TEST METHOD PROCEDURE
Prequalification of Normal Human Epidermal Keratinocyte Growth Medium

In Vitro Cytotoxicity Validation Study
Phase III

January 28, 2004

Prepared by

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

Based on Standard Operating Procedure Recommendations from an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Institute of Environmental Health Sciences (NIEHS)
National Institutes of Health (NIH)
U.S. Public Health Service
Department of Health and Human Services

I. PROPOSAL

The following document provides the guidelines and testing requirements for qualifying lots of Keratinocyte Basal Medium without Ca^{++} (KBM® [CAMBREX/Clonetics # CC-3104]) and the medium supplements (SingleQuots® [CAMBREX/Clonetics # CC-4131]) for use in the normal human epidermal keratinocyte (NHK) neutral red uptake (NRU) assays for Phase III of the In Vitro Cytotoxicity Validation Study. The medium and supplements will be tested so as to demonstrate their ability to perform adequately in the NHK NRU assay prior to purchase by the validation study laboratories for use in Phase III.

The Testing Facility will request the quality control test data from CAMBREX/Clonetics for each potential lot of medium and supplements. Based upon the QC test data, the Testing Facility will purchase and test the one or two most current lots of medium and supplements in stock with CAMBREX/Clonetics that appear to have the potential to support NHK cultures according to the requirements of the In Vitro Cytotoxicity Validation Study NHK neutral red uptake assay.

This test method procedure is based on the Phase III NHK NRU protocol (IIVS Protocol No. SP100066) and outlines the procedures needed for performing the cytotoxicity test specifically for prequalifying NHK culture medium. The test method procedure and NHK NRU protocol support the *in vitro* validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM) and sponsored by NIEHS, U.S. Environmental Protection Agency, and ECVAM. This test method procedure applies to all personnel involved with performing media/supplement testing.

A. NHK Neutral Red Uptake Cytotoxicity Test

The NHK NRU test will be performed to analyze NHK growth characteristics and the *in vitro* toxicity of Sodium Lauryl Sulfate (SLS), as measured by the IC_{50}, with each NHK medium/supplement being tested.

The Testing Facility will select the lots of medium/supplements and combinations based on the maximum available quantity and shelf life, as well as growth test results provided by Cambrex. Potential medium testing/supplement combinations are:

- One lot of medium/one lot of SingleQuots®: Test the lot of medium using the lot of SingleQuots® (one test of three plates).
- Two or more lots of medium/one lot of SingleQuots®: Test each lot of medium using the one lot of SingleQuots® (one test of three plates for each lot of medium)
- One lot of medium/two or more lots of SingleQuots®: Test the lot of medium using each lot of SingleQuots® (one test of three plates for each lot of SingleQuots®).

NHK cultures will be established using each medium/supplement combination, and will be subcultured on 3 different days into 96-well plates for three subsequent SLS cytotoxicity tests using each appropriate test medium/supplement combination.

B. Testing Conditions

The work will be performed in the IIVS Good Laboratory Practice (GLP)-compliant laboratories, but will not be performed in full compliance with national and international GLP guidelines, and neither a protocol nor an audited report will be generated.

The Study Director will provide recommendations and appropriate test data for acceptance/rejection of the tested media/supplements to the Study Management Team (SMT).

The Testing Facility will maintain the following documentation: study workbooks noting all methods and procedures; logs for general laboratory procedures and equipment (e.g., media preparation, SLS preparation, incubator function); electronic and paper formats of all optical density data obtained from the spectrophotometer plate reader; electronic and paper format of all calculations of ICx values and other derived data.

II. SPONSOR

A. Name: National Institute of Environmental Health Sciences (NIEHS); The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

B. Address: P.O. Box 12233
Research Triangle Park, NC 27709

C. Representative: Molly Vallant, Project Officer, NIEHS

D. Study Management Team Representatives: William Stokes, Silvia Casati, Raymond Tice, Judy Strickland, Michael Paris

III. IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Substances: Keratinocyte Basal Medium without Ca^{++} (KBM®, Clonetics CC-3104)

KBM® SingleQuots® (Clonetics CC-4131)

B. Controls: Positive: Sodium Lauryl Sulfate
Vehicle (Negative): Assay medium

IV. TESTING FACILITY AND KEY PERSONNEL

- Name: Institute for In Vitro Sciences, Inc.
- Address: 21 Firstfield Road, Suite 220
  Gaithersburg, Maryland 20878
- Study Director: Hans Raabe, M.S.
- Laboratory Technician(s): Greg Mun, B.A., Laboratory Manager
  Robin Anderson, B.S.
  Filomena Diaco, B.S.
  Gregory Moyer, B.S.
  Massod Rahimi, B.S.
  Angela Sizemore, B.S.
  Teri Beth Wallace, B.S.

