

Antibody Production/Neoplasia

Item 2 – Guidelines for the Production of Polyclonal and Monoclonal Antibodies in Rodents and Rabbits

Monoclonal and polyclonal antibodies are important reagents utilized in a variety of experimental techniques in almost every scientific discipline. *In vitro* methods have been successfully developed for large-scale monoclonal antibody production; however, animals continue to be an important source of monoclonal and polyclonal antibodies, especially in the research setting. The purpose of these guidelines is to provide methods with demonstrated success that also minimize pain and distress for the laboratory animals employed in these techniques when *in vitro* methods are not appropriate.

Adjuvants

Immunogens (substances to which antibodies are desired) are rarely sufficiently antigenic to directly induce a satisfactory immune response; therefore, they are usually administered in conjunction with adjuvants to enhance the inflammatory response at the site of administration. Historically, the single most commonly used adjuvant in the research setting has been Freund's adjuvant.

Freund's adjuvant is a water-in-oil emulsion consisting primarily of mineral oil. The resultant emulsion is very viscous and can be difficult to inject. Freund's adjuvants are available in two forms: **complete** which contains killed *Mycobacterium tuberculosis*, or **incomplete** without the additional bacterial component. The oil acts as a repository which releases the immunogen over time. The mycobacterial cell wall is a potent immune enhancer. Freund's Complete Adjuvant (FCA) is extremely inflammatory and may only be used once. Anaphylactic reactions can occur when FCA is used more than once and these reactions may be fatal. **Only Incomplete Freund's Adjuvant should be used for booster immunizations.**

FCA is known to commonly produce undesirable side effects. Granuloma formation, tissue necrosis and sloughing, abscessation and fever are routinely seen. Other deleterious systemic effects, such as polyarteritis, have been reported. FCA is considered a human hazard since accidental self-inoculation or splashing in the eye have been shown to cause painful lesions not easily treated, as well as sensitization to tuberculin which negates future skin testing.

As a result of investigation into alternative adjuvants for clinical use and studies into the mechanism of action of adjuvants, new agents, some of which are purified products, have been developed. Many of these new adjuvants are suitable and highly effective for research use. A few examples include Hunter's TiterMax, *Bordetella pertussis*, *E. coli* LPS, liposomes, the RIBI adjuvant system and Adjuvax. Although it is not within the scope of this document to discuss all of the many excellent alternatives which are currently available, Hunter's TiterMax and RIBI adjuvant system are two examples worthy of discussion.

Hunter's TiterMax combines the benefits of a potent synthetic adjuvant (copolymer CRL8-41) with those of a microparticulate-stabilized, water-in-oil emulsion containing a metabolizable non-toxic oil, squalene. TiterMax solves the toxicity problem posed by Freund's because it contains no protein, peptide, bacterial or plant component. TiterMax contains no mineral oil or *Mycobacterium tuberculosis*; therefore it will not cause tuberculin hypersensitivity, granulomas or adjuvant arthritis.

The RIBI adjuvant system (RAS) developed by RIBI ImmunoChem Research, Inc., has been used successfully as an alternative to Freund's adjuvant. RAS is an oil-in-water emulsion and is less viscous, therefore easier to handle, than Freund's. Similar to TiterMax, the metabolizable oil employed is squalene. RAS includes the following two immunoenhancers: 1) monophosphoral lipid A (MPL) which is the minimal structure obtained from Gram-negative lipopolysaccharide endotoxin to retain immunostimulating properties without the toxic side effects and 2) trehalose dimycolate, a derivative from *Mycobacterium tuberculosis* which has potent adjuvant activities without allergenic and toxic side effects.

The source of antigen preparation must be considered before selecting an adjuvant and immunizing an animal subject. Many immunogens are identified and isolated from polyacrylamide gels. Ideally, the immunogen should be eluted from the gel before immunization. If this is not possible, the gel should be trimmed to minimize the amount of gel administered. Polyacrylamide is inflammatory and has adjuvant properties. It should **never** be administered in conjunction with Freund's as it is too inflammatory and could result in severe, painful reactions. The same is also true for nitrocellulose. Only Incomplete Freund's or other less inflammatory adjuvants should be utilized in conjunction with either polyacrylamide or nitrocellulose administration.

