



Australian Government
National Health and
Medical Research Council

N H M R C

Guidelines for Monoclonal Antibody Production

2008

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ISBN Online: 1864963468

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Preamble

NHMRC has developed the *Guidelines on Monoclonal Antibody Production* (the Guidelines) to assist institutional Animal Ethics Committees (AECs) in the evaluation of applications involving monoclonal antibody (MAb) production.

These guidelines were developed in response to recognition of the growing need for further emphasis on the principles of *replacement* and *refinement* as they apply to the use of animals for the production of MAbs.

This document should be read in conjunction with the *Australian code of practice for the care and use of animals for scientific purposes, 7th edition, 2004* (the Code). The Code is available from the NHMRC website at: <http://www.nhmrc.gov.au/publications/synopses/ea16syn.htm>

These guidelines are based upon the following primary considerations:

- The production of MAbs using animals involves procedures which have the potential to produce significant pain and distress.
- Section 1.8 of the Code states that ‘Techniques which replace or complement the use of animals in scientific and teaching activities must be sought and used whenever possible.’
- *In vitro* methods exist which can replace the ascites fluid method in most experimental applications without compromising the aims of the study. These methods are available and are better or equal to the ascites method in terms of antibody quality.

There are three stages in the production of MAbs:

- a. The immunisation stage – the induction of antibody-producing cells *in vivo*.
- a. The testing and selection of antibody-producing cells *in vitro*.
- b. The *in vitro* or *in vivo* propagation of selected cells to produce MAbs. *In vitro* cells are propagated in a bioreactor. *In vivo* cells are propagated in mice to generate ascites fluid containing MAbs.

In most circumstances, use of mice will continue to be required in the immunisation stage.

NHMRC advises that the ascites fluid method of monoclonal antibody production is no longer acceptable, except in *extremely* rare cases where *in vitro* methods are shown to be unsuitable.

Accordingly, AECs are expected to critically evaluate any proposed use of the ascites fluid method. Before approval of proposals which include the ascites method, AECs must determine that:

- a. The proposed use is scientifically justified.
- b. *In vitro* methods have been shown to be unsuitable for the specific monoclonal antibody which is the subject of the application, and this has been verified by an independent institution with expertise in the *in vitro* production of MAbs.
- c. Methods that avoid or minimise discomfort, distress and pain have been incorporated into the design of the application.

The guidelines do not accept increased cost of in vitro methods as reasonable justification for the in vivo method. Fulfilment of these AEC responsibilities, with appropriate documentation, is considered central to an institution’s compliance with the Code.

Definitions and abbreviations

Adjuvant	A substance added to a vaccine to improve the immune response so that less vaccine is needed to provide protection or other desired effect such as the production of antibody.
AEC	Animal Ethics Committee
Antibody	A protein substance produced by the body's immune system in response to a foreign substance, or antigen, and which binds specifically to that particular substance and specific sites or antigenic determinants on that substance.
Antigen	A substance inducing an immune response in an animal and reacting in it. Antigens are usually, but not necessarily, foreign to the animal in which they produce immune reactions.
Approved project	A project approved by a properly constituted AEC, on the basis of a written proposal.
Ascites method	The use of living mice for the propagation of a hybridoma cell line within the abdominal cavity and the subsequent collection of the resulting ascitic fluid or peritoneal exudate containing MABs.
B cell	B cells are one of the two main classes of lymphocytes, the white blood cells produced by lymphoid tissue. B cells or B-lymphocytes, are responsible for the production of antibody and the subsets of T-lymphocytes (helper, suppressor and cytotoxic T-cells) are responsible for cell-mediated immunity and stimulating the transformation of B-lymphocytes.
CFA	Complete Freund's adjuvant: An adjuvant composed of mineral oil, a surfactant (Arlacel A) and heat killed mycobacteria used to prepare a water-in-oil emulsion of an antigen that will produce a vigorous immune response when injected into an animal.
The Code	<i>Australian code of practice for the care and use of animals for scientific purposes</i> (NHMRC 2004).
ELISA	Enzyme Linked Immuno Sorbent Assay: An assay in which antigen or antibody is detected by an enzyme-linked reagent that converts a colourless substance into a coloured or fluorogenic product. ELISAs commonly employ microwell plates for the detection of antigens and may be performed with the direct, indirect, or sandwich method. The results of ELISAs may be expressed qualitatively or quantitatively and can be measured by a spectrophotometer or luminometer. When optimised, the intensity of colour or light generated, is directly proportional to the amount of reactant present.
Hybridoma	A cell hybrid formed between a myeloma cell (a neoplastic plasma or immunoglobulin-producing cell) and an antibody-producing cell, which is used for raising monoclonal antibodies.
IFA	Incomplete Freund's adjuvant: An adjuvant composed of mineral oil, a surfactant (Arlacel A) and without heat killed mycobacteria. IFA is used to prepare water-in-oil emulsions of antigens that favour antibody production rather than cell-mediated immunity when injected into an animal.

