2795 **be expected to exactly match a toxicity category determined *in vivo* because**
2796 **the next time the animal test is run it might also fail to match the toxicity**
2797 **classification of the first animal test.**

2798

2799 One other interesting piece of information can be found in the results in Table
2800 4-16, and that is the EPA toxicity categories which would be assigned to this list of
2801 personal care and cosmetics products. The usual assumption is that EPA Category I
2802 materials are extremely toxic, such as undiluted commercial pesticides, or strong
2803 bleaches or acids. However, here we see that common products that are used
2804 routinely around the head and face are able to elicit Category I classifications. Even
2805 a labeled baby shampoo is categorized as an EPA Category 1! It is possible that
2806 many of these personal care products are actually potential severe eye irritants for

2807   humans. However, this does not seem likely, or we would have seen a tremendous
2808   number of severe eye injuries from misuse (or even correct use) of the products. A
2809   more likely possibility is that the EPA scoring scale is quite overprotective of the
2810   human response. This is an important concept to keep in mind when assessing the
2811   predictive capacity of the *in vitro* tests described in this BRD. When assessing the
2812   validity of a new method it is always necessary to make some judgment concerning
2813   just how many underpredictions of the Draize-defined toxicity classifications can be
2814   accepted. Knowing how this set of personal care products scored in the Draize eye
2815   irritation test may assist in making realistic assessments.
2816

2817   4.8.2 Historic references on reliability of the Draize test

2818
2819   Additional information addressing the variability inherent in the Draize test
2820   can be found in (Weil and Scala 1971; Marzulli and Ruggles 1973; Choksi,
2821   Haseman et al. 2005; Prinsen 2006).

2822 # 5 Test method data and results

2823

2824   Since the testing strategy described in this BRD consists of three separate
2825 test methods, the Cytosensor method, the EpiOcular method and the BCOP test
2826 method, information concerning the data and the protocols used to generate the
2827 data will be described under the appropriate headings for each test method in turn.

2828 ## 5.1 Description of the test method protocols used to generate data

2829

2830   The number of unique materials with *in vivo* and *in vitro* paired data is
2831 described by Table 5-1 for each assay system. The materials tested in the
2832 Cytosensor assay were not tested in any other *in vitro* assay system. The CTFA
2833 cytosensor study used the same 25 unique materials in both the Draize and LVET *in*
2834 *vivo* systems. The Colipa study and the CTFA study had some overlap of materials,
2835 but the materials were either reformulated or separately sourced with several year's
2836 time between the studies – thus it would be questionable to consider them
2837 "identical" materials. Thirty unique materials were tested in both the EpiOcular and
2838 BCOP assay systems. These materials are listed under EpiOcular, BCOP, and the
2839 EpiOcular & BCOP assays below.
2840

2841 **Table 5-1 Description of number of unique materials tested in each assay system with**
2842 **corresponding *in vivo* data.**
2843

Assay	Study	*In Vivo* Data	Materials	Comments
Cytosensor	LVET Only Section 6.1.1	LVET	105 unique	Not tested in any other *in vitro* assay.
	CTFA Phase III Section 6.1.2.1	Draize / LVET	25 unique / 25 unique	Same 25 materials were tested in the Draize and LVET. Not tested in any other *in vitro* assay.
	COLIPA Section 6.1.2.2	Draize	20 unique	Not tested in any other *in vitro* assay.
EpiOcular	Different Companies Section 6.2.1	Draize / LVET	30 unique / 25 unique	Different materials tested in the Draize and LVET. 30 materials (all from the Draize study) were also tested in the BCOP assay.
BCOP	Different Companies Section 6.3.2.2.1	Draize / LVET	66 unique / 2 unique	30 materials (all from the Draize study) were also tested in the EpiOcular assay

2844

2845 ## 5.1.1 Cytosensor method

2846

2847   The Cytosensor data submitted by the participating companies for anti-
2848 microbial cleaning products (and similar formulations) were generated by at least
2849 two different protocols. One was the protocol designed for the silicon
2850 microphysiometer, the predecessor instrument to the Cytosensor, which is
2851 described in Section 2.2.1. This protocol uses a 500 second exposure to cells grown

2852 on a cover slip (see Section 2.2.1 for further explanation). For ease in combining
2853 data so that a comprehensive prediction model for both instruments could be
2854 developed, data from this protocol were transformed to Cytosensor data by an
2855 algorithm described in Section 2.2.1.1.
2856
2857   The second protocol used to generate anti-microbial cleaning products data
2858 was the standard Cytosensor protocol used by both the Procter & Gamble Company
2859 and the Institute for *In Vitro* Sciences, Inc. This protocol uses an 810 second
2860 exposure to cells grown on a Transwell membrane (see Section 2.2.1 for further
2861 explanation), and is presented in Annex A1.
2862
2863   Also included in this BRD are data generated from the CTFA Phase III
2864 evaluation study on surfactant-based formulations (Gettings, Lordo et al. 1996).
2865 This study used the Silicon Microphysiometer protocol (500 sec exposure).
2866
2867   Data from a second large validation study which used surfactants and
2868 surfactant-based formulations (some of which were prepared to be identical to the
2869 ones used in the CTFA evaluation) – the COLIPA eye irritation study (Brantom,
2870 Bruner et al. 1997) - used the Cytosensor protocol (810 sec exposure) which is
2871 contained in Annex A2.

2872 5.1.2 EpiOcular method

2873
2874   The EpiOcular data submitted by the participating companies for anti-
2875 microbial cleaning products (and similar formulations) were all generated by a single
2876 protocol which was developed by the Procter & Gamble Company and
2877 Microbiological Associates/IIVS. This protocol uses the EpiOcular tissue model
2878 (MatTek Corporation, Ashland, MA) and is contained in Annex A3. See Section
2879 2.2.2.2 for more details on the protocol.

2880 5.1.3 BCOP method

2881
2882   The BCOP data submitted by the participating companies for anti-microbial
2883 cleaning products (and similar formulations) were all generated by a common
2884 protocol which is contained in Annex A2. This is essentially identical to the
2885 "ICCVAM Recommended BCOP Test Method Protocol" which is contained in
2886 ICCVAM's test method evaluation report following their review of 4 methods to
2887 detect ocular corrosives and severe irritants. The standard exposure time in this
2888 protocol is 10 minutes; however, some data are included in this BRD where the
2889 corneas were exposed for only three minutes. In fact, it was determined that the 10
2890 minute exposure often overpredicted cleaning formulations which contained >5%
2891 solvent. The animal derived toxicity categories were more accurately predicted by a
2892 three minute score. Therefore, we suggest that formulations containing >5% solvent
2893 be evaluated with a three minute exposure protocol. It is indicated in the text where
2894 these types of data are being discussed.
2895

2896   Some interlaboratory variability data are presented in this BRD which were
2897 extracted from the ICCVAM BRD on the BCOP assay. The protocols which were
2898 used to generate these data are described in the ICCVAM BRD.
2899

2900 5.2 Availability of copies of original data used to evaluate the predictive
2901 capacity and reliability of the three test methods
2902

2903 5.2.1 Cytosensor data
2904

2905   For the main analysis of predictive capacity, data from the Cytosensor were
2906 submitted by participating companies along with spreadsheets containing the results
2907 of animal studies. In some cases, the original reports from the animal studies were
2908 submitted. The spreadsheets containing the data are appended to this BRD (Annex
2909 C2), and the actual reports can be made available to ICCVAM or the EPA upon
2910 request.
2911

2912   For the supplemental information that was used for predictive capacity and
2913 reliability (results from the CTFA Phase III evaluation and the COLIPA study), the
2914 raw animal data from the CTFA Phase III evaluation are available, but only
2915 subsequent transcriptions are available for the COLIPA study. Raw data from the *in*
2916 *vitro* portion of these two studies can be supplied if desired.
2917

2918 5.2.2 EpiOcular data
2919

2920   Raw data for both the *in vitro* and *in vivo* studies reported for the EpiOcular
2921 method are available upon the request of ICCVAM or the EPA.
2922

2923   Raw data from the Colgate-Palmolive sponsored validation of the EpiOcular
2924 test method (used here for interlaboratory reliability information) can be made
2925 available to ICCVAM or the EPA upon request.

2926 5.2.3 BCOP data
2927

2928   Raw data for both the *in vitro* and *in vivo* studies reported for the BCOP
2929 method are available upon the request of ICCVAM or the EPA.
2930

2931   Raw data from some of the ancillary studies taken from the ICCVAM BCOP
2932 BRD (ICCVAM 2006) may be available from the NICEATM archives.
2933

2934
2935
5.3 Summary of results and prediction models used to evaluate the data

2936
2937   The development of the prediction models for each of the test methods is
2938   described in the data analysis section of this BRD (Section 6.0).
2939

2940   ### 5.3.1 Cytosensor test method

2941
2942   Participating companies submitted Cytosensor data for ~275 test samples
2943   having formulations similar to those found in typical cleaning product formulations.
2944   After evaluating the animal data (all LVET data for these samples) it was found that
2945   the data were insufficient to accurately calculate EPA toxicity categories for 170
2946   materials due to termination of the animal test prior to 21 days or individual animal
2947   data were not provided. Thus 108 materials remained for which there were both
2948   EPA categories and Cytosensor MRD_{50} information. Three of these materials were
2949   described as having oxidizing properties and had been tested in the Cytosensor
2950   before other studies conducted by the participating companies indicated that
2951   oxidizing products often cause a delayed ocular response which is best observed in
2952   the BCOP assay. Therefore, the oxidizing materials were not used in the analysis of
2953   the Cytosensor performance, leaving 105 unique materials which could be used to
2954   gauge the performance of the Cytosensor. Coded information on the 105 materials
2955   is given in Table 5-2. Full formulation information on the materials can be traced
2956   using the code to identify the appropriate information in Annex B3.
2957
2958   In addition to the company submissions, we were able to obtain Cytosensor
2959   and rabbit raw data from 25 materials from the CTFA Phase III eye irritation
2960   evaluation study (Gettings, Lordo et al. 1996) on surfactants and surfactant
2961   containing materials (Table 5-4). Both LVET and Draize test data were obtained for
2962   all 25 materials allowing a comparison of these two rabbit eye test methodologies
2963   for deriving the cut-offs needed for a prediction model. A list of the formulations is
2964   included in Annex B4.
2965
2966   In order to obtain additional information on the performance characteristics of
2967   the CM assay when the traditional Draize test was used to define the EPA and GHS
2968   toxicity classification of the formulations, we obtained raw data from a COLIPA-
2969   sponsored study (Brantom, Bruner et al. 1997; Harbell, Osborne et al. 1999) which
2970   tested a range of surfactant-containing formulations including 12 surfactants and 7
2971   surfactant-containing materials (Table 5-5). The traditional Draize methodology was
2972   used to define the toxicity classifications of the chemicals and formulations.
2973
2974   ### 5.3.1.1 Company Cytosensor data submissions paired with data from the LVET assay

2975
2976   Table 5-2 lists the 105 unique formulations for which both Cytosensor data
2977   and rabbit LVET data exist. Table 5-3 summarizes the number of formulations which

2978   fall into each each of the predetermined "buckets". Both GHS and EPA toxicity
2979   categories are listed along with the Cytosensor MRD_{50} value. Where 6-rabbit tests
2980   were used, the distribution of 3-rabbit subgroups are listed to indicate the level of
2981   variability associated with the final category assignment. See Section 4.8.1 for a
2982   discussion of this type of analysis. The protocol used to generate the paired data
2983   was the standard Cytosensor protocol used by both the Procter & Gamble Company
2984   and the Institute for *In Vitro* Sciences, Inc. This protocol uses an 810 second
2985   exposure to cells grown on a Transwell membrane (see Section 2.2.1 for further
2986   explanation), and is presented in Annex A1.

Table 5-2 Results of 105 unique materials tested in the Cytosensor assay and the rabbit LVET assay. Four of the materials were tested twice in the LVET assay and have toxicity categories from both tests listed.

Code Number	Physical State	Formulation Type			Cytosensor MRD$_{50}$	*In vivo* GHS	*In vivo* EPA	GHS Categories (3 rabbit subgroups)				EPA Categories (3 rabbit subgroups)			
		#1	#2	#3	(mg/mL)	(LVET)	(LVET)	1	2A	2B	NI	I	II	III	IV
1001	liquid	SU	SO		0.435	Non-irritant	Category III	0	0	0	20	0	0	10	10
1002	liquid	SU	SO		0.535	Non-irritant	Category III	0	0	3	17	0	0	20	0
1003	liquid	SU	SO		0.44	Category 2A	Category II	0	16	4	0	0	16	4	0
1004	liquid	SU	SO		0.421	Category 2B	Category III	0	0	1	0	0	0	1	0
1005	liquid	SU	SO		0.411	Category 2A	Category II	0	1	0	0	0	1	0	0
1006	liquid	SU	SO		0.443	Non-irritant	Category III	0	0	0	1	0	0	1	0
1007	liquid	SU	SO		0.428	Category 2B	Category III	0	0	1	0	0	0	1	0
1008	liquid	SU	SO		0.272	Category 2B	Category III	0	0	1	0	0	0	1	0
1009	liquid	SU	SO		0.465	Non-irritant	Category III	0	0	0	20	0	0	19	1
1010	liquid	SU	SO		0.456	Category 1	Category I	1	0	0	0	1	0	0	0
1011	liquid	SU	SO		0.44	Category 1	Category I	1	0	0	0	1	0	0	0
1012	liquid	SU	SO		0.415	Category 2A	Category II	0	1	0	0	0	1	0	0
1013	liquid	SU	SO		0.426	Category 2B	Category III	0	0	1	0	0	0	1	0
1014	liquid	SU			0.444	Non-irritant	Category III	0	0	0	1	0	0	1	0
1015	liquid	SU	SO		0.412	Non-irritant	Category III	0	0	0	1	0	0	1	0
1016	liquid	SU	SO		0.272	Category 2B	Category III	0	0	1	0	0	0	1	0
1017	liquid	SU			0.432	Category 2B	Category III	0	0	1	0	0	0	1	0
1018	liquid	SU			0.465	Category 2B	Category III	0	0	1	0	0	0	1	0
1019	liquid	SU	SO		0.276	Category 1	Category I	1	0	0	0	1	0	0	0
1020	liquid	SU	SO		0.296	Category 1	Category I	1	0	0	0	1	0	0	0
1021	granular	SU	AL		0.19	Non-irritant	Category III	0	0	0	20	0	0	16	4
1022	liquid	SU	SO		0.51	Category 2A	Category I	10	9	1	0	10	9	1	0
1023 (2nd test for 1022)	liquid	SU	SO		0.51	Category 2A	Category III	4	15	1	0	0	0	20	0
1024	liquid	SU	SO		0.2	Category 1	Category I	1	0	0	0	1	0	0	0
1025	liquid	SU	SO		0.829	Non-irritant	Category III	0	0	0	20	0	0	20	0
1026	viscous	SU	SO		0.434	Category 2B	Category III	0	0	20	0	0	0	20	0

Code Number	Physical State	Formulation Type			Cytosensor MRD$_{50}$	In vivo GHS	In vivo EPA	GHS Categories (3 rabbit subgroups)				EPA Categories (3 rabbit subgroups)			
		#1	#2	#3	(mg/mL)	(LVET)	(LVET)	1	2A	2B	NI	I	II	III	IV
1027	liquid	SU	SO		0.44	Non-irritant	Category III	0	0	1	19	0	0	20	0
1028	liquid	SU	SO		0.46	Category 2A	Category III	0	1	0	0	0	0	1	0
1029	liquid	SU	SO		0.45	Category 2B	Category III	0	0	1	0	0	0	1	0
1030	liquid	SU	SO		0.6	Category 2B	Category III	0	0	1	0	0	0	1	0
1031	liquid	SU	SO		0.5	Category 2B	Category III	0	0	1	0	0	0	1	0
1032	liquid	SU	SO		0.96	Category 2A	Category III	0	1	0	0	0	0	1	0
1033 (2nd test for 1032)	liquid	SU	SO		0.96	Category 2B	Category III	0	0	1	0	0	0	1	0
1034	liquid	SU	SO		0.67	Non-irritant	Category III	0	0	0	1	0	0	1	0
1035	liquid	SU	SO		63.9	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1036	liquid	SU	SO		0.79	Non-irritant	Category III	0	0	0	1	0	0	1	0
1037	polymer	SU	AL		9.043	Non-irritant	Category III	0	0	0	1	0	0	1	0
1038	built add	SU	AL		8.916	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1039	liquid	SU	SO		0.26	Category 1	Category I	1	0	0	0	1	0	0	0
1040	liquid	SU	SO		0.76	Category 2A	Category II	0	1	0	0	0	1	0	0
1041	liquid	SU	SO		0.22	Category 2A	Category II	0	1	0	0	0	1	0	0
1042	viscous	SU	SO	AL	22.7	Non-irritant	Category III	0	0	0	1	0	0	1	0
1043	liquid	SU	SO		0.407	Category 2A	Category II	0	20	0	0	0	10	10	0
1044	liquid	SU	SO		0.428	Category 2A	Category II	0	20	0	0	0	19	1	0
1045	liquid	SU	SO		0.344	Category 2A	Category III	0	19	1	0	0	0	20	0
1046	liquid	SU	SO		0.264	Category 2A	Category II	0	1	0	0	0	1	0	0
1047	cream	SU	SO		0.286	Non-irritant	Category III	0	0	0	1	0	0	1	0
1048	liquid	SU	AC		5.81	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1049	liquid	SU	AC		6.02	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1050	liquid	SU	SO	AC	4.99	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1051	liquid	SU	SO		7.103	Category 2B	Category III	0	0	1	0	0	0	1	0
1052	viscous liquid	SU			1.354	Non-irritant	Category III	0	0	0	1	0	0	1	0
1053	liquid	SU			0.0808	Category 2B	Category III	0	0	1	0	0	0	1	0
1054	liquid	SU			0.0773	Category 2B	Category III	0	0	1	0	0	0	1	0
1055	liquid	SU			0.638	Category 2A	Category II	0	1	0	0	0	1	0	0
1056	liquid	SU			0.817	Category 2A	Category II	0	1	0	0	0	1	0	0

Code Number	Physical State	Formulation Type			Cytosensor MRD$_{50}$	*In vivo* GHS	*In vivo* EPA	GHS Categories (3 rabbit subgroups)				EPA Categories (3 rabbit subgroups)			
		#1	#2	#3	(mg/mL)	(LVET)	(LVET)	1	2A	2B	NI	I	II	III	IV
1057 (2nd test for 1056)	liquid	SU			0.817	Category 1	Category I	1	0	0	0	1	0	0	0
1058	liquid	SU			0.81	Category 2A	Category II	0	1	0	0	0	1	0	0
1059	liquid	SU			0.787	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1060	liquid	SU			0.9	Non-irritant	Category III	0	0	0	1	0	0	1	0
1061	cream	SU			26.733	Non-irritant	Category III	0	0	0	1	0	0	1	0
1062	cream	SU			46.5	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1063	cream	SU			43.1	Non-irritant	Category III	0	0	0	1	0	0	1	0
1064	liquid	SU			0.501	Non-irritant	Category III	0	0	0	1	0	0	1	0
1065	liquid	SU	SO		300	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1066	liquid	SU			3.8	Non-irritant	Category III	0	0	0	1	0	0	1	0
1067	liquid	SU			2.573	Non-irritant	Category III	0	0	0	1	0	0	1	0
1068	liquid	SU			4.308	Non-irritant	Category III	0	0	0	1	0	0	1	0
1069	liquid	SU			0.556	Non-irritant	Category III	0	0	0	1	0	0	1	0
1070	liquid	SU			1.96	Non-irritant	Category III	0	0	0	1	0	0	1	0
1071	liquid	SU			0.66	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1072	solid/flakes	SU			3.718	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1074	cream	SU			4.19	Non-irritant	Category III	0	0	0	1	0	0	1	0
1075	solid	SU			10.96	Non-irritant	Category IV	0	0	0	20	0	0	0	20
1076	liquid	SU			0.63	Non-irritant	Category III	0	0	4	16	0	0	20	0
1077	liquid	SU			0.63	Category 1	Category I	1	0	0	0	1	0	0	0
1078	gel	SU			0.49	Non-irritant	Category III	0	0	6	14	0	0	20	0
1079	liquid	SU			0.708	Category 2B	Category III	0	0	1	0	0	0	1	0
1080 (2nd test for 1079)	liquid	SU			0.708	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1081	liquid	SU			0.717	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1082	liquid	SU			2.019	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1083	liquid	SU			1.43	Non-irritant	Category III	0	0	0	1	0	0	1	0
1084	liquid	SU			3.86	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1085	liquid	SU			15.18	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1086	liquid	SU			0.93	Category 2B	Category III	0	0	17	3	0	0	20	0
1087	liquid	SU			2.49	Non-irritant	Category IV	0	0	0	1	0	0	0	1

Code Number	Physical State	Formulation Type			Cytosensor MRD$_{50}$	In vivo GHS	In vivo EPA	GHS Categories (3 rabbit subgroups)				EPA Categories (3 rabbit subgroups)			
		#1	#2	#3	(mg/mL)	(LVET)	(LVET)	1	2A	2B	NI	I	II	III	IV
1088	liquid	SO	AL		48.48	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1089	cream	SO			20.652	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1090	liquid	SO			8.085	Non-irritant	Category III	0	0	0	1	0	0	1	0
1091	liquid	AC	SU		6.41	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1092	liquid	SO	SU		300	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1093	liquid	SO			5.97	Category 2B	Category III	0	0	1	0	0	0	1	0
1094	liquid	SO			142.857	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1095	liquid	SO			69.842	Non-irritant	Category III	0	0	0	1	0	0	1	0
1096	liquid	SO			22.438	Non-irritant	Category III	0	0	0	1	0	0	1	0
1097	liquid	SO			22.172	Non-irritant	Category III	0	0	0	1	0	0	1	0
1098	creamy liquid	SO			20.68	Non-irritant	Category IV	0	0	0	20	0	0	0	20
1099	creamy liquid	SO	SU		3.96	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1100	liquid	SO			18.834	Non-irritant	Category III	0	0	0	1	0	0	1	0
1101	liquid	SO			16.581	Non-irritant	Category III	0	0	0	1	0	0	1	0
1102	liquid	SO	SU		0.92	Category 2B	Category III	0	0	1	0	0	0	1	0
1103	semi-viscous liquid	SO	SU		21.9	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1104	liquid	AL	SO		41.5	Non-irritant	Category III	0	0	0	1	0	0	1	0
1105	liquid	AL	SO		69.63	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1106	liquid	AL	SO		52.13	Non-irritant	Category III	0	0	0	1	0	0	1	0
1107	liquid	AL	SO		21.4	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1108	viscous liquid	SO	SU	AC	2.2	Non-irritant	Category IV	0	0	0	1	0	0	0	1
1109	thin liquid	SO	AC	SU	3.377	Non-irritant	Category III	0	0	0	1	0	0	1	0
1110	liquid	SO	AC		30.365	Non-irritant	Category III	0	0	0	1	0	0	1	0

AC=Acid, AL=Alkaline (base), SO=Solvent, SU=Surfactant

2788   Table 5-3 gives the distribution of materials in Table 5-2. It is obvious that the
2789   distribution of product categories is relatively uneven, but follows a pattern similar to
2790   that of the types of anti-microbial cleaning products on the market (personal
2791   communication, P&G).
2792
2793   **Table 5-3 Distribution of product categories originally submitted with both animal eye**
2794   **irritation data and Cytosensor data.**
2795

Product Categories	Number of products tested
Surfactants	82
Acids	1
Bases	4
Solvents	18
Total	**105**

2796

2797   5.3.1.2 CTFA Phase III study (Gettings, Lordo et al. 1996)

2798
2799   The CTFA Phase III study was chosen for inclusion in this BRD since it is
2800   helpful to understand how the Draize and the LVET perform on a set of materials
2801   (surfactant-based personal care products) for which there are CM data and which
2802   are similar to those materials contained in this BRD. The animal data can be found
2803   in Annexes C3-C6.
2804
2805   The CTFA Phase III study (Gettings, Lordo et al. 1996) was an evaluation
2806   program of a number of *in vitro* eye irritation tests. The project's original goal was to
2807   determine how well the *in vitro* tests predicted the Draize MAS scores for 25
2808   surfactant-based personal care products, but a secondary analysis conducted at the
2809   conclusion of the primary study included LVET MAS scores as well. The reference
2810   data for the CTFA Phase III study are arguably the most useful of the animal data
2811   from any of the studies in this BRD. Data from both the Draize and LVET assays
2812   were obtained under GLP-compliant conditions and with a randomized block design
2813   utilizing three male and three female rabbits for each chemical. There are several
2814   advantages to the block design: 1) it simulates to some extent within lab day-to-day
2815   variability since for each chemical not all rabbits are dosed on the same day, and 2)
2816   it eliminates some of the scoring bias since the scorers read each animal
2817   independently and are unaware of which six rabbits were treated with the same test
2818   article. However, the main positive point about the study is that the *in vitro* and *in*
2819   *vivo* assays were run nearly concurrently (separated only by a few weeks) using
2820   samples from the same batch of chemical or formulation. The one negative point to
2821   this study is that ocular anesthesia was used during the rabbit test (both Draize and
2822   LVET) and to the best of our knowledge none of the other animal assays in this
2823   BRD used ocular anesthesia. There are reports that rabbits given ocular anesthesia
2824   may have a more intense ocular reaction than animals treated without anesthesia,
2825   *e.g.,* Gunderson & Liebmann (1944).
2826

2827   Only one laboratory (Microbiological Associates, Inc., Rockville, MD)
2828 contributed CM data for this study. All 25 chemicals in the study were deemed
2829 compatible for testing with the CM. An overall summary of the CTFA Phase III study
2830 including the chemical identities, animal scores, and *in vitro* scores is given in Table
2831 5-4. Although these studies were conducted with the silicon microphysiometer, for
2832 ease of comparison with the other studies in this section of the BRD, the *in vitro*
2833 MRD_{50} values have been converted to CM values using the relationship presented
2834 in Section 2.2.1.1.
2835
2836   Table 5-4 shows that in the CTFA Phase III study most materials (16/25;
2837 64%) are assigned the same EPA toxicity category by either the LVET or the Draize
2838 test, supporting the fact that Draize and LVET are not all that different. The total
2839 concordance is 64%, with 12% differing by one category and 24% differing by 2
2840 categories.
2841
2842   A similar analysis by GHS categories shows that there is 64% concordance,
2843 with 4% differing by one category, 16% differing by 2 categories and 16% differing
2844 by three categories.
2845
2846

2847 **Table 5-4 Summary of Cytosensor data from the CTFA Phase III study using toxicity**
2848 **classifications determined by both the Draize Rabbit Test and the Low Volume Eye Test for**
2849 **surfactant-containing materials (Gettings, Lordo et al. 1996)**

2850

CTFA Phase III Cytosensor In Vitro Data									
DRAIZE & LVET *In Vivo* Eye Classifications									
CTFA chemical number	Substance	Test Code	Concentration Tested	*In Vivo* GHS[1,2] (DRAIZE)	*In Vivo* GHS[1,2] (LVET)	*In Vivo* EPA[3,4] (DRAIZE)	*In Vivo* EPA[3,4] (LVET)	DRAIZE[9] MMAS	CM converted value MRD$_{50}$ (mg/mL)
1	Shampoo 7	HZA	100%	Category 1	No category	Category I	Category III	37.8	1.18
2	Liquid Soap 1	HZB	25%	No category	No category	Category III	Category IV	20.7	2.80
3	Shampoo 1	HZC	25%	No category	No category	Category III	Category III	36.0	1.72
4	Shampoo 5	HZD	25%	No category	No category	Category III	Category III	19.5	2.78
5	Gel Cleanser	HZE	100%	No category	No category	Category I	Category III	22	3.19
6	Baby Shampoo 2	HZF	100%	Category 1	No category	Category I	Category III	37.5	1.50
7	Shampoo 8	HZG	25%	No category	No category	Category III	Category III	17.8	2.80
8	Eye Makeup re.	HZH	100%	No category	No category	Category IV	Category IV	2.3	20.0
9	Skin Cleaner	HZI	100%	Category 1	Category 2B	Category I	Category I	41.0	1.09
10	Mild Shampoo	HZJ	100%	No category	No category	Category IV	Category IV	8.2	6.38
11	Bubble bath	HZK	100%	Category 1	Category 2B	Category I	Category I	39.7	0.97
12	Foam Bath	HZL	100%	Category 1	No category	Category I	Category III	37.8	1.09
13	Shampoo 3	HZM	25%	No category	No category	Category III	Category III	12.7	3.11
14	Shampoo 6	HZN	25%	No category	No category	Category III	Category III	18.0	2.56
15	Baby Shampoo 1	HZP	100%	No category	No category	Category III	Category III	11.7	2.45
16	Cleansing Gel	HZQ	100%	No category	No category	Category III	Category IV	17.2	5.85
17	Facial Cleansing Foa	HZR	25%	No category	No category	Category I	Category III	39.0	5.60
18	Shower Gel	HZS	100%	Category 1	Category 2B	Category I	Category I	41.4	1.13
19	Polishing Scrub	HZT	100%	No category	No category	Category IV	Category IV	7.0	30.9
20	Hand Soap	HZU	25%	No category	No category	Category III	Category III	33.7	4.85
21	Shampoo 4	HZV	25%	No category	No category	Category III	Category III	25.2	2.34
22	Liquid Soap 2	HZW	25%	2B	No category	Category III	Category III	31.0	2.64
23	Shampoo 2	HZX	100%	Category 1	No category	Category I	Category III	40.0	1.20
24	Shampoo AntiD	HZY	100%	Category 1	Category 2B	Category I	Category II	43.0	1.14
25	Facial Cleanser	HZZ	100%	No category	No category	Category IV	Category IV	3.7	>168.9

[1]GHS=Globally Harmonized System (UN [2003])

[2]Eye Irritant Category 1 = irreversible effects on the eye/serious damage to the eye; Category 2A = reversible effects on the eye/irritating to the eyes; Category 2B = reversible effects on the eye/mildly irritating to the eyes; No category = no effects on the eye

[3]EPA=U.S. Environmental Protection Agency (EPA [1996]).

[4]Toxicity Category I for the Primary Eye Irritation Study = Corrosive, or corneal involvement or irritation not reversible within 21 days; Category II = Corneal involvement or irritation clearing in 8-21 days; Category III = Corneal involvement or irritation clearing in 1-7 days; Category IV: minimal effects clearing in less than 24 hr

2851 [5]MMAS scores reported in Gettings et al. (1996)
2852

2853   5.3.1.3 COLIPA Validation study for eye irritation

2854

2855   In 1995/1996 the European Cosmetics, Toiletry and Perfumery Association
2856   (COLIPA) sponsored an international validation study of *in vitro* eye irritation
2857   methods (Brantom, Bruner et al. 1997). The COLIPA study used a set of 55
2858   cosmetic formulations and ingredients - a large proportion of which were pure
2859   surfactants or surfactant based formulations - to assess the ability of *in vitro*
2860   methods to predict eye irritation potential. Two laboratories conducted the CM assay
2861   according to a standardized protocol (Annex A2) which used an 810 second
2862   exposure time. Raw data from the studies conducted by Microbiological Associates,
2863   Inc. and CellTox AB were obtained from the archives of the Institute for *In Vitro*
2864   Sciences, Inc. Mean data from these two laboratories for each chemical are
2865   presented in Table 5-5.

2866

2867   The reference data for the COLIPA study came from three main sources; two
2868   for the neat chemicals and one for the formulations. The data for the chemicals
2869   came from the ECETOC data bank (ECETOC 1992) and the EU isolated cornea
2870   study (Gautheron, Giroux et al. 1994). All of these data are now available in a new
2871   edition of the ECETOC data bank (ECETOC 1998). The raw animal data are also
2872   found in Annexes C7&C8.

2873

2874   Thirty-two formulations were used in the COLIPA study, and the Draize
2875   scores for these formulations come from Draize tests conducted contemporaneously
2876   with this study. The formulations were newly prepared for the COLIPA study, but
2877   most were based on formulations that had been tested in Phases I, II, and III of the
2878   CTFA evaluation program (Feder, Lordo et al. 1991; Gettings, Dipasquale et al.
2879   1994; Gettings, Lordo et al. 1996). Thus, it is likely that for the formulations, the *in*
2880   *vitro* tests were challenged with exactly the same material as the *in vivo* test. The
2881   same cannot be said for the chemicals since historical data were used for them.
2882   Because the evaluation of formulations (anti-microbial cleaning products) is the
2883   focus of this BRD, only the results with the formulations, or with pure surfactants,
2884   from the COLIPA study will be addressed here,

2885

2886   There were 19 surfactants and surfactant-containing materials which had
2887   data from the two participating CM laboratories. An overall summary of the COLIPA
2888   study including the chemical identities, animal scores and *in vitro* scores (averages
2889   from MA and CellTox AB) are given in Table 5-5. The formulations are included in
2890   Annex B5.

2891
2892

2893 **Table 5-5 Summary of Cytosensor and *in vivo* data from the COLIPA study which includes**
2894 **average values (see footnotes) from MA and CellTox AB laboratories (Brantom, Bruner et al.**
2895 **1997).**
2896

	COLIPA study - Surfactants and Sufactant-based Formulations *Cytosensor and In Vivo* Eye Irritation Classifications						
COLIPA chemical number	Substance	Concentration Tested	n. of animals	*In Vivo* GHS[1,2]	*In Vivo* EPA[3,4]	ECETOC MMAS Score[5]	Average MRD$_{50}$ (mg/mL)
5	Shampoo no. 1 - normal	100%	3	Category 1	Category I	33.3	0.735
6	Eye make-up remover	100%	3	No Category	Category IV	0.7	93.5
11	Polyethylene glycol 400	100%	6	No Category	Category IV	0.0	306.4
13	Triton X-100	1%	3	No Category	Category III	1.7	19.0
15	Tween 20	100%	4	No Category	Category III	4.0	6.50
17	Sodium lauryl sulphate	3%	6	No Category	Category III	16.0	3.00
20	Triton X-100 [2]	5%	6	Category 2A	Category III	32.3	3.54
21	Benzalkonium chloride [1]	1%	4	Category 2A	Category I	34.3	4.22
21	Benzalkonium chloride [2]	1%	6	Category 1	Category I	56.3	4.22
23	Sodium lauryl sulphate	15%	6	Category 1	Category I	59.2	0.513
24	Sodium lauryl sulphate	30%	6	Category 2A	Category II	60.5	0.312*
25	Triton X-100	10%	6	Category 1	Category II	59.0	1.85
26	Benzalkonium chloride	5%	4	Category 1	Category I	83.8	1.095
27	Benzalkonium chloride	10%	3	Category 1	Category I	108.0	0.314
28	Pump deodorant / antiperspirant	100%	3	No Category	Category III	14.7	33.54
34	Gel cleanser	100%	3	No Category	Category III	15.7	5.58
36	Shampoo - baby	100%	3	Category 1	Category I	36.0	2.33
39	Liquid soap no.1	100%	3	Category 1	Category I	37.0	0.78
49	Skin cleanser	100%	3	Category 1	Category I	34.3	0.70
52	Cetylpyridinium bromide	6%	4	Category I	Category I	85.8	1.36*

* - MA value only, CellTox AB designated unsuitable for testing

[1]GHS=Globally Harmonized System (UN [2003])

[2]Eye Irritant Category 1 = irreversible effects on the eye/serious damage to the eye; Category 2A = reversible effects on the eye/irritating to the eyes; Category 2B = reversible effects on the eye/mildly irritating to the eyes; No category

[3]EPA=U.S. Environmental Protection Agency (EPA [1996])

[4]Toxicity Category I for the Primary Eye Irritation Study = Corrosive, or corneal involvement or irritation not reversible within 21 days;

2897 [5]MMAS scores reported in Harbell et al. (1999)

2898

2899

2900   ## 5.3.2 EpiOcular

2901

2902   Participating companies submitted EpiOcular data for 61 test samples having
2903   formulations similar to those found in typical cleaning product formulations. The raw
2904   animal data can be found in Annex C1. After evaluating the animal data (both LVET
2905   data and Draize data) it was found that the animal data were insufficient to
2906   accurately calculate EPA toxicity Categories for 6 materials due to termination of the
2907   animal test prior to 21 days or individual animal data were not provided. Thus 55
2908   materials remained for which there were both EPA Categories and EpiOcular ET_{50}
2909   information. Twenty-five materials were paired with LVET data (Table 5-8) and 30
2910   were paired with Draize data (Table 5-6). Tables 5-7 and 5-9 give the distribution of
2911   materials in Tables 5-6 and 5-8, respectively.

2912

2913   Data from another set of studies conducted to validate the EpiOcular assay
2914   were also submitted for this BRD. Seventy-three surfactants or surfactant-based
2915   materials (or dilutions of materials) were tested in these studies. However, the
2916   EpiOcular protocol used in those studies differs (a dilution of the test material was
2917   performed before the testing) from the protocol being proposed in this BRD;
2918   therefore, these studies will be presented only as supporting information for
2919   interlaboratory reproducibility (Section 7.2.3).

2920

2921   **Table 5-6 EpiOcular data paired with the Draize test**

Code Number	In Vivo Dosing Volume	Formulation Type			In Vivo GHS (DRAIZE)	In Vivo EPA (DRAIZE)	GHS Categories (3 rabbit subgroups)				EPA Categories (3 rabbit subgroups)				EpiOcular ET_{50} (min)
		#1	#2	#3			1	2A	2B	NI	I	II	III	IV	
H	0.1	AL	SU		Non-irritant	Category II	0	9	1	10	0	10	10	0	9.4
I	0.1	SU	AL		Non-irritant	Category III	0	0	0	20	0	0	10	10	12
J	0.1	SU			Non-irritant	Category III	0	0	0	20	0	0	20	0	19.3
K	0.1	RC	SU		Non-irritant	Category IV	0	0	0	20	0	0	0	20	> 240
P	0.1	Phenolic	AL		Non-irritant	Category IV	0	0	0	1	0	0	0	1	125.8
R	0.1	SU			Non-irritant	Category IV	0	0	0	20	0	0	0	20	> 240
T	0.1	AC			Non-irritant	Category IV	0	0	0	1	0	0	0	1	31.6
W	0.1	SU			Non-irritant	Category IV	0	0	0	20	0	0	0	20	39.6
CJ	84 mg solid				Category 1	Category I	1	0	0	0	1	0	0	0	2.9
AG	0.1	AL			Category 1	Category I	20	0	0	0	20	0	0	0	<0.17
AH	0.1	AL	SU		Category 1	Category I	18	2	0	0	19	0	1	0	0.4
AI	0.03	AL	SU		Category 1	Category I	16	4	0	0	16	0	4	0	<0.17
AJ	0.03	AL	SU		Category 1	Category I	20	0	0	0	20	0	0	0	<0.17
AK	0.1	AL	SO	SU	Category 1	Category I	20	0	0	0	20	0	0	0	<0.17
AL	0.03	AL	SO	SU	Category 2A	Category I	10	10	0	0	10	0	10	0	<0.17
AM	0.1	SO	AL		Category 1	Category I	20	0	0	0	20	0	0	0	<0.17
AN	0.03	AL	SU		Category 1	Category I	19	1	0	0	16	4	0	0	1.5
AO	0.03	AL	SO	SU	Category 1	Category I	20	0	0	0	20	0	0	0	<0.17
AP	0.03	AL	SU		Category 1	Category I	16	4	0	0	16	0	4	0	<0.17
AT	0.1	RC	AL		Category 1	Category I	20	0	0	0	19	1	0	0	<1
AU	0.1	RC	AL		Category 1	Category I	20	0	0	0	20	0	0	0	<1
AV	0.1	RC	AL		Category 1	Category I	1	0	0	0	1	0	0	0	<1
AX	0.03	SO	AL		Category 1	Category I	19	1	0	0	16	3	1	0	<0.17
BB	0.1	SO			SCNM	Category IV	0	0	0	0	0	0	0	20	>240
BE	0.1	AC	SU		Non-irritant	Category III	9	0	0	11	0	0	16	4	4
BJ	0.1	AL	SU		Non-irritant	Category III	0	0	10	10	0	0	20	0	2.1
BK	0.1	SO			Non-irritant	Category III	0	0	0	1	0	0	1	0	9.4
BM	0.1	SO			Non-irritant	Category IV	0	0	0	20	0	0	0	20	4.9
BL	0.1	SO			Non-irritant	Category IV	0	0	0	20	0	0	0	20	6.7
BN	0.1	SU			Non-irritant	Category IV	0	0	0	1	0	0	0	1	1.8

2922   AC = Acid; AL = Alkaline (base); RC = Reactive Chemistry (Oxidizer); SO = Solvent; SU = Surfactant; SCNM = Study Criteria Not Met

2923
2924   **Table 5-7 Distribution of product categories for EpiOcular data paired with the Draize test**
2925

Product Categories	Number of products tested
Surfactants	5
Acids	2
Alkaline	11
Oxidizers	4
Solvent	6
Other	2
Total	**30**

2926
2927
2928   **Table 5-8 EpiOcular data paired with LVET data**

EpiOcular Data Paired With LVET - Defined Toxicity Categories																
Code Number	Formulation Type			*In Vivo* GHS	*In Vivo* EPA	GHS Categories (3 rabbit subgroups)				EPA Categories (3 rabbit subgroups)				EpiOcular ET_{50} (min)	LVET MAS
	#1	#2	#3	(LVET)	(LVET)	1	2A	2B	NI	I	II	III	IV		
CY	SU	SO		Category 1	Category I	1	0	0	0	1	0	0	0	2.85	
DC	RC	SU		Category 1	Category I	1	0	0	0	1	0	0	0	1.1	59.67
DH	RC	SU		Category 1	Category I	1	0	0	0	1	0	0	0	0.7	60
DD	RC	SU		Category 2A	Category II	0	1	0	0	0	1	0	0	0.9	49.333
CK	SU			Non-irritant	Category III	0	0	0	1	0	0	1	0	21.75	6
CN	SO			Category 2B	Category III	0	0	1	0	0	0	1	0	49.5	18.333
CQ	SU			Non-irritant	Category III	0	0	0	1	0	0	1	0	29.5	13
CS	SU			Non-irritant	Category III	0	0	0	1	0	0	1	0	23.8	4
CU*	SU	AL		Non-irritant	Category III	0	0	0	20	0	0	16	4	20.25	5.5
CV	RC	AL	SU	Category 2A	Category III	0	1	0	0	0	0	1	0	16	11.5
CW*	SU	SO		Non-irritant	Category III	0	0	0	20	0	0	20	0	13.7	10.2
CX	SU	SO		Non-irritant	Category III	0	0	0	1	0	0	1	0	11.2	21.7
DB	RC	AL		Non-irritant	Category III	0	0	0	1	0	0	1	0	1.7	7
DG*	SU	SO		Category 2B	Category III	0	0	20	0	0	0	20	0	0.75	27.2
DI*	SU			Non-irritant	Category III	0	0	4	16	0	0	20	0	0.484	17.2
DK	RC	AL		Category 2A	Category III	0	1	0	0	0	0	1	0	0.167	33
CO	SO			Non-irritant	Category IV	0	0	0	1	0	0	0	1	47.6	4
CP	SU	SO		Non-irritant	Category IV	0	0	0	1	0	0	0	1	29.5	0
CR	SU	SO		Non-irritant	Category IV	0	0	0	1	0	0	0	1	26.1	2.667
CT	SU	SO		Non-irritant	Category IV	0	0	0	1	0	0	0	1	20.8	2.667
CZ	RC	SO	AC	Non-irritant	Category IV				1				1	2.1	0
DA	RC	SU	SO	Non-irritant	Category IV				1				1	1.9	0
DE	RC	SO		Non-irritant	Category IV				1				1	0.85	0
DF	SO			Non-irritant	Category IV	0	0	0	1	0	0	0	1	0.8	1.333
DJ	SO			Non-irritant	Category IV	0	0	0	1	0	0	0	1	0.45	1.333

* Six animal subgroups were used to determine GHS and EPA categories
2929   AC = Acid; AL = Alkaline (base); RC = Reactive Chemistry (Oxidizer); SO = Solvent; SU = Surfactant

2930
2931   **Table 5-9 Distribution of product categories for EpiOcular data paired with the LVET test**

Product Categories	Number of products tested
Surfactants	12
Acids	0
Alkaline	0
Oxidizers	9
Solvent	4
Other	0
Total	**25**

2932

2933   ## 5.3.3 BCOP

2934   ### 5.3.3.1 Data from participating companies

2935

2936   Participating companies submitted BCOP data for 38 test samples having
2937   formulations similar to those found in typical cleaning product formulations. The raw
2938   animal data can be found in Annex C1. After evaluating the animal data (all Draize
2939   data for these samples), it was found that they were insufficient to accurately
2940   calculate EPA toxicity Categories for 8 materials due to termination of the animal
2941   test prior to 21 days or individual animal data were not provided. Thus 30 materials
2942   remained for which there were both EPA Categories and BCOP information. These
2943   30 materials are highlighted in Table 5-10.

2944

2945   In addition to the company submissions, we were able to obtain raw data
2946   from 25 materials from the CTFA Phase III study (Gettings, Lordo et al. 1996) on
2947   surfactants and surfactant containing materials (which are similar to the materials
2948   used in many anti-microbial cleaning products). Both LVET and Draize test data
2949   were obtained for all 25 materials allowing a comparison between these two rabbit
2950   eye test methodologies.

2951

2952   We were also able to obtain raw data from the European Commission/British
2953   Home Office (EC/HO) study (Balls, Botham et al. 1995) which tested a range of
2954   materials including 15 surfactants. All animal studies (historically derived data) were
2955   conducted with the traditional Draize methodology.   Table 5-10 details the BCOP
2956   data from participating companies paired with Draize-defined toxicity categories.
2957   Table 5-11 gives the distribution of the BCOP data from Table 5-10.

2958
2959

2960
2961
2962

Table 5-10 BCOP data from participating companies paired with Draize-defined toxicity categories (with the exception of two materials which were defined using the LVET assay). Highlighted materials were the original 30 materials submitted.

