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DOE SPECIFICATION

BERYLLIUM LYMPHOCYTE PROLIFERATION TESTING (BeLPT)

U.S. Department of Energy
Washington, D.C. 20585

AREA SDMP

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1. Use of this purchase specification is not mandatory. Users should review the document and determine if it meets the user's purpose.

2. Comments (recommendations, additions, and deletions) and any pertinent data that may be of use in improving this document should be addressed to: DOE Beryllium Lymphocyte Proliferation Testing Writing Group, c/o Daniela Stricklin, AU-13 /GTN, U.S. Department of Energy, 19901 Germantown Road, Germantown, MD 20874-1290.

3. This document was developed from a draft protocol "Testing for Sensitivity to Beryllium and Investigating Chronic Beryllium Disease" that was distributed in June 2000 by Dr. Frederick Miller, Chairman of the Committee to Accredit Beryllium Sensitivity Testing (CABST) Committee.

4. The 2019 updates to the protocol were developed with input from laboratories currently performing the BeLPT in the US. The laboratories are affiliated with Oak Ridge Associated Universities (ORAU), National Jewish Health Center, and Cleveland Clinic.

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1. SCOPE

This specification is for beryllium lymphocyte proliferation tests (BeLPT) used for detecting whether an individual has developed a **sensitization to beryllium** and for clinical evaluation and diagnosis of patients for chronic beryllium disease (CBD). However, a lung biopsy is needed to fully establish the presence of CBD. This specification should be used in all contracts with laboratories for the purchase of BeLPT Services.

2. BACKGROUND

Beryllium (Be) is a lightweight metal that can cause a chronic hypersensitivity-like immune response that leads to a chronic lung disease called berylliosis or chronic beryllium disease (CBD). The disability associated with CBD is primarily due to lung damage caused by an immune response to beryllium retained in the lung. Development of this disease process is a function of beryllium exposure, an individual's ability to mount a beryllium-specific, cell-mediated immune response to beryllium (called sensitivity), their ability to develop granulomatous responses and possibly other factors.

Testing for an individual's sensitivity to beryllium using an *in vitro* assay is currently used as a screening assay for beryllium exposed workers, as part of the diagnostic criteria for CBD, and for surveillance in identifying potentially unhealthy working conditions. The following protocols have been developed to optimize and standardize beryllium sensitivity testing through the lymphocyte proliferation assay.

Due to the complexity of the test, the procedures described here require training and experience to render reproducible and reliable results. Individuals requesting studies dealing with beryllium sensitization or CBD status should use facilities with an established and continuing record of satisfactory performance.

3. PRINCIPLE

When a T cell antigen receptor recognizes a specific antigenic substance bound to a human leukocyte antigen (HLA) Class I or II molecule, the T cell responds in a variety of ways (for example secreting inflammatory cytokines and/or undergoing cell division). Each T lymphocyte expresses only a single T cell antigen receptor. When sufficient numbers of specific T cells are present (usually greater than l/10,000), the response of specific T cells can be detected clinically by either *in vitro* or *in vivo* testing. The *in vivo* response is usually measured as a delayed hypersensitivity skin test. The *in vitro* T cell response is normally measured by recording the proliferation response of the cells to beryllium *in vitro*. When an individual has a clinically measurable response to a specific antigen, the individual is said to be sensitive or hypersensitive to that antigen. In the case of Be hypersensitivity, the metal binds to some host proteins and change the conformation such that in a conducive HLA background, an immune response will be triggered.

Beryllium can be a component of antigens and is associated with a granulomatous hypersensitivity disorder in a small number (up to 15%) of individuals in an exposed population. Large numbers of CD4+ T-lymphocytes accumulate in the lung in chronic beryllium disease (Rossman et al, 1988, and Saltini et al, 1989). The reactivity of these lymphocytes to beryllium provides a specific and sensitive laboratory test for differentiating chronic beryllium disease from sarcoidosis (Rossman et al, 1988). In chronic beryllium disease, the beryllium reactivity of lymphocytes obtained by bronchoalveolar lavage is generally greater than the reactivity of lymphocytes obtained from the peripheral blood. By measuring the reactivity of lymphocytes obtained from the peripheral blood, beryllium sensitivity can also be detected in workers without disease (Kreiss et al, 1993). Some of the workers without disease that have been identified by positive blood proliferation responses to beryllium have gone on to develop disease. The rate of CBD varies according to industry, with higher exposure settings resulting in higher CBD rates.

4. APPLICATION

Beryllium sensitivity testing is used as a screening tool for beryllium sensitization and possible chronic beryllium disease, as a surveillance tool for identifying potentially hazardous working conditions, and as part of the diagnostic criteria for CBD. The blood lymphocyte proliferation test for beryllium sensitization (BeLPT) is a screening test with a sensitivity and specificity that is not clearly defined at this time. This is attributable to the fact that the populations of normal people have not had lung biopsy and bronchoalveolar lavage to include or exclude chronic beryllium disease. The positive predictive value (PPV) of two positive or abnormal BeLPTs for beryllium sensitization is estimated to be 97-98%. However, the PPV for predicting CBD is less-well established and has ranged from 35 to 65 percent in different cohorts (NRC 2008). The bronchoalveolar lavage lymphocyte proliferation test (BAL-LPT) is the preferred test for beryllium sensitization as part of the diagnostic criteria for identifying pulmonary CBD. The blood BeLPT may be more reliable in detection of extra-pulmonary CBD.

