DB-ALM Protocol n° 80 : Chicken Enucleated Eye Test (CEET) / Isolated Chicken Eye (ICE) test

Eye Irritation

The isolated eye of a chicken is exposed to the test compound and assessed for corneal swelling, corneal opacity and fluorescein retention in order to evaluate the eye irritation potential of the compound. *(Validation study protocol)*

Résumé

The Chicken Enucleated Eye Test (CEET) (also referenced as the Isolated Chicken Eye (ICE) test)) is an organotypic test for eye irritation testing originally developed by Prinsen and Koëter (1993) as a modification to the Isolated Rabbit Eye (IRE) test described by Burton et al. (1981) using slaughterhouse material instead of laboratory animals. In this protocol corneal swelling, corneal opacity and fluorescein retention are used to evaluate the potential ocular irritancy of a test substance.

A review document of **Isolated Chicken Eye (ICE) test** is available as “Method Summary” in the DB-ALM.

Experimental Description

Endpoint and Endpoint Measurement:

CORNEAL OPACITY: subjectively scored

CORNEAL THICKNESS: expressed as corneal swelling in%

FLUORESCEIN RETENTION: subjectively scored

Experimental System(s):

EYE ENUCLEATED (chicken): freshly isolated chicken eye

Basic Procedure

Liquid materials are applied with a micropipette in a standard dosing volume of 30 ml. Pastes may be softened by means of a warm water bath (70 ^{0}C), collected with the micropipette and applied after cooling down to a lukewarm temperature. This procedure is also applicable when dealing with highly viscous liquids, if they cannot be handled properly at room temperature.
The procedure followed should be noted on the scoring form.
Solids, ground to a fine powder if necessary, are applied by powdering the entire surface of the cornea with a standard amount of 30 mg.
In addition, prior to or during testing, the hydrophobicity or hydrophilicity of liquid materials should be established. This can easily be done by putting some of the compound in a beaker with water and observing whether or not the compound mixes with the water.

Data Analysis/Prediction Model

A classification system is available for more details see the **Procedure details** section of this DB-ALM Protocol.

Test Compounds and Results Summary

Chemicals (including acids, surfactants, solvents, and metal salts).

Discussion

This protocol was used in an international validation study on alternatives to the Draize eye irritation test for the classification and labelling of chemicals. In this project, nine promising non-animal alternative methods to the Draize Eye Test were selected to undergo final validation. Each of the nine methods was examined by four laboratories and about 60 compounds were tested. The goal of the project was to select those methods which could serve as a valid prescreen or complete replacement of the Draize Eye Test.

The Enucleated Eye Test (EET) with isolated eyes of rabbits has been recognized as a valuable alternative to the Draize eye irritation test, because it represents a test system nearest to the *in vivo* test, without the need to use live animals (Burton *et al.*,1981; Price and Andrews, 1985). However, it is preferable to use slaughterhouse waste tissue as a source of eyes for this procedure. Of the possible eye-donor species, such as the cow, the pig, or the chicken, the latter has been found to be the most suitable for the assessment of eye irritation potential of test materials (Prinsen and Koëter, 1990, 1992, 1993).

In the Chicken Enucleated Eye Test (CEET), the test compound is applied in one single dose onto the corneas of isolated eyes, which are obtained from slaughterhouse animals after these have been killed. Three parameters are measured to disclose possible adverse eye effects, namely corneal thickness (expressed as corneal swelling), corneal opacity and fluorescein retention of damaged epithelial cells of the cornea. The measurement of corneal swelling in this assay guarantees a highly objective parameter, which enables the investigator to discriminate the damaging effects of test materials very precisely (Mishima, 1968; Burton, 1972; Jacobs and Martens, 1990). In combination with the measurement of corneal opacity and fluorescein retention, though assessed by subjective observation, but being accurately measured by the use of a slit-lamp microscope, a reliable evaluation of the eye irritation potential of test materials can be achieved (Prinsen and Koëter, 1993).

Status

The CEET using this DB-ALM Protocol (no. 80) participated in the EC/HO (European Commission/Home Office) validation study (Balls et al., 1995). In this project, nine promising non-animal alternative methods to the Draize Eye Test were selected to undergo final validation. Each of the nine methods was examined by four laboratories and about 60 compounds were tested. The goal of the project was to select those methods which could serve as a valid pre-screen or complete replacement of the Draize Eye Test (Draize *et al.*, 1944).

