

**CODE OF PRACTICE FOR THE  
IMMUNISATION OF LABORATORY ANIMALS**

Amended second version  
August 2000

Working party on immunisation procedures  
Keuringsdienst van Waren  
(*Inspectorate for Health Protection and Veterinary Public Health*)

This code of practice has been translated into English under the authority of the animal welfare officer of the University of Groningen, the Netherlands, December 2007



## FOREWORD

Immunisation is used on a wide scale in bio-medical, biological and chemical research for the purposes of:

- preparing specific polyclonal immune sera
- generating specific B-cells needed to develop hybridomas for the production of monoclonal antibodies
- research into a) the protective effects of vaccines, b) potential protective antigens and c) vaccine quality control
- basic immunological research
- the induction of disease models.

The immunisation of laboratory animals will always involve a risk of distress. Various factors affect the severity of any distress, including the nature of the antigen, the possible application of an adjuvant and the immunisation method (frequency, injection route and site, volume). In 1990, the former Veterinary Chief Inspectorate set up the Working party on immunisation procedures to look into this. Amongst other tasks, the working party was asked to draw up an inventory of the immunisation procedures being applied throughout the country, the adjuvants being used and the level of distress undergone by the animals. There turned out to be no general consensus about immunisation protocols for the various objectives. Most people were using the procedures that to some extent applied within the research institute concerned or which were described in the relevant literature.

One of the working party's foremost tasks was to compile guidelines for immunising laboratory animals. This resulted in a **Code of Practice for the immunisation of laboratory animals**. The Code was published more than 7 years ago, in 1993, by the former Veterinary Chief Inspectorate and sent to institutes holding permits, animal experimentation experts, researchers and the Committees on Animal Experimentation.

The Code **aims** to lay down guidelines that have been approved by experts, and sets out immunisation procedures whereby an optimum immune response will be achieved with a minimum of distress for the laboratory animals. In this respect, the Code serves as a guide for researchers. The Code can also serve as an important instrument to help Committees on Animal Experimentation and laboratory animal experts assess the admissibility of animal experiments. A Code of this kind is by definition a reflection of the body of scientific insight and experience prevailing at a particular time.

A survey carried out by the Inspectorate amongst laboratory animal experts, researchers, Committees on Animal Experimentation and bio-technicians in 1996 showed that the Code had become effective on a wide scale. In a number of research institutes, the Code has led to discussions about and amendments to immunisation protocols. However, the survey also revealed that many users were not implementing parts of the Code fully and/or correctly.

On 31 March 1998, a symposium entitled 'Immunising laboratory animals: does the Code of Practice need a booster?' was held in Lunteren. This symposium revolved around the question of whether recent experiences and altered scientific insights warranted amending the Code. In view of reactions given by people using the existing Code and the conclusions of the symposium, the Inspectorate decided to devise a second version of the Code. The Inspectorate asked the Working party on immunisation procedures to develop this second version.

The working party currently comprises the following members:

- A.E.J.M. van den Bogaard, Maastricht University
- Ms. C.J.J. Coenen-de Roo, N.V. Organon, Oss

- C.F.M. Hendriksen, RIVM (*Netherlands National Institute for Public Health and the Environment*), Bilthoven
- L.A.T. Hilgers, ID (*Institute for Animal Science and Health*), Lelystad
- Ms. P.P.A.M. Leenaars, RIVM, Bilthoven
- W.A. de Leeuw, Keuringsdienst van Waren Oost (*Inspectorate for Health Protection and Veterinary Public Health East*), Zutphen
- H. Snippe, Utrecht University, Utrecht.

These people are participating in the working party at the request of the Inspectorate and on the basis of their specific expertise. They are, however, acting independently in a personal capacity and are not bound by instructions.

You are now reading the amended second version of the Code of Practice for the Immunisation of Laboratory Animals.

The Inspectorate hopes that this Code will be as well received by the target group as the original version.

It should be emphasized that the Code does not only apply to immunisation for the purpose of producing antibodies. It also applies to immunisation for other purposes, as expressed in the introduction to this foreword. If the research concerned makes it necessary to deviate from the Code, this must be explicitly explained and justified in the relevant research plan.

At the Inspectorate's request, the working party will remain intact to monitor scientific developments critically and, should it be necessary, amend the Code of Practice. The working party will also continue to function as an oracle and sounding board for those using the Code.