BCOP Data Paired With DRAIZE - Defined Toxicity Categories

Code Number	In Vivo Dosing Volume	Formulation Type #1	#2	#3	In Vivo GHS (DRAIZE)	In Vivo EPA (DRAIZE)	GHS Categories (3 rabbit subgroups) 1	2A	2B	NI	EPA Categories (3 rabbit subgroups) I	II	III	IV	BCOP IV Score 10 min	3 min
A	0.1	SU	AL		Category 1	Category I	16	4	0	0	16	4	0	0	206.9	132.8
B	0.1	SU	AL		Category 1	Category I	20	0	0	0	20	0	0	0	152.2	108
C	0.1	RC	SU	AC	Category 1	Category I	16	0	0	4	16	0	4	0	29.7	10.3
D	0.1	AC			Category 1	Category I	1	0	0	0	1	0	0	0	187.7	67.5
E	0.1	SU	AL		Category 1	Category I	1	0	0	0	1	0	0	0	196.2	110.5
F	0.1	RC	SU	AC	Category 1	Category I	1	0	0	0	1	0	0	0	360.8	18.2
G	0.1	SU/SO			Category 1	Category I	20	0	0	0	20	0	0	0	139.7	133.3
H	0.1	AL	SU		Non-irritant	Category II	0	9	1	10	0	10	10	0	14	2.85
I	0.1	SU	AL		Non-irritant	Category III	0	0	0	20	0	0	10	10	0.6	-0.3
J	0.1	SU			Non-irritant	Category III	0	0	0	20	0	0	20	0	7.7	2.6
K	0.1	RC	SU		Non-irritant	Category IV	0	0	0	20	0	0	0	20	0.3	0
L	0.1	SU			Non-irritant	Category III	0	0	0	20	0	0	19	1	5.5	2.8
M	0.1	SU	AL		Non-irritant	Category III	0	0	10	10	0	0	20	0	55.7	15.3
N	0.1	RC			Non-irritant	Category III	0	0	0	1	0	0	1	0	152.7	7.2
O	0.1	SU	AL		Non-irritant	Category IV	0	0	0	20	0	0	0	20	7.2	2.6
P	0.1	Phenolic	AL		Non-irritant	Category IV	0	0	0	1	0	0	0	1	1.1	-0.3
Q	0.1	SU			Non-irritant	Category IV	0	0	0	1	0	0	0	1	13.5	3.3
R	0.1	SU			Non-irritant	Category IV	0	0	0	20	0	0	0	20	0.2	-0.6
S	0.1	AC	SU		Non-irritant	Category IV	0	0	0	1	0	0	0	1	18.8	9.2
T	0.1	AC			Non-irritant	Category IV	0	0	0	1	0	0	0	1	1.8	0
U	0.1	SU	AL		Non-irritant	Category IV	0	0	0	20	0	0	0	20	3.4	2.9
V	0.1	SU/SO			Non-irritant	Category IV	0	0	0	20	0	0	0	20	20.8	3.5
W	0.1	SU			Non-irritant	Category IV	0	0	0	20	0	0	0	20	5.7	3.5
X	0.1	RC	AL		Category 2A	Category I	9	8	0	3	10	0	10	0	81.9	41.7
Y	0.1	RC	AL		Category 2A	Category II	0	19	1	0	0	16	4	0	74.9	65
Z	0.1	SO			Category 2A	Category II	0	1	0	0	0	1	0	0	31.6	12.8
AB	0.1	SU			Category 1	Category I	20	0	0	0	20	0	0	0	90	51.9
AC	0.1	AC			Category 1	Category I	15	0	1	4	16	0	4	0	134.8	101
AD	0.1	SU			Category 1	Category I	20	0	0	0	20	0	0	0	113.1	79
AE	0.1	AL			Category 1	Category I	1	0	0	0	1	0	0	0	66.7	37.8
AF	0.1	AC			Non-irritant	Category IV	0	0	0	1	0	0	0	1	9.6	2.5
AG	0.1	AL			Category 1	Category I	20	0	0	0	20	0	0	0	391.9	
AH	0.1	AL	SU		Category 1	Category I	18	2	0	0	19	0	1	0	255.7	
AI	0.03	AL	SU		Category 1	Category I	16	4	0	0	16	0	4	0	354.7	
AJ	0.03	AL	SU		Category 1	Category I	20	0	0	0	20	0	0	0	357.1	
AK	0.1	AL	SO	SU	Category 1	Category I	20	0	0	0	20	0	0	0	444.3	
AL	0.03	AL	SO	SU	Category 2A	Category I	10	10	0	0	10	0	10	0	353.6	
AM	0.1	SO	AL		Category 1	Category I	20	0	0	0	20	0	0	0	135.8	
AN	0.03	AL	SU		Category 1	Category I	19	1	0	0	16	4	0	0	113.5	
AO	0.03	AL	SO	SU	Category 1	Category I	20	0	0	0	20	0	0	0	216.2	
AP	0.03	AL	SU		Category 1	Category I	16	4	0	0	16	0	4	0	393.3	
AQ	0.1	RC	AL	SU	Category 1	Category I	20	0	0	0	20	0	0	0	84.9	47.8
AR	0.1	RC	AL	SU	Category 1	Category I	20	0	0	0	20	0	0	0	116.1	72.1
AS	0.1	RC	AL	SU	Category 1	Category I	20	0	0	0	19	1	0	0	79.8	36.6
AT	0.1	RC	AL		Category 1	Category I	20	0	0	0	19	1	0	0	85.6	49.8
AU	0.1	RC	AL		Category 1	Category I	20	0	0	0	20	0	0	0	122.2	64.5
AV	0.1	RC	AL		Category 1	Category I	1	0	0	0	1	0	0	0	191.8	68.8
AW	0.1	RC	AL		Category 1	Category I	19	1	0	0	19	1	0	0	43.1	29.6
AX	0.03	SO	AL		Category 1	Category I	19	1	0	0	16	3	1	0	157.3	
AY	0.1	RC	AL		Category 1	Category I	1	0	0	0	1	0	0	0	194.3	79.7
BB	0.1	SO			SCNM	Category IV	0	0	0	0	0	0	0	20	2	0
BD	0.1	SO	SU	AL	Non-irritant	Category III	0	0	0	20	0	0	20	0	18.3	2.6
BE	0.1	AC	SU		Non-irritant	Category III	9	0	0	11	0	0	16	4	15	
BF	0.1	SO	AC	SU	Category 2A	Category III	0	1	0	0	0	0	1	0	63.5	30.4
BJ	0.1	AL	SU		Non-irritant	Category III	0	0	10	10	0	0	20	0	78.3	
BK	0.1	SO			Non-irritant	Category III	0	0	0	1	0	0	1	0	6.7	2.6
BL	0.1	SO			Non-irritant	Category IV	0	0	0	20	0	0	0	20	6	7.7
BM	0.1	SO			Non-irritant	Category IV	0	0	0	20	0	0	0	20	25.4	11.6
BN	0.1	SU			Non-irritant	Category IV	0	0	0	1	0	0	0	1	13.5	
BP	0.1	SO			Non-irritant	Category IV	0	0	0	1	0	0	0	1	19.1	3.9
BQ	0.1	SO			Non-irritant	Category IV	0	0	0	1	0	0	0	1	33.6	16.8
BR	0.01*	SU	SO		Non-irritant	Category IV	0	0	0	20	0	0	0	20	23.2	4.5
BS	0.01*	RC	AL		Category 2A	Category III	0	1	0	0	0	0	1	0	273.6	121.3
CG	aerosol	AL	SU		Non-irritant	Category IV	0	0	0	1	0	0	0	1	3.9	3.5
CH	0.1	SO			Non-irritant	Category III	0	0	0	1	0	0	1	0	17.4	
EF		RC			Category 2A	Category II	0	20	0	0	0	10	10	0	104.8	54.5
EG	0.1	AC			Category 2A	Category II	0	1	0	0	0	1	0	0	71.8	57.5
BJ	0.1	AL	SU		Non-irritant	Category III	0	0	10	10	0	0	20	0	54.6	8.2

2963
2964   * = Materials tested in the LVET assay

2965   **Table 5-11 Distribution of materials conducted in the BCOP assay.**

Product Categories	Number of products tested
Surfactants	18
Acids	7
Alkaline	14
Oxidizers	16
Solvent	12
Other	1
Total	**68**

2966

2967 ## 5.4 Use of coded chemicals and compliance with GLP Guidelines

2968

2969 ### 5.4.1 Company-submitted anti-microbial cleaning product *in vitro* data

2970

2971   Because some of the *in vitro* data were submitted to IIVS in spreadsheets, it
2972 was impossible to determine which data were generated under GLP compliance and
2973 which were not. However, all of the BCOP data (Section 6.3.2.2.1) generated after
2974 the original submissions were conducted with full GLP compliance.

2975

2976   Essentially all of the company-submitted *in vitro* data generated for anti-
2977 microbial cleaning products and similar formulations were generated using coded
2978 chemicals.

2979

2980 ### 5.4.2 Data obtained from secondary sources

2981

2982   Both *in vitro* and *in vivo* data obtained from publications or internal records for
2983 the CTFA Phase III study (Gettings, Lordo et al. 1996) were generated with full GLP
2984 compliance. Coded test materials were used for both the *in vitro* and *in vivo* portion
2985 of this study.

2986

2987   *In vitro* data from the COLIPA study (Brantom, Bruner et al. 1997) were
2988 generated with full GLP compliance, but some of the *in vivo* data were obtained
2989 from historical sources so it could not be determined whether or not all of these tests
2990 were done with GLP compliance. The data for formulations conducted in the
2991 COLIPA study were generated with coded test materials, but it could not be
2992 determined if all of the substances were tested as coded materials.

2993

6 Test Method Predictive Capacity

2994

2995   Prediction models for each of the three *in vitro* assays were constructed
2996 using the same approach (a graphical one). For each model all the paired *in vitro*
2997 and *in vivo* data provided were used, and the *in vitro* data were plotted against the *in*
2998 *vivo*-defined toxicity category (both EPA and GHS). In some cases only LVET data
2999 was available and in other cases only Draize data. Generally each type of data was
3000 analyzed separately, although we generally concluded that the prediction models
3001 were the same regardless of the *in vivo* assay used.

3002

3003   Once the data were graphed, cut-off lines were fitted by eye to provide the
3004 "best" predictions. A description of these cut-offs then became the prediction model.
3005 Our strategy in setting the cut-offs was to minimize under predictions of toxicity at
3006 the expense of over predictions. Of course, over and under predictions are
3007 somewhat arbitrary terms since we have shown earlier in this BRD (Section 4.8.1)
3008 that repeated three-rabbit eye irritation tests do not necessarily provide identical
3009 toxicity classifications. In other words, a second rabbit test may over or under
3010 predict the first test.

3011

3012   Although data from the testing of anti-microbial cleaning products (and
3013 related cleaning products) were primarily used to set the cut-offs, additional data
3014 from chemically related formulations and some pure substances (e.g. surfactants)
3015 were used to provide supporting information for our decisions.

6.1 Cytosensor predictive capacity

3016

3017

6.1.1 Using the LVET assay to define a prediction model for the CM

3018

3019

EPA Labeling Categories

3020

3021

3022   The distribution of product categories originally submitted with both animal
3023 eye irritation data (LVET) and Cytosensor *in vitro* data is shown in Table 6-1. It can
3024 be seen that there were significantly more surfactants than any other product
3025 category tested with the Cytosensor. No oxidizing formulations were tested using
3026 the Cytosensor.

3027

3028   **Table 6-1 Distribution of product categories originally submitted with both**
3029   **animal eye irritation data and Cytosensor *in vitro* data.**

Product Categories	Number of products tested
Oxidizers	0
Surfactants	82
Acids	1
Bases	4
Solvents	18
Total	105

3030

3031   As the first step towards determining a prediction model for CM data, we
3032   created a scatter plot showing the MRD_{50} for each material plotted against the EPA
3033   labeling category that had been determined by an LVET assay. Figure 6-1 shows
3034   the distribution of MRD_{50} values for all of the 105 antimicrobial cleaning products for
3035   which paired animal data and CM data were available). We then wished to
3036   determine if the results were distributed in such a way that the products with
3037   different EPA labeling categories could be easily separated. It is immediately
3038   apparent from Figure 6-1 that the distribution of MRD_{50} scores across the EPA
3039   labeling categories is not random. EPA Category I and II materials all have MRD_{50}'s
3040   <1 mg/mL., and only Category IV materials have MRD_{50} values >80 mg/mL. <u>This
3041   distribution allowed us to set cut-off values by eye for predicting EPA labeling
3042   categories.</u> We attempted to choose cut-offs conservatively with a bias towards
3043   having as few under predictions as was reasonable. No statistical methods were
3044   employed to construct the proposed prediction model.

3045

3046   Beginning with predictions of the most severe labeling categories, we found
3047   that it was not possible to envision a cut-off value that would distinguish Category I
3048   materials from Category II materials because of the significant overlap of their
3049 MRD_{50} values. Thus we chose to identify all materials in both of the highest toxicity
3050   categories with a conservatively set cut-off value of 2.0 mg/mL. A materials whose
3051 MRD_{50} value is <2.0 mg/mL will be labeled as an EPA I. MRD_{50} values of all
3052   Category I & II materials in this dataset fall below this cut-off. <u>Thus all materials with
3053 MRD_{50} values below 2.0 mg/mL must be given the most severe designation –
3054   Category I.</u>

3055

3056   Similarly, MRD_{50} values for EPA Category III and IV materials have
3057   significant overlap, although at least three of the Category IV materials have MRD_{50}
3058   scores ≥ 80 mg/mL. Thus it is possible to suggest an upper cut-off limit of MRD_{50} >
3059   80 mg/mL to separate some EPA Category IV materials from Category III materials.
3060   Materials whose MRD_{50} values are ≥2 mg/mL and <80 mg/mL are defined as being
3061   EPA Category III. <u>No animal-defined Category I or II materials are underpredicted
3062   by this proposed prediction model</u>. However, since many Category III materials and
3063   a few of the Category IV materials fall below the 2.0 mg/mL proposed cut-off for
3064   Category I materials, many EPA Category III and a few EPA Category IV materials
3065   will be over predicted, and hence over labeled. This outcome has been accepted by
3066   the manufacturers who have co-authored this BRD.

3067

3068   Figure 6-1 shows a plot of MRD_{50} values versus EPA category assignments
3069   (by LVET) with the above-proposed cut-off values added. Included in Figure 6-1 are
3070   three materials for which two sets of animal results were available. Data from both
3071   LVET trials have been included to underscore the variability of the animal test and
3072   indicate that no *in vitro* test can be expected to predict a given animal score any
3073   better than a second animal test itself might be expected to do. It can be seen that
3074   for Material 1022, for example, the results of the two animal tests differed by two full
3075   classifications (an EPA I versus an EPA III)! The two other materials each differed

3076 by a single category. Formulation 1056 was categorized as an EPA I in one trial
3077 and an EPA II in the second; formulation 1079 was categorized as an EPA III in one
3078 trial and an EPA IV in the second. A fourth material also had two sets of animal
3079 data reported, but this material is not specifically indicated since both of the animal
3080 tests predicted the same EPA category.
3081
3082
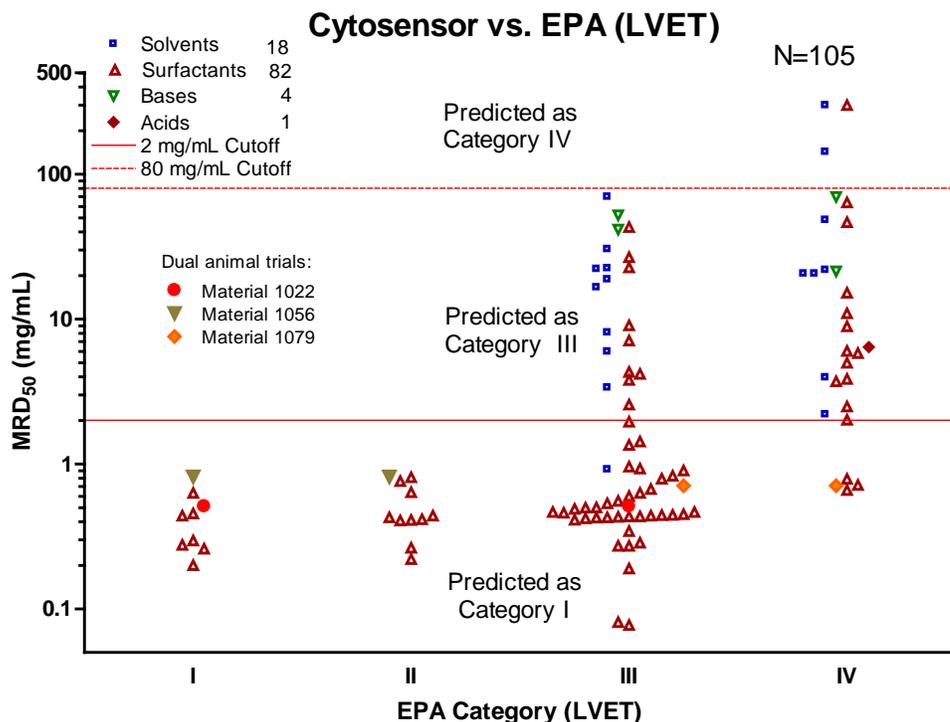
3083
3084 Figure 6-1 Cytosensor MRD$_{50}$ values plotted against EPA toxicity categories determined by
3085 the LVET. Suggested cut-off values with their predicted EPA categories are included. There
3086 are 105 unique materials; however, 3 materials are graphed with 2 different EPA categories
3087 since they were tested twice in the animal trials with different results each time.
3088

3089   The following contingency table (Table 6-2) gives an analysis of the
3090 performance based on the cut-offs shown in Figure 6-1. The data in this table
3091 indicate that the proposed cut-offs make this a very conservative model for the
3092 prediction of materials whose EPA toxicity category is greater than III. One hundred
3093 percent of the animal test determined EPA Categories I and II were captured by this
3094 model. There were no underpredictions of Category I or II materials. In addition
3095 there were no underpredictions of Category III materials; all were predicted as
3096 Category III or higher. The discordant results for the CM assay and EPA toxicity
3097 categories are shown in Table 6-3. There were no underpredictions of the EPA
3098 category for any material; however, 39% of solvents and 78% of surfactants were
3099 overpredicted.
3100

3101   What occurs as a consequence of the conservative cut-offs is that many
3102 materials are overpredicted relative to their toxicity category as determined by the

3103   animal test. All of the Category II materials are overpredicted as Category I's, and
3104   67% of the Category III materials are overpredicted as Category I's. Since the CM
3105   can't distinguish between Category I and Category II, Category I is assumed as the
3106   worst case for materials with MRD_{50}'s < 2 mg/ml. Eighty-nine percent of the
3107   Category IV materials are overpredicted as Category III (75%) or I (14%) materials.
3108
3109   **Table 6-2 Contingency table depicting the accuracy and predictivity of the CM assay for EPA**
3110   **toxicity categories (determined by positive responses in the LVET) using cut-off values of**
3111   **MRD_{50} > 80 mg/mL = IV, 80 mg/mL >MRD_{50} > 2 mg/mL = III, and MRD_{50} < 2 mg/mL = I. The**
3112   **model does not propose to differentiate between EPA Category I and II materials. The total**
3113   **number of materials is listed as 108 since the three materials with differing repeat animal**
3114   **scores were each scored twice.**
3115

LVET- Determined EPA Category	CM Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	9	0	0	9	100%	NA	0%
II	11	0	0	11	0%	100%	0%
III	40	20	0	60	33%	67%	0%
IV	4	21	3	28	11%	89%	NA
Total	64	41	3	108	30%		
Predictivity	14%	49%	100%				
Category under predicted	NA	0%	0%				
Category over predicted	86%	51%	NA				

3116

3117   The practical advantage of such a model is that the very low irritating
3118   materials (Category III's and IV's) can be easily identified and an appropriate toxicity
3119   category applied. This will clearly result in some over labeling (75% of animal-
3120   determined IV's will be over labeled as III's), but the participating companies have
3121   accepted that this degree of over labeling will occur. The EPA appears to concur
3122   with this type of approach since the EPA label Review Manual (2003) states (for
3123   primary eye irritation of Category IV) that "…the registrant may choose to use
3124   Category III labeling."
3125

3126   An additional analysis was conducted to compare the performance of the prediction
3127   model with each of the different product formulation types. Table 6-3 presents the
3128   under and overpredictions associated with each product type. It can be seen that
3129   none of the product types was underpredicted. The surfactants had the highest over
3130   prediction rate (78%), however the sample size for the other product classes,
3131   especially the acids and bases, was probably too low to make a meaningful
3132   comparison.
3133
3134
3135
3136
3137

3138 **Table 6-3 Prediction results for the CM assay and EPA toxicity categories by product**
3139 **formulation type. Number of each product tested and percentage (in parentheses).**
3140

	Solvents	Surfactants	Bases	Acids
Under predicted	0	0	0	0
Correctly Predicted	11 (61%)	19 (22%)	2 (50%)	0
Over Predicted	7 (39%)	66 (78%)	2 (50%)	1 (100%)

3141
3142
3143 GHS Labeling Categories
3144
3145   A similar exercise to that shown for developing an EPA category prediction
3146 model was conducted using GHS toxicity categories. Figure 6-2 shows the CM
3147 MRD_{50}'s plotted against LVET-determined GHS categories. It can be seen that a
3148 much different pattern results with a greater number of formulations classified as
3149 non irritating in the GHS system as compared to the number that fall into the EPA
3150 non irritating category of IV's. As a result, the cut-off between NI materials and the
3151 2B and higher categories was lowered to 10 mg/ml. The next lower cut-off to identify
3152 strongly irritating (GHS 1) materials could be set conservatively at 2 mg/ml, the
3153 same as was done for the EPA classification. Because of the overlap of MRD_{50}
3154 values for category 1 and 2A materials, no cut-off is proposed to separate these two
3155 groups. Thus materials with MRD_{50}'s <2.0 mg/ml will be categorized as 1's, those
3156 with MRD_{50}'s ≥ 2.0 and <10 mg/mL will be categorized as 2B's, and those materials
3157 with MRD50's ≥ 10 mg/mL will be categorized as 2A's.
3158
3159   Again materials with two sets of animal data are also indicated on the graph.
3160 Three of four replicated materials had differing GHS categories depending on the
3161 animal study used. Each of the three differed by one category between the two
3162 trials.
3163
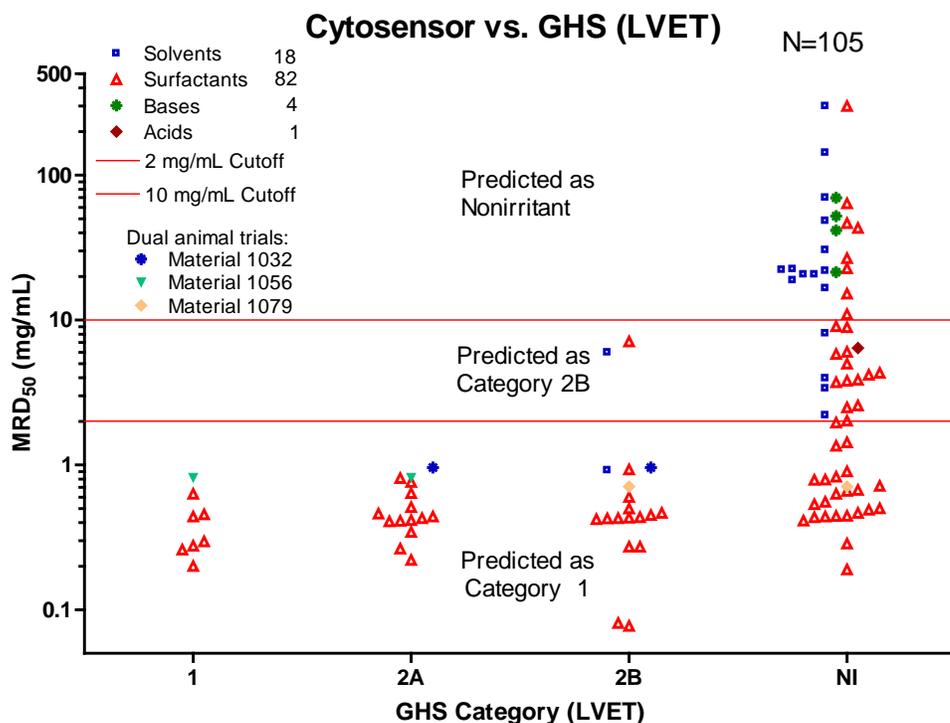
Figure 6-2 Cytosensor MRD$_{50}$ values plotted against GHS toxicity categories determined by the LVET. All materials except oxidizing formulations are graphed. Suggested cut-off values with their predicted GHS categories are included. There are 105 unique materials; however, 3 materials have 2 GHS categories each since they were tested twice in the animal trials.

The following contingency table (Table 6-4) gives an analysis of the performance based on the cut-offs shown in Figure 6-2. The data in this table indicate that the proposed cut-offs make this a very conservative model for the prediction of materials whose GHS toxicity category is greater than 2B. One hundred percent of the animal test-determined GHS Categories 1 and 2A were captured by this model. There were no underpredictions of Category 1 or 2A materials. In addition there were no underpredictions of Category 2B materials; all were predicted as Category 2B or higher. The discordant results for the CM assay and GHS toxicity categories are shown in Table 6-5. There were no underpredictions of the GHS category for any material; however, 28% of solvents and 80% of surfactants were overpredicted.

What occurs as a consequence of the conservative cut-offs is that many materials are overpredicted relative to their toxicity category as determined by the animal test. All of the Category 2A materials are overpredicted as Category 1's, and 89% of the Category 2B materials are overpredicted as Category 1's. Since the CM can't distinguish between Category 1 and Category 2A, Category 1 is assumed as the worst case for materials with MRD$_{50}$'s < 2 mg/ml. Sixty-four percent of the Nonirritant materials are overpredicted as Category 2B (27%) or 1 (36%) materials.

3191 **Table 6-4 Contingency table depicting the accuracy and predictivity of the CM assay for GHS**
3192 **toxicity categories (determined by positive responses in the LVET) using cut-off values of**
3193 **$MRD_{50} \geq 10$ mg/mL = NI, 10 mg/mL >$MRD_{50} \geq 2$ mg/mL = 2B, and $MRD_{50} < 2$ mg/mL = I. The**
3194 **model does not propose to identify GHS Category 2A materials. The total number of materials**
3195 **is listed as 108 since the three materials with differing repeat animal scores were each scored**
3196 **twice.**
3197

LVET- Determined GHS Category	CM Predicted GHS Category				Concordance	Toxicity over predicted	Toxicity under predicted
	1	2B	NI	Total			
1	8	0	0	8	100%	NA	0%
2A	15	0	0	15	0%	100%	0%
2B	17	2	0	19	11%	89%	0%
NI	24	18	24	66	36%	64%	NA
Total	64	20	24	108	31%		
Predictivity	13%	10%	100%				
Category under predicted	NA	0%	0%				
Category over predicted	88%	90%	NA				

3198
3199
3200 Over and under predictions by formulation type
3201
3202   An additional analysis was conducted to compare the performance of the
3203 prediction model with each of the different product formulation types. Table 6-5
3204 presents the under and overpredictions associated with each product type. It can be
3205 seen that none of the product types was underpredicted. The surfactants had the
3206 highest over prediction rate (80%), however the sample size for the other product
3207 classes, especially the acids and bases, was probably too low to make a meaningful
3208 comparison.
3209
3210 **Table 6-5 Number of discordant results (and percentages) for the CM assay and GHS toxicity**
3211 **categories.**
3212

	Solvents	Surfactants	Bases	Acids
Under predicted	0	0	0	0
Correctly Predicted	13 (72%)	17 (20%)	4 (100%)	0
Over Predicted	5 (28%)	68 (80%)	0	1 (100%)

3213

3214 6.1.1.1 Secondary analysis of acidic and alkaline materials
3215
3216   The first pass analysis described above utilized all of the submitted materials
3217 (with the exception of oxidizing formulations) for which adequate animal data were
3218 available to determine an EPA or GHS category. However, there has always been
3219 some concern that the CM should not be used for acidic or alkaline materials (pH ≤
3220 4.0 or ≥10.0). Therefore, we conducted a second analysis in which materials fitting

3221 the above acid or alkaline definitions (or for which one of the 3 product activity
3222 categories was described as acid or alkaline) were omitted from the database.
3223
3224   Table 6-6 describes the seventeen materials identified as fitting the
3225 description as acid or alkaline. It can be seen that all of the materials were EPA
3226 Category III or IV materials and that none of the materials were underpredicted by
3227 the CM assay as might be hypothesized from the operation of the CM (cells
3228 exposed to an increasing dilution series of the test material which might quickly
3229 change the pH).
3230
3231 **Table 6-6 Distribution of EPA categories for the 17 materials from the CM database classified**
3232 **as acid or alkaline.**
3233

LVET-defined EPA Category	CM-defined EPA Category		
	I	III	IV
III	1	7	0
IV	0	9	0

3234
3235   Figure 6-3 shows the distribution of MRD_{50} values for the non-acidic, non-
3236 alkaline materials plotted against EPA labeling categories (determined by the
3237 LVET). Even with the seventeen acidic/alkaline materials removed, there is not a
3238 significant change in the distribution among EPA determined categories. The same
3239 cut-off values as determined for Figure 6-1 were used.
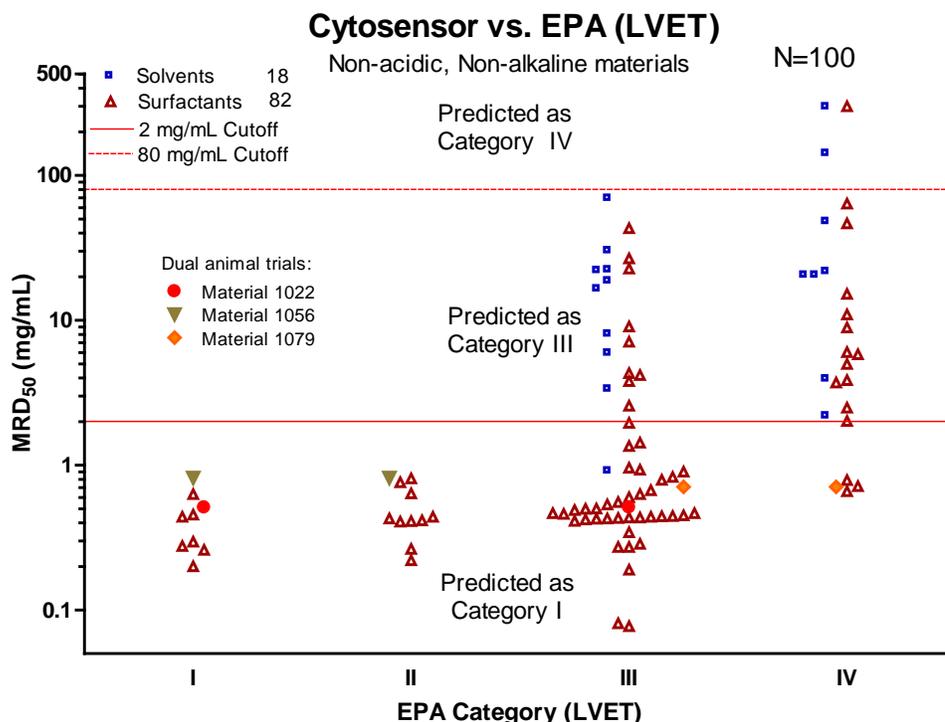
3240
3241 **Figure 6-3 Cytosensor MRD_{50} values plotted against EPA toxicity categories determined by**
3242 **the LVET. Only non-acidic, non-alkaline materials are graphed. Suggested cut-off values with**
3243 **their predicted EPA categories are included. There are 100 unique materials; however, 3**
3244 **materials have 2 values since they were tested twice in the animal trials.**

3245
3246   As expected from the results shown in Table 6-7, the performance of the CM
3247 assay is very similar when the acid and alkaline materials are removed (Table 6-7
3248 versus Table 6-2). There are still no underpredictions and while positive predictive
3249 values increase somewhat, the concordance decreases somewhat (due to the
3250 removal of 2 Category III materials which were correctly predicted by the CM). <u>Thus</u>
3251 <u>we do not feel that acid or alkaline materials need to be excluded from analysis by</u>
3252 <u>the Cytosensor and propose to keep them in the applicability domain for the CM</u>
3253 <u>assay</u>. The discordant results for the CM assay and EPA toxicity categories without
3254 acid/alkaline materials are shown in Table 6-8. There were no underpredictions of
3255 the EPA category for any material; however, 39% of solvents and 78% of
3256 surfactants were still overpredicted.
3257
3258 **Table 6-7 Contingency table depicting the accuracy and predictivity of the CM assay for EPA**
3259 **toxicity categories (determined by positive responses in the LVET) of <u>non-acidic</u>, <u>non-alkaline</u>**
3260 **materials using cut-off values of $MRD_{50} \geq 80$ mg/mL = IV, 80 mg/mL >$MRD_{50} \geq 2$ mg/mL = III,**
3261 **and $MRD_{50} < 2$ mg/mL = I. The model does not propose to identify EPA Category II materials.**
3262

LVET- Determined EPA Category	CM Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	9	0	0	9	100%	NA	0%
II	11	0	0	11	0%	100%	0%
III	40	18	0	58	31%	69%	0%
IV	4	18	3	25	12%	88%	NA
Total	64	36	3	103	29.1%		
Predictivity	14.1%	50%	100%				
Category under predicted	NA	0%	0%				
Category over predicted	85.9%	50%	NA				

3263
3264   A similar exercise was conducted using GHS toxicity categories. Figure 6-4
3265 shows the CM MRD_{50}'s plotted against LVET-determined GHS categories with the
3266 seventeen acidic/alkaline materials removed. Even with the seventeen
3267 acidic/alkaline materials removed, there is not a significant change in the distribution
3268 among GHS determined categories. The same cut-off values as determined for
3269 Figure 6-2 were used.
3270
3271
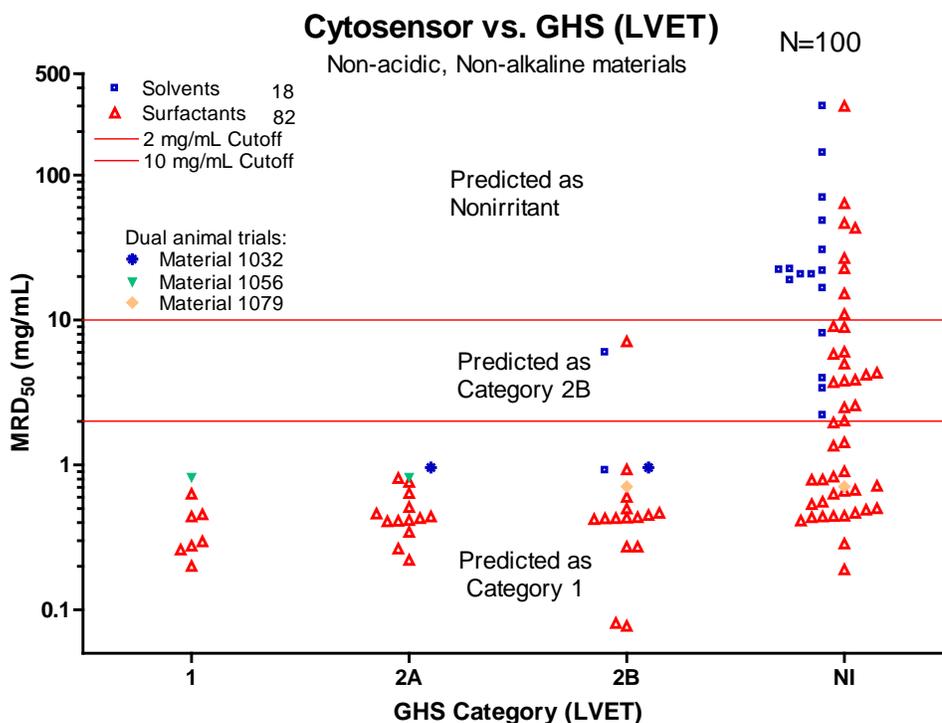
Figure 6-4 Cytosensor MRD_{50} values plotted against GHS toxicity categories determined by the LVET. Only non-acidic, non-alkaline materials are graphed. Suggested cut-off values with their predicted GHS categories are included. There are 100 unique materials; however, 3 materials have 2 values since they were tested twice in the animal trials.

As expected from the results shown in Table 6-9, the performance of the CM assay is very similar when the acid and alkaline materials are removed (Table 6-9 versus Table 6-4). There are still no underpredictions and while positive predictive value increase somewhat, the concordance decreases somewhat (due to the removal of 4 Nonirritant materials which were correctly predicted by the CM). Thus we do not feel that acid or alkaline materials need to be excluded from analysis by the Cytosensor and propose to keep them in the applicability domain for the CM assay.

3288 **Table 6-8 Contingency table depicting the accuracy and predictivity of the CM assay for GHS**
3289 **toxicity cate8gories (determined by positive responses in the LVET) using cut-off values of**
3290 **MRD$_{50}$ ≥ 10 mg/mL = NI, 10 mg/mL >MRD$_{50}$ ≥ 2 mg/mL = 2B, and MRD$_{50}$ < 2 mg/mL = I. The**
3291 **model does not propose to identify GHS Category 2A materials.**
3292

LVET- Determined GHS Category	CM Predicted GHS Category				Concordance	Toxicity over predicted	Toxicity under predicted
	1	2B	NI	Total			
1	8	0	0	8	100%	NA	0%
2A	15	0	0	15	0%	100%	0%
2B	17	2	0	19	11%	89%	0%
NI	24	17	20	61	33%	67%	NA
Total	64	19	20	103	29%		
Predictivity	13%	11%	100%				
Category under predicted	NA	0%	0%				
Category over predicted	87%	89%	NA				

3293

3294 6.1.2 Using the Draize assay to define a prediction model for the CM

3295

3296   Since the above analyses were conducted with EPA or GHS categories
3297 determined by the LVET, we next evaluated whether similar prediction models
3298 would have been developed if the traditional Draize test were used to obtain EPA
3299 classifications. It is known that the LVET gives somewhat lower MAS scores than
3300 does the Draize test, but the LVET is still more sensitive – and thus overpredictive –
3301 of the human response (see discussion in Section 4.7). We found two studies which
3302 used materials (surfactants and surfactant-containing formulations) similar to those
3303 which are the focus of this BRD (anti-microbial cleaning products). One of the two
3304 studies - the CTFA Phase III study - is important because it uses both LVET and
3305 Draize evaluation of surfactant-containing products; hence the results using the two
3306 methods can be directly compared for an identical set of formulations (see Table 5-
3307 4). The second study – the COLIPA study - used only the Draize test for
3308 characterization, but it contained some formulations similar to those which are being
3309 used in this BRD.

3310

3311 6.1.2.1 CTFA Phase III Evaluation

3312

3313   Previous analysis (Cytosensor BRD prepared for ECVAM) of the CTFA
3314 Phase III study indicated that the lower cut-off value to identify EPA Category I
3315 materials should be set at 2 mg/mL, identical to that which we have proposed in the
3316 preceding analysis of the data submitted specifically for this BRD. There are some
3317 differences in the chemical makeup of the two data sets, but they do overlap
3318 considerably in the type of chemical formulation, both data sets being highly biased

3319   towards surfactant-based formulations. The distribution of product categories
3320   submitted with CTFA Phase II *in vitro* and Cytosensor data is shown in Table 6-11.
3321
3322   **Table 6-9 Distribution of product categories originally**
3323   **submitted with both animal eye irritation data and CTFA**
3324   **Phase III *in vitro* data.**

Product Categories	Number of products tested
Oxidizers	0
Surfactants	25
Acids	0
Bases	0
Solvents	0
Total	25

3325
3326   Further analysis of this study brings up the importance of being aware of the
3327   variability of the animal test in making EPA toxicity category decisions. Figure 6-5
3328   shows that there are 2 Category I materials (identified as Facial Cleaning Foam and
3329   Gel Cleanser) which would be identified as being underpredicted (relative to the
3330   Draize classifications) by the CM assay (they both have MRD_{50} values >2 mg/mL).
3331   In order to begin to understand these apparent underpredictions, the individual
3332   animal scores for both six-rabbit tests were examined. Since the EPA currently
3333   accepts the results from three rabbit tests, we parsed the 6- rabbit test data into 20
3334   unique, but equally likely, subgroups of three rabbit results. EPA grading criteria
3335   were then applied to each of the three-rabbit subgroups and an EPA toxicity
3336   Category determined (see Table 4-16). For the Gel Cleanser, even though the six-
3337   rabbit calculation gave a Category I result, only ten (of twenty) three-rabbit
3338   subgroups received a score of Category I; <u>the other ten received a score of</u>
3339   <u>Category III</u>. The same results were found for the Facial Cleaning Foam; ten three-
3340   rabbit subgroups received a score of Category I, and the other ten received a score
3341   of Category III. Thus if the test were performed repeatedly on the two materials
3342   using today's three-rabbit test standard, 50% of the time the materials would be
3343   graded as Category III and 50% of the time they would be graded as Category I - a
3344   difference of 2 toxicity classification grades! Thus it is extremely hard to say that the
3345   CM truly underpredicts the irritation potential of these two materials.
3346
3347   An additional insight from the CTFA Phase III study is the apparent over
3348   classification of the surfactant-based personal care products relative to their
3349   intended use (often on the face and around the eyes). A large number of these
3350   commonly used personal care products fall into EPA Category I (10 out of 25) when
3351   they are tested using the Draize test; however, they are categorized somewhat
3352   lower, and possibly more realistically when using the LVET.
3353
3354   It appears from an examination of the Figure 6-5 and Tables 6-12 & 6-14 that
3355   a decision on where to place the cut-off values would be very similar whether the
3356   LVET or the Draize data were used as the basis.
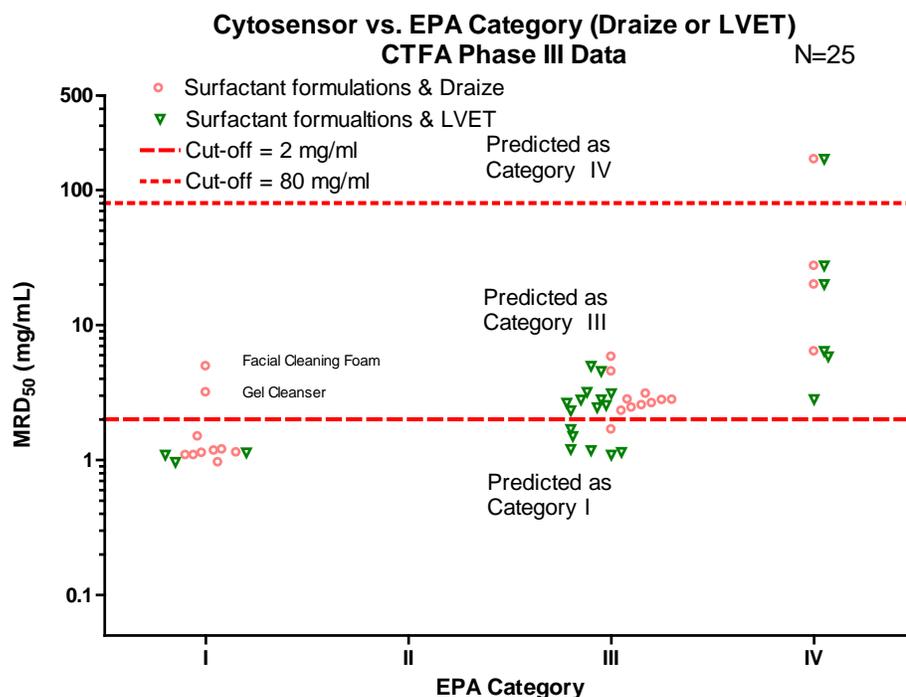
3357
3358 **Figure 6-5 Plot of CM data versus both LVET- and Draize-defined EPA Categories for the 25**
3359 **surfactant-based personal care products tested in the CTFA Phase III (Gettings, Lordo et al.**
3360 **1996) evaluation using cut-off values of $MRD_{50} \geq 80$ mg/mL = IV, 80 mg/mL $>MRD_{50} \geq 2$ mg/mL**
3361 **= III, and $MRD_{50} < 2$ mg/mL = I. The model does not propose to identify EPA Category II**
3362 **materials.**
3363

3364   The following contingency tables (Table 6-12 & 6-14) give an analysis of the
3365 performance based on the cut-offs shown in Figure 6-5 for the LVET-determined
3366 EPA category or the Draize-determined EPA category, respectively. One hundred
3367 percent of the LVET-determined EPA Category I materials were captured by this
3368 model; however, 20% of the Draize-determined EPA Category I materials were
3369 underpredicted by the CM. In contrast, 38% of LVET-determined EPA Category III
3370 materials were overpredicted, whereas, only 9% of Draize-determined EPA
3371 Category III materials were overpredicted. The discordant results for the CM assay
3372 and EPA toxicity categories are shown in Table 6-13 & 6-15. There were no
3373 underpredictions of the LVET-determined EPA category, but 8% of Draize-
3374 determined EPA category was underpredicted. There was a significant amount of
3375 overprediction for both LVET and Draize–determined EPA categories mainly due to
3376 the EPA Category IV materials being overpredicted as Category III.
3377
3378

3379 **Table 6-10 Contingency table presenting the accuracy and predictivity of the CM for EPA**
3380 **toxicity categories (LVET-determined) for the 25 surfactant-based personal care products in**
3381 **the CTFA Phase III study (Gettings, Lordo et al. 1996).**
3382

LVET- Determined EPA Category	LVET Category Predicted by CM				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	3	0	0	3	100%	NA	0%
II	0	0	0	0	0%	0%	0%
III	6	10	0	16	63%	38%	0%
IV	0	5	1	6	17%	83%	NA
Total	9	15	1	25	56%		
Predictivity	33%	67%	100%				
Category under predicted	NA	0%	0%				
Category over predicted	67%	33%	NA				

3383

3384   Table 6-11 shows the overall results based on product category – in this
3385 instance for surfactants only.
3386
3387
3388 **Table 6-11 Discordant results for the CTFA CM study**
3389 **and EPA toxicity categories (LVET-determined).**

	Surfactants
Under predicted	0
Correctly Predicted	14
Over Predicted	11

3390
3391
3392 **Table 6-12 Contingency table presenting the accuracy and predictivity of the CM for EPA**
3393 **toxicity categories(Draize-determined) for the 25 surfactant-based personal care products in**
3394 **the CTFA Phase III study (Gettings, Lordo et al. 1996).**
3395

Draize- Determined EPA Category	Draize Category Predicted by CM				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	8	2	0	10	80%	NA	20%
II	0	0	0	0	0%	0%	0%
III	1	10	0	11	91%	9%	0%
IV	0	3	1	4	25%	75%	NA
Total	9	15	1	25	76%		
Predictivity	89%	67%	100%				
Category under predicted	NA	13%	0%				
Category over predicted	11%	20%	NA				

3396
3397

3398   Table 6-13 shows the overall results based on product category – in this
3399   instance for surfactants only.

3400
3401   **Table 6-13 Discordant results for the CTFA CM study**
3402   **and EPA toxicity categories (Draize-determined).**

	Surfactants
Under predicted	2 (8%)
Correctly Predicted	19 (76%)
Over Predicted	4 (16%)

3403
3404

3405   6.1.2.2 COLIPA Evaluation

3406
3407   The distribution of product categories for the COLIPA *in vitro* and Cytosensor
3408   data is shown in Table 6-14.   The COLIPA evaluation was for surfactant and
3409   surfactant-containing materials only.

3410
3411   **Table 6-14 Distribution of product categories originally submitted**
3412   **with both animal eye irritation data and COLIPA *in vitro* data.**
3413

Product Categories	Number of products tested
Oxidizers	0
Surfactants	19
Acids	0
Bases	0
Solvents	0
Total	19

3414
3415   Figures 6-6 & 6-7 show MRD_{50} scores obtained in the COLIPA evaluation of
3416   *in vitro* assays for eye irritation. The cut-off values for MRD_{50} scores have been
3417   empirically chosen to identify, where possible, the various toxicity categories.   In
3418   attempting to select cut-off values we first tried those that were chosen from the
3419   CTFA Phase III studies (see preceding sections). Since these appeared adequate,
3420   we continued the analysis with these values for the sake of consistency. As with the
3421   CTFA Phase III studies, in the case of the GHS system and the EPA system which
3422   have 4 categories, the overlap of MRD_{50} response was so large that it was deemed
3423   impossible to differentiate between the two middle categories (either EPA II and III
3424   or GHS 2A and 2B) from each other. This analysis was made even more difficult
3425   because of the distribution of the toxicity classifications. There were only two GHS
3426   Draize determined 2A or 2B materials. Hence only upper (to possible identify non-
3427   irritants) and lower (to possibly identify severe irritants) cut-off values are shown.

