

**TEST METHOD PROTOCOL
for Solubility Determination**

***In Vitro* Cytotoxicity Validation Study
Phase III**

September 24, 2003

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**

TEST METHOD PROTOCOL

Solubility Determination Phase III

I. PURPOSE

The purpose of this study is to evaluate the cytotoxicity of test chemicals using the BALB/c 3T3 Neutral Red Uptake (NRU) and normal human keratinocyte (NHK) cytotoxicity tests. The data will be used to evaluate the intra- and inter-laboratory reproducibility of the assay and effectiveness of the cytotoxicity assay to predict the starting doses for rodent acute oral systemic toxicity assays. This test method protocol outlines the procedures for performing solubility determinations for 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 protocol applies to all personnel involved with performing the solubility testing.

A. Solubility Test

The solubility tests will be performed to determine the best solvent to use for each of the 60 blinded/coded test chemicals to be tested in the 3T3 and NHK NRU cytotoxicity tests

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: *Named Representative*

III. IDENTIFICATION OF TEST SUBSTANCES AND SOLVENTS

- A. Test Chemicals: *60 Coded Chemicals (60)*
- B. Solvents: Chemical Dilution Medium for 3T3 assay (See **Section VII.B.1**)
Treatment Medium for NHK assay (See **Section VII.B.2**)

IV. TESTING FACILITY AND KEY PERSONNEL

A. Facility Information

- 1) Name:
- 2) Address:
- 3) Study Director:
- 4) Laboratory Technician(s):
- 5) Scientific Advisor:
- 6) Quality Assurance Director:
- 7) Safety Manager:

8) Facility Management:

B. Test Schedule

- 1) Proposed Experimental Initiation Date:
- 2) Proposed Experimental Completion Date:
- 3) Proposed Report Date:

V. TEST SYSTEM

The solubility test procedure is based on attempting to dissolve chemicals in various solvents with an increasingly rigorous mechanical techniques. The solvents to be used, in the order of preference, are cell culture media, DMSO, and ethanol. Solubility shall be determined in a step-wise procedure that involves attempting to dissolve a test chemical in the solvents (in the order of preference) at relatively high concentrations using the sequence of mechanical procedures (**Section VII.C.2.a**). If the chemical does not dissolve, the volume of solvent is increased so as to decrease the concentration by a factor of 10, and then the sequence of mechanical procedures are repeated in an attempt to solubilize the chemical at the lower concentrations.

Determination of whether a chemical has dissolved is based entirely on visual observation. A chemical has dissolved if the solution is clear and shows no signs of cloudiness or precipitation.

VI. DEFINITIONS

- A. *Soluble*: Chemical exists in a clear solution without visible cloudiness or precipitate.
- B. *Documentation*: all methods and procedures will be noted in a Study Workbook; logs will be maintained for general laboratory procedures and equipment (e.g., media preparation, solubility testing, laboratory balance calibration); solubility reports will be in electronic and paper format; all data will be archived.

VII. PROCEDURES

A. Materials

1. Technical Equipment

[Note: Suggested brand names/vendors are listed in parentheses. Equivalents may be used.]

- a) Water bath: 37°C ± 1°C
- b) Glass tubes with caps (e.g., 5 mL)
- c) Laboratory balance
- d) Pipetting aid
- e) Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette), dilution block
- f) Waterbath sonicator
- g) Dry heat block (optional)

2. Chemicals, Media, and Sera

- a) Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine; should have high glucose [4.5gm/l] (e.g., ICN-Flow Cat. No. 12-332-54)
- b) L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49)
- c) Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49)
- d) Dimethyl sulfoxide (DMSO), U.S.P. analytical grade (Store under nitrogen @ -20°C)
- e) Ethanol (ETOH), U.S.P. analytical grade (100 %, non-denatured for test chemical preparation; 95 % can be used for the desorb solution)
- f) Keratinocyte Basal Medium without Ca⁺⁺ (KBM□, Clonetics CC-3104) that is completed by adding the KBM□ SingleQuotes□ (Clonetics CC-4131) to achieve the proper concentrations of epidermal growth factor, insulin, hydrocortisone, antimicrobial agents, bovine pituitary extract, and calcium (e.g., Clonetics Calcium SingleQuotes□, 300 mM CaCl₂, Clonetics # CC-4202).