5. TRITIATED THYMIDINE UPTAKE PROCEDURE FOR DETERMINING BERYLLIUM SENSITIZATION

5.1 EQUIPMENT

5.1.1. Beta radiation Liquid Scintillation Counter (LSC) such as TopCount, MicroBeta or similar instrument that count beta emissions from harvested filters

5.1.2. Liquid Scintillation Counter for swipe surveillance of lab with appropriate vials and swipes.

5.1.3. Class II Biological safety cabinet

5.1.4. Cell Counter that counts the number of cells present in a solution

5.1.5. Centrifuges with 50ml conical centrifuge tube capacity

5.1.6. Harvester (96 well) that will aspirate cells in culture plates onto a filter for beta counting

5.1.7. Incubators for cell culture at 37ºC and 5-10% CO_2 with approximately 70% humidity

5.1.8 Multi channel and Single channel pipettors and sterile tips.

5.1.7. Liquid Handling System (optional) to fill tissue culture plates with reagents.

5.2 REAGENTS

5.2.1 Beryllium sulfate, 0.2 M $BeSO_4$ x 4 H_2O.

5.2.2. RPMI-1640 medium with 25 mM HEPES buffer and 200 mM L-glutamine

5.2.3. Human serum, Type AB, male, heat inactivated to eliminate complement activity: Each lot MUST BE TESTED for its ability to (a) support lymphocyte proliferation and exhibit good growth in control wells; (2) demonstrate low toxicity or cell killing; (3) not be overly mitogenic demonstrated by high control counts; (4) exhibit low spontaneous incorporation of thymidine; and (5) exhibit good response to known abnormal. It is aliquoted and stored at -20°C until use. It should not be refrozen after aliquot is thawed. Reference range for each lot of serum is necessary.

Note: Some laboratories may elect to use alternatives to the serum referenced above. However, an alternative serum should be tested for the same attributes listed here and its performance documented prior to use.

5.2.4. Penicillin-streptomycin, 100 U and 50µgm/ml respectively or 50 µg/ml gentamicin sulfate

5.2.5. L-glutamine-200 mM (100X), liquid such as Gibco Cat. No.25030-081. Store at -5 to -20°C. Thaw. Store thawed reagent at refrigerator temperature. Good for 2-4 weeks. Protect from light. A pH near 7.4 is advisable since higher pH has been observed to result in poor cell growth. Check Certificate of Analysis. See SDS for hazards.

5.2.6. Phosphate-buffered balanced salt solution (PBS) such as Hank's, Dulbecco's, etc..

5.2.7. Tritiated Thymidine (thymidine, [methyl-^{3}H]) (specific activity 2-10 Ci/mM).

5.2.8. Positive Growth Controls known to stimulate T cell lymphocytes. Choose two of the following: plant mitogens such as phytohemagglutinin (PHA; preferred), and concanavalin-A (ConA) and/or recall antigens suitable for population such as Candida albicans allergenic extract or tetanus toxoid. These growth controls

should be prepared in a concentration known to stimulate lymphocyte proliferation optimally. Titrating of these controls to determine optimal concentration is advised.

5.2.9. Density gradient solution for blood mononuclear cell separation (i.e., Ficoll-Hypaque, Histopaque, etc.). Once opened, store solutions at 4°C and warm to room temperature before use.

5.2.10. Liquid Scintillation Counter Cocktail compatible with Liquid Scintillation. Counters in use in 5.1.1.

5.2.11. Cell counter controls for quality check of instrument. Low Abnormal, Normal, and High Abnormal controls should be included.

5.3 PREPARATION OF REAGENTS

5.3.1. Beryllium Sulfate (0.2 M $BeSO_4$ x 4 H_2O). Store at room temperature. Same for other salts of beryllium if used. Aliquot a working amount of 0.2 M $BeSO_4$ and sterilize by filtration with a 0.22 µm Millipore filter. Prepare $BeSO_4$ dilutions fresh for each assay using the 0.2 M stock solution using aseptic techniques.

5.3.3.1. Make a 1:10 dilution of $BeSO_4$ solution with phosphate buffered saline solution, Ca++ and Mg++ free.

5.3.3.2. Continue making serial dilutions with RPMI-1640 medium with 25 mM HEPES buffer and 200 mM L-glutamine to create three dilutions of 2 µM $BeSO_4$, 20 µM $BeSO_4$, and 200 µM $BeSO_4$. When added to wells, this will give 1, 10, and 100 µM $BeSO_4$ concentrations of beryllium.

5.3.2. Complete Growth Medium (CGM) should be prepared using sterile technique to a final concentration of 10% human AB serum, 1% L-glutamine, and 1% penicillin/streptomycin using RPMI-1640 medium with 25mM HEPES buffer and 200 mM L-glutamine. Store at 4°C and label with a "Use By" date. Some laboratories may prefer to make 2X or double concentration of complete growth media (2X CGM) with 20% AB serum, 2% L-glutamine and 2% Pen/Strep in RPMI-1640 medium with 25mM HEPES buffer and 200 mM L-glutamine which is then added to equal volume of substrate in the tissue culture plates.

