2.0 ICE TEST METHOD PROTOCOL COMPONENTS

2.1 Overview of How the ICE Test Method is Conducted

As discussed in **Section 1.0,** the enucleated eye test using rabbit eyes (i.e., the IRE test method) was introduced by Burton et al. (1981) as a prescreen for severe eye irritants. The ICE protocol, first described by Prinsen and Koëter (1993), was developed based on the IRE test method. For this reason, most of the test method components remained the same for the ICE test method, although the dose-volume of the test substance was modified for the chicken eye. **Appendix A** provides a comparative summary of test method components for all ICE protocols reviewed in this BRD.

In the ICE test method, three parameters are evaluated to measure the extent of eye damage following exposure to a chemical substance: corneal swelling, corneal opacity, and fluorescein retention. While the latter two parameters involve a qualitative assessment done by slit-lamp microscopic examination, analysis of corneal swelling is measured quantitatively, potentially providing improved precision and reduced interlaboratory variability compared to the *in vivo* rabbit eye test, which relies only on qualitative measurements.

During an ICE study, a test substance is applied to the corneas of enucleated chicken eyes, isolated from chickens processed for human consumption. Chicken heads are transported from the slaughterhouse to the laboratory within two hours, and eyes are immediately dissected and placed in the superfusion apparatus, where isotonic saline is supplied onto the cornea through a steel tube attached to a peristaltic pump. Test substances are applied as a single dose (30 µL for liquids, or 30 mg for solids) for 10 seconds, followed by rinsing with 20 mL isotonic saline. Corneal reactions are measured at regular intervals up to four hours post-treatment, and mean values for each parameter (corneal swelling, corneal opacity, and fluorescein retention) are determined; fluorescein retention is evaluated at 30 minutes post-treatment only. Based on the maximum mean values[1] of these measurements, the irritation potential of the test substance is defined within a range from nonirritating to severely irritating.

2.2 Description and Rationale for the Test Method Components

The ICE test method protocol has remained virtually unchanged since its initial publication in 1993. The protocol was used by three other laboratories (Shell, Unilever, and Rhône-Poulenc) in the European Commission (EC)/British Home Office (HO) validation study (Balls et al. 1995). The laboratory at Rhône-Poulenc (currently Bayer CropScience) is still using the ICE test method. Many of the essential protocol components are based on historical use, and rationales for their inclusion are not known.

[1] For each endpoint, the mean of three eyes is recorded for each time point and the largest mean value is used for scoring.

2.2.1 Materials, Equipment, and Supplies Needed

2.2.1.1 *Sources of Chicken Eyes*

Historically, chickens obtained from a slaughterhouse have been used for this assay because they are killed for human consumption, eliminating the need for laboratory animals. A local source of chickens (preferably spring chickens of either sex, approximately seven weeks old, approximately 2.5-3.0 kg, breed not specified), close to the laboratory should be located, so chicken heads can be transferred to the laboratory and processed within two hours after the birds are killed. Although a controlled study to evaluate the optimum chicken age has not been done, the age and weight of the chickens specified represents that of spring chickens traditionally processed by a poultry slaughterhouse. Unpublished studies on adult chickens show no significant differences in results (Prinsen M., personal communication). The lack of an age difference is presumed to be due to the physiological properties of the cornea in general, which deviates only slightly during the lifespan of the chicken. Although a formal study to determine the optimum window of time to transport the heads to the laboratory has not been conducted, two hours appears to produce consistent results (Prinsen M, personal communication). However, given the quality control measures taken once the eyes reach the laboratory (i.e., baseline fluorescein retention and corneal thickness measurements), it appears that longer transport times could be considered.

2.2.1.2 *Preparation of the Eyes*

Because eyes can be more precisely dissected in the laboratory, intact heads are transported there from the slaughterhouse, at ambient temperature in plastic boxes humidified with tissues moistened with isotonic saline or water. Transportation of the eyes in the intact chicken head provides effective protection from external damage during transport while humidified transportation boxes prevent desiccation. The temperature range during transport is not considered critical because of quality control measures done before an eye is used in an assay (Prinsen M, personal communication). The post-mortem eyelid closure reflex provides an efficient barrier to external contaminants, desiccation and physical injury during transportation. However, the effect of hypoxia on the eye resulting from closed eyelids has not been studied. It is unclear if less elapsed time between the animal's death and study initiation would improve results.

