

Use of SRBC antibody responses for immunotoxicity testing

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Abstract

The production of antigen-specific antibodies represents a major defense mechanism of humoral immune responses and involves the cooperation and interaction of several immune cell types: antigen presenting cells, T helper cells, and B cells. Thus, there are several cells or cell products (e.g., interleukins) that may be altered following xenobiotic exposure, making assays that evaluate the production of antigen specific antibody a relatively comprehensive and sensitive assessment of immune function. Data suggest that the primary antibody response to SRBC may be one of the most sensitive endpoints available to assess chemical-induced alterations to the immune system. As a result, this endpoint has become the cornerstone of several recently established guidelines for assessing the potential immunotoxicity of xenobiotics. Five types of antibody may be produced in a humoral immune response (i.e., IgGs of various subtypes, IgM, IgD, IgA, or IgE). For immunotoxicity assessment, the focus has primarily been on assays that assess production of IgM antibodies. Although a number of assays have been developed to evaluate antibody production, the antibody forming cell (AFC) assay and enzyme-linked immunosorbent assay (ELISA) are the two most frequently employed to evaluate the potential immunotoxicity of a xenobiotic. In this manuscript, background information, as well as the pros and cons of each of these assays are discussed and detailed methods on conducting each assay are provided.

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1. Introduction

The immune system is a very complex and regulated organ system that involves the cooperation and interaction of a number of different cell types, cell products, tissues, and organs. This unique organization may contribute to the immune system as a target organ for xenobiotics. Xenobiotic-induced dysfunction of the mammalian immune system has been well documented [1–3]. The responses of the immune system can be divided into two categories: innate and acquired. Innate responses do not require previous sensitization to an antigen and include: phagocytic cells (macrophages, monocytes, polymorphonuclear cells) and cellular products like interferons. Acquired or specific immune responses are dependent on processes generated

by previous exposure to an antigen. The acquired immune response includes both cell-mediated and humoral immunity.

The production of antigen-specific antibodies represents a major defense mechanism of humoral immune responses and protects against infectious agents by neutralizing viruses, destroying targets by antibody-dependent cellular cytotoxicity and complement-mediated lysis, or by enhancing phagocytosis through opsonization. Several days following antigen exposure, antigen-specific antibodies, predominantly of the IgM isotype, are generated and released into the general circulation by specific plasma cells (terminally differentiated, antibody-secreting B cells). The generation of an antibody response involves the cooperation and interaction of a number of immune cell types. These include antigen presenting cells (APCs), such as macrophages or dendritic cells, T helper cells and B cells. The APCs uptake the antigen and subsequently process and present it in

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association with major histocompatibility complex (MHC) class II molecules to antigen specific T helper cells. The T helper cells produce a variety of cytokines (e.g., IL-2, IL-4, IL-6, interferon-gamma) that then help B-cells to proliferate and differentiate into antibody producing plasma cells. There are five types of antibody that may be produced by B-cells. These include IgGs of various subtypes, IgM, IgD, IgA, and IgE. For immunotoxicity assessment, the focus has primarily been on assays to assess IgM antibodies. Thus, there are numerous targets that may be altered following xenobiotic exposure, making assays that evaluate antigen specific antibody production a relatively comprehensive and sensitive assessment of immune function.

Luster et al. [4] demonstrated the importance of assessing humoral immune function in an immunotoxicological testing battery. These investigators reported that the quantification of a specific antibody-forming response to SRBC provided one of the best predictors of immunotoxicity in mice. In a subsequent study, 51 chemicals were evaluated in mice using a number of immunotoxicological endpoints. The results of this study indicated that the highest correlations with immunotoxic potential occurred for the splenic IgM antibody forming cell (AFC; or plaque forming cell assay [PFC]) assay to SRBC and cell surface marker analysis [5]. These data suggest that the primary antibody response to SRBC may be one of the most sensitive endpoints available to assess chemical-induced alterations to the immune system.

Although the immune system has been shown to be an important target organ of xenobiotic-induced toxicity [2,3], it has only been relatively recently that regulatory agencies around the world have established guidelines for immunotoxicology testing. As the assessment of the primary antibody response to a T-dependent antigen (e.g., SRBC) has been reported to provide one of the best predictors of immunotoxicity in mice [5], it has become the cornerstone of several guidelines for assessing the potential immunotoxicity of xenobiotics. For example, the United States Environmental Protection Agency [6] in 1998 published, and subsequently finalized in 2005, guidelines requiring chemicals used as pesticides to initially undergo an evaluation of the primary humoral immune response to a T-dependent antigen (i.e., SRBC) utilizing either the AFC assay or ELISA following test article exposure for 28 days.

Evaluating pharmaceuticals for their ability to alter the antigen-specific antibody response, on the other hand, is typically determined on a case-by-case basis. For example, the United States Food and Drug Administration [7] suggest considering follow-up studies to investigate mechanism(s) of immunotoxicity that may include evaluating the antibody response to a T-dependent antigen among other endpoints if (1) there is evidence of immunotoxicity in repeat dose toxicology studies; (2) the test article is used for the treatment of HIV or related disease; (3) the test article or metabolites accumulate or are retained in

reticuloendothelial tissues (i.e., there are pharmacokinetic effects; 4) there are data suggestive of immunosuppression that occur in clinical trials. In Europe, conventional pharmaceuticals (not biotechnology derived or vaccines) under CPMP guidance [8] must undergo an initial 28-day screening study in which the primary humoral immune response to a T-dependent antigen (e.g., SRBC) is conducted if an analysis of lymphocyte subsets and natural killer cell activity are unavailable. Additional studies are conducted on a case-by-case basis, which consist of functional assays to further define immunological changes and may include a measure of the primary antibody response if not evaluated in the initial screening study. Although a number of assays have been developed to evaluate antibody production (e.g., radial immunodiffusion, hemagglutination, immunoelectrophoresis, enzyme-linked immunospot (ELISPOT) assay, the AFC assay and enzyme-linked immunosorbent assay (ELISA)) are the two most frequently employed to evaluate the potential immunotoxicity of a xenobiotic.

