Appendix A

Standard Operating Procedures for the LLNA: DA Test Method

[This Page Intentionally Left Blank]

Appendix A1

Standard Operating Procedures/Protocol for the LLNA: DA Test Method

[This Page Intentionally Left Blank]

These are the standard operating procedures performed during the two-phased interlaboratory test method validation study (Omori et al. 2008) for the murine local lymph node assay (LLNA) modified by Daicel Chemical Industries, Ltd., based on adenosine triphosphate content (ATP; referred to hereafter as the "LLNA: DA") as confirmed by the LLNA: DA Validation Committee and provided by the study director.[1] These procedures are intended for tests conducted to evaluate a single test substance. Although the standard operating procedures detailed herein are specific for the interlaboratory test method validation study, the substances tested in the intralaboratory validation study followed a technically similar LLNA: DA test method protocol (Idehara et al. 2008; Idehara unpublished data).

1.0 Preparation of Equipment and Materials

Prepare the experimental equipment, materials, and reagents given in **Table A-1**. Luminometer tubes, 15 mL test tubes, 50 mL test tubes, petri dishes, and slide glass should be disposable. The underlined items will be provided by the LLNA: DA Validation Committee but in some cases, a luminometer will be furnished by the test facilities. All other materials will be provided by the test facilities.

Table A-1 List of Required Equipment, Materials and Reagents

Name of Equipment, Material, or Reagent	Manufacturer	Comment (Trade Name, Model Number, etc.)
Luminometer	Kikkoman Corporation, Japan	LUMITESTER C-100 Detection Range: $4x10^{-12}$ – $1x10^{-6}$ M Upper Limit: 1,000,000 RLU
Luminometer tubes	Kikkoman Corporation, Japan	Polypropylene, sterilized
15 mL test tubes	IWAKI brand	Polypropylene, sterilized
50 mL test tubes	IWAKI brand	Polypropylene, sterilized
Petri dish	Corning Incorporated	Cell culture dish, sterilized
Cell scraper	Costar brand	Disposable cell scraper, sterilized
Slide glass	Matsunami	Micro slide glass
Vortex mixer		
Analytical balance		For body weight measurements (readability of at least 0.1 g)

[1] 2/6/2006: Confirmed by LLNA: DA Validation Committee; 2/17/2006: Revised by Takashi Omori; 2/19/2006: Revised by Takashi Omori; 3/27/2006: Revised by Takashi Omori; 4/2/2006: Revised by Takashi Omori; 12/2/2006: Revised by Takashi Omori.

Name of Equipment, Material, or Reagent	Manufacturer	Comment (Trade Name, Model Number, etc.)
Analytical balance		For lymph node weight measurements (readability of at least 0.1 mg)
Brush	Ikkyuen	Osho
Phosphate buffered saline	Invitrogen Gibco™	pH 7.2, sterilized
Luciferin-luciferase reagent	Kikkoman Corporation, Japan	CheckLite™ 250 Plus[1]
Cages		Capable of housing four mice, with feed and water dispensers
Micropipette		For applying test solutions (25 μL), handling phosphate buffered saline (1000 μL), tissue suspension (20 μL), cell suspension (100 μL), and dissolved Luciferin-luciferase solution (100 μL)
Micropipette tips		Sterilized
Dissecting instruments		Large and small tweezers, scissors, surgical holder, injection needle and holder
Timer		With second display
General laboratory materials		Cotton, antiseptic solution, paper towel, clean sheet, test tube rack, microtube rack

Abbreviations: etc. = et cetera; g = grams; M = molar; mg = milligrams; μL = microliter; mL = milliliter; RLU = relative luminescence units.

[1]For the substances tested in the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008), the ATP content for potassium dichromate was measured by the CheckLite™ 250 Plus Kit (Kikkoman Corporation, Japan) but that for all other substances was determined using the ViaLight® HS Kit (Lonza Rockland, Inc., USA).

2.0 Preparations Prior to Delivery of Animals

The animals to be used in the tests are young adult female mice (nulliparous and non-pregnant) of the CBA/JNCrlj strain, aged between eight to twelve weeks prior to application of test and control substances. The animals will be provided by the LLNA: DA Validation Committee. Preparations should be made according to the standards of the test facilities to begin acclimatizing the animals once they have arrived on the previously agreed upon date of delivery.