Immunization Techniques

The quantity and quality of the antibodies produced will be affected by a number of factors: the site of injection, amount administered per site, amount administered per animal, adjuvant system used, and the frequency and total number of booster immunizations. One must also recognize that these factors impact on the animal subjects. Specific recommendations for immunization techniques are provided in Appendix I which will typically induce high-quality antibody production while minimizing the adverse effects on the animal.

Subcutaneous administration is the preferred route. When administering subcutaneous injections, the use of multiple injection sites containing small volumes is more beneficial from both a humane and scientific perspective. This distributes the immunogen over a larger surface area allowing for more processing by the immune system resulting in higher titers while reducing the incidence of severe local inflammatory response and abscessation. Intraperitoneal immunization is permissible in small rodents and should only be administered as a single injection. Deep intramuscular injections are discouraged in all species as the resultant inflammatory response may result in pain during ambulation. Intradermal and footpad injections are highly discouraged and **must be specifically requested and approved by the Institutional Animal Care and Use Committee (IACUC)**.

There is no specific recommendation limiting the number of booster immunizations. Frequency of administration should be based on the rationale that time is required for animals to process immunogens and mount a response. Animals should not be boosted if adverse effects from prior immunizations are apparent. Analgesia may be necessary at any time if painful, adverse reactions occur. Any animal displaying behavioral changes or lesions should be brought to the attention of the veterinary staff immediately.

Bleeding Techniques

The collection of blood for antibody harvest is a critical aspect of the immunization technique. The goal of collection is to obtain a suitable volume while minimizing physiologic effects on the animal.

The volume of blood that can be collected is limited by the size of the subject. A **maximum** blood volume equal to 1% of the animal's body weight should be collected with a **minimum** interval of three weeks between collections. Collection of this volume will minimize hypovolemia and allow the animal time to regenerate red cell mass prior to the next collection. If volumes of blood greater than these are collected, the animal may go into shock. If the frequency of collection is shortened, anemia may develop. Specific recommendations for collection volumes by species are provided in Appendix I.

The recommended technique for collecting blood is determined by species. For survival collection in rodents, the orbital venous sinus or plexus, lateral tail veins or ventral tail artery are all suitable. Rodents **must** be anesthetized prior to orbital bleeding as this is considered a painful/distressful procedure (see Appendix II for Rodent Anesthesia Cocktail). Rabbits are routinely bled from the marginal ear vein or

central auricular artery. Acepromazine is commonly used for sedation and vessel dilation prior to auricular bleeding in rabbits (see Appendix II for dosages). The use of xylene for vasodilation and “nicking” the ear vein for blood collection are **not allowed**. At euthanasia, larger volumes of blood may be collected (up to 70% of the total blood volume possible, depending on species). Cardiac puncture is **only** allowed as a terminal procedure under general anesthesia (see Appendix II for dosages).

If unfamiliar with any of these techniques or if using a different species than those described, please contact the Department of Laboratory Animal Resources (DLAR) at 464-6563 to schedule consultation and/or training. For a fee, DLAR also provides complete polyclonal antibody production services to Upstate investigators; performing all immunizations and blood collections. Contact DLAR at the number above for more specific information on this service.

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Appendix I

RECOMMENDED PROCEDURES FOR IMMUNIZATION OF RABBITS AND RODENTS

Note: DLAR staff can provide all immunizations and blood sample collections.

Immunization

RODENTS:

1. Injection site should be shaved and cleansed with an alcohol swab (70%) to reduce potential for infection. Use only sterile needles for administering the adjuvant/antigen inoculum. Adjuvant/immunogen emulsion should be in a 1:1 ratio.
2. Injection Sites:

Subcutaneous (preferred)
Intraperitoneal
Intravenous (without adjuvant)
3. Injection Volumes (total volume should not exceed 0.5 ml):

Subcutaneous: ≤ 0.2 ml per site (2-4 sites)
Intraperitoneal: < 0.25 ml (mouse); < 0.5 ml (rat)
Intravenous: < 0.5 ml
4. Intradermal or footpad injections are highly discouraged and require prior specific approval by the IACUC.
5. Alternatives to Freund's Complete Adjuvant, such as the RIBI adjuvant system or Hunter's TiterMax should be considered.
6. If Freund's Complete Adjuvant must be used, it should only be used once. Subsequent boosters should employ only Incomplete Freund's.
7. Immunogen should be separated from polyacrylamide or nitrocellulose if possible. If not, the amount of adjuvant should be reduced. Complete Freund's should never be used with polyacrylamide or nitrocellulose.

