Florida State University
Monoclonal Antibody Production in the Mouse

Monoclonal antibodies are important tools both for basic research as well as for medical treatment. Monoclonal antibodies are created by first immunizing an animal (typically a mouse) with the antigen of interest, harvesting its spleen after determination of successful polyclonal antibody production, fusion of splenic cells in vitro with an immortalized cell line and then subsequent production of large amounts of monoclonal antibodies either *in vivo* or *in vitro*. Development of a suitable hybridoma cell line and subsequent monoclonal antibody production has the capacity to cause inflammation, tissue necrosis, discomfort, pain and potentially death in the mouse. These guidelines have been established to minimize any discomfort associated with this process. Please refer also to Antibody Production - Adjuvants, Immunization Routes for Antibody Production, and Immunization Guidelines for Research Animals for further information related to this subject.

- Alternative *in vitro* methods for monoclonal antibody production **must** be considered. *In vivo* production (hybridoma/ascites) must be scientifically justified and approved by the ACUC. At this time (2001) reliable and consistent *in vitro* methods for hybridoma generation are not available. However, many *in vitro* alternatives to the ascites method for monoclonal antibody production are available and must be considered.

- Select the appropriate mouse strain for the experimental purpose. Consider MHC haplotype, B- and T-cell receptors genes and expression of various regulatory immunomodulatory proteins. As most hybridomas are derived from fusion with BALB/c tissue, Balb/c mice are the strain most commonly used.

Hybridoma Development

1. Select the appropriate adjuvant for immunizing mice in the hybridoma stage. Consider using incomplete Freund's adjuvant (IFA) or another adjuvant (e.g. RIBI, squalene) before using complete Freund's adjuvant (CFA). Some antigens are suitable for intravenous injection and do not require an adjuvant. If CFA is used, it may be used for only the initial dose. All boosters except the last should use IFA as an adjuvant (if CFA is the initial adjuvant).

2. All antigens should be processed to minimize microbial contamination prior to mixing with adjuvant. Millipore filtration with an appropriate membrane (0.22 micron) is recommended (filter with minimal protein adsorption and minimal disruption of protein conformation). The amount of antigen will depend on its antigenicity, purity and availability. In general, 10-200 micrograms of antigen are used per booster with 10-50 micrograms the most commonly used amount.

3. Immunization for hybridoma production should take into consideration route and volume. Subcutaneous and intraperitoneal routes are most commonly used. For subcutaneous immunization, no more than 0.3 ml total volume should be injected each time; no more than 0.05 ml should be injected per site. No more than 0.5 ml total volume should be injected intraperitoneally per booster. Intramuscular injections are discouraged due to the limited muscle mass of the mouse. Intradermal injections are limited to 0.01-0.05 ml per site with a total volume of 0.2 ml. Intravenous injections can be used for aqueous antigens (particulate or saline soluble) that do not require adjuvant. Total volume injected intravenously should not exceed 0.5 ml per booster. For personnel not experienced in the handling and injection of mice, please consult the LAR veterinarian for assistance and training. Footpad injections are not permitted without scientific justification and appropriate approval from the ACUC. If permitted, injection is only allowed for one hind foot per animal. Front feet may not be used since rodents use their front feet for grasping and manipulating food.

4. Animals injected either intraperitoneally or via a footpad should be observed daily for discomfort (minimum of 5 days). Animals in distress or pain should have appropriate analgesics administered. Animals in extreme discomfort should be terminated. Consult the LAR veterinarian for appropriate treatment options.

5. As a guideline, 2-4 injections of antigen are given at intervals of 2-8 weeks between boosters. Boosters should be given at 21-28 days intervals. Boostering at less than 2 week intervals is not productive and contributes stress to the animal. Test bleeds should be done approximately 10-24 days after boostering. Very often a final boost with antigen only (no adjuvant) is done either IP or IV and the animal is sacrificed 3-4 days later. These are guidelines only. Absolute time frames should be dictated by the investigator's experience or pilot studies.

6. Several methods are available for blood collection. Procedures for blood collection should be included in the investigator's protocol and approved by the ACUC. For assistance and training, consult the LAR veterinarian.

7. The method of euthanasia should be appropriate for rodents (as outlined in the *2000 Report of the AVMA Panel on Euthanasia)* and approved by the ACUC. For training or assistance, consult the LAR veterinarian.

8. Further work for cell fusion and hybridoma selection is performed *in vitro* where possible; ascites production may only be performed if in vitro methods have proven inadequate (see below) and has been approved by the ACUC.