Additionally, the CEET (and data obtained using this Protocol) was evaluated in the Interagency Regulatory Alternatives Group (IRAG) working group 1 (organotypic models) (Chamberlain et al., 1997) and by the expert panel of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (ICCVAM, 2005). In 2006, ICCVAM issued a report endorsing the use of the Isolated Chicken Eye (ICE) test, with certain limitations, as a screening test to identify substances as ocular corrosives and severe irritants in a tiered-testing strategy, using a weight-of-evidence approach, for regulatory hazard classification (ICCVAM, 2006).

Based on the positive outcome of the ICCVAM retrospective study (ICCVAM, 2006) **the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed (ESAC, 2007) the ICE test method for the use in appropriate circumstances and with certain limitations as a screening test to identify substances** as ocular corrosives and severe irritants as determined by the U.S. EPA, EU (R41) and UN GHS within the context of a sequential testing strategy for eye irritation and corrosion (OECD Test Guideline 405, OECD 2012 and Method B.5 of Annex V of the Directive 67/548/EEC, EU 2004, which was later incorporated into the Annex to Commission Regulation 440/2008/EC, EU 2008).

The OECD Test Guideline No 438: Isolated Chicked Eye Test Method for Identifying Ocular Corrosives and Severe Irritants was adopted in September 2009 (OECD, 2009).

The new protocol version that allows the performance of the ICE test in compliance with the provisions of the OECD TG 438 is currently under preparation and it will be soon available in the DB-ALM.

Last update: September 2009

PROCEDURE DETAILS, *April 1994 **

Chicken Enucleated Eye Test (CEET) / Isolated Chicken Eye (ICE) test
DB-ALM Protocol n° 80

The protocol presents the standard operation procedure used in the Home Office UK/EEC Validation Study for Alternatives to the Draize Test. Additional information added in the course of producing this Protocol, e.g. this note, is presented in italics. In addition, Annex 3, the representative data presented in Table 1 and the list of bibliographical references were not part of the original study SOP. An English language version of the SOP for this test system, as used at TNO, is available on request from Dr. Prinsen.

* The US ICCVAM (2006), the EU ECVAM (ESAC, 2007) and OECD (2009) refer to this Protocol version in their statements, studies and/or regulation. Therefore, the herewith included Standard Operating Procedure will be maintained in the current format and content for documentation purposes. Considering all the changes introduced during these studies conducted by the organisations indicated above which finally led to the regulatory acceptance of this method, a new protocol version is currently under preparation and will become available as a new Protocol in the DB-ALM.

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Principle
The test compound is applied onto the corneas of at least three eyes in one single dose. Prior to dosing, each test eye provides its own baseline values for the assessment of corneal effects. One untreated eye serves as a control of the experimental conditions. The reactions of the corneas are examined at regular intervals for up to four hours after treatment. Based on the mean scores for corneal swelling, corneal opacity and fluorescein retention, an assessment of the eye irritation potential of the compound, ranging from non- to severely irritating, can be made.

Experimental design
In the past, the Enucleated Eye Test has been performed in three of the four laboratories participating in the validation study with this method, using different types of equipment, such as the slit-lamp microscope. Different slit-lamp microscopes and depth measuring devices can give rise to a variation in the swelling ranges obtained. However, ranking of the compounds according to the swelling figures should be similar, regardless of the apparatus.

Since a variation in equipment and in the source of the chickens cannot be avoided, only general directions with respect to these items can be given in the protocol.

Chicken eyes are carefully dissected and placed in a superfusion apparatus using the following procedure:

First the eyelids are carefully removed without damaging the cornea, and a small drop of fluorescein sodium (0.5-2%) is applied to the corneal surface for a few seconds and subsequently rinsed off with isotonic saline at ambient temperature.

N.B.. both 0.5% and 2% concentrations of fluorescein were used by laboratories in the EEC/Colipa study. A 2% concentration is recommended, and is available as Fluorescein sodium BP 2% w/v (Minims, Smith and Nephew Ltd., Romford, England). Usually a flush of 5 ml isotonic saline is

sufficient to rinse away the fluorescein that has not been retained by the cornea, but a longer rinsing period may be used if required.