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## CODE OF PRACTICE FOR THE IMMUNISATION OF LABORATORY ANIMALS

### 1. GENERAL CONSIDERATIONS

The aim when immunising laboratory animals is to find a method of preparing and administering an antigen that will prompt an optimum immune response. The nature of the optimum response depends on the question being addressed: generating humoral and/or cellular responses or inducing certain diseases, as the case may be. The basic principle of this Code is that users should aspire to a method of immunisation that involves a minimum of distress to the laboratory animals concerned.

### 2. IMMUNISATION PROTOCOL

The aim of immunisation can be:

- to prepare specific polyclonal immune sera
- to generate specific B-cells needed to develop hybridomas for the production of monoclonal antibodies
- research into a) the protective effects of vaccines, b) potential protective antigens and c) vaccine quality control
- basic immunological research
- induction of disease models.

When devising an immunisation protocol, the aim of the immunisation will inevitably affect decisions about the immunisation route, the volume needed, the dosage of the antigen used, the adjuvant<sup>1</sup> needed and the number of times the animals will be immunised.

It goes without saying that procedures relating to experiments with animals may only be carried out by skilled and qualified personnel. The research plan must be drawn up by a researcher qualified in this area.

#### a) using an adjuvant

In the following situations, an adjuvant may be applied to optimise the response:

1. when the antigen has poor immunogenicity
2. when the antigen is not widely available
3. when using autologous proteins
4. when steering an immune response.

When formulating an immunisation protocol, the researcher should make sure that he is aware of the composition of the adjuvant and the negative side effects on the animals in relation to the route of administration (see table 1). If adjuvants causing a serious level of distress in relation to the route of administration are to be used, the case must be argued in the research plan.

The solution or emulsion<sup>2</sup> being injected should preferably be sterile. Preparation should take place aseptically. The needles or cannulas being used must be sterile. The inoculum should preferably be isotonic and at least at room temperature.

For preparing and analysing emulsions, see Appendix 1.

#### b) route, injection site, volume and number of immunisations

Antigens can be administered in an aqueous solution or as an emulsion. The following section describes the most common routes for immunisation, on the assumption of administering aqueous

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<sup>1</sup> A compound or a combination of compounds administered together with an antigen to heighten the immune response to the antigen.

<sup>2</sup> A formulation of at least two immiscible liquids, usually an aqueous and an oil-based substance, whereby droplets of one liquid are dispersed into the other.

solutions and emulsions (see table 2). The basic principle is that the volume being administered should never exceed the maximum permitted amount. Please refer to table 2 for details.

### **Subcutaneous (SC)**

This is the most common administration route and is the best option for routine immunisation. A subcutaneous injection should be given in a loose fold of skin (e.g. neck, flank, groin), but avoiding spots used when handling the animal. In principle, the antigen-adjuvant mixture should always be administered in the same place. Injecting into multiple sites (maximum of 4) or variant injection sites should be explained in the research plan.

### **Oral (PO)**

In mice and rats, an antigen solution should be administered orally by means of a bulbed cannula. The cannula is pushed down into the stomach via the palatum of the mouth. Make sure that the length and width of the cannula are correct. The length can be gauged by measuring from the tip of the nose to the diaphragm, just below the sternum. This method of administration may only be deployed by experienced staff.

### **Intranasal (IN)**

This administration route using an aqueous solution is used for immunisation and inducing tolerance. An antigen in an aqueous environment is administered by means of a pipette or a micro-cannula at the entrance to a nostril or via a droplet onto the nose of an animal held in an upright position. A short-term anaesthetic with isoflurane, halothane or CO<sub>2</sub> must be administered. Ether should not be used.

### **Intracutaneous (IC) / intradermal (ID)**

Emulsions should not be used for intracutaneous injections, as this can cause ulceration. Aqueous solutions can be used for intracutaneous injections. If an intracutaneous injection with an emulsion is strictly necessary, this must be explained in the research plan. Emulsions may only be used for rabbits and larger animals.

The use of intracutaneous injections is subject to four conditions:

- the injection volume may not exceed 0.05 ml per injection site
- if injections are being administered in more than one location, sufficient space must be left between the injection sites to accommodate the expected reaction
- the number of injection sites should be restricted (to a maximum of 4)
- injections should be administered in the animal's back to minimise the risk of ulceration.

### **Intramuscular (IM)**

Intramuscular injections are not the best option for mice, rats and guinea-pigs. If intramuscular injections must be given, the reasons must be explicitly stated in the research plan. In these cases, the thigh muscle is the preferred injection site.