9. Hybridomas and other tumor cell lines should be tested for contamination with viruses or mycoplasmas prior to further use in vivo.

Antigens		Adjuvant
Proteins	10-100 micrograms per injection, per animal	Freund's (CFA/IFA) or alum
Cells	0.5 - 5.0 x 107 cells per injection, per animal	Freund's

Washington University School of Medicine Hybridoma Center Immunization Schedule			
Day	**Manipulation**	**Adjuvant**	**Site**
0	Primary Immunization	CFA	SC
14	First booster	IFA	SC
28	Second booster	IFA	SC
36	Titer test bleed		IV
42	Rest prior to fusion or third booster	IFA	SC
56	Final boost	None	IV
59	Harvest spleen and fuse		

Ascites Monoclonal Antibody Production

1. Alternative *in vitro* methods (batch tissue-culture methods, semi-permeable membrane systems) for monoclonal antibody production **must** be considered. *In vivo* production (hybridoma /ascites) should be considered the exception rather than the rule, be documented and scientifically justified in writing in the protocol and approved by the ACUC. The following may be justifications for *in vivo* monoclonal antibody production but evidence of the justification must be provided to the ACUC:
    - When a supernatant of a dense hybridoma culture grown for 7-10 days (stationary batch method) yields a monoclonal antibody concentration of less than 5 mg/ml, or if other systems are used and concentrations obtained are less than 500 mg/ml (hollow fiber system) or 300 mg/ml (semi-permeable membrane system).
    - When more than 5 mg of monoclonal antibody produced by each of five or more different hybridoma cell lines is needed simultaneously. It is technically difficult to produce this amount of monoclonal antibody since it requires more monitoring and processing capability than the average laborartory can achieve.
    - When analysis of monoclonal produced in tissue culture reveals that a desired antibody function is diminished or lost.
    - When a hybridoma cell line grows and is productive only in the animal.
    - When more than 50 mg of functional monoclonal antibody is needed, and previous poor performance of the cell line indicates that hollow-fiber reactors, small-volume membrane-based fermentors, or other techniques cannot meet this need during optimal growth and production.

For further discussion, see Scientific Needs for Mouse Ascites Production of mAb.

2. Appropriate selection of mice is important. Studies suggest that the age range of 6-11 weeks is the optimum for ascites production.

3. A baseline weight should be obtained from all mice prior to priming. Weight should be marked on the cage card along with the date of priming.

4. Priming with either pristane or IFA via an intraperitoneal injection is performed (priming). Maximum volume of injection must not exceed 0.5 ml. It is preferable to use 0.1-0.2 ml for pristane and 0.25-0.5 ml for IFA. Studies have shown that smaller volumes of pristane cause less distress with no significant differences in ascites production. Consideration should be given to the use of incomplete Freund's adjuvant (IFA) as an alternative to pristane. Use of IFA may cause less distress and allow inoculation of hybridoma cells as early as one day after priming.

5. Hybridomas cells are injected after a suitable period (as short as 3 days, as long weeks). Number of hybridoma cells injected are approximately 1×10^6 as this has been shown to lead to increased survival times. Cells should be suspended in sterile physiologic solution. Total volume or cell suspension should not exceed 0.5 ml. Injections must be performed aseptically.

6. Animals should be checked at least every other day after cell injection. Once abdominal distention has been noted, frequency of monitoring may need to be increased. Observations should include posture, activity, food and water intake, respiratory pattern, body condition (e.g. rough hair coat, severe abdominal distention, pale ears or eyes). Weight should not exceed a 20% increase over baseline. Appropriate steps should be taken once signs of distress are noted. Please consult with the LAR veterinarian for further guidance should any of these signs be noted.

7. Ascitic fluid harvesting (paracentesis or abdominal taps). Fluid should be harvested prior to abdominal distension becoming so severe as to cause discomfort or impair normal activity. Fluid should be harvested after antiseptic preparation of the site to prevent bacterial contamination. Needle size should be 20 gauge or smaller. Experienced personnel may perform this procedure without anesthesia, however untrained personnel must perform the procedure only under anesthesia and with hands-on guidance from experienced personnel.

8. The number of taps is limited to 3. The date of each tap must be listed on the cage card. The third tap should be performed under anesthesia or after euthanasia. An increase in the number of taps permitted may be obtained after demonstration to

the ACUC that the specific tumor line does not cause significant pain or distress to the animal in the proposed time frame.