3428
3429   For the COLIPA GHS data set (Figure 6-6), it appeared a cut-off value of
3430   >10 mg/mL might be appropriate to identify the GHS nonirritants from the more
3431   irritating materials while a higher cut-off of 80 mg/ml seemed appropriate to use with
3432   the EPA classifications. The cut-off of <2 mg/ml was retained for identifying both
3433   GHS 1 or EPA I materials. However, as seen in most of the previous analyses, there

3434   were very few materials in the GHS 2A or 2B, or EPA II categories. This makes it
3435   difficult to determine exactly where the cut-off between these intermediate irritating
3436   categories and the mild categories lies. Additionally the EPA classification had only
3437   two Category IV materials, again making a decision for a cut-off problematic. Hence
3438   only upper (to possible identify non-irritants) and lower (to possibly identify severe
3439   irritants) cut-off values are shown on the scatter plots. Products falling between
3440   these limits are considered Category III.
3441

3442   When CM MRD_{50}'s were plotted against the EPA categorization scheme
3443   (Figure 6-7), there were two Category I materials (labeled #21 and #36 on the
3444   scatter plot) that appeared to be underpredicted as Category III's. However, material
3445   #21 (1% benzalkonium chloride) has two sets of animal test data reported in the
3446   ECETOC eye irritation report (ECETOC 1992) from which the COLIPA study took its
3447   *in vivo* data. We chose to graph the highest category data, but the EPA category of
3448   the replicate animal test was a Category 3 – the same as was estimated by the
3449   Cytosensor MRD_{50}. When the second underpredicted EPA Category 1 material was
3450   decoded it was found to be a baby shampoo formulation. Thus the two EPA
3451   Category I "underpredictions" may not be as much of a concern as first suspected.
3452
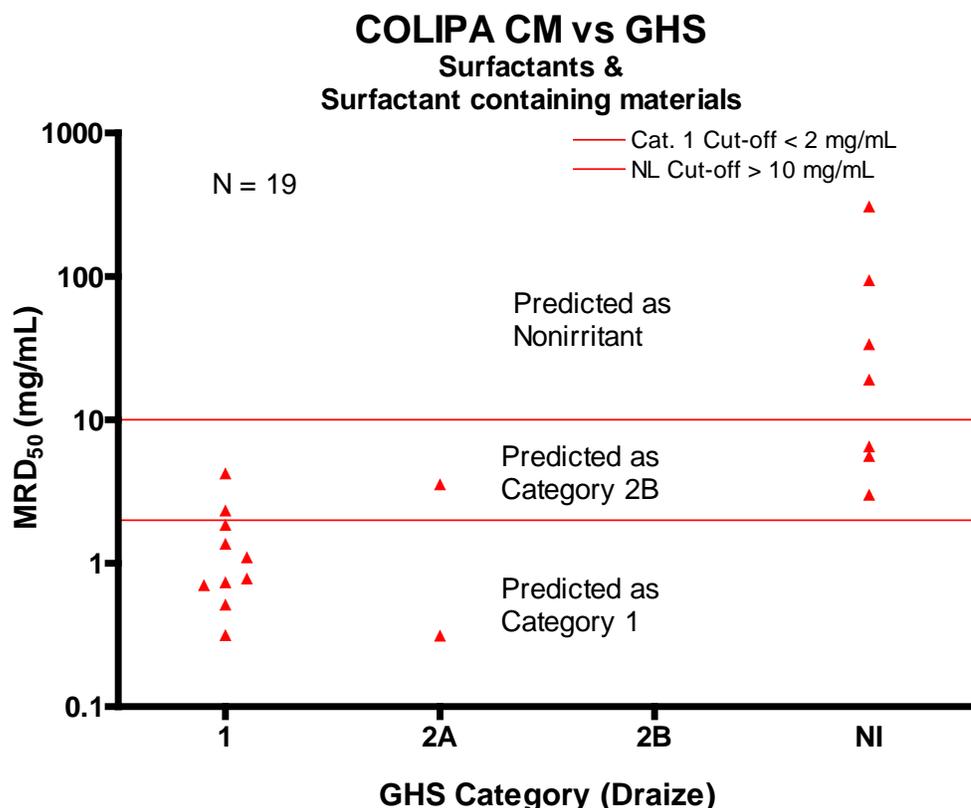
3453
3454 **Figure 6-6 Surfactant and surfactant-containing formulation results of the COLIPA study**
3455 **related to GHS classification. Data points indicate the mean MRD_{50} for both laboratories (with**
3456 **the exception of two data points where only one laboratory made the determination). In some**
3457 **cases data points have been slightly offset along the X-axis in order to clearly separate them**
3458 **from data of similar magnitude.**
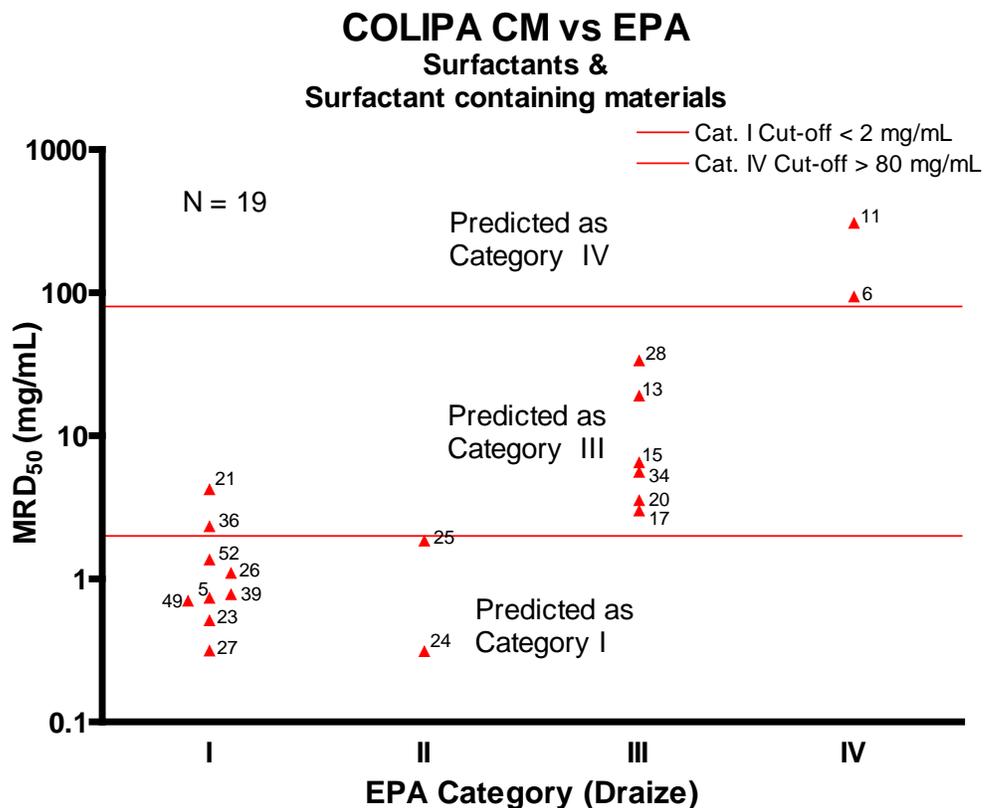
COLIPA CM vs EPA
Surfactants &
Surfactant containing materials

Figure 6-7 Surfactant and surfactant-containing formulation results of the COLIPA study related to EPA classification. Data points indicate the mean MRD_{50} for both laboratories with the exception of 24 and 52 which were done in one laboratory only. In some cases data points have been slightly offset along the X-axis in order to clearly separate them from data of similar magnitude. The individual materials can be identified by comparing the numbers adjacent to the symbols with the numbering code given in Table 5.3.1.3.

Contingency Tables 6-15 & 6-17 give an analysis of the performance based on the cut-offs show in Figures 6-6 & 6-7, respectively. It appears from the graphs that the CM does not have the ability to clearly separate the surfactants or surfactant-containing materials used in the COLIPA study into the four Draize test defined GHS or EPA Categories. However, severe irritants seem to be reasonably predicted when MRD_{50} scores of less than 2 are used. Using this lower cut-off value, there is a high positive predictive value for GHS Category 1 (80%; 8 of 10 materials) and EPA Category I (78%; 7 of 9 materials). There also seems to be good predictivity for EPA Category III materials and possibly for the Category IV materials as well.

Even though the positive predictive value was high using a lower cut-off of MRD_{50} <2 mg/ml, the sensitivity was lower, with several chemicals being underpredicted by at least one toxicity category by the GHS, and EPA classification system. Overpredictions of mild materials (GHS Nonirritant, and EPA IV), did not occur as often. One very important conclusion from both the CTFA Phase III study and the COLIPA study is that the prediction model (cut-off values) determined for

3484   the CM using the traditional Draize assay is identical to the prediction model
3485   determined using the LVET assay.
3486

3487   The discordant results for the CM assay and the GHS & EPA toxicity
3488   categories are shown in Tables 6-16 & 6-18, respectively. The majority of the
3489   materials were correctly predicted with 63% correctly predicted with the GHS
3490   category and 79% correctly predicted with the EPA category. The amount of
3491   underprediction was 16% for the GHS category and 11% for the EPA category.
3492

3493   **Table 6-15 COLIPA surfactant and surfactant containing materials. Contingency table**
3494   **depicting the concordance and predictivity of the CM assay for GHS toxicity classifications**
3495   **when the cut-off values shown in Figure 6-6 are applied.**
3496

Draize Determined GHS Category	GHS Category Predicted by CM				Concordance	Toxicity Overpredicted	Toxicity Underpredicted
	1	2B	NI	Total			
1	8	2	0	10	80%	NA	20%
2A	1	1	0	2	0%	50%	50%
2B	0	0	0	0	0%	0%	0%
NI	0	3	4	7	57.1%	42.9%	NA
Total	9	6	4	19	63.1%		
Predictivity	88.9%	0%	100.0%				
Category Underpredicted	NA	50%	0%				
Category Overpredicted	11.1%	50%	NA				

3497

3498   **Table 6-16 Discordant results for the COLIPA CM**
3499   **study and GHS toxicity categories.**

	Surfactants
Under predicted	3 (16%)
Correctly Predicted	12 (63%)
Over Predicted	4 (21%)

3500

3501   **Table 6-17 COLIPA surfactant and surfactant containing materials - Contingency table**
3502   **depicting the concordance and predictivity of the CM assay for EPA toxicity classifications**
3503   **when the cut-off values shown in Figure 6-7 are applied.**
3504

Draize Determined EPA Category	EPA Category Predicted By CM				Concordance	Toxicity Overpredicted	Toxicity Underpredicted
	I	III	IV	Total			
I	7	2	0	9	77.8%	NA	22.2%
II	2	0	0	2	0%	100%	0%
III	0	6	0	6	100%	0%	0%
IV	0	0	2	2	100%	0%	NA
Total	9	8	2	19	78.9%		
Predictivity	77.8%	75%	100%				
Category Underpredicted	NA	25%	0%				
Category Overpredicted	22.2%	0%	NA				

3505

3506
3507

**Table 6-18 Discordant results for the COLIPA CM
study and EPA toxicity categories.**

	Surfactants
Under predicted	2 (11%)
Correctly Predicted	15 (78%)
Over Predicted	2 (11%)

3508

3509 ## 6.1.3 Cytosensor studies without animal data

3510

3511   Many companies do not currently conduct rabbit eye irritation tests on
3512 cleaning products; hence, many *in vitro* study data were submitted to this BRD
3513 without accompanying animal data. We compared the distribution of these scores
3514 using the same cut-off values for classification that were used in the analysis of the
3515 predictive capacity of the CM for LVET-determined EPA toxicity Categories (see, for
3516 example, Table 6-2). Figure 6-8 shows the distribution of CM scores for the products
3517 without animal data. Using the previous suggested cut-offs ($MRD_{50} \geq 80$ mg/mL =
3518 IV, 80 mg/mL $>MRD_{50} \geq 2$ mg/mL = III, and $MRD_{50} < 2$ mg/mL = I), 1.9% of the
3519 materials would be Category IV's, 24.7% would be Category III's and 73.5% would
3520 be Category I. This compares to the products with paired animal and CM data
3521 analyzed in Table 6-2 where the materials assigned to categories by CM scores
3522 were 2.8% Category IV's, 38% Category III's and 59% Category I's. Thus it appears
3523 that the distribution of CM-measured toxicities for the set of materials used to
3524 determine cut-off values (those which were tested with both the rabbit test and the
3525 CM test) were somewhat less irritating than those which were tested in the CM
3526 alone.
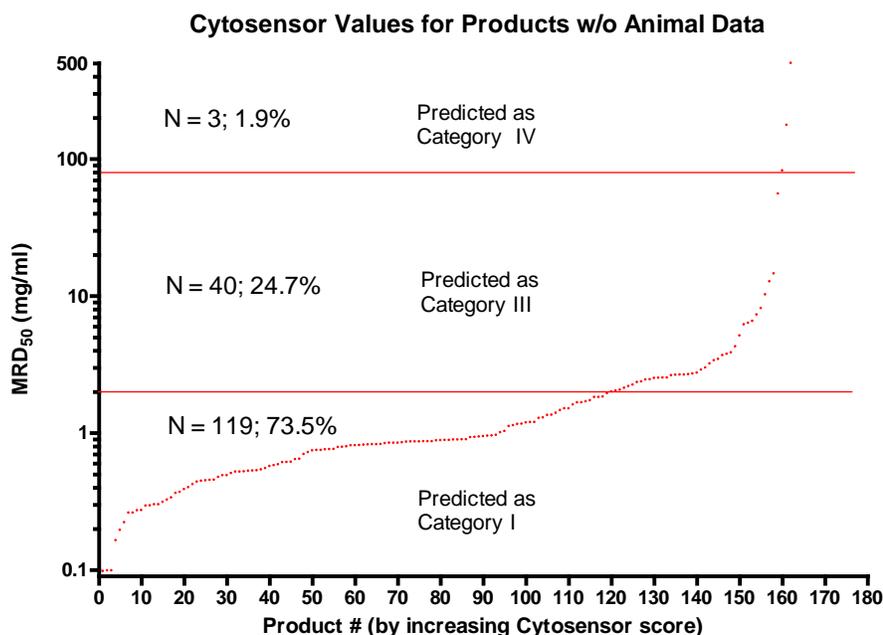
3527
3528 **Figure 6-8 Distribution of CM scores for the products without animal data using cut-offs of
3529 $MRD_{50} \geq 80$ mg/mL = IV, 80 mg/mL $>MRD_{50} \geq 2$ mg/mL = III, and $MRD_{50} < 2$ mg/mL = I.**
3530

3531   6.1.4 Conclusion for the Cytosensor assay

3532

3533   The Cytosensor assay appears to be most useful at the less irritating portion of
3534   the irritation spectrum. It is capable of identifying both Category III and IV materials,
3535   although most Category IV materials will be overpredicted as Category III materials.
3536   <u>None of the 105 materials cleaning products were under predicted for LVET-defined</u>
3537   <u>EPA toxicity categories</u>. Over predictions were much more frequent, but this was
3538   driven by the fact that the CM assay seems incapable of clearly differentiating
3539   between Category I and Category II materials. In fact many Category III materials
3540   (67%) were also over predicted as EPA Category I. The corporate participants have
3541   agreed that this outcome of over labeling some materials is acceptable to them.

3542

3543   Similar results were found with the prediction model for GHS categories, with the
3544   exception that 36% of the GHS Non-irritating materials were clearly identified as
3545   such by the CM. However only 11% of the GHS category 2B materials were
3546   correctly identified; the rest were over predicted as Category 1.

3547

3548   The corporate participants in this program have agreed that the outcome is
3549   acceptable to them. Another assay (we propose BCOP) will be used as a second
3550   tier test to differentiate EPA Category I from EPA Category II (and lower) materials,
3551   if needed.

3552

3553   <u>Again it is important to note that the prediction model for both the GHS and</u>
3554   <u>EPA toxicity categories is the same whether determined by the Draize assay or the</u>
3555   <u>LVET assay.</u>

3556

3557   **Historical knowledge of the performance of the Cytosensor assay plus**
3558   t**he preceding analysis of the Cytosensor data in this BRD have led us to the**
3559   **following recommendations:**

3560

3561   1) **Anti-microbial cleaning products having an oxidizing chemistry**
3562   **should not be tested with the Cytosensor assay.**

3563

3564   2) **Only fully water soluble anti-microbial cleaning products can be**
3565   **tested with the Cytosensor assay.**

3566

3567   3) **If the anti-microbial cleaning product has an MRD$_{50}$ score of <2**
3568   **mg/ml, it is classified as EPA Category I or GHS Category 1.**

3569

3570   4) **If the anti-microbial cleaning product has an MRD$_{50}$ score of ≥2**
3571   **mg/ml, but < 80 mg/ml, it is classified as EPA Category III. If the anti-**
3572   **microbial cleaning product has an MRD$_{50}$ score of ≥2 mg/ml, but <10**
3573   **mg/ml, it is classified as GHS Category 2B.**

3574

3575   5) **If the anti-microbial cleaning product has an MRD$_{50}$ score of ≥80**
3576   **mg/ml, it is classified as EPA Category IV. If the anti-microbial**

3577   **cleaning product has an MRD$_{50}$ score of ≥10 mg/ml, it is classified**
3578   **GHS Category NI.**
3579

3580   6) **(Optional) To determine if an anti-microbial cleaning product which**
3581   **was categorized as either EPA I or GHS 1 is actually an EPA II or a**
3582   **GHS 2A, it should be further tested in the BCOP assay.**

3583

3584

6.2 EpiOcular predictive capacity

6.2.1 Company submissions

EPA Labeling Categories (LVET-determined)

Table 6-21 gives the distribution of product categories originally submitted with both animal eye irritation data (LVET) and EpiOcular *in vitro* data. This distribution is more highly weighted to formulations having oxidizing chemistry than is the total data submitted by participating companies for all of the other *in vitro* tests.

Table 6-19 Distribution of product categories originally submitted with both animal eye irritation data (LVET) and EpiOcular data.

Product Categories	Number of products tested
Oxidizers	9
Surfactants	12
Solvents	4
Total	25

Figure 6-9 shows the full distribution of ET_{50} values for all of the 25 materials for which data were available when plotted against EPA labeling categories (determined by the LVET). EPA categories are not equally represented since only one Category II material and three Category I materials are present. This is not surprising since this method was not intended for identifying more severe irritants. It is immediately apparent from Figure 6-9 that the distribution of ET_{50} scores across the EPA labeling categories is not random. EPA Category I materials have ET_{50}'s <4 min, while most EPA Category III and IV materials have ET_{50}'s > 10 min. This distribution allowed us to set cut-off values by eye for predicting EPA labeling categories. We attempted to choose cut-offs conservatively with a bias towards having as few under predictions as was reasonable. No statistical methods were employed to construct the proposed prediction model. Thus – for this somewhat limited data set - all materials in the highest toxicity category can be identified with a cut-off value of 4 min. However, a number of the Category III and IV materials also fall below this ET_{50} value.

ET_{50} values for EPA Category III and IV materials have significant overlap. Thus it is not possible from this data set to suggest an upper cut-off limit to separate EPA Category IV materials from Category III materials. However, materials having ET_{50} values above ~ 70 min would likely be Category IV materials. The consequence of this is that many EPA Category III and a few EPA Category IV materials would be overpredicted. Figure 6-9 shows a plot of ET_{50} values versus EPA category classification (by LVET) with the above proposed cut-off values added.

3624
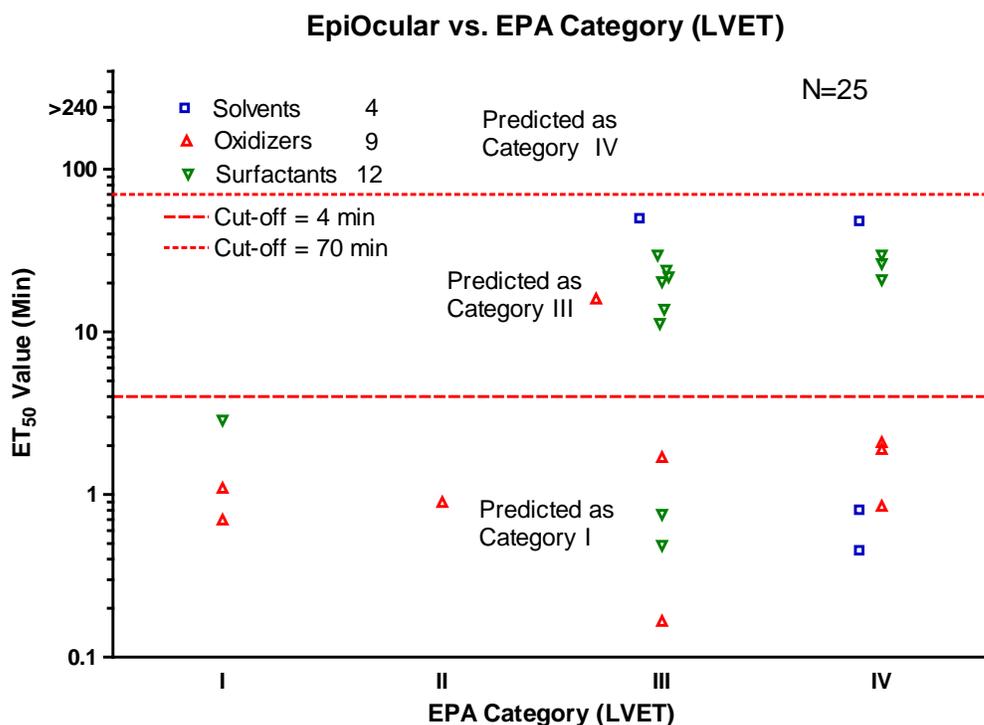
EpiOcular vs. EPA Category (LVET)

3625
3626 **Figure 6-9 EpiOcular ET$_{50}$ values plotted against EPA categories determined by the LVET.**
3627 **Suggested cut-off values with their predicted EPA categories are included.**
3628

3629   The contingency table (Table 6-20) gives an analysis of the performance
3630 based on the cut-offs shown in Figure 6-9. The data in this table indicate that the
3631 proposed cut-offs make this a very conservative model for the prediction of
3632 materials whose EPA toxicity category is greater than III. There was a significant
3633 amount of overprediction for EPA Category IV materials (100%); however, there
3634 were no underpredictions for any of the EPA categories.

3635
3636 **Table 6-20 Contingency table depicting the accuracy and predictivity of the EpiOcular assay**
3637 **for EPA toxicity categories (determined by the LVET) using cut-off values of ET$_{50}$ ≥ 70 min =**
3638 **IV, and ET$_{50}$ < 4 min = I. ET$_{50}$ values ≥4 min and <70 min are predicted to be EPA III. The model**
3639 **does not propose to identify EPA Category II materials.**

LVET- Determined EPA Category	EpiOcular Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	3	0	0	3	100%	NA	0%
II	1	0	0	1	0%	100%	0%
III	4	8	0	12	67%	33%	0%
IV	5	4	0	9	0%	100%	NA
Total	13	12	0	25	44%		
Predictivity	23%	67%	0%				
Category under predicted	NA	0%	0%				
Category over predicted	77%	33%	NA				

3640
3641   An additional analysis was conducted to compare the performance of the
3642   prediction model with each of the different product formulation types. Table 6-21
3643   presents the under and overpredictions associated with each product type. It can be
3644   seen that none of the product types was underpredicted; however, 75% of solvents,
3645   42% of surfactants, and 67% of oxidizers were overpredicted
3646
3647   **Table 6-21 Prediction results for the EO assay and EPA toxicity categories**
3648   **by product formulation type. Number of each product tested and percentage**
3649   **(in parentheses)..**
3650

	Solvents	Surfactants	Oxidizers
Under predicted	0	0	0
Correctly Predicted	1 (25%)	7 (58%)	3 (33%)
Over Predicted	3 (75%)	5 (42%)	6 (67%)

3651
3652
3653   It appears that almost all of the oxidizing formulations (8 out of 9) are
3654   predicted to be Category I materials by the EpiOcular assay, even though their *in*
3655   *vivo* irritation potential appears to vary considerably (from Category IV to Category I)
3656   in the animal test. This may be a reflection of the epithelial-only nature of the
3657   EpiOcular tissue. In this model, it may be possible for the oxidizing formulations to
3658   kill almost all of the EpiOcular tissue *in vitro* (and thus have the highest score
3659   possible which would be a Category I), while *in vivo* the material might penetrate
3660   only a small way past the epithelium into the stroma and thus cause a toxicity that
3661   would be a Category III or at the most a Category II. *Because of these significant*
3662   *(all of the over predictions were by at least 2 toxicity categories) and consistent*
3663   *overpredictions, we suggest that oxidizers be tested only in the BCOP assay.*
3664   Figure 6-10 shows the distribution of full ET_{50} values for all of the 16 materials for
3665   which data were available when plotted against EPA labeling categories
3666   (determined by the LVET) without the oxidizers.

EpiOcular vs EPA Category (LVET)
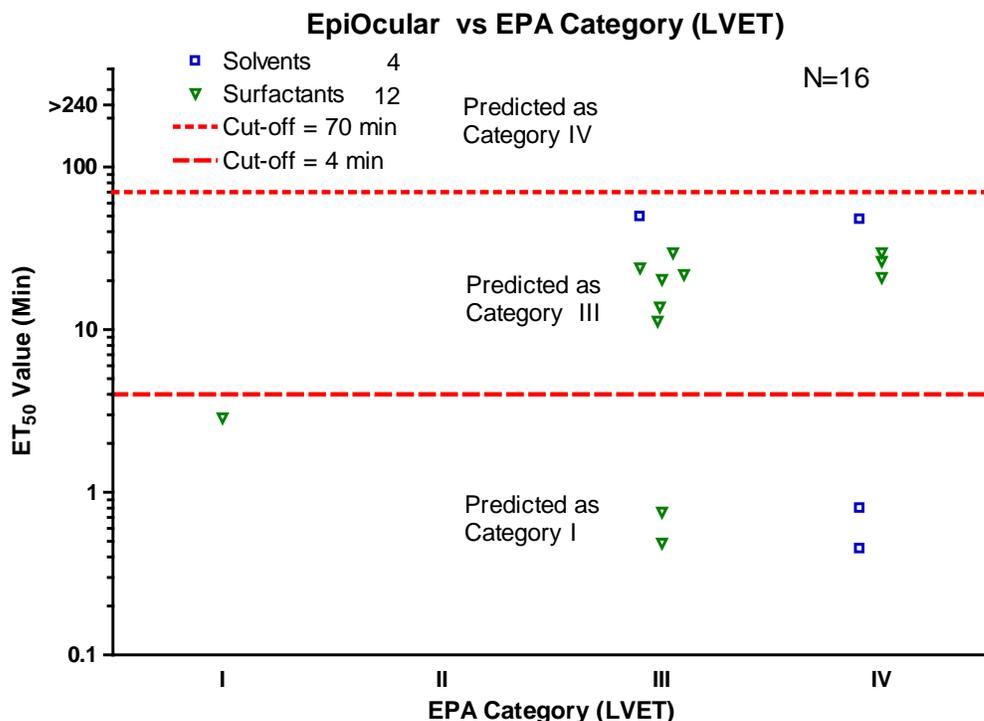
3667
3668 **Figure 6-10 EpiOcular ET$_{50}$ values plotted against EPA categories determined by the LVET.**
3669 **Oxidizers have been removed since they will be tested only in the BCOP assay. Suggested**
3670 **cut-off values with their predicted EPA categories are included.**
3671

3672   The contingency table (Table 6-24) gives an analysis of the performance
3673 based on the cut-offs shown in Figure 6-10. The data in this table indicate that the
3674 proposed cut-offs make this a very conservative model for the prediction of
3675 materials whose EPA toxicity category is greater than III with the caveat that no
3676 Category II materials were available for this analysis. All animal test-determined
3677 EPA Category I formulations were captured by this model. There were no
3678 underpredictions of Category I materials. In addition there were no underpredictions
3679 of Category III materials; all were predicted as Category III or higher.
3680

3681   What occurs as a consequence of the conservative cut-offs is that many
3682 materials are overpredicted relative to their toxicity category as determined by the
3683 animal test (LVET). Twenty-two percent of the Category III materials are
3684 overpredicted as Category I's, and 100% of the Category IV materials are
3685 overpredicted as Category III or I materials.
3686
3687

3688 **Table 6-22 Contingency table depicting the accuracy and predictivity of the EpiOcular assay**
3689 **for EPA toxicity categories (determined by the LVET) using cut-off values of ET$_{50}$ ≥ 70 min =**
3690 **ET$_{50}$ values ≥4 min and <70 min are predicted to be EPA III IV, and ET$_{50}$ < 4 min = I. ET$_{50}$**
3691 **values ≥4 min and <70 min are predicted to be EPA III. The model does not propose to identify**
3692 **EPA Category II materials.**
3693

LVET- Determined EPA Category	EpiOcular Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	1	0	0	1	100%	NA	0%
II	0	0	0	0	0%	0%	0%
III	2	7	0	9	78%	22%	0%
IV	2	4	0	6	0%	100%	NA
Total	5	11	0	16	50%		
Predictivity	20%	64%	0%				
Category under predicted	NA	0%	0%				
Category over predicted	80%	36%	NA				

3694
3695
3696   The discordant results for the EpiOcular assay and EPA toxicity categories
3697 without oxidizers are shown in Table 6-23. Because the prediction model was not
3698 changed, the results for the solvents and surfactants remain the same as in Table 6-
3699 21.
3700
3701   **Table 6-23 Prediction results for the EO assay and EPA toxicity categories by**
3702   **product formulation type. Number of each product tested and percentage (in**
3703   **parentheses).**
3704

	Solvents	Surfactants
Under predicted	0	0
Correctly Predicted	1 (25%)	7 (58%)
Over Predicted	3 (75%)	5 (42%)

3705
3706   The practical advantage of such a model is that the very low irritating
3707 materials (Category III's and IV's) can be identified and an appropriate toxicity
3708 category applied. This will clearly result in some over labeling (67% of animal-
3709 determined IV's will be over labeled as III's and 33% as I's), but the participating
3710 companies have accepted this degree of over labeling will occur. Alternatively, all of
3711 the EO predicted Category I materials could be retested in the BCOP assay. The
3712 EPA appears to concur with this type of approach, at least for over labeling by one
3713 category, since the EPA label Review Manual (2003) states (for primary eye
3714 irritation of Category IV) that "…the registrant may choose to use Category III
3715 labeling."
3716

<u>GHS Labeling Categories (LVET-determined)</u>

It can be seen from Figure 6-11 that the distribution of ET_{50} scores across the GHS labeling categories is not random. All GHS Category 1 materials have ET_{50}'s <4 min, while most GHS Category 2B and Nonirritant materials have ET_{50}'s > 10 min. Thus – for this somewhat limited data set - all materials in the highest toxicity category can be identified with a cut-off value of 4 min. However, a number of the Category 2B and Nonirritant materials also fall below this ET_{50} value.

ET_{50} values for GHS Category 2B and Nonirritant materials have considerable overlap. However, due to the limited number of Category 2B data points, it is not possible from this data set to suggest an upper cut-off limit to separate GHS Category 2B materials from Nonirritant materials. Materials having ET_{50} values above ~ 70 min would likely be Nonirritant materials. The consequence of this is that many GHS Category 2B and a few Nonirritant materials would be overpredicted.
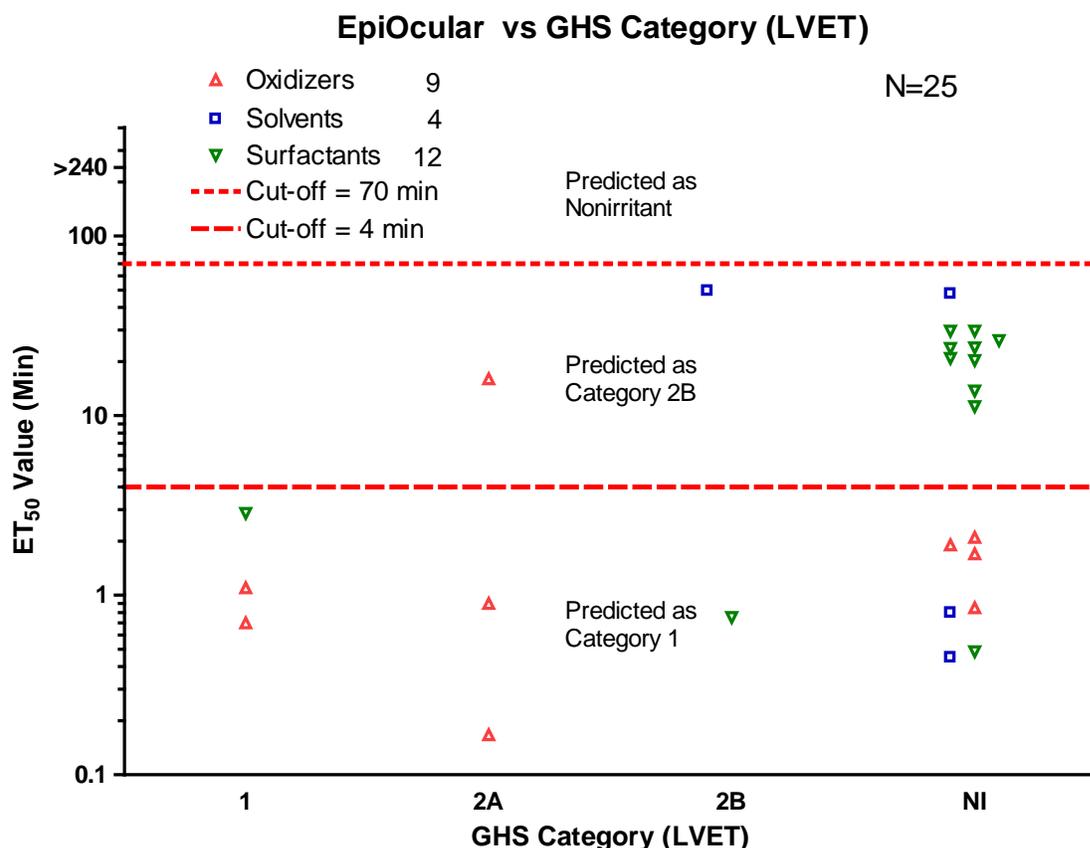
Figure 6-11 EpiOcular ET_{50} values plotted against GHS categories determined by the LVET. Suggested cut-off values with their predicted GHS categories are included.

3739   The contingency table (Table 6-24) gives an analysis of the performance
3740 based on the cut-offs shown in Figure 6-11. The data in this table indicate that the
3741 proposed cut-offs make this a very conservative model for the prediction of
3742 materials whose GHS toxicity category is greater than 2B. There was a significant
3743 amount of overprediction for GHS Nonirritant materials (100%).
3744
3745 **Table 6-24 Contingency table depicting the accuracy and predictivity of the EpiOcular assay**
3746 **for GHS toxicity categories (determined by the LVET) using cut-off values of ET_{50} ≥ 70 min =**
3747 **NL and ET_{50} < 4 min =1. The model does not propose to identify GHS Category 2A materials.**

| LVET- Determined GHS Category | EpiOcular Predicted GHS Category | | | | | Toxicity over | Toxicity under |
	1	2B	NI	Total	Concordance	predicted	predicted
1	3	0	0	3	100%	NA	0%
2A	2	1	0	3	0%	67%	33%
2B	1	1	0	2	50%	50%	0%
NI	7	10	0	17	0%	100%	NA
Total	13	12	0	25	16%		
Predictivity	23%	8%	0%				
Category under predicted	NA	8%	0%				
Category over predicted	77%	83%	NA				

3748
3749
3750   An additional analysis was conducted to compare the performance of the
3751 prediction model with each of the different product formulation types. Table 6-25
3752 presents the under and overpredictions associated with each product type. It can be
3753 seen that none of the solvents or surfactants were underpredicted, but one of the
3754 oxidizers was underpredicted. However, 75% of solvents, 92% of surfactants, and
3755 67% of oxidizers were overpredicted.
3756
3757   **Table 6-25 Prediction results for the EO assay and GHS toxicity categories by product**
3758   **formulation type. Number of each product tested and percentage (in parentheses).**
3759

	Solvents	Surfactants	Oxidizers
Under predicted	0	0	1 (11%)
Correctly Predicted	1 (25%)	1 (8%)	2 (22%)
Over Predicted	3 (75%)	11 (92%)	6 (67%)

3760
3761   It appears that almost all of the oxidizing formulations (8 out of 9) are
3762 predicted to be GHS Category 1 materials by the EpiOcular assay, even though
3763 their *in vivo* irritation potential appears to vary considerably (from Nonirritant to
3764 Category 1) in the animal test. Because of these significant and consistent
3765 overpredictions, the data set we again analyzed without the oxidizing formulations
3766 (Figure 6-12).
3767
3768

EpiOcular vs GHS Category (LVET)

N=16

▫ Solvents	4
▽ Surfactants	12

- - - Cut-off = 70 min
— — Cut-off = 4 min

Predicted as Nonirritant

Predicted as Category 2B
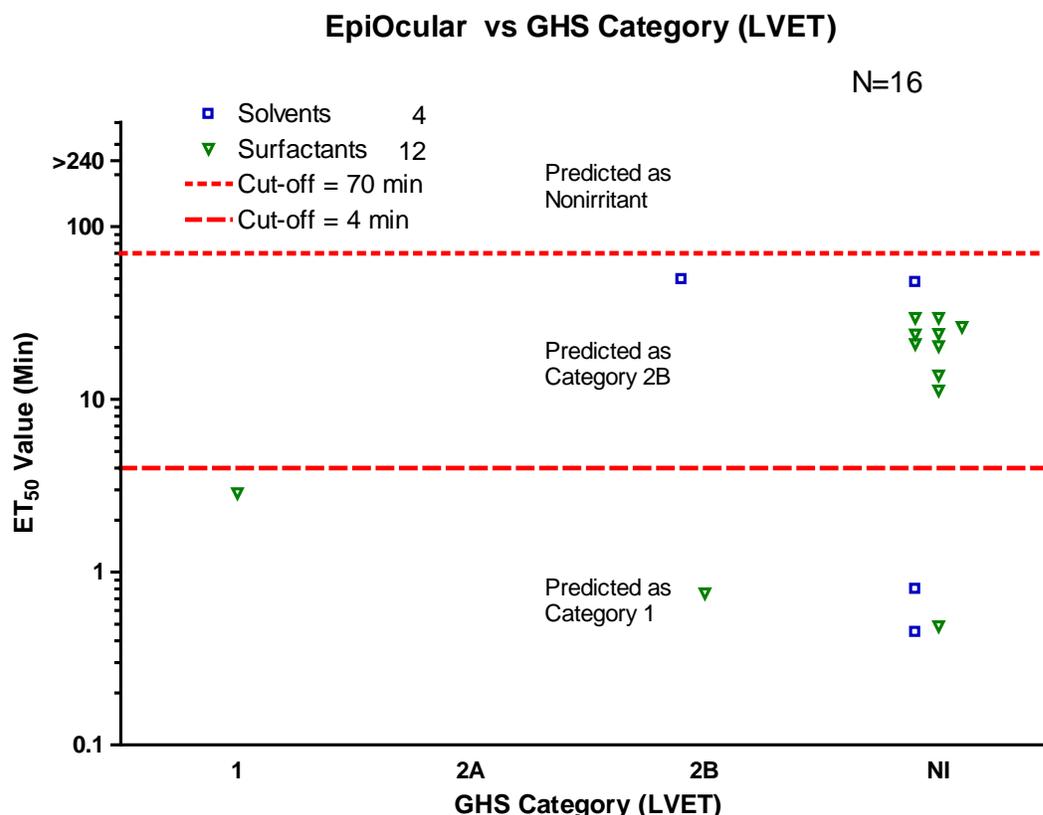
Predicted as Category 1

Figure 6-12 EpiOcular ET$_{50}$ values plotted against GHS categories determined by the LVET. Oxidizers have been removed since they will be tested only in the BCOP assay. Suggested cut-off values with their predicted GHS categories are included.

The contingency table (Table 6-26) gives an analysis of the performance based on the cut-offs shown in Figure 6-12. The data in this table indicate that the proposed cut-offs make this a very conservative model for the prediction of materials whose GHS toxicity category is greater than Category 2B with the caveat that no Category 2A materials were available for this analysis. All animal test-determined GHS Category 1 formulations were captured by this model. There were no underpredictions of Category 1 materials. In addition there were no underpredictions of Category 2B materials; all were predicted as Category 2B or higher.

What occurs as a consequence of the conservative cut-offs is that many materials are overpredicted relative to their toxicity category as determined by the animal test (LVET). Fifty percent of the Category 2B materials are overpredicted as Category 1's, and 100% of the Nonirritant materials are overpredicted as Category 2B or 1 materials

3791 **Table 6-26 Contingency table depicting the accuracy and predictivity of the EpiOcular assay**
3792 **for GHS toxicity categories (determined by the LVET) using cut-off values of ET_{50} ≥ 70 min =**
3793 **NL and ET_{50} < 4 min =1. The model does not propose to identify GHS Category 2A materials.**

LVET- Determined GHS Category	EpiOcular Predicted GHS Category			Total	Concordance	Toxicity over predicted	Toxicity under predicted
	1	2B	NI				
1	1	0	0	1	100%	NA	0%
2A	0	0	0	0	0%	0%	0%
2B	1	1	0	2	50%	50%	0%
NI	3	10	0	13	0%	100%	NA
Total	5	11	0	16	13%		
Predictivity	20%	9%	0%				
Category under predicted	NA	0%	0%				
Category over predicted	80%	91%	NA				

3794
3795
3796   The discordant results for the EpiOcular assay and GHS toxicity categories
3797 without oxidizers are shown in Table 6-27. Since the prediction model did not
3798 change from the previous analysis, there was no change for the other formulation
3799 types from the analysis in Table 6-25.
3800
3801   **Table 6-27 Prediction results for the EO assay and GHS toxicity categories by product**
3802   **formulation type. Number of each product tested and percentage (in parentheses).**
3803

	Solvents	Surfactants
Under predicted	0	0
Correctly Predicted	1 (25%)	1 (8%)
Over Predicted	3 (75%)	11 (92%)

3804
3805   The practical advantage of such a model is that the very low irritating
3806 materials (Category 2B and Nonirritants) can be identified and an appropriate
3807 toxicity category applied. This will clearly result in some over labeling (77% of
3808 animal-determined Nonirritants will be over labeled as Category 2B and 23% as
3809 Category 1), but the participating companies have accepted this degree of over
3810 labeling will occur. Alternatively, all of the EO predicted Category 1 materials could
3811 be retested in the BCOP assay.
3812
3813 EPA Labeling Categories (Draize-determined)
3814
3815   The above discussion of EPA and GHS toxicity categories (as determined by
3816 the LVET assay) utilizes a relatively small data set. However, additional EO data
3817 were available from company participants which were paired with Draize-determined
3818 EPA and GHS categories. The distribution of product categories for the additional
3819 data points is shown in Table 6-28.
3820
3821

3822
3823
3824

Table 6-28 Distribution of product categories originally submitted with both animal eye irritation data (Draize) and EpiOcular data.