B. Preparations of Media and Solutions

[Note: All solutions glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures will be adequately documented. Completed media formulations should be kept at approximately 2-8° C and stored for no longer than two weeks.]

1. 3T3 Chemical Dilution Medium

DMEM (buffered with sodium bicarbonate) supplemented with (final concentrations in DMEM are quoted):

4 mM	Glutamine
200 IU/mL	Penicillin
200 µg/mL	Streptomycin

2. NHK Treatment Medium

KBM□ (Clonetics CC-3104) supplemented with KBM□ SingleQuotes□ (Clonetics CC-4131) and Clonetics Calcium SingleQuotes□ (CC-4202) to make 500 mL medium. Final concentration of supplements in medium are:

0.0001 ng/mL	Human recombinant epidermal growth factor
5 □g/mL	Insulin
0.5 □g/mL	Hydrocortisone
30 □g/mL	Gentamicin
15 ng/mL	Amphotericin B
0.10 mM	Calcium
30 □g/mL	Bovine pituitary extract

NOTE:

KBM□ SingleQuotes□ contain the following stock concentrations and volumes:

0.1 ng/mL	hEGF	0.5 mL
5.0 mg/mL	Insulin	0.5 mL
0.5 mg/mL	Hydrocortisone	0.5 mL
30 mg/mL	Gentamicin, 15 ug/mL Amphotericin-B	0.5 mL

7.5 mg/mL Bovine Pituitary Extract (BPE) 2.0 mL

Clonetics Calcium SingleQuotes are 2 mL of 300 mM calcium.

165 µl of solution per 500 mL calcium-free medium equals 0.10 mM calcium in the medium.

C. Determination of Solubility

The preference of solvent for dissolving test chemicals is medium, DMSO, and then ethanol. Solubility shall be determined in a step-wise procedure that involves attempting to dissolve a test chemical at a relatively high concentration with the sequence of mechanical procedures specified in **Section VII.C.2.a**. If the chemical does not dissolve, the volume of solvent is increased so as to decrease the concentration by a factor of 10, and then the sequence of mechanical procedures in **Section VII.C.2.a** are repeated in an attempt to solubilize the chemical at the lower concentrations. For testing solubility in medium, the starting concentration is 20,000 µg/ml (i.e., 20 mg/mL) in Tier 1, but for DMSO and ethanol the starting concentration is 200,000 µg/ml (i.e., 200 mg/mL) in Tier 2. Weighing out chemical for each solvent (i.e., medium, DMSO, ethanol) can be done all at once, if convenient, but solubility testing (at each tier that calls for more than one solvent) is designed to be sequential - medium, then DMSO, then ethanol – in accordance with the solvent hierarchy (see **Figure 1**). This allows for testing to stop, rather than continue testing with less preferred solvents, if the test chemical dissolves in a more preferred solvent. For example, if a chemical is soluble in medium at a particular tier, testing may stop. Likewise, if a chemical is soluble in DMSO at any tier, testing need not continue with ethanol. However, since the issue of primary importance is testing the solvents and concentrations of test chemical required by any one tier, sequential testing of solvents may be abandoned if the lab can test more efficiently in another way.

1. Method

- a) Tier 1 begins with testing 20 mg/mL each in Chemical Dilution Medium and Treatment Medium (see **Table 1**). For each medium, weigh approximately 10 mg (10,000 µg) of the test chemical into glass tubes. Document the chemical weight. Add approximately 0.5 mL of each medium into its respective tube so that the concentration is 20,000 µg/ml (20 mg/mL). Mix the solution as specified in **Section VII.C.2.a**. If complete solubility is achieved in each medium, then additional solubility procedures are not needed.
- b) If the test chemical is insoluble in either Chemical Dilution Medium or Treatment Medium, proceed to Tier 2 by adding enough medium, approximately 4.5 mL, to attempt to dissolve the chemical at 2 mg/mL by using the sequence of mixing procedures specified in **Section VII.C.2.a**. If the test chemical dissolves in medium at 2 mg/mL, no further procedures are necessary. If the test chemical does NOT dissolve in one medium or the other (if both are tested in this tier), weigh out approximately 100 mg test chemical in a second glass tube and add enough DMSO to make the total volume approximately 0.5 mL (for 200 mg/mL) and attempt to dissolve the chemical as specified in **Section VII.C.2.a**. If the test chemical does not dissolve in DMSO, weigh out approximately 100 mg test chemical in another glass tube and add enough ethanol to make the total volume approximately 0.5 mL (for 200 mg/mL) and attempt to dissolve the chemical as specified in **Section VII.C.2.a**. If the chemical is soluble in either solvent, no additional solubility procedures are needed.