MAb	Monoclonal antibody: The antibody from a single clone of an antibody-producing cell used to form a hybridoma. Monoclonal antibodies comprise a single species of antibody molecule that bind to the same antigenic determinant on an antigen. Polyclonal antibodies are produced from multiple clones of antibody-forming cells and contain multiple species of antibody molecules that may bind to different antigenic determinants on an antigen. Polyclonal antibodies are the general result of immunisation of whole animals.
NHMRC	National Health and Medical Research Council
PCR	Polymerase chain reaction: An analytical and research method for quickly making many copies of a specific segment of DNA in an exponential rather than linear manner.
Priming	A treatment that does not in itself elicit a large response from a system but which expands the capacity to respond to a second stimulus. In immune responses, a first dose of an antigen, the priming dose, induces immunological memory or the capacity to react more extensively to subsequent, or booster, doses of the antigen.
Pristane	A saturated hydrocarbon (2,6,10,14-tetramethylpentadecane) that is purified from mineral oil or extracted from shark liver. Pristane can be carcinogenic and will induce a lupus-like auto-immune syndrome in mice and a form of experimental arthritis.
Seroconversion	The development of antibody in serum in response to an animal's exposure to an antigen.
Western Blot	A method of identifying proteins and peptides that have been electrophoretically separated and transferred onto nitrocellulose or nylon membranes. Also called immunoblotting because specific antibodies, which labelled with enzymes or radionuclides, are the agents used for identification of proteins or peptides.

1 Introduction

1.1 Purpose

These guidelines apply to:

- AECs, institutions, and investigators undertaking or overseeing the production of MABs for scientific, teaching or commercial purposes.
- All new or intended projects.
- Modifications to existing projects. Projects approved prior to the adoption of these guidelines may continue to use the method detailed in the original project application. However, if a project is extended to include production of MABs *not* specified in the original application these guidelines must be followed. After expiration of an approved protocol, all renewals must comply with these guidelines.

These guidelines are not intended as a comprehensive source of information about MAB production. They do not apply to MABs routinely available as reagents from commercial outlets. Institutions are encouraged to make appropriate enquiries about the production methods of MABs when they purchase these from other institutions in order to ensure that the production method used by the producing institution meets the ethical standards required by these guidelines.

1.2 Target audience

These guidelines were developed to assist institutional AECs in the evaluation of applications involving MAB production to ensure that only *in vitro* methods of production are used, except where *in vitro* methods have been shown to be unsuccessful in the production of a specific MAB from a given cell line.

1.3 Scope of the guidelines

The guidelines recognise that the use of animals for MAB production will continue to be required during the initial, immunisation stage of production, in the induction of antibody-producing cells *in vivo*.

The previous version of these guidelines (2001) provided six exceptional circumstances in which it may have been necessary to use *in vivo* production of MABs in the propagation stage. It is now considered that technological advances in the field of MAB production have rendered those exceptions redundant. Appendix 1 in these guidelines outlines the previous exceptional circumstances and the reasons why these are no longer considered acceptable reasons to use *in vivo* MAB production. In extremely rare circumstances, the ascites method may be used but *only after* the hybridoma has been sent to a laboratory which has expertise in *in vitro* MAB production, and that laboratory was unsuccessful in producing MABs *in vitro* (see 2.2.3).