Six cages capable of holding four animals each should be prepared prior to the end of acclimatization.[2] The cages should be labeled as listed in **Table A-2**. The symbol "X" represents the code of the test substance to be provided. Mark the label using the letter indicated on the datasheets provided prior to the test. The animal test group numbers are also indicated on the datasheets. The numbers should be confirmed and the cages labeled with care. This test will be performed two or three times, so it is important to include the test number on the labels.

Table A-2 Preparation of Test Group Cages

Test Group Number	Label
Group 1	Acetone: Olive Oil (4:1)
Group 2	Positive Control
Group 3	Vehicle
Group 4	Test Substance "X" – Low Concentration
Group 5	Test Substance "X" – Medium Concentration
Group 6	Test Substance "X" – High Concentration

"X" represents the code of the test substance provided by the study management team.

3.0 Delivery, Acclimatization and Animal Assignment

On the date of delivery, 25 animals will arrive and acclimatization should begin immediately. Acclimatization should be performed according to the standards of the test facilities. The animals should be acclimatized for at least five days, but no more than 16 days.

After acclimatization healthy animals with no observable skin lesions or other abnormalities should be randomly assigned to six groups of four animals each using randomly generated numbers. After assigning the animals to groups, four animals each should be placed in the six cages prepared as described in **Section 2.0**. Any animals remaining after the assignment of 24 should be omitted from the test. Should there be fewer than 24 animals with no observed abnormalities, three animals should be assigned to each group beginning with the test group with the highest number until all of the animals are assigned.

[2] For the substances tested in the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished data), at least three animals per dose group were used (i.e., in most cases, 4 animals per control group and three animals per test substance group).

From the delivery of the animals to the end of the test procedures the temperature of the animal housing facility should be maintained at 22ºC (±3ºC) with a relative humidity of 30-70%. The animals should be housed with a light: dark cycle of 12 hours light: 12 hours dark and should be given food and water *ad libitum*. Any deviations from the standard housing and feeding procedures should be recorded.

4.0 Confirmation of Test Materials

When the test materials sent by the LLNA: DA Validation Committee arrive, confirm that the inventory document matches the contents.

The labels for each of the treatments (acetone: olive oil [4:1], positive control, vehicle, and low, medium and high concentrations of test substances) include a test substance code and a group number. After confirming that these codes match the datasheet, arrange the treatments in a test tube rack according to group number. Sodium lauryl sulfate (SLS) solution will arrive in one tube. Apportion 3 mL of SLS solution to each of the accompanying empty test tubes, mark each tube with the group number, and arrange the tubes in order in the test tube rack.

The treatments should be refrigerated immediately and only removed when beginning the test. Refrigeration of the solutions used in these procedures should be between 0-10ºC, and preferably between 2-8ºC, except when instructed differently. Should there be specific instructions as to the handling of the solutions, the instructions will be included with the materials shipment and they should be followed. For instance:

- SLS (CASRN: 151-21-3) is a 1% solution and should be kept at room temperature
- Acetone: olive oil is 4:1 volume to volume ratio
- Positive control is a 25% acetone: olive oil (4:1) solution of hexyl cinnamic aldehyde (CASRN: 101-86-0)

5.0 Procedures on Test Days 1, 2, 3 and 7

5.1 Day 1

Mark the animals on the tail with their test group number and a number from one to four. Weigh the animals and record their weight to the nearest 0.1 g on the test forms.

Remove the test materials from the refrigerator. Should the materials arrive with instructions to heat or sonicate the treatments prior to application, perform these procedures as instructed.

5.1.1 Pre-treatment with 1% SLS Solution

Beginning with Group 1 and proceeding in order to Group 6, the SLS solution should be applied with a brush to the dorsum of both ears of the mice. The number of the SLS solution used should match the test group number. The brush should be dipped in the SLS solution and applied to the dorsum of one ear using a petting motion, covering the entire dorsum with four to five strokes. Dip the brush again in the SLS solution and apply the solution to the dorsum of the other ear in the same manner.

Record the time when beginning to apply SLS solution to Group 1 and when completing application to Group 6. The application procedure should be performed continuously without delay for Groups 1 through 6.