2. AFC or plaque forming cell (PFC) assay

2.1. Background

Jerne and Nordin developed the hemolytic plaque assay in 1963 to measure the number of IgM antibody-forming cells specific to SRBC [9]. Although the assay has been subsequently modified, it essentially involves the removal of spleens from animals that have been immunized with SRBC. For rodents, spleens are typically removed 4–6 days following immunization and a single cell suspension is then produced by gentle agitation (e.g., rubbing between the frosted ends of glass slides). Spleen cells are then mixed with SRBC and complement in a semisolid media, such as agar, and then placed on petri dishes, covered with a glass cover slip, and incubated for 3 h at 37 °C. During incubation of the petri dishes, B cells produce IgM antibody specific for the SRBC, which then bind to SRBC membrane antigens and produce complement-mediated lysis of the SRBC and the subsequent formation of plaques (i.e., clear areas of hemolysis around each antibody forming cell) (Fig. 1). The plaques are then visually quantitated and the resulting data expressed as SRBC-specific IgM AFC (or PFC)/spleen or IgM AFC (or PFC)/million spleen cells. Data are expressed as both AFC/spleen and AFC/million cells in order to help elucidate the potential contribution of test article-induced alterations of spleen cell numbers on the AFC response. Following a second immunization with SRBC, the secondary IgG AFC immune response can then be assessed using a slight modification of this assay [10].

The AFC assay does not quantitate the amount of antibody produced, but rather the number of specific antibody-producing B cells or plasma cells in a particular tissue (i.e., the spleen), and thus, does not account for antibody produced in other sites (e.g., bone marrow, lymph

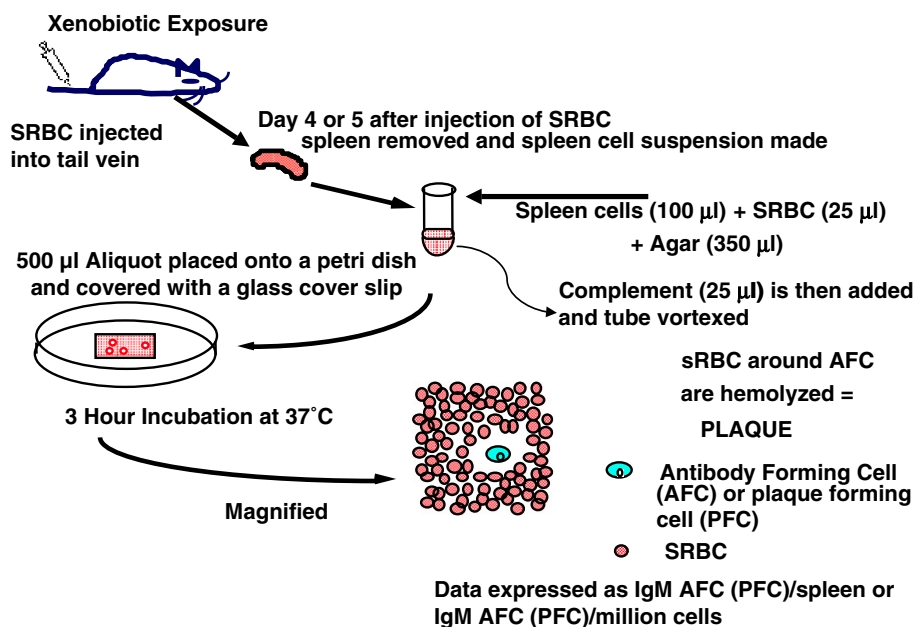


Fig. 1. Measurement of the in vivo AFC response to SRBC in mice and rats.

nodes). Therefore, it is difficult to extrapolate results of the AFC assay to human tests since serum antibody concentrations are typically measured in humans. In addition, the AFC assay requires the sacrifice of the animal and therefore limits experimental design relative to exposure. The AFC assay is also very labor intensive, costly, and not conducive to automation, although the ELISPOT assay, which can be conducted in nitrocellulose bottom 96-well plates, may be automated [11]. The AFC assay, however, is well-characterized and has been validated in a number of laboratories for its sensitivity and reproducibility and is recommended by the US National Toxicology Program to assess the humoral immune response [4,5]. The AFC assay can also be conducted entirely in vitro using immunocompetent cells obtained from either treated or naive animals. In the latter case, the immunocompetent cells are exposed to the test article and SRBC for the first time in tissue culture. In addition, this approach permits separation-reconstitution studies to identify the primary cell type(s) targeted by a xenobiotic, as well as a means to potentially distinguish between xenobiotic-induced direct or indirect effects (e.g., neuroendocrine alterations) on the immune system [10].

Many of the procedures, such as assessment of the primary humoral immune response to SRBC, that have been developed for assessing xenobiotic-induced alterations of the immune system of adult rodents have undergone extensive evaluation [5]. Recently, however, increasing attention has been focused on the effects of xenobiotics on the developing immune systems of the fetus and newborn [12–15]. Whether the SRBC primary antibody response would be useful in assessing the functionally immature immune system of young animals (e.g.,

neonates) was investigated by Ladics et al. [16]. Ten-day-old rat pups were unable to generate a primary IgM response to SRBC using the AFC assay due to the apparent immature status of their immune systems; however, results with weanlings suggest that an antibody response to SRBC of sufficient magnitude (i.e., within the historical control range for adults) can be demonstrated with the AFC assay.

2.2. SRBC preparation and immunization of rats and mice for both the AFC assay and ELISA

2.2.1. Equipment and reagents

1. Refrigerated sterile sheep blood in Alsevers (Covance Pipersville, PA or other commercial supplier; we prefer to use the same sheep from the same supplier, when possible)
2. Refrigerated phosphate-buffered saline (PBS; Sigma)
3. Refrigerated tabletop centrifuge
4. Cell counting instrument (e.g., Advia™ 120 Hematology System)
5. 50 mL plastic centrifuge tubes with caps
6. Ice bucket and ice
7. Adjustable micropipettes and tips
8. Heating pads
9. Syringes and 25-gauge needles

2.2.2. Procedure

1. Gently invert the bottle of sheep blood until it is thoroughly mixed. Transfer 5 mL sheep blood into a 50 mL centrifuge tube.
2. QS (bring the total volume) to approximately 50 mL with PBS and invert tube to mix.