Nathan Wilt, B.S.

V. PROCEDURES

A. Materials

NHK cells used for this procedure will come from the same lot of NHK cells used in Phases I and II of the validation study. Equipment, chemicals, and other media will be the same as in IIVS Protocol No. SP100066.

B. Preparations of Media and Solutions

All media and solutions will be prepared as in IIVS Protocol No. SP100066.

C. Methods

All culture procedures will be performed as in IIVS Protocol No. SP100066..

NHK cultures will be established with cryopreserved cells seeded into individual tissue culture flasks using the existing medium/supplement combination (the "control" medium) and each test medium/supplement combination. It may be acceptable to suspend freshly-thawed cells initially into 9 mL of control medium. The cell suspension will then be added to culture flasks containing pre-warmed control or test medium. The cells will be subcultured on three different days into 96-well plates for three subsequent NRU tests using each appropriate test medium/ supplement combination and control.

D. Preparation of SLS

The preparation of SLS (IIVS code 02AD92) will follow the procedures in Sections VII.D.1.a, b, and d of IIVS Protocol No. SP100066. SLS will be dissolved only in Routine Culture Medium. Determination of the pH will follow Section VII.D.2.

Preparation of SLS concentrations/dilutions will follow the main experiment procedures specifically for testing compounds in Routine Culture Medium as outlined in Section VII.D.3.b of IIVS Protocol No. SP100066. The concentrations/dilutions should be the same or similar to those used for SLS as a positive control in Phase II of the validation study.

E. Test Procedure

The 96-well plate configuration will be the same as that outlined in Section VII.E.1 of IIVS Protocol No. SP100066. The C_1 test concentration will be the highest SLS concentration. Application of the SLS, subsequent toxicity testing, and measurement of NRU will follow procedures outlined in Sections VII.E.2.a and b and Section VII.4 of IIVS Protocol No. SP100066.

Cells cultured in control medium and in each test medium/supplement combination will be tested in parallel for their sensitivity to SLS.

F. Microscopic Evaluation

Observations of the cell cultures in the culture flasks, as well as in the 96-well plates will be performed and documented and should include cell morphology (e.g., overall appearance, colony formation and proliferation, presence of mitotic figures, and distribution). Representative observations of the cultures in the culture flasks will be performed every working day. Representative observations of the cultures in the 96-well plates will be performed daily prior to treatment with SLS; at the end of the 48 hour treatment incubation;

and during the neutral red incubation period (to evaluate relative neutral red uptake in the vehicle control cultures).

Changes in morphology of the cells due to cytotoxic effects of the SLS (prior to measurement of NRU) should be recorded as per procedures outlined in Section VII.E.3 of IIVS Protocol No. SP100066.

G. Data Analysis and Test Evaluation

Data analysis will be performed as in Section VII.F of IIVS Protocol No. SP100066. The following parameters will be evaluated to determine whether the NHK media and supplements are adequate to support the NHK NRU assay:

1) SLS IC_{50}

2) r^2 (coefficient of determination) value calculated for the Hill model fit (i.e., from PRISM® software.

3) Difference between the mean of all vehicle controls (VC) and (a) the left mean VC, and (b) the right mean VC.

4) Number of points between 0 % and 50.0 % viability and between 50.0 % 100 % viability.

5) Mean corrected $OD_{540\text{-}550}$ of the VCs.

6) Cell morphology and confluence of the VCs at the end of the 48 h treatment

The Study Director will utilize all observed growth characteristics and test results to determine whether the media/supplements perform adequately, and provide the test data and a recommendation for the use or rejection of the media/supplements to the SMT. IIVS will request CAMBREX/Clonetics reserve a portion of an acceptable lot based on estimates of media needed by the three laboratories.

V. REFERENCES

IIVS Protocol No. SP100066. Test Method Protocol for the NHK Neutral Red Uptake Cytotoxicity Test. A Test for Basal Cytotoxicity for an In Vitro Validation Study. November 11, 2003. Prepared by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).

VI. APPROVAL

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SPONSOR REPRESENTATIVE **DATE**

(Print or type name)

_____ _____

Testing Facility STUDY DIRECTOR **DATE**
(Print or type name)

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