Six brushes should be prepared and numbered, using only one brush for each test group. When performing the same application procedure on Days 2, 3, and 7 there is the possibility of brush contamination due to residual solution on the mouse auricula. It is important to switch brushes after finishing application for one group and check the number of the next brush before proceeding to the next group. After use, the brushes should be washed thoroughly and made available for the next day.

5.1.2 Test Substance Application

One hour after starting the SLS solution application, the numbered treatments should be applied to the auriculae of the mice, beginning with Group 1 and ending with Group 6. Using a micropipette or similar device, 25 μL of the test solution should be dripped slowly on the dorsum of one of the mouse's ears, covering the dorsum entirely. Again take up 25μL of treatment solution and apply it in the same manner to the dorsum of the mouse's other ear.

When applying the treatments, micropipette tips should be changed for each test group. After completing application for one test group, remove the tip and spray the end of the micropipette with an alcohol mist and wipe to avoid contamination.

Record the time when beginning to apply the test solution to Group 1 and when completing application to Group 6. The application procedure should be performed continuously without delay for Groups 1 through 6.

Immediately after completing application the test materials should be refrigerated.

5.1.3 General Information on the 1% SLS Pre-treatment and Test Substance Application

The objective of the application procedure is to first apply SLS solution to the entirety of the dorsum of the ear and then to apply a prescribed amount of test solution to the same area. Using ether anesthesia ensures ease and accuracy of the procedure. However, special care should be taken to avoid taking the life of the animals in the course of anesthesia. If one technician immobilizes the animal and extends the ear with tweezers while the other technician applies the solution, the procedure can be performed with accuracy without using anesthesia. If this approach is used six pairs of tweezers should be prepared, one for each group, to avoid contamination. Alternatively, the tweezers should be wiped with an alcohol swab after application is completed for each test group.

5.2 Days 2 and 3

Apply SLS solution and treatments using the same procedures as for Day 1.

When performing the application procedures the animals should be observed carefully for necrosis, hardening, hyperplasia or erythema of the auricula, as well as piloerection, or a decrease in locomotor activity. Any such abnormalities observed should be recorded on the test forms.

5.3 Day 7

On Day 7 the same procedures should be performed as on Days 2 and 3.

Excision of the auricular lymph nodes will be performed from 24 to 30 hours after the start of application on Day 7. It is therefore recommended that application procedures on Day 7 begin in the morning or early afternoon.

6.0 Procedure on Test Day 8 (Excision of Auricular Lymph Nodes and ATP Assay)

6.1 Laboratory Preparation

Forty-eight 15 mL test tubes should each be filled with 1.98 mL of phosphate buffered saline (PBS). The dispensing of PBS should be conducted under aseptic manipulation. Dispense a minimum of 24 mL of PBS in a 50 mL test tube. Pipetting should be under aseptic manipulation.

Dissolve the luciferin-luciferase reagent according to the ATP assay kit instructions (at least 4.8 mL are required). The ATP assay kit provided, CheckLite™ 250 Plus, includes five bottles each of Luciferin-luciferase reagent, solvent water, and ATP releasing agent. Using one bottle of each type, create a solution according to the instructions (approximately 5.5 mL). Shield the assay solutions from light using aluminum foil and refrigerate until the time of use. Immediately before using, return to room temperature and remove the foil prior to use. Dispense 0.1 mL of the ATP releasing agent included in the ATP assay kit to each of the 48 luminometer tubes. ATP assay kit reagents should be dispensed using sterilized pipette tips under aseptic manipulation to avoid contamination with ATP and microorganisms.

6.2 Body Weight Measurement

Weigh the mice and record their body weights to the nearest 0.1 g on the test forms.

6.3 Auricular Lymph Node Excision and Weight Measurement

Perform procedures in **Sections 6.3**, **6.4** and **6.5** within 24 to 30 hours after the start of treatment application on Day 7. The necessary materials for procedures in **Sections 6.3**, **6.4** and **6.5** are given in **Annex I**.