3. Centrifuge at approximately 550g for 10–12 min at 2–8 °C.
4. Pour off supernatant, “flick” the tube to break up the pellet, and resuspend the pellet to 10 mL with PBS.
5. Place at least 400 μ L washed SRBC per tube from step 4 in each of two tubes and obtain a cell count from each tube.
6. After removing the volume needed in step 5 from the 10 mL resuspended pellet, QS the resuspended pellet to approximately 50 mL with PBS.
7. Centrifuge at approximately 550g for 10–12 min at 2–8 °C.
8. Pour off supernatant and prepare 4×10^8 SRBC/mL (rats) or 1×10^9 SRBC/mL (mice), based on cell counts obtained in step 5 and calculations listed below.
9. SRBC preparation is kept on ice until needed. The preparation must be used the day it is prepared.
10. Prior to injection of SRBC, animals [we typically use male and female Crl:CD(SD) BR rats or Crl:CD-1 (ICR) BR mice; while the female B6C3F1 mouse has also been extensively utilized [5,10] are place in plastic cages on heating pads for 15 min to cause venous dilation. SRBC are then injected into the tail vein through a 25-gauge needle.
11. Inject 0.5 mL per rat or 0.2 mL per mouse of the washed SRBCs prepared in step 8 above into the tail vein.
12. When needed for evaluation of the secondary IgG response, repeat the injection of SRBCs approximately 1 week after the first injection.

2.2.3. Calculations

1. Take the mean of 2 separate counts, which are expressed in 10^6 U (cells)/ μ L.
 2. Multiply the mean of the counts (step 1) by 1000, or 1×10^3 , to get SRBC/mL.
 3. Multiply the number in step 2 by 10 mL (initial volume of resuspended pellet from which the cell count samples were taken).
 4. Divide this number by 4×10^9 cells/mL for rats or 1×10^9 cells/mL for mice.
 5. QS the washed stock SRBC (step 8 in Section 2.2.2) to the number of mL determined in step 4.
 6. To prepare a sufficient volume of 4×10^8 cells/mL for injection of rats, multiply the number of rats to be injected $\times 0.5$ mL/rat and allow an additional quantity in the total volume. Once the needed volume is determined, prepare a 1:10 dilution of 4×10^9 cells/mL by adding 1 mL of 4×10^9 cells/mL for every 9 mL PBS. The volume of 1×10^9 cells/mL for injection of mice prepared in step 5 should be sufficient since each mouse is to be injected with 0.2 mL SRBC. However, to ensure that the volume is adequate, multiply the number of mice to be injected $\times 0.2$ mL per mouse and allow an additional quantity in the total volume.
- ### 2.3. In vivo AFC response to SRBC
- #### 2.3.1. Equipment and reagents
1. Hanks' Balanced Salt Solution with Hepes (HBSS/H)-500 mL
 - a. 10 $\times$ HBSS (Gibco #14060-024)
 - b. deionized water
 - c. 7.5% NaHCO₃ (Gibco #25080-011)
 - d. 1 M Hepes buffer (Gibco #15630-015)

50 mL of 10 $\times$ HBSS; 425 mL deionized water; 2.35 mL of 7.5% NaHCO₃; 10 mL of 1 M Hepes buffer; adjust pH to 7.4; QS to 500 mL with deionized water; store at 2–8 °C
 2. Guinea pig complement (GPC; Gibco)
 3. Guinea pig complement restoring solution (Gibco) [add 3 mL to 1 bottle of lyophilized GPC and place 250 μ L aliquots into snap-cap tubes and freeze until ready to use (1 tube for $\sim$ 30 plates). On the day of the assay, thaw a sufficient number of tubes and add 750 μ L HBSS/H per tube.
 4. DEAE Dextran (Diethylaminoethyl-dextran) stock solution
 - a. DEAE dextran powder (Sigma)
 - b. 0.85% saline, warmed (Sigma)

Add 900 mg DEAE dextran powder to 30 mL warm 0.85% saline in a 50 mL centrifuge tube. Vortex until all the powder dissolves and adjust pH to 6.9. Store in refrigerator after preparation is complete, and adjust pH at each use.
 5. Agar
 - a. HBSS/H
 - b. Bacto-Agar (Difco)
 - c. DEAE Dextran stock solution

Place 100 mL HBSS/H into a 250 mL Erlenmeyer flask with a stir bar and heat on a hot plate. Slowly add 500 mg of Bacto-Agar. Once the solution begins to boil, add 1.6 mL DEAE Dextran stock solution. Allow the agar to boil and then remove from the heat and place in a preheated 47 °C water bath until ready to use.
 6. SRBC preparation for use in the AFC assay (2×10^9 cells/mL)
 - a. SRBC from a commercial source (e.g., Covance, Pipersville, PA)
 - b. Phosphate-buffered saline (PBS)

Place 5 mL stock SRBC in a 50 mL centrifuge tube. QS to 50 mL with PBS and centrifuge at 550g for 10 min at $\sim$ 4 °C. Pour off supernatant and resuspend pellet to 10 mL with PBS. Remove a volume sufficient for counting on a cell counter (e.g., 3 mL) and then QS the remaining portion to 50 mL with PBS. Centrifuge (2nd wash) as described above and while the 2nd wash is being done, obtain cell counts and calculate volume for adjustment of cells to 2×10^9 cells/mL.