1.4 Welfare of animals in research

Encapsulated in the Code is the need in scientific and teaching activities to consider the principles of:

- Replacement of animals with other methods
- Reduction in the number of animals used
- Refinement of techniques used to reduce the impact on animals.

The Code provides general principles for the care and use of animals, specifies the responsibilities of investigators and institutions, and details the terms of reference, membership and operation of institutional AECs.

Techniques which replace or complement the use of animals in scientific and teaching activities must be sought and used wherever possible. Studies must be scientifically and statistically valid, and must use only the minimum number of animals necessary.

AECs must ensure that all animal care and use within the institution is conducted in compliance with the Code and incorporates the principles of replacement, reduction and refinement. It is the responsibility of AECs to ensure that only those scientific or teaching activities which conform to the requirements of all relevant sections of the Code and of legislation are approved.

Written proposals for the consideration of AECs should include reasons why animals are necessary for the project and, in particular, why techniques which do not use animals have been rejected as unsuitable. They should also include a description of experimental, surgical and related procedures, including dose and route of any substance administered.

The production of MABs using animals involves procedures which have the potential to produce significant pain and distress. These guidelines arose in response to a growing awareness that a method of MAB production exists which can replace the use of the animals for one of the stages of MAB production. This method replaces the *in vivo* propagation of selected cells to produce MABs, with an *in vitro* method. The guidelines will assist AECs to determine whether any proposed use of the *in vivo* method is justified.

2 Guidelines

2.1 Replacement

2.1.1 Use of the ascites method

In vitro MAb production is the accepted method. The ascites method may only be used after it has been shown that *in vitro* methods are unsuitable for the production of the specific antibody required for the proposed project. The hybridoma must have been sent to a laboratory which has expertise in *in vitro* MAb production (see 2.2.3). Specific approval must be given by the AEC.

2.1.2 The role of AECs in assessing applications to produce MAbs

- a. AECs should recognise that all hybridomas are unique, and that no single technique is suitable for all; in extremely rare circumstances successful production may depend on use of the ascites method. However, an assumption, or conclusion, of a need for the ascites method should not be applied to broad classes of antibodies but only to individual hybridoma lines for which *in vitro* techniques have failed. The ascites method should only be used as a final recourse after having sought the assistance of an institution with expertise in the *in vitro* production of MAbs.
- b. AECs should not accept increased cost of *in vitro* methods as a reasonable justification to allow use of the ascites method.
- c. AECs must ensure that researchers do not request an external source to produce custom-made MAbs for them using the ascites method.

2.1.3 Facilities for *in vitro* MAb production

There are a number of different methods available to maximise antibody production *in vitro*, including a variety of new culture media which stimulate different growth characteristics that may specifically suit individual clones. It is also essential to check that the cell line is a stable secreting clone and that all cells in the population are actively secreting antibody. This can easily be checked by one round of sub-cloning and testing by appropriate means (ELISA, Western Blot, immuno staining).

Access to appropriate facilities and equipment is essential to the success of *in vitro* MAb production methods. Investigators lacking experience or resources must seek assistance from experienced providers of *in vitro* MAb expertise. If the advice provided is still insufficient to allow successful production of MAbs, the hybridoma must be sent to an appropriate facility for at least a pilot study to determine the optimum *in vitro* growth and secretion conditions for that particular hybridoma.

Institutions with sufficient demand should consider establishing a centralised or 'core' facility for MAb production using *in vitro* methods.