Immediately after sacrificing the mice with ether anesthesia excise completely all auricular lymph nodes for each ear (there can be one or two auricular lymph nodes) as illustrated in **Figure A-1**. Place the excised lymph nodes for one animal in a disposable petri dish and immediately measure the wet weight to the nearest 0.1 mg with an analytical balance.

6.4 Preparation of Cell Suspension

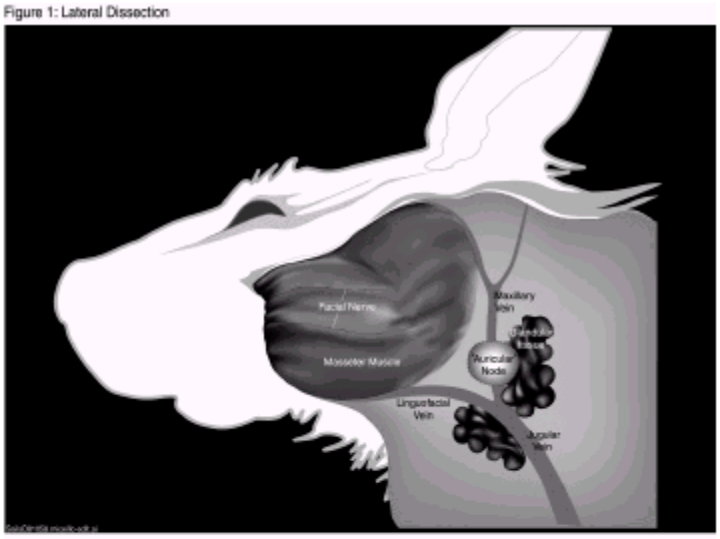
The lymph nodes from one animal should be sandwiched between two pieces of slide glass and light pressure should be applied to crush the nodes (**Figure A-2**). After confirming that the tissue has spread out thinly pull the two slides apart. Suspend the tissue on both pieces of slide glass in 1 mL of PBS. As illustrated in **Figure A-3**, each piece of slide glass should be held at an angle over the petri dish and rinsed with PBS while the tissue is scraped off of the

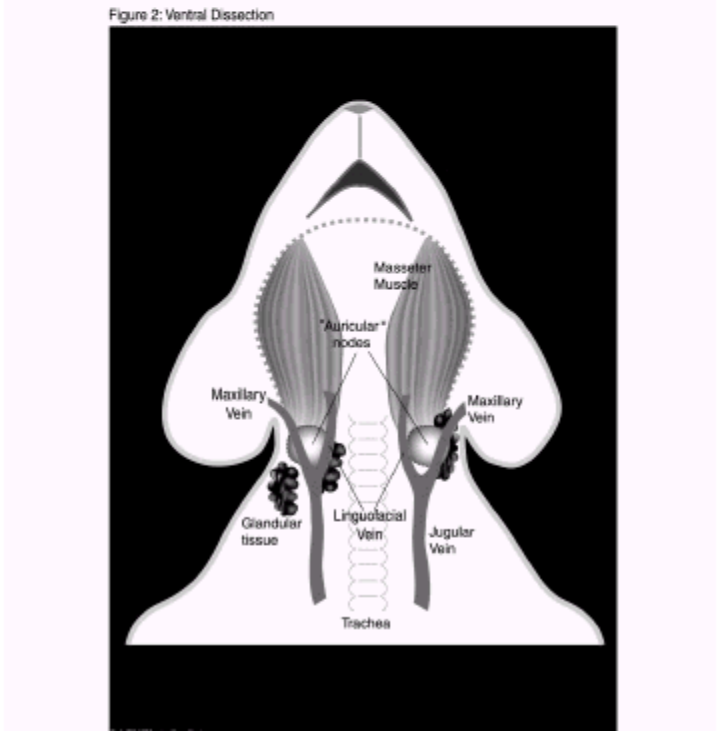
glass with repeated movements of a cell scraper. One mL of PBS should be used for rinsing both slides.

The tissue suspension in the petri dish should be homogenized lightly with the cell scraper, and 20 µL of the suspension should be taken up with a micropipette, taking care not to take up the membrane that is visible to the eye. The pipetted suspension should be added to 1.98 mL of PBS and homogenized well. This will be cell suspension No. 1. Again take up 20µL of the suspension in the petri dish, add to 1.98 mL of PBS, and homogenize well. This will be cell suspension No. 2.