7. Disposable and sterile 100 × 15 mm plastic petri dishes for plaquing and rat spleen mashing and small 60 × 15 mm petri dishes for mouse spleen mashing (Falcon)
8. Glass microscope slides with frosted ends
9. 4 × 50 mm; 1.5 thickness, glass cover slips (Fisher) to plaque samples
10. 37 °C incubator
11. Hot plate with magnetic stir bar capability
12. Cell counter (e.g., Advia™ 120 Hematology System) or hemacytometer
13. Vortexer
14. Belco colony counter
15. Refrigerated centrifuge
16. 15 or 50 mL capped centrifuge tubes
17. 12 × 75-mm glass culture tubes (borosilicate; Fisher) used for plaquing
18. 70% Ethanol
19. Disposable and sterile 5 mL capped tubes (12 × 75 mm, Fisher) for preparation of complement solution and SRBC solution for plaquing.
20. Surgical scissors and forceps
21. Ice bucket and ice
22. Pipettes—5 and 10 mL and pipet-aid
23. Pasteur pipettes and bulbs
24. Adjustable micropipettes and tips
25. 5- or 10-cm³ disposable syringes
26. Erlenmeyer flask
27. pH meter
28. Magnetic stir bars

2.3.2. Procedure (sterility not required)

1. *Sacrifice of animals/spleen cell preparation:* Four days after immunization with SRBC (peak day of response observed in our laboratory; each laboratory should determine their own peak day of response), mice and rats are sacrificed by CO₂ asphyxiation, and their undersides sprayed with ethanol until the fur is wet and spleens are removed, weighed, and placed in 3 or 6 mL of cold HBSS/H, respectively. Spleen cell suspensions are prepared by initially cutting the spleen into several pieces (rats) and then gently mashing the spleen or spleen pieces between the frosted ends of glass microscope slides until most of the spleen cells have been removed from the capsule. Alternatively, the plunger end of a 5- or 10-cm³ disposable syringe can be used.
2. *Spleen cell preparation:* The spleen cells are transferred to a sterile conical centrifuge tube using a Pasteur pipette and bulb and the screw cap replaced. While on ice, the spleen cell suspensions are allowed to sit for 3 min to allow fragments of capsule to settle to the bottom of the tube. Once the debris has settled, the cells are transferred to a new sterile conical centrifuge tube, leaving behind the debris, and capped. The mouse and rat spleen cell suspensions are centrifuged for 10 min at 550g at ~4 °C to pellet the cells. The supernatant is decanted and the cell pellet resuspended in either 3 or 6 mL cold

HBSS/H for mice and rats, respectively and kept on ice until the AFC response is measured (i.e., plaqued) (See steps 6 and 7).

3. *Preparation for plaquing:* Place petri dishes in 37 °C incubator to warm and prepare agar as described above. The warmed agar is then dispensed in 0.35 mL aliquots into glass culture tubes, 2–3 per animal (i.e., 2–3 dilutions per spleen, 1 tube per dilution) that have been set up in racks in a 37 °C water bath.
4. The optimal dilutions for IgM plaques may vary depending on the xenobiotic with which the animal has been dosed, as well as the concentration of test material utilized and the number of days animals were dosed. Suggested dilutions of spleen cells in HBSS/H for vehicle control animals are 1:200 and 1:300, while the suggested dilutions for animals dosed with test compounds are 1:25, 1:100, or 1:300.
5. SRBC (25 µL; 2 × 10⁹ SRBC/mL) are added to the tubes of agar in the water bath, a few tubes at a time. An aliquot (100 µL) of each diluted spleen cell suspension is added to each glass tube in the water bath containing agar and SRBC. Last, 25 µ of guinea pig complement is then added and the tube immediately vortexed.
6. *Plaquing:* The tube contents (~500 µL) are then poured into a 100 × 15-mm petri dish and the solution is covered by a large glass cover slip. Both the top and bottom of the petri dish can be utilized. Samples that spread unevenly or incompletely need to be redone immediately. When the agar is solid (~3 min), the top dish is placed on the bottom dish and placed in a 37 °C dry heat incubator for 3 h.
7. After 3 h, the number of AFCs (or plaques) are counted with a Belco colony counter or alternatively, a dissecting microscope. When counting plaques, it is important to avoid counting bubbles in the agar. If too many plaques are observed, making them difficult to count, the samples can be plaqued again using a larger dilution of the original spleen cell suspension.
8. While plates are incubating, cell counts may be taken for each spleen cell suspension using a cell counter.
9. *Calculations:* Data are typically expressed as AFC/spleen or AFC/10⁶ splenocytes. The calculations to be made include the following: AFC per mL in original suspension, AFC/spleen, and AFC/10⁶ splenocytes. Since the number of AFCs or plaques was evaluated in a 100 µL sample of the original cell suspension, the count × 10 × the dilution factor = AFC/mL in the original cell suspension. The AFC/spleen is then calculated by multiplying the AFC/mL by the total volume of the original cell suspension. The AFC/mL divided by 10⁶ cells/mL (from the cell counts) = AFC/10⁶ spleen cells.

2.4. In vitro AFC response to SRBC

2.4.1. Equipment and reagents

1. Roswell Park Memorial Institute (RPMI-1640; Gibco)

2. Complete RPMI-1640 media for tissue culture
 - a. 10× RPMI-1640 (Gibco)
 - b. deionized water
 - c. 10% fetal bovine serum (FBS) (e.g., Hyclone; recommended that a number of serum from several different vendors be screened to find one that yields an optimal response)
 - d. 1% penicillin–streptomycin–fungizone solution (Penn-strep; Hazelton Research Laboratories)
 - e. 1% L-glutamine
 - f. 2-Mercaptoethanol (2-ME; Bio-Rad)
3. Cell culture feed mix (composed of essential and non-essential amino acids, L-glutamine, dextrose, NaHCO₃, pen-strep, and FBS) in minimal essential media.
4. Guinea pig complement (GPC; Gibco)
5. Guinea pig complement restoring solution (Gibco) Prepared as described above for the in vivo AFC response.
6. DEAE Dextran (Diethylaminoethyl-dextran) stock solution
 - a. DEAE dextran powder (Sigma)
 - b. 0.85% saline, warmed
Prepared as described above for the in vivo AFC response.
7. Agar (prepared as described for the in vivo AFC response)
 - a. HBSS/H
 - b. Bacto-Agar (Difco)
 - c. DEAE Dextran stock solution
8. SRBC preparation for use in the AFC assay (2×10^9 cells/mL) (prepared as described above for the in vivo AFC response).
9. Disposable and sterile 100 × 15 mm plastic petri dishes for plaquing.
10. Syringe plunger from a 10-cm³ syringe
11. 24 × 40 mm; 1.5 thickness, glass cover slips (Fisher) to plaque samples
12. 37°C incubator
13. Hot plate with magnetic stir bar capability
14. Cell counter (e.g., Advia™ 120 Hematology System) or hemacytometer
15. Vortexer
16. Bellco colony counter
17. Refrigerated centrifuge
18. 15 or 50 mL disposable and sterile capped conical centrifuge tubes (Fisher)
19. 12 × 75-mm glass culture tubes (borosilicate; Fisher) used for plaquing
20. Disposable and sterile 60 × 15 mm plastic petri dishes (for spleen mashing)
21. Disposable and sterile 5 mL capped tubes (12 × 75 mm, Fisher) for preparation of complement solution and SRBC solution for plaquing.
22. Blood gas mixture (Mishell-Dutton gas; 10% CO₂, 7% O₂, and 83% N₂).
23. Ventilated laminar flow hood
24. 70% Ethanol
25. Tissue culture boxes
26. Surgical scissors and forceps
27. Ice bucket and ice
28. Pipettes—5 and 10 mL and pipet-aid
29. Pasteur pipettes and bulbs
30. 48-well sterile culture plates with lids
31. Adjustable micropipettes and sterile tips
32. pH meter