Before inspection, the eyelids are carefully excised, taking care not to damage the cornea. Corneal integrity is quickly assessed with a drop of 2% (w/v) sodium fluorescein (British Pharmacopoeia - BP) applied to the corneal surface for a few seconds, and then rinsed with isotonic saline. Although an exact exposure duration is not used, the fluorescein should not remain on the corneal surface for more than a few (i.e., 1-3) seconds. The treated cornea is then examined for fluorescein retention by damaged corneal epithelial cells and for corneal opacity with a slit-lamp microscope. If undamaged, the eye is further dissected from the skull, taking care not to damage the cornea. Because of the firmness of the chicken eye sclera, dissection is simple. The eyeball is pulled from the orbit by holding the nictitating membrane firmly with surgical forceps, and the eye muscles are cut with a bent, blunt-tipped scissor. It is important to avoid causing corneal damage due to excessive pressure (e.g., compression artifacts). When the eye is removed from the orbit, a visible portion of the optic nerve should be left attached. If the optic nerve is detached from the eye during dissection, a hole can be created at the surface of the posterior portion of the eye, resulting in a change in

turgor pressure by exposing the posterior chamber to the external environment. Once removed from the orbit, the eye is placed on an absorbent pad and the nictitating membrane and other connective tissue are cut away.

2.2.1.3 *ICE Experimental Setup*

Each eye is mounted in a custom-built stainless steel clamp (**Figure 2-1**), with the cornea positioned vertically and then transferred to a chamber in a custom-built superfusion apparatus (**Figure 2-2**). Photographs and line drawings of the clamp and superfusion apparatus are available from M. Prinsen at TNO and can be used to craft similar clamps.

Figure 2-1 Custom-Built Stainless Steel Eye Clamp for the ICE Test Method

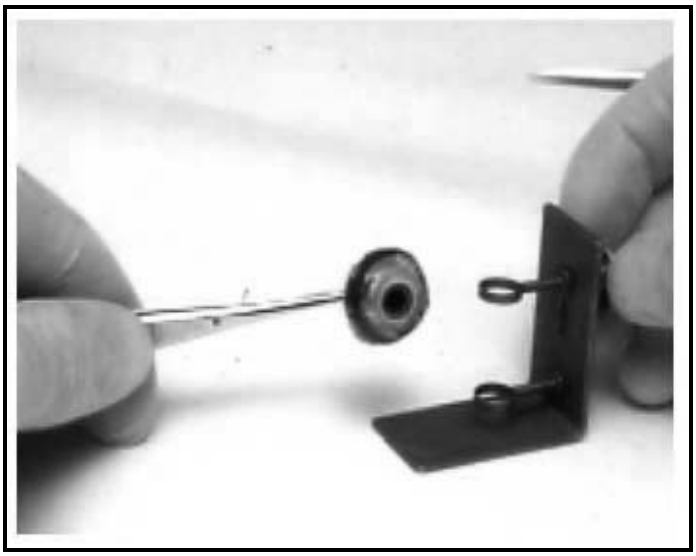
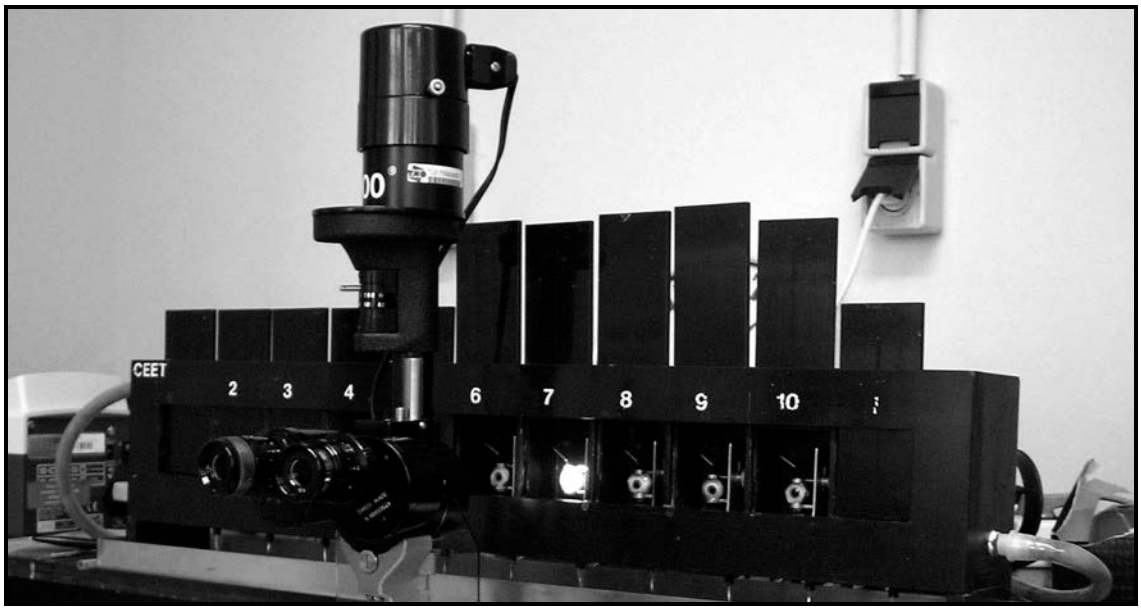


