



University of Maryland College Park Animal Care and Use Policy

Polyclonal Antibody Production Guidelines

Introduction

Since the commonly used Freund’s adjuvants can result in significant pain or stress, investigators are expected to consider alternatives such as Ribi Adjuvant System®, and Hunter’s TiterMax®. Investigators should consider using commercial vendors as a source of polyclonal antibodies. Although many adjuvants are non-pharmaceutical grade agents, the pharmaceutical-grade components should be used when available and that methods to preclude bacterial contamination be employed (e.g., antigen filtration prior to mixing with the adjuvant).

Selection of the adjuvant

1. Freund's Mineral Oil Adjuvant Emulsions

Freund's complete adjuvant (FCA) is a mixture of a non-metabolizable oil (mineral oil), a surfactant (mannide monooleate), and killed mycobacteria (Mycobacteria tuberculosis or M. butyricum). Freund's incomplete adjuvant (FIA) is the same as FCA except that it does not contain killed mycobacteria. Freund's adjuvants are prepared as water-in-oil emulsions by combining approximately equal volumes of adjuvant and aqueous antigen solution in such a way that the oil becomes the continuous phase. If properly mixed, the antigen will be distributed over a large surface area, which increases the potential for interaction with relevant antigen presenting cells in vivo. Moreover, like other water-in-oil adjuvants, FCA and FIA exert an antigenic depot effect and therefore sequester and release the antigen over long periods of time, thus resulting in a prolonged antibody response. Both formulations can result in adverse reactions resulting from the stimulation of cell-mediated immune responses. FCA is particularly toxic when injected into laboratory animals because the host response to the non-metabolizable oil and the mycobacteria can result in both local and disseminated granulomatous reactions. Less severe inflammatory reactions result when: a) the concentration of mycobacteria in FCA is less than 0.5 mg/ml; b) more concentrated aqueous antigen solutions are added, resulting in an antigen-rich emulsion and reduction in the quantity of emulsion injected; c) multiple injection sites with minimal volumes of emulsion are injected at any one site; and d) the injection sites are separated to avoid fusion of inflammatory lesions.

In addition, FCA is to be used only for weakly immunogenic antigens and only for initial immunizations. If booster immunizations are necessary, FIA must be used instead of FCA. The recommended volumes per of CFA-Antigen Emulsion per site and route are as follows:

Species	Subcutaneous	Intramuscular	Footpad	Intradermal	Intraperitoneal
Mouse	0.1 ml/site 4 sites max	Not Permitted	0.05 ml max *Justification Required	0.05 ml/site 4 sites max	0.25 ml max
Rat	0.2 ml/site 4 sites max	Not Permitted	0.1 ml max *Justification Required	0.05 ml/site 4 sites max	0.5 ml max
Rabbit	0.25 ml/site 4 sites max	0.25 ml/site 4 sites max Justification Required	**Not Recommended	0.05 ml/site 1.0 ml total	Not Permitted

*Justification required for use; only one foot; only hind foot

** Rabbits do not have a true footpad

2. Ribi Adjuvant Systems (RAS)

The manufacturer offers different mixtures of oil, detergent, and immunostimulator(s) from which to choose. Three RAS formulations, each containing different detoxified bacterial products as immunostimulators, are commercially available: a) mycobacterial trehalose dimycolate (TDM) emulsion recommended for use with strong immunogens in mice, guinea pigs, and rats; b) Gram-negative bacterial monophosphoryl lipid A (MPL®) + TDM emulsion recommended for use in mice, guinea pigs, and rats; and c) MPL® + TDM + mycobacterial cell wall skeleton (CWS) emulsion recommended for use in rabbits and large animals (e.g., goats). Adding aqueous antigen to the adjuvant and vortexing the mixture forms stable oil-in-water emulsions. In general, Ribi's adjuvant emulsions, like other oil-in-water emulsions, are more suitable for hydrophobic or amphipathic protein antigens than for hydrophilic proteins since the effectiveness of RAS is dependent on antigen absorption to the oil droplets in the oil-in-water emulsion. The inflammatory response associated with adjuvant use is minimized by utilizing metabolizable oil (squalene) and detoxified bacterial components as immunostimulators. Furthermore, since significantly less oil is needed to form stable oil-in-water emulsions (e.g., RAS) than for stable water-in-oil emulsions (e.g., Freund's-type adjuvants), Ribi's adjuvant causes less tissue damage when injected into animals. It should be noted, however, that since RAS has less of an antigenic depot effect than water-in-oil emulsions, more frequent booster injections are usually needed for an adequate antibody response. Immunization protocols recommended by the RAS manufacturer are:

*MPL® + TDM Emulsion, R-700 or TDM, R-760**

Mouse: 0.2 ml dose, 0.1 ml administered subcutaneously in two sites

Guinea Pig, Rat: 0.4 ml dose, 0.2 ml administered subcutaneously in two sites

*Use with strong immunogens

Inject on day 0, boost on day 21, and test bleed on day 26 or 28. If necessary, booster injections may be given every 21 days using the same formulation. For monoclonal antibodies, boost intravenously (or intraperitoneally) with saline/antigen only 14 days after last adjuvant/antigen injection. Remove spleen for fusion three days following intravenous injection.