These procedures should be performed while wearing gloves and a mask and micropipette tips should be sterile. Detailed step-by-step procedures are given in **Annex II**.

232 **Figure A-1 Auricular lymph nodes[3]**





233

234

[3] Taken from ICCVAM IWG LLNA Protocol (ICCVAM 2001)

Figure A-2 Preparation of cell suspension

Lymph nodes from each animal are sandwiched between two pieces of slide glass and light pressure is applied to crush the nodes.

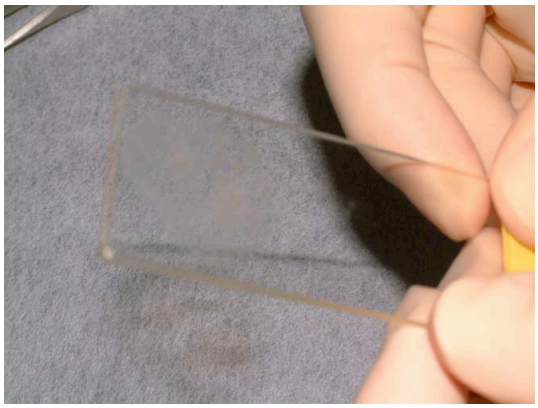
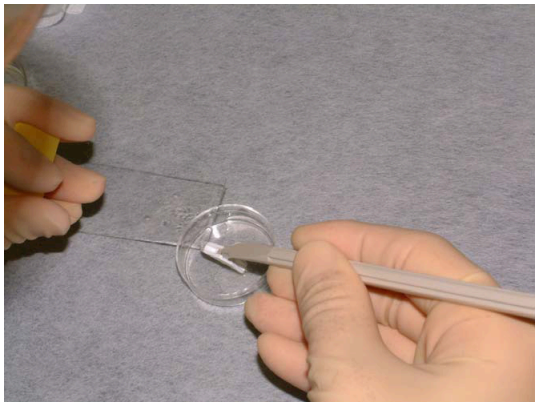


Figure A-3 Preparation of cell suspension

Rinse with PBS while scraping the tissue off of the glass with a cell scraper. Repeat the scraping motion, scooping up liquid from the petri dish as need. Use 1 mL of PBS for the nodes of each animal.



6.5 ATP Assay

Prepare 48 luminometer tubes in advance by dispensing 0.1 mL of the ATP releasing reagent provided to each tube. Add 0.1 mL of each homogenized cell suspension to the luminometer tubes and homogenize. After allowing the solution in the tube to stand for approximately 20 seconds, add 0.1 mL of the Luciferin-luciferase solution, promptly homogenize and place in the luminometer. The amount of bioluminescence (RLU; relative luminescence units) measured over 10 seconds will be displayed. Record this measurement on the test forms.

The amount of bioluminescence begins to decrease immediately after adding the Luciferin-luciferase solution. It is therefore important that the series of procedures from the addition of Luciferin-luciferase solution to switching on the luminometer are performed as quickly as possible, ideally with the same rhythm.

These procedures should be performed while wearing gloves and a mask and micropipette tips should be sterile. The detailed procedures are given in **Annex III**.

7.0 Points of Caution on Procedures from Excision to ATP Assay

The ATP content of the lymph node decreases over time after the sacrifice of the animal. It is therefore desirable that the time elapsed between sacrifice of the animal and ATP assay is uniform for each animal. The series of procedures from excision to ATP assay must be performed rapidly and without delay.

If these procedures are performed by one technician, the animals should be sacrificed one at a time. If there are multiple technicians, it is possible to divide tasks and sacrifice the animals one group at a time. If two technicians perform the procedures, steps in **Section 6.3** should be performed by one individual and steps in **Sections 6.4** and **6.5** should be performed by the other. If three technicians perform the procedures, steps in **Sections 6.3**, **6.4** and **6.5** can each be handled by one individual. If multiple technicians are involved, it is important that the timing of excision is carefully planned so that there are no delays in subsequent steps.

8.0 Data Entry

Input the body weights on Day 1 and Day 8, the lymph node weight, and the amount of ATP bioluminescence into the designated Excel file.