### **Intraperitoneal (IP)**

Aqueous solutions can be administered to all laboratory animals via the intraperitoneal route, for example as an alternative to the intravenous route. In exceptional circumstances, it may be necessary to administer an emulsion via the intraperitoneal route. This is only customary in mice and rats. In view of the possible side effects, the volumes injected in these cases may not exceed 0.2 ml. Other restrictions apply to the use of other adjuvants (see table 1). The use of an intraperitoneal injection for an emulsion must be explained in the research plan.

### **Foot**

Injections into the foot are by definition damaging to laboratory animals! Injections with emulsions in particular can cause serious distress. An antigen may only be injected into the foot if the need to do so can be scientifically argued. Injections should preferably be administered SC via the sole of the foot, as this provides the most room. These injections must be administered by people authorised to do so and with experience of injection routes of this kind.

Only one hind leg per animal may be used for immunisation and the animals must be kept in a cage with extra thick bedding.

### **Intravenous (IV)**

Intravenous injections with emulsions are lethal. Booster immunisations carry an increased risk of lethal allergic reactions. It is recommended that one animal should be tested before giving the booster immunisation to an entire group of animals.

### **Base of the tail**

This route is used, *inter alia*, to induce arthritis in mice and rats. An emulsion or an aqueous antigen solution is injected SC via the tail and across the tendon insertion at the base of the tail.

## **3. INSPECTION AFTER IMMUNISATION**

With regard to monitoring the welfare of the animals concerned, the guidelines incorporated into the Code of Practice for monitoring the welfare of laboratory animals (2000) should be observed. If immunisation is expected to cause serious distress, the laboratory animals must be given extra checks above and beyond the routine daily inspections. In other words, the nature and frequency of the inspections must be modified. A working protocol must be available per experiment for all staff concerned. This working protocol must at least specify the critical points in the experiment, the expected symptoms of distress, the criteria governing the decision to destroy the animals involved and the responsibilities of the various members of staff. Special attention must be paid to aspects such as the general appearance of the animal, physical hygiene, body temperature, food and water intake, weight, injection site, etc. Euthanasia should be considered in the event of serious distress. Modified accommodation is also a possibility. After every immunisation, animals must be observed and if applicable, a post-mortem examination carried out to ascertain whether the predicted degree of distress corresponds with the actual level of distress.

## **4. TAKING BLOOD SAMPLES**

If the aim of the immunisation is to produce antibodies, taking (repeated) blood samples will be an important part of the experiment. A test sample must be taken 7 to 10 days after the booster injection. The titre must be measured in the usual way the same or the next day. If the titre is satisfactory, blood must be taken between 3 days and 4 weeks of the test sample.

Blood samples must be taken in accordance with the BVA/FRAME/RSPCA/UFAW guidelines (1993). These guidelines have general international approval and relate to aspects including the way the blood is taken, the place it is taken, the width of the needle, the maximum volumes that may be taken. In addition to these guidelines, please refer to publications by McGuill and Rowan (1989) and by Bertens et al. (1995) (see table 3).

When maximum volumes are being taken, they may not be taken more frequently than 1 x per 2 weeks.

Table 1

Classification of distress caused by the most commonly used adjuvants in relation to the administration route.