MPL® + TDM + CWS Emulsion, R-730

Rabbit: 1.0 ml dose, 0.05 ml administered intradermally in six sites, 0.3 ml administered intramuscularly into each hind leg, and 0.1 ml administered subcutaneously in the neck region.

Inject on day 0, boost on day 28, and test bleed on day 38 and 42. If necessary, booster injections may be given every 28 days using the same formulation. Note: Injecting more frequently than every 28 days may result in a reduction in the immune response.

3. Hunter's TiterMax®

TiterMax® is a water-in-oil emulsion, similar to Freund's adjuvants, containing metabolizable oil (squalene) and a non-ionic surfactant (copolymer of polyoxyethylene and polyoxypropylene). Most adjuvant activity is attributed to the surfactant's ability to activate and bind certain complement components, which putatively target the antigen to follicular dendritic cells in the spleen and lymph nodes.

Some studies suggest that TiterMax® is superior to Freund's adjuvants with some protein antigens, particularly in rabbits and mice. Compared with FCA, TiterMax® can be used in smaller quantities for initial injection, which minimizes the inflammatory reaction at the injection site(s), and less frequent booster injections are required.

Recommended routes of administration and injection:

Species	Injection Route	Total Injections	Volume per Injection
Mouse	IM	2	0.02 ml
	SubQ	1	0.04 ml
Rat	IM	2	0.05 ml
Guinea Pig	IM	2	0.05 ml
	SubQ	4	0.05 ml
Rabbit	IM	2	0.04 ml
	SubQ	4	0.10 ml
	ID	10	0.04 ml

4. Montanide ISA Adjuvants®

A group of oil/surfactant-based adjuvants where surfactants are combined with a non-metabolizable and/or metabolizable oil. Components undergo quality control to protect against contaminants yielding excessive inflammation. Performance of the ISA 50 and ISA 70 were found to be similar to Freund's incomplete adjuvant for antibody production, but with less inflammatory response. The surfactant in ISA 50 and ISA 70 is a major component of the Freund's adjuvant surfactant, mannide oleate.

5. Syntex Adjuvant Formulation (SAF)®

Recently developed as a Freund's complete adjuvant alternative, this pre-formed oil-in-water emulsion is stabilized by Tween 80 and pluronic polyoxyethylene/olyoxypropylene block copolymer L121. SAF uses a low toxicity, high stimulatory derivative of muramyl dipeptide, thr-MDP, and the metabolizable oil squalene.

Antigen Preparation

Antigen preparations should be free of extraneous microbial contamination. Millipore filtration of the antigen prior to mixing with the adjuvant is recommended. The presence of byproducts, such as polyacrylamide gel, should be avoided because of their inflammatory or toxic properties. Avoid pH extremes, particulate matter, and contamination with chemicals such as SDS, urea, acetic acid, or other solvents or potentially toxic agents. Special precautions may be necessary if the antigen is a viable microbe.

Antigen-Adjuvant Emulsions

If FCA is used, the mycobacteria should first be resuspended by vortexing or shaking. One part or less of Freund's adjuvant to one part antigen (v/v) is recommended. Two sterile luer-lock syringes, one containing Freund's adjuvant and one containing the antigen, preferably in sterile saline, are used for these purposes. Glass syringes or plastic disposable syringes without rubber plungers are preferred as the oil reacts with the rubber plunger on plastic disposable syringes. The antigen solution is injected into the adjuvant through a 3-way stopcock or mixing cannula, and the emulsion is prepared by pushing the solution back and forth between the syringes for several minutes. An emulsion is properly prepared when it becomes thick, is difficult to inject back and forth through the cannula, and will not separate on standing; a droplet placed into a saline solution should not disperse. Emulsification is enhanced by using cold (4°C) adjuvant. Failure of the preparation to emulsify may be due to antigen contamination with SDS or organic solvents. For antigen-adjuvant preparations using adjuvants other than Freund's, manufacturer's instructions must be followed.

Animal Selection

Rabbits are the most commonly used laboratory animal species for polyclonal antibody production. Because of the inherent value of these experiments and the animals in which they are conducted, combined with the stress associated with these immunization protocols, specific-pathogen free rabbits must be used. These animals are readily available commercially and their use dramatically reduces the morbidity and mortality frequently documented in rabbits infected with microbial pathogens, especially *Pasteurella multocida*.

Young (2.5 – 3.0 kg) rabbits are preferred. Older rabbits are not as useful for antibody production as the ability to respond to new antigens declines with age. Female rabbits are more often used due to their docile nature.