2.2 Refinement

2.2.1 Refinement of immunisation

(induction of antibody-producing lymphoid cells):

- a. Site of injection – Sub-cutaneous or intra-peritoneal routes of administration of antigen are recommended in mice. Intravenous injection is also an appropriate route of administration for soluble antigen without adjuvants. AECs should be aware that the use of intradermal, intramuscular, or foot pad routes of administration of antigen containing adjuvant in the mouse requires special justification and monitoring for evidence of unwanted side effects. Injected animals must be monitored in an appropriate way as per 2.2.16 (x) of the Code.
- b. Use of adjuvant – All adjuvants are irritants and should only be injected by an experienced technician. Careful monitoring of the animals is essential to detect any unwanted side effects. Freund's adjuvant should not be used where it is possible to use no adjuvant or less irritant adjuvants. When Freund's adjuvant is used, the recommended volume is no more than 100µl per injection site (see 2.2.1a above) in mice. Complete Freund's adjuvant must not be used more than once in an individual mouse. Managing the site of Freund's injection is important to spread the depot and to reduce the chance of granulomata or abscesses.
- c. Inoculation Schedule – There is no specific right or wrong immunisation schedule to optimise the production of secreting B cells in the mouse. The use of adjuvants, the timing of antigen presentation, and the frequency of inoculation can all be used to encourage the creation of cells producing a particular class of antibody.
- d. Total volume of inoculum – The combined adjuvant +antigen volume should not exceed 200µl in mice (max 100µl Freund's).
- e. Blood sampling – The method of blood sampling of animals should be assessed by the AEC. The need to perform more than two survival test bleeds should be justified. The competence of the technician should be taken into account when approving a method of bleeding (See NHMRC, 2008, *Guidelines to promote the wellbeing of animals used for scientific purposes: The assessment and alleviation of pain and distress in research animals*, Part III D).

2.2.2 Decontamination of infected hybridomas

Where a cell line is contaminated with yeast or bacteria, it is acceptable to put hybridoma cells subcutaneously or into the abdomen of an immunocompetent mouse, and for the resultant rescued cells to be harvested, back into appropriate media. This must be used only if back-up cultures are unavailable from any other source.

2.2.3 Refinements of the ascites method of production (*in vivo* propagation of hybridoma clones and collection of ascitic fluid)

In the unlikely event that the ascites method is justifiable, the investigator must provide full documentation of the *in vitro* procedures attempted in the expert external laboratory. A most important aspect of the ascites method of MAb production is the utilisation of skilled, competent, technical/scientific staff experienced in the handling and monitoring of mice and with the techniques used. Staff must be capable of recognising signs of distress in mice and be responsible for taking action when necessary.

- a. Priming – Use the lowest possible dose of priming agent required to achieve satisfactory results. For pristane, the volume should not exceed 200µl. The proposed use of more than 200µl should not be approved without justification.
- b. Collection of ascitic fluid – Monitoring of animals to avoid pain and distress is critical during the accumulation of ascitic fluid. The excessive build up of fluid can cause distress, for example when the gait of the animal is visibly impaired. It can also mask a loss of body weight. Ascitic fluid should be collected only after the animal has been humanely killed.
- c. Holding period – Mice should be held no more than 28 days after hybridoma inoculation. However, accumulation of sufficient ascites fluid normally occurs within 14 days. An animal displaying loss of condition, pain or distress, must be humanely killed immediately.
- d. AEC responsibility – AECs must be prepared to justify instances of approval of the ascites method of MAb production to relevant state and national authorities. Records giving the reasons for such approvals must be kept.
- e. Information required by the AEC should include:
 - the nature of the *in vitro* methods which were use
 - the laboratories where they were attempted
 - possible technical explanations for why they failed.