Annex I: Equipment and Reagents Used for the Experimental Procedures in Sections 6.3, 6.4, and 6.5

For the equipment and reagents underlined below, the items provided by the LLNA: DA Validation Committee should be used. In the event the test facility provides a luminometer, it can be used.

6.3 Auricular Lymph Node Excision and Weight Measurement

Dissecting instruments set (Tweezers, scissors, surgical holder, injection needle and holder)

Antiseptic solution

Cotton

Petri dish (24)

Analytical balance (readability of at least 0.1 mg)

6.4 Preparation of Cell Suspension

15 mL test tubes with 1.98 mL phosphate buffered saline (PBS) (48)

50 mL test tubes with at least 24 mL PBS (1)

Slide glass (48)

Tweezers (1)

Micropipette 1000 µL (1) (Volume to be measured: 1 mL)

Micropipette 100 µL (1) (Volume to be measured: 20 µL)

Cell scraper (1)

Sterilized pipette tips for 1000 µL micropipette (24) and for 100 µL micropipette (24)

Vortex mixer (1)

Paper towels

Clean sheet

Test tube rack

6.5 ATP Assay

Luminometer tubes with 0.1 mL ATP releasing agent (48)

15 mL test tube with dissolved luciferin-luciferase solution (1)

Micropipette - 100μL or 200 μL (2) (Volume to be measured: 0.1 mL)

Sterilized micropipette tips (96)

Timer (with second display) (1)

Luminometer (1)

Vortex mixer (can use same mixer listed under **Section 6.4** Preparation of Cell Suspension)

Test tube rack

Luminometer tube rack (microtube rack)

Annex II: Preparation of Cell Suspension for the Experimental Procedures in Section 6.4

1. Cover the laboratory bench with a clean sheet and place one piece of slide glass on the sheet.
2. After measuring the lymph node weights, use tweezers to move the lymph nodes from one animal from the petri dish to the center of the slide glass.
3. Place another piece of slide glass on top.
4. Pick up the two sandwiched pieces of slide glass. Squeeze the two pieces in the center to crush the lymph nodes. (Apply only light pressure. Too much pressure can break the cells.)
5. Confirm that the tissue has spread out thinly between the two slides and place the sandwiched slides on the clean sheet.
6. Fasten a tip on the 1000 μL micropipette and draw 1 mL phosphate buffered saline (PBS) from the 50 mL tube.
7. Remove the upper slide glass from the sandwiched slides and place it on the clean sheet with the side that was in contact with the lymph node tissue facing up. The other slide glass should be held at an angle in the petri dish, the side with lymph node tissue affixed facing forward, and washed with 1 mL PBS.
8. Dispose of the 1000 μL micropipette tip.
9. Scrape the tissue off of the glass with a cell scraper, scooping up PBS from the petri dish and repeating the scraping motion. Confirm that there is no tissue, or only trace amounts of tissue, left on the slide before disposing of the slide glass.
10. Pick up the slide glass laid aside at step 7; scrape the tissue off in the same manner and dispose of the slide glass. Note that it becomes difficult to scrape the tissue off of the slide glass once it has dried. Perform steps 4 through 10 without delay. The scraping should be performed while keeping the area of the slide glass to which the lymph node tissue is affixed sufficiently wet with PBS from the petri dish.

11. The tissue suspension in the petri dish should be homogenized lightly with the cell scraper. If large pieces of tissue are observed, stir with the cell scraper to break up the pieces and obtain a uniform solution.

12. Wipe the cell scraper with a paper towel. (The cell scraper will be used for the next animal.)

13. Fasten a tip to the 100 µL micropipette, tilt the petri dish at an angle and mix the suspension by pipetting in and out several times. Take up 20 µL of the suspension with the pipette, taking care not to take up any membrane that is visible to the eye.