Then, the head with the fluorescein-treated cornea is examined with a slit-lamp microscope (Slit-lamp 900 CN, Haag-Streit AG, Liebefeld-Bern, Switzerland) to ensure that the cornea is not damaged. If undamaged, the eye is further dissected from the head without damaging the eye or cornea. Care is taken to remove the eyeball from the orbit without cutting off the optical nerve too short. The enucleated eye is placed in a clamp (stainless steel clamp available from TNO, Zeist, the Netherlands) with the cornea positioned vertically and transferred to a chamber of the superfusion apparatus (TNO, Zeist). The clamp holding the eye is positioned in such a way that the entire cornea is supplied with isotonic saline from a bent stainless steel tube, at a rate of c. 0.10-0.15 ml/min (peristaltic pump, Desaga STA 131900, Heidelberg, Germany). The chambers of the superfusion apparatus and the saline are temperature-controlled at 32 +/- 1.5^{0}C (water pump, Thermomix 1441, B.Braun Melsungen AG, Melsungen, Germany).

At least four eyes are selected, and, after being placed in the superfusion apparatus, are examined again with the slit-lamp microscope to ensure that they are not damaged. Corneal thickness is accurately measured at the corneal apex, using a Depth Measuring Device (Depth Measuring Attachment no. II for the Haag-Streit slit-lamp microscope) and is expressed in instrument units. Eyes with a corneal thickness deviating more than 10% from the mean value for the eyes, eyes that are unacceptably stained with fluorescein (score higher than 0.5, indicating the cornea to be permeable), or eyes that show any other signs of damage, are rejected as test eyes and replaced.

After an equilibration period of 45-60 minutes, the corneal thickness of the eyes is measured again to determine the zero reference value for corneal swelling calculations. At time t = 0, i.e. immediately after the zero reference measurement, the test substance is applied to the eye. For this purpose, the clamp holding the eye is placed outside the chamber with the cornea facing upwards. Liquid materials are applied in amounts of 0.03 ml from a micropipette, in such a way that the entire surface of the cornea is bathed with the test substance. Solids, ground to a fine powder if necessary, are applied in an amount of 30 mg by powdering the entire surface of the cornea.

After an exposure period of 10 seconds, the corneal surface is rinsed thoroughly with 20 ml of isotonic saline at ambient temperature. The eye in the holder is then returned to its chamber. This procedure is repeated for each test eye. The test substance is tested on at least three eyes; one eye is treated in a similar way with isotonic saline only and serves as a control. The control eye and test eyes are examined at 30, 75, 120, 180 and 240 minutes after treatment, using the criteria and scoring system given in Annex 1. Fluorescein retention is only determined at 30 minutes after treatment. All examinations are carried out with the slit-lamp microscope and are noted on the scoring form given in Annex 2.

N.B.. The dissection process can usually be mastered within a reasonable period, although some training will be required. The number of compounds that can be run on one day will depend on the number of chambers in the superfusion apparatus and on the number of eyes used to test each compound. The superfusion apparatus used at the TNO laboratory had eleven chambers and five eyes are used per compound, allowing two compounds to be run each day, with the eleventh eye serving as the control. Other laboratories use three test eyes per compound and thus can test three compounds per day. The number of tests can easily be increased if more chambers are available.

Annex 3 outlines the classification system used at TNO. A CEET Index was used to analyze data from the participating laboratories in the EEC/Colipa study. This was based on the addition of the maximum mean scores of corneal swelling, corneal opacity and fluorescein retention. The opacity and fluorescein scores were equally weighted in the index when compared to the maximum swelling % obtained. At TNO, the highest swelling observed is usually c. 60%, therefore the max. corneal opacity and fluorescein scores are multiplied by a factor of 20. This was used as the basis for the calculation of provisional CEET scores in the EEC/Colipa study. This way of scoring proved to be quite useful for statistical evaluation of the CEET results and for comparison with in vivo rabbit eye scores. However, it must be noted that the use of different sources for eyes and different thickness measurement devices may result in a variation in the obtained ranges of swelling. In such cases, a correction factor may need to be applied.

ANNEX 1

CRITERIA AND SCORING SYSTEM FOR CORNEAL EFFECTS

The following criteria and scoring systems are applied for the assessment of possible effects:

Corneal swelling
Corneal swelling, expressed as a percentage, is calculated according to the following formula:

$$\frac{\text{corneal thickness at time } t - \text{corneal thickness at time } t = 0}{\text{corneal thickness at } t = 0} \times 100\%$$

The mean percentage of swelling for all test eyes is calculated for the observation time points of 30, 75, 120, 180 and 240 minutes.