Product Categories	Number of products tested
Oxidizers	4
Surfactants	5
Solvents	6
Bases	11
Acids	1
Other	2
Total	**29**

3825
3826   Figure 6-13 presents the additional data identified by their designated product
3827 categories. Since the distribution pattern seemed to be similar to what was seen
3828 earlier, the same cut-off values as were suggested by the previous analysis of the
3829 LVET-determined EPA Categories were applied to this data set.
3830
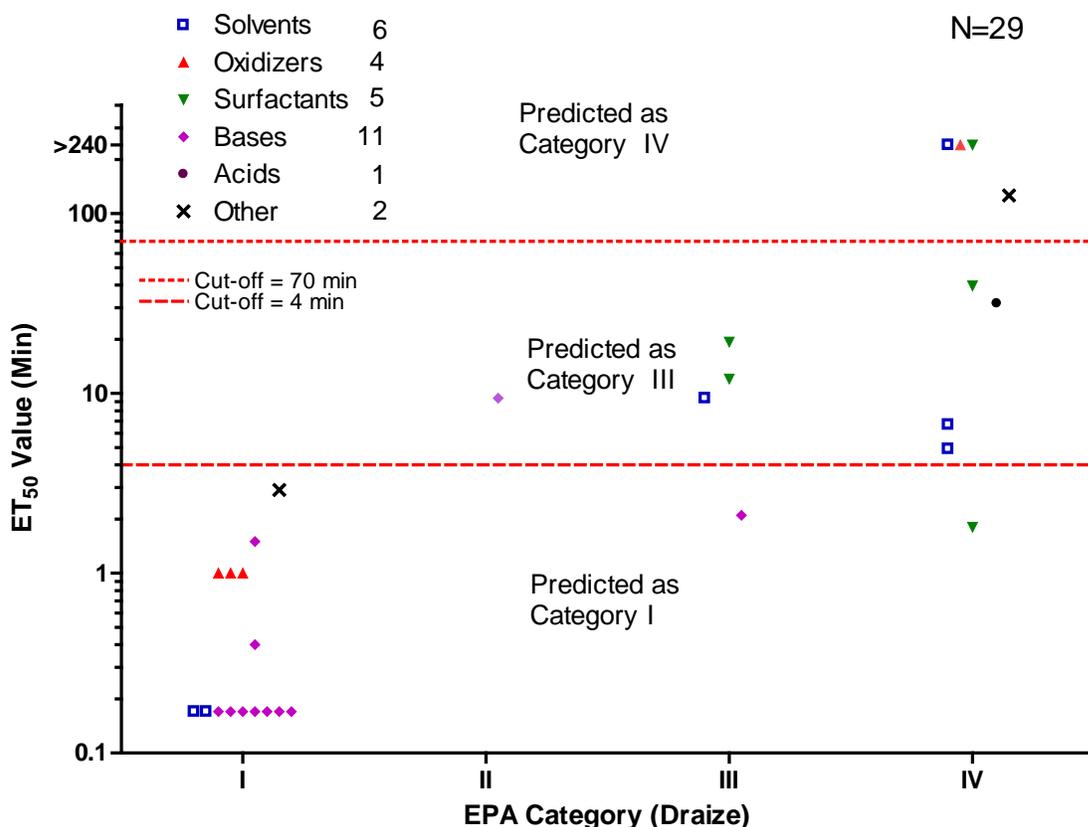
3831
3832 **Figure 6-13 EpiOcular ET_{50} values plotted against EPA categories determined by the Draize**
3833 **test. Suggested cut-off values with their predicted EPA categories are included.**
3834

3835   Again a contingency table was generated to quantitate over and
3836 underpredictions. This is shown as Table 6-29. The data in this table indicate that
3837 the proposed cut-offs are slightly less conservative than that shown with LVET-
3838 designated EPA categories. The Category I materials are correctly predicted, but
3839 the single Category II material is underpredicted. One of the Category III materials is
3840 overpredicted, but the remainder of the Category III materials are appropriately
3841 identified. Forty-four percent of the Category IV materials are overpredicted as III's
3842 and 11% (1 material) are overpredicted as I's.
3843
3844 **Table 6-29 Contingency table depicting the accuracy and predictivity of the EpiOcular assay**
3845 **for EPA toxicity categories (determined by the Draize test) using cut-off values of $ET_{50} \geq 70$**
3846 **min = IV, and $ET_{50} < 4$ min = I. The model does not propose to identify EPA Category II**
3847 **materials.**
3848

Draize- Determined EPA Category	EpiOcular Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	15	0	0	15	100%	NA	0%
II	0	1	0	1	0%	0%	100%
III	1	3	0	4	75%	25%	0%
IV	1	4	4	9	44%	56%	NA
Total	17	8	4	29	76%		
Predictivity	88%	38%	100%				
Category under predicted	NA	12%	0%				
Category over predicted	12%	50%	NA				

3849
3850   The discordant results for the EpiOcular assay and the Draize-derived EPA
3851 toxicity categories are shown in Table 6-30. There was one underprediction of the
3852 EPA category for a base material; however, 33% of solvents, 40% of surfactants,
3853 9% of bases, and 100% of acids were overpredicted.
3854
3855   **Table 6-30 Prediction results for the EO assay and EPA toxicity categories by product**
3856   **formulation type. Number of each product tested and percentage (in parentheses).**
3857

	Solvents	Surfactants	Oxidizers	Bases	Acids	Other
Under predicted	0	0	0	1 (9%)	0	0
Correctly Predicted	4 (67%)	3 (60%)	4 (100%)	9 (82%)	0	2 (100%)
Over Predicted	2 (33%)	2 (40%)	0	1 (9%)	1 (100%)	0

3858
3859   In this case (a different set of formulations; the Draize test used to determine
3860 EPA hazard categories) the oxidizing formulations appear to have been correctly
3861 predicted by the EO assay with the proposed cut-offs mentioned earlier. However,
3862 to parallel the analysis of the preceding section, the oxidizing formulations were
3863 removed and the data set re-evaluated in Figure 6-14.
3864
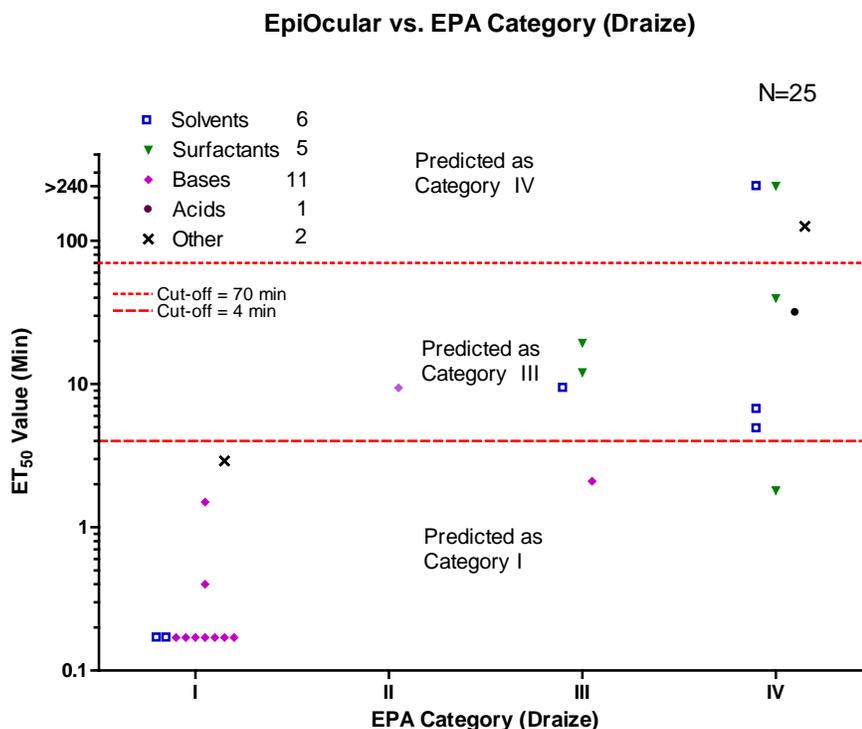
EpiOcular vs. EPA Category (Draize)

3865
3866
3867   **Figure 6-14 EpiOcular ET$_{50}$ values plotted against EPA categories determined by the Draize**
3868   **test. Oxidizers have been removed since they will be tested only in the BCOP assay.**
3869   **Suggested cut-off values with their predicted EPA categories are included.**
3870
3871   Again a contingency table was generated to quantitate over and
3872   underpredictions. This is shown as Table 6-31. The data in this table indicate that
3873   the proposed cut-offs are slightly less conservative than that shown with LVET-
3874   designated EPA categories. The Category I materials are correctly predicted, but
3875   the single Category II material is underpredicted. One of the Category III materials is
3876   overpredicted, but the remainder of the Category III materials are appropriately
3877   identified. Fifty percent of the Category IV materials are overpredicted as III's and
3878   thirteen percent are overpredicted as I's.
3879
3880

3881 **Table 6-31 Contingency table depicting the accuracy and predictivity of the EpiOcular assay**
3882 **for EPA toxicity categories (determined by the Draize test) using cut-off values of $ET_{50} \geq 70$**
3883 **min = IV, and $ET_{50} < 4$ min = I. The model does not propose to identify EPA Category II**
3884 **materials.**
3885

Draize- Determined EPA Category	EpiOcular Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	III	IV	Total			
I	12	0	0	12	100%	NA	0%
II	0	1	0	1	0%	0%	100%
III	1	3	0	4	75%	25%	0%
IV	1	4	3	8	38%	63%	NA
Total	14	8	3	25	72%		
Predictivity	86%	38%	100%				
Category under predicted	NA	12%	0%				
Category over predicted	14%	50%	NA				

3886
3887   The discordant results by formulation type for the EpiOcular assay and
3888 Draize-derived EPA toxicity categories are shown in Table 6-32. Since the
3889 prediction model did not change, the results for all formulation types other than
3890 oxidizers did not change from the analysis shown in Table 6-30.
3891
3892 **Table 6-32 Prediction results for the EO assay and EPA toxicity categories by product**
3893 **formulation type. Number of each product tested and percentage (in parentheses).**
3894

	Solvents	Surfactants	Bases	Acids	Other
Under predicted	0	0	1 (9%)	0	0
Correctly Predicted	4 (67%)	3 (60%)	9 (82%)	0	2 (100%)
Over Predicted	2 (33%)	2 (40%)	1 (9%)	1 (100%)	0

3895
3896
3897 GHS Labeling Categories (Draize-determined)
3898
3899   The same data set of 29 additional materials discussed above was also
3900 evaluated for the prediction of GHS categories, however only 28 materials had
3901 sufficient animal data to provide a GHS classification. Figure 6-15 shows the
3902 distribution of the materials with respect to GHS category and EpiOcular ET_{50} value.
3903

EpiOcular vs. GHS Category (Draize)
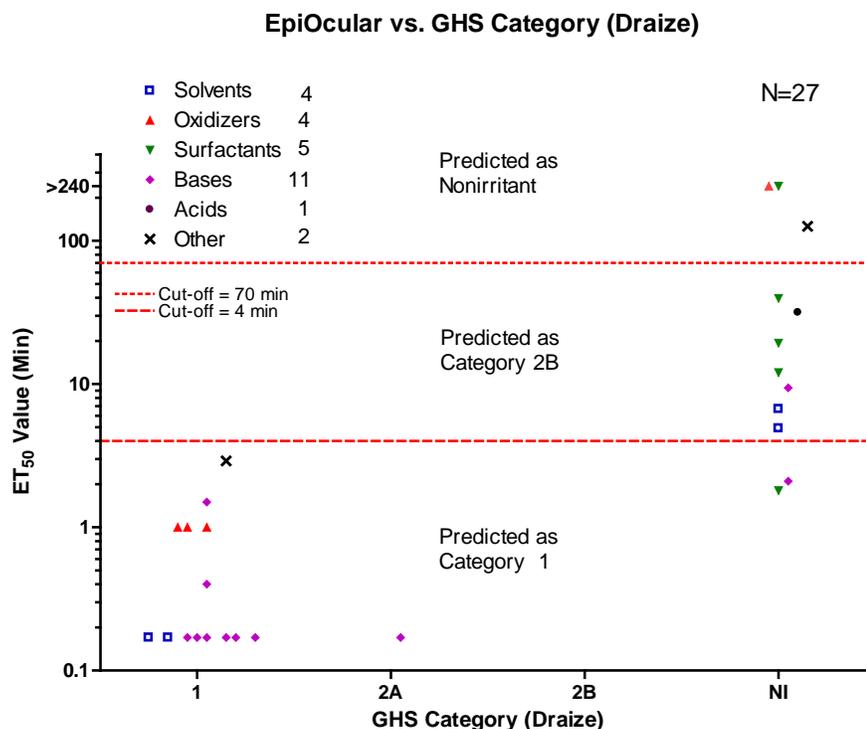
3904
3905 **Figure 6-15 EpiOcular ET$_{50}$ values plotted against GHS categories determined by the Draize.**
3906 **Suggested cut-off values with their predicted GHS categories are included.**
3907

3908   A contingency table was generated to quantitate over and underpredictions
3909 for the GHS labeling. This is shown as Table 6-35. The data in this table indicate
3910 that the proposed cut-offs are slightly less conservative than that shown with LVET-
3911 designated GHS categories. The Category 1 materials are correctly predicted, but
3912 the single Category 2A material is overpredicted. Fify-eight percent of the Nonirritant
3913 materials are overpredicted as Category 2B.
3914

3915 **Table 6-33 Contingency table depicting the accuracy and predictivity of the EpiOcular assay**
3916 **for GHS toxicity categories (determined by the LVET) using cut-off values of ET$_{50}$ ≥ 70 min =**
3917 **NL and ET$_{50}$ < 4 min =1. The model does not propose to identify GHS Category 2A materials.**
3918

Draize- Determined GHS Category	EpiOcular Predicted GHS Category				Concordance	Toxicity over predicted	Toxicity under predicted
	1	**2B**	**NI**	**Total**			
1	14	0	0	14	100%	NA	0%
2A	1	0	0	1	0%	100%	0%
2B	0	0	0	0	NA	NA	NA
NI	2	7	3	12	25%	75%	NA
Total	17	7	3	27	63%		
Predictivity	82%	0%	100%				
Category under predicted	NA	0%	0%				
Category over predicted	18%	100%	NA				

3919

3920   The discordant results for the EpiOcular assay and Draize-derived GHS
3921 toxicity categories are shown in Table 6-34. There were no underpredictions of the
3922 GHS category for any material; however, 50% of solvents, 80% of surfactants, 27%
3923 of bases, and 100% of acids were overpredicted.
3924
3925   **Table 6-34 Discordant results for the EpiOcular assay and GHS toxicity categories.**

	Solvents	Surfactants	Oxidizers	Bases	Acids	Other
Under predicted	0	0	0	0	0	0
Correctly Predicted	2 (50%)	1 (25%)	4 (100%)	8 (73%)	0	2 (100%)
Over Predicted	2 (50%)	4 (75%)	0	3 (27%)	1 (100%)	0

3926
3927   The oxidizing formulations appear to have been correctly predicted by the EO
3928 assay with the proposed cut-offs mentioned earlier. However, the oxidizing
3929 formulations were removed and the data set re-evaluated in Figure 6-16.
3930

EpiOcular vs. GHS Category (Draize)
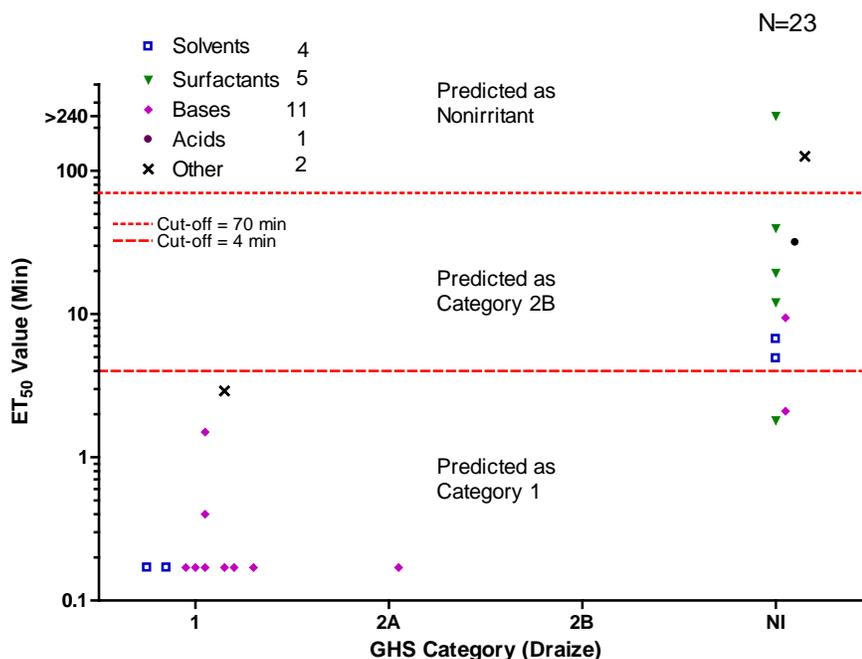
3931
3932 **Figure 6-16 EpiOcular ET_{50} values plotted against GHS categories determined by the Draize.**
3933 **Oxidizers have been removed since they will be tested only in the BCOP assay. Suggested**
3934 **cut-off values with their predicted GHS categories are included.**
3935
3936   A contingency table was generated to quantitate over and underpredictions
3937 for the Draize-derived GHS labeling. This is shown as Table 6-35. The data in this
3938 table indicate that the proposed cut-offs are slightly less conservative than that
3939 shown with LVET-designated GHS categories. The Category 1 materials are
3940 correctly predicted, but the single Category 2A material is overpredicted. Sixty-four
3941 percent of the Nonirritant materials are overpredicted as Category 2B.
3942

3943 **Table 6-35 Contingency table depicting the accuracy and predictivity of the EpiOcular assay**
3944 **for GHS toxicity categories (determined by the LVET) using cut-off values of ET_{50} > 70 min =**
3945 **NI and ET_{50} < 4 min =1. The model does not propose to identify GHS Category 2A materials.**
3946

Draize- Determined GHS Category	EpiOcular Predicted GHS Category				Concordance	Toxicity over predicted	Toxicity under predicted
	1	2B	NI	Total			
1	11	0	0	11	100%	NA	0%
2A	1	0	0	1	0%	100%	100%
2B	0	0	0	0	NA	NA	NA
NI	2	7	2	11	18%	82%	NA
Total	14	7	2	23	57%		
Predictivity	79%	0%	100%				
Category under predicted	NA	0%	0%				
Category over predicted	21%	100%	NA				

3947
3948   The discordant results by formulation type for the EpiOcular assay and
3949 Draize-derived GHS toxicity categories are shown in Table 6-36. Since the
3950 prediction model did not change, the results for all formulation types other than
3951 oxidizers did not change from the analysis shown in Table 6-34.
3952
3953   **Table 6-36 Discordant results for the EpiOcular assay and GHS toxicity categories.**

	Solvents	Surfactants	Bases	Acids	Other
Under predicted	0	0	0	0	0
Correctly Predicted	2 (50%)	1 (25%)	8 (73%)	0	2 (100%)
Over Predicted	2 (50%)	4 (75%)	3 (27%)	1 (100%)	0

3954

3955 6.2.2 Conclusion for EpiOcular studies
3956
3957   Products used in the analysis of the EO performance had either Draize-
3958 derived or LVET-derived EPA and GHS toxicity categories. The performance of the
3959 EO assay varied somewhat depending on which of the *in vivo* assays was used,
3960 however this difference may also have been due to a different distribution of
3961 products. None the less the prediction model was determined to be the same
3962 regardless of the *in vivo* assay type. Thus the following summary is based on the
3963 combination of results from both *in vivo* assays.
3964
3965   The EpiOcular assay (as has been suggested by several reports (Stern,
3966 Klausner et al. 1998; Jones, Budynsky et al. 2001)) appears to be most useful at the
3967 less irritating portion of the toxicity spectrum. It is capable of identifying both EPA
3968 Category III and IV materials, although most Category IV materials will be
3969 overpredicted as Category III materials. Only one of the forty-one materials (2%)
3970 was under predicted for EPA toxicity categories. Over predictions were much more
3971 frequent. The corporate participants have agreed that this outcome of over labeling
3972 some materials is acceptable to them.

3973   Very similar results were found with the prediction model for GHS categories.
3974   There were no under predictions of GHS toxicity categories regardless of whether
3975   the categories were Draize-determined or LVET-determined.
3976
3977   Another assay (we propose BCOP) will be used as a second tier test to
3978   differentiate EPA Category I from Category II and less irritating materials, if needed.
3979
3980   **Historical knowledge of the performance of the EpiOcular assay plus**
3981   **the preceding analysis of the EpiOcular data in this BRD have led us to the**
3982   **following recommendations:**
3983
3984   1) **Anti-microbial cleaning products having an oxidizing chemistry**
3985   **should not be tested with the EpiOcular assay.**
3986
3987   2) **Both water soluble and water insoluble anti-microbial cleaning**
3988   **products can be tested with the EpiOcular assay.**
3989
3990   3) **If the anti-microbial cleaning product has an ET_{50} score of <4**
3991   **minutes, it is classified as EPA Category I or GHS Category 1.**
3992
3993   4) **If the anti-microbial cleaning product has an ET_{50} score of ≥4**
3994   **minutes, but <70 minutes, it is classified as EPA Category III or GHS**
3995   **Category 2B.**
3996
3997   5) **If the anti-microbial cleaning product has an ET_{50} score of ≥70**
3998   **minutes, it is classified as EPA Category IV or GHS Category NI.**
3999
4000   6) **(Optional) To determine if an anti-microbial cleaning product which**
4001   **was categorized as either EPA I or GHS 1 is actually an EPA II or a**
4002   **GHS 2A, it should be further tested in the BCOP assay.**
4003

4004   ## 6.3 BCOP predictive capacity

4005   ### 6.3.1 Overview

4006
4007   As opposed to the Cytosensor and EpiOcular assays which were conducted
4008   almost entirely in a retrospective fashion, i.e. both the *in vitro* and *in vivo* data had
4009   been determined before the beginning of this project, the analysis of the BCOP
4010   assay proceeded sequentially with *in vitro* data being produced prospectively
4011   throughout the analysis period. An initial set of paired animal and BCOP data was
4012   used to set potential cut-off values for the various EPA categories. Subsequently
4013   new materials were received from many of the participants under code and these
4014   materials were tested in a blind fashion under GLP-compliant conditions at IIVS.
4015   Many of these materials had additional histopathological analysis which was
4016   conducted either at IIVS or at a subcontractor who had been trained in
4017   histopathological analysis of bovine corneas. The histopathological analysis was
4018   conducted while the materials were still under code.

4019   ### 6.3.2 Analysis using only BCOP *in vitro* scores (no histopathology)

4020   #### 6.3.2.1 Original company data submissions

4021
4022   Table 6-37 gives the distribution of materials in Figure 6-17. As seen with the
4023   analysis of the Cytosensor and the EpiOcular assays, the distribution of product
4024   categories is relatively uneven. Surfactants appear to be under represented when
4025   compared to the information available for the other two *in vitro* assays.
4026
4027   **Table 6-37 Distribution of product categories originally submitted**
4028   **with both animal eye irritation data and BCOP *in vitro* data.**
4029

Product Categories	Number of products tested
Oxidizers	8
Surfactants	1
Acids	0
Bases	10
Solvents	9
Total	28

4030
4031
4032   Figure 6-17 shows the distribution of the initial 28 BCOP *in vitro* scores
4033   plotted against EPA labeling categories (determined by the Draize test). Six of these
4034   materials were tested in a modified Draize protocol with a reduced volume (0.03 ml),
4035   but since the results were scored as Category I even though a reduced volume was
4036   used, it was decided that it was valid to use these data in the analysis.
4037
4038   It is apparent from Figure 6-17 that the distribution of BCOP *in vitro* scores
4039   across the EPA labeling categories is not random. Most EPA Category I materials

4040   have *in vitro* scores higher than 100 and none fall below 40. In contrast all EPA
4041   Category IV materials have scores below 35. EPA Category III materials are widely
4042   spread between *in vitro* scores of 20 and 80. <u>This distribution allowed us to set cut-</u>
4043   <u>off values by eye for predicting EPA labeling categories.</u> We attempted to choose
4044   cut-offs conservatively with a bias towards having as few under predictions as was
4045   reasonable. No statistical methods were employed to construct the proposed
4046   prediction model.
4047
4048   It appears that all but one of the Category 1 materials is identified with a cut-
4049   off greater than an *in vitro* score of 75. It appears that it is not possible to
4050   differentiate between III's and IV's with a cut-off value, but both III's and IV's might
4051   be identified with a cut-off of below an *in vitro* score of 35. Since the BCOP assay
4052   does not differentiate between materials in the mild irritancy range as well as the
4053   other assays in this BRD, a second assay such as EO or CM may be used to
4054   demonstrate an EPA Category IV. Figure 6-17 shows a plot of BCOP *in vitro* scores
4055   versus EPA category classifications (as determined by the Draize test) with the
4056   above proposed cut-off values added.
4057

BCOP Scores vs. EPA Category
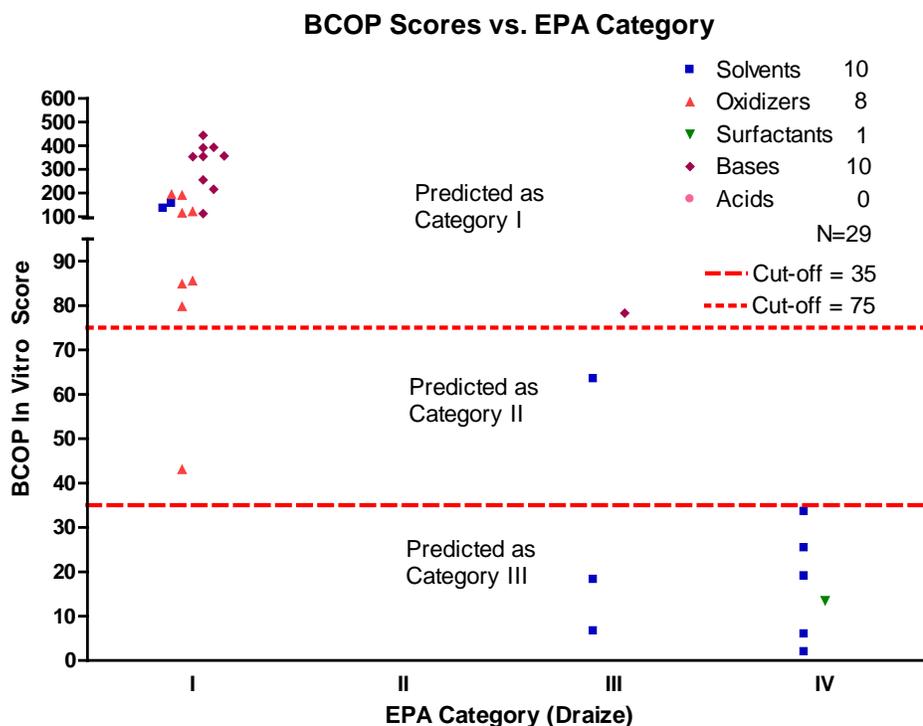
4058
4059
4060   **Figure 6-17 BCOP *in vitro* scores plotted against EPA categories determined by the Draize**
4061   **test. Proposed cut-off values with their predicted EPA categories are included.**
4062
4063   The following contingency table (Table 6-38) gives an analysis of the
4064   performance based on the preliminary cut-offs shown in Figure 6-17. The data in
4065   this table indicate that the proposed cut-offs make this a conservative model for the
4066   prediction of materials whose EPA Category is I. Ninety-five percent of the *in vivo-*

4067 determined EPA Category I materials were captured by this model. There was only
4068 one underprediction of a Category I material. However, there were no EPA Category
4069 II materials in this initial data set; therefore, it is impossible to determine how well
4070 the predicted cut-offs actually predict Category II materials.
4071
4072   What occurs as a consequence of the conservative cut-offs is that many less
4073 irritating materials are overpredicted relative to their toxicity category as determined
4074 by the Draize animal test. With this prediction model all of the Category IV materials
4075 are overpredicted as Category III's, and 50% of the Category III materials are
4076 overpredicted as Category II's or Category I's.
4077
4078 **Table 6-38 Contingency table (based on Figure 6-17) depicting the accuracy and predictivity**
4079 **of the BCOP assay for EPA toxicity categories (determined by the Draize test) using cut-off**
4080 **values of *in vitro* score ≥ 75 = I, 75 > BCOP *in vitro* score ≥ 35 = II, and BCOP *in vitro* score <**
4081 **35 = III. Although the model does propose to identify EPA Category II materials, there are no**
4082 **Category II's in the data set to test the hypothesis. The model does not propose to identify**
4083 **Category IV materials.**
4084

Draize- Determined EPA Category	BCOP Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	II	III	Total			
I	18	1	0	19	94.7%	NA	5.3%
II	0	0	0	0	0%	0%	0%
III	1	1	2	4	50%	50%	0%
IV	0	0	6	6	0%	100%	NA
Total	19	2	8	29	69%		
Predictivity	94.7%	0%	25.0%				
Category under predicted	NA	50%	0%				
Category over predicted	5.3%	50%	75.0%				

4085
4086   The discordant results assessed by product formulation for the BCOP assay
4087 and EPA toxicity categories are shown in Table 6-39. There was one
4088 underprediction of the EPA category for oxidizing materials; however, 60% of
4089 solvents, 100% of surfactants, and 10% of bases were overpredicted.
4090
4091   **Table 6-39 Prediction results for the BCOP assay and EPA toxicity categories by product**
4092   **formulation type. Number of each product tested and percentage (in parentheses).**
4093

	Solvents	Surfactants	Oxidizers	Bases	Acids
Under predicted	0	0	1 (12%)	0	0
Correctly Predicted	4 (40%)	0	7 (88%)	9 (90%)	0
Over Predicted	6 (60%)	1 (100%)	0	1 (10%)	0

4094
4095   The practical advantage of such a model is that the very irritating materials
4096 (Category I's) can be easily identified and an appropriate toxicity category applied.
4097 This will clearly result in some over labeling (all of *in vivo* EPA Category IV's would
4098 be over labeled as III's), unless a second tier test was used to differentiate the

4099 Category IV's from the Category III's. The participating companies have accepted
4100 that this degree of over labeling of Category IV's will occur. As stated before, the
4101 EPA appears to concur with this type of approach since the EPA label Review
4102 Manual (2003) states (for primary eye irritation of Category IV) that "…the registrant
4103 may choose to use Category III labeling."

4104 6.3.2.2 Further analysis

4105 6.3.2.2.1 Additional materials tested and analyzed by EPA category
4106
4107   In order to test the validity of the proposed BCOP prediction model, additional
4108 cleaning products were solicited from the companies participating in creating this
4109 submission. The greatest emphasis was placed on obtaining Category II materials
4110 since none were present in the first set of submissions. However, obtaining
4111 additional compounds to test proved difficult since many of the formulations for
4112 which full animal data were available were no longer being marketed and thus would
4113 only be available if the material were reformulated specifically for this project.
4114 Additionally, obtaining EPA Category II formulations was problematic because few
4115 Category II cleaning products appear to be currently marketed (personal experience
4116 of submitter who searched retail stores). The highly aggressive Category I materials
4117 are common since they are often highly concentrated industrial and institutional
4118 cleaning products. Consumer products, on the other hand, are generally sold in a
4119 more dilute form and are less irritating (Categories III and IV) than the industrial and
4120 institutional products (personal communication, manufacturers participating in this
4121 project).
4122
4123   Thirty-seven additional materials (only 36 had sufficient data to obtain GHS
4124 hazard categories) for which *in vivo* rabbit data (Draize or LVET) already existed
4125 were eventually submitted during the course of this project for testing in the BCOP
4126 assay. Each of these materials was submitted in a coded form so that the laboratory
4127 conducting the BCOP assay (IIVS) would not be aware of the EPA classification
4128 already assigned to the product by the animal test.
4129
4130   When the 37 new materials (including more surfactants, as we had needed)
4131 and their BCOP scores were added to the database, a new plot was constructed of
4132 the BCOP *in vitro* scores versus the EPA categories (Figure 6-18). It appeared that
4133 an *in vitro* score of 75 was still a satisfactory cut-off to separate EPA Category I
4134 materials from Category II materials. **Thus the additional of the 37 new data
4135 points verified the originally postulated prediction model – an important
4136 outcome when trying to validate a prediction model.** With the addition of five
4137 EPA Category II materials it appeared that the cut-off for conservatively separating
4138 Category II from Category III materials should be lowered to an *in vitro* score of 25.
4139 This allows three of the five Category II materials to be correctly identified, as well
4140 as ensures that the three low-scoring Category I materials would not be
4141 underpredicted by more than one toxicity category.
4142

BCOP Scores vs. EPA Category
10 minute exposure
(all materials with 10 minute data)
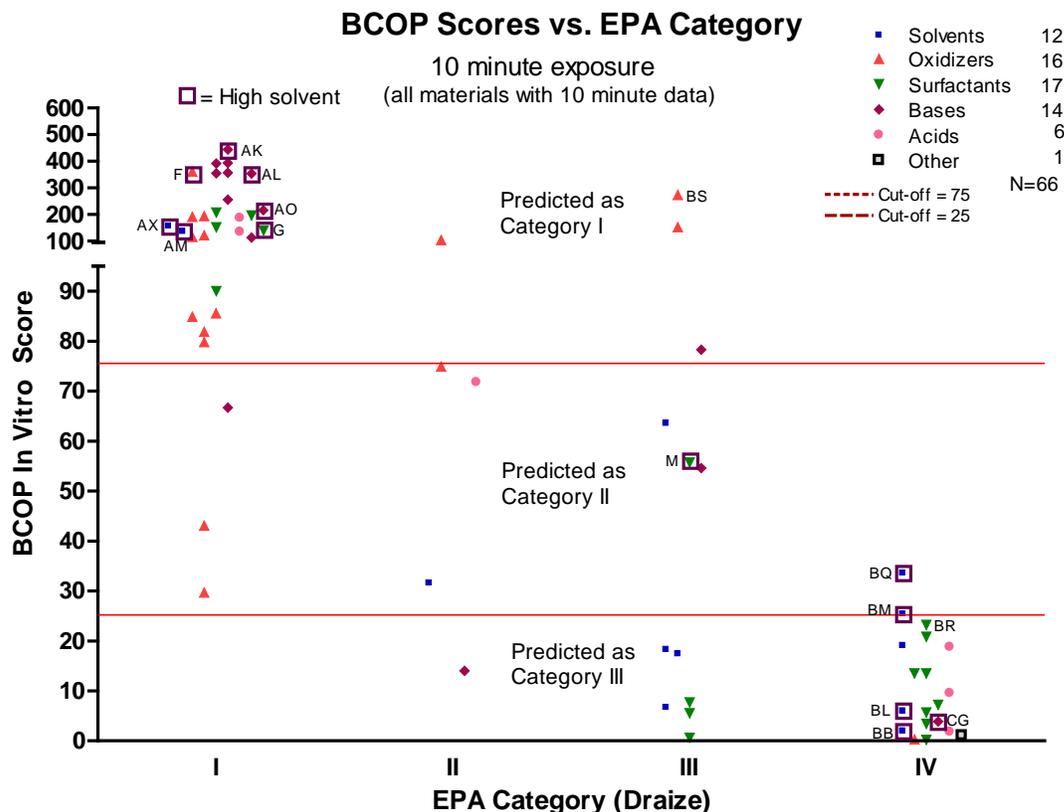
4143
4144
4145 **Figure 6-18 BCOP *in vitro* scores plotted against EPA categories determined by the Draize**
4146 **test. Proposed cut-off values with their predicted EPA categories are included. The EPA**
4147 **toxicity categories for test materials BR and BS were determined by using the results of an**
4148 **LVET assay. The discussion of the materials labeled as "High solvent" occurs later in this**
4149 **chapter.**
4150
4151   A contingency table (Table 6-40) was constructed using the information from
4152 Figure 6-18. The results show that the BCOP assay performs well at identifying
4153 Category I materials (positive predictive value of 87.1%) while also having high
4154 sensitivity (90%) for Category I materials.
4155
4156

4157 **Table 6-40 Contingency table (based on Figure 6-18) depicting the accuracy and predictivity**
4158 **of the BCOP assay for EPA classification (determined by the Draize test) using cut-off values**
4159 **of *in vitro* score > 75 = I, 75 > BCOP *in vitro* score > 25 = II, and BCOP *in vitro* score < 25 = III.**
4160 **The model does not propose to identify Category IV materials.**
4161

Draize- Determined EPA Category	BCOP Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	II	III	Total			
I	27	3	0	30	90%	NA	10%
II	1	3	1	5	60%	20%	20%
III	3	3	6	12	50%	50%	0%
IV	0	2	17	19	0%	100%	NA
Total	31	11	24	66	54.5%		
Predictivity	87.1%	27.3%	25%				
Category under predicted	NA	27.3%	4%				
Category over predicted	12.9%	45.5%	71%				

4162

4163   The discordant results assessed by product formulation for the BCOP assay
4164 and EPA toxicity categories are shown in Table 6-41. There were five
4165 underpredictions of the EPA category for base and oxidizing materials; however,
4166 50% of solvents, 53% of surfactants, 19% of oxidizers, 21% of bases, 50% of acids,
4167 and 100% of other materials were overpredicted.
4168
4169   **Table 6-41 Prediction results for the BCOP assay and EPA toxicity categories by product**
4170   **formulation type. Number of each product tested and percentage (in parentheses).**

	Solvents	Surfactants	Oxidizers	Bases	Acids	Other
Under predicted	0	0	3 (19%)	2 (14%)	0	0
Correctly Predicted	6 (50%)	8 (47%)	10 (62%)	9 (64%)	3 (50%)	0
Over Predicted	6 (50%)	9 (53%)	3 (19%)	3 (21%)	3 (50%)	1 (100%)

4171

4172 6.3.2.2.2 Additional materials tested and analyzed by GHS toxicity category
4173
4174   When the 36 new materials (only 37 had sufficient raw data to calculate GHS
4175 hazard classifications) and their BCOP scores were added to the database, a new
4176 plot was constructed of the BCOP *in vitro* scores versus the GHS categories (Figure
4177 6-19). It appeared that an *in vitro* score of 75 was a satisfactory cut-off to separate
4178 GHS Category 1 materials from GHS Category 2A materials. With the addition of six
4179 GHS Category 2A materials it appeared that the cut-off for conservatively separating
4180 GHS Category 2A from Category 2B materials should be an *in vitro* score of 25,
4181 identical to the EPA toxicity categories II/III cut-off. This allows four out of eight GHS
4182 Category 2A materials to be correctly identified, as well as ensures that the three
4183 low-scoring GHS Category 1 materials would not be underpredicted by more than
4184 one toxicity category.
4185
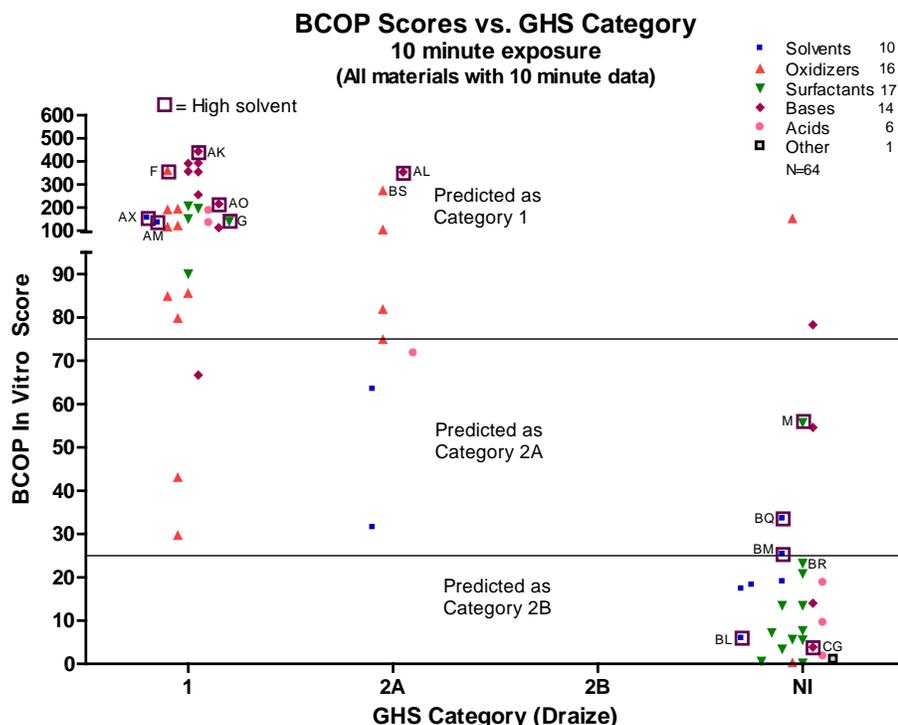
BCOP Scores vs. GHS Category
10 minute exposure
(All materials with 10 minute data)

4186
4187 **Figure 6-19 BCOP *in vitro* scores plotted against GHS categories determined by the Draize**
4188 **test. Proposed cut-off values with their predicted GHS categories are included. The EPA**
4189 **categories for test materials BR and BS were determined by using the results of an LVET**
4190 **assay. The discussion of the materials labeled as "High solvent" occurs later in this chapter.**
4191
4192   A contingency table (Table 6-42) was constructed using the information from
4193 Figure 6-19. The results show that the BCOP assay performs well at identifying EPA
4194 Category 1 materials (positive predictive value of (81%) while also having high
4195 sensitivity (89%) for Category I materials.
4196
4197 **Table 6-42 Contingency table (based on Figure 6-19) depicting the accuracy and predictivity**
4198 **of the BCOP assay for GHS toxicity categories (determined by the Draize test) using cut-off**
4199 **values of a BCOP *in vitro* score ≥ 75 = 1, 75 > BCOP *in vitro* score ≥ 25 = 2A, and a BCOP *in***
4200 ***vitro* score < 25 = 2B. The model does not propose to identify Category NL materials.**
4201

Draize- Determined GHS Category	BCOP Predicted GHS Category				Concordance	Toxicity over predicted	Toxicity under predicted
	1	**2A**	**2B**	**Total**			
1	25	3	0	28	89.3%	NA	10.7%
2A	4	4	0	8	50%	50%	0%
2B	0	0	0	0	0%	0%	0%
NI	2	4	22	28	0%	100%	NA
Total	31	11	22	64	45.3%		
Predictivity	80.6%	36.4%	0%				
Category under predicted	NA	27.3%	0%				
Category over predicted	19.4%	36.4%	100%				

4202   The discordant results assessed by product formulation for the BCOP assay
4203   and GHS toxicity categories are shown in Table 6-43. There were three
4204   underpredictions of the GHS category for base and oxidizing materials; however,
4205   60% of solvents, 71% of surfactants, 31% of oxidizers, 36% of bases, 54% of acids,
4206   and 100% of other materials were overpredicted.
4207
4208   **Table 6-43 Prediction results for the BCOP assay and GHS toxicity categories by product**
4209   **formulation type. Number of each product tested and percentage (in parentheses).**

	Solvents	Surfactants	Oxidizers	Bases	Acids	Other
Under predicted	0	0	2 (13%)	1 (7%)	0	0
Correctly Predicted	4 (40%)	5 (29%)	9 (56%)	8 (57%)	3 (50%)	0
Over Predicted	6 (60%)	12 (71%)	5 (31%)	5 (36%)	3 (50%)	1 (100%)

4210

4211   6.3.2.2.3 Analysis of anti-microbial cleaning formulations with high solvent
4212   concentrations
4213
4214   In the analysis presented in Figures 6-18 & 6-19, we noticed that several
4215   formulations classified as being based on a solvent chemistry for cleaning were
4216   overpredicted relative to their Draize-based classification. This phenomena of some
4217   solvents being overpredicted has been observed before and was mentioned in the
4218   conclusions of the ICCVAM/NICEATM review of the BCOP assay which states in
4219   Section 6.2.1 that:"The accuracy analysis indicated that alcohols are often
4220   overpredicted (50% to 56% [7/14 to 9/16] false positive rate depending on the
4221   classification system used) in the BCOP test method."
4222
4223   The formulations that are solvent-based generally contain glycol ethers or
4224   ethanol as the solvent. We examined the formulation list (see Annex B) for all the
4225   test materials which were listed as containing some amount of either "solvent" or
4226   "glycol ether", no matter what the percentage or whether they were actually
4227   categorized as "solvent" by the submitter. Thirty-one such materials were identified.
4228   These materials were then identified on the scatter plots of BCOP scores versus
4229   Draize categories, and it was found that three of these materials were overpredicted
4230   (one by one category, two by two categories). A further analysis showed that these
4231   three materials all contained either "solvent" or glycol ethers at a concentration >5%.
4232   In total, there were 13 materials that had solvent concentrations above 5%. We
4233   gave these 13 materials a new designation of "High Solvent". The identities of the
4234   High Solvent materials are shown in Figures 6-18 and 6-19 by red boxes.
4235
4236   Because of earlier indications that some solvent-containing materials might
4237   be overpredicted, IIVS – for the last several years – has tested such materials in the
4238   BCOP assay using two different exposure times: 3 minutes and 10 minutes. We
4239   have generally noticed that the three minute exposure gives a better prediction of
4240   the actual irritancy potential than does the 10 minute exposure. Eight of the thirteen
4241   "High Solvents" had three minute exposure data, and when we graphed these
4242   values we found that all three of the overpredicted formulations were now correctly
4243   predicted (Figure 6-20).   Five high solvent materials which had been correctly

4244   predicted as EPA Category I materials (all had BCOP scores between 157.3 and
4245   444.3) could not be included since no three minute data had been collected when
4246   these materials were originally tested. None of the five of the materials were still
4247   available from the submitter and it was deemed too difficult to reformulate them.
4248

BCOP Scores vs. EPA Category
High Solvents (>5% alcohol/solvent)
(3 minute exposure)
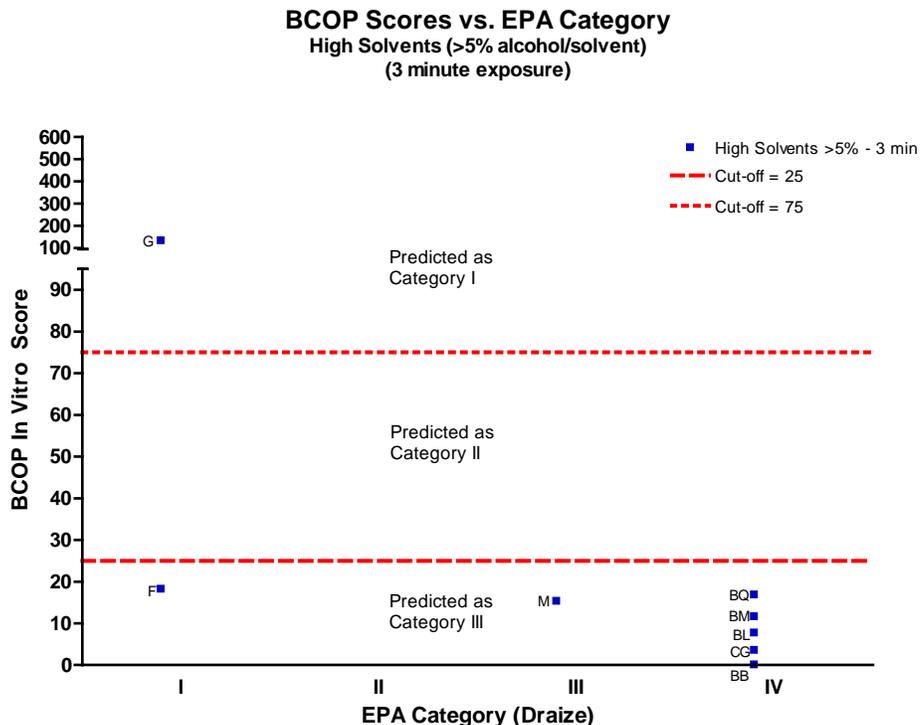
4249
4250   **Figure 6-20 BCOP *in vitro* scores (3 minute exposure) for High Solvents are plotted against**
4251   **EPA categories determined by the Draize test. Five High Solvent materials had 10 minute data**
4252   **only and therefore are not included in this graph. Proposed cut-off values with their predicted**
4253   **EPA categories are included.**
4254
4255   The remaining non-high solvent materials were then graphed as before using
4256   their 10 minute exposure time values (Figure 6-21). There are only 55 data points
4257   on Figure 6-21 because the thirteen high solvent materials are excluded.
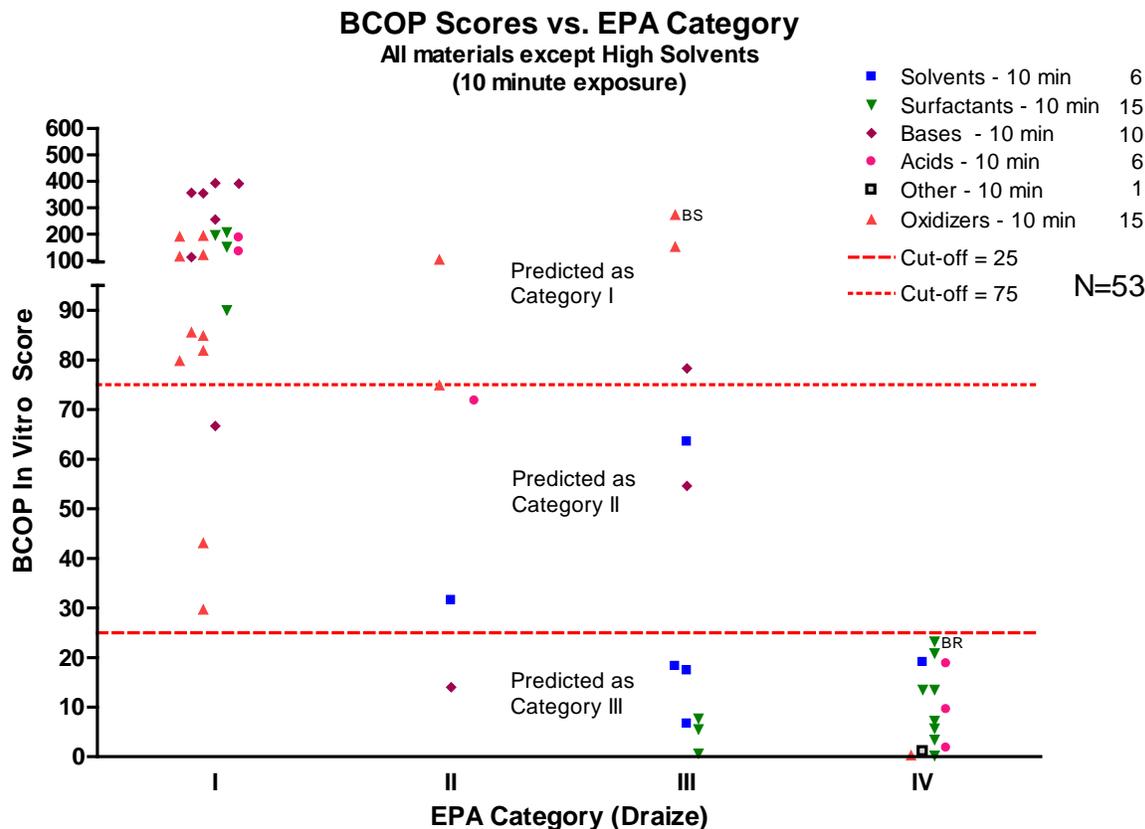
4258
4259 **Figure 6-21 BCOP *in vitro* scores for non-High Solvent materials plotted against EPA**
4260 **categories determined by the Draize test. Proposed cut-off values with their predicted EPA**
4261 **categories are included. The EPA categories for test materials BR and BS were determined**
4262 **by using the results of an LVET assay.**
4263
4264   A contingency table (Table 6-44) was then created for the EPA categorization
4265 by combining the results of Figures 6-20 & 6-21. The results from this analysis are
4266 reasonably similar to that of Table 6-40 where all of the materials were recorded
4267 using their 10 minute exposure values. Using the High Solvent approach the
4268 positive predictivity for Categories I, II and III were 84%, 38% and 25%, respectively;
4269 while using the prior approach the predictivites for these categories were 87.1%,
4270 27.3% and 28%, respectively. Thus there was some gain in the predictivity of
4271 Category II materials. However, percentages of underpredicted Category I materials
4272 increased from 9.7% to 16%, primarily because one Category I material (High
4273 Solvent) was misidentified as a Category III and five previously correctly predicted
4274 (using ten minute data) Category I High Solvent materials could not be used in the
4275 analysis since they had no 3 minute exposure data.
4276
4277