2.4.2. Procedure (sterility required)

1. *Sacrifice of animals:* The bench top, scissors, and forceps are thoroughly sprayed with ethanol to sterilize them. Test article treated or naive mice are sacrificed by CO₂ asphyxiation and their undersides sprayed with ethanol until the fur is thoroughly wet, while under a ventilated laminar flow hood. Using sterile technique under a ventilated laminar flow hood, spleens are removed, weighed, and placed in 15 mL conical centrifuge tubes containing 3 mL of cold, sterile HBSS/H and the screw cap immediately replaced. If using spleen cells from naive mice and subsequently exposing them to test article for the first time in tissue culture, the suspensions from all the spleens are pooled. Typically, four mice would be required to provide enough cells for each 48-well plate (i.e., $5\text{--}7.5 \times 10^6$ spleen cells/well). The scissors and forceps must be rinsed again with ethanol after cutting through the skin before removal of the spleen, as the skin is not sterile. The instruments should also be thoroughly rinsed with ethanol between each animal.
2. *Spleen cell preparation:* The syringe plunger from a 10-cm³ syringe is sterilized under a laminar flow hood by spraying it with 70% ethanol. Spleen cell suspensions are prepared under a laminar flow hood by initially placing each spleen into a sterile petri dish (note: HBSS/H used to initially collect spleens is discarded), 3–4 mL of RPMI complete media is added, and the spleens gently mashed with the sterile syringe plunger until most of the spleen cells have been separated from the capsule. The spleen cells are transferred to a sterile conical centrifuge tube using a Pasteur pipette and bulb and the screw cap replaced. While on ice, the spleen cell suspensions are allowed to sit for 3 min to allow fragments of capsule to settle to the bottom of the tube. Once the debris has settled, the cells are transferred to a new sterile conical centrifuge tube, leaving behind the debris, and capped. The mouse spleen cell suspensions are centrifuged for 10 min at 550g at ~4 °C to pellet the cells. The supernatant is decanted and the cell pellet resuspended in 2 mL (or desired volume) of RPMI complete media and kept on ice until used.
3. *Immunization with SRBC:* A cell count is then taken for each spleen cell suspension. The spleen cell suspensions are then adjusted with RPMI complete media to 1.0×10^7 cells per mL and 500 μL is then added to each well of a 48-well culture plate. The tops of 48-well

- culture plates should be marked with an indelible ink pen to indicate the various treatment groups. At least four wells per treatment group should be included. SRBC are adjusted to a stock concentration of 1×10^9 SRBC per mL. To immunize the culture wells, 6.5 μ L of SRBC stock are added. After SRBC are added, each well is then gently resuspended with a Pasteur pipette, using a new pipette for each treatment group.
4. The culture plates are then placed in a tissue culture box. We use a stainless steel apparatus that measures 6 5/8 in. $\times$ 8 7/8 in. $\times$ 5 7/8 in., has a removable 0.5-in. plexiglass front panel and a pressure gauge. Each tissue culture box is then gassed with a commercially available “blood gas” mixture until the interior pressure is maintained at 5.5 psi and placed on a rocker platform (7 cpm) in a temperature controlled environment (37 °C).
 5. After 24 h, the boxes are removed and the plates are placed in a laminar flow hood. Each well receives one drop of “feed mix”, while maintaining the sterility of the wells. The plates are placed back in the boxes, gassed again, and returned to the rocker platforms at 37 °C.
 6. **Plaques:** Five days after immunization (peak day of the in vitro AFC response observed in our laboratory) of the cell cultures with SRBC, each culture well is plaqued. Preparation for plaquing an in vitro AFC response to SRBC is identical to that described previously for an in vivo AFC response except that 50 μ L of the spleen cell suspension from each well is plaqued and the amount of agar aliquoted to each 12 $\times$ 75-mm glass tube is 400 μ L. The solution of SRBC, spleen cells, agar, and guinea pig complement (total volume of 500 μ L) is vortexed.
 7. A 200 μ L aliquot of the solution is placed on one half of a 100 $\times$ 15-mm petri dish and covered by a small glass coverslip (24 $\times$ 40; 1.5 thick). A second sample can be

plaqued on the other half of the top petri plate, while two additional samples can be placed in the bottom half of the plate. This procedure is repeated until all of the sample wells have been plaqued. The remainder of this assay is as described for the in vivo AFC response (Section 2.3.2; steps 7 and 8).

8. **Calculations:** While the plates are incubating, cell counts are taken for the spleen cell suspension in each well. The viability of the cells in each well is also determined. The calculations to be made include the following: AFC per mL in original suspension, AFC/culture, AFC/ 10^6 splenocytes and percent viability. The calculations for the first three parameters are conducted as described above for the in vivo AFC response, utilizing the appropriate dilutions. Historically, the viability of our spleen cell cultures after 5 days of incubation has ranged from 50 to 70%.