Adjuvants	Distress <sup>a</sup>								
	Route								
	Oral	Intranasal	Subcutaneous	Base of tail	Intraperitoneal	Intramuscular	Intracutaneous	Foot	Intravenous <sup>c</sup>
<b>Emulsions</b>									
Freund complete adjuvant	- <sup>e</sup>	- <sup>e</sup>	3,4	4,5	5	5	5	5	- <sup>d</sup>
Water-in-mineral oil emulsion	- <sup>e</sup>	- <sup>e</sup>	3	4,5	4	4	5	5	- <sup>d</sup>
Freund incomplete adjuvant	- <sup>e</sup>	- <sup>e</sup>	3	4,5	4	4	5	5	- <sup>d</sup>
Specol <sup>TM</sup>	- <sup>e</sup>	- <sup>e</sup>	3	4,5	4	4	5	5	- <sup>d</sup>
Montanide ISA <sup>TM b</sup>	- <sup>e</sup>	- <sup>e</sup>	3	4,5	4	4	5	5	- <sup>d</sup>
Titermax <sup>TM</sup>	- <sup>e</sup>	- <sup>e</sup>	3	4	4	4	4	5	- <sup>d</sup>
Mineral oil-in-water emulsion	- <sup>e</sup>	- <sup>e</sup>	3	4	4	4	5	5	5
Non-mineral oil-in-water emulsion	- <sup>e</sup>	- <sup>e</sup>	2	4	4	3	4	5	5
Squalane-in-water	- <sup>e</sup>	- <sup>e</sup>	3	4	4	4	4	5	5
Ribi adjuvant system <sup>TM</sup>	- <sup>e</sup>	- <sup>e</sup>	3	4	4	4	4	5	5
<b>Insoluble salts</b>									
Al(OH) <sub>3</sub>	1	1	2	2,3	3	3	4	3	5
Al(PO) <sub>4</sub>	1	1	2	2,3	3	3	4	3	5
Aluin	1	1	2	2,3	3	3	4	3	5
<b>Surface-active substances</b>									
DDA	1	1	2	2	2	2	3	3	5
Liposomes	1	1	1	2	1	2	2	3	2
Non-ionic block polymers <sup>b</sup>	1	1	2	2,3	2,3	2,3	3,4	3,4	5
Saponin or Quil A	1	1	2	2,3	2-4	2	4	3	- <sup>d</sup>
ISCOMs	1	1	2	2	2-4	2	2	3	2,3
<b>Polymers</b>									
Polyacryl acids <sup>b</sup>	1	1,2	2	2,3	3	2	3	3	5
Carbopol <sup>TM</sup>	1	1,2	2	2,3	3	2	3	3	5
Polysaccharides <sup>b</sup>	1	1	2	2	2	2	2	2	2-4
Dextran sulphate <sup>c</sup>	1	2	2	2,3	3,4	3	4	4	5
<b>Microbial products</b>									
Lipopolysaccharide <sup>b,c</sup>	1	1	2,3	2,3	3,4	2,3	3,4	3,4	4,5
Glycolipids <sup>b,c</sup>	1	1	2,3	2,3	3,4	2,3	3	3,4	2-5
Glycopeptides <sup>b,c</sup>	1	1	2,3	2,3	3,4	2,3	3	3,4	2-5
<b>Other</b>									
Micro-particles <sup>b,c</sup>	1	1,2	2,3	2-4	2,3	2-4	2-4	3,4	2-4
Cytokines <sup>b,c</sup>	1	1-3	2-4	2-4	2-4	2-4	2-4	2-4	2-4
Aqueous	1	1	1	1	1	1	1	2	1

a distress score: 1 = slight; 2 = slight/moderate; 3 = moderate; 4 = moderate/serious; 5 = serious; 6 = very serious

b distress partly dependent on product specifications.

c distress largely dependent on dosage.

d Water-in-oil emulsions can be administered intravenously.

e Only used in specific situations. Distress dependent on product and dosage. Good observation required.

Table 2a:

Route and maximum volume of the inoculum per injection site for **small laboratory animals****N.B. Keep volumes to a minimum when using emulsions!**

	<b>mouse</b> 20-25 g	<b>rat</b> 250 g	<b>guinea pig</b> 350 g	<b>Rabbit</b> 2.5 kg
<b>oral (PO)</b>	using bulbed cannula			
max. volume (ml)	0.5-1.0	2.0	5.0	7.5
tube gauge (mm)	1.0	2.0	2.0	5.0
<b>intranasal (IN)</b>	in or on nostril, under short anaesthetic			
max. volume (µl)	2*10µl	2*15µl	‡	‡
tube gauge (mm)	0.45	0.6		
<b>subcutaneous (SC)</b>	neck	neck	neck	neck/back
max. volume (ml)	0.5 (aqueous) 0.1 (emulsion)	0.5-1 <sup>1)</sup>	1.0	1.5 (aqueous) 0.25(emulsion)
gauge of needle	26G	25G	25G	24G
<b>intracutaneous (IC)</b>	under the skin on the back			
max. volume (ml)	0.05 per injection site for animal types			
needle gauge	27G-30G	25G	25G	25G
<b>intramuscular (IM)</b>	muscle mass in hind legs (thigh muscle)			
max. volume (ml)	0.05	0.1	0.1	0.2-0.5 <sup>1)</sup>
needle gauge	26G	25G	25G	25G
<b>intraperitoneal (IP)</b>	lateral from the median line at navel level			
max. volume (ml)	1.0	5.0	5-10 <sup>1)</sup>	10-20 <sup>1)</sup> (aqueous)
needle gauge	25G	25G	23G	21G
<b>Foot</b>				
max. volume (ml)	0.05	0.05	n/a	n/a
needle gauge	30G	27G		
<b>intravenous (IV)</b>	tail vein	tail vein	front leg vein	ear vein
max. volume (ml)	0.2	0.5	0.5-1 <sup>1)</sup>	1-5 <sup>1)</sup>
needle gauge	25G-27G	23G-25G	22G-27G	21G-23G
<b>base of tail</b>	SC via the tail to the base of the tail			
max. volume (ml)	0.1	0.2	n/a	n/a
needle gauge	25G	23G-25G		