Figure 2-2 Superfusion Apparatus for the ICE Test Method



The clamp is positioned in the multi-chambered superfusion apparatus so the entire cornea is moistened with isotonic saline from a bent stainless steel tube via a peristaltic pump at a rate of approximately 0.10-0.15 mL (2 or 3 drops)/minute. This flow rate was adopted from that used in the IRE test method and has been used extensively in the test method developer's laboratory. The isotonic saline is temperature-controlled to 32 ± 1.5°C , because the steel tube passes through the warm-water mantle of the superfusion apparatus. After placement in the superfusion apparatus, the corneas are again examined with the slit-lamp microscope to ensure that they have not been damaged during the procedure.

Corneal thickness is measured at the corneal apex using an optical pachymeter on the slit-lamp microscope. A slit-lamp system is preferred to ultrasound-based instruments (e.g., ultrasonic pachymeter) because the latter requires direct contact of a probe with the cornea, risking injury to the corneal surface, especially if the test substance has already damaged it. Eyes with a corneal thickness deviating more than 10% from the mean value for the eyes, eyes with a fluorescein retention score of > 0.5 (indicating corneal permeability), or eyes showing corneal opacity or any other signs of damage are rejected and replaced. Based on historical use of the ICE in the developer's laboratory, these values appear to accurately represent the range seen with the average, untreated, and undamaged chicken eye. Once all eyes have been examined and approved, they are equilibrated for 45 to 60 minutes prior to dosing. Unpublished observations have shown that the duration of equilibration is not critical, and may be allowed to extend longer if necessary. In addition, as the chamber doors are opened frequently during the test period and the corneas are frequently rinsed with isotonic saline at ambient temperature during the assay, temperature fluctuations seem to have little effect on the integrity of the cornea, as evidenced by the results obtained with the negative control eyes (Prinsen M, personal communication).

Originally, five eyes were used per test substance (Prinsen and Köeter 1993), but later publications included as few as three eyes per test substance (Balls et al. 1995; Prinsen 1996, Prinsen 2000; Prinsen 2005). Reducing the number of treated eyes from five to three does not appear to have decreased the performance of the ICE test method (Prinsen M, personal communication).

2.2.2 Dose-Selection Procedures, Including the Need for Any Dose Range-Finding Studies or Acute Toxicity Data Prior to Conducting a Study

Dose-selection procedures are not relevant to this *in vitro* assay. Test substances are applied as neat chemicals (solids or liquids), if possible, or as received in the case of proprietary mixtures or formulations. If dilution is required, test substances are diluted with an aqueous vehicle.

2.2.3 Endpoints Measured

The control and test eyes are examined pre-treatment and at 30, 75, 120, 180, and 240 minutes after treatment using the criteria and scoring system described in **Section 2.2.6**. These time points provide an adequate number of measurements over the four-hour treatment period while leaving sufficient time between measurements for the requisite observations to be made for all eyes.

The endpoints evaluated are corneal opacity, corneal swelling, fluorescein retention (corneal permeability) and morphological effects (e.g., pitting or loosening of the epithelium). Corneal opacity and corneal thickness are evaluated at each time point. Because fluorescein retention reflects initial damage and does not change over time, it is determined only at 30 minutes. If the test substance adheres to the cornea and precludes determination at 30 minutes, fluorescein retention may be assessed at a later time point.

After the final examination at four hours, eyes are typically preserved in 4% neutral buffered formaldehyde for histopathological examination (if necessary or requested). It is widely recognized that microscopic assessment of effects can provide additional information to be added to an overall assessment of toxicity. However, the expense of such a detailed examination may not be warranted in all cases (e.g., if the outcome of the test is clearly negative or clearly positive). Instead, histopathological effects could most efficiently be used to resolve borderline responses by determining the depth-of-injury (Maurer et al. 2002).

2.2.4 Duration of Exposure

2.2.4.1 *Quantity of Test Substance Applied*

A liquid test substance is applied at 0.03 mL with a micropipette, so that the entire corneal surface is bathed. A solid test substance is applied at 0.03 g as a fine powder (grinding may be necessary), and evenly distributed over the corneal surface. Using a fine powder ensures more uniform coverage of the corneal surface. Excess test substance could result in a "piling up" effect, which could preclude uniform coverage of the corneal surface (i.e., some test substance may not come into contact with the surface for the entire exposure period). These quantities were originally chosen because the diameter of the chicken cornea is approximately 30% that of the rabbit cornea (the standard quantities used in the IRE are 0.1 mL or 0.1 g).

