

1. PURPOSE

This Standard Operating Procedure (SOP) describes the procedures for producing monoclonal antibodies in mice.

2. RESPONSIBILITY

Principal Investigator (PI) and veterinary care staff.

3. MATERIALS

- 3.1. Adjuvant
- 3.2. Antigen
- 3.3. Appropriate syringes and needles for injection
- 3.4. Blood collection materials
- 3.5. Scale
- 3.6. 70% ethanol
- 3.7. Sterile surgical instruments (forceps & scissors)
- 3.8. Container for ascites fluid collection (8 oz specimen container or 50 cc centrifuge tube)
- 3.9. Centrifuge tubes, 15 cc
- 3.10. Centrifuge
- 3.11. Wooden stir sticks
- 3.12. Transfer pipettes

4. CONSIDERATIONS

- 4.1. The production of monoclonal in mice by the ascites method raises several issues of concern regarding the potential for severe and unnecessary pain and suffering for the animals. A number of *in vitro* replacements for the rodent ascites method of monoclonal antibody production have been developed. Every attempt should be made to obtain material already available or to use an *in vitro* method for production of monoclonal antibodies.
- 4.2. Before, producing monoclonal antibodies *in vivo*, the PI needs to justify to the Facility Animal Care Committee (FACC) in the Animal Use Protocol why *in vitro* production is not suitable. The following procedures can only be applied if the FACC accepts the justification and approves the *in vivo* production of monoclonal antibodies.

5. IMMUNIZATION PROTOCOL FOR PREPARATION OF HYBRIDOMA CELLS

- 5.1. The antigen must be:
 - 5.1.1. Non-toxic
 - 5.1.2. Sterile
 - 5.1.3. Free of pyrogens
 - 5.1.4. pH within physiological limits
 - 5.1.5. Easily passed through a 25G needle

NOTE: Proteins in polyacrylamide gel may cause adverse reaction at the site of injection. Use another method of purification or a dilution when possible.

- 5.2. For each scheduled immunization, prepare a sample consisting of a maximum of 50 micrograms of antigen in sterile PBS (or animal compatible buffer) in a volume of 50 μL per mouse.
- 5.3. Adjuvant:
 - 5.3.1. Use an adjuvant to increase the immunological response to poor antigens.
NOTE: When used with a strong antigen, the adjuvant may induce an overt local inflammatory response.
 - 5.3.2. If using Freund's Complete Adjuvant (FCA), use **ONLY ONCE** for the primary injection. Do not repeat. Use only FCA with a concentration of 0.5 mg/ml of mycobacteria or less. Use Freund's Incomplete Adjuvant (FIA) for all secondary immunizations.
- 5.4. Recordkeeping:
 - 5.4.1. Record the following information in the medical record of the animal.
 - 5.4.1.1. Name of antigen
 - 5.4.1.2. Adjuvant used
 - 5.4.1.3. Route of administration
 - 5.4.1.4. Site(s) of injection
 - 5.4.1.5. Volume injected
 - 5.4.1.6. Date of injection
 - 5.4.2. Record blood collection volume and site as well as body weight, general condition and appearance of the injection sites.
- 5.5. Animal selection:
 - 5.5.1. Use 3-5 young (approximately 8 weeks of age), adult female mice per antigen.
 - 5.5.2. Consider the source of parental myeloma cells to be used in the fusion process when selecting the strain. The mice used should be the same strain both for immunization to produce the hybridoma clone and for subsequent monoclonal antibody production, so that the tissue used is histocompatible.
 - 5.5.3. Allow a minimum of 7 days of acclimation after the arrival of the animals.
 - 5.5.4. Identify animals using ear punches.
- 5.6. Pre-immune blood sample:
 - 5.6.1. Collect approximately 100 μL of blood from the saphenous or submandibular vein.
 - 5.6.2. Centrifuge and freeze serum at -20°C .
- 5.7. Primary immunization:
 - 5.7.1. Combine the 50 μL antigen sample with 50 μL of adjuvant. Emulsify until it no longer separates.
 - 5.7.2. Injection:
 - 5.7.2.1. Inject half the sample (50 μL) intraperitoneally and inject the other half of the sample (50 μL) subcutaneously.
 - 5.7.2.2. Alternatively, inject the sample subcutaneously into 2 sites, 50 μL per site.
 - 5.7.2.3. Do not contaminate the needle track with resulting intradermic or intramuscular deposition of the mixture. Before removing the needle, pull back on the plunger slightly to prevent the leakage of adjuvant into the dermal layer.
- 5.8. Wait 3 weeks to build up a primary immunological response.
- 5.9. Secondary immunization (21 days after primary immunization):
 - 5.9.1. **DO NOT REPEAT FREUND'S COMPLETE ADJUVANT (FCA).** If FCA was used in primary immunization, use Freund's Incomplete Adjuvant (FIA), or another adjuvant.
 - 5.9.2. Give booster injections in the vicinity of the initial sites as long as there is no indication of inflammatory reaction from the initial injection.
 - 5.9.3. Proceed as indicated for the primary immunization in section 5.7.

- 5.10. Titer determination:
 - 5.10.1. Collect a 100 µL blood sample from the saphenous or submandibular vein 7 days after secondary immunization as in section 5.6.
- 5.11. Repeat secondary immunization and titer determination every 3 to 4 weeks. In most cases, the antibody titer reaches an acceptable level after two boosts.
- 5.12. Pre-fusion immunization for hybridoma preparation:
 - 5.12.1. 3 days before fusion with myeloma cells inject 50 µl of antigen (without adjuvant) intraperitoneally.
- 5.13. Euthanize mice as per Rodent Euthanasia SOP. Collect spleen and prepare cells for fusion and proceed according to desired fusion, screening, cloning and propagation protocols.
- 5.14. Animal monitoring:
 - 5.14.1. Observe animals for a minimum of 15 minutes post-injection for any abnormal reactions.