9. Consideration should be given to fluid volume replacement to avoid hypovolemic shock if large volumes of ascitic fluid are removed. Warm saline or lactated ringers solution (2-3 ml) may be given subcutaneously at the time of paracentesis.

10. Mice should be monitored for 20-30 minutes following a tap and again later on the same day.

11. Euthanasia criteria (death is not an acceptable endpoint):
    - Gross abdominal distension is present causing difficulty in breathing.
    - Skin of abdomen is gray-green (check the ventral surface daily).
    - Volume of ascites (by weight) exceeds 20% of baseline body weight.
    - Paracentesis does not relieve abdominal distension (distension is due to solid tumor growth).
    - Animal is lethargic, dehydrated, not eating or drinking, maintains a hunched posture, has sunken flanks or has a ruffled haircoat.
    - Animal is moribund (with or without intervention, death is inevitable).

Note: Each cell line is unique (some are more aggressive than others) and may not perform as others with which the investigator has previous experience. Pilot studies are recommended for new cell lines to determine the growth characteristics of the cell line and optimum time frame prior to use in a larger number of animals.

References:

Small Scale Monoclonal Antibody Production. 1999. Special Edition Lab Animal.

Amyx, H.L. 1987 Control of animal pain and distress in antibody production and infectious disease studies. JAVMA, 191(10):1287-1289.

Bennett, B., Check, I.J., Olsen, M.R. and Hunter, R.L. 1992 A comparison of commercially available adjuvants for use in research. Journal of Immunological Methods, 153:31-40.

Brodeur, B.R., Tsang, P. and Larose, Y. 1984 Parameters affecting ascites tumour formation in mice and monoclonal antibody production. Journal of Immunological Methods, 71:265-272.

Gilette, R.W. 1987 Alternatives tro pristane priming for ascitic fluid and monoclonal prodcution. Journal of Immunology 99:21-23.

Goettel-Connolly, C. 1997/1998 Alternatives in monoclonal antibody production workshop. AWIC Newsletter, 8(3-4):21.

Hanly W. C., Artwohl, J.E. and Bennett, B.T. 1995 Review of Polyclonal antibody production in mammals and poultry. ILAR Journal, 37(3):93-118.

Heidel, J. 1997 Monoclonal Antibody Production in Gas-Permeable Tissue culture Bags Using Serumfree Media. Center for Alternatives to Animal Testing: Alternative in Monoclonal Antibody Production 8:18-20.

ILAR (Institute for Laboratory Animal Research), National Research Council. 1999 Monoclonal Antibody Production. A Report of the Committee on Methods of Producing Monoclonal Antibodies. National Academy Press.
Jackson, L.R. and Fox, J.G. 1995. Institutional policies and guidelines on adjuvants and antibody production. ILAR Journal, 37(3): 141-152.
Jackson, L.R., Trudel L.J., Fox, J.G. and Lipman, N.S. 1996 Evaluation of hollow fiber bioreactors as an alternative to murine ascites production for small scale monoclonal antibody production. Journal of Immunological Methods 189:217-231.
Jennings, V.M. 1995. Review of selected adjuvants used in antibody production. ILAR Journal, 37(3):119-125.
Leenaars, M and Hendriksen, D.F.M. Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations. ILAR Journal, 46 (3).
Lipman N.S., Trudel, L.J., Murphy, J.C. and Sahali, Y. 1992 Comparison of immune response potentiation and in vivo inflammatory effects of Freund's and RIBI adjuvants in mice. Laboratory Animal Science, 42(2):193-197.
McArdle, J. 1997/1998 Alternatives to ascites production of monoclonal antibodies. AW IC Newsletter, 8(3-4):1-2,15-18.
McGuill, M.W. and Rowan, A. N. 1989 Refinement of monoclonal antibody production and well-being. ILAR Journal, 31(1):7-10.
Peterson, N. and Peavey, J. 1998 Practical application of in vitro monoclonal antibody production. Contemporary Topics in Laboratory Animal Science 37:61-66.
Peterson, N. 2005 Advances in Monoclonal Antibody Technology: Genetic Engineering of Mice, Cells, and Immunoglobulins. ILAR Journal, 46(3):314-319.
Smith, C.P., Jensen, D., Allen, T. and Kreger, M. (Eds.) 1997 Information Resources for Adjuvants and Antibody Production. U. S. Dept. of Agriculture.