2.2.4.2 *Application of the Test Substance*

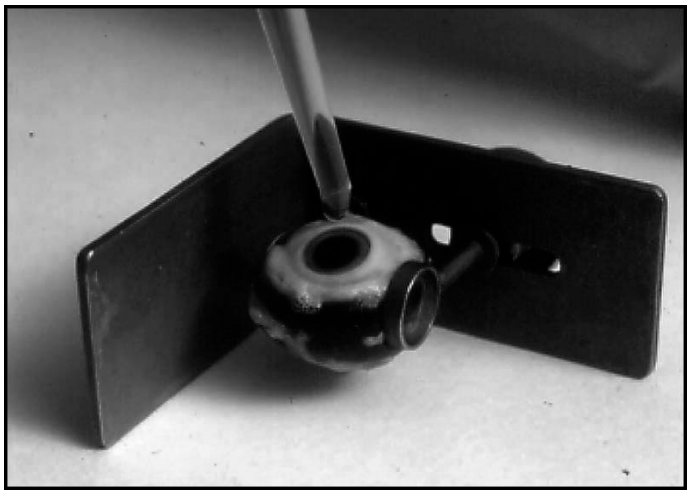
After an equilibration period, a zero reference corneal thickness measurement is taken to establish a baseline (i.e., time = 0) for comparison to post-treatment measurements. Immediately following the zero reference measurement, each eye (in its holder) is removed from the superfusion apparatus, placed in a horizontal position, and the test substance is applied to the cornea (**Figure 2-3**). The test substance is applied for 10 seconds, rinsed from the eye with 20 mL isotonic saline at ambient temperature, then the eye (in its holder) is returned to the superfusion apparatus in the original upright position. During dosing, the clamp holding the eye is removed from the superfusion apparatus and placed on tissue paper with the cornea facing upwards. This position maximizes uniform contact of the test substance with the corneal surface.

The time of application was chosen based on the IRE study design. According to Burton et al. (1981), a 10-second exposure was chosen after experimentation demonstrated that this time interval produced the best discrimination between irritant and non-irritant substances.

2.2.5 Known Limits of Use

Like the *in vivo* rabbit eye test, the ICE test method has been used to test a wide range of substances with various physicochemical characteristics (Prinsen and Koëter 1993;

Figure 2-3 Application of the Test Substance in the ICE Test Method



Prinsen 1996; Prinsen 2000; Prinsen 2005). However, some substances may require an alternative testing strategy due to their increased potential for yielding results that are not predictive of an *in vivo* response (Balls et al. 1995).

One such class of substances is hydrophobic compounds. Because of the aqueous environment under which the assay is conducted, very hydrophobic compounds may not fully contact the corneal surface, which could potentially result in an underprediction of the *in vivo* response. To ensure adequate contact with the cornea, it may be necessary to employ multiple exposures, or to remove as much isotonic saline as feasible from the corneal surface prior to application. Such measures would require additional protocol optimization to ensure that the accuracy of the results was improved.

Solid substances (e.g., powders, pastes) may also cause discordant responses in the ICE test method. In some cases, solids may adhere to the corneal surface and rinsing may fail to detach them. Residual solid substance may preclude accurate assessment of endpoints. More extensive rinsing (i.e., by using an increased rinse volume or by increasing the number of rinses) may be applied and, in general, residual test substance will be removed within one hour (Prinsen M, personal communication).

The ICE test method can only provide information on the ability of a test substance to interact with the cornea (i.e., damage to conjunctival tissue is not assessed). However, a direct relationship between conjunctival damage and corneal swelling has been reported by Burton (1972). Based on this relationship, it may be inferred that only very mildly irritating substances are capable of inducing conjunctival effects in the absence of corneal effects (Prinsen M, personal communication). Therefore, focusing solely on corneal effects does not appear to limit the effectiveness of ICE with respect to predicting corrosives and severe irritants.

Finally, the availability of a poultry abattoir close to the testing laboratory may be a limiting factor. However, because the test method is essentially portable and is a relatively quick procedure, the experimental set-up could be moved to the slaughterhouse (Balls et al. 1995).

2.2.6 Nature of the Response Assessed

2.2.6.1 *Data to be Collected*

As noted in **Section 2.2.3**, corneal endpoints observed in the ICE are opacity, swelling, fluorescein retention, and morphological changes. Response severity is graded at each time point. Numerical and descriptive data are collected. Numerical data includes scores for opacity, thickness, and fluorescein retention, while descriptive data represents morphological and histopathological results. Therefore, the responses assessed in this test method are both qualitative and quantitative.