4278 **Table 6-44 Contingency table (based on a combination of the results from Figure 6-20 & 6-21)**
4279 **depicting the accuracy and predictivity of the BCOP assay for EPA toxicity categories**
4280 **(determined by the Draize test) using cut-off values of a BCOP *in vitro* score ≥ 75 = I, 75 >**
4281 **BCOP *in vitro* score ≥ 25 = II, and a BCOP *in vitro* score < 25 = III. The model does not propose**
4282 **to identify Category IV materials.**
4283

Draize- Determined EPA Category	BCOP Predicted EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	II	III	Total			
I	21	3	1	25	84%	NA	16%
II	1	3	1	5	60%	20%	20%
III	3	2	7	12	58%	42%	0%
IV	0	0	19	19	0%	100%	NA
Total	25	8	28	63	49%		
Predictivity	84%	38%	25%				
Category under predicted	NA	38%	7%				
Category over predicted	16%	25%	68%				

4284
4285   The discordant results assessed by product formulation for the BCOP assay
4286 and EPA toxicity categories are shown in Table 6-45. There were five
4287 underpredictions of the EPA category for base, oxidizing, and high solvent
4288 materials; however, 33% of solvents, 53% of surfactants, 27% of oxidizers, 20% of
4289 bases, 50% of acids, and 100% of other materials. Sixty-three percent of high
4290 solvents were overpredicted, but only by a single toxicity category.
4291
4292 **Table 6-45 Prediction results for the BCOP assay and EPA toxicity categories by product**
4293 **formulation type. Number of each product tested and percentage (in parentheses).**
4294

	Solvents	Surfactants	Oxidizers	Bases	Acids	Other	High Solvents
Under predicted	0	0	2 (13%)	2 (20%)	0	0	1 (12%)
Correctly Predicted	4 (67%)	8 (50%)	9 (60%)	6 (60%)	4 (57%)	0	2 (25%)
Over Predicted	2 (33%)	8 (50%)	4 (27%)	2 (20%)	3 (43%)	1 (100%)	5 (63%)

4295
4296   The same analysis of using 3 minute data for the High Solvent materials was
4297 conducted using GHS categories. Figure 6-22 shows the results using the High
4298 Solvents, and Figure 6-23 shows the results with the rest of the materials. Again
4299 three previously correctly predicted High Solvent Category I materials could not be
4300 used since there was no three minute exposure data for them.
4301

BCOP Scores vs. GHS Category
High Solvents (>5% alcohol/solvent)
(3 minute exposure)
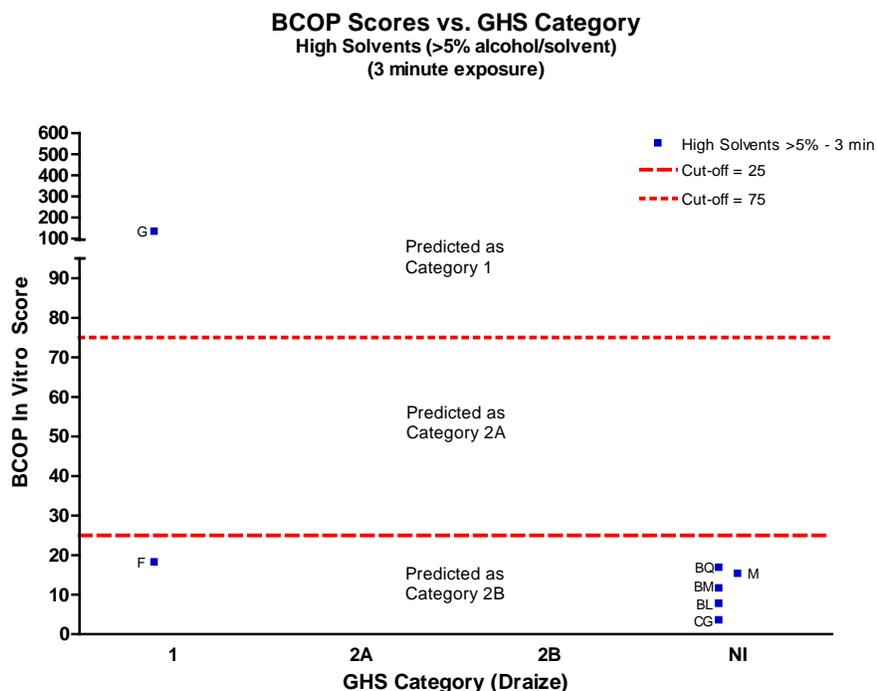
4302
4303 **Figure 6-22 BCOP *in vitro* scores plotted against GHS categories determined by the Draize**
4304 **test. Five materials had only 10 minute data and therefore are not included on this graph.**
4305 **Proposed cut-off values with their predicted GHS categories are included. Test material BB is**
4306 **not included due to the study criteria not being met for the GHS category.**

BCOP Scores vs. GHS Category
All materials except High Solvents
(10 minute exposure)
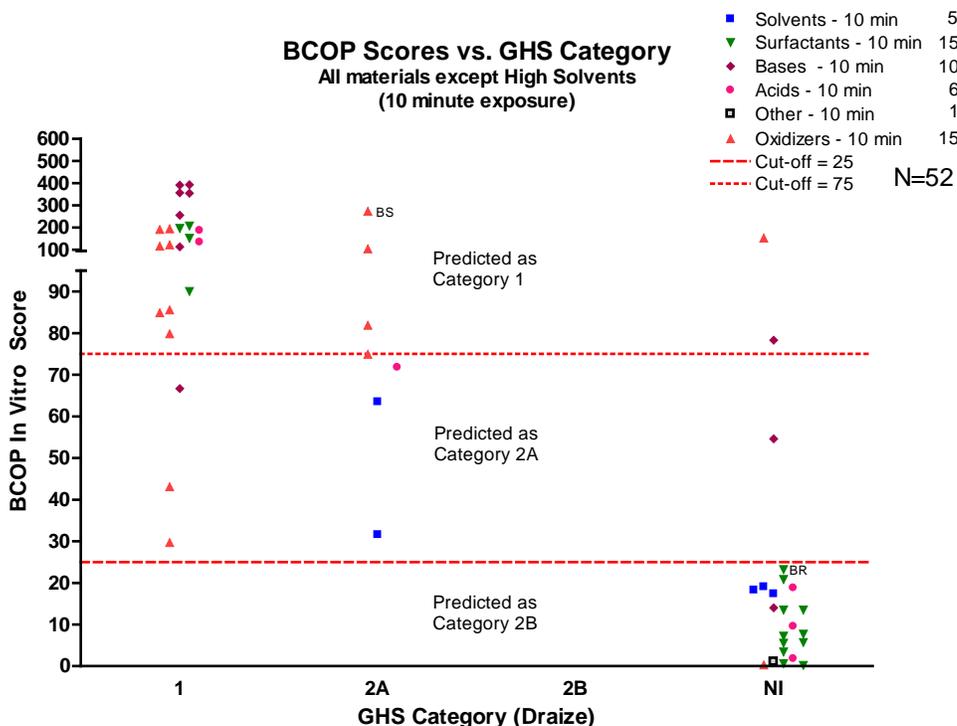
4307
4308 **Figure 6-23 BCOP *in vitro* scores plotted against GHS categories determined by the Draize**
4309 **test. Proposed cut-off values with their predicted GHS categories are included. The EPA**
4310 **categories of test materials BR and BS were determined using the LVET assay.**

4311   Table 6-46 shows the results of a contingency analysis of the GHS
4312   conducted by combining the results from both graphs. As can be seen by comparing
4313   with the previous GHS category analysis in Table 6-42, the predictivity improved
4314   slightly from the original analysis, but the underprediction of Category 1 materials
4315   increased slightly from 11% to 17%, primarily because one Category 1 material
4316   (High Solvent) was misidentified as a Category 2B and five previously correctly
4317   predicted (using ten minute data) Category 1 High Solvent materials could not be
4318   used in the analysis since they had no 3 minute exposure data.
4319
4320   **Table 6-46 Contingency table (based on Figure 6-22 & 6-23) depicting the accuracy and**
4321   **predictivity of the BCOP assay for GHS toxicity categories (determined by the Draize test)**
4322   **using cut-off values of a BCOP *in vitro* score ≥ 75 = 1, 75 > BCOP *in vitro* score ≥ 25 = 2A, and**
4323   **a BCOP *in vitro* score < 25 = 2B. The model does not propose to identify Category NL**
4324   **materials.**

Draize- Determined GHS Category	BCOP Predicted GHS Category				Concordance	Toxicity over predicted	Toxicity under predicted
	1	2A	2B	Total			
1	20	3	1	24	83%	NA	17%
2A	3	4	0	7	57%	43%	0%
2B	0	0	0	0	0%	0%	0%
NI	2	1	25	28	0%	100%	NA
Total	25	8	26	59	41%		
Predictivity	80%	50%	0%				
Category under predicted	NA	38%	4%				
Category over predicted	20%	13%	96%				

4325
4326   The discordant results assessed by product formulation for the BCOP assay
4327   and GHS toxicity categories are shown in Table 6-47. There were four
4328   underpredictions of the EPA category for base, oxidizing, and high solvent
4329   materials; however, 60% of solvents, 73% of surfactants, 36% of oxidizers, 43% of
4330   bases, 50% of acids, 100% of other materials, and 71% of high solvents were
4331   overpredicted.
4332
4333   **Table 6-47 Prediction results for the BCOP assay and EPA toxicity categories by product**
4334   **formulation type. Number of each product tested and percentage (in parentheses).**
4335

	Solvents	Surfactants	Oxidizers	Bases	Acids	Other	High Solvents
Under predicted	0	0	2 (13%)	1 (10%)	0	0	1 (14%)
Correctly Predicted	2 (40%)	4 (27%)	8 (53%)	6 (60%)	3 (50%)	0	1 (14%)
Over Predicted	3 (60%)	11 (73%)	5 (33%)	3 (30%)	3 (50%)	1 (100%)	5 (71%)

4336

4337   6.3.3 Histopathology Analysis
4338

4339   We have previously reported (Curren, Evans et al. 2000) that certain
4340   materials, especially those with oxidizing chemistry, may be under estimated when
4341   relying only on the *in vitro* score. Often these materials cause cellular changes in the

4342 cornea that are not manifested *in vitro* as damage by the conventional measures of
4343 opacity and permeability. Presumably similar changes *in vivo* do result in visible
4344 changes to the eye as a result of secondary recruitment and resulting migration of
4345 inflammatory cells into the corneal stroma. Thus we decided to do additional
4346 analysis of the predictive capacity of the BCOP assay by adding data from parallel
4347 studies of the histopathology of the treated corneas.
4348
4349   Additional rationale for the use of histopathology comes from the pioneering
4350 work of Drs. James Jester and Jim Maurer ((Maurer, Parker et al. 2002) who have
4351 shown that the area and depth of the initial ocular injury is a major predictor of the
4352 final lesion and its potential for recovery. A more complete description of this
4353 hypothesis and its relationship to the BCOP assay can be found in Annex G (Draft
4354 BCOP Histopathology Guidance Document).
4355
4356   For seventeen of the antimicrobial cleaning product materials, the treated
4357 bovine corneas were fixed, sectioned and stained for histopathological examination.
4358 The corneas were evaluated either by the staff of IIVS or by a subcontractor (a
4359 Board-certified veterinary pathologist) trained in histological analysis of bovine
4360 corneas. A detailed description of the types of lesions observed can be found in
4361 Annex G.
4362
4363   Histology was evaluated and described for the: 1) Upper, middle and lower
4364 epithelium; 2) Upper, middle, and lower stroma; and 3) Endothelium. Table 6-50
4365 relates the histological damage observed in a cornea to a specific EPA or GHS
4366 category. Decisions as to the category assigned were based primarily on the depth
4367 of injury. For the epithelium, this was measured primarily by tissue loss or the
4368 presence of necrotic cells. For the stroma, damage was characterized by 1)
4369 abnormal chromatin condensation or vacuolated nuclei in the keratocytes, 2)
4370 significant increase in collagen matrix vacuolization, or 3) loss of keratocytes.
4371 Damage to the endothelium was evidenced by loss of cells or increased cellular
4372 vacuolization.
4373
4374

4375   **Table 6-48 Scoring chart for histologically apparent damage and proposed EPA and GHS**
4376   **toxicity category.**

4377

Extent of Damage	Suggested EPA Category	Suggested GHS Category
Cell loss or damage extending no further than midway through the epithelium.	IV	NL
Cellular damage or collagen matrix damage extending no further than the upper third of the stroma	III	2B
Cellular damage or collagen matrix damage extending no further than two-thirds of the way through the stroma	II	2A
Cellular damage or collagen matrix damage extending into the lower third of the stroma and/or causing damage to the endothelial cells.	I	1

4378
4379   Table 6-49 identifies the materials used to treat the corneas, the BCOP *in*
4380   *vitro* score, the histology results, and the final determination of the EPA toxicity
4381   category.

4382
4383   **Table 6-49 Integration of histopathology results with BCOP *in vitro* scores to give final EPA**
4384   **toxicity category classification (based on prediction model of Figure 6-18). Test material code**
4385   **letters appear in Figure 6-24 & 6-25.**

Test Material	EPA Category by Draize Test	BCOP *In vitro* Score 10 min/3 min	EPA Category by Prediction Model	Histology Results 10 min/3 min	EPA Category (Based on Histopathology) 10 min/3 min	Final EPA Category 10 min/3 min
1 (V)	IV	20.8	III	Damage observed mid-stroma	II	II
2 (I)	III	0.6	III	Upper epithelium lost	IV	III
3 (H)	II	9.2	III	Damage into lower third of stroma	I	I
4 (F) High Solvent	I	514/18.2	I/III	Damage into lower third of stroma/Damage into lower third of stroma	I/I	I/I

5 (C)	I	29.7	II	Damage into lower third of stroma	I	I
6 (X)	I	81.9	I	Damage to lower stroma	I	I
7 (Y)	II	74.9	II	Damage to lower stroma	I	I
8 (Z)	II	31.6	II	Damage to Upper Half of stroma	II	II
9 (AV)	I	191.8	I	Damage into lower third of stroma	I	I
10 (AW)	I	43.1	II	Damage greater than 50% depth of stroma	II	II
11 (BJ)	III	54.6	II	Damage through upper 2/3s of stroma	II	II
12 (AE)	I	66.7	II	Damage through top half of stroma	II	II
13 (CG) High Solvent	IV	3.9/3.5	III/III	Damage into upper quarter of stroma/Upper epithelium lost	III/IV	III/III
14 (N)	III	152.7	I	Damage into lower third of stroma	I	I
15 (BS)	III (LVET)	278.1	I	Damage into lower third of stroma	I	I
16 (BR)	IV	23.2	III	Damage through upper third of stroma	III	III
17(EG)	II	71.8	II	Damage into lower third of stroma	I	I

4386

4387   6.3.3.1   Analysis of the predictive capacity of BCOP including histological
4388   evaluation for EPA hazard classifications

4389

4390   Using the results of the above histological observations, a further analysis of
4391   the predictive capacity for EPA toxicity categories of the combination of BCOP *in
4392   vitro* score and histopathology was performed. The EPA toxicity categories are
4393   plotted against the *in vitro* score (using the same cut-offs as previously described)
4394   for both the High Solvents (Figure 6-24) and the remaining materials (Figure 6-25).

4395   The materials which underwent histology analysis are circled and their final
4396   predicted toxicity category (as determined by Table 6-49) shown.
4397
4398
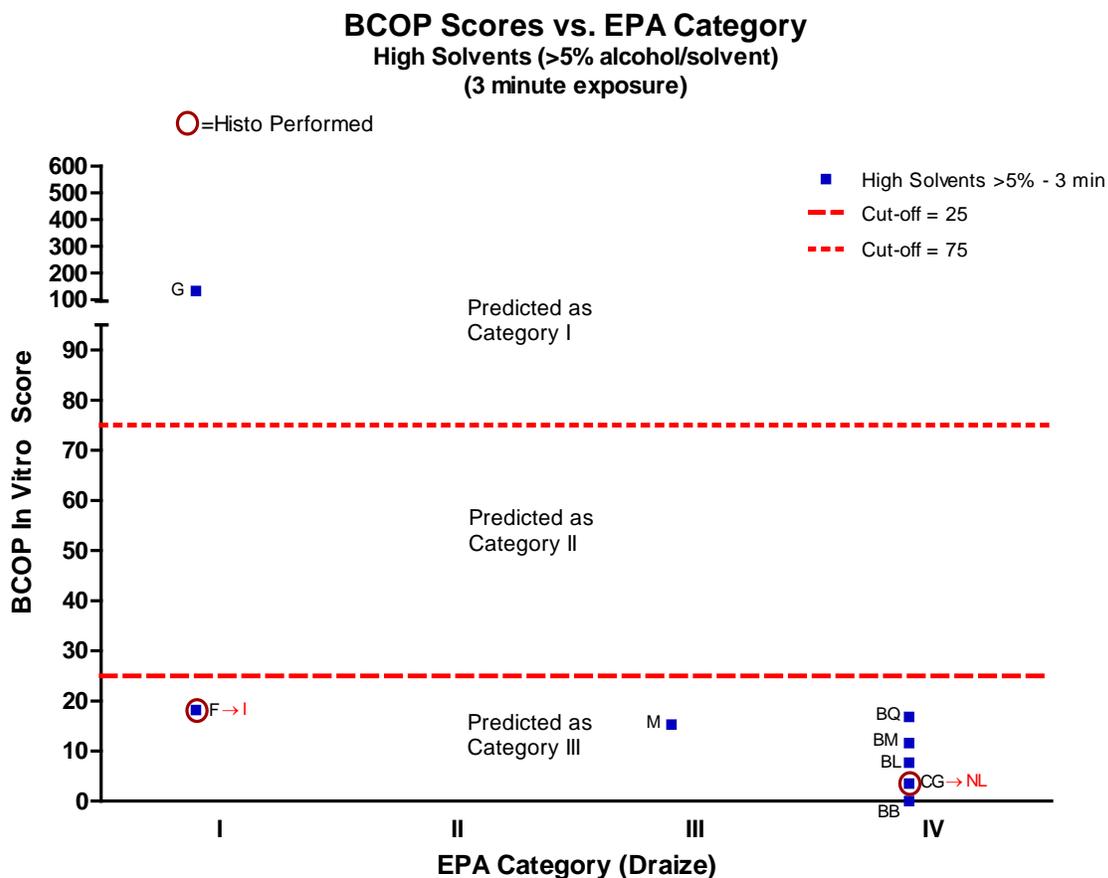
4399
4400   **Figure 6-24 BCOP *in vitro* scores (3 minute exposure) for High Solvent formulations plotted**
4401   **against EPA categories determined by the Draize test. Five High Solvent materials had 10**
4402   **minute data only and therefore are not included in this graph. Materials with histology-**
4403   **determined EPA categories are circled with the final category indicated.**
4404

BCOP Scores vs. EPA Category
All materials except High Solvents
(10 minute exposure)
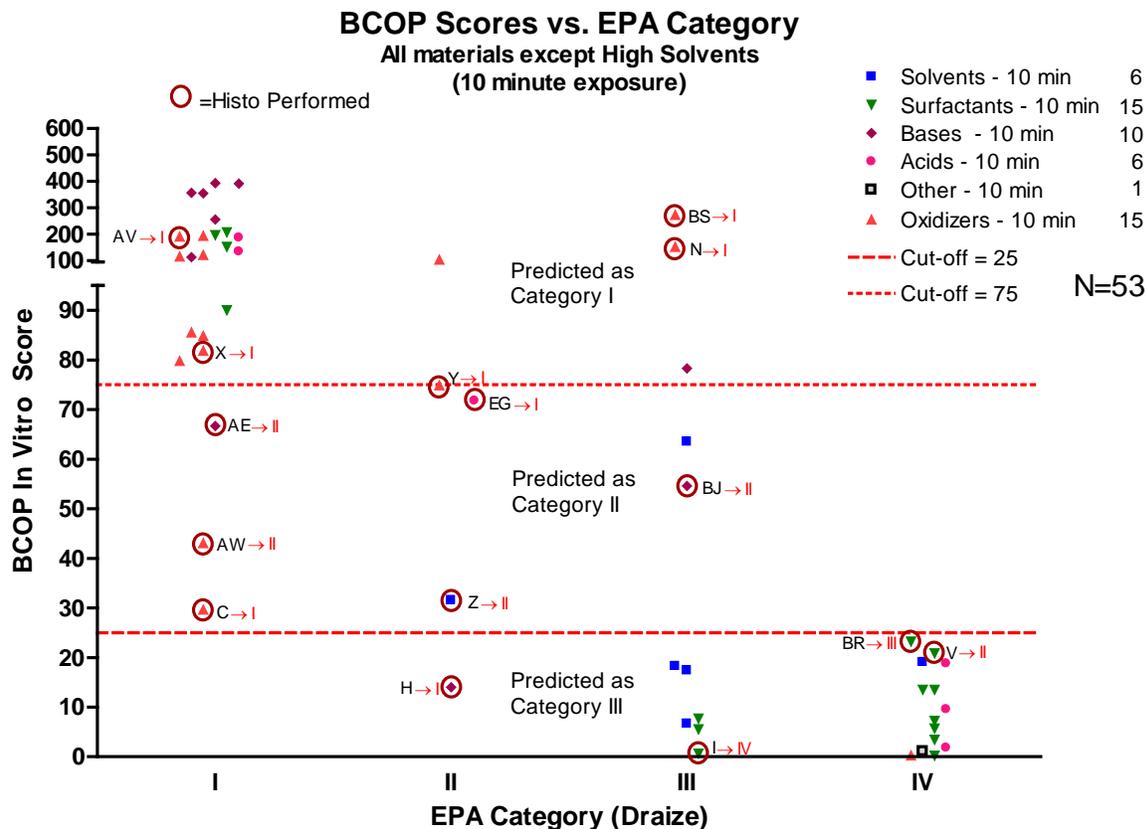
4405
4406 **Figure 6-25 BCOP *in vitro* scores plotted against EPA categories determined by the Draize**
4407 **test. Proposed cut-off values with their predicted EPA categories are included. The EPA**
4408 **categories of test materials BR and BS were determined using the LVET assay.**
4409
4410
4411   The contingency Table 6-50 shows the results of these analyses. It can be
4412 seen that adding histopathology analysis to the BCOP *in vitro* score leads to fewer
4413 EPA toxicity categories being underestimated. The sensitivity of the assay
4414 detecting EPA category I's improves to 92% (23 of 25 Category I's identified) from
4415 84% (Table 6-44). Similarly the underprediction of EPA Category II's improves from
4416 20% (Table 6-44) with BCOP *in vitro* score only, to 0% when histopathology is
4417 added.
4418
4419

4420 **Table 6-50 Contingency table (based on Figure 6-24 & 6-25) depicting the accuracy and**
4421 **predictivity of the BCOP assay for EPA toxicity categories (determined by the Draize test)**
4422 **using cut-off values of a BCOP *in vitro* score ≥ 75 = I, 75 > BCOP *in vitro* score ≥ 25 = II, and a**
4423 **BCOP *in vitro* score < 25 = III, plus histopathological evaluation. The model does not propose**
4424 **to identify Category IV materials.**
4425

Draize- Determined EPA Category	BCOP Predicted (with histology) EPA Category				Concordance	Toxicity over predicted	Toxicity under predicted
	I	II	III	Total			
I	23	2	0	25	92%	NA	8%
II	4	1	0	5	20%	80%	0%
III	3	2	7	12	58%	42%	0%
IV	0	1	18	19	0%	100%	NA
Total	30	6	25	61	51%		
Predictivity	77%	17%	28%				
Category under predicted	NA	33%	0%				
Category over predicted	23%	50%	72%				

4426
4427   The discordant results assessed by type of formulation for the BCOP assay
4428 with histology and EPA toxicity categories are shown in Table 6-51. There was one
4429 underprediction each of the EPA category for bases and oxidizing materials;
4430 however, 50% of solvents, 53% of surfactants, 33% of oxidizers, 30% of bases, 67%
4431 of acids, and 100% of other materials were overpredicted.

4432

4433   **Table 6-51 Discordant results for the BCOP assay and EPA toxicity categories.**

	Solvents	Surfactants	Oxidizers	Bases	Acids	Other
Under predicted	0	0	1 (7%)	1 (10%)	0	0
Correctly Predicted	7 (50%)	7 (47%)	9 (60%)	6 (60%)	2 (33%)	0
Over Predicted	7 (50%)	8 (53%)	5 (33%)	3 (30%)	4 (67%)	1 (100%)

4434

4435 6.3.3.2   Analysis by GHS category for BCOP including histological evaluation

4436

4437   Using the results of the histological observations, an analysis of the predictive
4438 capacity for GHS toxicity categories of the combination of BCOP *in vitro* score and
4439 histopathology was performed. The GHS toxicity categories are plotted against the
4440 *in vitro* score (using the same cut-offs as previously described) for both the High
4441 Solvents (Figure 6-26) and the remaining materials (Figure 6-27). The materials
4442 which underwent histology analysis are circled and their final predicted toxicity
4443 category (as determined by Table 6-49) shown.
4444

BCOP Scores vs. GHS Category
High Solvents (>5% alcohol/solvent)
(3 minute exposure)
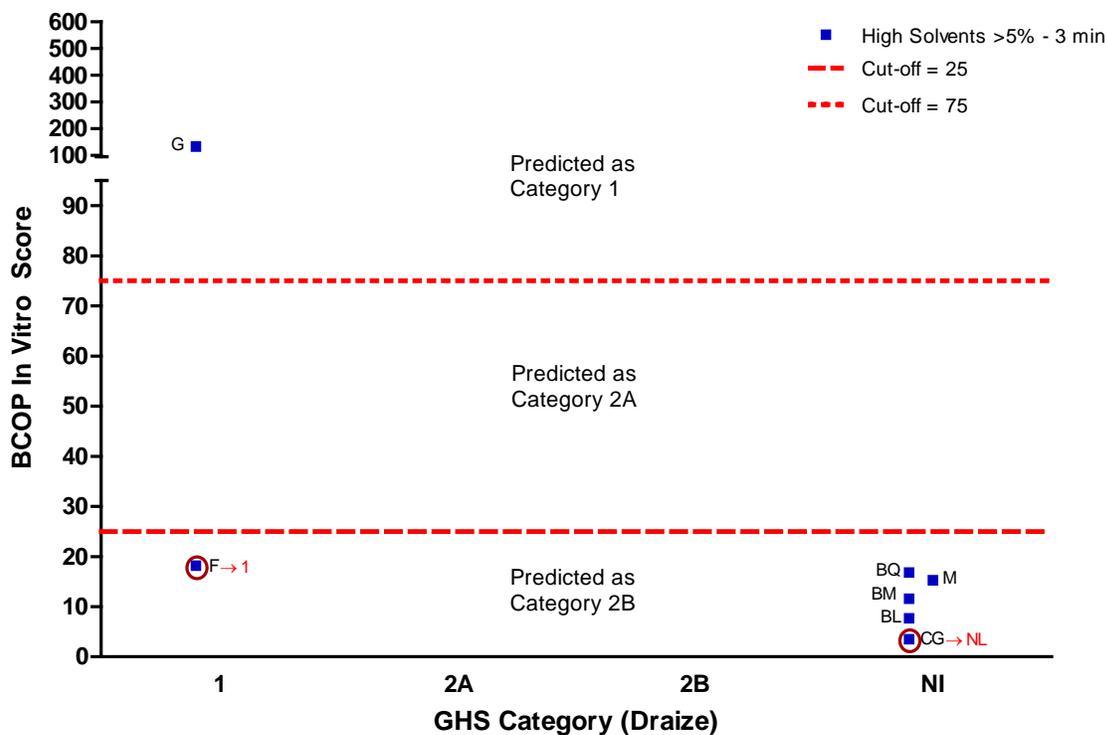
4445
4446 **Figure 6-26 BCOP *in vitro* scores (3 minute exposure) for High Solvent formulations plotted**
4447 **against GHS categories determined by the Draize test. Five High Solvent materials had only**
4448 **10 minute data and therefore are not included on this graph. Proposed cut-off values with**
4449 **their predicted GHS categories are included. Materials with histology-determined EPA**
4450 **categories are circled with the final category indicated. Test material BB is not included due**
4451 **to the study criteria not being met for the GHS category.**
4452
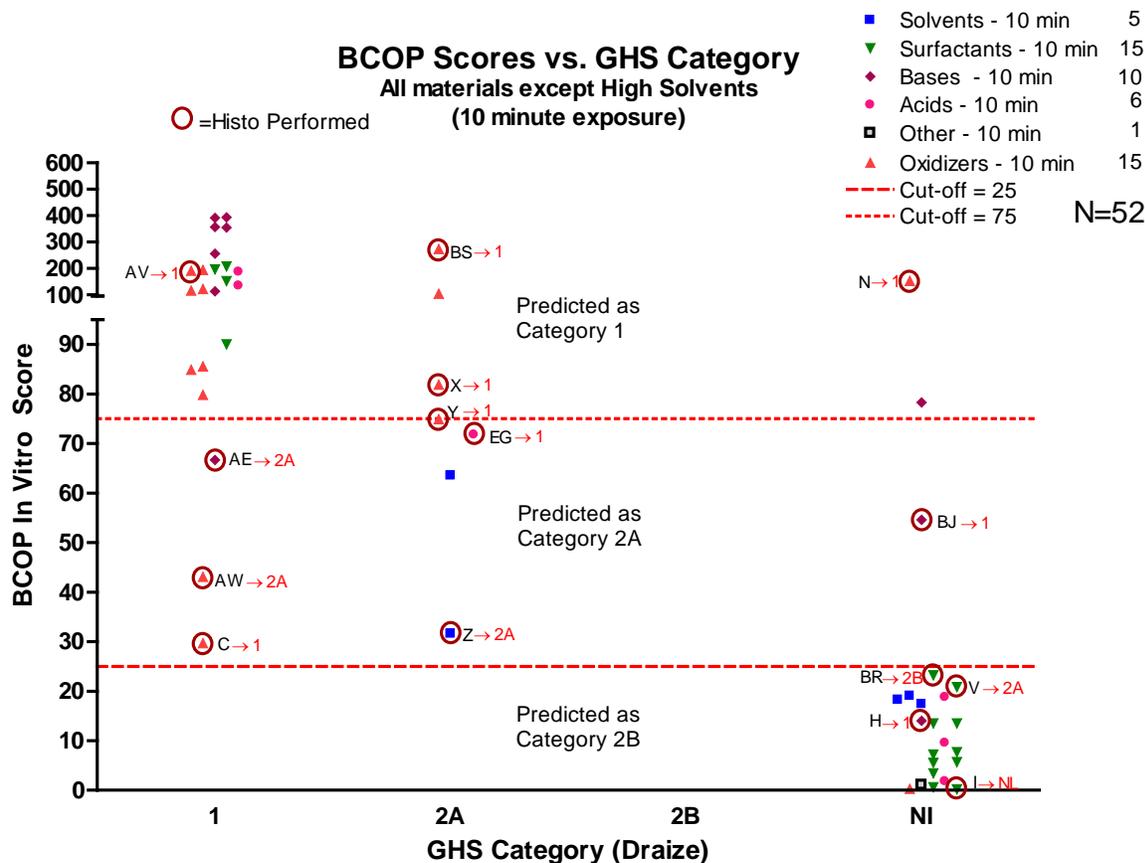
Figure 6-27 BCOP *in vitro* scores for non-High solvent materials plotted against GHS categories determined by the Draize test. Proposed cut-off values with their predicted GHS categories are included. The EPA categories of test materials BR and BS were determined using the LVET assay. Materials with histology-determined EPA categories are circled with the final category indicated.

The contingency Table 6-52 shows the results of these analyses. It can be seen that adding histopathology analysis to the BCOP *in vitro* score leads to fewer GHS toxicity categories being underestimated. The sensitivity of the assay for EPA category I's improves to 92% (22 of 24 Category 1's identified) from 83% (Table 6-46). However, the overprediction of GHS 2A materials increases from 43% (Table 6-46) to 71%.

4468 **Table 6-52 Contingency table (based on Figure 6-26 & 6-27) depicting the accuracy and**
4469 **predictivity of the BCOP assay for GHS toxicity categories (determined by the Draize test)**
4470 **using cut-off values of a BCOP *in vitro* score ≥ 75 = 1, 75 > BCOP *in vitro* score ≥ 25 = 2A, and**
4471 **a BCOP *in vitro* score < 25 = 2B. The model does not propose to identify Nonirritant materials.**

Draize- Determined GHS Category	BCOP Predicted (with histology) GHS Category				Concordance	Toxicity over predicted	Toxicity under predicted
	1	2A	2B	Total			
1	22	2	0	24	92%	NA	8%
2A	5	2	0	7	29%	71%	0%
2B	0	0	0	0	0%	0%	0%
NI	4	1	23	28	0%	100%	NA
Total	31	5	23	59	41%		
Predictivity	71%	40%	0%				
Category under predicted	NA	40%	0%				
Category over predicted	29%	20%	100%				

4472

4473   The discordant results assessed by type of formulation for the BCOP assay
4474 with histology and GHS toxicity categories are shown in Table 6-53. There was one
4475 underprediction each of the GHS category for bases and oxidizing materials;
4476 however, 67% of solvents, 73% of surfactants, 40% of oxidizers, 30% of bases, 67%
4477 of acids, and 100% of other materials were overpredicted.

4478

4479   **Table 6-53 Discordant results for the BCOP assay and GHS toxicity categories.**

	Solvents	Surfactants	Oxidizers	Bases	Acids	Other
Under predicted	0	0	1 (7%)	1 (10%)	0	0
Correctly Predicted	4 (33%)	4 (27%)	8 (53%)	6 (60%)	2 (33%)	0
Over Predicted	8 (67%)	11 (73%)	6 (40%)	3 (30%)	4 (67%)	1 (100%)

4480

4481 6.3.3.3 Conclusions from analysis of the BCOP predictive capacity

4482

4483   The BCOP assay appears to be most useful at the most irritating portion of the
4484 irritation spectrum. It is capable of identifying EPA toxicity category I, II, and III
4485 materials. When histology was considered, only two of the sixty-one materials (3%)
4486 were under predicted for EPA toxicity categories, and these were only under
4487 predicted by a single toxicity category. Over predictions were much more frequent,
4488 but this was driven by the fact that the BCOP assay seems incapable of clearly
4489 differentiating between Category III and Category IV materials. In fact 64% (18 of
4490 28) of the over predictions were the result of EPA category IV materials being
4491 predicted as EPA category III materials. Very similar results were found with the
4492 prediction model for GHS categories. Sixty-seven percent (22 of 33) of the over
4493 predictions were Non Irritating materials over predicted as category 2B's. The
4494 corporate participants have agreed that this outcome of over labeling some
4495 materials is acceptable to them.

4496

4497   Many High Solvent (>5% solvent) materials were overpredicted using the
4498   traditional 10 minute exposure. Predictions improved when a three minute exposure
4499   was used, and this shorter exposure is recommended for future use with high
4500   Solvent formulations.
4501
4502   Importantly, when BCOP testing (and selected histopathology) was conducted
4503   on 37 new antimicrobial cleaning product formulations, the results fit the pattern of
4504   the originally hypothesized prediction model. Thus the preliminary hypothesis was
4505   supported, lending considerable weight to the validity of this prediction model.
4506
4507   We report that histopathology can be performed on treated corneas – this allows
4508   for possible underpredictions to be discovered. Another assay (we propose
4509   Cytosensor or EpiOcular) can be used as a second tier test to differentiate EPA
4510   Category III from Category IV and less irritating materials, if needed. This will reduce
4511   the over prediction rate of the entire testing strategy.
4512
4513   **The preceding analysis of the BCOP data has led us to the following**
4514   **recommendations:**
4515
4516   1)   **In general, when testing anti-microbial cleaning product**
4517   **formulations, the BCOP assay should be conducted with a ten**
4518   **minute exposure.**
4519
4520   2)   **If the anti-microbial cleaning product contains a solvent at the level**
4521   **of 5% or greater, it should be tested with a three minute exposure.**
4522
4523   3)   **All anti-microbial cleaning products having an *In Vitro* Score ≥75**
4524   **should be classified as an EPA Category I or a GHS Category 1. <u>No**
4525   **histopathology needs to be conducted.</u>**
4526
4527   4)   **Anti-microbial cleaning products having an *In Vitro* Score <75 and ≥**
4528   **25 are given a preliminary classification of EPA Category II or GHS**
4529   **Category 2A. They should be further assessed with a**
4530   **histopathological evaluation and given the final categorization of**
4531   **whichever determination (in vitro score or histological evaluation)**
4532   **is more severe.**
4533
4534   5)   **Anti-microbial cleaning products having an *In Vitro* Score <25 are**
4535   **given a preliminary classification of EPA Category III or GHS**
4536   **Category 2B. They should be further assessed with a**
4537   **histopathological evaluation and given the final categorization of**
4538   **whichever determination (in vitro score or histological evaluation)**
4539   **is more severe.**
4540
4541   6)   **(Optional) To determine if an anti-microbial cleaning product which**
4542   **was categorized as either EPA III or GHS 2B is actually an EPA IV**

4543   **or a GHS NI, it should be further tested in either the Cytosensor or**
4544   **EpiOcular assays.**

4545

4546   6.4 Strategic testing approach

4547

4548   Data from each of the three proposed assays shows that they each have a
4549   set of strengths and weaknesses. What is especially apparent is that the Cytosensor
4550   and EpiOcular assays do not have the ability to clearly separate Category I and II
4551   materials from each other. However, both are able to identify a proportion of the
4552   very mild EPA category IV or GHS NI materials. Thus the utility of these two assays
4553   is in the mild end of the irritation spectrum.

4554

4555   The BCOP assay, in contrast, is able to separate the Category II materials
4556   from the Category I materials, but it is not able to differentiate between Category III
4557   materials and the Category IV materials.

4558

4559   The strategy we propose is a tiered testing process that can be initiated with
4560   any of the three assays (as long as the test material is physically compatible with
4561   that assay). Thus for a suspected highly aggressive material one would start with
4562   the BCOP assay. This test might immediately identify the material as a Category I or
4563   II material, in which case the testing would end. However, the BCOP assay might
4564   merely identify the material as less than a Category II (either a Category III or
4565   Category IV). If the manufacturer does not need to know whether or not it might be a
4566   Category IV, the testing again could stop with the material being labeled a Category
4567   III material. On the other hand if the manufacturer thought the material might be a
4568   Category IV, the testing could move to the next tier of EpiOcular or Cytosensor
4569   assay where the predictive capacity of the latter two assays in the mild range may
4570   appropriately identify the toxicity category.

4571

4572   Alternatively, testing could start with either the EpiOcular or the Cytosensor
4573   assay which have the ability to classify a material as a Category III, Category IV, or
4574   greater than a Category III. Since these assays cannot differentiate between
4575   Category II and Category I materials, the material would have to be labeled
4576   Category I if no more testing was desired. However, the material could be tested in
4577   the next tier (BCOP assay) if it was important to the manufacturer to know whether
4578   the material might be a Category II.

4579

4580   **The second test in this tiered approach is always an option. If the exact**
4581   **irritation category is not required, and the manufacturer can accept an over**
4582   **prediction for a material whose *in vitro* score was ambiguous, then a single**
4583   **test can always provide the necessary information.**

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7 Test Method Reliability

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4588   Test method reliability will be assessed by reporting on the intralaboratory
4589   repeatability I (runs conducted in a single laboratory within a short period of time
4590   [days]), intralaboratory reproducibility II (runs conducted in a single laboratory within
4591   an extended period of time [months]) and interlaboratory reproducibility (between-
4592   laboratory repeatability). Typically the reliability of a method is assessed utilizing the
4593   data sets contained within the BRD. However, in this submission there are
4594   insufficient examples of repeated studies to provide a rigorous assessment of
4595   reproducibility for each of the methods. Therefore, information from other sources (a
4596   Background Review Document of the Cytosensor submitted to ECVAM, a
4597   Background Review Document for the EpiOcular method submitted to ECVAM and
4598   a Background Review Document on the Bovine Cornea Opacity and Permeability
4599   Test Method prepared by NICEATM which has been reviewed by an Expert Panel)
4600   will be presented as evidence for reproducibility. Where data to assess
4601   reproducibility are available within this submission they will be appropriately utilized.
4602   Table 7-1 details the study, reported results, number of replicates, and format of the
4603   available data for each type of variability study described in Section 7.

4604

4605   The reliability of each of the three methods proposed for this testing strategy
4606   will be addressed individually below. For most of the examples, reproducibility is
4607   assessed by calculating the CV for MRD_{50}, ET_{50}, or *in vitro* score values obtained
4608   from identical materials.

4609

4610   **Table 7-1 Description of the results reported for each variability study.**

Studies	Results Reported					
	Variability	*In Vitro* Test	Number of test substances	No. of operators	No. of assays	Format of data (raw data, summary results, other)
EC/HO	Intralaboratory	Cytosensor	35	Unknown	3 - 5	Summary; Mean
COLIPA	Intralaboratory	Cytosensor	26 and 29[1]	Unknown	≥3 for each of 2 labs	Raw data; Mean, SD and CV
20 chemicals from EC/HO and the COLIPA study run by same lab	Intralaboratory	Cytosensor	16	Unknown	≥3	See above for individual studies
Microbiological Associates/IIVS positive control	Intralaboratory	Cytosensor	1	Multiple	629	Raw data; mean; SD and CV
Formulations submitted for this BRD	Intralaboratory (within run and between experiment)	EpiOcular	15	Unknown	1 – 3 exp. 2 rep./exp.	Raw data; Mean, SD and CV
MatTek/IIVS positive control	Intralaboratory	EpiOcular	1 tested multiple times	Multiple	Unknown[2]	Mean, SD and CV
Colgate-Palmolive Phase II & III	Interlaboratory	EpiOcular	19 – 4 labs 54 – 2 labs	Unknown	2 - 4	Raw data; Mean, SD and CV
Formulations submitted for this BRD	Intralaboratory (within run)	BCOP	75	Multiple	3 - 5	Raw data; Mean, SD and CV
Formulations submitted for this BRD	Intralaboratory (between experiments)	BCOP	5	Multiple	2 - 6	Mean, SD, and CV
Gautheron et al.	Interlaboratory	BCOP	51	Multiple	4-12	Mean
Balls et al.	Interlaboratory	BCOP	59	Multiple	Unknown	Mean, CV
Southee et al.	Interlaboratory	BCOP	16	Multiple	Unknown	Mean, CV

4611   [1]One lab tested 26 materials while a second lab tested 29 materials
4612   [2]The same material was evaluated in two labs over a nine year period. The exact number of experiments is unknown.

