

SOP: AB107.1
Modified: 7/27/22 KE

Monoclonal Antibody (MAB) Production Using a Murine Model (Immunizations, Bleeds, and Blood Processing)

Materials:

1. Purified recombinant or native *Mtb* antigens
2. 6 naïve BALB/c mice (female)
3. BD Lo-Dose U-100 Insulin Syringe, 1mL, 28 G x 1/2 in, 500/cs (VWR, Cat# BD329420)
 - **Best for immunizations, small and easy to use**
4. BD Microtainer Tubes (Z – NO additives), red, with microguard closure, 50/pk (Fisher Scientific, Cat# 365963)
5. Disposable scalpel blades (*tail bleeds*), blade #10, 100/pk (VWR, Cat# 21909-378)
6. Fisherbrand™ Color-Coded Capillary Tubes, Non-heparinized Tube, blue, 100/pk (Fisher Scientific, Cat# 22-260943)
 - **Optional, to aid in bleeding**
7. Butler Schein Animal Health KWIK STOP STYPTIC POWDER, 14 g/ bottle (Fisher Scientific, Cat# NC0220287)
 - **Optional, to aid in bleeding**
8. 1 mL **OR** 3 mL syringes
 - **to mix antigen and adjuvant with emulsifying needle**
9. Emulsifying needle
 - **cleaned with DI water and 70% ethanol THEN sterilized**
10. Titermax Gold adjuvant, 1 mL/ bottle (Thomas Scientific, Cat# C939E38)
11. Incomplete Freund's Adjuvant, 10mL/bottle (Sigma, Cat# F5506-10ML)
 - **Optional, can be used instead of Titermax Gold**
12. Appropriate equipment for CO₂ asphyxiation
 - **Ask LAR – part of their tours of the facilities and training for mice**
13. Biohazard bags for mice after dissection
 - **Ask LAR – mouse corpses to be disposed of appropriately in LAR facility (appropriate location)**
14. Autoclave tape
 - **One piece to tie up biohazard bags for mouse corpse disposal**
 - **A second piece to be labeled appropriately for disposal (ask LAR for appropriate labeling)**
15. Ethanol resistant markers
16. Biosafety cabinet
 - **For collection of spleen**
17. 0.65 mL Eppendorf tubes **OR** 1.7 mL Eppendorf tubes
18. Mini centrifuge (for smaller volumes) – **reach up to 10,000 x g**
19. 37°C incubator
20. 4°C fridge
21. -70°C freezer

Immunization & Bleeding Protocol (Note 1):

1. **All animal work must be done in accordance/compliance with the approved IACUC protocol (Kuali protocol #1281). Make sure to read IACUC protocol thoroughly prior to starting project.**
2. **Make sure all required training is done prior to starting project.** Below is a list (not exhaustive) of trainings for animal work. **Please check with supervisor to determine ALL training needs.**
 - Animal Care and Use at Colorado State University **and** through IACUC (online)
 - EHS CSU Occupational health Program Risk Assessment and Training Assignment (online)
 - Blood-borne Pathogens Precautions and Training (online)
 - Basic Research Techniques with Mice (in-person)
 - Tour the LAR (**Painter** at main campus or **RIC Basement** at Foothills campus) with Veterinarian/LAR staff in charge
 - Obtain access to LAR w/ RamCard
 - Get access to Kuali Protocols and Animal Ordering system

3. _____ Make a calendar *for immunizations and boosters, immunization conditions, and bleeds*. Table 1 below demonstrates a typical timeline for MAB production, *in compliance with approved IACUC protocol*.

Table 1: Immunization and Bleed Schedule (Note 2)

	Pre-Bleed (Naïve)	Prime - Inject #1	Boost #1 - Inject #2	Test Bleed (#1)	Boost #2 - Inject #3	Test Bleed (#2)	Boost #3 - Immunization #4 (IF needed)	Test Bleed (#3) (IF needed)	Final Boost (Immunization #4 or 5, depending)
Timeline	“Day 0”	Day 1	Day 15	Day 25	Day 29	Day 39	Day 43	Day 53	Depends
IACUC	--	7-10 days after naïve bleed	8-16 days after “Prime” immunization	7-10 days after “Boost #1” immunization	8-16 days after “Boost #1” immunization	7-10 days after “Boost #2” Immunization	8-16 days after “Boost #2” immunization	7-10 days after “Boost #3” Immunization	7-9 days after “Boost #2 or Boos #3” immunization
Immunization Conditions (dose, adjuvant, injection site)	N/A	100 µg antigen w/equal volume Titermax Gold (total of 100 µL of emulsion); SubQ on neck	100 µg antigen w/equal volume Titermax Gold (total of 100 µL of emulsion); SubQ on right leg	N/A	100 µg antigen w/equal volume Titermax Gold (total of 100 µL of emulsion); SubQ on left leg	N/A	50 µg antigen given IP, <i>NO adjuvant</i> , 100 µL total volume	N/A	50 µg antigen given IP, <i>NO adjuvant</i> , 100 µL total volume