2.2.7 Appropriate Controls and the Basis for their Selection

2.2.7.1 *Negative Controls*

The recommended negative control in all of the published ICE protocols is isotonic saline. This control appears most suitable since the test method is conducted using isotonic saline to moisten the enucleated chicken eyes as well as for the requisite rinsing steps. Treating the negative control eyes with isotonic saline instead of test substance ensures that any mechanical alterations (i.e., those not related to the test substance) and the general conditions maintained by the superfusion apparatus are properly controlled. In cases where the test substance is solubilized or diluted, the most common vehicle recommended is deionized or distilled water. Other vehicles may be used if demonstrated to be useful to the assay. However, inclusion of alternate vehicles should be adequately controlled in the experimental design.

2.2.7.2 *Positive Controls*

As discussed by Harbell and Curren (2002), the function of the positive control is to ensure the test system is operating within normal limits and each experiment is properly executed so toxic effects of interest can be properly detected. A concurrent positive control is included in each experiment to develop a historical database. Results from the concurrent positive control are compared to the historical control range, which is used to determine whether a particular experiment is acceptable. Because the positive control should allow for detection of an over- or under-response in the assay, the selected positive control should not produce responses at either the extreme low or the extreme high end of assay response.

None of the published ICE protocols recommend the use of a concurrent positive control substance. The rationale for excluding such a control has been based on the historical success with the ICE method in the developer's laboratory (Prinsen M, personal communication).

2.2.7.3 *Solvent Controls*

Solvent controls are recommended when solvents other than deionized water or saline are used to dissolve test substances, in order to demonstrate that the solvent is not interfering with the test system.

2.2.7.4 *Benchmark Controls*

Benchmark controls may be useful for demonstrating that the test method is functioning properly for detecting the ocular irritancy potential of chemicals of a specific chemical class or with a specific range of responses, or for evaluating the relative irritancy potential.

2.2.8 Acceptable Range of Control Responses and the Basis for the Acceptable Ranges

2.2.8.1 *Negative Controls*

An acceptable range of negative control responses is an irritancy classification of nonirritating (**Section 2.2.13**). Because an aqueous medium (isotonic saline) is used to moisten and rinse the chicken eyes, isotonic saline or distilled water may be used as the vehicle when a test substance is diluted. Therefore, eyes exposed to isotonic saline typically serve as both the negative and vehicle controls.

2.2.8.2 *Positive Controls*

Because positive controls have not been traditionally used in this test method, a defined range of responses has not been previously described. However, it would seem prudent that the positive control substance produce an Irritancy Score (**Section 2.2.13**) that is appropriate based on its historical classification as a severe irritant in the *in vivo* rabbit eye test. If adequate historical ICE test method data are not available for a particular positive control, pilot studies may have to be conducted to provide this information.

2.2.8.3 *Solvent Controls*

If another solvent is used to dissolve or dilute a test substance, separate vehicle and negative (isotonic saline) controls should be included in the experiment. In this case, the vehicle control should also produce a nonirritating response.

2.2.8.4 *Benchmark Controls*

Benchmark controls may be useful in demonstrating that the test method is functioning properly for detecting the ocular irritancy potential of chemicals of a specific chemical class or a specific range of responses, or for evaluating the relative irritancy potential of an ocular irritant. Therefore, the response of the benchmark should be consistent across multiple experiments.

2.2.9 Nature of the Data to be Collected and the Methods Used for Data Collection

The severity level for each study endpoint is evaluated at each time point. The following criteria and scoring system are applied for the assessment of possible effects:

2.2.9.1 *Corneal Swelling*

Corneal swelling is determined from corneal thickness measurements made with an optical pachymeter on a slit-lamp microscope. It is expressed as a percentage and is calculated from corneal thickness measurements according to the following formula:

$$\left(\frac{corneal\ thickness\ at\ time\ t \quad - \quad corneal\ thickness\ at\ time = 0}{corneal\ thickness\ at\ time = 0} \right) \times 100$$

The mean percentage of swelling for all eyes exposed to the test substance is calculated for each observation time point. Based on the highest mean score for corneal swelling, as observed at any time point, an overall category score is then given for each test substance.

2.2.9.2 *Corneal Opacity*

Corneal opacity is determined with slit-lamp examination, by scoring the area of the cornea that is most densely opacified.