Corneal opacity
Opacity degrees of density (the most dense area is used for scoring)

0 = no opacity
0.5 = very faint opacity
1 = scattered or diffuse areas, details of iris clearly visible
2 = easily discernible translucent area, details of iris slightly obscured
3 = severe corneal opacity, no specific details of iris visible, size of pupil barely discernible
4 = complete corneal opacity, iris invisible

The mean corneal opacity value for all test eyes is calculated for the observation time points of 30, 75, 120, 180 and 240 minutes.

Fluorescein retention

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

The mean fluorescein retention value for all test eyes is calculated for the observation time point of 30, minutes only. If desired, or in case of test substances that have adhered to the cornea, fluorescein retention can be determined at t = 240 min or whenever the test compound is removed.

N.B.. In cases when all the test substance cannot be removed by the initial rinsing, further attempts to rinse it off are made at each observation time

point.

Morphological effects
These include "pitting" of corneal epithelial cells, "loosening" of epithelium, "roughening" of the corneal surface and "sticking" of the test substance to the cornea. These findings can vary in severity and they may occur simultaneously. The classification of these findings is subjective to the interpretation of the investigator.

ANNEX 2

TNO NUTRITION - TOXICOLOGY AND NUTRITION INSTITUTE

ITV/IRR/006 F1
TITLE: SCORING FORM CHICKEN ENUCLEATED EYE TEST (CEET)

TEST COMPOUND: *PROJECT NO.:* *DATE OF TESTING AND SIGNATURE:*	*LIQUID:**YES/NO** VISCOUS: **YES/NO** WARMED: **YES/NO*** *SOLID:**YES/NO** GROUND: **YES/NO*** *HYDROPHILIC/HYDROPHOBIC* *APPEARANCE:*

EYE NO.	*CORNEAL THICKNESS IN INSTRUMENT UNITS AT t =< /SPAN >*							*CORNEAL OPACITY SCORES AT t =< /SPAN >*						*OTHER EFFECTS*	*FLUORESCEIN RETENTION*		
actual time:->	*-45*	*0*	*30*	*75*	*120*	*180*	*240*	*0*	*30*	*75*	*120*	*180*	*240*		*0*	*30*	
1																	
sw%																	
2																	
sw%																	
3																	
sw%																	
4																	
sw%																	
5																	
sw%																	
6																	
sw%																	
init.[3]																	
mean[1]																	
sem[2]																	
CATEGORY :																	
CLASSIFICATION :																	

sw% = corneal swelling percentage; [1] = mean of the test eyes; [2] = standard error of the mean;
[3] = initials investigator; C = control eye (to be designated on the form)

REMARKS:

ANNEX 3

TNO CLASSIFICATION SYSTEM APPLIED TO THE CHICKEN ENUCLEATED EYE TEST

1. SEVERITY OF EFFECTS
On the basis of the severity of the observed findings for corneal swelling, corneal opacity and fluorescein retention, the effects are divided into four categories: I = none; II = slight; III = moderate; IV = severe.

Interpretation of corneal swelling, corneal opacity and fluorescein retention and classification into the four categories is done according to the following methodology:

Corneal swelling (TNO classification)

mean corneal swelling %	*category*
0 - 5	*I*
6 - 12	*II*
13 - 18 (>75 min. after treatment)	*II*
(<75 min. after treatment)	*III*
19 - 26	*III*
27 - 32 (>75 min. after treatment)	*III*
(<75 min. after treatment)	*IV*
32	*IV*

NOTE: This classification scheme is only applicable, if the range of swelling percentages obtained is approximately between 0% and 70%. Different slit lamp microscopes and depth measuring devices can give rise to a variation of the swelling range. Therefore, each laboratory must design a category system for corneal swelling using the principles described above, i.e. using the four categories: none, slight, moderate, severe.

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 min after treatment*	*category*
0.0 - 0.5	*I*
0.6 - 1.5	*II*
1.6 - 2.5	*III*
2.6 - 4.0	*IV*

2. ASSESSMENT OF THE *EX VIVO* EYE IRRITANCY CLASSIFICATION
The *ex vivo* irritancy classification is assessed by reading the irritancy classification that corresponds to the combination of the category obtained for corneal swelling, corneal opacity and fluorescein retention, which are presented in the scheme below:

Classification	*Combination of the three categories*
A. Not irritating	*3 x* ***I*** *2 x* ***I****, 1 x* ***II***
B. Slightly irritating	*3 x* ***II*** *2 x* ***II****, 1 x* ***I*** *2 x* ***II****, 1 x* ***III*** *2 x* ***I****, 1 x* ***IV****[1]* *1 x* ***I****, 1 x* ***II****, 1 x* ***III****[1]*
C. Moderately irritating	*3 x* ***III*** *2 x* ***III****, 1 x* ***II*** *2 x* ***III****, 1 x* ***IV****[2]* *2 x* ***III****, 1 x* ***I****[1]* *2 x* ***II****, 1 x* ***IV****[1]* *1 x* ***II****, 1 x* ***III****, 1 x* ***IV****[1]*
D. 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]* ***immediate corneal opacity score 3*** ***corneal opacity score 4*** ***severe loosening of epithelium***

[1] Combinations of categories less likely to occur.