RABBITS:

1. Injection site should be shaved and cleansed with an alcohol swab (70%) to reduce potential for infection. Use only sterile needles for administering the adjuvant/antigen inoculum. Adjuvant/immunogen emulsion should be in a 1:1 ratio.
2. Injection Sites:

Subcutaneous (preferred)
Intravenous (without adjuvant)
3. Injection Volumes (total volume should not exceed 1-2 ml):

Subcutaneous: ≤ 0.5 ml per site (3-5 sites)
Intravenous: < 2 ml

4. Subcutaneous injections should be given in the dorsal thoracic and lumbar regions. The cervical region should be avoided since the skin in this area is commonly used for handling. Intradermal or footpad injections are highly discouraged and require prior specific approval by the IACUC.
5. Alternatives to Freund's Complete Adjuvant, such as the RIBI adjuvant system or Hunter's TiterMax should be considered.
6. If Freund's Complete Adjuvant must be used, it should only be used once. Subsequent boosters should employ only Incomplete Freund's.
7. Immunogen should be separated from polyacrylamide or nitrocellulose if possible. If not, the amount of adjuvant should be reduced. Complete Freund's should never be used with polyacrylamide or nitrocellulose.

Bleeding Techniques

Only a maximum of 1% of the animal's body weight should be collected at one time (unless terminal). There should be at least 3 weeks between collections.

RODENTS:

1. Submandibular: Preferred method. 20-gauge needle or lancet. No anesthesia required.
2. Lateral Tail Vein: 25 or 26-gauge needle and syringe. The use of a tranquilizing agent to relax the rodent and promote vasodilation is recommended.
3. Retro-orbital: Hematocrit tube or Pasteur pipette. General anesthesia required. IACUC approval required.
4. Cardiac Puncture: Terminal procedure only. General anesthesia required.

RABBITS:

1. Marginal Ear Vein or Central Auricular Artery: 21-25-gauge butterfly needle and syringe. The use of a tranquilizing agent to relax the rabbit and promote vasodilation is recommended.
2. Cardiac Puncture: Terminal procedure only. General anesthesia required.

Appendix II

RODENT ANESTHESIA COCKTAIL AND RABBIT TRANQUILIZATION

RODENT ANESTHESIA COCKTAIL

Take 10 ml sterile vial and mix drugs as below:

Ketamine (100 mg/ml) 1.2 ml

Xylazine (100 mg/ml) 0.1 ml

Sterile Saline 8.2 ml

Label bottle (K/X) with earliest expiry date of any drug/saline used to make cocktail.

Mice: 120 mg/kg ketamine and 10 mg/kg xylazine

Suggested dose of administration: (For accurate dosing, weigh mice right before procedure.)

Mouse weight in grams	Dose for intraperitoneal (I.P.) injection
10 gm	0.1 ml
20 gm	0.2 ml
30 gm	0.3 ml

Rat: Suggested dose of administration:

Rat weight in grams	Dose for intraperitoneal (I.P.) injection
100 gm	0.7 ml
200 gm	1.4 ml
300 gm	2.1 ml

Note: Ketamine is a DEA III scheduled drug and must be kept in double-locked area with appropriate records of administration

- Calculated dose **usually** provides 45-60 minutes of surgical plane of anesthesia (each animal is different and dosage may have to be adjusted)
- For non-invasive procedures (imaging etc.) **three quarters** of calculated dose may be used.
- If you know/suspect your strain of mice is sensitive to K/X, start with only **three quarters** of calculated dose.
- If re-dosing is necessary, use only **one quarter** of the calculated cocktail to prevent the risk of anesthetic overdose leading to death.
- Atipamizole at 1-1.5 mg/kg could be used to reverse xylazine.
- Atropine may be administered at dose of 0.04 mg/kg SQ, 5-10 minutes prior to K/X cocktail administration, to prevent significant cardiac depression.
- Yobine (Yohimbine 2 mg/ml) at a dosage of 102 mg/kg I.P. could be used to reverse xylazine. Effect usually rapid, within 1-3 minutes.

RABBIT TRANQUILIZATION

Acepromazine (10 mg/ml) at a dosage of 2 mg/kg (SQ or IM) **or** 1 mg/kg (IV)

This is given 10-15 minutes prior to ear vein or artery bleeding. Will provide sedation to the rabbit and cause vasodilation, making blood collection easier for both rabbit and collector.

(Rev 6/10/21)