Appendix A

LLNA: BrdU-ELISA Protocol

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1.0 Introduction/Principle

This document describes the recommended standard operating procedure for the non-radioisotopic modification of the LLNA, which is based on BrdU incorporation in place of tritiated thymidine to measure lymph node cell proliferation. This document is based on the protocol used in the JSAAE multi-laboratory validation study of the LLNA: BrdU-ELISA, *Recommended Standard Operating Procedure for the Non-Radioisotopic Local Lymph Node Assay using BrdU-ELISA (Non-RI LLNA), version 1.20, July 31, 2008*, by Masahiro Takeyoshi, Ph.D., Chemicals Evaluation and Research Institute, Japan. This

2.0 Description of the Method

The method is practically identical to the standard LLNA methodology excluding the use of BrdU and colorimetric detection. A single intraperitoneal injection (5 mg/mouse per injection) of BrdU is made on day 4. This administration schedule was decided as the most effective labeling protocol to yield maximum SI values based on preliminary study data with several different protocols. Approximately 24 h after the BrdU injection, the auricular lymph nodes are removed, weighed, and stored at -20˚C until analysis using an enzyme-linked immunosorbent assay to measure the level of BrdU incorporation (BrdU-ELISA).

The cell proliferation response is measured by a commercial BrdU detection kit (i.e., Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany; Cat. No. 11 647 229 001). To perform the BrdU-ELISA, the lymph nodes are crushed, passed through a #70 nylon mesh. The lymph node cells (LNC) from individual animals are suspended in 15 ml of physiological saline. The cell suspension is added to the wells of a flatbottom microplate in triplicate. After fixation and denaturation of the LNC, anti-BrdU antibody is added to each well, and after rinsing, substrate solution containing tetramethylbenzidine (TMB) is added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is defined as the BrdU labeling index.

2.1 Animals

2.1.1 Animal source

Young adult female mice (nulliparous and non-pregnant) of the CBA/JN or other recommended mouse strains, such as CBA/Ca or CBA/J strain, should be used at age 8-12 weeks. All animals should be age matched (preferably within a one-week time frame).

2.1.2 Quarantine and Acclimation

Healthy animals in good general condition on arrival should be quarantined for more than five days. During the quarantine and acclimation period, clinical signs, body weights and excrement of the animals should be observed.

2.1.3 Grouping

Animals confirmed to be in good health with favorable body weight gains during the quarantine and acclimation period should be allocated to groups by a stratified randomization or other appropriate methods before the start of the study.

2.1.4 Identification

Animals should be identified by colored marks on the tails, ear tags, or other appropriate methods.

2.1.5 Animal Husbandry

The animals should be housed in an animal room maintained at a temperature of 22±3°C and a relative humidity of 30-70%. The rooms should be artificially lighted for 12 h daily, and the animals should be given free access to conventional laboratory diet and drinking water.

2.2 Chemicals and Vehicle

2.2.1 Vehicle

The solvent/vehicle should be selected on the basis of maximizing the test concentrations while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone/olive oil (4:1 v/v), DMF, MEK, propylene glycol, and DMSO, but others may be used.

2.2.2 Test Chemicals

Solid test substances should be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be prepared daily unless stability data demonstrate the acceptability of storage.

2.2.3 Controls

Concurrent negative (vehicle) and positive controls should be included in each test. Positive control (50% HCA, CAS RN. 101-86-0) should be used to ensure the appropriate performance of the assay. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) >2 over the negative (vehicle) control group.

2.2.4 Dose selection

Doses are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. The maximum concentration tested should be the highest achievable level while avoiding overt systemic toxicity and excessive local irritation. All test solutions should be prepared in a day of application unless the stability is confirmed in advance.

2.2.5 Preparation of BrdU

BrdU should be accurately weighed and dissolved in physiological saline for injection) to make 10 mg/ml solution. The BrdU solution should be sterilized by a commercial filtration system (i.e. MILLEX®-HV, MILLIPORE etc.). The BrdU solution can be prepared before administration and stored in a freezer below - 20°C until use.

2.3 Animal Experiment

2.3.1 Grouping

A minimum of four successfully treated animals is used per dose group, with a minimum of three consecutive concentrations of the test substance plus a negative (vehicle) control and a positive control group.

Table 1 Structure of LLNA: BrdU-ELISA Test Groups

Group	Number of Animals
Negative (vehicle) control	4
Positive control (50% HCA)	4
Test substance-low dose	4
Test substance-middle dose	4
Test substance-high dose	4

2.3.2 Sensitization Procedure

Apply 25µl of test solution to the dorsum of both ears of the mice using micro volume pipette daily for three consecutive days.

2.3.3 BrdU Administration

A single intraperitoneal injection of 0.5 ml of BrdU solution (5 mg/mouse/injection) should be given to the mice 48 hours (h) after the final sensitization.

2.3.4 General Condition

Clinical signs should be observed at least once a day.

2.3.5 Body Weights

Body weights should be measured on the day of the first test substance application and on the lymph nodes are collected.

2.3.6 Collection of Lymph Nodes And Measurement of Lymph Node Weight

Approximately 24 h after BrdU injection, the auricular lymph nodes should be removed. The lymph nodes should be carefully dissected and trimmed of fascia and fat, weighed, and stored individually in a 1.5 ml centrifuge tube at -20°C until the ELISA is performed.

