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

RESEARCH ANIMAL RESOURCE CENTER
MEMORIAL SLOAN-KETTERING CANCER CENTER
WEILL CORNELL MEDICAL COLLEGE

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Monoclonal and polyclonal antibodies are important reagents utilized in a variety of experimental techniques in almost every scientific discipline. Additionally, the application of monoclonal antibodies to clinical use in both diagnostic and treatment arenas is increasing. Historically the production of antibodies has been based as much on hearsay as it has on carefully evaluated scientific study. Many of the methods employed have changed little since their first description. Animals continue to be an important source of monoclonal and polyclonal antibodies especially in the research setting. However, in vitro methods have been successfully developed for production of monoclonal antibodies from hybridomas in both large and small-scale systems. MSKCC has a core laboratory that produces and purifies monoclonal antibodies from established hybridomas as well as assists researchers in the development of new antibodies that meet their scientific needs. Please call (646) 888-2331 or visit their website at https://www.mskcc.org/research-advantage/core-facilities/monoclonal-antibody-core-facility for additional information. The purpose of these Guidelines is to provide methods with demonstrated success that also minimize pain and distress for the laboratory animals utilized for antibody production.

Before antibody production is initiated, careful consideration should be given to the species selected, time frame, and materials required. The use of alternative methods should also be considered, such as phage display. For example, many successful in vitro techniques have been developed to produce antibodies with comparable affinity and function to those produced in vivo as ascites. Antibodies to many targets are available commercially and may preclude the need to use animals for further antibody production.

ANTIGENS/IMMUNOGENS

A robust humoral response can be generated by presenting the target to the host in different ways, most commonly as purified proteins or peptides. Peptides are generally not immunogenic so they are typically conjugated to highly immunogenic proteins, such as keyhole limpet hemocyanin or diphtheria-toxoid so they can be recognized by the immune system. To present the native protein, cells containing the antigen or a DNA immunization protocol in which the protein is expressed by the host, can be used. The reference, Antibodies, A Laboratory Manual (Second Edition) is a comprehensive reference for generating antibodies and outlines the advantages and limitations of each approach.

Most immunogens are prepared and presented in a buffer. When the buffer has toxic components, they need to be 10 fold less than the LD50 and the animals should be observed frequently for known toxic effects.

ADJUVANTS

Adjuvants are vehicles employed to enhance the immune response. Substances to which antibodies are desired--immunogens--are rarely sufficiently antigenic to induce a satisfactory immune response. Therefore these immunogens are administered in conjunction with adjuvants to enhance the response. Adjuvants work through a number of mechanisms but many cause a moderate to
severe inflammatory response at the site of administration. Freund's adjuvant, which is commonly used in the research setting, is extremely effective but may produce painful inflammatory side effects if not administered properly. 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 mycobacteria, 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. Complete Freund's adjuvant (CFA) is extremely inflammatory and alternatives to this adjuvant should always be considered. When use is scientifically justifiable, CFA should only be used once. Only Freund's incomplete adjuvant should be used for booster immunizations. Repeat administration of the mycobacterial antigens in CFA frequently leads to abscessation. Custom formulations of CFA, containing more than 0.1 mg/ml of mycobacteria, should not be utilized unless scientifically justified.

Investigations into alternative adjuvants for clinical use and studies investigating the mechanism of adjuvant action have led to the development of several new agents. It is not within the scope of this document to discuss all of the excellent alternatives that are available, such as the Sigma Adjuvant System® (formerly known as RIBI adjuvant system), TiterMax, Adjuplex™ Vaccine Adjuvant, and Alum), the first two are discussed here and select references are provided in the bibliography. A Research Animal Resource Center (RARC) veterinarian can also be contacted for additional information at RARC_VS@mskcc.org or RARC_VS@med.cornell.edu as well as the Antibody and Bioresource Core Facility at skiabcf@mskcc.org.