Score		*Observation*
0	=	No opacity
0.5	=	Very faint opacity
1	=	Scattered or diffuse areas; details of the iris are clearly visible
2	=	Easily discernible translucent area; details of the iris are slightly obscured
3	=	Severe corneal opacity; no specific details of the iris are visible; size of the pupil is barely discernible
4	=	Complete corneal opacity; iris invisible

The mean corneal opacity value for all test eyes is calculated for each observation time point. Based on the highest mean score for corneal opacity, as observed at any time point, an overall category score is then given for each test substance.

2.2.9.3 *Fluorescein Retention*

The mean fluorescein retention value for all test eyes is calculated for the 30-minute observation time point only. When test substances have adhered to the cornea, fluorescein retention can be determined whenever the test substance has been adequately removed.

Score		*Observation*
0	=	No fluorescein retention
0.5	=	Very minor single cell staining
1	=	Single cell staining scattered throughout the treated area of the cornea
2	=	Focal or confluent dense single cell staining
3	=	Confluent large areas of the cornea retaining fluorescein

2.2.9.4 *Morphological Effects*

These effects include *pitting* of corneal epithelial cells, *loosening* of the epithelium, *roughening* of the corneal surface and *sticking* of the test substance to the cornea. These findings can vary in severity and may occur simultaneously. The classification of these findings is subjective according to the investigator's interpretation. On the basis of severity of the observed findings, these effects are divided into four categories: 1 = none; 2 = slight; 3 = moderate; 4 = severe. Multiple observers within a single laboratory should ensure that consistency is maintained in assigning scores. A histopathological evaluation may also be done to elucidate more detailed evidence of damage, or lack thereof.

2.2.9.5 *Methods for Collection of Numerical Data*

Qualitative corneal opacity measurements are made with a slit-lamp microscope, and a score assigned based on the scale provided in **Section 2.2.9.2**. The most densely opacified area of the cornea is used for scoring. Fluorescein retention is also a qualitative measurement, evaluated using a slit-lamp microscope. Numerical values are assigned according to the scale

provided in **Section 2.2.9.3**. Corneal thickness is a quantitative measurement that requires either a slit-lamp microscope equipped with an optical pachymeter, or an ultrasonic pachymeter. The slit-lamp system is generally preferred to an ultrasonic pachymeter, as the probe for the latter must be in contact with the cornea during the measurement, which can increase the risk of corneal damage, especially if it is already damaged by irritant exposure. Corneal thickness measurements are used to calculate corneal swelling relative to baseline measurements (**Section 2.2.9.1**).

The severity of corneal damage has been reported to be proportional to the severity of corneal irritation (Burton et al. 1972). The scoring method for each endpoint has been correlated to the EU regulatory classification system (EU 2001) for comparison to *in vivo* results (See **Section 2.2.13**).

2.2.10 Type of Media in Which Data Are Stored

Although not specifically mentioned in published ICE protocols, it is reasonable to assume that data can be collected either by hand, or by directly entering it into a computer spreadsheet. Handwritten data requires subsequent computer entry in order to perform the requisite mathematical calculations (**Section 2.2.12**). Data should be routinely backed up on the source computer hard drive as well as an external disk drive.

Data from the test method should be stored and archived in a manner consistent with international GLP guidelines (OECD 1998; EPA 2003a, 2003b; FDA 2003). Materials that should be retained include, but are not limited to, raw data, documentation, protocols, final reports, records and reports of the maintenance and calibration of apparatus, validation documentation for computerized systems, the historical file of all Standard Operating Procedures, and environmental monitoring records. The archives should be organized and indexed to expedite information retrieval and storage conditions should minimize document deterioration. An individual should be identified as responsible for these data archives.

GLP guidelines are nationally and internationally recognized rules designed to ensure high-quality laboratory records. They provide a standardized approach for reporting and archiving laboratory data and records, and the test protocol, in order to ensure the integrity, reliability, and accountability of a study (OECD 1998; EPA 2003a, 2003b; FDA 2003).

2.2.11 Measures of Variability

As indicated in **Section 2.2.9**, both numerical and descriptive data are generated by the ICE test method. Variability of numerical ICE test data is typically assessed through calculation of the mean along with the standard error of the mean for each numerical endpoint. Descriptive data may also provide an additional, qualitative measure of variability.