2.4 BrdU-ELISA

The incorporation of BrdU into lymph node cells should be determined using a commercial cell proliferation assay kit (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany; Cat. No. 11 647 229 001) after they are crushed and suspended in

physiological saline. The absorbance is defined as the BrdU labeling index. Follow the instructions in the assay kit.

2.5 Preparation of Reagents in the BrdU-ELISA Kit

The assay method should be according to the instruction manual in the assay kit excluding preparation of the BrdU labeling solution.

2.5.1 Peroxidase Conjugated Anti-BrdU Antibody (Anti-BrdU-POD) Stock Solution

Dissolve Anti-BrdU-POD (bottle 3) in 1.1 ml double distilled water for 10 minutes and mix thoroughly. This solution can be stored at 2-8°C for several months. For long-term storage it is recommended to store the solution in aliquots at -15 to -25°C.

2.5.2 Anti-BrdU-POD Working Solution

Dilute Anti-BrdU-POD stock solution 1:100 with antibody dilution solution (bottle 4). For one 96-well microtiter plate dilute 100 ml Anti-BrdU-POD stock solution in 10 ml antibody dilution solution (bottle 4). Prepare shortly before use.

2.5.3 Washing Solution

Dilute washing buffer concentrate (bottle 5) 1:10 with double distilled water. For one 96-well microtiter plate, dilute 10 ml washing buffer concentrate (bottle 5) with 90 ml double distilled water. This solution can be stored at 2-8°C for several weeks.

2.6 Preparation of Cell Suspension of Lymph Nodes

The procedure for preparing the LNC suspension is a critical step of this assay; it is most important to crush the lymph node and suspend the LNC completely. Every technician should establish the skill in advance. The lymph nodes in negative control animals are very small, so careful operation is required to avoid an artificial effect on SI values.

2.6.1 Optimizing Assay Condition

Mean absorbance of negative (vehicle) control group should be within 0.1-0.2. Because the absorbance depends on the combination of assay apparatus and the target volume of the LNC suspension, every laboratory should decide their own optimal target volume of LNC suspension in advance so that the absorbance of the negative control is within 0.1-0.2. The volume is expected to be approximately 15 ml. The volume of the LNC suspension for all

test animals should be adjusted to the optimized volume.

2.6.2 Preparation of LNC Suspension

A small amount (approximately 0.3 ml) of physiological saline should be added to the centrifuge tube that contains the collected lymph node. The lymph node should be crushed with a disposable plastic pestle to make the LNC suspension. The LNC suspension should be passed through a #70 nylon mesh and adjusted to the optimal target volume in a 50 ml Falcon tube.

[Note: Although a crushing apparatus other than a plastic pestle can be used to prepare the LNC, the target volume of the LNC suspension should be adjusted to the optimized volume.]

2.7 Assay Flow (BrdU-ELISA)

1. The cell suspension (100 µl) is added to the wells of a flat-bottom microplate (three wells per sample) after mixing thoroughly with a Vortex. Simultaneously, three blank wells should be prepared by adding 100 µl of physiological saline.
2. After filling all sample wells and blank wells, the plate should be centrifuged at 300 x *g* for 10 minutes.
3. Remove 3/4 of the supernatant volume. Great care should be taken so that the LNC are not aspirated.
4. The assay plate should be dried completely in a hot-air oven.
5. Add 200 µl of Fix-Denat solution and allow plate to stand for 30 minutes at room temperature.
6. Remove the Fix-Denat solution completely.
7. Add 100 µl of anti-BrdU-POD antibody working solution and allow it to react for 1 h.
8. Remove the anti-BrdU-POD antibody solution completely.
9. Add 200 µl of wash solution into each well, and wash the well by pippetting

10 times. Discard the wash solution completely.

10. The wash step (Step 9) should be repeated twice (three times in total).

11. Add 100 □l of TMB substrate solution and let it stand for 15 minutes at room temperature in a dark place.

12. Measure an absorbance (ABS) at 370 nm with a reference wavelength of 492 nm. When using stop solution (1M sulfuric acid, 25 µl/well), measure ABS at 450 nm with a reference wavelength of 690 nm.

3.0 Calculation of Results

BrdU labeling index and Stimulation Index (SI) are defined as follows:

3.1 Without Stop Solution

BrdU labeling index = $(ABS_{370}-ABS_{blank370}) - (ABS_{490}-ABS_{blank490})$

3.2 With Stop Solution

BrdU labeling index = $(ABS_{450}-ABS_{blank450}) - (ABS_{650}-ABS_{blank650})$

3.3 Stimulation Index

$$\text{Stimulation Index (SI)} = \frac{\text{BrdU labeling index for each test animal}}{\text{Mean BrdU labeling index for concurrent vehicle control group}}$$

4.0 Evaluation of Results

4.1 Success Criteria for Each Experiment

Employing the optimized assay condition described previously, the mean SI for the positive control group (50% HCA) should be equal to or greater than 2. If not, all data derived from the experiment should not be used for evaluation.

4.2 Evaluation of the Results

The mean BrdU labeling index for each animal should be calculated based on the results of BrdU ELISA. The SI for each animal should be calculated by dividing of the mean BrdU labeling index for each treated animal by the mean BrdU labeling index of the concurrent vehicle control group. A positive response is defined as mean SI of the test group ≥2.

5.0 References

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