5.3.3. Tritiated Thymidine, (thymidine, [methyl-^{3}H]). Note: tritiated thymidine is a radioactive material, therefore, appropriate safety precautions must be applied to prevent spills, to properly label, and to dispose of contaminated pipettes, vials, microtiter plates and other items in contact with this substance as well as all liquid wastes. Laboratory-specific requirements for technologist training and waste disposal should be developed in consultation with the appropriate radiation safety officer. Prepare a new lot number of tritiated thymidine with in RPMI-1640 medium with 25 mM HEPES buffer and 200 mM L-glutamine to make a concentration that will deliver 1 µCi ^{3}H to each well.

5.3.4 Mitogens should be prepared in a concentration known to stimulate lymphocyte proliferation optimally (i.e., ~30 µg/ml in culture and 10 µg/ml in culture for PHA and ConA, respectively). It is recommended that a titration of the mitogens or antigens be done to determine concentration for optimal growth.

5.4. QUALITY CONTROL

5.4.1. New lots of AB serum are tested for ability to support a response to beryllium. Specifically, the serum should produce stimulation indexes of approximately 1 (but must be < 3) in beryllium-challenged wells for non-sensitized subjects. Also, it should demonstrate low toxicity or cell-killing [i.e., not more than one standardized Ln (SI) (See B.4) should be less than –3.0] in the presence of beryllium. The serum should NOT stimulate excessive lymphocyte proliferation in control wells. Serum lots must exhibit lymphocyte proliferation greater in beryllium-challenged wells than non-beryllium wells for sensitized persons [i.e. greater than 2.5 standardized Ln (SI).

5.4.2. The Liquid scintillation counter should be calibrated at regular intervals according to the instrument manufacturer's specifications to ensure optimal performance. Documentation of the calibration should be kept in a readily accessible format.

5.4.3. The harvester is checked once or twice daily for radiation contamination, adequate aspiration and washing by harvesting a blank plate through the harvesting process. It is counted on the LSC and all counts must be below established background range for harvester to be placed into use.

5.4.4. The cell counter should be calibrated at regular intervals and regular maintenance done according to manufacturer's specifications. Cell count controls should be run each day of instrument's use. Instrument is taken out of use if controls are not within range. Documentation is kept of performance, maintenance and repairs.

5.4.5. All pipettes, incubators, centrifuges and timers are checked for accuracy according to manufacturer specifications. Documentation is kept of performance, maintenance and repairs.

5.4.6. Each culture plate contains blank wells. Mean counts per minute (CPM) for the blank wells should not exceed the normal range of background counts for the counting instrument used. Documentation of the normal range of background counts should be maintained.

5.4.7. Each new technologist should set up approximately 10 to 20 tests in duplicate with other experienced technologists. This can be done using blood submitted, since about 25% of the samples will have enough cells. This should include an abnormal BeLPT if blood is available. Laboratory must follow an established protocol to evaluate new technologists based on statistics described in Appendix B. Annual performance evaluations of staff can be performed similarly.

5.4.8. The laboratory should have knowledge of and adherence to applicable standards

for dealing with chemical, biological, radiological hazards, and waste handling procedures. Documentation should be maintained on required training for laboratory personnel in the areas of radiation hazards, blood-borne pathogens, and chemical hazards.

5.4.9. Lack of commercial controls for beryllium sensitivity makes it difficult to have a proficiency program. Split sample proficiency using cells drawn from same person at same time and distributed to different laboratories is recommended as alternative. Records of outcomes are analyzed and kept.

5.4.10. The laboratory should maintain a log of samples received containing, at a minimum: name and/or identification number, date received, time in transit if greater than 24 hours, and unusual circumstances (e.g., more than 24 hours old, hemolyzed, tubes extremely hot or cold, etc.).

5.4.11. It shall be the responsibility of the Laboratory Director or designee to assure that the laboratory meets an external standard of quality assurance and quality control and that the laboratory is in compliance with appropriate State and Federal regulations. Clinical Laboratory Improvement Act (CLIA) certification or equivalent such as the College of American Pathologists (CAP) accreditation is required.

6. BeLPT: BLOOD SPECIMEN

6.1. SAMPLE COLLECTION

Three 10 ml containers of blood collected in sodium heparin are sent at room temperature to arrive at the testing site preferably within 24 to 30 hours (but no more than 48 hours) of being drawn. Receipt of samples as soon as possible after blood draw is preferred since the test is a functional assay. Appropriate insulating material should be used to maintain satisfactory temperature control and to avoid extreme temperature fluctuation of the cells and maximize cell viability during shipping.

The laboratory should have an established policy for how a sample is labelled and if the labeling is acceptable. At a minimum, the tubes should be labeled with two identifiers which match identifiers on paper work (name, SSN, badge or employee number), date and initial of person obtaining sample. An encrypted ID is acceptable if applicable to a single patient and known only to the requesting clinic.

