

**THE UNIVERSITY OF MELBOURNE  
ANIMAL WELFARE COMMITTEE**

**GUIDELINES ON THE USE OF FREUND'S ADJUVANT**

**Introduction**

There has been much debate in recent years about the use of Freund's adjuvants for raising antibodies in animals. This is because of the tendency for animals to develop undesirable and painful side-effects such as large inflammatory lesions, tissue necrosis and infections at the site of each injection, and the accidental risk to workers of self inoculation if complete Freund's adjuvants (CFA) is handled carelessly. These effects can be reduced or eliminated by the use of appropriate sites and routes of administration, adequate separation of injection sites, and the use of small amount of inoculum per site. Despite this, investigators are encouraged to use alternative adjuvants where available. When an investigator wishes to use Freund's Adjuvants, proper justification must be given to the AEC

**Policy**

All scientific procedures carried out on animals must comply with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (2004).

**Recommended Method for the Preparation of Freund's Adjuvants**

***Safety Considerations for Workers***

CFA consists of a standard concentration of heat-killed organisms of *Mycobacterium tuberculosis* suspended in oil and an emulsifier.

Some investigators are highly sensitive to CFA (as well as to the various antigens used) and are at considerable risk of developing an acute hypersensitivity reaction as well as a severe local reaction at the site of injection should accidental inoculation occur. For this reason caution must be exercised when handling CFA, whether in preparing the mix or injecting it into the animal. Because the emulsion prepared can be extremely viscous, and there is always the risk of accidental exposure to the harmful affects of CFA when mixing or injecting, protective clothing must be worn ie gloves, a gown and protective glasses or goggles. It is also most important that mixing be carried out using aseptic technique. If accidental inoculation occurs, medical advice should be sought.

### ***Method for Mixing Antigen and Adjuvant [CFA or Incomplete Freund's Adjuvant (IFA)]***

The antigen should be suspended in 0.85% sterile saline (100–300 µg protein/ml). The final ratio for the volumes of antigen and adjuvant should be slightly more than 1:1 in favour of the antigen (some users recommend a ratio of 2:1 or even 3:1 in favour of the antigen). In preparing this mixture, the intention is to form a stable water-in-oil emulsion where the discrete aqueous phase is suspended in microdroplets within the continuous oil phase.

For making small volumes of emulsion (<10 mL), the appropriate Luer-lok glass syringe size fitted with an 18 or 19 gauge needle and bijou bottle can be used. A portion of the aqueous component (less than 20%) is added to the total volume of adjuvant, drawn into the syringe, then vigorously expelled at least 5 times. A second aliquot of the aqueous component is then added as above until all the aqueous component has been incorporated into the emulsion. Alternatively, emulsification can be achieved by using two sterile Luer-lok syringes connected through a sterile three-way tap. The adjuvant /immunogen solution is then forced through repeatedly between the two syringes. Mixing is continued until the emulsion becomes first milky, and then pasty. Insufficient mixing is a major cause of immunisation failure.

For larger volumes, a Sorvall Omnimixer may be used. Total volumes of antigen and adjuvant are added to the bowl and the contents mixed at high speed. Care must be taken to immerse the bowl in an ice-bath while mixing in order to avoid over-heating.

### **Technique for Injecting the Antigen Adjuvant Mix into the Rabbit**

*The only acceptable route for the administration of CFA to rabbits is the subcutaneous route.*

Use of the intradermal and intramuscular routes is no longer permitted. This is because of the risk of ulceration and infection of the skin (intradermal route) and of damaging the sciatic nerve or blood vessels in the hind legs and the fact that any abscess development is not readily detectable by the intramuscular route).

### ***Priming the Animal with CFA***

*A non-immune bleed should be carried out before the primary immunisation. The volume of blood collected should represent no more than 1% of the animal's bodyweight.*

The site/s to be injected should be swabbed with 70% alcohol. No more than 1.0 mL of the CFA emulsion should be injected per rabbit and this should be divided over 4–8 sites. A 21–24 gauge sterile needle should be used and a volume no greater than 0.25 mL may be administered at any one site.

Injection sites must be separated from each other widely enough to ensure continued blood

supply to adjacent area of skin and subcutis. Each site should be massaged gently following injection.

It is important to remember that injections require a reasonable degree of skill to administer and should not be undertaken by a novice without assistance from a trained member of staff.

Dose sites should be located along the postero-dorsal aspect of the rabbit's back (taking care to avoid the scruff of the neck which is used for catching and restraining the animal). The location of dose sites in this manner will help to reduce the risk of infection through the animal scratching, and ensure that any adverse reaction to injections can be readily identified for treatment by staff caring for animals.

### ***Boosting with IFA***

*CFA must never be used more than once in an animal.*

For subsequent injections with IFA the following guidelines apply.

One mL of IFA emulsion should be administered 4–6 weeks following the initial CFA dose, preferably subcutaneously. A test bleed (volume no more than 1% of the animal's bodyweight) from the central ear artery should be taken 7 to 14 days later. In the same manner, further doses and bleeds can be undertaken, provided that immunisation intervals are no less than 4 weeks apart.

The total length of time the animals are to be kept in the immunisation program must be justified to the animal ethics committee and will be considered on a case-by-case basis.

### **Guidelines for the Bleeding of Rabbits**

A baseline pre-immune serum sample should always be taken before work commences. The volume of blood removed should be no more than 1% of the animal's bodyweight for this or any subsequent bleed from a conscious animal. Partial replacement of the volume removed (using for example 0.9% sodium chloride or Hartmann's solution injected subcutaneously) in addition to ensuring that bleeding is carried out every 4-6 weeks only, will greatly facilitate the recovery of animals. For survival bleeds the recommended route of collection is the central ear artery.

For the final bleed-out, a general anaesthetic should be administered and cardiac puncture used. The animal should then be euthanased.

### **Monitoring**

Animals should be checked daily, including weekends and public holidays. The general appearance of the animals should be observed for signs of pain or distress and the injection sites inspected for lesions like swelling, ulceration or abscessation.

If the injection site(s) develop lesions, appropriate treatment and careful monitoring are required.

### **Disposal of Carcasses at the Conclusion of the Experiment**

It is a requirement that any product or part thereof which has been imported into Australia from overseas for use in animals be disposed of appropriately. Carcasses in which Freund's Adjuvants have been used must be double bagged and given to the Animal Facility Manager for disposal through Mediwaste. Adjuvants must not be used in animals destined for the human food chain.

### **Alternatives to CFA**

<http://www.antibodyresource.com/>

<http://research.uiowa.edu/animal/?get=adjuvant>

<http://altweb.jhsph.edu/>