- c) If the chemical is NOT soluble in one or both media, DMSO, or ethanol at Tier 2, then continue to Tier 3 in Table 1 by adding enough solvent to increase the volume of the three (or four) Tier 2 solutions by 10 and attempt to solubilize again using the sequence of mixing procedures in **Section VII.C.2.a**. If the test chemical dissolves, no additional solubility procedures are necessary. If the test chemical does NOT dissolve, continue with Tier 4 and, if necessary, Tier 5 using DMSO and ethanol. Tier 4 begins by diluting the Tier 3 samples with DMSO or ethanol to bring the total volume to 50 mL. The mixing procedures in **Section VII.C.2.a** are again followed to attempt to solubilize the chemical. Tier 5 is performed, if necessary, by weighing out another two samples of test chemical at ~10 mg each and adding ~50 mL DMSO or ethanol for a 200 μ g/mL solution, and following the mixing procedures in **Section VII.C.2.a**.

Example: If complete solubility is not achieved at 20,000 μ g/mL in either Chemical Dilution Medium or Treatment Medium at Tier 1 using the mixing procedures specified in **Section VII.C.2.a**, then the procedure continues to Tier 2 by diluting the solution to 5 mL (with either of the appropriate media) and mixing again as specified in **Section VII.C.2.a**. If the chemical is not soluble in Chemical Dilution Medium or Treatment Medium, two samples of ~ 100 mg test chemical are weighed to attempt to solubilize in DMSO and ethanol at 200,000 μ g/mL (i.e., 200 mg/mL). Solutions are mixed following the sequence of procedures prescribed in **Section VII.C.2.a** in an attempt to dissolve. If solubility is not achieved at Tier 2, then the solutions (Chemical Dilution Medium and/or Treatment Medium, DMSO, and ethanol) prepared in Tier 2 are diluted by 10 so as to test 200 μ g/mL in media, and 20,000 μ g/mL in DMSO and ethanol. This advances the procedure to Tier 3. Solutions are again mixed as prescribed in **Section VII.C.2.a** in an attempt to dissolve. If solubility is not achieved in Tier 3, the procedure continues to Tier 4, and to 5 if necessary (see **Figure 1** and **Table 1**).

Table 1. Determination of Solubility in Chemical Dilution Medium, Treatment Medium, DMSO, or Ethanol

TIER	1	2	3	4	5
Total Volume Chemical Dilution Medium/Treatment Medium	0.5 mL	5 mL	50 mL		
Concentration of Test Chemical (Add ~10 mg to a tube. Add enough medium to equal the first volume. Dilute to subsequent volumes if necessary.)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	200 µg/mL (0.20 mg/mL)		
Total Volume DMSO/Ethanol		0.5 mL	5 mL	50 mL	
Concentration of Test Chemical (Add ~100 mg to a large tube. Add enough DMSO or ethanol to equal the first volume. Dilute with subsequent volumes if necessary.)		200,000 µg/mL (200 mg/mL)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	
Total Volume DMSO/Ethanol					50 mL
Concentration of Test Chemical (Add ~10 mg to a large tube. Add enough DMSO or ethanol to equal 50 mL.)					200 µg/mL (0.2 mg/mL)
Equivalent Concentration on Cells	10,000 µg/mL (10 mg/mL)	1000 µg/mL (1 mg/mL)	100 µg/mL (0.1 mg/mL)	10 µg/mL (0.01 mg/mL)	1 µg/mL (0.001 mg/mL)

[NOTE: The amounts of test chemical weighed and Chemical Dilution Medium and Treatment Medium added may be modified from the amounts given above, provided that the targeted concentrations specified for each tier are tested.]