4613   ## 7.1 Cytosensor

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4615   ### 7.1.1 Cytosensor intralaboratory repeatability I

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4617   The within-run reproducibility could not be assessed for the materials
4618   submitted for this study because the Cytosensor reports were not readily available.
4619   However, within-run reproducibility has been demonstrated for the Cytosensor in
4620   several large international validation studies as presented in a BRD submitted to
4621   ECVAM. Table 7-2 presents the results extracted from the BRD for the EC/HO
4622   validation study (Balls, Botham et al. 1995) for a group of 32 materials (a mixture of
4623   surfactant and non-surfactant materials). For this study the mean CV was 38.9%
4624   and the median CV was 30.5%. The distribution of product categories for the within-
4625   laboratory reproducibility of the CM is shown in Table 7-3.
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4631 **Table 7-2 Within-laboratory reproducibility of CM from archived data that was originally obtained at Microbiological Associates, Inc. for**
4632 **the EC/HO study (Balls, Botham et al. 1995). The protocol utilized the CM using Transwells and an 810 second exposure time. At least**
4633 **triplicate runs were performed.**
4634

Chemical	Formulation Type	Values used to calculate the reported Mean MRD_{50} (mg/mL)					Values not used to calculate the reported Mean MRD_{50} (mg/mL)					Results using the values used to calculate the reported mean				Results using all values other than > or < values to calculate mean[a]			
		Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Range Finding Assay	Assay A	Assay B	Assay C	Assay D	Average MRD_{50} (mg/mL)	N	SD	CV (%)	Average MRD_{50} (mg/mL)	N	SD	CV (%)
2,5-Dimethylohexanediol	SO		151.36	151.36	165.96		>5.00					156.22	3	8.43	5.4	156.22	3	8.43	5.4
Acetone	SO		144.54	165.96	114.82		>5.00					141.77	3	25.68	18.1	141.77	3	25.68	18.1
Ammonium nitrate	Other		169.82	125.89	144.54		>5.00					146.75	3	22.05	15.0	146.75	3	22.05	15.0
Benzalkonium chloride	SU			0.48	0.43	0.50	0.35	>3.50	<3.00			0.47	3	0.04	8.2	0.44	4	0.07	15.6
Benzalkonium chloride	SU		0.95	0.98	1.38		3.01					1.10	3	0.24	21.7	1.58	4	0.97	61.5
Benzalkonium chloride [1]/[2]	SU		5.01	3.89	7.08		3.02					5.33	3	1.62	30.4	4.75	4	1.75	36.9
Cetylpyridinium bromide	SU		0.98	1.15	0.93		3.09					1.02	3	0.11	11.1	1.54	4	1.04	67.6
Cetylpyridinium bromide	SU		1.26	0.72	2.69		1.20					1.56	3	1.02	65.3	1.47	4	0.85	57.8
Cetylpyridinium bromide	SU		128.82	91.20	91.20		>5.00					103.74	3	21.72	20.9	103.74	3	21.72	20.9
Ethanol*	SO		162.18	67.61	147.91		>5.00					125.90	3	50.98	40.5	125.90	3	50.98	40.5
Ethyl acetate	SO			53.70			>4.70	>4.75	>4.75			53.70	1			53.70	1		
Gammabutyrolactone	Other		93.33	144.54	112.20		>5.00					116.69	3	25.90	22.2	116.69	3	25.90	22.2
Glycerol	SO		218.78		131.83	204.17	>5.00		>5.38			184.93	3	46.56	25.2	184.93	3	46.56	25.2
Imidazole*	SU			23.44	22.39	23.44	24.55	>5.25				23.09	3	0.61	2.6	23.45	4	0.88	3.8
Isobutanol	SO		29.51	28.84	27.54		4.60					28.63	3	1.00	3.5	22.62	4	12.04	53.2
Isopropanol	SO		89.13	93.33	91.20		4.74					91.22	3	2.10	2.3	69.60	4	43.27	62.2
L-Aspartic acid	AC		0.85	2.63	0.71		2.75					1.40	3	1.07	76.7	1.74	4	1.11	63.8
Methyl acetate	SO		89.13	93.33	93.33		4.98					91.93	3	2.43	2.6	70.19	4	43.52	62.0
Methyl ethyl ketone	SO		53.70	45.71	52.48		4.82					50.63	3	4.31	8.5	39.18	4	23.17	59.1
Potassium cyanate	Other		50.12	30.90	30.20		26.92					37.07	3	11.30	30.5	34.53	4	10.53	30.5
Promethazine HCl	Other		1.66	1.48	1.32		1.02					1.49	3	0.17	11.5	1.37	4	0.27	19.7
Pyridine	Other		28.84	30.20	30.90		21.88					29.98	3	1.05	3.5	27.96	4	4.14	14.8
Sodium hydroxide	AL		0.87	2.24	2.09		7.24					1.73	3	0.75	43.3	3.11	4	2.82	90.7
Sodium hydroxide	AL		14.13	26.92	8.32	21.88	4.47					17.81	4	8.23	46.2	15.14	4	9.29	61.4
Sodium lauryl sulfate	SU		3.02	2.88		3.24	3.09					3.05	3	0.18	5.8	3.06	4	0.15	4.8
Sodium lauryl sulfate	SU		0.81	0.54	0.49		2.95					0.61	3	0.17	28.5	1.20	4	1.18	98.3
Thiourea	Other		50.12	48.98	53.70		>5.00					50.93	3	2.47	4.8	50.93	3	2.47	4.8
Trichloroacetic acid	AC		1.82	5.13	1.62		3.09					2.86	3	1.97	69.0	2.92	4	1.61	55.3
Trichloroacetic acid	AC		15.85	9.77	16.98		3.93					14.20	3	3.88	27.3	11.63	4	6.03	51.9
Triton X-100	SU		1.35	2.57	2.19		2.95					2.04	3	0.62	30.7	2.26	4	0.69	30.3
Triton X-100 [1]/[2]	SU		4.57	2.82	3.02		3.16					3.47	3	0.96	27.6	3.39	4	0.80	23.5
Tween 20	SU		7.76	4.79	4.57		7.41					5.71	3	1.78	31.3	6.13	4	1.69	27.5
[b]		not irr. at sol. (4.00)					>4.00	>4.00	>4.00	>4.00									
[b]		not irr. at sol. (4.00)					>4.00	>4.00	>4.00	>4.00									
[b]		not irr. at sol. (5.50)					>5.00	5.02	>5.50	>5.50	>5.50					5.02			
Mean															23.9				38.9
Median															21.7				30.5

[a] mean calculated using the values from assays 2-5, plus range finding assay if an actual value was obtained, i.e. < and > values were omitted.
[b] the identity of these chemicals is unknown
4635 AC - Acid, AL - Alkaline, SO - Solvent, SU - Surfactant

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Table 7-3 Distribution of product categories for the within-laboratory reproducibility of the CM.

Product Categories	Number of products tested
Surfactants	12
Acids	3
Bases	2
Solvents	9
Other	6
Unknown	3
Total	**35**

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4640   Results from a second international validation study organized by the
4641 European cosmetics trade association COLIPA are presented in Tables 7-4 through
4642 7-7. These data come from both surfactant materials (Tables 7-4 and 7-6) and non-
4643 surfactant materials (Tables 7-5 and 7-7). Two different laboratories participated in
4644 this study and the individual results for each are presented. It can be seen that the
4645 first laboratory had a mean CV of 19.7% for the surfactant materials and a mean CV
4646 of 15.4% for the non-surfactant materials. The second laboratory had a mean CV of
4647 14.3% for the surfactant materials and a mean CV of 10.4% for the non-surfactant
4648 materials.   The distribution of product categories for the within-laboratory
4649 reproducibility of the COLIPA study is shown in Table 7-8.

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4651   For more details of each of these studies plus within-run repeatability from
4652 several additional studies please see Section 3. Within-laboratory reproducibility in
4653 the Cytosensor BRD. This can be provided to ICCVAM by the authors of this BRD
4654 after its review by ECVAM, or ICCVAM can request it directly from ECVAM at any
4655 time.

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4658 **Table 7-4   Surfactant Materials – COLIPA Within-laboratory reproducibility of CM from**
4659 **archived Microbiological Associates, Inc. data created for the COLIPA study for surfactant**
4660 **materials (Brantom, Bruner et al. 1997; Harbell, Osborne et al. 1999). The protocol utilized**
4661 **L929 cells and an 810 second exposure. Twenty-nine total materials were tested.**

Substance	Formulation Type	MRD_{50} (mg/ml)	SD	CV (%)	Number of replicates
Shampoo #1 normal	**SU**	**0.75**	**0.21**	**28.7**	**3**
Eye make-up remover	**SU**	**87.77**	**1.17**	**1.3**	**3**
Triton X-100 1%	**SU**	**21.17**	**4.21**	**19.9**	**3**
Tween 20	**SU**	**9.50**	**5.31**	**55.9**	**3**
SLS 3%	**SU**	**3.23**	**0.65**	**20.2**	**3**
Triton X-100 5%	**SU**	**4.66**	**0.52**	**11.1**	**3**
Benzalkonium chloride 1%	**SU**	**4.11**	**0.89**	**21.6**	**3**
SLS 15%	**SU**	**0.52**	**0.02**	**3.5**	**3**
SLS 30%	**SU**	**0.31**	**0.02**	**5.8**	**3**
Triton X-100 10%	**SU**	**2.47**	**0.57**	**23.0**	**3**
Benzalkonium chloride 5%	**SU**	**0.81**	**0.10**	**12.7**	**3**
Benzalkonium chloride 10%	**SU**	**0.32**	**0.07**	**21.0**	**3**
Pump Deodorant	**SU**	**19.35**	**9.38**	**48.5**	**3**
Gel cleaner	**SU**	**5.68**	**2.37**	**41.8**	**3**
Shampoo - baby	**SU**	**2.51**	**0.96**	**38.1**	**3**
Hair styling lotion	**SU**	**164.82**	**7.98**	**4.8**	**3**
Liquid soap #1	**SU**	**0.88**	**0.03**	**3.5**	**3**
Mouthwash	**SU**	**37.84**	**3.55**	**9.4**	**3**
Skin cleaner	**SU**	**0.63**	**0.10**	**16.3**	**3**
Cetylpyridinium bromide 6%	**SU**	**1.36**	**0.20**	**14.5**	**3**
Polyethylene glycol 400	**SU**	**296.50**	**34.17**	**11.5**	**3**
Mean				19.7	
Median				16.3	

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Table 7-5 Non-Surfactant Materials – COLIPA Within-laboratory reproducibility of CM from archived Microbiological Associates, Inc. data created for the COLIPA study for non-surfactant materials (Brantom, Bruner et al. 1997; Harbell, Osborne et al. 1999). The protocol utilized L929 cells and an 810 second exposure. Twenty-nine materials were tested.

Substance	Formulation Type	MRD$_{50}$ (mg/ml)	SD	CV (%)	Number of replicates
Methyl ethyl ketone	SO	54.18	3.16	5.8	3
Imidazole	SU	18.84	5.52	29.3	3
Propylene glycol		265.07	3.54	1.3	3
Glycerol	SO	214.83	25.35	11.8	3
Sodium hydroxide 1%	AL	9.09	1.00	11.0	3
Isopropanol	SO	52.59	17.20	32.7	3
Sodium hydroxide 10%	AL	4.33	0.15	3.5	3
Trichloroacetic acid 30%	AC	1.12	0.31	28.1	3
Mean				15.4	
Median				11.4	

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Table 7-6 Surfactant Materials – COLIPA Within-laboratory reproducibility of CM from archived CellTox AB data created for the COLIPA study for surfactant materials (Brantom, Bruner et al. 1997; Harbell, Osborne et al. 1999). The protocol utilized L929 cells and an 810 second exposure. Twenty-six materials were tested.

Substance	Formulation Type	MRD$_{50}$ (mg/ml)	SD	CV (%)	Number of replicates
Shampoo #1 normal	SU	0.72	0.06	8.1	3
Eye make-up remover	SU	99.31	1.00	1.0	3
Triton X-100 1%	SU	16.79	0.73	4.3	3
Tween 20	SU	3.49	0.62	17.7	3
SLS 3%	SU	2.78	0.07	2.7	3
Triton X-100 5%	SU	2.42	0.07	2.7	3
Benzalkonium chloride 1%	SU	4.33	1.19	27.4	3
SLS 15%	SU	0.51	0.02	3.3	3
Triton X-100 10%	SU	1.24	0.28	22.9	3
Benzalkonium chloride 5%	SU	1.38	0.12	8.9	3
Benzalkonium chloride 10%	SU	0.31	0.05	16.4	3
Pump Deodorant	SU	47.74	28.34	59.4	3
Gel cleaner	SU	5.47	1.20	22.0	3
Shampoo - baby	SU	2.15	0.73	33.7	3
Hair styling lotion	SU	292.01	6.07	2.1	3
Liquid soap #1	SU	0.68	0.10	14.0	3
Mouthwash	SU	46.85	9.20	19.6	3
Skin cleaner	SU	0.76	0.05	6.0	3
Polyethylene glycol 400	SU	316.23	0.00	0.0	3
Mean				14.3	
Median				8.9	

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4675 **Table 7-7 Non-Surfactant Materials – COLIPA Within-laboratory reproducibility of CM from**
4676 **archived CellTox AB data created for the COLIPA study for surfactant materials (Brantom,**
4677 **Bruner et al. 1997; Harbell, Osborne et al. 1999). The protocol utilized L929 cells and an 810**
4678 **second exposure. Twenty-six materials were tested.**

Substance	Formulation Type	MRD_{50} (mg/ml)	SD	CV (%)	Number of replicates
Imidazole	**SU**	**26.03**	**0.99**	**3.8**	**3**
Propylene glycol		**218.86**	**7.59**	**3.5**	**3**
Glycerol	**SO**	**208.70**	**3.06**	**1.5**	**3**
Isopropanol	**SO**	**124.51**	**25.26**	**20.3**	**3**
Sodium hydroxide 1%	**AL**	**13.59**	**5.11**	**37.6**	**3**
Sodium hydroxide 10%	**AL**	**0.60**	**0.01**	**1.9**	**3**
Trichloroacetic acid 30%	**AC**	**1.24**	**0.05**	**4.2**	**3**
Mean				10.4	
Median				3.8	

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4680   **Table 7-8 Distribution of product categories for the within-**
4681   **laboratory reproducibility of the COLIPA study**

Product Categories	Number of products tested
Surfactants	21
Acids	1
Bases	2
Solvents	3
Other	
Total	**27**

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4683 ## 7.1.2 Cytosensor intralaboratory reproducibility II

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4685   There were no examples of intralaboratory reproducibility for studies
4686 submitted specifically for this BRD. However, there is one example illustrated in the
4687 Cytosensor BRD which was submitted to ECVAM. This example was created by
4688 observing that some identical materials were tested in both the EC/HO validation
4689 study for eye irritation and the COLIPA-sponsored validation study which occurred
4690 approximately 21 months later. Although the materials are listed by the same name
4691 in each study, it is unclear whether the materials were actually identical (as far as
4692 purity and the presence of contaminants) since they were procured at a different
4693 time and possibly from different sources.

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4695   Table 7-9 presents the results for 11 surfactant materials tested by one
4696 laboratory during the EC/HO study and the COLIPA study. Both cetylpyridinium
4697 bromide (10%) and polyethylene glycol 400 were deemed incompatible with the test
4698 system in one study, but not in the other. They are both listed to show that there is
4699 some variability associated with determining whether or not a material is compatible
4700 with the test apparatus. Similarly for the non-surfactant materials shown in Table 7-
4701 10, ethyl acetate was considered incompatible with the Cytosensor in one study but
4702 not in the other. It can be seen that mean CV for the 9 surfactant material tested in
4703 both studies was 17.4%, and the mean CV for the 7 non-surfactant materials tested
4704 in both studies was 32.5%.   The distribution of product categories for the

4705   intralaboratory reproducibility of the COLIPA and EC/HO comparison is shown in
4706   Table 7-11.
4707   **Table 7-9 Surfactant materials - Comparison of the MRD$_{50}$ values for testing conducted**
4708   **approximately 21 months apart**

Substance	Formulation Type	COLIPA Mean MRD$_{50}$ (mg/mL) [CV%] MA	EC/HO Mean MRD$_{50}$ (mg/mL) [CV%] SM 31	Mean MRD$_{50}$ (mg/mL)	SD	CV (%)
Tween 20	SU	9.50 [55.9]	5.53 [31.3]	7.50	2.83	37.7
Sodium lauryl sulphate 3%	SU	3.23 [20.2]	3.04 [6.0]	3.13	0.15	4.8
Triton X-100 5%	SU	4.66 [11.1]	3.39 [27.6]	4.03	0.90	22.3
Benzalkonium chloride 1%	SU	4.11 [21.6]	5.16 [30.4]	4.62	0.72	15.6
Sodium lauryl sulphate 15%	SU	0.517 [3.5]	0.60 [28.5]	0.56	0.06	10.9
Triton X-100 10%	SU	2.47 [23.0]	1.96 [30.7]	2.21	0.37	16.6
Benzalkonium chloride 5%	SU	0.811 [12.7]	1.09 [21.7]	0.96	0.20	21.4
Benzalkonium chloride 10%	SU	0.321 [21.0]	0.47 [8.5]	0.39	0.10	26.3
Cetylpyridinium bromide 6%	SU	1.36 [14.5]	1.35 [65.3]	1.35	0.01	0.6
Cetylpyridinium bromide 10%	SU	*	1.02 [11.1]	*	*	*
Polyethylene glycol 400	SU	296.5 [11.5]	*	*	*	*
Mean		**[19.5]**	**[26.11]**			**17.4**
Median		**[17.4]**	**[28.05]**			**16.6**

4709   * - Material determined to be unsuitable for testing

4710

4711   **Table 7-10 Non-surfactant materials - Comparison of the MRD$_{50}$ values for testing conducted**
4712   **approximately 21 months apart**

Substance	Formulation Type	COLIPA Mean MRD$_{50}$ (mg/mL) [CV%] MA	EC/HO Mean MRD$_{50}$ (mg/mL) [CV%] SM 31	Mean MRD$_{50}$ (mg/mL)	SD	CV (%)
Imidazole	SU	18.8 [29.3]	23.1 [2.7]	20.95	3.04	14.5
Glycerol	SO	214.8 [11.8]	180.7 [26.6]	197.75	24.11	12.2
Sodium hydroxide 1%	AL	9.09 [11.0]	16.2 [50.0]	12.65	5.03	39.8
Isopropanol	SO	52.6 [32.7]	91.2 [2.3]	71.90	27.29	38.0
Methyl ethyl ketone	SO	54.2 [5.8]	50.5 [8.5]	52.35	2.62	5.0
Sodium hydroxide 10%	AL	4.33 [3.5]	1.60 [43.3]	2.97	1.93	65.1
Trichloroacetic acid 30%	AC	1.12 [28.1]	2.47 [69.0]	1.80	0.95	53.2
n-Butyl acetate	SO	*	*	*	*	*
Ethyl acetate	SO	*	53.7	*	*	*
Mean		**[17.5]**	**[28.9]**			**32.5**
Median		**[11.8]**	**[26.6]**			**22.6**

4713   * - Material determined to be unsuitable for testing

4714

4715   **Table 7-11 Distribution of product categories for the**
4716   **intralaboratory reproducibility of the CM.**

Product Categories	Number of products tested
Surfactants	11
Acids	1
Bases	2
Solvents	5
Other	0
Total	**19**

4717

4718   Another data set that can give information about intralaboratory
4719   reproducibility is the response of a single material over time. The following
4720   description is extracted from the Cytosensor BRD submitted to ECVAM.
4721

4722   "The CM instrument was first used by the *in vitro* toxicology staff at
4723   Microbiological Associates, Inc. in 1994. At that time the practice of maintaining a
4724   graphical record of the results of the positive control material – 10% SLS in sterile,
4725   deionized water – was begun (Figure 7-1). This practice has continued through the
4726   transfer of the instrument and staff to the Institute for *In Vitro* Sciences, Inc. in 1997,
4727   and continues to this day. Table 7-12 presents a summary of the results for 629
4728   assays conducted over a 12 plus year period as well as the results from the last 94
4729   assays conducted over the last two years. That little change has occurred in the
4730   absolute MRD_{50} in the last 12 years can be inferred from the 12 year average of
4731   0.0799 mg/mL versus the last two year's average of 0.0775 mg/mL. The average
4732   CV calculated over the last 12 years is 14.3%. Over the last approximately 2 years
4733   the average CV has increased to 18.9%.
4734

4735   **Table 7-12 Positive Control Data of SLS completed at IIVS**

Substance	Dates	No. of Assays	Mean MRD_{50} (mg/mL)	SD	CV (%)
SLS	April, 14 1994 – June 30, 2006	629	0.0799	0.011	14.3
SLS	March 2, 2004 - June 30, 2006	94	0.0775	0.015	18.9

4736

4737   SLS MRD_{50} values are plotted on a control graph with upper and lower cut-off
4738   ranges graphed at two SD of all data (March 2004 – June 2006). Assays performed
4739   on days when the MRD_{50} fell outside of the two SD range (5 points on this graph)
4740   were repeated. Because on some days more than one SLS control was run, some
4741   points may overlap such that it may appear that fewer than 94 values are plotted.
4742

4743   It appears from these data that there is good long term with-in lab
4744   reproducibility for a single material."
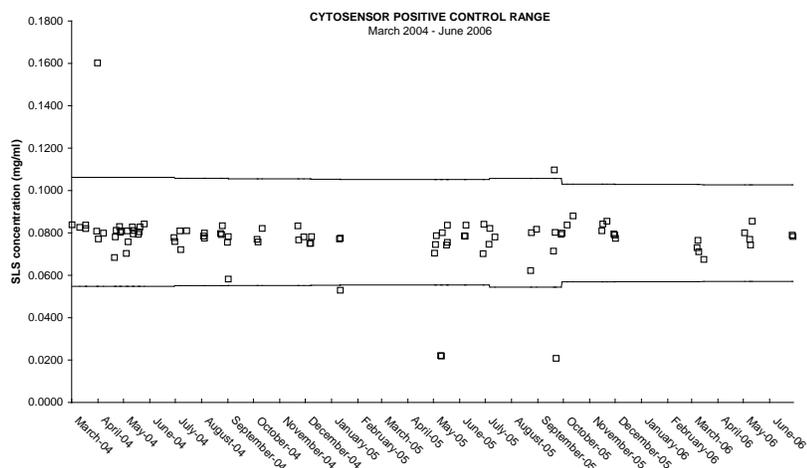
CYTOSENSOR POSITIVE CONTROL RANGE
March 2004 - June 2006

Figure 7-1   Graph of 10% SLS (positive control) MRD$_{50}$ values obtained at IIVS over a 28-month period.

Additional information on the intralaboratory reproducibility can be found in Section 3. Within-laboratory reproducibility of the Cytosensor BRD submitted to ECVAM.

7.1.3 Cytosensor interlaboratory reproducibility

There were no examples of interlaboratory reproducibility for studies submitted specifically for this BRD. However, there are two main examples which are presented in the Cytosensor BRD submitted to ECVAM. One is from the EC/HO international validation study for eye irritation where four Cytosensor laboratories participated and the other is from the COLIPA validation study which had two Cytosensor laboratories participating.

The results from the EC/HO study are broken down into two categories: 1) surfactants (Table 7-13), and 2) non-surfactant materials (Table 7-14). For the 11 surfactants (only one laboratory found that polyethylene glycol 400 was compatible with the Cytosensor) the mean CV for the 4 laboratories was 37.0% and for the non-surfactants was 50.6%. Not all laboratories found that all the non-surfactant materials met the testing criteria, so the number of laboratories testing each of the 32 materials ranged from 1 to 4. The distribution of product categories for the interlaboratory reproducibility of the EC/HO study is shown in Table 7-15.

4778 **Table 7-13 Surfactant Materials - Between-laboratories reproducibility of CM results from**
4779 **EC/HO study.**
4780

Chemical	Formulation Type	Conc. tested	MRD$_{50}$ Values (mg/mL)				Mean MRD$_{50}$ (mg/mL)	SD	CV (%)
			CM 30	CM 31	CM 32	CM 33			
Cetylpyridinium bromide	SU	10%	0.78	1.02	2.34	0.89	1.26	0.73	58.2
Cetylpyridinium bromide	SU	6%	0.6	1.35	0.44	1.11	0.87	0.43	48.8
Benzalkonium chloride	SU	5%	1.15	1.09	0.98	1.28	1.13	0.12	11.1
Benzalkonium chloride	SU	10%	0.26	0.47	0.38	0.44	0.39	0.09	24.2
Triton X-100	SU	10%	1.61	1.96	1.50	2.22	1.82	0.33	18.0
Sodium lauryl sulfate	SU	15%	0.62	0.60	0.51	0.74	0.62	0.10	15.5
Benzalkonium chloride [1]/[2]	SU	1%	4.71	5.16	4.65	3.58	4.53	0.67	14.8
Triton X-100 [1]/[2]	SU	5%	1.90	3.39	5.09	2.53	3.23	1.39	43.0
Sodium lauryl sulfate	SU	3%	2.71	3.04	3.74	3.64	3.28	0.49	15.0
Tween 20	SU	100%	1.52	5.53	4.98	1.06	3.27	2.31	70.5
Cetylpyridinium bromide	SU	0.10%	48.19	102.33	7.76	180.30	84.65	74.62	88.1
Polyethylene glycol 400	SU	100%	*	*	*	363.92	*	*	*
Mean									**37.0**
Median									**24.2**

4781 * Participating laboratory did not test the chemical because it determined that chemical was not
4782 compatible with the test system.

4783

4784

4785 **Table 7-14 Non-surfactant materials - Between-laboratories reproducibility of CM results from**
4786 **EC/HO study.**

Chemical	Formulation Type	Conc. tested	MRD$_{50}$ Values (mg/mL)				Mean MRD$_{50}$ (mg/mL)	SD	CV (%)
			CM 30	CM 31	CM 32	CM 33			
Sodium hydroxide	AL	10%	2.28	1.60	2.67	2.49	2.26	0.47	20.8
Trichloroacetic acid	AC	30%	1.69	2.47	0.81	2.20	1.79	0.73	40.7
Captan 90 concentrate		100%	*	*	*	*	*	*	*
Chlorhexidine		100%	*	*	*	*	*	*	*
Cyclohexanol	SO	100%	15.49	*	0.58	*	8.03	10.5	131.3
Quinacrine		100%	*	*	1.08	*	*	*	*
Promethazine HCl		100%	1.35	1.48	0.81	1.45	1.27	0.31	24.4
Parafluoraniline		100%	*	*	3.47	*	*	*	*
Acetone	SO	100%	153.82	140.28	139.00	162.18	148.82	11.15	7.5
n-Hexanol	SO	100%	*	*	*	*	*	*	*
1-Naphthalene acetic acid		100%	12.11	*	*	*	*	*	*
Sodium oxalate		100%	*	*	*	*	*	*	*
Isobutanol	SO	100%	28.84	28.64	22.54	31.62	27.91	3.83	13.7
Imidazole	SU	100%	22.75	23.07	0.18	48.75	23.69	19.85	83.8
2-Ethyl-1-hexanol	SO	100%	*	*	*	*	*	*	*
4-Carboxybenzaldehyde		100%	*	*	*	*	*	*	*
Methyl ethyl ketone	SO	100%	55.72	50.47	78.16	47.97	58.08	13.77	23.7
Pyridine		100%	1.54	29.99	15.92	31.48	19.73	14.01	71.0
1-Naphthalene acetic acid		100%	*	*	*	*	*	*	*
2,2-Dimethylbutanoic acid	AC	100%	*	*	*	*	*	*	*
Gammabutyrolactone		100%	79.98	114.82	0.91	179.47	93.79	74.39	79.3
Thiourea		100%	50.12	50.93	*	47.97	49.68	1.53	3.1
n-Octanol	SO	100%	*	*	*	*	*	*	*
Methyl acetate	SO	100%	61.09	91.83	116.14	109.65	94.68	24.64	26.0
L-Aspartic acid	AC	100%	1.11	1.17	*	*	1.14	0.04	3.6
Benzoyl-L-tartaric acid		100%	0.81	*	*	*	*	*	*
Potassium cyanate		100%	28.18	36.06	9.40	50.82	31.11	17.25	55.4
Isopropanol	SO	100%	83.18	91.20	87.10	143.55	101.26	28.39	28.0
Sodium perborate, 4H$_2$0		100%	0.11	*	*	3.27	1.69	2.24	132.6
Dibenzyl phosphate	AC	100%	0.75	*	*	*	*	*	*
2,5-Dimethylohexanediol	SO	100%	75.21	155.96	6.21	156.31	98.67	72.25	73.2
Methyl cyanoacetate		100%	42.95	*	0.13	*	21.54	30.28	140.5
Sodium hydroxide	AL	1%	28.18	16.22	32.36	31.62	27.1	7.48	27.6
Ethanol	SO	100%	97.05	117.49	123.03	110.41	111.99	11.22	10.0
2,6-Dichlorobenzoyl chloride		100%	*	*	*	*	*	*	*
Ammonium nitrate		100%	40.27	145.55	27.99	*	71.27	64.62	90.7
Ethyl-2-methylacetoacetate		100%	*	*	0.40	*	*	*	*

Chemical	Formulation Type	Conc. tested	MRD$_{50}$ Values (mg/mL)				Mean MRD$_{50}$ (mg/mL)	SD	CV (%)
			CM 30	CM 31	CM 32	CM 33			
Ethyl acetate	SO	100%	*	53.70	*	*	*	*	*
Maneb		100%	*	*	*	*	*	*	*
Fomesafen		100%	*	*	*	*	*	*	*
Tetraaminopyrimidine sulfate		100%	1.05	*	*	*	*	*	*
Toluene		100%	*	*	*	*	*	*	*
n-Butyl acetate		100%	*	*	*	*	*	*	*
Trichloroacetic acid	AC	3%	13.90	13.80	16.29	16.11	15.03	1.36	9.0
Methyl isobutyl ketone		100%	*	*	0.81	*	*	*	*
Ethyl trimethyl acetate		100%	*	*	*	*	*	*	*
Methylcyclopentane		100%	*	*	*	*	*	*	*
Glycerol	AL	100%	121.62	180.72	8.26	208.93	129.88	88.87	68.4
Mean									**50.6**
Median									**28.0**
Mean when all four labs tested material									**39.0**

4787   * Participating laboratory did not test the chemical because it determined that chemical was not
4788   compatible with the test system.
4789
4790   **Table 7-15 Distribution of product categories for the**
4791   **interlaboratory reproducibility of the EC/HO study.**

Product Categories	Number of products tested
Surfactants	12
Acids	5
Bases	3
Solvents	12
Other	
Total	

4792
4793
4794   For more details of each of these studies plus additional information on
4795   interlaboratory reproducibility please see Section 5. Between-laboratory
4796   reproducibility in the Cytosensor BRD.
4797
4798   The results from the COLIPA study are broken down into three categories: 1)
4799   surfactants (Table 7-16), 2) surfactant-based formulations and mixtures (Table 7-
4800   17), and 3) non-surfactants, ingredients and mixtures (Table 7-18). For the 13
4801   surfactants, both laboratories tested 10, but one laboratory found 2 materials that
4802   did not meet the testing criteria. The mean CV for the 2 laboratories was 23.3% for
4803   the surfactants, 16.5% for the 7 surfactant-based formulations and mixtures and
4804   32.5% for the 9 non-surfactants. The distribution of product categories for the
4805   interlaboratory reproducibility of the COLIPA study is shown in Table 7-19.
4806
4807

4808 **Table 7-16 Surfactant Materials - Between-laboratories reproducibility of Cytosensor**
4809 **Microphysiometer results from COLIPA study.**

Chemical	Formulation Tested	Conc. tested	MRD$_{50}$ Values (mg/mL)		Mean MRD$_{50}$ (mg/mL)	SD	CV (%)
			MA	**CT AB**			
Triton X-100 1%	SU	1%	21.17	16.79	18.98	3.1	16.3
Tween 20	SU	100%	9.5	3.49	6.50	4.25	65.4
SLS 3%	SU	3%	3.23	2.78	3.00	0.32	10.6
Triton X-100 5%	SU	5%	4.66	2.42	3.54	1.58	44.7
Benzalkonium chloride 1%	SU	5%	4.11	4.33	4.22	0.16	3.7
SLS 15%	SU	1%	0.52	0.51	0.51	0.01	1.0
SLS 30%	SU	100%	0.31	*	*		
Triton X-100 10%	SU	15%	2.47	1.24	1.85	0.87	46.8
Benzalkonium chloride 5%	SU	30%	0.81	1.38	1.1	0.4	36.7
Benzalkonium chloride 10%	SU	10%	0.32	0.31	0.31	0.01	3.2
Cetylpyridinium bromide 6%	SU	100%	1.36	*	*		
Cetylpyridinium bromide 10%	SU	100%	*	*			
Polyethylene glycol 400	SU	100%	296.5	316.23	306.36	13.95	4.6
Mean							**23.3**
Median							**13.5**

4810 * - Participating laboratory did not test the chemical because it determined that chemical was not
4811 compatible with the test system.

4812
4813

4814 **Table 7-17 Surfactant based formulations and mixtures - Between-laboratories reproducibility**
4815 **of Cytosensor Microphysiometer results from COLIPA study.**

Chemical	Formulation Tested	Conc. tested	MRD_{50} Values (mg/mL)		Mean MRD_{50} (mg/mL)	SD	CV (%)
			MA	CT AB			
Perfumed skin lotion	SU	100%	*	*			
Eye make-up remover	SU	100%	87.77	99.31	93.54	8.16	8.7
Hair dye base F#1	SU	100%	*	*			
Pump Deodorant	SU	5%	19.35	47.74	33.54	20.08	59.9
Emulsion antiperspirant	SU	100%	*	*			
Gel cleaner	SU	100%	5.68	5.47	5.58	0.15	2.6
Sunscreen SPF 15	SU	100%	*	*			
Hydrophilic ointment	SU	100%	*	*			
Hair conditioner	SU	100%	*	*			
Moisturiser with sunscreen	SU	100%	*	*			
Hair dye base form #3	SU	100%	*	*			
Polishing scrub	SU	100%	*	*			
Shampoo #1 normal	SU	100%	0.75	0.72	0.74	0.02	2.2
Hand cleaner	SU	100%	*	*			
Hand soap	SU	100%	*	*			
Shampoo - baby	SU	100%	2.51	2.15	2.33	0.25	10.8
Liquid soap #1	SU	100%	0.88	0.68	0.78	0.14	18.5
Shampoo antidandruff	SU	100%	*	*			
Shampoo 2-in-1	SU	100%	*	*			
Cleansing foam III	SU	100%	*	*			
Shower gel	SU	100%	*	*			
Skin cleaner	SU	100%	0.63	0.76	0.7	0.09	13.0
Mean							**16.5**
Median							**10.8**

4816 * - Participating laboratory did not test the chemical because it determined that chemical was not
4817 compatible with the test system.
4818
4819

4820 **Table 7-18 Non-Surfactants, ingredients, and mixtures – Between-laboratories reproducibility**
4821 **of Cytosensor Microphysiometer results from COLIPA study.**

Chemical	Formulation Tested	Conc. Tested	MRD_{50} Values (mg/mL)		Mean MRD_{50} (mg/mL)	SD	CV (%)
			MA	CT AB			
Blush		100%	*	*			
Eye liner		100%	*	*			
n-Butyl acetate		100%	*	*			
Imidazole		100%	18.84	26.03	22.43	5.09	22.7
Propylene glycol		100%	265.07	218.86	241.97	32.67	13.5
Glycerol	SO	100%	214.83	208.7	211.77	4.34	2.0
Ethyl acetate		100%	*	*			
Sodium hydroxide 1%	AL	1%	9.09	13.59	11.34	3.19	28.1
Isopropanol	SO	100%	52.59	124.51	88.55	50.86	57.4
Methyl ethyl ketone		1%	54.18	*	54.18		
Sunscreen lotion		10%	*	*			
Cologne		100%	*	*			
Eye shadow		100%	*	*			
Mascara		100%	*	*			
Hair styling lotion		100%	164.82	292.01	228.41	89.94	39.4
Mouthwash		100%	37.84	46.85	42.35	6.37	15.0
Toothpaste		100%	*	*			
Hair dye base form #2		100%	*	*			
Sodium hydroxide 10%	AL	6%	4.33	0.6	2.47	2.64	106.9
Trichloroacetic acid 30%	AC	30%	1.12	1.24	1.18	0.09	7.3
Mean							**32.5**
Median							**22.7**

4822 * - Participating laboratory did not test the chemical because it determined that chemical was not
4823 compatible with the test system.
4824
4825 **Table 7-19 Distribution of product categories for the**
4826 **interlaboratory reproducibility of the COLIPA study.**

Product Categories	Number of products tested
Surfactants	22
Acids	1
Bases	2
Solvents	2
Other	15
Total	**41**

4827

4828   ## 7.2 EpiOcular model

4829   ### 7.2.1 EpiOcular intralaboratory repeatability for antimicrobial cleaning
4830   products submitted by participating companies (within run and between
4831   experiments)

4832

4833   The within-run reproducibility can be assessed for some (15) of the
4834   formulations submitted for this study (Table 7-20). Studies were completed with at
4835   least 4 exposure times and on duplicate tissues for each exposure time. The
4836   distribution of product categories for the intralaboratory repeatability of the
4837   EpiOcular assay is shown in Table 7-21.

4838

4839   **Table 7-20 EpiOcular intralaboratory repeatability both within run and between experiments**

Code	Class	Assay Date	EPA Cat.	GHS Cat.	Conc.	ET_{50} (min)	Exp. Time (min)	Tissue 1 (% Survival)	Tissue 2 (% Survival)	Mean (%)	S.D.	CV (%)
H	AL	12/07/05	II	2A	Neat	9.4	20	28.4	26.7	**27.5**	1.2	4.5
							10	45.9	44.5	**45.2**	1.0	2.2
							5	82.9	86.9	**84.9**	2.9	3.4
							1	104.3	98.9	**101.6**	3.8	3.8
H	AL	04/05/06	II	2A	Neat	9.8	20	13.9	28.9	**21.4**	10.6	49.5
							12	37.4	22.2	**29.8**	10.8	36.0
							10	27.3	70.1	**48.7**	30.2	62.1
							5	76.8	80.9	**78.8**	2.8	3.6
							1	89.4	87.6	**88.5**	1.3	1.5
H	AL	04/05/06	II	2A	Neat	9.1	20	17.6	25.4	**21.5**	5.5	25.6
							12	15.1	29.9	**22.5**	10.5	46.7
							10	29.8	56.6	**43.2**	18.9	43.9
							5	78.3	81.9	**80.1**	2.5	3.1
							1	90.4	92.0	**91.2**	1.1	1.2
J	SU	12/07/05	III	2B	Neat	19.3	45	19.3	23.4	**21.4**	2.9	13.7
							20	46.1	48.4	**47.3**	1.6	3.4
							10	80.4	89.6	**85.0**	6.5	7.6
							5	95.9	93.8	**94.9**	1.5	1.5
K	RC	12/07/05	IV	NI	Neat	> 240	240	93.7	98.0	**95.9**	3.1	3.2
							90	99.6	109.6	**104.6**	7.0	6.7
							45	105.6	114.5	**110.1**	6.3	5.7
							20	98.8	107.9	**103.4**	6.4	6.2
P	AL	12/07/05	IV	NI	Neat	125.8	240	21.0	27.4	**24.2**	4.5	18.7
							90	57.6	58.5	**58.1**	0.7	1.1
							45	88.4	92.1	**90.3**	2.6	2.9
							20	126.8	127.9	**127.4**	0.8	0.6
P	AL	04/05/06	IV	NI	Neat	74.0	240	9.2	16.2	**12.7**	5.0	39.2
							90	36.9	37.6	**37.3**	0.5	1.4
							45	72.6	73.4	**73.0**	0.5	0.7
							20	125.1	119.8	**122.5**	3.7	3.1

4840

4841

Code	Class	Assay Date	EPA Cat.	GHS Cat.	Conc.	ET$_{50}$ (min)	Exp. Time (min)	Tissue 1 (% Survival)	Tissue 2 (% Survival)	Mean (%)	S.D.	CV (%)
R	SU	12/07/05	IV	NI	Neat	> 240	240	83.5	82.0	**82.8**	1.1	1.3
							90	96.8	80.8	**88.8**	11.3	12.8
							45	110.9	97.2	**104.1**	9.7	9.3
							20	92.8	99.1	**96.0**	4.4	4.6
T	AC	12/07/05	IV	NI	Neat	31.6	240	8.3	8.8	**8.5**	0.4	4.1
							90	8.5	9.5	**9.0**	0.7	7.3
							45	19.6	20.7	**20.1**	0.8	4.0
							20	75.7	76.2	**76.0**	0.3	0.4
W	SU	12/07/05	IV	NI	Neat	39.6	45	41.1	48.8	**44.9**	5.4	12.1
							20	62.6	74.3	**68.5**	8.3	12.1
							10	84.4	90.8	**87.6**	4.5	5.2
							5	96.2	104.8	**100.5**	6.1	6.1
W	SU	04/05/06	IV	NI	Neat	43.3	90	14.9	29.1	**22.0**	10.0	45.4
							45	52.2	43.9	**48.0**	5.9	12.2
							20	78.5	77.6	**78.1**	0.6	0.8
							5	91.0	88.7	**89.8**	1.6	1.8
V	SU	12/07/05	IV	NL	Neat	12.0	20	25.8	25.0	**25.4**	0.6	2.3
							10	53.1	58.8	**56.0**	4.0	7.2
							5	68.4	69.1	**68.8**	0.5	0.7
							1	103.6	97.1	**100.3**	4.6	4.6
AT	RC	12/07/05	I	1	Neat	<1	20	0.8	0.8	**0.8**	0.0	5.9
							10	0.7	0.6	**0.6**	0.1	14.9
							5	4.9	6.9	**5.9**	1.4	23.6
							1	9.0	14.3	**11.6**	3.7	32.2
AU	RC	12/07/05	I	1	Neat	<1	20	0.9	0.9	**0.9**	0.0	0.0
							10	0.8	1.0	**0.9**	0.1	10.5
							5	3.8	2.7	**3.2**	0.8	23.8
							1	5.1	7.7	**6.4**	1.9	29.3
AV	RC	12/07/05	I	1	Neat	<1	20	1.1	0.8	**0.9**	0.1	15.2
							10	1.1	1.8	**1.5**	0.5	32.9
							5	2.4	1.9	**2.1**	0.4	18.0
							1	3.8	8.1	**6.0**	3.0	50.6
BB	SO	12/07/05	IV	SCN M	Neat	>240	240	96.7	101.6	**99.2**	3.5	3.5
							90	104.6	106.6	**105.6**	1.4	1.3
							45	90.7	101.6	**96.1**	7.7	8.0
							20	104.0	96.4	**100.2**	5.4	5.4
BK		12/07/05	III	NI	Neat	9.4	20	37.8	32.8	**35.3**	3.5	10.1
							10	40.5	49.1	**44.8**	6.1	13.6
							5	82.7	91.7	**87.2**	6.4	7.3
							1	107.5	106.2	**106.8**	0.9	0.9
BM	SO	12/07/05	IV	NI	Neat	4.9	20	14.5	16.3	**15.4**	1.2	8.0
							10	18.4	18.4	**18.4**	0.0	0.0
							5	43.0	53.7	**48.4**	7.6	15.7
							1	97.6	99.7	**98.7**	1.4	1.4

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Code	Class	Assay Date	EPA Cat.	GHS Cat.	Conc.	ET$_{50}$ (min)	Exp. Time (min)	Tissue 1 (% Survival)	Tissue 2 (% Survival)	Mean (%)	S.D.	CV (%)
BL	SO	12/07/05	IV	NI	Neat	6.7	20	37.3	34.7	**36.0**	1.8	5.1
							10	34.7	29.4	**32.1**	3.8	11.8
							5	60.7	58.0	**59.4**	1.9	3.2
							1	100.8	104.5	**102.6**	2.6	2.6

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Table 7-21 Distribution of product categories for the intralaboratory repeatability of the EpiOcular assay.

Product Categories	Number of products tested
Surfactants	4
Acids	1
Bases	2
Solvents	3
Oxidizers	4
Other	1
Total	**15**

4847 7.2.2 EpiOcular intralaboratory reproducibility (between experiments)

4848

4849   Intralaboratory reproducibility for EpiOcular can be estimated from the repeat
4850 testing of a single material (0.3% Triton X-100) over a nine year period (Table 7-22).
4851 Percent CV for the two laboratories combined was 20.7% and for a single laboratory
4852 (IIVS) was 22.2% (eight years only). The standard deviation range for the 0.3%
4853 Triton X-100 over a nine year period is described in Table 7-23.

4854
4855 **Table 7-22 Intralaboratory reproducibility of EpiOcular tissue over a nine year period from
4856 1997 through 2005 for two different laboratories.**

Test Material	Mean ET$_{50}$ Value (min)	Standard Deviation (min)	CV (%)
0.3% Triton X-100 (Combined data from MatTek and IIVS)	26.1	5.4	20.7
0.3% Triton X-100 (IIVS only-through Oct., 2004)	27.0	6.0	22.2

4857
4858 **Table 7-23 Standard deviation range for 0.3% Triton X-100 for EpiOcular tissue over a nine
4859 year period.**

SD Range	1997 (%)	1998 (%)	1999 (%)	2000 (%)	2001 (%)	2002 (%)	2003 (%)	2004 (%)	2005 (%)	1997-2005 YTD (%)
0.0 to 0.5	41	52	36	29	35	32	36	33	47	38
0.5 to 1.0	35	26	31	25	36	22	26	27	35	29
1.0 to 1.5	20	17	24	27	20	31	25	19	15	22
0.0 to 1.5	95	95	92	81	91	85	87	79	97	89
1.5 to 2.0	5	5	8	18	9	15	13	21	3	11
# Production Lots	>50	>50	>50	>50	>50	>50	>50	>50	>50	>500
Average ET$_{50}$ (min)	22.9	25.0	22.1	20.7	22.9	22.5	24.1	22.2	24.77	23.00

4860   Additional information on the intralaboratory reproducibility of EpiOcular can
4861   be found in the BRD prepared for ECVAM.