Specimens should not be frozen, refrigerated or exposed to high temperatures during storage or shipping. All blood shipments should comply with IATA 650 regulations for shipping diagnostic substances, Category B, Exempt Human Specimens.

6.2. SAMPLE PREPARATION

6.2.1. Upon receiving a sample, carefully layer blood on density gradient and centrifuge

according to density gradient manufacturer's recommendations to maximize the yield of mononuclear cells. Aspirate the cell interface with a sterile pipette into a sterile 50 ml conical tube, add sterile phosphate-buffered balanced salt solution and centrifuge. Decant supernatant. Re-suspend the cell button and repeat wash step for a total of 3 washes.

6.2.2. Perform a cell count to according to cell counter's instruction. Determine the amount of Complete Growth Medium (CGM) to add so that the final cell concentration is 2.5 x 10^6 cells/ml.

6.3 PLATE PREPARATION

6.3.1. A 96 well tissue culture plate with a capacity of 300 µl is used. Each tissue culture plate will receive 4 repetitions of 100 µl each of cell suspension in CGM and reagents either RPMI 1640 for unstimulated controls, $BeSO_4$ for beryllium sensitivity, or mitogen/antigen for lymphocyte positive growth controls. A total of 200 µl will be in each well. Use pipette and sterile tips to inoculate wells.

Two separate culture plates are needed with differing incubation times ranging from 3 to 7 days for controls and beryllium sulfate. Mitogen/antigen culture plates are incubated for the optimal growth of each. Incubation is carried out at 37°C in an atmosphere of 5-10% carbon dioxide-air with sufficient humidity to prevent drying out of wells.

The plate arrangement calls for the cell suspension in CGM to be added to unstimulated control wells of RPMI 1640, the three $BeSO_4$ concentrations wells and mitogen and/or antigen wells as follows:

6.3.1.1. Eight to 12 unstimulated control wells consisting of cell suspension in CGM added to equal amount of RPMI1640 to be incubated for two separate times from 4 days to 7 days;

6.3.1.2. Four treated wells for each of the 3 concentrations of $BeSO_4$, to be incubated for two separate times from 4 days to 7 days;

6.3.1.3. Four each of mitogen/antigen wells harvested on the optimal day for each stimulant.

6.4 PULSING WITH TRITIATED THYMIDINE

At 6 to 18 hours prior to end of incubation period, add 1 µCi tritiated thymidine to each well and re-incubate for approximately 6 additional hours. At end of incubation period, plates may be harvested or frozen at -20°C until ready for harvest.

6.5 HARVESTING CULTURES AND COUNTING ON LSC

Cultures are harvested using a 96 well harvester and appropriate filter plates for the harvester and beta counter used. Culture plates are aspirated by vacuum onto a filter in the filter plate. All unbound tritiated thymidine is aspirated into a waste container during the wash cycles leaving only tritiated thymidine bound to cells on the filter plates. Filter plates are processed according to the manufacturer's protocol using a suitable cocktail for the Liquid Scintillation Beta Counter. Counts are expressed in counts per minute (CPM). Counting time should be at least one minute per well for a beta emission counter.

7. BeLPT: BRONCHOALVEOLAR LAVAGE (BAL) SPECIMEN

The procedure for performing assay for tritiated thymidine uptake for determining beryllium sensitivity in bronchoalveolar lavage (BAL) specimens, or BAL-BeLPT is as follows:

7.1. SAMPLE COLLECTION

7.1.1. For the BAL-BeLPT, the specimen is a saline lavage obtained during bronchoscopy. It is recommended that samples be taken from the wedged position in lingula or right middle lobe. The lavage is performed before the biopsy to avoid contamination with blood. The lavage volume needs to be sufficient to ensure enough cells are recovered to perform the LPT. The specimen must be transported to the laboratory immediately. Temperature should be kept between 15 to 25°C using insulation if necessary.

7.1.2. Specimens that cannot be set up for proliferation testing that day must be centrifuged and cell button suspended in RPMI-1640 with antibiotics and processed within 36 hours (no more than one night should pass before the cells are placed into culture for proliferation assays). The test typically requires a minimum of 1 x 10^6 cells, but 7-10 x 10^6 cells is recommended. Samples should be labelled according to laboratory-established protocol set forth for blood samples (see 6.1).

Laboratories may require advance notice for accepting a BAL BeLPT.

7.2 SAMPLE PREPARATION – IN HOUSE

Note: It may be difficult to obtain sufficient cells to achieve the required final cell concentration. Accordingly, the assay should be performed on the original lavage cell preparation and a density gradient separation is done only if there are sufficient cells--rare. The cell recovery in the gradient is considerably less than 100%. Laboratory experience in cell recovery should be the guide. In patients with end-stage lung disease there are often increased numbers of neutrophils which can be removed by density gradient centrifugation (if PMN>20%).

7.2.1. Note volume of lavage on summary sheet. Give a brief description of the fluid (cloudy, bloody, etc.).

7.2.2. Mix BAL and remove 2 - 3 ml of lavage for differential if needed.

7.2.3. Transfer lavage to 50 ml centrifuge tube(s), and centrifuge for 20 minutes at 400 x g. Wash the cells 2-3 times with sterile phosphate-buffered balanced salt (PBS) solution to be certain all surfactant proteins are removed.