Figure 1. Solubility Flow Chart

TIER 1

STEP 1:	20 mg/mL test chemical (TC) in 0.5 mL Chemical Dilution Medium and Treatment Medium: <ul style="list-style-type: none"> • if TC soluble in both media, then <u>STOP</u>. • if TC insoluble in one medium, then go to STEP 2.
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TIER 2

STEP 2:	2 mg/mL TC in medium (one or both) – increase volume from STEP 1 by 10 (i.e., to 5 mL) <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble in one medium, then go to STEP 3.
STEP 3:	200 mg/mL TC in DMSO <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 200 mg/mL in ETOH. <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • If TC insoluble, go to STEP 4.

TIER 3

STEP 4:	0.2 mg/mL TC in medium (one or both) – increase volume from STEP 2 by 10 (i.e., to 50 mL) <ul style="list-style-type: none"> • if TC soluble in both media, then <u>STOP</u>. • if TC insoluble in one medium, test at 20 mg/mL in DMSO – increase volume from STEP 3 by 10 (i.e., to 5 mL). <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 20 mg/mL in ETOH – increase volume from STEP 3 by 10 (i.e., to 5 mL). <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, then go to STEP 5.
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TIER 4

STEP 5:	2 mg/mL TC in DMSO – increase volume from STEP 4 by 10 (i.e., to 50 mL) <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 2 mg/mL in ETOH – increase volume from STEP 4 by 10 (i.e., to 50 mL). <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, then go to STEP 6.
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TIER 5

STEP 6:	0.2 mg/mL TC in 50 mL DMSO <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 0.2 mg/mL in 50 mL ETOH <ul style="list-style-type: none"> • <u>STOP</u>
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2. Mechanical Procedures

- a) The following hierarchy of mixing procedures will be followed to dissolve the test chemical:
 - 1) Add test chemical to solvent as in Tier 1 of **Table 1**. (Test chemical and solvent should be at room temperature.)
 - 2) Gently mix at room temperature. Vortex the tube (1 –2 minutes).
 - 3) If test chemical hasn't dissolved, use waterbath sonication for up to 5 minutes.
 - 4) If test chemical is not dissolved after sonication, then warm solution to 37°C for 5 - 60 min. This can be performed by warming tubes in a 37°C water bath or in a CO₂ incubator at 37°C. The solution may be stirred during warming (stirring in a CO₂ incubator will help maintain proper pH).
 - 5) Proceed to Tier 2 (and Tiers 3-5, if necessary of Table 1 and repeat procedures 2-4).
- b) The preference of solvent for dissolving test chemicals is Chemical Dilution Medium or Treatment Medium, DMSO, and then ethanol. Thus, if all solvents for a particular tier are tested simultaneously and a test chemical dissolves in more than one solvent, then the choice of solvent follows this hierarchy. For example, if, at any tier, a chemical is soluble in Chemical Dilution Medium and DMSO, but not in Treatment Medium or ethanol, the choice of solvent would be medium for the 3T3 assay and DMSO for the NHK assay. If the chemical were insoluble in both media, but soluble in DMSO and ethanol, the choice of solvent would be DMSO for both assays.

After the lab has determined the preferred solvent for the test chemical and before proceeding to the cytotoxicity testing, the Study Director will submit the solubility test results (laboratory worksheets are preferable), and discuss the solvent selection with the Study Management Team (SMT) of the validation study. The SMT will provide direction on the solvent to be used in each assay for each chemical prior to cytotoxicity testing. If the laboratory has attempted all solubility testing without success, then the SMT will provide additional guidance for achieving test chemical solubility. The SMT anticipates that all validation study test chemicals will be tested in the NRU assays.

The Testing Facility shall forward the results from the solubility tests assay to the SMT through the designated contacts in electronic format and hard copy upon completion of testing. The SMT will be directly responsible for the statistical analyses of the Validation Study data.

VIII. REFERENCES

U. S. Environmental Protection Agency. 1996. Product Properties Test Guidelines. OPPTS 803.7840. Water Solubility: Column Elution Method; Shake Flask Method. EPA712-C-96-041, Prevention, Pesticides and Toxic Substances, Washington DC.

IX. APPROVAL

SPONSOR REPRESENTATIVE
(Print or type name)

DATE

Test Facility STUDY DIRECTOR
(Print or type name)

DATE