3. SRBC-specific IgM ELISA

3.1. Background

Compared to the AFC assay, the SRBC-specific IgM ELISA is relatively new, with the first publications occurring in the late 1980s to early 1990s [17–19]. The SRBC-specific IgM ELISA utilizes solubilized hemoglobin-free SRBC membranes and measures antigen specific antibody found in the serum of a subject generated from all antibody-producing tissues (i.e., spleen, lymph nodes, bone marrow), reflecting the systemic immunity of the host (Fig. 2). The ELISA can also be designed to measure any class of antibody. The ELISA is more cost-effective and less time consuming in comparison to the AFC assay and can be automated, as ELISAs are typically performed in 96-

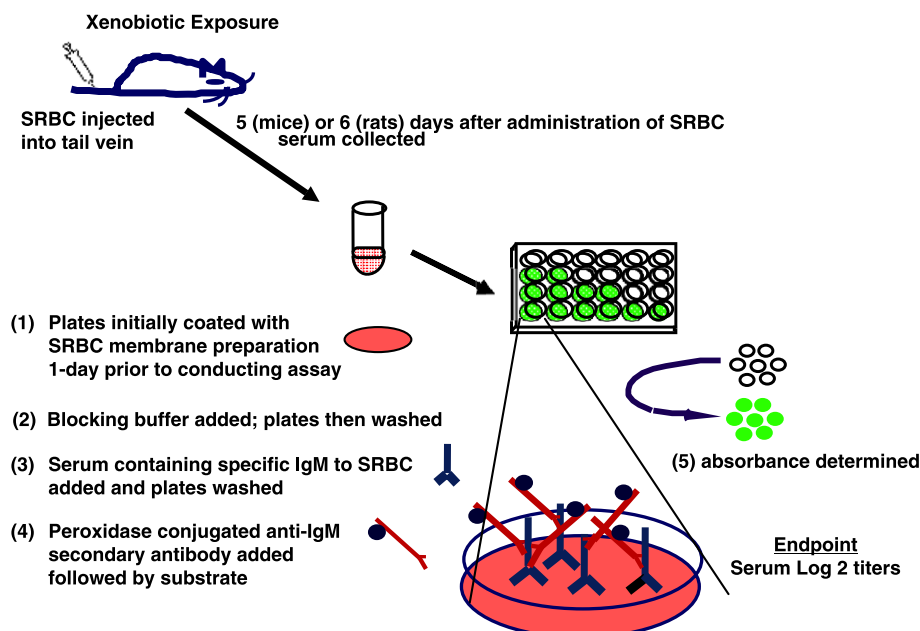


Fig. 2. Measurement of the SRBC-Specific IgM ELISA in mice and rats.

well microtiter plates. In addition, the ELISA allows for a number of serum samples to be taken from the same animal and samples may be frozen for later assay. As a result, a time course of humoral immune function can be conducted, a recovery period following test article administration can be evaluated or, upon rechallenge with antigen, a secondary IgG-mediated immune response measured. One current limitation of the ELISA is the availability of reagents for species other than rats, mice, and primates. Further, in contrast to the AFC assay, the ELISA is not conducive for mechanistic approaches (e.g., separation-reconstitution studies) to study xenobiotic-induced alterations of the immune system. Similar to the AFC assay, 10-day-old rat pups were unable to generate a primary IgM response to SRBC using the ELISA. Unlike the AFC assay, however, a high background response was observed with weanlings using the SRBC-specific IgM ELISA, which may preclude its use with these age animals [16].

3.2. Preparation of hemoglobin-free sheep red blood cell membranes for serum IgM ELISA

3.2.1. Equipment and reagents

1. Sterile, defibrinated sheep blood containing no Alsever's solution
2. Saline (0.85%) (Sigma)
3. 5 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA (Sigma)
4. 0.1% Sodium dodecyl sulfate (SDS) in PBS (Sigma)
5. Mid-speed refrigerated centrifuge with fixed angle rotor
6. Low-speed refrigerated centrifuge with swinging bucket rotor
7. Round-bottom ultracentrifuge tubes
8. 12,000–14,000 molecular weight dialysis tubing
9. Pipettes—5 and 10 mL and pipet-aid
10. Adjustable micropipettes and tips

3.2.2. Procedure (sterility is not necessary)

All centrifugation is done at 2–8 °C. All solutions with the exception of 0.1% SDS in PBS are used at 2–8 °C.

1. Transfer sheep blood into as many round-bottom ultracentrifuge tubes as needed. Centrifuge in a swinging bucket rotor at approximately 1000g for approximately 15 min. Aspirate the supernatant.
2. Fill each tube with saline and re-suspend the pellet. Centrifuge in a swinging bucket rotor at approximately 1000g for approximately 15 min. Aspirate the supernatant. Repeat this procedure twice, for a total of three washes. Remove the buffy coat prior to the third wash.
3. Following the third wash, fill each tube with Tris-EDTA to lyse the red cells. Centrifuge in a fixed angle rotor at approximately 25,000g for approximately 30 min. Decant the supernatant carefully, as

the pellet will be difficult to see due to the dark color of the supernatant. Re-suspend the entire pellet and fill each tube with Tris-EDTA. Repeat Tris-EDTA wash as many times as necessary until the supernatant is clear to light pink. During the initial Tris-EDTA washes, do not combine the contents of the tubes.

4. When most of the hemoglobin is removed, re-suspend the pellets. Pool the contents of the tubes. Remove the dark, fibrinous portion of the pellet by filtering the suspension through a gauze pad and perform a final wash with Tris-EDTA. The pellets may be stored at 2–8 °C if all of the Tris-EDTA washes cannot be conducted in one day.
5. Re-suspend the final pellet (fluffy white layer of membrane) with approximately three times its volume of 0.1% SDS in PBS. Dialyze the suspension for at least 24 h at room temperature against SDS/PBS. Change the dialysis buffer at least two times.
6. Measure the protein content of the dialyzed solution using the BioRad protein assay (see below).
7. If necessary, dilute the SRBC membrane preparation to a concentration of approximately 1 mg/mL with SDS/PBS.
8. Aliquot the SRBC membrane preparation and store at approximately –20 °C for up to 6 months.