4862   7.2.3 EpiOcular interlaboratory reproducibility
4863
4864   Data on interlaboratory reproducibility can be found in the BRD prepared for
4865   ECVAM. Two specific examples from two phases of a validation study conducted for
4866   Colgate-Palmolive are shown below. This validation study was conducted with
4867   surfactants and surfactant-containing products to investigate a different prediction
4868   model than is presented in this BRD. The prediction model tested in Phase II (Table
4869   7-24) and Phase III (Table 7-25) is based on Draize MAS scores, and consequently
4870   the reproducibility comparisons are based on predicted MAS scores, not directly on
4871 ET_{50} values. However, the values given do reflect the reproducibility that can be
4872   expected using ET_{50} values as is done in this BRD. It can be seen that the mean
4873   %CV in Phase II (4 laboratories) was 18.1% and in Phase III (2 labs) was 11.8%.
4874   The distribution of product categories for the interlaboratory reproducibility of the
4875   EpiOcular assay is shown in Table 7-26.
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4878 **Table 7-24 Interlaboratory reproducibility of four laboratories in the Colgate-Palmolive Phase**
4879 **II validation study.**

Test Material	Formulation Type	Predicted Draize Score				Average	SD	CV (%)
		Lab 1	Lab 2	Lab 3	Lab 4			
Shampoo #1 (2 in 1)	SU	14.5	16.7	18.9	18.2	17.1	1.9	11.4
Shampoo #2 (Regular)	SU	28.6	27.2	31.3	30.6	29.4	1.9	6.4
Shampoo #3 (Regular)	SU	29.7	25.5	30.5	32.4	29.5	2.9	9.9
Dishwashing Liquid	SU	79.7	58.2	35.1	97	67.5	26.8	39.7
All purpose cleaner	SU	23.5	20.5	21.2	28.1	23.3	3.4	14.7
Disinfectant cleaner	SU	40	36.4	45.7	34.8	39.2	4.8	12.3
Sodium linear alkylbenzene sulfonate	SU	36.1	39.8	38.4	40.5	38.7	1.9	5.0
30% Dimethyltetradecylamine oxide	SU	58.3	88.3	84.7	50.2	70.4	19.0	27.0
1.5% branched alkyldimethylamine	SU	21.6	22.3	26.4	23.8	23.5	2.1	9.0
PPG-5 Ceteth-20	SU	3.1	3.4	4.8	3.6	3.7	0.7	20.0
C9-11 Alcohol ethoxylate EO6:1	SU	61.7	53.7	33.7	32.1	45.3	14.7	32.5
C12-14 Alcohol ethoxylate 2EO	SU	6	4.9	9.9	7.1	7.0	2.1	30.8
C12-16 Alcohol ethoxylate 3EO	SU	8.7	10.3	11.2	9.8	10.0	1.0	10.4
2.46% Lauryl hydroxysultaine	SU	24.2	25.1	27.3	23.5	25.0	1.7	6.6
10% Polyoxyethylene (10) oleyl ether	SU	1.8	3.1	3.1	4.3	3.1	1.0	33.2
3.2% Benzalkonium chloride	SU	71.8	60.3	78.8	62.2	68.3	8.6	12.6
36% Sodium methyl 2-sulfonate & disodium 2-sulfolaurate	SU	35.4	39.8	36.2	34.8	36.6	2.2	6.1
2.4% Imidazolium compound	SU	1.8	2.5	2.8	1.8	2.2	0.5	22.7
C12-15 Alcohol ethoxylate EO7:1	SU	6.2	5	8.7	10.7	7.7	2.6	33.4
Mean CV (%)								**18.1**
Median CV (%)								**12.6**

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4881 **Table 7-25 Interlaboratory reproducibility of two laboratories in the Colgate-Palmolive Phase**
4882 **III validation study.**
4883

Test Material	Formulation Type	Concentration Tested	Predicted Draize Score		Average	SD	CV (%)
			Lab 1	Lab 2			
1-decanaminium, N-decyl-N,N-dimethyl, Cl-	SU	50%	97	97	97.0	0.0	0.0
20% Myristalkonium chloride/ 20% Quaternium-14	SU	100%	97	92.2	94.6	3.4	3.6
Alkyldimethyl benzyl ammonium Cl-	SU	5%	60	84	72.0	17.0	23.6
Ammonium lauryl sulfate	SU	12%	25.5	25.4	25.5	0.1	0.3
Ammonium lauryl sulfate	SU	28%	34.3	29.4	31.9	3.5	10.9
Ammonium nonoxyl-4 sulfate	SU	10%	17.5	19.4	18.5	1.3	7.3
Behentrimonium methosulfate & cetearyl alcohol	SU	100%	1.8	1.8	1.8	0.0	0.0

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Test Material	Formulation Type	Concentration Tested	Predicted Draize Score		Average	SD	CV (%)
			Lab 1	Lab 2			
Benzalkonium chloride	SU	0.10%	1.8	1.8	1.8	0.0	0.0
Benzalkonium chloride	SU	0.50%	18.1	24.2	21.2	4.3	20.4
Benzalkonium chloride	SU	1%	27.9	35.5	31.7	5.4	17.0
Benzalkonium chloride	SU	2.50%	66.4	65.8	66.1	0.4	0.6
Benzalkonium chloride	SU	5%	68.3	96.5	82.4	19.9	24.2
Benzalkonium chloride	SU	10%	90.2	97	93.6	4.8	5.1
Benzethonium chloride	SU	3.20%	42.1	56.5	49.3	10.2	20.7
Benzethonium chloride	SU	1.00%	29.2	41.7	35.5	8.8	24.9
Branched alkyldimethylamine	SU	1.50%	16.8	20.4	18.6	2.5	13.7
Branched alkyldimethylamine	SU	30%	97	97	97.0	0.0	0.0
C10-12 Alcohol ethoxylate (PO)	SU	100%	87.6	80.6	84.1	4.9	5.9
Ceteareth-12	SU	100%	1.8	4.1	3.0	1.6	55.1
Cetrimonium chloride	SU	2.50%	22.2	19.7	21.0	1.8	8.4
Cetyl alcohol	SU	100%	1.8	1.8	1.8	0.0	0.0
Cetylpyridinium bromide	SU	10%	25.2	24.8	25.0	0.3	1.1
Cetylpyridinium bromide	SU	0.1%	1.8	1.8	1.8	0.0	0.0
Cetylpyridinium bromide	SU	1%	10.2	17.8	14.0	5.4	38.4
Cocamidopropyl betaine	SU	10%	23.7	32.6	28.2	6.3	22.4
Cocamidopropyl betaine	SU	30%	44.1	46.4	45.3	1.6	3.6
Decyl glucoside	SU	10%	21.1	23	22.1	1.3	6.1
Didecyldimethyl ammonium chloride (DDAC)	SU	1%	32.5	39.9	36.2	5.2	14.5
Didecyldimethyl ammonium chloride (DDAC)	SU	3.20%	62.9	72.9	67.9	7.1	10.4
Didecyldimethyl ammonium chloride (DDAC)	SU	5%	9	14.8	11.9	4.1	34.5
Lauryl glucoside	SU	12%	2.5	3.4	3.0	0.6	21.6
Myristalkonium chloride/Quaternium-14/Ethanol	SU	3%	40.2	59.5	49.9	13.6	27.4
Myristalkonium chloride/Quaternium-14/Ethanol	SU	20%	62.8	97	79.9	24.2	30.3
PPG-5-Ceteth 20	SU	100%	1.8	3.5	2.7	1.2	45.4
Quaternium-18	SU	100%	1.8	1.8	1.8	0.0	0.0
Shampoo #4	SU	10%	14.3	15.3	14.8	0.7	4.8
Sodium C14-16 olefin sulfonate	SU	10%	19.2	20	19.6	0.6	2.9
Sodium ether sulfate 3EO	SU	30%	30.6	30.5	30.6	0.1	0.2
Sodium laureth sulfate	SU	12%	18.5	21	19.8	1.8	9.0
Sodium laureth sulfate	SU	25%	23.7	27.1	25.4	2.4	9.5
Sodium lauroyl sarcosinate	SU	10%	24.8	23.2	24.0	1.1	4.7
Sodium lauroyl sarcosinate	SU	30%	33	35.1	34.1	1.5	4.4
Sodium lauryl sulfate	SU	3%	23.2	24	23.6	0.6	2.4
Sodium lauryl sulfate	SU	10%	30.3	33.4	31.9	2.2	6.9
Sodium lauryl sulfate	SU	15%	34.7	36.8	35.8	1.5	4.2
Sodium lauryl sulfate	SU	20%	39.6	41.8	40.7	1.6	3.8
Sodium lauryl sulfate	SU	30%	39.6	47.3	43.5	5.4	12.5
Sodium methyl 2-sulfonate & disodium 2-sulfolaurate	SU	39%	33.4	35.3	34.4	1.3	3.9

Test Material	Formulation Type	Concentration Tested	Predicted Draize Score		Average	SD	CV (%)
			Lab 1	Lab 2			
TEA-lauryl sulfate	SU	20%	26.5	32.1	29.3	4.0	13.5
Triton X-100	SU	1%	9.7	12.1	10.9	1.7	15.6
Triton X-100	SU	2.50%	24.1	22.8	23.5	0.9	3.9
Triton X-100	SU	5%	36.6	46	41.3	6.6	16.1
Triton X-100	SU	10%	51.8	53.7	52.8	1.3	2.5
Triton X-100	SU	20%	50.2	63.8	57.0	9.6	16.9
Mean CV (%)							**11.8**
Median CV (%)							**7.1**

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Table 7-26 Distribution of product categories for the interlaboratory reproducibility of the EpiOcular assay.

Product Categories	Number of products tested
Surfactants	73 (including unique dilutions of products)
Acids	0
Bases	0
Solvents	0
Other	0
Total	**73**

4889   ## 7.3 BCOP assay

4890   ## 7.3.1 BCOP intralaboratory repeatability

4891   ### 7.3.1.1 BCOP within-run reproducibility for antimicrobial cleaning products data
4892

4893   Data from 75 runs (255 corneas; 3-5 corneas per run) of the BCOP assay for
4894   antimicrobial cleaning products are presented in Table 7-27. This table displays, for
4895   each test material run, the mean value and the mean %CV for the three main
4896   parameters of the BCOP assay – opacity, permeability, and *in vitro* score. Also
4897   presented is the overall mean of the %CV for each of the parameters. The
4898   distribution of product categories for the within-run reproducibility of the BCOP
4899   assay is shown in Table 7-28.
4900

4901   BCOP scores in the very low range (arbitrarily set in this BRD as ≤10) can
4902   often generate high %CV's, but these %CV's have no practical use in evaluating the
4903   utility of the assay since the individual measurements may only differ by one or two
4904   units on a scale that reaches into the hundreds. For example, the three opacity
4905   readings of 0, 2, 3 yield an extraordinarily high %CV of 91.7% even though the three
4906   values are essentially the same when the overall scale is considered. Therefore,
4907   %CV's from materials whose average *in vitro* score is ≤10 (first section of Table 7-
4908   27) will be considered separately from the %CV's of those materials whose average
4909   *in vitro* score is >10 (second section of Table 7-27)..
4910

4911   Table 7-27 indicates that there is a large difference between the %CV's for
4912   BCOP values where the average *In Vitro* Score is ≤ 10 and those cases where it is
4913   >10. The average %CV's for opacity values, permeability values and *in vitro* scores,
4914   in the first case, are 266%, 167.1% and 66.4%, respectively. However, in the
4915   second case they are much lower: 27.9%, 24.1% and 18.3%, respectively. It is clear
4916   that where small opacity values are recorded (the first case), the percent CV is
4917   really meaningless as a way of judging reproducibility. However, in the second case
4918   with higher numbers the average %CV's indicate that the BCOP assay has a high
4919   within run reproducibility.
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4924   **Table 7-27 BCOP within run reproducibility**

Materials with an average *In Vitro* Score ≤10										
Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
H	AL	43	-0.3	0.348	4.9	Non-irritant	Category III	1732.1%	61.8%	67.8%
		44	-0.3	0.444	6.4					
		45	0.7	1.047	16.4					
		avg	0.0	0.613	9.2					
		S.D.	0.6	0.379	6.3					
		% CV	1732.1%	61.8%	67.8%					
I	SU	34	-1.3	0.088	0.0	Non-irritant	Category III	57.7%	13.4%	127.7%
		35	-1.3	0.111	0.3					
		36	-0.3	0.113	1.4					
		avg	-1.0	0.104	0.6					
		S.D.	0.6	0.014	0.7					
		% CV	57.7%	13.4%	127.7%					
J	SU	23	6.3	0.085	7.6	Non-irritant	Category III	32.7%	24.1%	22.5%
		24	3.3	0.098	4.8					
		25	4.3	0.134	6.3					
		avg	4.7	0.105	6.2					
		S.D.	1.5	0.025	1.4					
		% CV	32.7%	24.1%	22.5%					
K	RC	21	0.3	-0.001	0.3	Non-irritant	Category IV	0.0%	692.8%	10.2%
		22	0.3	0.003	0.4					
		24	0.3	-0.001	0.3					
		avg	0.3	0.000	0.3					
		S.D.	0.0	0.002	0.0					
		% CV	0.0%	692.8%	10.2%					
L	SU	17	3.7	0.035	4.2	Non-irritant	Category III	21.4%	76.6%	28.6%
		18	5.7	0.106	7.3					
		19	4.7	0.028	5.1					
		avg	4.7	0.056	5.5					
		S.D.	1.0	0.043	1.6					
		% CV	21.4%	76.6%	28.6%					

Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	In vitro Score	In vivo GHS Cat	In vivo EPA Cat	Opacity CV	Perm. CV	In vitro Score CV
O	SU	14	7.3	0.030	7.8	Non-irritant	Category IV	17.3%	26.9%	14.0%
		15	7.3	0.032	7.8					
		16	5.3	0.048	6.1					
		avg	6.7	0.037	7.2					
		S.D.	1.2	0.010	1.0					
		% CV	17.3%	26.9%	14.0%					
P	AL	29	1.3	0.019	1.6	Non-irritant	Category IV	57.7%	168.8%	61.9%
		30	1.3	0.001	1.3					
		31	0.3	0.000	0.3					
		avg	1.0	0.006	1.1					
		S.D.	0.6	0.011	0.7					
		% CV	57.7%	168.8%	61.9%					
R	SU	35	0.3	0.019	0.6	Non-irritant	Category IV	1732.1%	60.5%	261.8%
		36	0.3	0.005	0.4					
		46	-0.7	0.022	-0.4					
		avg	0.0	0.015	0.2					
		S.D.	0.6	0.009	0.5					
		% CV	1732.1%	60.5%	261.8%					
T	AC	27	-0.3	0.008	-0.2	Non-irritant	Category IV	103.9%	21.5%	96.8%
		28	2.7	0.012	2.8					
		29	2.7	0.009	2.8					
		avg	1.7	0.010	1.8					
		S.D.	1.7	0.002	1.8					
		% CV	103.9%	21.5%	96.8%					
U	SU	26	2.7	0.050	3.4	Non-irritant	Category IV	24.7%	55.1%	29.9%
		27	2.7	0.120	4.5					
		28	1.7	0.050	2.4					
		avg	2.3	0.073	3.4					
		S.D.	0.6	0.040	1.0					
		% CV	24.7%	55.1%	29.9%					

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4926

Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	In vitro Score	In vivo GHS Cat	In vivo EPA Cat	Opacity CV	Perm. CV	In vitro Score CV
W	SU	45	3.7	0.074	4.8	Non-irritant	Category IV	37.1%	5.3%	30.6%
		47	6.7	0.073	7.8					
		48	3.7	0.067	4.7					
		avg	4.7	0.071	5.7					
		S.D.	1.7	0.004	1.8					
		% CV	37.1%	5.3%	30.6%					
AF	AC	34	13.0	0.013	13.2	Non-irritant	Category IV	34.4%	33.3%	32.4%
		37	8.0	0.021	8.3					
		38	7.0	0.026	7.4					
		avg	9.3	0.020	9.6					
		S.D.	3.2	0.007	3.1					
		% CV	34.4%	33.3%	32.4%					
BB	SO	25	1.0	-0.004	0.9	SCNM	Category IV	132.3%	-1249.0%	135.5%
		26	0.0	-0.002	0.0					
		28	5.0	0.004	5.1					
		avg	2.0	0.000	2.0					
		S.D.	2.6	0.004	2.7					
		% CV	132.3%	-1249.0%	135.5%					
BK	SO	29	2.0	0.414	8.2	Non-irritant	Category III	88.2%	60.2%	45.9%
		30	6.0	0.178	8.7					
		31	1.0	0.143	3.1					
		avg	3.0	0.245	6.7					
		S.D.	2.6	0.147	3.1					
		% CV	88.2%	60.2%	45.9%					
BL	SO	14	1.0	0.294	5.4	Non-irritant	Category IV	142.0%	32.8%	51.8%
		16	0.0	0.211	3.2					
		17	7.0	0.151	9.3					
		avg	2.7	0.219	6.0					
		S.D.	3.8	0.072	3.1					
		% CV	142.0%	32.8%	51.8%					

Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
CG	AL	12	5.3	0.027	5.7	Non-irritant	Category IV	41.7%	90.7%	44.3%
		13	2.3	0.001	2.4					
		14	3.3	0.014	3.5					
		avg	3.7	0.014	3.9					
		S.D.	1.5	0.013	1.7					
		% CV	41.7%	90.7%	44.3%					
Mean CV for materials with an i*n vitro* score less than 10								**266%**	**167.1%**	**66.4%**

Materials with an average *In Vitro* Score > 10										
Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
H	AL	15	0.3	0.614	9.5	Non-irritant	Category III	57.7%	27.4%	27.3%
		17	1.3	0.914	15.0					
		18	1.3	0.560	9.7					
		avg	1.0	0.696	11.4					
		S.D.	0.6	0.191	3.1					
		% CV	57.7%	27.4%	27.3%					
H	AL	48	2.7	0.401	8.7	Non-irritant	Category III	66.6%	41.6%	46.1%
		49	2.7	0.783	14.4					
		50	7.7	0.999	22.7					
		avg	4.3	0.728	15.2					
		S.D.	2.9	0.303	7.0					
		% CV	66.6%	41.6%	46.1%					
H	AL	37	2.7	0.698	13.1	Non-irritant	Category III	60.0%	16.6%	9.4%
		38	1.7	0.673	11.8					
		40	0.7	0.902	14.2					
		avg	1.7	0.758	13.0					
		S.D.	1.0	0.126	1.2					
		% CV	60.0%	16.6%	9.4%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
H	AL	50	1.3	0.911	15.0	Non-irritant	Category III	43.3%	37.2%	26.7%
		51	3.3	0.425	9.7					
		52	3.3	0.899	16.8					
		avg	2.7	0.745	13.8					
		S.D.	1.2	0.277	3.7					
		% CV	43.3%	37.2%	26.7%					
H	AL	29	2.7	1.101	19.2	Non-irritant	Category III	86.6%	15.4%	31.3%
		32	2.7	0.884	15.9					
		33	10.7	1.205	28.7					
		avg	5.3	1.064	21.3					
		S.D.	4.6	0.164	6.7					
		% CV	86.6%	15.4%	31.3%					
Q	SU	42	8.7	0.223	12.0	Non-irritant	Category IV	6.4%	66.4%	26.3%
		43	8.7	0.150	10.9					
		44	9.7	0.525	17.5					
		avg	9.0	0.299	13.5					
		S.D.	0.6	0.199	3.6					
		% CV	6.4%	66.4%	26.3%					
V	SU	19	4.7	1.090	21.0	Non-irritant	Category IV	11.5%	10.3%	5.1%
		20	5.7	0.928	19.6					
		21	4.7	1.132	21.6					
		avg	5.0	1.050	20.8					
		S.D.	0.6	0.108	1.1					
		% CV	11.5%	10.3%	5.1%					
X	RC	19	7.7	4.772	79.3	Category 2A	Category I	39.7%	4.4%	5.2%
		21	8.7	5.207	86.8					
		22	3.7	5.067	79.7					
		avg	6.7	5.016	81.9					
		S.D.	2.6	0.222	4.2					
		% CV	39.7%	4.4%	5.2%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
Z	SO	39	6.0	1.358	26.4	Category 2A	Category II	24.7%	17.8%	14.7%
		41	6.0	1.942	35.1					
		43	9.0	1.627	33.4					
		avg	7.0	1.642	31.6					
		S.D.	1.7	0.292	4.6					
		% CV	24.7%	17.8%	14.7%					
AQ	RC	11	4.7	5.055	80.5	Category 1	Category I	57.7%	12.1%	8.1%
		12	4.7	5.875	92.8					
		13	11.7	4.640	81.3					
		avg	7.0	5.190	84.9					
		S.D.	4.0	0.628	6.9					
		% CV	57.7%	12.1%	8.1%					
AS	RC	27	3.7	4.860	76.6	Category 1	Category I	61.1%	18.7%	14.0%
		28	3.7	5.905	92.2					
		29	9.7	4.065	70.6					
		avg	5.7	4.944	79.8					
		S.D.	3.5	0.923	11.2					
		% CV	61.1%	18.7%	14.0%					
AT	RC	34	2.7	5.870	90.7	Category 1	Category I	19.2%	9.9%	8.8%
		35	2.7	5.760	89.1					
		36	3.7	4.880	76.9					
		avg	3.0	5.504	85.6					
		S.D.	0.6	0.543	7.6					
		% CV	19.2%	9.9%	8.8%					
AW	RC	29	4.7	4.000	64.7	Category 1	Category I	13.3%	26.1%	25.1%
		30	3.7	3.775	60.3					
		34	4.7	5.950	93.9					
		avg	4.3	4.575	73.0					
		S.D.	0.6	1.196	18.3					
		% CV	13.3%	26.1%	25.1%					

Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
BD	SO	16	6.7	1.210	24.9	Non-irritant	Category III	170.8%	12.0%	31.9%
		17	-1.3	0.994	13.6					
		18	1.7	0.984	16.5					
		avg	2.4	1.063	18.3					
		S.D.	4.0	0.128	5.8					
		% CV	170.8%	12.0%	31.9%					
BP	SO	11	7.0	0.848	19.7	Non-irritant	Category IV	7.5%	20.7%	11.3%
		12	8.0	0.865	21.0					
		13	8.0	0.583	16.7					
		avg	7.7	0.765	19.1					
		S.D.	0.6	0.158	2.2					
		% CV	7.5%	20.7%	11.3%					
A	SU	25	175.7	2.925	219.5	Category 1	Category I	6.0%	15.3%	5.3%
		26	166.7	2.245	200.3					
		28	155.7	3.005	200.7					
		avg	166.0	2.725	206.9					
		S.D.	10.0	0.418	11.0					
		% CV	6.0%	15.3%	5.3%					
B	SU	11	138.7	0.946	152.9	Category 1	Category I	2.2%	7.4%	1.5%
		12	135.7	0.932	149.6					
		13	141.7	0.824	154.0					
		avg	138.7	0.901	152.2					
		S.D.	3.0	0.067	2.3					
		% CV	2.2%	7.4%	1.5%					
C	RC	47	16.3	0.460	23.2	Category 1	Category I	21.7%	47.0%	30.9%
		48	16.3	0.624	25.7					
		49	23.3	1.124	40.2					
		avg	18.7	0.736	29.7					
		S.D.	4.0	0.346	9.2					
		% CV	21.7%	47.0%	30.9%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
D	AC	15	179.7	0.001	179.7	Category 1	Category I	4.3%	43.3%	4.3%
		16	187.7	0.003	187.7					
		18	195.7	0.003	195.7					
		avg	187.7	0.003	187.7					
		S.D.	8.0	0.001	8.0					
		% CV	4.3%	43.3%	4.3%					
E	SU	15	172.7	2.196	205.6	Category 1	Category I	1.8%	21.2%	4.2%
		16	168.7	1.442	190.3					
		19	166.7	1.741	192.8					
		avg	169.3	1.793	196.2					
		S.D.	3.1	0.380	8.2					
		% CV	1.8%	21.2%	4.2%					
F	RC	32^	22.7	2.053	53.5	Category 1	Category I	81.0%	167.7%	73.8%
		35	513.7	0.044	514.3					
		37	513.7	0.001	513.7					
		avg	350.0	0.699	360.5					
		S.D.	283.5	1.173	265.9					
		% CV	81.0%	167.7%	73.8%					
F	RC	22	410.7	0.157	413.0	Category 1	Category I	145.9%	78.5%	118.5%
		24	25.7	1.848	53.4					
		26	22.7	2.273	56.8					
		avg	153.0	1.426	174.4					
		S.D.	223.2	1.119	206.7					
		% CV	145.9%	78.5%	118.5%					
G	SU	12	88.7	3.530	141.6	Category 1	Category I	12.8%	4.0%	9.5%
		13	96.7	3.680	151.9					
		14	74.7	3.395	125.6					
		avg	86.7	3.535	139.7					
		S.D.	11.1	0.143	13.2					
		% CV	12.8%	4.0%	9.5%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
M	SU	32	25.3	1.334	45.3	Non-irritant	Category III	20.3%	30.1%	17.2%
		35	38.3	1.281	57.5					
		37	32.3	2.131	64.3					
		avg	32.0	1.582	55.7					
		S.D.	6.5	0.476	9.6					
		% CV	20.3%	30.1%	17.2%					
N	RC	15	6.7	0.997	21.6	Non-irritant	Category III	164.5%	84.4%	148.2%
		16	7.7	0.991	22.5					
		17	413.7	0.017	413.9					
		avg	142.7	0.668	152.7					
		S.D.	234.7	0.564	226.2					
		% CV	164.5%	84.4%	148.2%					
S	AC	22	12.7	0.302	17.2	Non-irritant	Category IV	11.8%	18.7%	10.3%
		23	14.7	0.417	20.9					
		24	11.7	0.434	18.2					
		avg	13.0	0.385	18.8					
		S.D.	1.5	0.072	1.9					
		% CV	11.8%	18.7%	10.3%					
Y	RC	28	35.7	2.612	74.9	Category 2A	Category II	9.4%	20.6%	9.7%
		29	29.7	3.497	82.1					
		33	31.7	2.397	67.6					
		avg	32.3	2.836	74.9					
		S.D.	3.1	0.583	7.3					
		% CV	9.4%	20.6%	9.7%					
AB	SU	27	85.0	1.434	106.5	Category 1	Category I	24.0%	13.5%	16.0%
		28	54.0	1.770	80.5					
		29	62.0	1.394	82.9					
		avg	67.0	1.532	90.0					
		S.D.	16.1	0.207	14.4					
		% CV	24.0%	13.5%	16.0%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
AC	AC	25	113.0	1.289	132.3	Category 1	Category I	32.2%	31.7%	32.0%
		26	150.0	1.941	179.1					
		27	77.0	1.067	93.0					
		avg	113.3	1.432	134.8					
		S.D.	36.5	0.454	43.1					
		% CV	32.2%	31.7%	32.0%					
AD	SU	18	89.0	1.409	110.1	Category 1	Category I	3.1%	2.9%	2.3%
		19	94.0	1.338	114.1					
		20	94.0	1.406	115.1					
		avg	92.3	1.384	113.1					
		S.D.	2.9	0.040	2.6					
		% CV	3.1%	2.9%	2.3%					
AE	AL	17	43.0	1.455	64.8	Category 1	Category I	3.7%	23.8%	9.7%
		18	42.0	2.126	73.9					
		20	40.0	1.424	61.4					
		avg	41.7	1.668	66.7					
		S.D.	1.5	0.397	6.5					
		% CV	3.7%	23.8%	9.7%					
AG	AL	6	340.7	3.487	393.0	Category 1	Category I	3.6%	6.7%	2.7%
		7	343.0	3.217	391.3					
		8	329.3	3.192	377.2					
		9	363.7	2.887	407.0					
		10	344.3	3.127	391.2					
		avg	344.2	3.182	391.9					
		S.D.	12.4	0.215	10.6					
		% CV	3.6%	6.7%	2.7%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
AH	AL	6	278.3	1.869	306.3	Category 1	Category I	29.1%	8.6%	25.7%
		7	111.3	1.994	141.2					
		8	254.0	2.189	286.8					
		9	243.3	2.214	276.5					
		10	240.0	1.844	267.7					
		avg	225.4	2.022	255.7					
		S.D.	65.5	0.174	65.6					
		% CV	29.1%	8.6%	25.7%					
AI	AL	6	325.3	2.498	362.8	Category 1	Category I	4.3%	16.0%	5.2%
		7	299.0	2.478	336.2					
		8	306.3	2.108	337.9					
		9	332.0	3.238	380.6					
		10	314.3	2.773	355.9					
		avg	315.4	2.619	354.7					
		S.D.	13.5	0.419	18.4					
		% CV	4.3%	16.0%	5.2%					
AJ	AL	1	289.7	2.289	324.0	Category 1	Category I	9.8%	14.0%	9.4%
		2	312.7	2.234	346.2					
		3	354.3	1.944	383.5					
		4	300.7	1.999	330.7					
		5	360.0	2.734	401.0					
		avg	323.5	2.240	357.1					
		S.D.	31.9	0.313	33.7					
		% CV	9.8%	14.0%	9.4%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
AK	AL	16	401.7	2.869	444.7	Category 1	Category I	2.0%	12.6%	1.9%
		17	396.0	2.404	432.1					
		18	410.7	2.034	441.2					
		19	417.3	2.439	453.9					
		20	409.7	2.659	449.6					
		avg	407.1	2.481	444.3					
		S.D.	8.3	0.312	8.4					
		% CV	2.0%	12.6%	1.9%					
AL	AL	6	325.3	1.818	352.6	Category 2A	Category I	16.7%	20.4%	13.9%
		7	325.7	2.918	369.5					
		8	222.7	3.163	270.1					
		9	349.7	2.853	392.5					
		21	348.3	2.363	383.7					
		avg	314.3	2.623	353.7					
		S.D.	52.6	0.536	49.1					
		% CV	16.7%	20.4%	13.9%					
AM	SO	1	89.0	2.267	123.0	Category 1	Category I	6.6%	19.5%	9.4%
		2	103.0	2.517	140.8					
		3	98.0	2.612	137.2					
		4	96.0	1.887	124.3					
		5	105.7	3.212	153.9					
		avg	98.3	2.499	135.8					
		S.D.	6.5	0.487	12.7					
		% CV	6.6%	19.5%	9.4%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
AN	AL	1	88.0	1.173	105.6	Category 1	Category I	12.0%	16.0%	11.7%
		2	98.3	1.418	119.6					
		3	105.0	1.058	120.9					
		4	79.7	0.973	94.3					
		5	106.7	1.363	127.1					
		avg	95.5	1.197	113.5					
		S.D.	11.5	0.191	13.3					
		% CV	12.0%	16.0%	11.7%					
AO	AL	11	176.0	2.594	214.9	Category 1	Category I	7.9%	6.1%	6.3%
		12	159.7	2.314	194.4					
		13	192.7	2.289	227.0					
		14	194.7	2.244	228.4					
		15	179.3	2.459	216.2					
		avg	180.5	2.380	216.2					
		S.D.	14.2	0.144	13.6					
		% CV	7.9%	6.1%	6.3%					
AP	AL	16	358.3	3.431	409.8	Category 1	Category I	4.2%	14.2%	4.3%
		17	360.3	2.421	396.6					
		18	343.0	2.966	387.5					
		19	325.0	2.826	367.4					
		20	353.7	3.421	405.0					
		avg	348.1	3.013	393.3					
		S.D.	14.5	0.427	16.8					
		% CV	4.2%	14.2%	4.3%					
AR	RC	18	42.7	4.715	113.4	Category 1	Category I	12.0%	3.0%	3.2%
		19	45.7	4.590	114.5					
		20	53.7	4.440	120.3					
		avg	47.3	4.582	116.1					
		S.D.	5.7	0.138	3.7					
		% CV	12.0%	3.0%	3.2%					

Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
AU	RC	40	50.7	4.285	114.9	Category 1	Category I	8.0%	11.3%	9.7%
		42	48.7	4.465	115.6					
		44	56.7	5.280	135.9					
		avg	52.0	4.677	122.2					
		S.D.	4.2	0.530	11.9					
		% CV	8.0%	11.3%	9.7%					
AV	RC	48	95.7	6.240	189.3	Category 1	Category I	4.6%	8.0%	4.6%
		49	104.7	6.465	201.6					
		51	101.7	5.530	184.6					
		avg	100.7	6.079	191.8					
		S.D.	4.6	0.488	8.8					
		% CV	4.6%	8.0%	4.6%					
AV	RC	19	91.7	3.965	151.1	Category 1	Category I	16.9%	11.6%	13.6%
		20	126.7	4.810	198.8					
		22	101.7	4.950	175.9					
		avg	106.7	4.575	175.3					
		S.D.	18.0	0.533	23.8					
		% CV	16.9%	11.6%	13.6%					
AX	SO	11	154.3	2.273	188.4	Category 1	Category I	15.1%	10.2%	11.3%
		12	116.7	2.218	150.0					
		13	109.0	2.318	143.8					
		14	121.7	2.073	152.8					
		15	110.7	2.708	151.3					
		avg	122.5	2.318	157.3					
		S.D.	18.5	0.237	17.7					
		% CV	15.1%	10.2%	11.3%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
AX	SO	1	124.0	2.264	158.0	Category 1	Category I	4.8%	4.2%	3.7%
		2	133.3	2.379	169.0					
		3	136.3	2.239	169.9					
		4	129.0	2.179	161.7					
		5	121.3	2.404	157.4					
		avg	128.8	2.293	163.2					
		S.D.	6.2	0.095	6.0					
		% CV	4.8%	4.2%	3.7%					
AY	RC	41	129.7	5.405	210.7	Category 1	Category I	4.9%	15.4%	8.0%
		42	117.7	4.535	185.7					
		43	123.7	6.195	216.6					
		avg	123.7	5.379	204.3					
		S.D.	6.0	0.830	16.4					
		% CV	4.9%	15.4%	8.0%					
BE	AC	1	5.0	0.290	9.4	Non-irritant	Category III	40.1%	22.5%	32.5%
		2	11.0	0.318	15.8					
		3	12.3	0.240	15.9					
		4	8.0	0.255	11.8					
		5	16.0	0.412	22.2					
		avg	10.5	0.303	15.0					
		S.D.	4.2	0.068	4.9					
		% CV	40.1%	22.5%	32.5%					
BF	SO	35	48.0	1.140	65.1	Category 2A	Category III	18.6%	20.2%	6.5%
		36	33.0	1.722	58.8					
		37	44.0	1.502	66.5					
		avg	41.7	1.455	63.5					
		S.D.	7.8	0.294	4.1					
		% CV	18.6%	20.2%	6.5%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
BJ	AL	11	66.7	0.757	78.1	Non-irritant	Category III	9.6%	32.4%	10.2%
		12	67.0	0.777	78.7					
		13	54.7	1.146	71.9					
		14	61.3	0.676	71.4					
		15	70.3	1.396	91.2					
		avg	64.0	0.950	78.3					
		S.D.	6.1	0.308	8.0					
		% CV	9.6%	32.4%	10.2%					
BJ	AL	7	75.0	1.326	94.9	Non-irritant	Category III	4.2%	9.1%	4.9%
		8	70.5	1.341	90.6					
		9	76.5	1.556	99.8					
		avg	74.0	1.408	95.1					
		S.D.	3.1	0.129	4.6					
		% CV	4.2%	9.1%	4.9%					
BM	SO	32	16.0	0.584	24.8	Non-irritant	Category IV	16.1%	59.4%	12.9%
		36	18.0	0.301	22.5					
		37	13.0	1.065	29.0					
		avg	15.7	0.650	25.4					
		S.D.	2.5	0.386	3.3					
		% CV	16.1%	59.4%	12.9%					
BN	SU	1	10.3	0.325	15.2	Non-irritant	Category IV	24.2%	36.0%	21.8%
		2	9.0	0.147	11.2					
		3	5.7	0.369	11.2					
		4	11.0	0.467	18.0					
		5	7.7	0.306	12.3					
		avg	8.7	0.323	13.6					
		S.D.	2.1	0.116	3.0					
		% CV	24.2%	36.0%	21.8%					

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Test Material Code	Formulation Type	Cornea Number	Opacity	Permeability	*In vitro* Score	*In vivo* GHS Cat	*In vivo* EPA Cat	Opacity CV	Perm. CV	*In vitro* Score CV
BQ	SO	20	32.0	0.110	33.7	Non-irritant	Category IV	6.9%	48.9%	6.8%
		22	31.0	0.320	35.8					
		23	28.0	0.214	31.2					
		avg	30.3	0.215	33.6					
		S.D.	2.1	0.105	2.3					
		% CV	6.9%	48.9%	6.8%					
BR	SU	43	15.3	0.832	27.8	Non-irritant	Category IV	60.5%	3.7%	30.7%
		46	14.3	0.822	26.7					
		47	3.3	0.776	15.0					
		avg	11.0	0.810	23.2					
		S.D.	6.7	0.030	7.1					
		% CV	60.5%	3.7%	30.7%					
BS	RC	10	208.5	3.478	260.7	Category 2A	Category III	3.7%	15.0%	4.1%
		11	223.5	3.733	279.5					
		12	211.5	4.608	280.6					
		avg	214.5	3.940	273.6					
		S.D.	7.9	0.593	11.2					
		% CV	3.7%	15.0%	4.1%					
EF	RC	40	27.7	5.157	105.0	Category 2A	Category II	19.7%	13.5%	13.4%
		41	18.7	4.797	90.6					
		42	25.7	6.197	118.6					
		avg	24.0	5.384	104.8					
		S.D.	4.7	0.727	14.0					
		% CV	19.7%	13.5%	13.4%					
EG	AC	26	35.7	2.257	69.5	Category 2A	Category II	11.3%	14.0%	4.1%
		27	41.7	2.232	75.2					
		32	44.7	1.742	70.8					
		avg	40.7	2.077	71.8					
		S.D.	4.6	0.290	2.9					
		% CV	11.3%	14.0%	4.1%					
Mean CV for materials with *in vitro* scores greater than 10								**27.9%**	**24.1%**	**18.3%**

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Table 7-28 Distribution of product categories for the within-run reproducibility of the BCOP assay. Some products have repeat tests.

Product Categories	Number of products tested
Surfactants	18
Acids	7
Bases	20
Solvents	12
Oxidizers	18
Other	0
Total	**75**

4952   7.3.1.2 BCOP within-run reproducibility for a wide range of materials
4953
4954   The BCOP within-run variability has been assessed in the Bovine Corneal
4955   Opacity and Permeability Test Method Background Review Document prepared by
4956   NICEATM (appended to this report). The within-run variability for the single
4957   parameter of *in vitro* score is presented in Tables 7-1 to 7-3, 7-7 and 7-9 of that
4958   document. It should be noted that in each of the tables the mean %CV is
4959   significantly influenced by several CV's of greater than 100 generated by the very
4960   low overall scores of very mild materials. For example, in Table 7-1 if the last four
4961   CV's generated from the extremely low scores of the mild materials are ignored, the
4962   mean %CV falls from 48.3% to 18.6%!

4963   7.3.2 BCOP intralaboratory reproducibility
4964

4965   The BCOP interlaboratory reproducibility can be evaluated based not only on
4966   data from studies on antimicrobial cleaning products that were submitted to support
4967   this specific BRD, but also on the information contained in the Bovine Corneal
4968   Opacity and Permeability Test Method Background Review Document prepared by
4969   NICEATM.

4970   7.3.2.1 BCOP intralaboratory reproducibility for antimicrobial cleaning products data
4971

4972   Table 7-29 presents intralaboratory reproducibility data for 5 different anti-
4973   microbial cleaning products tested from 2 – 6 times in the same laboratory. It can be
4974   seen that the individual %CV's range from 2.6 – 49.2%, and the mean
4975   intralaboratory %CV for the 5 materials is 20.3%. The highest CV of 49.2% is the
4976   result of two extremely high *in vitro* scores, already well above the proposed cutoff
4977   of 75 for EPA I or GHS 1 toxicity categories. Thus we consider the impact of this
4978   high CV to be negligible. The distribution of product categories for the
4979   intralaboratory reproducibility for antimicrobial cleaning products is shown in Table
4980   7-30.
4981

4982   **Table 7-29 Intralaboratory reproducibility for 5 antimicrobial cleaning products. See Table 7-**
4983   **27 for individual cornea scores.**

Substance	Formulation Type	Mean *In vitro* Irritancy Score (n = 3-5 corneas)	No. of Exp.	Mean	S.D.	%CV
F	RC	360.5 174.4	2	267.5	131.6	49.2%
H	AL	9.2 11.4 15.2 13 13.8 21.3	6	14.0	4.1	29.6%
AV	RC	191.8 175.3	2	183.6	11.7	6.4%
AX	SO	157.3 163.2	2	160.3	4.2	2.6%
BJ	AL	78.3 95.1	2	86.7	11.9	13.7%
Mean %CV						**20.3%**
Median %CV						**13.7%**

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Table 7-30 Distribution of product categories for the intralaboratory reproducibility for antimicrobial cleaning products.

Product Categories	Number of products tested
Surfactants	0
Acids	0
Bases	2
Solvents	1
Oxidizers	2
Other	0
Total	**5**

4988   7.3.2.2 BCOP intralaboratory reproducibility for a wide range of materials
4989

4990   The BCOP intralaboratory variability has been assessed in the Bovine
4991   Corneal Opacity and Permeability Test Method Background Review Document
4992   prepared by NICEATM. The intralaboratory variability for the single parameter of *in*
4993   *vitro* score is presented in Tables 7-4 to 7-6 of that document. The mean %CV's for
4994   each of the respective tables are: 12.6%, 14.8% and 14.0%
4995

4996   7.3.3 BCOP interlaboratory reproducibility

4997   The Bovine Corneal Opacity and Permeability Test Method Background
4998   Review Document prepared by NICEATM is the best source of interlaboratory
4999   reproducibility information. This document presents data from an interlaboratory
5000   study by Gautheron (1994) (Table 7-31), Balls et al (1995) (Table 7-33) and
5001   Southee (1998) (Table 7-35). These data are very useful since the in vitro BCOP
5002   data for all of the anti-microbial cleaning products presented in this document were
5003   all conducted in one laboratory (IIVS). Thus interlaboratory variability for the specific
5004   BCOP antimicrobial cleaning products data could not be assessed.

5005   All three of the above studies are analyzed by %CV of the mean results of
5006   the participating laboratories. This is a reasonable way of assessing variability when
5007   the results can vary over wide ranges, *e.g.,* as it does with the Cytosensor assay,
5008   but it can be extremely biased when used to characterize assays which are
5009   constrained at the lower end of irritancy by scores which range around zero.
5010   Fluctuations in these scores which are meaningless relative to the entire scoring
5011   scale (approximately 500 for the BCOP assay) result in large CV's which
5012   inappropriately influence the overall CV of a study by raising the average CV
5013   significantly. For example, BCOP scores of 1, 2, and 4 are all indicative of
5014   essentially no toxicity and could be said to actually all represent the same score.
5015   However, a calculation of the mean and CV of these three values results in a mean
5016   of 2.3 and a CV of **65%**! That same variation of one to three units at higher irritancy,
5017   *e.g.,* scores of 150, 151, and 154, results in a mean of 152.3 and a CV of **1.0%**! To
5018   get an accurate measurement of the true variability of scores, one should scan the
5019   range of scores that are being considered and put more weight on the CV's that
5020   appear at mid-range than on the scores at the low end of the scale.

5021   The Gautheron study reported data from 11-12 labs. The %CV's were
5022   consistently around 30% – 50% (median CV = 46.9%); however, the mean CV was
5023   considerably higher at 167%. Inspection of the table shows that this high value is
5024   mainly the result of the CV's from mean values which are <5. The distribution of
5025   product categories for the interlaboratory reproducibility for the Gautheron study is
5026   shown in Table 7-32

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5028   **Table 7-31 Coefficient of Variation Analysis of the Interlaboratory Variability of the BCOP Test**
5029   **Method for Gautheron *et al.* (1994)[1]**

Substance	Formulation Type	Mean Irritancy Score	No. of Labs	%CV	Prediction
2-Ethoxyethanol	SO	91.3	12	16.5	Severe
2,4-Pentanedione	SO	59.8	12	24	Severe
Allyl alcohol	SO	156	12	27	Severe
Imidazole		87.9	12	28.5	Severe
Furan		56	12	29.4	Severe
Benzethonium chloride	SU	133.9	11	31.7	Severe
Butyrolactone		45.6	12	32.2	Moderate
Cyclohexanone	SO	105.6	11	33.3	Severe
2-Methoxyethanol	SO	63.5	11	33.6	Severe
Laurylsulfobetaine	SU	80.6	11	34	Severe
Ethyl acetoacetate		31.8	11	34.9	Moderate
Gluconolactone		76.6	11	35	Severe
Methylisobutyl ketone	SO	19.9	11	36	Mild
Pyridine	SO	112.8	11	38.4	Severe
Ethanol	SO	60.7	11	39.1	Severe
3-Glycidoxypropyltrimethoxysilane		16.6	12	40	Moderate
N-Lauroylsarcosine, sodium salt	SU	50	11	41.7	Moderate
Octanol	SO	47.4	11	41.7	Moderate
Deoxycholic acid, sodium salt	SU	93.5	12	43	Severe
2-Aminophenol		7	12	43.5	Mild
Hexadecyltrimethylammonium bromide		66.4	11	45.2	Severe
1-Phenyl-3-pyrazolidone		12.9	12	46.5	Mild
Dibenzoyl-L-tartaric acid		120.5	11	46.8	Severe
Dimethyl sulfoxide	SO	11.4	11	46.9	Mild
1-Nitropropane	SO	7.6	12	46.9	Mild
1 ,2,4-Trimethylbenzene		16.1	12	47	Mild
Propyl-4-hydroxybenzoate		7.9	11	48	Mild
Promethazine hydrochloride		112.4	11	49.3	Severe
1 ,2,3-Trichloropropane	SO	47.5	11	50.3	Moderate
Diacetone alcohol	SO	53.5	11	50.8	Moderate
Methanol	SO	84.2	11	55.7	Severe
2,4-Dichloro-5-sulfamoylbenzoic acid		26.3	12	58.5	Moderate
Sodium oxalate		4.8	12	66	Mild
Quinacrine		31.1	11	74.8	Moderate
Petroleum ether	SO	5.5	12	75.4	Mild
Dimethylbiguanide		2.9	11	82	Mild
Magnesium carbonate		3	11	83	Mild
Triethanolamine	SO	2.2	11	101.5	Mild

Substance	Formulation Type	Mean Irritancy Score	No. of Labs	%CV	Prediction
Aluminum hydroxide		6.8	12	107	Mild
Tetraaminopyrimidine sulfate		6	11	107	Mild
Hexane	SO	1.4	12	143	Mild
Iminodibenzyl		2.4	11	177.5	Mild
2-Mercaptopyrimidine		-1.25	12	208	Mild
Triton X-155	SU	0.55	11	276	Mild
DL-Glutamic acid		0.58	12	330.6	Mild
Anthracene		-0.33	12	430	Mild
Betaine monohydrate		0.92	12	432	Mild
MYRJ-45	SU	-0.18	11	962	Mild
EDTA di-potassium salt		-0.33	12	1009	Mild
BRIJ-35	SU	-0.09	11	1280	Mild
Phenylbutazone		-0.17	12	1325	Mild
Mean CV(%)					**167.6 (all substances)** **84 (excluding MYRJ-45, EDTA, BRIJ-35, phenylbutazone)**
Median CV(%)					**46.9**

5030 [1] *Substances organized by increasing %CV.*
5031
5032 **Table 7-32 Distribution (estimated) of product categories for the**
5033 **interlaboratory reproducibility for the Gautheron study.**

Product Categories	Number of products tested
Surfactants	7
Acids	Unknown
Bases	Unknown
Solvents	16
Oxidizers	Unknown
Other	Unknown
Total	**23**

5034
5035   The Balls *et al.* study reported data from 5 labs. The %CV's were consistently
5036 around 25%-35% (median CV = 30.6%); however, the mean CV was considerably
5037 higher at 125%. Again, inspection of the table shows that this very high value is
5038 mainly the result of the CV's from mean values which are <5. The distribution of
5039 product categories for the interlaboratory reproducibility for the Balls study is shown
5040 in Table 7-34.
5041
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5043 **Table 7-33 Coefficient of Variation Analysis of the Interlaboratory Variability of the BCOP Test**
5044 **Method for Balls *et al.* (1995)**
5045

Substance	Formulation Type	Mean Irritancy Score	No. of Labs	%CV	Prediction
1 -Naphthalene acetic acid, Na salt		149.2	5	7.6	Severe
Benzalkonium chloride (10%)	SU	136.5	5	10.9	Severe
Sodium hydroxide (1%)	AL	150	5	12.3	Severe
Cetylpyridinium bromide (6%)	SU	71.2	5	12.7	Severe
Acetone	SO	123	5	14	Severe
Imidazole		112.7	5	14.5	Severe
Benzalkonium chloride (5%)	SU	128.5	5	15.6	Severe
Methyl acetate	SO	54.9	5	17.4	Moderate
Sodium hydroxide (10%)	AL	271.9	5	17.6	Severe
Toluene	SO	35.6	5	18.1	Moderate
Chlorhexidine		114	5	18.3	Severe
Trichloroacetic acid (3 0%)	AC	264	5	18.7	Severe
Dibenzyl phosphate	SO	378	5	18.8	Severe
2,2-Dimethylbutanoic acid	AC	111.9	5	19.5	Severe
Pyridine	SO	148	5	20.1	Severe
Promethazine hydrochloride		121.4	5	20.4	Severe
Trichloroacetic acid (3%)	AC	75.9	5	21.1	Severe
Benzalkonium chloride (1 %)	SU	88.8	5	21.7	Severe
Parafluoraniline		30.4	5	21.7	Moderate
Methyl ethyl ketone	SO	70.4	5	22.6	Severe
4-Carboxybenzaldehyde		78.3	5	24	Severe
Ethanol	SO	70.6	5	24.1	Severe
Cetylpyridinium bromide (10%)	SU	72	5	24.2	Severe
Triton X-100 (5 %)	SU	78.3	5	24.2	Severe
Triton X-100 (10 %)	SU	70.3	5	25.3	Severe
Isobutanol	SO	56	5	26.1	Severe
n-Hexanol	SO	61.9	5	27	Severe
Sodium lauryl sulfate (15 %)	SU	63.3	5	28	Severe
Cyclohexanol	SO	60.1	5	28.5	Severe
2,6-Dichlorobenzoyl chloride		10.4	5	30.6	Mild
Sodium lauryl sulfate (3 %)	SU	25.8	5	30.9	Mild
Isopropanol	SO	57.9	5	31.3	Severe
Sodium perborate		97	5	35.8	Severe
Methyl isobutyl ketone	SO	12.6	5	36	Mild
1-Naphthalene acetic acid		78.1	5	37.4	Severe
Butyl acetate	SO	34.6	5	38.4	Moderate
Methyl cyanoacetate		12.2	5	39.2	Mild
Ethyl acetate	SO	32	5	40.5	Moderate
Potassium cyanate		15	5	40.9	Mild
2,5-Dimethylhexanediol	SO	20.8	5	41.6	Mild
Benzoyl-L-tartaric acid		169.6	5	43	Severe
gamma-Butyrolactone	SO	60.7	5	45	Severe
Tetraaminopyrimidine sulfate		15.1	5	46.3	Mild
Methylcyclopentane	SO	2.8	5	47.8	Mild
2-Ethyl-1-hexanol	SO	39.8	5	48.2	Moderate
Cetylpyridinium bromide (0.1%)	SU	9.2	5	51.4	Mild

Substance		Mean Irritancy Score	No. of Labs	%CV	Prediction
Maneb		40.5	5	58.3	Moderate
n-Octanol	SO	40.9	5	58.8	Moderate
Ethyl-2-methylacetoacetate		14.4	5	65.3	Mild
Ethyl trimethyl acetate	SO	17.8	5	66.3	Mild
Ammonium nitrate		9.8	5	69.7	Mild
L-Aspartic acid		1.3	5	73.6	Mild
Captan 90 concentrate		43.8	5	75.8	Moderate
Quinacrine		1.6	5	76.9	Mild
Fomesafen		60.7	5	89.4	Severe
Sodium oxalate		14	5	143	Mild
Polyethylene glycol 400	SU	1.1	5	145	Mild
Glycerol	SO	0.26	5	712	Mild
Tween 20	SU	-0.04	5	4511	Mild
Mean %CV					**125 (all test substances) 50 (excluding Tween 20)**
Median %CV					**30.6**

5046   [1]*Substances organized by increasing %CV.*

5047

5048   **Table 7-34 Distribution of product categories (estimated) for**
5049   **the interlaboratory reproducibility for the Balls study.**

Product Categories	Number of products tested
Surfactants	12
Acids	1
Bases	2
Solvents	21
Oxidizers	Unknown
Other	Unknown
Total	**36**

5050

5051   The Southee *et al.* study reported data from 3 labs. The% CV's were
5052   consistently around 15% - 25%% (median CV = 22.8%); however, the mean CV
5053   was higher at 32%. Again, inspection of the table shows that this higher value is
5054   mainly the result of the CV's from mean values which are <5. The distribution of
5055   product categories for the interlaboratory reproducibility for the Southee study is
5056   shown for Table 7-36.

