2.0 HET-CAM Test Method Protocol Components

The HET-CAM protocol, first described by Luepke (1985), uses a vascular fetal membrane, the chorioallantoic membrane (CAM), which is composed of the fused chorion and allantois. The CAM has been proposed as a model for a living membrane (such as the conjunctiva) since it comprises a functional vasculature. Additionally, evaluation of coagulation (i.e., protein denaturation) may reflect corneal damage that may be produced by the test substance. The acute effects induced by a test substance on the small blood vessels and proteins of this soft tissue membrane are proposed to be similar to effects induced by the same test substance in the eye of a treated rabbit.

Since the initial description of the HET-CAM test method, several studies have been conducted to evaluate the feasibility of using HET-CAM as a complete replacement for the *in vivo* rabbit ocular test. Most of these reports describe a HET-CAM test method protocol that is similar, but not identical, to the original protocol. These differences include the breed of hen from which eggs are obtained, the endpoints evaluated, data collection procedures, and methods used to analyze the data.

To date, no single HET-CAM test method protocol has gained wide acceptance as a standardized protocol. However, for a general description of how the HET-CAM test method is conducted, see ICCVAM (2006a). Briefly, during a HET-CAM study, the test substance is applied to the surface of the CAM. The CAM is subsequently evaluated for development of irritant endpoints (hemorrhage [bleeding], vascular lysis [blood vessel disintegration], and coagulation [intra-and extravascular protein denaturation]. Depending on the method used to collect data on the endpoints (e.g., time to development, severity of observed effect) qualitative assessments of the irritation potential of test substances are made. As detailed in **Section 6.0**, analyses of each of the multiple HET-CAM analysis methods indicates that the Irritation Score (A) (IS[A]) analysis method achieved the best performance when evaluating substances not labeled as irritants. Therefore, the IS(A) method is described here. For a description of the other HET-CAM analysis methods (i.e., Q-score, mtc10, ITS, and S-score), see ICCVAM (2006a).

2.1 The Irritation Score (IS) Analysis Method

For those test method protocols that assigned a score to each of the endpoints evaluated at preset time intervals, the values assigned to each endpoint were totaled to give an IS value for the test substance (i.e., IS[A] analysis method). The possible IS values range from 0 (for test substances that do not induce development of any of the toxic endpoints of interest over the range of time intervals) to 21 (for test substances that induced development of all three toxic endpoints within 30 seconds of application of the test substance) (Luepke 1985).

As described in Leupke (1985), after the application of the test substance, the CAM, the blood vessels, including the capillary system, and the albumen are examined and scored for irritant effects (lysis, haemorrhage, coagulation) at 0.5, 2 and 5 minutes after treatment (**Table 2-1**); longer observation times give no additional important information, but need further incubation and a humid chamber. These scores are summed to give a single numerical value indicating the irritation potential of the test substance on a scale with a maximum value of 21.

Table 2-1 Scoring Scheme for Irritation Testing with the HET-CAM Test Method

Effect	Score		
	0.5 min	**2 min**	**5 min**
Lysis	5	3	1
Haemorrhage	7	5	3
Coagulation	9	7	5

For those test method protocols that noted the time that a specific endpoint was first observed, the IS value was calculated (i.e., IS[B] analysis method) using the formula (Kalweit et al. 1987, 1990):

$$\left(\left(\frac{(301 - \textit{Hemorrhage time})}{300}\right) \times 5\right) + \left(\left(\frac{(301 - \textit{Lysis time})}{300}\right) \times 7\right) + \left(\left(\frac{(301 - \textit{Coagulation time})}{300}\right) \times 9\right)$$

where:

Hemorrhage time = time (in seconds) of the first appearance of blood hemorrhages

Lysis time = time (in seconds) of the first appearance of vessel lysis

Coagulation time = time (in seconds) of the first appearance of protein coagulation

The IS value, when calculated using this formula, has a maximal value of 21.
When the development of hyperemia, injection, or another toxic endpoint was evaluated instead of vessel lysis, the time to first appearance for the alternative endpoint replaced the lysis time point.

2.1.1 IS Classification Scheme

For studies that used the analysis methods developed by Luepke (1985) or Kalweit et al. (1987, 1990), the ocular irritancy classification scheme described in **Table 2-2** was used for the accuracy analysis presented in this BRD (see **Section 6.0**). The rationale for the decision criteria used in this classification scheme were not provided and the correlation of these categories to irritancy categories described by the EPA (1996), GHS (UN 2003), and EU (2001) classification systems is unknown.

Table 2-2 IS Classification Scheme Used to Classify Substances for Accuracy Analysis[1]

HET-CAM Score Range	Irritation Category
0 to 0.9	Not Labeled
1 to 4.9	Slight Irritation
5 to 8.9	Moderate Irritation
9 to 21	Severe Irritation

[1]According to Luepke (1985) and Kalweit et al. (1987, 1990).