3.3. BioRad protein assay

3.3.1. Equipment and reagents

1. Borosilicate glass tubes (capable of holding at least 7 mL)
2. Disposable cuvettes
3. Protein standard—1 mg/mL bovine serum albumin (Sigma #A-7888)
4. Biorad Dye solution
5. Vortex
6. Pipettors—100 µL, 1000 µL and tips
7. 2 mL cryovials
8. Repeat Pipettor (capable of delivering 5.0 mL accurately)
9. Spectrophotometer (e.g., Beckman model DU 7500 spectrophotometer)

3.3.2. Procedure

1. Defrost biologic samples and BSA standard (1 mg/mL BSA). While the samples and BSA standard are defrosting, label tubes for the standard curve (blank, 10, 25, 50, 75, and 100 µg) and biologic samples. Each sample is done in duplicate.
2. Turn on both the visible lamp and the UV.
3. Dilute biologic samples so that the absorbance falls near the middle of the standard curve. For instance, rat and mouse liver peroxisomes are generally diluted 1:10 (900 µL dH₂O: 100 µL unknown) in 2 mL cryovials. For other samples, the appropriate dilutions need to be determined empirically.

4. Dilute the Biorad Dye solution 1:5. Five milliliters of diluted Biorad Dye solution is added to each tube, therefore, 100 mL dye diluted with 400 mL deionized water will be sufficient to assay 100 tubes.
5. Add 50 μ L of diluted unknown and 50 μ L dH₂O to the appropriately labeled borosilicate tubes. The volume of sample assayed may differ depending on the protein concentration of your sample. This must be determined empirically for each sample.
6. Add the appropriate amounts to the standard tubes as shown below:

Standard curve example

STD (μ g)	dH ₂ O (μ L)	BSA (μ L)
0(Blank)	100	0
10	90	10
25	75	25
50	50	50
75	25	75
100	0	100

7. Add 5 mL of diluted Biorad Dye to all tubes. Vortex all tubes. Let tubes incubate for 5 min to allow time for color development.
8. Fill each cuvette 2/3 full and read the standard curve and samples on a spectrophotometer (e.g., Beckman DU 7500 spectrophotometer) at 595 nm for 0.5 s.

3.4. SRBC specific IgM ELISA

3.4.1. Equipment and reagents

1. Flat-bottom microtiter plates
2. Hemoglobin-free SRBC membrane preparation (from Section 3.2)
3. Phosphate-buffered saline (PBS) (Sigma)
4. 0.05% Tween 20 in deionized water (wash solution) (Sigma)
5. 4% milk solution (blocking buffer)
6. 0.05% Tween 20 in PBS (serum diluent)
7. Mouse or rat sera collected 5 or 6 days, respectively, after immunization with SRBC (peak day of primary antibody response observed in our laboratory; each investigator should determine peak day of response in their own laboratory).
8. Horseradish peroxidase conjugated, affinity purified goat anti-rat or anti-mouse IgM (secondary antibody; Accurate Chemical and Scientific Co. or other commercial source).
9. Peroxidase substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt; Sigma)
10. 3% Oxalic acid (stop solution; Sigma)
11. 96-well plate washer (e.g., Dynex Technologies, Inc, Chantilly, VA)
12. 96-well plate reader (e.g., Dynex Technologies, Inc.)
13. Computer software (e.g., Revelation Software, Dynex Technologies, Inc.)
14. Pipettes—5 and 10 mL and pipet-aid
15. Adjustable micropipettes and tips
16. Multi-well pipettor

3.4.2. Procedure (sterility is not necessary)

1. ELISA plate preparation (coating with SRBC membrane prepared in Section 3.2). Plate(s) must be coated and then stored refrigerated for a minimum of 16 h.
 - a. SRBC membrane preparation is thawed and diluted to desired concentration in PBS.
 - b. Flat-bottom microtiter plates are coated with 125 μ L per well of diluted SRBC membrane preparation. Cover and incubate in the refrigerator.
2. Wash and block steps
 - a. On the day of the IgM ELISA, remove the plate from the refrigerator, decant, wash three times with 300 μ L of wash solution using a 96-well plate washer, then blot dry.
 - b. Coat each well with 200 μ L of blocking buffer. Cover and incubate at room temperature for approximately 1 h.
3. Serum preparation/addition
 - a. Dilute the serum samples to the desired starting concentration in serum diluent, preparing a sufficient volume to run a single replicate of each serum. This should be done during the incubation step in 2b above.
 - b. After incubation of the blocking buffer is complete, decant and wash the plate as described in step 2a.
 - c. Add 100 μ L of serum diluent to each well, excluding those wells which will be used as the first wells in the serial dilutions. Column 1 wells are used as blanks and receive no test serum, only 100 μ L of serum diluent. Add 200 μ L per well of the diluted serum sample to the first well in the serial dilutions.
 - d. Initiate the serial dilutions by taking 100 μ L from the well containing the diluted serum sample and performing 2-fold serial dilutions in the remaining wells (there should be a total of 10 dilutions per serum sample). When the serial dilutions are complete, each well should contain 100 μ L of a serum dilution. Cover the plate and incubate for approximately 1 h at room temperature.
4. Secondary antibody addition
 - a. Thaw the secondary antibody and dilute to the desired concentration using blocking buffer during serum incubation. If necessary, store refrigerated until needed.
 - b. After incubation of the serum is complete, decant and wash the plate as described in step 2a.
 - c. Add 100 μ L of the secondary antibody solution to each well. Cover the plate and incubate for approximately 1 h at room temperature.

5. Peroxidase substrate addition

- a. Prepare the peroxidase substrate near the end of step 4c incubation, allowing sufficient time for the tablet(s) to dissolve.
- b. After incubation of the secondary antibody is complete, decant and wash the plate as described in step 2a.

