

FLSC Standard Operating Procedure for Ascites Fluid Production

Ascites production is a controversial procedure for the production of monoclonal antibodies. Several animal rights groups have requested a ban on in vivo methods of monoclonal antibody production. In response to these and other concerns, the National Academy of Science released findings in 1999 that supported the use of in vitro methods whenever possible. They also acknowledged that there are legitimate circumstances for the use of ascites production in animals. The use of monoclonal antibodies is a powerful research tool, and the use of animals is indispensable to the establishment of monoclonal antibody producing cell lines. Once these cell lines are established, the use of an in vitro method is encouraged. Because the production of ascites for monoclonal antibodies has the capacity to induce severe distress, pain and even death in mice, FLSC has adopted a SOP for Ascites Fluid Production. This SOP was written to decrease the pain and distress to the animals and also for those caring for them.

A. Mice used for ascites production are female Balb/c, unless otherwise specified. Retired breeders are superior to younger animals since the abdominal distention associated with pregnancy is similar to mAb production, resulting in greater volumes of ascites fluids obtained with less distress to the animals.¹

B. Peritoneal Priming of Mice²

Pristane priming - Inject 0.2 ml pristane (2, 6, 10, 14 - tetramethylpentadecane, Sigma Chemical Co.) using a 25 g x 5/8" needle per mouse intraperitoneally (IP). Wait 10 days and no longer than 30 days before injecting cells into pristaned mice.

OR

Freund's Incomplete Adjuvant priming - inject 0.3 ml IP, wait 1-3 days to inject with cell line.

C. Cell Preparation

1. Only cell lines demonstrated to be free of murine pathogens will be permitted (refer to IACUC Policy, "Testing of Tumors and Cell Lines").
2. One confluent 75 cm tissue culture flask contains enough cells to inject 4-5 mice. Make sure the cells are healthy and that they are not overcrowded.
3. Centrifuge the cells in a 50 ml centrifuge tube at 400 g.
4. Discard the supernatant and gently resuspend the cells in PBS (Physiologically Buffered Saline) so that the number of hybridoma cells range from 1×10^5 to 1×10^7 . Resuspend so that the injected volume is not greater than 0.15 mL/ mouse.
5. Draw cells up in a 1 ml syringe. Cells will settle quickly, therefore, just prior to injecting the mice, "roll" the syringe briefly to re-suspend them. Inject 0.1 ml cells per mouse using a 22 gauge needle, intra peritoneally (IP) in the lower right quadrant of the abdomen.
6. All mice must be weighed and the weight recorded on the cage card prior to inoculation. The cage card will contain the following information on the front: baseline weight, maximum weight for 1st tap, injection volume. On the back of the card the following will be recorded: date abdominal distention occurs, daily weights, date of 1st tap, and volume of 1st tap, and subsequent tap weights and dates.

D. Animal Monitoring

Ascites production can take as little as 5 days or as long as 1 month, however it usually occurs within 7 to 10 days. Mice must be checked daily for abdominal distension. Once abdominal distension begins, the mice must be weighed daily. Abdominal paracentesis must occur before a 25% increase in weight over the baseline established at inoculation. Should the PI request multiple taps, no more than 4 taps may be performed³. The mouse must always be sacrificed by CO₂ and cervical dislocation for the fourth tap.

The following guidelines will be followed for all survival abdominal paracentesis:

1. If abdominal distension is not relieved by abdominal paracentesis, abdominal palpation will be performed to identify the presence of a solid tumor.
2. Euthanasia will be performed if one or more of the following signs are present:
 - The skin of the abdomen is grey-green in color.
 - Abdominal distension is causing dyspnea (difficult breathing) and it is not relieved by tapping the abdomen.
 - Presence of solid tumor growth in the abdomen
 - Ascites fluid is bloody or has high particulate matter content.
3. If signs of shock are present after paracentesis, (tachypnea, pallor evident on ears/muzzle/tail, inactivity, hunched posture) 2 - 3 mLs of warmed saline will be given SQ in multiple sites with an injection volume not to exceed 0.50 mL per site. The animal will be placed on a heating pad and observed for 30 minutes. If signs of shock are not transient and persist beyond 30 minutes euthanasia will be performed.

E. Collecting Ascites Fluid

Survival Collection - 1st – 3rd abdominal paracentesis

1. Aseptic technique should be used in the collection of ascites fluid.
2. Disinfect the abdominal surface with 70% alcohol.
3. Use the smallest sterile needle possible (18-22 g) to allow for good flow.
4. Needle insertion should be in the lower left quadrant of the abdomen, opposite the site of inoculation.
5. Rotation of the needle and adjusting the depth of insertion will allow for optimizing rate of collection.
6. Allow the ascites fluid to drip from the needle hub into a sterile collection tube. Do Not attach syringes or pipettes to aspirate fluid - doing so will injure the mouse, requiring euthanasia.
7. Manual restraint is usually sufficient for this procedure. The mouse is scruffed and held with the body in a vertical position with the head pointing upward. Inexperienced personnel may anesthetize the mice using isoflurane, but must understand that there is an increased risk of anesthetic death.
8. Five to eight ml. can be collected in the first tap. The mouse can be tapped again after a couple of days, when the abdomen is again distended.
9. The mouse should be sacrificed with CO₂, if clinical signs suggestive of impending death are observed; including anorexia, lethargy, unkempt appearance, and hypothermia (cool to the touch). The ascites can then be recovered using the non-survival collection procedure.

Non-Survival Collection – 4th abdominal paracentesis

1. The mouse is euthanized with CO₂ followed by cervical dislocation.
2. Aseptic technique should be used in the collection of ascites fluid.
3. Disinfect the abdominal surface with 70% alcohol.
4. With the animal in dorsal recumbency, the skin of the abdomen is lifted and a small cut is made through the skin midway between the pelvis and the xiphoid. Gently grasp the muscle layer and make a cut into the abdominal cavity. Without dropping the muscle, insert a 1 cc syringe (without a needle) along the muscle wall and aspirate the ascites fluid. Fluid is placed in a sterile collection tube. The cut can be enlarged to facilitate fluid collection.
5. It is generally found that equivalent volumes of fluid can be obtained by a single harvest after euthanasia as compared to repeat taps of live animals.

F. Ascites Fluid Handling and Storage

1. Handling and storage of ascites fluid is the responsibility of the investigator. FLSC staff will notify the investigator when the collection of fluid is completed and ready for pick-up.

2. Let the ascites fluid stand at room temperature for 30 minutes.
3. Centrifuge to separate the serum from other blood components. Transfer the straw-colored fluid (serum) to a sterile tube with a sterile transfer pipette or syringe.
3. Store at -70°C until the time of purification.

References

¹ *In vitro production of monoclonal antibodies in high concentration in a new and easy to handle modular minifermenter.* Falkenberg, F.W., Weichert, W.H., Krane, M., et al (1995). *Journal of Immunological Methods* 179:13-29.

² "*Refinement of Monoclonal Antibody Production and Animal Well-Being*" *ILAR News*, Vol. 31, No. 1, Winter, 1989.

³ *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 1999.

The procedures in this protocol are within the guidelines established by the *Current Protocols in Immunology*. "Antibody Detection and Preparation", page 2.6.1-2.6.5.