1) The Sigma Adjuvant System® (formerly known as RIBI adjuvant system) was developed by RIBI ImmunoChem Research Inc., Hamilton, MT has been used successfully as an alternative to Freund's adjuvants. RAS is an oil-in-water emulsion, and is less viscous and easier to handle than Freund's. The oil employed is squalene and is metabolizable, in contrast to the mineral oil used in Freund’s. RAS employs two immunoenhancers in their system: a.) monophosphoral lipid A (MPL), the minimal structure obtained from Gram-negative lipopolysacharide endotoxin which retains immunostimulating properties without the toxic side effects, and b.) trehalose dimycolate, a derivative from Mycobacterium tuberculosis which has potent adjuvant activities without allergenic and toxic side effects. Although RAS is more expensive than Freund's it has been shown to be more effective in particular species in particular research applications.

2) Titermax, distributed by CytRx Corporation, Norcross, GA, is a copolymer consisting of blocks of hydrophilic polyoxyethylene and hydrophobic polyoxypropylene, which when mixed with squalene, water and a microparticulate stabilizer results in a water-in-oil emulsion. The copolymers fold such that the hydrophobic portion is attached to the surface of the oil droplet while the adhesive hydrophilic portion extends from the surface into the water phase. The hydrophilic portion binds antigen, complement, and cytokines which are presented to antigen presenting cells, enhancing the immune response. The emulsion provides a repository for slow release of the materials. Titermax has been shown to be effective in a number of immunization regimens.

The source of antigen preparation must be considered before selecting an adjuvant and immunizing the animal subject. Many immunogens are identified and isolated from polycrylamide gels. Ideally the immunogen should be eluted from the gel before immunization. If elution is not possible, the gel should be trimmed so that the least amount of gel will be administered. Millipore
filtration of the gel may also be used to decrease the amount of gel present. The gel can also be lyophilized and re-suspended in sterile saline. If lyophilization is not possible, the gel should be ground into the smallest possible pieces, using a tissue grinder, before administering in sterile saline or incomplete Freund’s. Polyacrylamide is inflammatory and has adjuvant properties. It should never be administered in conjunction with complete Freund’s as it is too inflammatory and painful. The same is true for nitrocellulose; only incomplete Freund’s or other non-inflammatory adjuvants should be utilized in conjunction with its administration.

Regardless of the adjuvant used, the following general principles should always be followed. The adjuvant should be stored according to manufacturer guidelines. The antigen-adjuvant emulsion must be prepared using sterile technique and must be injected aseptically to prevent contamination and potential abscessation. Adverse effects should be anticipated and monitored for and appropriate treatment instituted as soon as necessary.

Many adjuvants create local inflammation at the injection site and/or may cause masses that can resemble tumors. In these cases, many of the principles in RARC’s “Guidelines for the Utilization of Rodents in Experimental Neoplasia and Ascites Production” will also be applicable, considering that these masses can occupy a significant amount of space. Masses should not exceed 10% of body weight and any masses that are ulcerated or impeding movement are likely compromising the animal’s welfare. In these cases, humane euthanasia is recommended unless these conditions are scientifically justified and are explicitly stated in the animal use protocol and approved by the Institutional Animal Care and Use Committee (IACUC).

IMMUNIZATION TECHNIQUES

The site of injection, the amount of material administered per site and per animal, the adjuvant system utilized, and the frequency and total number of booster immunizations will affect the quantity and quality of antibodies harvested. The site(s) of injection should be chosen with care in order to avoid areas that may compromise the normal movement or handling of the animal (e.g., intradermal injections in the neck scruff of a rabbit). The recommendations for immunization techniques provided in Appendix I ensure high quality antibody production while limiting the adverse effects on the animal.

When administering subcutaneous and/or intradermal injections, the use of multiple injection sites containing small volumes of the immunogen/adjuvant is more beneficial from both humane and scientific perspectives. This technique distributes the immunogen/adjuvant over a larger surface area for the immune system to process the antigen resulting in higher titers, but also reduces the incidence of a severe local inflammatory response and abscessation. Injection sites should be sufficiently separated to prevent the potential coalescing of the inflammatory lesions, which may result in disruption of blood supply to the area, with subsequent formation of draining abscesses, or occasionally, tissue sloughing.