7.2.4. The cells are counted similar to procedure in 6.2.2 above except final concentration should be 1.0 x10^6 cells/ml. Note: Since there is no mononuclear separation step with BAL, the cellular components of the final suspension consist of multiple cell types in addition to lymphocytes. Note: higher percentage of lymphocytes (usually > 15%) in BAL is associated with higher chance of a positive test result.

7.3 SAMPLE PREPARATION – OUTSIDE SOURCE

Contact testing laboratory prior to collecting specimens. Specimens should not be collected or delivered on weekends or holidays.

7.3.1. Mix fluid and remove 3-5 ml of lavage for cell differential or provide cytospin prep or results of CBC differential if done at site.

7.3.2. Transfer remaining sample into 50 ml conical centrifuge tubes.

7.3.3. Centrifuge the specimen 1400 rpm x 10 minutes.

7.3.4. Carefully remove the supernatant. Gently resuspend the cell pellet.

7.3.5. Add 19 ml of RPMI + 1 ml of Penicillin-Streptomycin.

7.3.6. Seal specimen for shipping. Recommend placing parafilm over cap.

7.3.7. Ship according to IATA regulations.

7.3.8. Notify Laboratory of shipment and tracking number.

7.3.9. NOTE: Samples must processed within 36 hours.

7.4 PLATE PREPARATION

7.4.1. Concentrations of beryllium sulfate are made fresh just prior to plate preparation. See section 6.3. Laboratories may differ in concentrations to use. At least 3 concentrations on each of 2-3 different incubation periods between 4 and 7days[1] should be performed.

7.4.2. Each LPT must have:

8-12 wells of RPMI for un-stimulated controls to be harvested with the

[1] Some labs use incubation periods between 2-3 days and 4-5 days.

accompanying beryllium treated wells.

4 beryllium treated wells for each concentrations of $BeSO_4$ to be harvested.

4 Mitogen/antigen #1wells (harvest on the optimal day).

4 Mitogen/antigen #2 wells (harvest on the optimal day).

7.4.3. If cell yield is low, the minimum acceptable assay is:

8 unstimulated controls

4 beryllium treated wells for each concentrations of BeSO4 to be harvested

1 well of each mitogen/antigen #1 and #2 for total of 2 wells

7.4.4. Each well will receive equal amounts of cell suspension in CGM and reagents (RPMI 1640, $BeSO_4$, and mitogen/ antigen). 100µl each for a total of 200 µl in each well is a suggested amount. This provides 1 x 10^5 cells/well

7.4.5. Incubation, addition of tritiated thymidine, harvesting and counting are same as in section 6 above.

7.4.6. BAL Differential.[2] BAL preparations from normal individuals usually consist of 80- 90% alveolar macrophages. However, in beryllium disease higher percentages of lymphocytes are typically observed. A cytospin preparation of the BAL can be made and cells differentiated. At least 200 cells should be counted. Only peripheral blood cell types and histiocytes or alveolar macrophages are included. Other cellular components such as epithelial and bronchial lining cells are not. RBCs, bacteria, or yeast and fungi should be noted.

Lymphocytes _____%; Alveolar macrophages _____%

Eosinophils _____%; Granulocytes incl. neutrophils _____%

Basophils _____%;

Note presence of intracellular or extracellular bacteria, red blood cells, fungal elements and yeast. Also note presence of brownish intracellular material (indicates smoker) and nuclear debris (indicates problems with lavage procedure).

8. CALCULATIONS

The measurement of lymphocyte proliferation is based on beta particle counts from the tritiated thymidine. The data are analyzed by comparing counts from $BeSO_4$ exposed wells and mitogen/antigen exposed wells to the counts from unexposed control wells. The laboratory must also calculate the metrics it uses to determine if a test is acceptable, and whether the result is

[2] Some BeLPT testing laboratories have this analysis done separately by a clinical hematology laboratory.

normal, abnormal, or borderline. Electronic copies of count data and all calculations must be provided to the client upon request.

The key elements of the analytic approach include:

- Calculation of a stimulation index (SI) to compare the response in treated wells to the response in unexposed control wells for each of the treated sets ($BeSO_4$ concentrations and day combinations)
- Calculation of a serum specific cut-point for SI to identify biologically abnormal responses. The cut-point is determined from a statistical distribution of highest SIs from an unexposed reference population using an appropriate method to ensure that a minimum number of unexposed would exceed the limit (ex: 1 in 1000 or the 99.9%tile)
- Appropriate statistical method for determining if the SIs are statistically abnormal
- Calculation of positive controls to ensure the cells are healthy
- Calculation of a metric, such as coefficient of variation (CV), for replicate sets to ensure excess variance doesn't exist, i.e., the data are sufficiently close together to analyze

Comparison of the SIs to the calculated cut-point examines whether a patient's responses differ from those of individuals not exposed to beryllium, or a biological abnormal. In order to test whether a patient's exposed well counts and control well counts differ statistically, most approaches require that counts follow an approximately normal distribution. Thus, it is recommended that the natural log transformation should be used, but not necessary if methods robust to outliers are utilized. Calculation of positive controls and of the CV, or similar metric, ensures that the test is viable and will allow sound interpretation of results.