Separate animal medical health record must be kept for each rabbit. Information that must be recorded in the animal's record includes, but is not limited to, sedation/anesthetic events, blood collection, compound administration, and euthanasia. When administering any compound, the compound's name, volume administered, and the site(s) where administered must be recorded. Two to three rabbits per antigen are recommended. There may be variability of the antigenic response in different individuals.

Restraint

Use proper restraint methods during immunization and blood collection procedures to avoid animal and personnel injuries. Acclimating animals to handling and restraint procedures prior to the initiation of immunization or other experimental procedures reduces stress in animals and personnel. It is extremely important to train personnel to perform manual restraint techniques and to appropriately use commercially available restrainers. Rabbit sedation with acepromazine during immunization and blood collection reduces stress, enhances vasodilation, and can prevent injury to rabbits and personnel.

Immunization site selection, preparation and administration

Carefully select and prepare the immunization site to preclude unnecessary pain and distress during handling and restraint and to minimize infection. Parts of the animal commonly handled during physical restraint must not be immunized. These areas include the dorsal cervical/scapular and rump areas of rabbits and the dorsal cervical/scapular regions and tail base in rodents. Skin injection sites must be at least 1-3 inches apart, depending on the animal's size.

The site must be prepared in a sterile manner to reduce the likelihood of abscess formation. Adequate preparation of the immunization field can significantly reduce the likelihood of negative consequences. Generously clip the hair and wipe the site with alcohol. Clipping the hair and properly preparing injection sites will reduce the potential for the development of infection or abscess formation and facilitate injection site visualization, thus permitting appropriate treatment of any developing immunization site lesions. The use of sterile needles and syringes is mandatory to minimize microbial contamination.

For injections, it is essential to use a needle with a sufficiently wide bore (gauge) to permit the smooth passage of the emulsion into the animal. Attempts to force the emulsion through a small needle gauge will result in separation of the needle from the syringe and "spattering" of the emulsion.

Investigators proposing to use footpad and/or IP injections must provide scientific rationale to the IACUC for review prior to performing these procedures as part of an approved IACUC protocol. Footpad immunization of rodents or other species should not be used for routine immunizations. Injections may be given subcutaneously in the region of the footpad to achieve the desired immunologic effect. Utilizing the footpad for immunization of small rodents may be necessary in particular studies where isolating a draining lymph node, as a primary action site, is required. The well-being of subject animals should be addressed by procedures such as controlling the quantity of adjuvant instilled in the footpad, the use of only one foot per experimental animal, and housing on soft bedding rather than wire-bottom cages. Front feet should not be used for footpad immunization. If scientific justification is provided, the recommended maximum footpad injection volumes are 0.01-0.05 ml in mice and 0.10 ml for rats.

Post-injection observation

Investigators or their staff must observe animals daily, including weekends and holidays, for the following: pain, off feed, swelling, abscessation, fistula formation, infection, and/or ulceration at or near the immunization site(s). This ensures timely and appropriate immunization site assessment and/or therapy. All observations must be entered into the animal's medical record or on a pain score sheet.

Blood Collection

A pre-immunization blood sample should be obtained because rabbits may have closely related antibodies to something in the environment. Specific blood collection concerns pertaining to polyclonal antibody production include:

- Survival blood samples are generally collected via tail vein or retroorbital sinus from rodents under anesthesia and via the central ear artery in rabbits with appropriate sedation/tranquilization with acepromazine. Saphenous and facial vein use in rodents does not require anesthesia. Isoflurane is commonly used for inhalant anesthesia in rodents, with precision vaporizers located throughout the various ACS facilities.
- Blood collection from rabbit ears by transecting the artery or vein is prohibited, as is rodent blood collection via tail transection or serial tail transection.
- Xylene use is prohibited.
- Because of the risk of cardiac tamponade, pulmonary hemorrhage, and pneumothorax, intracardiac blood collection is limited to terminal procedures and is performed under general anesthesia in both rodents and rabbits.
- Enter the blood volume collected in the animal's medical record, along with any hematocrit results.

Typical schedule

Boosters are given at 2 – 4 week intervals. Blood collection should be done 7 – 10 days following boosters.

Week 1 Pre-immunization bleed
 Inject antigen and adjuvant

Week 3 Boost antigen and adjuvant
Week 5 Boost antigen and adjuvant
Week 6 Bleed

Alternative Techniques

Antibody production in chickens is an alternative to the use of other animals for polyclonal antibody production. Antibody production in chickens offers the advantage of obtaining antibody through a non-invasive method (egg yolk).

Another alternative method in rabbits consists of placing a subcutaneous whiffle ball chamber. Immunizations are made directly into the chamber and antibody-rich fluid is harvested from the chamber. Advantages cited for this technique include greater flexibility in immunogen preparation, minimal discomfort and minimal tissue reaction, ease of immunization and fluid collection, and recovery of large volumes of antibody-rich fluid with low cellularity and absence of lipids. This procedure does require surgical placement of the chamber.