2.2.12 Statistical or Non-Statistical Methods Used to Analyze the Resulting Data

2.2.12.1 *Irritation Index*

The severity level for each study endpoint (i.e., corneal swelling, corneal opacity and fluorescein retention) recorded at each time point is used to calculate the maximum mean score for each endpoint (**Section 2.2.9**), from which an irritation index can be determined. The irritation index is derived by calculating the sum of the maximum mean scores for each

endpoint. Corneal swelling at each time point is calculated as a percentage of corneal thickness at time = zero. An overall corneal swelling score is calculated based on both (1) the mean corneal swelling of all three eyes treated with the test substance and (2) the time when swelling first occurred. Corneal opacity is qualitatively scored at each time point on a scale from zero to four, with zero representing no opacity and four representing complete corneal opacity. The overall corneal opacity score is the highest mean score obtained across the five observation times for the three eyes treated with the test substance. Additionally, the mean retention of fluorescein among the three treated eyes is determined at 30 minutes. To derive the irritation index, the opacity and fluorescein retention scores are equally weighted relative to the maximum corneal swelling obtained. Historical data from the test method developer's laboratory indicates that the maximum swelling observed is approximately 60% to 80%. Therefore, the maximum opacity (score = 4) and fluorescein retention (score = 3) scores obtained with any particular test substance are multiplied by a factor of 20 in order to increase their weighting (Chamberlain et al. 1997). The irritation index has a possible range of 0 to 200.

2.2.13 Decision Criteria and the Basis for the Prediction Model Used to Classify a Test Chemical

2.2.13.1 *Interpretation of Endpoint Scores*

Once each endpoint has been scored, irritancy categories can be assigned based on a pre-determined range. The rationale for the values selected for each range is based on a logical subdivision of these values into the ocular irritancy categories of non, slight, moderate, or severe (Prinsen M, personal communication). Interpretation of corneal thickness, corneal opacity, and fluorescein retention using four irritancy categories is done according to the following scales:

Corneal Thickness

Mean Corneal Swelling (%)	Category
0 to 5	I
> 5 to 12	II
> 12 to 18 (>75 min after treatment)	II
> 12 to 18 (≤75 min after treatment)	III
> 18 to 26	III
> 26 to 32 (>75 min after treatment)	III
> 26 to 32 (≤75 min after treatment)	IV
> 32	IV

Corneal Opacity

Mean Maximum Opacity Score	Category
0.0-0.5	I
0.6-1.5	II
1.6-2.5	III
2.6-4.0	IV

Fluorescein Retention

Mean Fluorescein Retention Score at 30 Minutes Post-treatment	Category
0.0-0.5	I
0.6-1.5	II
1.6-2.5	III
2.6-3.0	IV

2.2.13.2 *Defining the Irritancy Classification*

The overall *in vitro* irritancy classification for a test substance is assessed by reading the irritancy classification that corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and fluorescein retention. For the purposes of this evaluation, which focuses specifically on the ability of ICE to identify corrosives and severe irritants, only the severe irritancy classification for a test substance is presented in the scheme below.

Classification	Combinations of the 3 Endpoints
Severely Irritating	3 x IV
	2 x IV, 1 x III
	2 x IV, 1 x II[1]
	2 x IV, 1 x I[1]
	Corneal opacity ≥ 3 at 30 min (≥ 2 eyes)
	Corneal opacity = 4 at any time point (≥ 2 eyes)
	Severe loosening of the epithelium (≥ 1 eye)

[1]Combinations less likely to occur.

Using similar combination schemes, ICE test results have also been used to predict the *in vivo* classification of substances according to both EU and GHS classification schemes (Prinsen M, personal communication). However, as indicated in **Section 2.2.6**, histopathology may be warranted in order to discriminate between effects that are on the borderline between severe and moderate irritation. If a mathematical comparison is desired, the irritation index described in **Section 2.2.12** may be calculated.

2.2.14 Information and Data that Will Be Included in the Study Report and Availability of Standard Forms for Data Collection and Submission

The test report should include the following, if relevant to the conduct of the study:

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known
- The CAS Registry Number (RN), if known
- Purity and composition of the substance or preparation (in percentage[s] by weight)
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., warming, grinding)
- Stability, if known

Information Concerning the Sponsor and the Test Facility

- Name and address of the Sponsor
- Name and address of the test facility
- Name and address of the Study Director

Justification of the Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time (e.g., periodic testing of proficiency substances, use of historical negative and positive control data)

Criteria for an Acceptable Test

- Acceptable concurrent negative control ranges based on historical data
- Acceptable concurrent positive control ranges based on historical data
- If applicable, acceptable concurrent benchmark control ranges based on historical data

Test Conditions

- Experimental starting and completion dates
- Details of test procedure used
- Test concentration(s) used
- Description of any modifications of the test procedure
- Reference to historical data of the model (e.g., negative and positive controls, proficiency substances, benchmark substances)
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples (e.g., irritancy scores for the test substance and the positive, negative, and benchmark controls, including data from replicate repeat experiments as appropriate, and means ± the standard deviation for each experiment)

Description of Other Effects Observed

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement indicates all inspections made during the study, and the dates any results were reported to the Study Director. This statement also serves to confirm that the final report reflects the raw data.