Two validated methods are provided here as example analytic approaches.

8.1 LEAST SQUARES METHOD

See Appendix B Calculation of Results using the Least Squares Method for more detailed information

a. For each treatment group, calculate a metric called a "stimulation index" (SI) that is a ratio of the response in treated wells to the response in unexposed control wells (see Appendix B.3).

b. For each treatment group, calculate a metric called a standardized natural log SI [standardized Ln(SI)] that uses the amount of well-to-well variance in the test to estimate the standard error of the Ln(SI). The standardized Ln(SI) is obtained by dividing the Ln(SI) by its standard error (see Appendix B.4-B.6). This standardized statistic indicates the extent to which the Ln(SI) differs from the reference value of zero. Large (i.e., greater than 2.5) positive values indicate a response to beryllium.

c. Establish a reference data set for each serum, and determine the Ln of the maximum SI for each BeLPT. Calculate the mean (M) and standard deviation (SD) of the Ln (maximum SI) for the reference data set (see Appendix B section B.10).

d. Calculate a metric called the "standardized maximum Ln (SI)." First, find the treatment group with the largest Ln (SI), i.e., the maximum Ln (SI). Next, subtract the value of M for the reference data set and divide by the standard deviation, i.e., [maximum Ln (SI)-M]/SD. This metric compares the strongest response for each BeLPT with the strongest responses from normal individuals in the reference data set.

8.2 ALTERNATIVE OUTLIER RESISTANT METHOD

An alternative outlier resistant method that calculates estimates of the SIs, standard deviations (SDs), and the coefficients of variation (CVs) could also be used. An example of another outlier resistant method is given in Frome 1996. To calculate the SI, the mean CPM of wells treated (stimulated) is divided by the mean control CPM (no stimulation):

$$SI = \frac{\text{mean CPM of wells with mitogen or antigen}}{\text{mean CPM of wells with media}}$$

Some laboratories use different statistical programs generate results. Alternative approaches are acceptable as long as the CV for the background is used to establish outliers and boundary conditions for normal, abnormal, borderline results. In all cases, established protocols for each laboratory should document the methodologies used.

9. RESULTS

The results of BeLPT tests shall be reported as uninterpretable, abnormal, normal, or borderline. Each term is defined as follows:

Normal: Cells display a normal response to beryllium sulfate.

Abnormal: Two or more Be concentrations greater than the stimulation index.

Borderline: One Be concentration is greater than the stimulation index.

Uninterpretable: Results that cannot be interpreted due to assay variations such as large statistical variability on cell growth, increased cell death in test wells, over-proliferation observed in control wells, or poor stimulation from mitogen.

Unacceptable sample: Unacceptable is used to describe samples that cannot be reliably assayed due to insufficient white blood cells isolated from original sample, or too small of blood volume, or late delivery (>48 hours) of blood sample to performing laboratory. In such cases, acquisition of a new sample is recommended for the BeLPT.

If a test is uninterpretable, a repeat is recommended. However, if an unacceptable result is reported to the beryllium registry, such results must be reported as unsatisfactory, listed as “UNSAT” according standard registry terminology. If a test is abnormal or borderline, then two duplicate repeat BeLPTs should be requested. Usually, these tests will be performed in two different laboratories or in one laboratory utilizing two different sera. If at least two of the three

BeLPT tests are abnormal, the patient is deemed beryllium sensitized.

Please note that guidance published by the American Thoracic Society (ATS) in 2014 reported the diagnostic criteria for beryllium sensitization to be (Balmes 2014):

1. Two abnormal blood BeLPT results; or
2. One abnormal and one borderline blood BeLPT; or
3. One abnormal BeLPT test of alveolar lung lavage cells; or
4. Three borderline abnormal blood BeLPTs.

Therefore, the criteria for reporting beryllium sensitization may be updated in a revision pending for the DOE's 10 CFR 850 on the Chronic Beryllium Disease Prevention Program.

The BeLPT shall be reported as unacceptable if any of the following criteria are NOT met:

a. Background counts should be within the acceptable level for the counting instrument (determined as part of the quality control procedures, typically less than 50 CPM).

b. Background counts for complete media should be done with equal volumes of RPMI and complete media processed according to blood LPT standard process. The control well counts should be at least 2 times higher than the mean of the background of complete media counts (as a qualitative measure).

c. Mitogen-stimulated or antigen-stimulated wells should clearly demonstrate lymphocyte proliferation [standardized Ln (SI) greater than 3.0]. Alternative is to determine mean SI for mitogens or antigens by testing normal control persons.

d. The internal variability for control wells or beryllium stimulated wells is acceptable. The standard deviation of natural log transformed count data for control wells should be less than 0.95, and for the Be treated wells it should be less than 1.5 when 12 control wells are used and 4 beryllium stimulated wells are used for each treatment group. These values are guidelines that are most useful when there is no evidence for a beryllium response.

e. At least half of the standardized Ln (SI)s are greater than -3 (i.e., no strong evidence of cell killing).