Intraperitoneal immunization, which is permissible only in rodents, should only be administered as a single immunization, with the total volume of material administered being limited by the volume of the abdominal cavity. Intraperitoneal immunization in mice and rats is known to induce a local and acute inflammatory reaction, behavioral changes, peritonitis and the development of ascitic fluid, and is generally not recommended for polyclonal antibody production. Subcutaneous
injection is preferred. Deep **intramuscular** immunizations in the rabbit are generally discouraged as immunization in this area may result in the development of fistulous tracks, muscle necrosis, and could result in the resultant inflammatory response may result in increased pain when the rabbit ambulates. The Institutional Animal Care and Use Committee’s at both MSKCC and WCMC, which review animal use protocols, may approve the technique under special situations when scientifically justified. The immunization of footpads in any species also requires scientific justification and specific IACUC approval and scientific justification. If utilized, only rear footpads should be used as rodents heavily rely on their front paws for locomotion and manipulation of food. In most cases, subcutaneous injection or hock immunizations are good substitutes and are less traumatic. Please consult the “**Guidelines for Handling, Restraint, Injection, and Blood Collection from Small Laboratory Animals**” for specific recommendations regarding injection techniques.

There is no recommendation limiting the number of **booster** immunizations. The frequency and number of injections should be minimized and should be based upon the rationale that time is required for animals to process the immunogen and respond accordingly. This response should be monitored by serology. Animals should not receive booster immunizations if adverse effects from a prior immunization are apparent. The pre-existing condition must be resolved before continuing the immunization schedule. If there are indications of prior local reactions, booster injections should be distant from previous injection sites and must never be given at the site of a granuloma or swelling induced by previous injections.

Animals should be monitored for 30-60 minutes following the initial immunization, 24 hours later and then a minimum of three times weekly until the time of sacrifice. Subcutaneous, intramuscular, and intraperitoneal injections are typically more difficult to monitor due to their deeper locations when compared to the intradermal or hock injections routes. For immunization-induced lesions, supportive therapy may include topical cleansing, antibiotic administration, topical vitamin A&D ointment, analgesic administration and/or the administration of antihistamines. Fluid replacement or nutritional supplements may be required if animals have sustained anorexia or decreased fluid intake. Animals that develop inflamed lesions, or that are anorexic, lethargic, or show other signs of discomfort, should be brought to the attention of the veterinary staff utilizing **EnCCoMPass Health** (MSKCC or WCMC).

Immunization protocols often include a final boost immunization to stimulate and prime the B cells for fusion with the myeloma cell line to create hybridomas or to produce a peak immune response at the time of tissue collection. Generally this is done without an adjuvant and is administered IV. In the rare cases when an adjuvant is desired or if the immunogen contains particulates, IP administration should be used. If given IV, it can result in vascular blockage. Although anaphylaxis is not common, administering diphenhydramine at (1mg/kg IP in mice, rats and hamsters or 2 mg/kg SC in rabbits) can mitigate this reaction. Additionally, animals require continuous monitoring for at least 30 minutes following injection. The early signs of anaphylaxis includes hunched posture and ruffled fur, increasing degrees of lethargy, labored respiration and cyanosis. An animal displaying these signs should receive diphenhydramine (1mg/kg IP in mice and rats; 2mg/kg SC in rabbits), and must be monitored for at least another 30 minute to ensure the animal has stabilized and observed again a few hours later. Immediate treatment is important, therefore medication should be readily available at the time of injection. A RARC veterinarian should be consulted immediately if anaphylaxis occurs.
In addition to the methods described above, non-traditional methods have been developed that may be of interest. For example, one alternative to intradermal immunization is the use of a gene gun, a device that uses compressed gas to propel DNA-coated gold beads into the cells of the epidermis. The DNA codes for the antigen of interest, which is recognized by dendritic cells, found in high numbers in the epidermis. The use of a subcutaneously implanted chamber is a potential alternative to long-term blood or ascites collection of polyclonal antibodies. The device is implanted surgically into the subcutaneous space. It collects serum that can be harvested with minimal restraint. Although these techniques require optimization, consideration should be given to using them as they may reduce animal use, minimize pain or distress, and still achieve the desired scientific goals.