[2] Combination can be considered a borderline case between moderately and severely irritating.

Where considerable inter-eye variation is found, the compound should be retested.

3. EC-CLASSIFICATION OF EYE IRRITANTS AND EXTRAPOLATION FROM *EX VIVO* RESULTS TO EC-CLASSIFICATION
According to Directive 83/467/EEC, Part II (B) of Appendix 6, Dangerous Substances Directive, 5th Adaptation, the *in vivo* irritancy grades are divided into three classes, i.e. non-irritating (NI), irritating (R36) and severely irritating (R41). Extrapolation from the *ex vivo* irritancy grade to the EC-classification will be carried out using scientific judgement, redividing the original four categories into three. The combinations of the three categories that are allowed for each of the three classifications are mentioned in the scheme below.

Classification	*Combinations of the three categories*
NI = not irritating (combination of A and B)	*3 x* ***I*** *3 x* ***II*** *2 x* ***I****, 1 x* ***II*** *2 x* ***II****, 1 x* ***I*** *1 x* ***I****, 1 x* ***II****, 1 x* ***III***[1]
R36 = irritating (combination of B and C)	*3 x* ***III*** *2 x* ***II****, 1 x* ***III*** *2 x* ***III****, 1 x* ***II*** *2 x* ***III****, 1 x* ***IV*** *2 x* ***I****, 1 x* ***IV***[1] *2 x* ***II****, 1 x* ***IV***[1] *2 x* ***III****, 1 x* ***I***[1] *1 x* ***II****, 1 x* ***III****, 1 x* ***IV***[1]
R41 = 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] ***immediate corneal opacity score 3*** ***corneal opacity score 4*** ***severe loosening of epithelium***

[1] Combinations of categories less likely to occur.

The combination of 3 x **II** can be considered as a borderline case between non-irritating and irritating.

The combination of 2 x **III** and 1 x **IV** can be considered as a borderline case between irritating and severely irritating.

Table 1: An example of the use of the TNO classification system to predict irritancy (data from Prinsen and Koëter, 1993)

Test compound	***Corneal swelling***	***Corneal opacity***	***Fluorescein retention***	***TNO classification***	***EC classification***

Acetic acid	*III*	*IV*	*IV*	*severe*	*R41*
Brij 35	*I*	*I*	*II*	*non*	*NI*
Benzalkonium chloride	*IV*	*IV*	*IV*	*severe*	*R41*
Dimethyl sulphoxide	*I*	*I*	*II*	*non*	*NI*
Sodium fluorescein	*I*	*I*	*I*	*non*	*NI*
Glycerol	*I*	*I*	*I*	*non*	*NI*
Triacetin	*I*	*I*	*I*	*non*	*NI*
Mercury (II) chloride	*IV*	*IV*	*III*	*severe*	*R41*
Silver (I) nitrate	*II*	*II*	*II*	*slight*	*NI*
NaOH	*IV*	*IV*	*IV*	*severe*	*R41*
Toluene	*I*	*II*	*II*	*slight*	*NI*
Triethanolamine	*I*	*II*	*II*	*slight*	*NI*
n-Hexane	I	I	I	non	NI
Chloroform	III	II	III	moderate	R36
2-Methoxy ethanol	II	III	III	moderate	R36
1-Butanol	IV	III	IV	severe	R41
Acetaldehyde	III	II	III	moderate	R36
2-Butoxyethyl acetate	I	II	II	slight	NI
SDS	III	II	II	severe	R41
Dibutyltin dichloride	IV	III	IV	severe	R41
Tributyltin chloride	IV	III	IV	severe	R41

N.B.. Silver (I) nitrate is a borderline case between NI and R36, 2-methoxy ethanol and acetaldehyde are borderline cases between R36 and R41, and SDS was upgraded from R36 to R41 because of an observed loosening of epithelium.

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