- c. Add 100 μ L of peroxidase substrate to each well. Cover the plate and incubate for approximately 45 min at room temperature.

6. Stop solution addition

- a. Routinely, at the end of the substrate incubation, the plate will be read without adding stop solution. However, when deemed necessary (e.g., if the plate reader

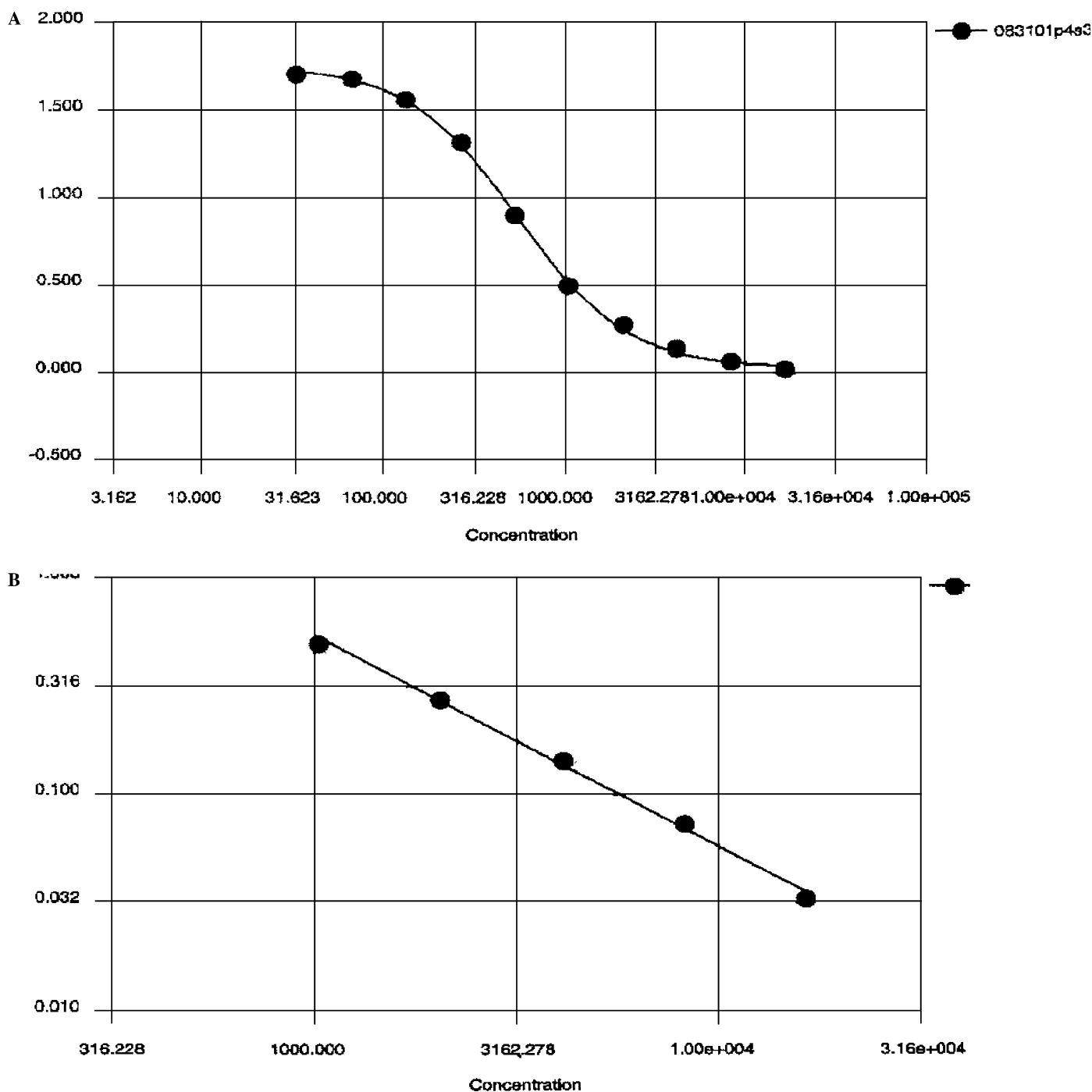


Fig. 3. SRBC-specific IgM ELISA criteria. (A) For each serum sample, create a semi-log graph and determine whether it is S-shaped; (B) Identify linear portion of curve using a log–log curve fit. Select at least one point above and at least one point below 0.5 and using a minimum of three points, obtain a slope not less than -0.600 but not to exceed -1.200 (a slope of -1.000 is optimal). Once the linear region of the curve is determined, interpolate at 0.5 OD. Data expressed as $\log_2$.

malfunctions), 50 μ L stop solution may be added at the end of the substrate incubation prior to reading the plate.

7. Reading the plate; data collection; calculations

- Read the plate at 405 nm.
- Collect data using Revelation Software Version 4.21 or equivalent software. The plate mean background value is automatically calculated from the designated blank wells and subtracted out by the program. Routinely, only a single replicate is run for each serum dilution.
- No standard is available. Therefore, for each serum sample, create a semi-log graph and determine whether it is S-shaped; then identify the linear portion of the curve by using a log–log curve fit (Fig. 3). Select at least one point above and at least one point below 0.5 OD and using a minimum of three points, obtain a slope not less than -0.600 but not to exceed -1.200 (a slope of -1.000 is optimal). Once the linear region of the curve is determined, interpolate at 0.5 OD and record the x value in whole units. The x value obtained represents the “titer” of the sample. The “titer” is defined as the reciprocal of the serum dilution which has an OD value of 0.5. If a serum is tested and no points have an OD value of ≥ 0.5 , the reciprocal of the starting serum dilution closest to an OD value of 0.5 will be used as the titer. Convert the serum titer to a $\log_2$ titer using the formula below and record the $\log_2$ titer in thousandth units. The titer and/or $\log_2$ titer data may be reported.

$\log_2$ of the titer is calculated as follows: $\log_2$ titer

$$= \frac{\log_{10}(\text{titer})}{0.301}$$

If a serum does not meet the criteria described above, the serum may be tested again using a starting dilution which may be either higher or lower than the previous starting dilution, or the data for that particular serum may be excluded.

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