<sup>1)</sup> depends on the weight of the animal

‡ maximum volume and tube gauge not known

needle gauge = width of needle, referred to as G (=Gauge)

30G = 0.30 mm      25G = 0.50 mm      22G = 0.70 mm      19G=1.00 mm

27G = 0.40 mm      24G = 0.55 mm      21G = 0.80 mm      18G=1.10 mm

26G = 0.45 mm      23G = 0.60 mm      20G = 0.90 mm

Table 2b:

Route and maximum volume of the inoculum per injection site for **large laboratory animals****N.B. When using an emulsion, use as small a volume as possible!**

	hen	dog	sheep	goat	cow
<b>subcutaneous (SC)</b>					
max. volume (ml)	0.5	1.0	2.0	2.0	2.0
needle gauge	23G <sup>1)</sup>	23-25G <sup>1)</sup>	18-21G <sup>1)</sup>	18-21G <sup>1)</sup>	18-21G <sup>1)</sup>
<b>intramuscular(IM)</b>					
max. volume (ml)	1.0	1.0	2.0	2.0	2.0
needle gauge	21-23G <sup>1)</sup>	23-25G <sup>1)</sup>	18-21G <sup>1)</sup>	18-21G <sup>1)</sup>	18-21G <sup>1)</sup>

<sup>1)</sup> depends on the weight of the animal

needle gauge = width of needle, referred to as G( =Gauge)

30G = 0.30 mm

27G = 0.40 mm

26G = 0.45 mm

25G = 0.50 mm

24G = 0.55 mm

23G = 0.60 mm

22G = 0.70 mm

21G = 0.80 mm

20G = 0.90 mm

19G = 1.00 mm

18G = 1.10 mm

Table 3:

Maximum volume of blood that can be removed (Bertens et al., 1995)

Animal type	Volume (ml)
Mouse	0.3
Hamster	0.3
Rat	2.0
Guinea pig	5.0
Rabbit	15#
Cat	20
Dog	100-500#
Monkey	20-200#
Pig	200-500#
Sheep	200-600#
Horse	500-7,000#

#Depends on body weight

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## PREPARATION AND ANALYSIS OF EMULSIONS FOR IMMUNISATION

1. Water-in-oil emulsions  
(e.g. Freund incomplete adjuvant, Freund complete adjuvant, Incomplete Seppic Adjuvant<sup>TM</sup>, and Specol).

Definition of Water-in-oil emulsion:

A formulation of water droplets in an oil phase, whereby the antigen is in the water phase or inner phase as the case may be.

When preparing formulations of water-in-oil, the antigen solution is dispersed in an oil that usually has a mineral basis. One or more detergents are added to the oil to stabilise the emulsion that has been made. There are various ways of preparing an emulsion on a small scale (e.g. by using two injection needles that have been connected). The water content can vary between a few % and 45%. Air in the water or oil phase can have an adverse effect on the quality of the product and the local reaction. The nature of the emulsion can be determined by testing its miscibility with water and oil; one drop of water-in-oil emulsion remains intact in water but disperses or mixes with oil. An impression of the size of the water droplets can be obtained by means of a microscope (400x or 1,000x magnification), whereby the emulsion can be diluted in oil.

2. Oil-in-water emulsions  
(e.g. Ribi Adjuvant System<sup>TM</sup>, Synthex Adjuvant Formulation, MF59 and sulfolipo-polysaccharide/squalane).

Definition of Oil-in-water emulsion:

A formulation of oil droplets in a water phase, whereby the antigen is in the water phase or outer phase as the case may be.

Preparing formulations on the basis of an oil-in-water adjuvant involves preparing the emulsion and then mixing or dissolving the antigen into the emulsion. The emulsion is stabilized by two or more detergents. The oil content can vary between a few % and 40%. The nature of the emulsion can be tested by determining its miscibility with water and oil; one drop of oil-in-water emulsion remains intact in oil but disperses or mixes with water. The size of the oil droplets is an indication of the quality and stability of the emulsion (or vaccine) and can be determined by means of a microscope (400x or 1,000x magnification), whereby the emulsion can be diluted in an aqueous solution.

Emulsions with a limited stability (particularly water-in-oil formulations) should be prepared shortly before they are needed.