A BeLPT shall be reported as an abnormal test if both of the following criteria are met:

a. The standardized maximum Ln (SI) is greater than 3.l.

b. At least two standardized Ln (SI) s are greater than 2.5 indicating a positive response to beryllium.

A large positive value of the standardized maximum SI indicates a "biological positive" test, i.e., it indicates by how many standard deviations this metric exceeds the typical maximum response for normal individuals in that serum. Two or more large standardized Ln (SI) s indicates a "statistically positive" test.

10. OTHER NOTES OF INTEREST

Safety data sheets (SDSs) are available for all chemicals and laboratory reagents and should be read by all personnel before performing this assay.

RPMI-1640, L-glutamine, penicillin-streptomycin, sterile phosphate buffered saline solution, and Ficol-Hypaque are sterile tissue culture reagents. When used with appropriate tissue culture laboratory practices, they pose no known safety, health, or disposal hazards.

Biodegradable scintillation cocktail usage requires good ventilation at the workplace and appropriate protective clothing, gloves, and safety glasses.

Human serum, type AB has been tested by an FDA approved method and found non-reactive for the presence of HBsAG and antibody to HIV by the supplier. However, it is derived from human source material and will be handled observing the same safety precautions used when handling any potentially infectious material.

Tritiated thymidine emits low energy beta radiation and shall be used with appropriate exposure controls and personal protective equipment. Policies for the use and disposal of radioactive reagents and laboratory ware are found in the guidelines prepared by the institution's safety and health protection offices and shall comply with accepted practices.

Beryllium sulfate is an extremely hazardous chemical. It is, based upon animal data, assumed to be a potential human carcinogen. It is a strong irritant; contact with skin, eyes, and mucous membranes must be avoided. Consult SDS before handling this chemical.

Computer software can be written for performing the calculations described in this specification. The procedure, however, is sufficiently straightforward so that simple programming can be used to implement it in any spreadsheet.

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APPENDIX A: List of Equipment and Supplies

- Plastic disposable sterile culture tubes, 16 x 125 mm, with screw cap
- Pipettes, serological with plug, plastic, sterile and individually wrapped (1, 5, and 10 ml)
- Sterile disposable reservoirs
- Multi-channel pipettors calibrated to deliver 100µl of cell suspension + CGM and 100µl of reagents into tissue culture plates.
- Microtiter 111 tissue culture plate and lid, 96 well, 0.32 ml/well (round or flat bottom)

  Note: Until such time as a definitive study showing a conclusive advantage to round vs. flat, either is usable without prejudice.
- Centrifuge
- CO_2 incubator
- Laminar flow hood
- Sterile, cotton plugged Pasteur pipettes
- Hemocytometer or Coulter Counter
- Microscope
- 15 ml and 50 ml conical centrifuge tubes
- 96 well cell harvester
- Gas ionization or liquid scintillation counter for detecting beta emissions from tritiated thymidine.

Note: The items above are recommended for simplicity. Equivalents are acceptable. Each laboratory conducts usual procedures to assure satisfactory operation of all equipment.

APPENDIX B: Calculation of Results using the Least Squares Method

The measurement of lymphocyte proliferation is based on beta particle counts from the tritiated thymidine. The data are analyzed by comparing counts from BeSO4 exposed wells and mitogen exposed wells to the counts from unexposed control wells. The laboratory must calculate metrics to determine if a test is acceptable and whether the result is normal, abnormal, or borderline. Two complementary metrics are used to evaluate the results of an LPT. The first metric examines whether patient results differ from those of individuals not exposed to beryllium (biological abnormal), and the second investigates whether a patient's exposed well counts and control well counts differ (statistical abnormal). For the least square (LSQ) approach of analyzing a lymphocyte proliferation test (LPT), the first step is to take the natural logarithm of all of the counts since it has been shown that the log counts are normally distributed and that the standard deviations of the log counts are constant within harvest days.

B.1 Checking for a Biologically Abnormal BeLPT

Because the pooled AB positive serum used to provide the human proteins needed for lymphocyte proliferation is known to be an important variable in the test system, cut-points used for interpreting test must be adjusted when a new batch of serum is used. Since these must be set before the first patient is tested, cut-points are set based on the results from unexposed volunteers who are presumed to be normal. At least 30 volunteers should be used. Stimulation indices (SIs) for each volunteer are calculated from the log transformed count data. Six SIs are calculated, one for each day at each $BeSO_4$ level using the formulas:

$$\ln(SI) = mean(\ln(exposed\ wells)) - mean(\ln(control\ wells))$$

$$SI = e^{\ln(SI)}$$

The SIs from the set of volunteers are analyzed to develop a test statistic used to determine whether SIs from patients are beyond the normal range. Since two of the six patient SIs must be beyond the normal range for an LPT to be classified as abnormal, the normal range is established by analyzing the distributions of Maximum SI. A maximum likelihood estimate of the cut-point that only one per 1000 unexposed volunteers would exceed is calculated using the formula:

Let $M = mean(\ln \max SIs)$ and $SD = standard\ deviation(\ln \max SIs)$. Then,

$$Cutpoint = e^{(M+3.1\times SD\times\sqrt{(\frac{N-1}{N})})}$$

To evaluate a patient's BeLPT, the formulas above are used to calculate six SIs, one for each day at each $BeSO_4$ level, along with SIs for PHA and CON-A. Each of the six treatment SIs is compared to the cut-point to assess biological abnormality.