14. Add the 20 µL of suspension to a 15 mL test tube containing 1.98 mL PBS. Pipette the solution and proceed to homogenize with the vortex mixer. (cell suspension No. 1)

15. Repeat steps 13 and 14 to prepare cell suspension No. 2.

16. Dispose of the 100 µL micropipette tip.

Annex III: ATP Assay for the Experimental Procedures in Section 6.5

1. Fasten a tip on the 100 μL (or 200 μL) micropipette and draw 0.1 mL of vortex-homogenized cell suspension No. 1.
2. To the luminometer tube filled with 0.1 mL ATP releasing reagent, add 0.1 mL of cell suspension No. 1, making sure to note the time with a timer. Dispose of the tip.
3. Homogenize with the vortex mixer and place in the luminometer tube rack.
4. Fasten a tip on a separate 100 μL (or 200 μL) micropipette and draw 0.1 mL of solution from the 15 mL tube containing dissolved Luciferin-luciferase reagent.
5. Take the luminometer tube from the rack and add 0.1 mL of Luciferin-luciferase solution to the luminometer tube 20 seconds after the time noted in step 2.
6. Promptly homogenize in the vortex mixer, place in the luminometer and turn on the switch. The amount of bioluminescence begins to decrease immediately after adding the Luciferin-luciferase solution. Step 6 should be performed as quickly as possible, ideally with the same rhythm.
7. Dispose of the tip.
8. After 10 seconds the amount of bioluminescence (RLU; relative luminescence units) will be displayed. Record this measurement on the test forms.
9. Repeat steps 1 through 8 for cell suspension No. 2, measure the bioluminescence and record.

Appendix A2

Results in the LLNA: DA Test Method for 1% Sodium Lauryl Sulfate (SLS) Pretreatment versus without 1% SLS Pretreatment

[This Page Intentionally Left Blank]

Appendix A2 Summary of Results in the LLNA: DA Test Method with 1% SLS Pretreatment versus without 1% SLS Pretreatment

Substance Name	Vehicle	Concentration (%)	SI[1] (+ SLS)	SI[1] (- SLS)	Calculated EC3[2] (%) (+ SLS)	Calculated EC3[2] (%) (- SLS)
2, 4-Dinitrochloro-benzene	AOO	0.03	2.10	1.88	0.05	0.06
		0.10	5.02	4.46		
		0.30	9.74	14.61		
Potassium dichromate	DMSO	0.1	2.61	2.54	0.15	0.22
		0.3	4.24	3.34		
		1.0	5.51	5.66		
Isoeugenol	AOO	1.0	2.05	1.32	2.46	4.24
		2.5	3.02	2.21		
		5.0	2.85	3.35		
Citral	AOO	5	1.93	1.88	7.4	10.4
		10	4.15	2.91		
		25	6.97	5.90		
Hexyl cinnamic aldehyde	AOO	5	1.51	0.99	7.5	8.8
		10	4.52	3.64		
		25	4.84	3.79		
Cinnamic alcohol	AOO	10	2.46	2.44	14.1	18.5
		25	4.40	3.43		
		50	6.36	4.01		
Hydroxycitronellal	AOO	10	1.98	1.49	15.8	19.8
		25	4.61	3.81		
		50	6.59	6.74		
Imidazolidinyl urea	DMF	10	2.36	2.54	20.3	33.0
		25	3.29	2.38		
		50	6.02	4.31		
Methyl methacrylate	AOO	25	0.73	1.11	NA	NA
		50	0.68	0.92		

Substance Name	Vehicle	Concentration (%)	SI[1] (+ SLS)	SI[1] (- SLS)	Calculated EC3[2] (%) (+ SLS)	Calculated EC3[2] (%) (- SLS)
		100	1.31	1.83		
Nickel (II) chloride	DMSO	2.5	1.53	0.98	NA	NA
		5.0	1.57	1.16		
		10.0	2.24	1.87		
Methyl salicylate	AOO	5	0.89	0.83	NA	NA
		10	1.59	1.32		
		25	1.69	2.34		
Salicylic acid	AOO	5	1.21	1.13	NA	NA
		10	2.05	1.29		
		25	2.48	2.44		
Sulfanilamide	DMF	10	1.08	0.92	NA	NA
		25	1.03	0.90		
		50	0.94	0.84		

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration required to produce a stimulation index of three; NA = not applicable; RLU = relative luminescence units; SI = stimulation index; SLS = sodium lauryl sulfate.

[1]SI determined from mean ATP content (RLU).

[2]EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

+ SLS = with pretreatment of 1% SLS prior to test substance application

- SLS = without pretreatment of 1% SLS prior to test substance application