3 References and further information

National Academy of Sciences and National Institutes of Health (US), 1999, *Monoclonal Antibody Production: A report of the Committee on Methods of Producing Monoclonal Antibodies, Institute for Laboratory Animal Research, National Research Council*, National Academy Press, Washington DC., available online at: <http://www.nih.gov/grants/policy/antibodies.pdf>

New South Wales Department of Primary Industries, 2003, *Guidelines for the Production of Monoclonal Antibodies* (Animal Research Review Panel 13), available online at: <http://www.agric.nsw.gov.au/reader/research-procedures/arrp-monoclonal-antibodies.htm>

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NHMRC, Department of Health and Ageing Office of the Gene Technology Regulator and Gene Technology Ethics Committee, 2008, *Guidelines for the generation, breeding, care and use of genetically modified and cloned animals for scientific purposes*, available online at: http://www.nhmrc.gov.au/publications/synopses/_files/ea17.pdf

Online resources

Scientists Center for Animal Welfare (SCAW) <http://www.scaw.com>

Appendix 1: Previously acceptable reasons for using the ascites method

In the 2001 version of these guidelines, the following six circumstances were listed as being *possibly* acceptable reasons for the use of the ascites method. It is now considered that technological advances in the field of MAb production have rendered these exceptions redundant, for the reasons outlined below.

If a researcher or institution is unable to produce MAbs using the *in vitro* method, the AEC must ensure that the hybridoma has been sent to an external laboratory with expertise in MAb production before approving the use of the ascites method.

1. The hybridoma cell line does not grow well *in vitro* and/or yields of antibody are very low.

Cell lines usually respond to one of the many media formulations and/or one of the culture methods that are now available. The usual reason a cell line does not secrete large amounts of antibody in culture, is that a percentage of the population is non-secreting (switched off). This can also happen to cell lines that were previously proven to be good producers. A solution is to sub-clone the cells to stabilise the population and remove the non-secreting cells. The enlistment of an experienced person is essential in such cases.

2. The MAb is to be used for experiments in mice where non-mouse proteins may confuse the results.

In vitro production systems without foetal calf serum operate successfully. Some chemically defined media have no protein at all.

3. Steps in purification of MAb have caused denaturation and loss of antibody activity.

Steps in purification of MAb can usually be modified to suit the stability of the antibody. Buffers at neutral pH or even alkaline pH can be used instead of acidic pH to elute antibody from affinity columns.

4. Specific glycosylation patterns from MAbs produced *in vitro* have adversely affected antibody activity.

Glycosylation patterns, as well as chemical stability, of the antibody can be selected for at the time of cloning. Usually, the final product of a fusion experiment can be tested for such characteristics and, if deemed inappropriate, the researcher should search for an alternative clone producing a more robust antibody.

5. It may be necessary to check the virus status of a hybridoma cell line by injecting it into mice and testing for seroconversion.

Polymerase chain reaction assays will detect viral DNA/RNA more effectively than serology will detect viable virus replication and seroconversion.

Appendix 2: Development of the guidelines

Revision of the 2001 guidelines

NHMRC first published Guidelines for Monoclonal Antibody Production in 2001. In line with NHMRC policy the document was revised in 2007-08. The document was revised by the Animal Welfare Committee with expert advice from Dr Alfio Comis, managing scientist at MAbSA Technologies (part of Adelaide University and Institute of Medical and Veterinary Science).

Consultation process

The revised draft was released for a public consultation period from 20 February 2008 – 26 March 2008. The consultation process was advertised nationally through the higher education supplement in *The Australian* and *The Weekend Australian*, as well as through NHMRC website and *Tracker* (fortnightly bulletin). Ten submissions were received through this process, and the guidelines were further revised in light of these comments.

Animal Welfare Committee membership

Dr Elizabeth Grant AM (Chair)	Category D A person who is independent of the institution and who has never been involved in the use of animals in scientific or teaching activities
A/Prof David Adams	Category A A person with qualifications in veterinary science
Dr Catherine Berglund	Category D A person who is independent of the institution and who has never been involved in the use of animals in scientific or teaching activities
Dr Tim Kuchel	Category A A person with qualifications in veterinary science
Prof Clive Phillips	Category B A suitably qualified person with substantial recent experience in the use of animals in science or teaching activities
Dr Carole Webb	Category C A person with demonstrable commitment to and established experience in furthering the welfare of animals
A/Professor Christine Wright	Category B A suitably qualified person with substantial recent experience in the use of animals in science or teaching activities

NHMRC staff

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