B.2 Checking for a Statistically Abnormal BeLPT

Test results from each patient are analyzed to assure that counts from beryllium exposed wells

are significantly different from those in control wells. A metric called a standardized log SI (Std Ln(SI)) is calculated using the formula:

$$Std\ \ln(SI) = \frac{\ln(SI)}{Pooled\ SE\ \ln(SI)}$$

Separate pooled standard errors are calculated for the 5-day and the 7-day SIs using the formulas:

$$s_p = \sqrt{\frac{\{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2 + (n_3 - 1){s_3}^2 + (n_4 - 1){s_4}^2\}}{\sum n_{1-4} - 4}}$$

$$SE_p = s_p \times \sqrt{(\frac{1}{n_1} + \frac{1}{n_j})};\ \text{j = 2, 3, or 4.}$$

Where s_p is pooled standard deviation se_p is pooled standard error, n_1 is the number of control well counts, and n_2, n3, and n_4 are the number of counts for the low, medium, and high levels of $BeSO_4$ exposed wells, respectively. When counts are complete there are 12 control counts and 4 treatment counts for each level. Therefore, $\sqrt{(}\ 1\backslash n_1 + 1\backslash n_2) = \sqrt{(}\ 1\backslash 12 + 1\backslash 4) = 0.577$. Under a null hypothesis of no treatment effect the StdLn(SIs) follow a Student's t-distribution based on n-p=20 degrees of freedom, where n is the total number of counts (24 for complete counts) and p is the number of means estimates (4 when three treatments). Since $t_{0.01,20} = 2.53$, StdLn(SIs) larger than 2.5 provide 99% confidence that treated wells are larger than control wells.

B.3 Assigning Outcomes of Abnormal and Borderline

If an LPT is not found to be unsatisfactory (see Assigning Unsatisfactory Outcomes below) or uninterpretable, the test will be assigned an outcome of abnormal or borderline. Extensive experience with repeated tests for individual workers has confirmed the following observation: if an LPT is biologically abnormal and also has two statistically abnormal StdLn(SIs) that occur in other day\treatment combinations, the outcome of the repeated test is generally normal. Therefore, an LPT outcome of abnormal should be assigned when the test is both biologically and statistically abnormal for at least two corresponding day\treatment combinations. An **abnormal** LPT must have not only two StdLn(SIs) greater than 2.5, but also these StdLn(SIs) must occur for the same day\treatment combinations as the two SIs that were greater than cutoff. A **borderline** outcome is given when there is one or two SIs greater than cutoff but only one corresponding StdLn (SIs) greater than 2.5. If a SI is greater that the cutoff, but the corresponding StdLn (SI) is not greater than 2.5, and the CV is acceptable, the lab director must be consulted regarding the validity and reportability of this SI.

B.4 Assigning Unsatisfactory Outcomes

An **unsatisfactory** outcome may result from high coefficients of variation (CVs), cell killing, fewer than five SIs, poor growth, over-proliferation seen in unexposed control well counts, poor mitogen response, and technical error or accident. All tests reported as unsatisfactory must be repeated.

B.5 Identifying LPTs Having Unsatisfactory CVs

Well-to-well variation is evaluated to assure that lymphocytes are not responding to a protein in the pooled serum in a way that masks a response to beryllium. CVs are calculated for each of the six day\treatment level combinations, for the day 5 and the day 7 unexposed control wells, and for the mitogen control wells to determine if these are excessively large. Since CVs on the original scale are the equivalent to the standard deviations on the log scale, the formula below is used to estimate theCVs is:

$$CV \sim Stdev(\ln(count_1, count_2, \ldots, count_j),$$

where for complete counts j=4 for $BeSO_4$ exposed wells and mitogen exposed wells and j=12 for unexposed control wells. An LPT is declared to be CVunsatisfactory if:

- Either of the two CVs from unexposed control well sets are greater than 1.00, or
- At least two CVs from treated wells are greater than 1.25.

Rules used to assign LPT outcomes were validated by retrospectively applying them to a set of 3321 LPTs tested in a single serum. The LPT results had been interpreted by the lab using a least absolute value (LAV) method that had been modified over time based on experience. The 3321 LPTs did not include any tests that had been given an LAV method outcome of uninterpretable or unsatisfactory for any reason other than CVunsatisfactory. LAV outcomes were distributed as follows.

Abnormal	178	(5.3%)
Borderline	109	(3.3%)
Normal	2898	(87.3%)
CVunsat	136	(4.1%)

B.6 Conclusion

The simpler Least Squares (LSQ) method of calculating metrics and applying rules to the interpretation of BeLPT results can be computerized to produce overall results similar to those arrived at by experts with experience using this test. The high false negative rate inherent to keeping the false positive rate acceptably low results in disagreement on the individuals declared borderline and CVunsat.

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CONCLUDING MATERIAL

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DOE-AU-13

Project Number:

SDMP-0029