6. MONOCLONAL ANTIBODY PRODUCTION (ASCITES)

- 6.1. The PI will determine the number of animals to be used for ascites production per cell line. On average, 5 mice are used for each line.
- 6.2. Animal Selection:
 - 6.2.1. Consider the source of parental myeloma cells to be used in the fusion process when selecting the strain. The mice used should be the same strain both for immunization to produce the hybridoma clone and for subsequent monoclonal antibody production, so that the tissue used is histocompatible.
 - 6.2.2. Retired breeder female mice offer an advantage for ascites production because of their previously stretched abdominal musculature.
 - 6.2.3. SCID retired breeder female mice may also be used.
- 6.3. Priming:
 - 6.3.1. The primer will suppress the immune system so that the growth of cells is not impaired within the abdominal cavity and will cause a chemical irritation causing peritonitis that results in secretion of serous fluid.
 - 6.3.2. Priming is done by injecting 0.3 mL of FIA, intra-peritoneally (IP), per mouse using a 23G needle because of the viscosity of the FIA.
 - 6.3.3. The primer should be injected one to 10 days before injection of hybridoma cells.
- 6.4. Hybridoma clones are prepared by the PI and injected at the desired time following priming.
 - 6.4.1. A maximum of 3×10^6 hybridoma cells suspended in a total volume of 1.0mL are required per mouse.
 - 6.4.2. Hybridoma cells are injected intraperitoneally using a 25G needle (smaller gauge needles may damage the cells).
- 6.5. Animal monitoring:
 - 6.5.1. All procedures, body weight, and any observations are recorded on animal monitoring sheets.
 - 6.5.2. Abdominal distension will usually be noted seven to 10 days after inoculation.
 - 6.5.3. An increase in body weight due to ascites fluid accumulation and/or tumor growth does not commonly produce pain or distress. However, weight gain should not exceed 20% of the original body weight. Mice are weighed at the following times:
 - 6.5.3.1. Before FIA injection
 - 6.5.3.2. Before injection of hybridoma cells
 - 6.5.3.3. Daily, four days following inoculation
 - 6.5.3.4. Twice daily, after the appearance of obvious abdominal swelling
 - 6.5.4. Other signs of distress may include:
 - 6.5.4.1. Decreased activity

- 6.5.4.2. Hunched appearance
- 6.5.4.3. Ruffled fur
- 6.5.4.4. Respiratory distress
- 6.5.4.5. Loss of body condition (weight loss is usually masked by accumulation of fluid in the abdomen)
- 6.5.5. Notify Veterinary Care staff when animals show signs of distress. Carprofen (20mg/kg), subcutaneous fluid administration, and the provision of wet food in the cage may alleviate minor distress.
- 6.5.6. Animals should be euthanized when weight gain reaches 20% or when obvious signs of pain or distress are noted.
- 6.5.7. Humane euthanasia is indicated if an abdominal tumor is palpated.
- 6.5.8. Survival tapping of the ascites fluid is avoided since it often results in hemorrhage, edema, or death.
- 6.6. Ascites fluid collection:
 - 6.6.1. Each mouse will yield from 2.0 mL to over 5.0 mL of ascites fluid.
 - 6.6.2. Euthanize animals as per Rodent Euthanasia SOP.
 - 6.6.3. Clean abdomen with 70% ethanol and pat dry.
 - 6.6.4. Make an incision in the skin over the abdomen while holding the mouse over the collection container.
 - 6.6.5. Drain all the fluid into container and transfer into centrifuge tube.
 - 6.6.6. Centrifuge ascites fluid at 1500 rpm for 5 minutes.
 - 6.6.7. Separate supernatant from blood cells and store at -80°C if necessary. If a fibrin clot has formed, break it up using two wooden stir sticks.
- 6.7. Animals will be kept for a maximum of 30 days. If ascites has not developed within this timeframe, animals will be euthanized as per Rodent Euthanasia SOP.

SOP REVISION HISTORY

DATE	NEW VERSION
2022.01.13	4.1. The production of monoclonal antibodies in mice by the ascites method raises several issues of concern regarding the potential for severe and unnecessary pain and suffering for the animals. A number of in vitro replacements for the rodent ascites method of monoclonal antibody production have been developed. Every attempt should be made to obtain material already available or to use an in vitro method for production of monoclonal antibodies.
2022.01.13	4.2. Before, producing monoclonal antibodies in vivo, the PI needs to justify to the Facility Animal Care Committee (FACC) in the Animal Use Protocol why in vitro production is not suitable in that specific case . The following procedures can only be applied if the FACC accepts the justification and approves the in vivo production of monoclonal antibodies
2022.01.13	5.1. The PI must prepare an antigen that is must be:
2022.01.13	5.2. The PI must provide one sample of the antigen For each scheduled immunization, each prepare a sample consisting of a maximum of 50 micrograms of antigen in sterile PBS (or animal compatible buffer) in a volume of 50µL per mouse.
2022.01.13	5.7.2. Wipe the injection site with chlorhexidine.
2022.01.13	5.7.2.3. Do not contaminate the needle track with resulting intradermic or intramuscular deposition of the mixture. Before removing the needle, with draw pull back on the plunger slightly to prevent the leakage of adjuvant into the dermal layer.
2022.03.10	6.5.5. Notify Veterinary Care staff when animals show signs of distress. Carprofen (20mg/kg), subcutaneous fluid administration, and the provision of wet food in the cage may alleviate minor distress.