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 of undamaged blood while minimizing adverse physiologic effects on the animal subject. The use of a collection technique that allows for future collections is also important.

The volume of blood that can be collected is limited by the size of the subject. Most species of animals can tolerate losing ≤15% of their total blood volume every two weeks (approximately 10ml/kg body weight). Collection of this volume will minimize hypovolemia and allow the animal to regenerate red cell mass before the next collection. Excessive frequency of bleeding or volume collection may cause anemia, or even hypovolemic shock in the animal. Specific collection volumes by species are provided in the Appendix. At sacrifice, larger blood volumes are easily attainable. Up to 70% of the total blood volume may be obtained depending on the species.

The recommended blood collection technique is determined by species. For survival collection in rodents, the orbital venous sinus, submandibular or lateral tail veins, or ventral tail artery are all suitable. The use of a cardiac collection technique is suitable in terminal procedures as long as the animal is completely unconscious and under general anesthesia.

Rabbits are routinely bled from either the lateral veins or central artery of the ear. Administration of acepromazine (0.5 mg/kg SC), a phenothiazine tranquilizer, is recommended prior to blood collection in the rabbit. Acepromazine will tranquilize the animal and causes vasodilation facilitating blood collection. The full effect of acepromazine takes 30-45 minutes. Topical anesthetics, such as lidocaine or EMLA cream may also be used. These agents take 30-60 minutes to reach full effect, therefore the use of both a tranquilizer and anesthetic requires advance planning. The use of xylene as a vasodilator or cutting the lateral aspect of the ear with a razor blade for blood collection are strictly forbidden. Oil of Wintergreen should be avoided because of personnel safety issues such as contact sensitivity. There are a number of animal restraining devices available to make collection easier. Contact Veterinary Services for additional information. Please consult the “Guidelines for the Utilization of Anesthetics and Analgesics in Small Laboratory Animals” and the “Guidelines for Handling, Restraint, Injection, and Blood Collection from Small Laboratory Animals” for specific recommendations on inducing general anesthesia and for a description of blood collection techniques. If you are unfamiliar with any of these techniques you may contact the Research Animal Resource Center’s Training Coordinator at rarc-eqa@mskcc.org. rarceqa@med.cornell.edu to schedule training. In addition, RARC staff
are available to perform these techniques, please see the Veterinary Services “Experimental Services Brochure” for more information.

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APPENDIX I

RECOMMENDED PROCEDURES FOR IMMUNIZATION OF RABBITS AND RODENTS

Immunization

Rodents:

1. Investigators should consider alternatives to complete Freund’s adjuvants such as muramyl dipeptide, B. pertussis antigen, Sigma Adjuvant System®, Titermax, etc. because they may be more or equally as effective as Freund’s adjuvants while causing less inflammation.
   - If the use of Complete Freund’s adjuvant (CFA) is required, it should be used only once. Subsequent boosters should employ incomplete Freund’s adjuvant. CFA should not contain more than 1 mg/ml killed mycobacterium.

2. Recommended injection sites: subcutaneous; intradermal; intraperitoneal (not with CFA); intravenous (no adjuvant).

3. Chemical restraint may be useful, especially with intradermal injections. If opting for sedation or anesthesia, guidelines can be found in RARC’s Guidelines for the Utilization of Anesthetics and Analgesics in Small Laboratory Animals.

Rabbits:

1. If the use of Complete Freund’s adjuvant (CFA) is required, it should be used only once; subsequent boosters should employ incomplete Freund's adjuvant. CFA should not contain more than 0.1 mg/ml killed mycobacterium.

2. Clipping hair from the injection area and aseptic preparation of the skin is required if immunizing intradermally or when using CFA.

3. Recommended injection sites: subcutaneous, intradermal, intravenous (no adjuvant); intramuscular (discouraged); foot pad injection is not allowed unless scientifically justified and approved by the IACUC.

4. Subcutaneous and intradermal injections should be given in dorsal thoracic and lumbar regions. Please avoid cervical region as this is where one would pick up the rabbit for handling.

5. Immunogens should be separated from polyacrylamide gels whenever possible or reduce the amount of adjuvant in the emulsion because polyacrylamide is extremely inflammatory.