Additional reporting requirements for GLP-compliant studies are provided in the relevant guidelines (e.g., OECD 1998; EPA 2003a, 2003b; FDA 2003).

A standard data collection form is provided in the INVITTOX protocol (INVITTOX 1994). Although this form was generated during earlier ICE studies, in which a total of five eyes were used per test substance, this form could easily be adapted to the presently recommended three eyes per test substance, as is presented in **Figure 2-4**.

2.3 Basis for Selection of the Test System

Several aspects of the chicken eye have been cited as advantages over other species for the enucleated eye test. Chickens are used widely as a food animal species, and therefore access to chicken eyes can be obtained relatively easily. Although rabbits also are used for human consumption, their role as a food animal species is much less prominent than that of beef, pork, or poultry. Consequently, the IRE test is often performed using animals from previous laboratory studies, and therefore the need for laboratory animals is still evident. Given their widespread availability in slaughterhouses, bovine and porcine eyes have also both been investigated as sources of enucleated eyes. The limitations of the bovine cornea in the isolated eye test are its dimensions and the thickness of the cornea, which makes the screening of mild irritants difficult. Apart from the dimensions of the cornea, the same applies for the pig cornea. In addition, due to the slaughtering process, obtaining eyes from processed cows and pigs, as well as the labor required to remove the eye, is more difficult than in chickens (Prinsen and Koëter 1993). The structure of the chicken cornea is comparable to the rabbit cornea and additionally has a well-developed Bowman's membrane, as does the human cornea. Finally, because of the dark black background provided by the iris of a chicken eye, changes in opacity are more easily discriminated than in the rabbit (Prinsen M, personal communication).

Figure 2-4 Sample Scoring Form for the ICE Test Method (Modified from INVITTOX 1994)

TEST COMPOUND:														LIQUID: YES/NO		VISCOUS: YES/NO	
PROJECT NO.:														SOLID: YES/NO		GROUND: YES/NO	
DATE OF TEST:														HYDROPHILIC/HYDROPHOBIC			
SIGNATURE:														WARMED: YES/NO			
														APPEARANCE:			
Eye No.	CORNEAL THICKNESS IN INSTRUMENTAL UNITS AT t =							CORNEAL OPACITY SCORES AT t =						OTHER EFFECTS	FLUORESCEIN RETENTION		
Time	-45	0	30	75	120	180	240	0	30	75	120	180	240		0	30	I.N.
1																	
%sw																	
2																	
%sw																	
3																	
%sw																	
initials																	
mean																	
SEM																	
category:																	
classification:																	

I.N. = if necessary
%sw = percent corneal swelling

2.4 Proprietary Components

The ICE does not employ any proprietary components. However, differences in slit-lamp systems for measurement of corneal swelling have been documented, and therefore different laboratories may have different corneal swelling values for the different irritancy categories. Therefore, when comparing ICE test data generated by different laboratories, a "correction factor" may be needed to compensate for these differences (i.e., ranking of substances according to corneal swelling figures should be similar, regardless of the apparatus) (INVITTOX 1994). However, to date, this issue has not been considered as a problem in study analysis. Therefore, the potential impact of this issue on the utility of the ICE test method cannot be addressed at this time.

2.5 Basis for Number of Replicate and Repeat Experiments

2.5.1 Within Experiment Replicates

Early studies involving the ICE test method used up to six eyes per test. Five eyes were used for the test substance-treated group, and one eye was used as a negative control (Prinsen and Koëter 1993). However, unpublished observations indicated that reducing the number of test substance-treated eyes to three did not adversely affect the assay results. Because the superfusion apparatus currently used in the developer's laboratory has a capacity for 11 eyes, it was more efficient to use three eyes per test substance, and therefore evaluate three test substances in a single experiment (Prinsen M, personal communication). Additional eyes may be included if there is a concern regarding equivocal results.

2.5.2 Experimental Replicates

None of the published reports indicated that repeating experiments is necessary.

2.6 Compliance with Good Laboratory Practice

Studies should be performed in compliance with GLP Guidelines (OECD 1998; EPA 2003a, 2003b; FDA 2003). Conducting studies in compliance with GLP guidelines increases confidence in the quality and reliability of test data. Furthermore, if data using these test methods are to be submitted to the EPA or FDA in response to Federal testing requirements, then compliance with appropriate GLP guidelines is required.

2.7 Study Acceptance Criteria

According to the published reports for the ICE method, the only criteria for an acceptable study is that the negative control gives an irritancy classification of nonirritating.