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5059   **Table 7-35 Coefficient of Variation Analysis of the Interlaboratory Variability of the BCOP Test**
5060   **Method for Southee (1998)**

Substance	Formulation Type	Mean Irritancy Score	No. of Labs	%CV	Prediction
Butyl cellosolve	SO	100.9	3	7.5	Severe
Benzalkonium chloride	SU	160	3	8.5	Severe
NaOH (10%)	AL	226	3	8.6	Severe
Imidazole		136.9	3	9.1	Severe
4-Carboxybenzaldehyde		46.7	3	9.5	Moderate
Parafluoroaniline		32.1	3	19.1	Moderate
Methyl ethyl ketone	SO	82.5	3	21.6	Severe
Ethanol	SO	48.7	3	22.1	Moderate
Ammonium nitrate		5.03	3	23.4	Mild
Hexadecyltrimethylammonium bromide (10%)		29.3	3	27.1	Moderate
Glycerol	SO	0.72	3	33.5	Mild
Propyl-4-hydroxybenzoate		6.9	3	37.7	Mild
Triton X-100 (5%)	SU	3.3	3	44.8	Mild
Sodium lauryl sulfate (15%)	SU	9.7	3	57.1	Mild
Tween 20	SU	0.23	3	79.8	Mild
Sodium oxalate		3.6	3	108.8	Mild
Mean %CV					**32.4**
Median %CV					**22.8**

5061   [1]*Substances organized by increasing %CV*
5062
5063   **Table 7-36 Distribution of product categories (estimated) for the**
5064   **interlaboratory reproducibility for the Southee study.**

Product Categories	Number of products tested
Surfactants	4
Acids	Unknown
Bases	1
Solvents	4
Oxidizers	Unknown
Other	Unknown
Total	**9**

5065

5066   Additional information on interlaboratory reproducibility is given in Section 7
5067   of the NICEATM BRD on the BCOP assay.

5068 # 8 Test Method Data Quality

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5070 ## 8.1 Adherence to National and International GLP Guidelines

5071
5072   ICCVAM has suggested (ICCVAM 1997) that all data submitted in support of
5073 a new method be generated by methodologies that adhere to national or
5074 international GLP guidelines. It could not be ascertained that all of the *in vitro* data
5075 contained in this BRD were generated under full GLP compliance, but where it could
5076 be ascertained, that information is contained in the spreadsheets that form the
5077 database from which this BRD was generated. All of the new *in vitro* data that were
5078 generated during the course of constructing this BRD were conducted with full GLP
5079 compliance.
5080

5081 ## 8.2 Data Quality Audits

5082
5083   No data quality audits were conducted for the purpose of this BRD. Complete
5084 GLP audits were, of course, conducted for the studies included in this BRD which
5085 are identified as having been conducted with full GLP compliance.
5086

5087 ## 8.3 Impact of Deviation from GLP Guidelines

5088
5089   The data were not evaluated for the effect of any GLP deviations that may
5090 have been noted. However, *in vitro* data were accompanied by information that
5091 Criteria for a Valid Test listed in the protocol had been fulfilled during the study.
5092

5093 ## 8.4 Availability of Laboratory Notebooks or Other Records

5094
5095   Study notebooks, final reports, and other background documents are
5096 available for the majority of *in vitro* studies reported here. These documents have
5097 not been included with this BRD, but they will be available in a confidential form for
5098 inspection upon the request of NICEATM or the EPA. Companies who submitted
5099 data for this BRD did so with the understanding that their identities would not be
5100 linked to any of the tested materials. Thus company identifiers will be removed from
5101 any study notebooks or final reports which are requested by NICEATM or the EPA
5102 for audit.
5103
5104

9 Other Scientific Reports and Reviews

5105

5106

5107   The three *in vitro* methodologies (Cytosensor, EpiOcular, and BCOP assay)
5108 that are addressed in this BRD have been the subject of three individual BRD's.
5109 Since two are still under review, only the BCOP BRD is appended to this BRD.

5110

5111   The Cytosensor BRD was created under contract for ECVAM by IIVS. It has
5112 undergone an independent data audit and has been reviewed for scientific content
5113 by an independent management team designated by ECVAM. The final review for
5114 validity of the method has not been completed but is underway.

5115

5116   A BRD for the EpiOcular model has been created under contract to the
5117 Colgate-Palmolive company by IIVS. It has been submitted to ECVAM and has
5118 undergone a preliminary review by the ocular toxicology task force. Modifications
5119 and additions have been made to the document at the request at the task force, and
5120 it was resubmitted to ECVAM in December 2007.

5121

5122   A BRD for the BCOP assay was created by NICEATM as part of their
5123 program to identify the "Current Status of *In Vitro* Test Methods for Identifying
5124 Ocular Corrosives and Severe Irritants". It has been reviewed and modified and
5125 exists on the internet at
5126   http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_brd_bcop.htm.

5127

5128   # 10 Animal Welfare Considerations

5129

5130   ## 10.1 How the proposed non-animal testing strategy will refine, reduce or
5131   replace animal use for the purpose of toxicity labeling of anti-microbial
5132   cleaning products

5133

5134   Currently the EPA Pesticides Program requires a rabbit eye irritation test to
5135   determine the correct toxicity labeling category for ocular irritation. This test requires
5136   between one (if the material is shown to be corrosive or severe) and three (to
5137   determine less severe categories) rabbits for each product submitted for
5138   registration. <u>The testing strategy proposed in this BRD will completely replace the</u>
5139   <u>use of laboratory animals in this registration process.</u> Thus, no animals will be
5140   subjected to pain and suffering for the purpose of determining the EPA labeling
5141   category for eye irritation.

5142

5143   In terms of overall animal use, the BCOP assay does use tissue from animals
5144   (cattle), but these animals have already been slaughtered for the purpose of food
5145   production at the time that the ocular tissue is obtained. The cattle undergo no
5146   additional pain or suffering during the harvesting of the corneal tissue. In addition,
5147   this tissue is normally discarded and would end up being wasted if it was not used to
5148   prevent suffering to live animals.

5149

5150   Neither the Cytosensor method nor the EpiOcular method use live animals. A
5151   long established mouse cell line is used in the Cytosensor assay, but no new
5152   animals have to be sacrificed to conduct the assay. The EpiOcular assay utilizes
5153   only human tissue, and thus completely avoids the use of non-human animals.

5154

5155   Thus, no animals are harmed or experience pain as a result of conducting
5156   any of the three *in vitro* ocular irritation assays proposed in this testing strategy.

5157
5158

5159   **11 Practical Considerations**

5160   11.1 Use by industry
5161
5162   All of the companies involved in this validation effort conduct safety
5163   evaluations of new cleaning products in a similar fashion to that shown in Figure 11-
5164   1. However, the toxicity evaluation portion may be conducted differently depending
5165   on the individual company's specific product types and corporate experience and
5166   expertise. The specific *in vitro* test or tests to be used are chosen with knowledge of
5167   the historical performance of specific types of product chemistry with specific *in vitro*
5168   tests. The test data that result may then be compared to previous product test
5169   results contained in an historical database.
5170
5171   Since no one company has managed to develop comprehensive experience
5172   with the *in vitro* ocular activities of the entire range of anti-microbial cleaning
5173   products (because each company manufactures only specific product lines), we
5174   have attempted in this BRD to combine the experience and knowledge of all the
5175   companies. Thus each company's specific experience with its product line has been
5176   combined with that of others to produce a broad, generalized approach which
5177   covers the range of product types which exist in today's market place and are
5178   anticipated to be marketed in the reasonable future.
5179
5180   Most of these companies have spent a number of years developing the data
5181   we have presented here in the course of creating a safety evaluation approach
5182   which protects consumers without the use of whole animal studies.
5183

General Approach to New Product Safety Evaluation

New Formula

Toxicity Assessment
- Oral Toxicity   -Eye Irritation
- Dermal Toxicity   -Skin Irritation
- Inhalation -   -Sensitization

Exposure Assessment
- Population   -Duration, frequency
- Concentration

Do we have enough information?

No

Yes

Do we have enough information?

Yes

No

Identify and conduct necessary studies and assays

Integrated Risk Assessment

Exposure Studies
- Computer modeling
- Real-life air monitoring

- **Label language**
- **Packaging requirements**
- **Registration approval**

Market New Product

5184
5185   **Figure 11-1 Process of safety evaluations**
5186
5187
5188   Each of the tests described in this BRD has a long history of use by industry.
5189   The history of use of the BCOP assay has been documented in detail by NICEATM
5190   in the Background Review Document (BRD) "Current Status of *In Vitro* Test
5191   Methods for Identifying Ocular Corrosives and Severe Irritants: Bovine Corneal
5192   Opacity and Permeability Test Method" (NIEHS 2006). The BCOP assay is used as
5193   an in-house screen to assess potential ocular irritation of a wide range of
5194   substances resulting from accidental exposure in the workplace or home (Sina
5195   1994; Swanson, Lake et al. 1995; Casterton, Potts et al. 1996; Chamberlain, Gad et
5196   al. 1997; Harbell and Curren 1998; Cater, Nusair et al. 2002; Cuellar, Lloyd et al.
5197   2003; Bailey, Freeman et al. 2004). A secondary application has been the use of the

5198   assay for product development.   By comparing new formulations with marketed
5199   materials of similar composition, manufacturers can evaluate the relative irritancy as
5200   part of an initial safety screening. The BRD cites specific instances to illustrate the
5201   applications of the assay for workplace safety (Chamberlain, Gad et al. 1997),
5202   product safety, and/or product development (Cuellar N and Swanson J, personal
5203   communications).   In both instances, *in vivo* confirmatory testing is reduced or may
5204   not be necessary. Details of the use of this assay can be found in Section 2.2.3.
5205
5206   The Cytosensor microphysiometer assay has been used by companies since
5207   its first introduction in the early 1990's (Bruner, Miller et al. 1991) to assess eye
5208   irritancy potential of liquid or water soluble ingredients or formulations. This
5209   information is sometimes combined with other available information in integrative
5210   toxicological evaluation, and final safety decisions are made based on this
5211   information. Details can be found in the background description Section 2.2.1.
5212
5213   The EpiOcular model is also used by industry as an *in vitro* assay to assess
5214   eye irritation potential (Ghassemi, Osborne et al. 1997; Stern, Klausner et al. 1998).
5215   As with the other two models, this information is sometimes combined with other
5216   available information in integrative toxicological evaluation, and final safety
5217   decisions are made based on this information. Details can be found in the
5218   background description Section 2.2.2.
5219

5220   ## 11.2 Ease of transferability

5221
5222   ECVAM has recently (Hartung, Bremer et al. 2004) discussed transferability
5223   of tests. In their manuscript describing a modular approach to validation, they state
5224   that transferability "should demonstrate that the test can be successfully repeated in
5225   a laboratory different from the one which has developed or which was involved in
5226   the optimization of the test". Ease of transferability is supported by evaluating
5227   interlaboratory reproducibility of the assay system. Details of interlaboratory
5228   reproducibility for each of the three *in vitro* methods addressed in this BRD are
5229   given in Section 7 of this BRD and in even greater detail in the accessory BRD that
5230   is appended to this report.
5231

5232   ### 11.2.1 Facilities and major fixed equipment for the Cytosensor test method

5233
5234   The major fixed equipment for the Cytosensor test method is the instrument
5235   itself. When purchased new the instrument was quite expensive (>$100,000), but as
5236   of Summer 2007 the instrument is no longer available from its former manufacturer,
5237   Molecular Devices, Inc. (Menlo Park, CA). In addition, Molecular Devices has stated
5238   that they will be able to sell disposable supplies for the machine only until their
5239   current supply lasts. At this time (Summer 2008), we have discovered that they have
5240   provided at least one user with the name of their third-party contractor(s) who
5241   manufactures the disposables for them. This user has found that purchasing the

5242 disposable supplies directly from the original manufacturer (not Molecular Devices)
5243 is possible, and he has shared the purchasing information with IIVS. None the less,
5244 in anticipation of the dropping of support for the instrument, IIVS has purchased a
5245 supply of disposables which should last for at least two years, and they have also
5246 obtained repair parts that are also likely sufficient to support the instrument for more
5247 than two years. Thus it is likely that testing can continue with the CM for a number of
5248 years.
5249
5250   More information on additional standard laboratory equipment needed to
5251 support this assay is given in detail in the Cytosensor BRD prepared for ECVAM
5252 which will be available after it has had a final review by ESAC.
5253

5254 11.2.2 Facilities and major fixed equipment for the EpiOcular test method
5255
5256   A general purpose tissue culture facility is required for the use of the
5257 EpiOcular model. There should be provisions for handling the cultures in a sterile
5258 environment as well as facilities for appropriately containing any toxic test materials
5259 that might be utilized in the test.
5260
5261   Major equipment would include a Class II Type A or B tissue culture hood,
5262 37°C humidified incubator, and an inverted microscope. A 96-well plate reader is
5263 highly desirable but not mandatory since a small spectrophotometer could also be
5264 used.
5265
5266   More information on additional standard laboratory equipment needed to
5267 support this assay is given in detail in the EpiOcular BRD prepared for ECVAM
5268 which will be attached to this BRD after it has had a final review by ESAC.
5269

5270 11.2.3 Facilities and major fixed equipment for the BCOP test method
5271
5272   The main facility requirements for the BCOP assay can be found in most any
5273 standard biology laboratory. Sterile handling of the tissue is not an absolute
5274 requirement and most experiments can be conducted on the bench top. Proper
5275 containment is, of course, needed anytime that extremely toxic materials are tested.
5276 One major piece of equipment required is the opacitometer which can be obtained
5277 from Stag Bio (Clermont, France). The price of the opacitometer has risen
5278 significantly over the years and is now quoted at ~$9000 per unit.
5279
5280   More information on additional standard laboratory equipment needed to
5281 support this assay is given in detail in the BCOP BRD (ICCVAM 2006) prepared by
5282 NICEATM which will be attached to this BRD after it has had a final review by
5283 ESAC.
5284

5285   11.3 Training required

5286
5287   Training in standard *in vitro* techniques and laboratory procedures is required
5288   for all of the three assays in order to assure that the assay is run correctly. Since it
5289   is likely that toxic materials may be tested in the assays, laboratory safety training
5290   should also be required before a technician is allowed to conduct any of the assays.

5291   11.3.1 Required level of training and expertise needed to conduct the
5292   Cytosensor assay

5293
5294   Two areas of training are especially important for the conduct of the
5295   Cytosensor assay. The first is general tissue culture technique needed to culture the
5296   L929 cells (or other cell lines) which are used as the target cells in the assay. The
5297   second is specific operation of the Cytosensor itself. Although many of the functions
5298   of the machine are programmed to occur automatically through the supplied
5299   CytoSoft program, the technician still needs to learn how to program the general
5300   parameters of each run into the controlling computer. This training is not arduous,
5301   but should be continued until the technician can reproducibly test 3 to 5 compatible
5302   materials with the Cytosensor such the values for the materials approach the
5303   historic mean for those materials tested in that laboratory.
5304

5305   11.3.2 Required level of training and expertise needed to conduct the
5306   EpiOcular assay

5307
5308   The techniques involved with the EpiOcular methodology are fairly standard
5309   for those trained within an *in vitro* toxicology laboratory. No specific expertise
5310   outside of that commonly used for tissue culture and toxic material handling is
5311   required.
5312
5313   Training for this specific method is required and is assisted by developing a
5314   detailed laboratory workbook that outlines the procedures and the data that need to
5315   be recorded at each step.
5316
5317   In the IIVS laboratory, each technician is required to demonstrate acceptable
5318   performance for the testing of five standard surfactant materials whose toxicities are
5319   well established.
5320

5321   11.3.3 Required level of training and expertise needed to conduct the BCOP
5322   assay

5323
5324   The following discussion of training for the BCOP assay is abstracted from
5325   the NICEATM-prepared BCOP BRD appended to this report.

5326
5327   "A training period of between two to three months is usually required for a technician
5328   with general laboratory skills to proficiently conduct all aspects of the standard
5329   BCOP assay with reasonably little supervision. The individual would need basic
5330   laboratory skills including
5331
5332   • Aseptic technique,
5333   • Knowledge and training in the preparation of dilutions,
5334   • Training in the use of an analytical balance,
5335   • Proficiency in the use of single channel pipettes
5336   • Calibration and use of the spectrophotometer
5337
5338   Specific laboratory skills would include
5339
5340   • Identification of a bovine cornea free of corneal defects
5341   • Excising the cornea from the bovine eye
5342   • Mounting the cornea in a corneal holder without damaging the epithelium or
5343   endothelium
5344   • Addition of media without air bubbles to the posterior and anterior chamber
5345   • Examination of mounted corneas for defects
5346   • Addition of test material to the corneal chamber
5347   • Washing the cornea (closed and open chamber) without inducing mechanical
5348   damage
5349   • Calibration and use of the opacitometer"
5350
5351   "Evaluation of a technician for proficiency in the assay is based upon the
5352   successful performance of the assay using positive and negative controls.
5353
5354   The histopathological evaluation of the corneas requires skills in the preparation
5355   and the evaluation of corneal tissue. Fixed corneas should be trimmed, embedded
5356   and stained by a qualified histology laboratory. Proficiency in the evaluation of the
5357   slides requires a training period of up to six months and is dependent on the
5358   experience of the individual."

5359   ## 11.4 Cost Considerations

5360
5361   A GLP-compliant CM assay conducted at IIVS is $2,050 (minimum of 2 test
5362   materials). Five or more materials run concurrently is $1,375 per test material.
5363   These prices are currently used at IIVS. We know of no other commercial sources
5364   for the CM assay.
5365
5366   A GLP-compliant EO assay (range-finding plus definitive assay; positive and
5367   negative control; duplicate tissues) conducted at IIVS is $3,700 for a single test
5368   article. Five or more materials run concurrently is $2,750 per test material. A second
5369   laboratory, MB Research Laboratories (Spinnerstown, PA), charges $2,200 per test

5370   article for two replicates at three time points and charges $3,225 for four time points
5371   for one test article.
5372
5373   The price for a GLP compliant BCOP assay at IIVS is approximately $1,850
5374   for a single test substance, including positive and negative controls. Histopathology
5375   can be performed on corneas from that same study for an additional $4,750. Costs
5376   per test substance can be reduced considerably with the performance of multiple
5377   materials run concurrently. For example, a single material tested in the BCOP assay
5378   plus histology would be approximately $6,600, two materials run concurrently would
5379   be approximately $3,900/test material, three materials run concurrently would be
5380   approximately $3,480/test material and four materials run concurrently would be
5381   approximately $3,300/test article. A second laboratory, MB Research Laboratories
5382   (Spinnerstown, PA), charges $1,000 per test article with no histology and $1,900 per
5383   test article with histology.
5384
5385   The price for a GLP compliant *in vivo* Driaze test ranges from approximately
5386 $1160 to $14,500 depending on the lab and the number of days the animals
5387   remained on study.
5388
5389   Unlike *in vivo* testing, *in vitro* testing lends itself to significant economies of
5390   scale when evaluating multiple materials concurrently.

5391   ## 11.5 Time Considerations

5392
5393   Timing for each of the three assays varies and is described for each below.
5394   These times should be compared with a typical *in vivo* rabbit eye test which would
5395   require a minimum of one to three days, although the assay must be extended up to
5396   21 days if certain lesions don't clear.
5397

5398   ### 11.5.1 Timing for Cytosensor test method

5399
5400   The Cytosensor assay can actually be conducted in a single day, including
5401   multiple runs of the test material. Completion of the final report would then take
5402   several more days.
5403

5404   ### 11.5.2 Timing for EpiOcular test method

5405
5406   The EpiOcular test generally takes one and one-half to two days in the
5407   laboratory to complete. A two week lead time is usually required to obtain the
5408   EpiOcular tissue from its manufacturer, MatTek Corporation. Again completion of
5409   the final report would take several more days.
5410

5411   11.5.3 Timing for BCOP test method
5412
5413   The standard BCOP assay can be completed in the laboratory in one day (an
5414   extended day may be necessary for certain protocol modifications). Completion of
5415   the final report would take several more days.
5416
5417   If histology is required, *e.g.,* if the BCOP score was <75, but >25, then
5418   turnaround time would be considerably extended. Currently at IIVS it can take
5419   several weeks to have the tissue processed and then more time to have the slides
5420   read by a pathologist.
5421
5422   Total time required for the assay if histology was require would be
5423   approximately four weeks.
5424

12 References

Araki, K., Y. Ohahsi, S. Knoshita, K. Hayahsi, Y. Kuwayama and Y. Tano (1994). "Epithelial Wound Healing in the Denervated Cornea." <u>Current Eye Research</u> **13**: 203-211.

Bailey, P., J. Freeman, R. Phillips and J. Merrill (2004). "Validation of the BCOP Assay as a Predictor of Ocular Irritation of Various Petrochemical Products." <u>Society of Toxicology 2004 Meeting</u>.

Baldwin, H. A., T. O. McDonald and C. H. Beasley (1973). "Slit examination of experimental animals eyes. II Grading scales and photographic evaluation of induced pathological conditions." <u>J Society of Cosmetic Chemistry</u> **24**: 181-195.

Balls, M., P. A. Botham, L. H. Bruner and H. Spielmann (1995). "The EC/HO International Validation Study on Alternatives to the Draize Eye Irritation Test." <u>Toxicology In Vitro</u> **9**(6): 871-929.

Beckley, J. H. (1965). "Comparative Eye Testing: Man vs. Animal." <u>Toxicol Appl Pharmacol</u> **7**: 93-101.

Beckley, J. H. (1969). "Use of the Rhesus Monkey for Predicting Human Response to Eye Irritants." <u>Toxicol Appl Pharmacol</u> **15**: 1-9.

Bell, G. H., D. Emslie-Smith and C. R. Paterson (1976). <u>Chapter 41: Special Senses</u>. Edinburgh.

Botham, P., R. Osborne, K. Atkinson, G. Carr, M. Cottin and R. G. Van Buskirk (1997). "IRAG Working Group 3. Cell Function-based Assays. Interagency Regulatory Alternatives Group." <u>Food and Chemical Toxicology</u> **35**(1): 67-77.

Brantom, P. G., L. H. Bruner, M. Chamberlain, O. De Silva, J. Dupuis, L. K. Earl, et al. (1997). "A Summary Report of the COLIPA International Validation Study on Alternatives to the Draize Rabbit Eye Irritation Test." <u>Toxicology In Vitro</u> **11**: 141-179.

Bruner, L. H., G. J. Carr, R. D. Curren and M. Chamberlain (1998). "Validation of Alternative Methods for Toxicity Testing." <u>Environ Health Perspect</u> **106 Suppl 2**: 477-84.

Bruner, L. H. and K. A. Kohrman (1993). <u>The Low Volume Eye Test as a Refinement to the Draize Eye Irritation Test</u>. Bethesda, Scientists Centre for Animal Welfare.

Bruner, L. H., K. M. Miller, J. C. Owicki, J. W. Parce and V. C. Muir (1991). "Testing Ocular Irritancy In Vitro with the Silicon Microphysiometer." <u>Toxicology In Vitro</u> **5**: 277-284.

Burdick, J., J. Merrill, T. Spangler, G. Moyer and J. Harbell (2002). "Use of Histological Examination in Bovine Corneal Opacity and Permeability (BCOP) Assay for Assessing the Ocular Irritation Potential of Fragranced Formulations." <u>The Toxicologist</u> **66**(1): 244.

Butler, J. M. and B. R. Hammond (1980). "The Effects of Sensory Denervation on the Response of the Rabbit Eye to prostaglandin E1, bradykinin and substance P." <u>British Journal of Pharmacology</u> **69**: 495-502.

5469   Casterton, P. L., L. Potts and B. Klein (1996). "A Novel Approach to Assessing Eye
5470   Irritation Potential Using the Bovine Corneal Opacity and Permeability
5471   Assay." J Toxicol-Cut and Ocular Toxicol **15**(2): 147-163.
5472   Cater, K. C., G. Mun, G. Moyer, J. Merrill and J. Harbell (2003). "Exploratory In Vitro
5473   Eye Irritation Study of Marketed Alkaline Dry Laundry Detergents by BCOP
5474   Assay and pH/reserve Alkalinity (RA) Parameters." The Toxicologist **72**: 220.
5475   Cater, K. C., T. Nusair, J. C. Merrill and J. W. Harbell (2002). "Exploratory In Vitro
5476   Eye Irritation Study of Marketed Alkaline Liquid Laundry Detergents by BCOP
5477   Assay and pH/reserve Alkalinity (RA) Parameters." The Toxicologist **66**: 244.
5478   Cater, K. C., H. A. Raabe, G. C. Mun and J. W. Harbell (2001). "Corporate
5479   Validation Program for Predicting Eye Irritation of Surfactant Formulations."
5480   The Toxicologist **60**(1): 99.
5481   Chamberlain, M., S. C. Gad, P. Gautheron and M. K. Prinsen (1997). "IRAG
5482   Working Group 1. Organotypic Models for the Assessment/prediction of
5483   Ocular Irritation. Interagency Regulatory Alternatives Group." Food Chem
5484   Toxicol **35**(1): 23-37.
5485   Chan, P. K. and A. W. Hayes (1994). "Acute Toxicity and Eye Irritancy." In
5486   Principles and Methods of Toxiology, 3rd Edition (ed Hayes A.W.): 579-647.
5487   Choksi, N., J. Haseman, D. Allen, R. Tice and W. Stokes (2005). Estimated
5488   Likelihood for Under- and Over-Classification for a Sequential Draize Rabbit
5489   Eye Test. Society of Toxicology 45th Annual Meeting, San Diego, CA.
5490   Chrai, S., T. F. Patton, A. Mehta and J. R. Robinson (1973). "Lacrimal and Instilled
5491   Fluid Dynamics in Rabbit Eyes." J Pharmascent Sci **62**: 1112-1121.
5492   Cooper, K. J., L. K. Earl, J. Harbell and H. Raabe (2001). "Prediction of Ocular
5493   Irritancy of Prototype Shampoo Formulations by the Isolated Rabbit Eye
5494   (IRE) Test and Bovine Corneal Opacity and Permeability (BCOP) Assay."
5495   Toxicol In Vitro **15**(2): 95-103.
5496   Cormier, e. M., J. E. Hunter, W. Billhimer, J. May and M. A. Farage (1995). "Use of
5497   Clinical and Consumer Eye Irritation Data to Evaluate the Low-volume Eye
5498   Test." J Toxicol-Cut and Ocular Toxicol **14**: 197-205.
5499   Cuellar, N., P. H. Lloyd, J. E. Swanson, J. Merrill, M. Clear, G. Mun, et al. (2003).
5500   "Evaluating the Eye Irritancy of Solvents in a Simple Fragrance Mixture with
5501   the Bovine Corneal Opacity and Permeability (BCOP) Assay." The
5502   Toxicologist **72**(1): 312.
5503   Cuellar, N., P. H. Lloyd, J. E. Swanson, J. Merrill, G. Mun, J. W. Harbell, et al.
5504   (2004). "Phase Two: Evaluating the Eye Irritancy of Solvents in a Single
5505   Fragrance Mixture with the Bovine Corneal Opacity and Permeability (BCOP)
5506   Assay." The Toxicologist **78**(1): 268.
5507   Curren, R., M. G. Evans, H. A. Raabe, R. R. Ruppalt and J. W. Harbell (2000). "An
5508   Histopathological Analysis of Damage to Bovine Corneas In Vitro by Selected
5509   Ocular Toxicants." The Toxicologist **54**(1): 188.
5510   Daston, G. P. and F. E. Freeberg (1991). Chapter 16 Ocular Irritation Testing.
5511   Columbus, Ohio, CRC Press.
5512   Dearman, R. J., M. Cumberbatch and I. Kimber (2003). "Cutaneous Cytokine
5513   Expression: Induction by Chemical Allergen and Paracrine Regulation."
5514   Journal of Toxicology-Cutaneous and Ocular Toxicology **22**: 69-86.

5515   DeSousa, D. J., A. A. Rouse and W. J. Smolon (1984). "Statistical Consequences of
5516   Reducing the Number of Rabbits Utilized in Eye Irritation Testing: Data on 67
5517   Petrochemicals." Toxicol Appl Pharmacol **76**: 234-242.
5518   Draize, J. H., G. Woodard and H. O. Calvery (1944). "Methods for the Study of
5519   Irritation and Toxicity of Substances Applied Topically to the Skin and
5520   Mucous   Membranes."   Journal   of   Pharmacology   and   Experimental
5521   Therapeutics **82**: 377-390.
5522   Draize, J. H., G. Woodward and H. O. Calvery (1944). "Methods for the study of
5523   irritation and toxicity of the substances applied to the skin and mucous
5524   membranes." J Pharmacol. **82**: 377-390.
5525   Dua, H. S., J. A. P. Gomes and A. Singh (1994). "Corneal Epithelium Wound
5526   Healing." British Journal of Ophthamology **78**: 401-408.
5527   ECETOC (1992). "ECETOC Technical Report No 48."
5528   ECETOC (1998). "ECETOC Technical Report No. 48 (2)."
5529   Ehlers, N. (1976). Pharmacology of the Conjunctival Sac. Drugs and Ocular
5530   Tissues, Second Meeting of the International Society for Eye Research.
5531   EPA (1998). "Health Effects Test Guideline, OPPTS 870.2400 Acute Eye Irritation.
5532   EPA 712-C-98-195." U.S. Environmental Protection Agency: Washington,
5533   DC.
5534   EPA (2003). "Label Review Manual. 3rd Edition.   EPA 735-B-03-001." U.S.
5535   Environmental Protection Agency: Washington, DC.
5536   Eurell, T. E., J. M. Sinn, P. A. Gerding and C. L. Alden (1991). "In Vitro Evaluation of
5537   Ocular Irritatns Using Corneal Protein Profiles." Toxicology and Applied
5538   Pharmacology **108**: 374-378.
5539   Evans, M. G. (1998). "Condiserations for Histopathological Examination of Bovine
5540   Corneal Tissue." In Vitro & Molecular Toxicology **11**: 335-337.
5541   Farquhar, M. G. and G. E. Palade (1963). "Junctional complexes in Various
5542   Epithelia. ." J Cell Biology **17**: 375-412.
5543   Feder, P. I., R. A. Lordo, D. M. B. Dipasquale, M. Chudkowski, J. L. Demetrulias, K.
5544   L. Hintze, et al. (1991). "The CTFA Evaluation of Alternatives Program: An
5545   Evaluation of *In Vitro* Alternatives to the Draize Primary Eye Irritation Test
5546   (Phase I) Hydro-alcoholic Formulations; (Part 1) Statistical Methods." In Vitro
5547   Toxicology **4**(4): 231-246.
5548   Freeberg, F. E., J. F. Griffith, R. D. Bruce and P. H. S. Bay (1984). "Correlation of
5549   Animal Test Methods with Human Experience for Household Products." J.
5550   Toxic. Cut. & Ocular Toxic **1**(3): 53-64.
5551   Freeberg, F. E., D. T. Hooker and J. F. Griffith (1986). "Correlation of Animal Test
5552   Methods with Human Experience for Household Products: An Update." J
5553   Toxicol-Cut and Ocular Toxicol **5**(2): 115-123.
5554   Freeberg, F. E., G. A. Nixon, P. J. Reer, J. E. Weaver, R. D. Bruce, J. F. Griffith, et
5555   al. (1986). "Human and Rabbit Eye Responses to Chemical Insult." Fund.
5556   Appl. Toxic. **7**: 626-634.
5557   Gautheron, P., J. Giroux, M. Cottin, A. Audegond, A. Morilla, L. Mayordomo-Blanco,
5558   et al. (1994). "Interlaboratory Assessment of the Bovine Corneal Opacity and
5559   Permeability (BCOP) Assay." Toxicology In Vitro **8**(3): 381-392.

5560   Gettings, S. D., L. C. Dipasquale, D. M. Bagley, P. L. Casterton, M. Chudkowski, R.
5561   D. Curren, et al. (1994). "The CTFA Evaluation of Aternatives Program: An
5562   Evaluation of *In Vitro* Alternatives to the Draize Primary Eye Irritation Test.
5563   (Phase II) Oil/Water Emulsions." Fund. Chem. Toxic. **32**(10): 943-976.
5564   Gettings, S. D., R. A. Lordo, K. L. Hintze, D. M. Bagley, P. L. Casterton, M.
5565   Chudkowski, et al. (1996). "The CTFA Evaluation of Alternatives Program: an
5566   evaluation of in vitro alternatives to the Draize primary eye irritation test.
5567   (Phase III) surfactant-based formulations." Food Chem Toxicol **34**(1): 79-117.
5568   Ghassemi, A., R. Osborne, K. Kohrman, M. Roddy, Her and B. Kanengiser (1997).
5569   "Demonstrating the Ocular Safety of an Eye Cosmetic Product Using
5570   Alternatives to Animal Eye Irritation Tests." The Toxicologist: Fundamental
5571   and Applied Toxicology Supplement **36**(1, Part 2): 43.
5572   Ghassemi, A., L. H. Sauers, L. Bruner, P. J. Reer and R. H. Hall (1993).
5573   "Demonstrating the Human Safety of a New Household Cleaning (HSC)
5574   Products Using Alternatives to the Draize Eye Irritation Test." Presentation
5575   made at the US Society of Toxicology Meeting.
5576   Gran, B. P., J. E. Swanson, J. Merrill and J. Harbell (2003). "Evaluation the Irritancy
5577   Potential of Sodium Percarbonate: A Case Study Using the Bovine Corneal
5578   Opacity and Permeability (BCOP) Assay." The Toxicologist **72**: 220.
5579   Griffith, J. F., G. A. Nixon, R. D. Bruce, P. J. Reer and E. Bannan (1980). "Dose-
5580   response Studies with Chemical Irritants in the Albino Rabbit Eye as a Basis
5581   for Selecting Optimum Testing Conditions for Predicting Hazard to the
5582   Human Eye." Toxicol Appl Pharmacol **55**: 501-513.
5583   Hackett, R. B. and T. O. McDonald (1994). "Mechanisms of Ocular Response to
5584   Irritants." Dermatotoxicology, 5th Editions, (ed Marzulli F.N, Maibach H.I.):
5585   299-306.
5586   Harbell, J., S. W. Koontz, R. W. Lewis, D. Lovell and D. Acosta (1997). "IRAG
5587   Working Group 4: Cell Cytotoxicity Assays." Food and Chemical Toxicology
5588   **35**: 79-126.
5589   Harbell, J. W. and R. D. Curren (1998). "The Bovine Corneal Opacity and
5590   Permeability Assay: Observation on Assay Performance." In Vitro &
5591   Molecular Toxicology **11(4)**: 337-341.
5592   Harbell, J. W., R. Osborne, G. J. Carr and A. Peterson (1999). "Assessment of the
5593   Cytosensor[TM] Microphysiometer Assay in the COLIPA In Vitro Eye Irritation
5594   Validation Study." Toxicology In Vitro **13**: 313-323.
5595   Hartung, T., S. Bremer, S. Casati, S. Coecke, R. Corvi, S. Fortaner, et al. (2004). "A
5596   Modular Approach to the ECVAM Principles on Test Validity " Atern Lab Anim
5597   **32**: 467-472.
5598   Hogan, M. J. and L. E. Zimmerman (1962). "Ophthalmic Pathology: An Atlas and
5599   Textbook, 2nd edition." Philadelphia: W.B. Saunders.
5600   Hubert, F. (1992). "The Eye (Rabbit/Human): Parameters to be Measured in the
5601   Field of Ocular Irritation." ATLA **20**: 476-479.
5602   ICCVAM (1997). "Validation and Regulatory Acceptance of Toxicological Test
5603   Methods: A Report of the ad hoc Interagency Coordinating committee on the
5604   Validation of Alternative Methods." NIH Publication No.: 97-3981. Research
5605   Triangle Park: National Toxicology Program.

5606   ICCVAM (2006). *In Vitro* Test Methods for Detecting Occular Corrosives and Severe
5607   Irritants.
5608   Jacobs, G. A. and M. A. Martens (1990). "Quantification of Eye Irritation Based
5609   Upon In Vitro Changes of Corneal Thickness." <u>ATLA</u> **17**: 255-262.
5610   Jester, J. V., L. Li, A. Molai and J. K. Maurer (2001). "Extent of initial corneal injury
5611   as a basis for alternative eye irritation tests." <u>Toxicol In Vitro</u> **15**(2): 115-30.
5612   Jester, J. V., W. M. Petroll, J. Bean, R. D. Parker, G. J. Carr, H. D. Cavanagh, et al.
5613   (1998). "Area and depth of surfactant-induced corneal injury predicts extent
5614   of subsequent ocular responses." <u>Invest Ophthalmol Vis Sci</u> **39**(13): 2610-25.
5615   Jones, P. A., E. Budynsky, K. J. Cooper, D. Decker, H. A. Griffiths and J. H. Fentem
5616   (2001). "Comparative Evaluation of Five In Vitro Tests for Assessing the Eye
5617   Irritation Potential of Hair-care Products." <u>ATLA</u> **29**: 669-692.
5618   Karson, C. N., K. F. Berman, E. F. Donnelly, W. B. Mendelson, J. E. Kleinman and
5619   R. J. Wyatt (1981). "Speaking, Thinking, and Blinking." <u>Psychiatry Research</u>
5620   **5**: 243-246.
5621   Katahira, J. H., H. Sugiyama, N. Inoue, Y. Horiguchi, M. Matsuda and N. Sugimoto
5622   (1997). "Clostridium Perfringens Enterotoxin Utilizes Two Structurally Related
5623   Membrane Proteins as Functional Receptors in Vivo." <u>J Biological Chemistry</u>
5624   **272**: 26652-26658.
5625   Klyce, S. D. and R. W. Beuerman (1988). "Structure and Function of the Cornea."
5626   <u>The Cornea</u>: 3-23.
5627   Lambert, L. A., W. A. Chambers, S. Green, K. C. Gupta, R. N. Hill, P. M. Hurley, et
5628   al. (1993). "The Use of Low-volume Dosing in the Eye Irritation Test." <u>Food</u>
5629   <u>and Chemical Toxicology</u> **31**: 99-103.
5630   Liebsch, M., D. Traue, C. Barrabas, H. Spielmann, P. Uphill, S. Wilkins, et al.
5631   (2000). "The ECVAM Prevalidation Study on the Use of EpiDerm for Skin
5632   Corrosivity Testing." <u>ATLA</u> **28**: 371-401.
5633   Mann, I. and B. D. Pullinger (1942). "A Study of Mustard Gas Lesions of the Eyes of
5634   Rabbits and Men." <u>Proc Roy Soc Med</u> **35**: 229-244.
5635   Marzulli, F. N. and D. I. Ruggles (1973). <u>J Assoc Off Anal Chem</u> **56**: 905.
5636   Maurer, J. K., H. F. Li, W. M. Petroll, R. D. Parker, H. D. Cavanagh and J. V. Jester
5637   (1997). "Confocal Microscopic Characterization of Initial Corneal Changes of
5638   Surfactant-induced Eye Irritation in the Rabbit." <u>Toxicol Appl Pharmacol</u>
5639   **143**(2): 291-300.
5640   Maurer, J. K. and R. D. Parker (1996). "Light Microscope Comparision of Surfactant
5641   Induced Eye Irritation i Rabbits and Rats at Three Hours and Recovery/day
5642   35." <u>Toxicologic Pathology</u> **24**: 403-411.
5643   Maurer, J. K., R. D. Parker and J. V. Jester (2002). "Extent of Initial Corneal Injury
5644   as the Mechanistic Basis for Ocular Irritation: Key Findings and
5645   Recommendations for the Development of Alternative Assays." <u>Regul Toxicol</u>
5646   <u>Pharmacol</u> **36**(1): 106-117.
5647   Maurer, J. L. and R. D. Parker (1996). "LIght Microscope Comparison of Surfactant-
5648   induced Eye Irritation in Rabbits and Rats at Three Hours and Recovery/day
5649   35." <u>Toxicologic Pathology</u> **6**: 464-477.

5650   McConnell, H. M., J. C. Owicki, J. W. Parce, D. L. Miller, G. T. Baxter, H. G. Wada,
5651   et al. (1992). "The cytosensor microphysiometer: biological applications of
5652   silicon technology." <u>Science</u> **257**(5078): 1906-12.
5653   Millichamp, N. J. (1999). "Species Specificity: Factors Affecting the Interpretation of
5654   Species Differences in Toxic Responses of Ocular Tissues. ." <u>Ophthalmic</u>
5655   <u>Toxicology, Second Edition, Target Organ Toxicology Series (Ed. GCY</u>
5656   <u>Chiou). Philadelphia: Taylor and Francis, 89-117</u>.
5657   Mishima, S., A. Gasset, S. D. Klyce and J. L. Baum (1966). "Determination of Tear
5658   Volume and Tear Flow." <u>Invest Ophthalmol Vis Sci</u> **5**: 264-276.
5659   NIEHS (2006). "Current Status of In Vitro Methods for Identifying Ocular Corrosives
5660   and Severe Irritations: Bovine Corneal Opactiy and Permeability Test
5661   Background Review Document." <u>NIH Publication No.</u> **06-4512**.
5662   Prinsen, M. K. (2006). "The Draize Eye Test and In Vitro Alternatives; A Left-handed
5663   Marriage?" <u>Toxicol In Vitro</u> **20**(1): 78-81.
5664   Rees, W. M., J. E. Swanson, J. Burdick, D. S. Hilgers and J. Harbell (2001).
5665   "Evaluating Toxic Synergism in Hypochlorite-containing Solutions Using the
5666   Bovien Cornea Opacity and Permeability (BCOP) Assay." <u>The Toxicologist</u>
5667   **60**: 99.
5668   Roggeband, R., M. York, M. Pericoi and W. Braun (2000). "Eye Irritation Responses
5669   in Rabbit and Man After Single Applications of Equal Volumes of Undiluted
5670   Model Liquid Detergent Products." <u>Food and Chemical Toxicology</u> **38**: 727-
5671   734.
5672   Sina, J. (1994). "Validation of the Bovine Corneal Opacity-Permeability Assay as a
5673   Predictor of Ocular Irritation Potential." <u>In Vitro Toxicol</u> **7**(3): 283-290.
5674   Sina, J. F., D. M. Galer, R. G. Sussman, P. Gautheron, E. V. Sargent, B. Leong, et
5675   al. (1995). "A collaborative Evaluation of Seven Alternatives to the Draize Eye
5676   Irritation Test Using Pharmaceutical Intermediates." <u>Fund. Appl. Toxic.</u> **26**:
5677   20-31.
5678   Stern, M., M. Klausner, R. Alvarado, K. J. Renskers and M. S. Dickens (1998).
5679   "Evaluation of the EpiOcular(TM) Tissue Model as an Alternative to the
5680   Draize Eye Irritation Test." <u>Toxicology In Vitro</u> **12**: 455-461.
5681   Swanson, J. E. and J. Harbell (2000). "Evaluating the Eye Irritancy Potential of
5682   Ethanolic Test Materials with the Bovine Corneal Opacity and Permeability
5683   Assay." <u>The Toxicologist</u> **54**(1): 188.
5684   Swanson, J. E., L. K. Lake, T. A. Donnelly, J. Harbell and J. Huggins (1995).
5685   "Prediction of Ocular Irritation of Full-Strength Cleaners and Strippers by
5686   Tissue Equivalent and Bovine Corneal Assays." <u>Toxicology - Cutaneous and</u>
5687   <u>Ocular Toxicology</u> **14**(3): 179-195.
5688   Swanson, J. E., B. T. White, B. P. Gran, J. Merrill and J. Harbell (2003). "Evaluating
5689   Oxidizing/reactive Cleaning Products in the Bovine Corneal Opacity and
5690   Permeability (BCOP) Assay." <u>The Toxicologist</u> **72**: 220-221.
5691   Swanston, D. W. (1985). "Assessment of the Validity of Animal Techniques in Eye
5692   Irritation Testing." <u>Food and Chemical Toxicology</u> **23**: 169-173.
5693   Thakur, A., A. Clegg and e. al (1997). "Modulation of Cytokine Production from an
5694   EpiOcular Corneal Cell Culture Model in Response to Staphylococcus
5695   Aureus Superantigen." <u>Aust N Z J Ophthalmol</u> **25 Suppl 1**: S43-45.

5696   Ubels, J. L. (1998). "Effect of Hydration on Opacity in the Bovine Corneal Opacity
5697   and Permeability (BCOP) Assay." <u>J. Toxicol. Cut & Ocular Toxicol.</u> **17**(4):
5698   197-220.
5699   UN (2003). "Globally Harmonised System of Classifcation and Labelling of
5700   Chemicals (GHS)." <u>New York & Geneva: United Nations Publications</u>.
5701   Van Meer, G., W. van Hof and I. nan Genderen (1992). "Tight Junctions and Polarity
5702   of Lipids " <u>Tight Junctions (ed Cereijido M.)</u>: 187-201.
5703   Walker, A. P. (1985). "A More Realistic Animal Technique for Predicting Human Eye
5704   Response." <u>Food Chem Toxicol</u> **23**: 175-178.
5705   Weil, C. S. and R. A. Scala (1971). "Study of Intra- and Interlaboratory Variability in
5706   the Results of Rabbit Eye and Skin Irritation Tests." <u>Toxicol Appl Pharmacol</u>
5707   **19**(2): 276-360.
5708   Wilhelmus, K. R. (2001). "The Draize Eye Test." <u>Surveys in Ophthalmology</u> **45**: 493-
5709   515.
5710
5711