6. Chemical restraint may be useful, especially when precision is important, such as with intradermal injections. If opting for sedation or anesthesia, guidelines can be found in RARC’s Large Animal Surgery Guidelines.
### Suggested Injection Volumes for Immunizing Animals Antigen Emulsion

<table>
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<tr>
<th>Species</th>
<th>SubQ (ml)</th>
<th>Intradermal (ml)</th>
<th>Intraperitoneal (ml)</th>
<th>Footpad (ml)</th>
<th>Hock (ml)</th>
<th>Intramuscular (ml)</th>
<th>Intravenous (ml)</th>
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<tr>
<td>Mouse</td>
<td>&lt;0.1</td>
<td>*</td>
<td>&lt;0.2**</td>
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<td>&lt;0.05**</td>
<td>&lt;0.05**</td>
<td>&lt;0.05**</td>
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<tr>
<td>Rat</td>
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<td>&lt;0.05**</td>
<td>&lt;0.5</td>
<td>&lt;0.1**</td>
<td>&lt;0.05**</td>
<td>&lt;0.1**</td>
<td>&lt;0.1**</td>
</tr>
<tr>
<td>Rabbit</td>
<td>&lt;0.25</td>
<td>&lt;0.05**</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>&lt;0.25**</td>
</tr>
</tbody>
</table>

*Not Recommended

**Only when justified and with IACUC approval

### Suggested Injection Volumes for Immunizing Animals with Antigen/Adjuvant NOT Containing CFA

<table>
<thead>
<tr>
<th>Species</th>
<th>SubQ (ml)</th>
<th>Intradermal (ml)</th>
<th>Intraperitoneal (ml)</th>
<th>Footpad (ml)</th>
<th>Hock (ml)</th>
<th>Intramuscular (ml)</th>
<th>Intravenous (ml)</th>
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<tbody>
<tr>
<td>Mouse</td>
<td>&lt;0.2</td>
<td>*</td>
<td>&lt;0.5</td>
<td>&lt;0.05**</td>
<td>&lt;0.05**</td>
<td>&lt;0.05**</td>
<td>&lt;0.5</td>
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<tr>
<td>Rat</td>
<td>&lt;0.2</td>
<td>&lt;0.05**</td>
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<td>&lt;0.1**</td>
<td>&lt;1.0</td>
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<tr>
<td>Rabbit</td>
<td>&lt;0.25</td>
<td>&lt;0.1**</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>&lt;0.25**</td>
<td>&lt;2.0</td>
</tr>
</tbody>
</table>

*Not Recommended

**Only when justified and with IACUC approval
Bleeding Techniques

Rodents:
1. Retro-orbital bleeding technique (general anesthesia required).
2. Submandibular blood collection
3. Cardiac puncture (general anesthesia required; terminal only).
4. Tail vein blood collection: Venipuncture can be performed with a needle and syringe, for larger samples in rats, or for mice the lateral tail vein can be nicked with the beveled edge of a needle or a surgical scalpel blade and sample collected using a blood or capillary tube.

Rabbits:
1. Marginal ear vein or central auricular artery by venipuncture (0.5 mg/kg SC of acepromazine should be administered for sedation and vasodilation).
2. Cardiac puncture (general anesthesia; terminal procedure only).
3. Laceration of the ear is not allowed.

Collection volume and frequency

Rodents and rabbits:
1. No more than 15% of the animal's total blood volume should be collected at any one time unless the bleeding procedure is terminal.
2. Bleeding frequency should not exceed 15% of the animal’s total blood volume every two weeks. Smaller volumes can be obtained at more frequent intervals, but should not exceed the limit of 15% every two weeks.

Maximum Blood Collection Volumes for Survival and Terminal Bleeds

<table>
<thead>
<tr>
<th></th>
<th>Total Blood Volume</th>
<th>Maximum Blood Collection Survival¹</th>
<th>Maximum Blood Collection Terminal²</th>
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<tbody>
<tr>
<td>Mouse</td>
<td>78 ml/kg</td>
<td>8-12 ml/kg</td>
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REFERENCES
(SELECT)