4. _____ Perform naïve bleed (tail bleed) once mice are acclimated to their new space (*this time period must be checked with LAR, but it’s usually a week*). **The volume of blood collected cannot exceed 0.5% of the animal weight per bleed (per IACUC protocol) which equates to ~ 100 µL per bleed.**
- _____ **Collect blood directly into microtainer tubes (Z- no additives)**
 - _____ Process blood (see **Blood Processing for Serum Collection Protocol** below)
5. _____ Perform prime (injection #1) and subsequent **first** booster immunization (injection #2). Immunize with 100 µg of antigen (per mouse) emulsified in an equal volume of Titermax Gold adjuvant for both injections (Notes 3 & 4). **These injections are both subcutaneous (SubQ)**
6. _____ Perform test bleed #1 (as in step 4).
- _____ **Collect blood directly into microtainer tubes (Z- no additives)**
 - _____ Process blood (see **Blood Processing for Serum Collection Protocol** below)
7. _____ Perform second booster immunization (injection #3). Immunize with 100 µg of antigen (per mouse) emulsified in an equal volume of Titermax Gold adjuvant for both injections (Notes 2 & 3). This injection
8. _____ Perform test bleed #2 (as in step 4).
- _____ **Collect blood directly into microtainer tubes (Z – no additives)**
 - _____ Process blood (see **Blood Processing for Serum Collection Protocol** below)
9. _____ **IF NEEDED, perform a third booster immunization (injection #4)**. Immunize with 50 µg of antigen (per mouse) **WITHOUT** adjuvant. **This injection is intraperitoneal (IP).**
10. _____ **IF NEEDED, perform a third test bleed (as in step 4)**.
- _____ **Collect blood directly into microtainer tubes (Z – no additives)**
 - _____ Process blood (see **Blood Processing for Serum Collection Protocol** below)
11. _____ Perform final booster immunization (Note 5). Immunize with 50 µg of antigen (per mouse) **WITHOUT** adjuvant. **This injection is intraperitoneal (IP). This final booster immunization is performed 7-9 days from penultimate injection (IACUC protocol).**
12. _____ **Euthanize mice according to IACUC protocol and following euthanasia protocols.**
13. _____ Collect spleens of mice 2-4 days (per IACUC protocol) **after final booster immunization.**
- Cardiac bleeds can be performed after euthanasia and during spleen collection
 - Collect blood directly into microtainer tubes (Z – no additives)**
 - See SOP: AB103.7 for monoclonal antibody fusion (Note 1)**

Blood Processing for Serum Collection Protocol:

- _____ Collect blood via **tail bleeds** (during injections) or **cardiac puncture** (*terminal, during spleen collection*) in red microtainer tubes (Z-no additives).

2. _____ Incubate blood in microtainer tube for 1 hr, 37°C.
3. _____ After incubation, flick tube to dislodge clot.
4. _____ Place microtainer tubes at 4°C, overnight.
5. _____ The next day, centrifuge microtainer tubes at 10,000 *x g* for 10 min, 4°C.
6. _____ Gently remove serum (top “clear” layer) and transfer serum into new a microcentrifuge tube.
7. _____ Centrifuge collected serum (usually still red with RBCs) a *second time* at 10,000 *x g* for 10 min, 4°C.
8. _____ Gently remove serum (top “clear” layer) and transfer serum into new a microcentrifuge tube.
9. _____ Aliquot serum into smaller volumes (for qualification and freeze/thawing).
10. _____ Freeze serum at -70°C.

Serum Qualification (Note 6):

1. _____ Qualify serum via Western blotting (SOP: SP011.3 or SP011a). *A serum titer of 1:1,000 with dark bands (appropriate molecular weight for antigen) on Western blots equates to sufficient immunization.*
2. _____ Qualify serum via indirect (colorimetric) ELISA (SOP: SP039.2). *A serum titer of 1:1,000 with an OD405 of 1 on ELISA equates to sufficient immunization.*

Notes:

1. **SOP: AB103.7 and this protocol go together for MAB production.** This protocol focuses on immunizations and bleeding of mice as well as blood processing. SOP: AB103.7 focuses on spleen collection and subsequent fusion.
2. Generally, three injections (immunizations) and one final booster (2 days before spleen collection) are performed for sufficient immunization. However, some mice may be highly reactive after two injection and could then move on to the final booster. Some mice may need more immunizations. ***Make sure that the number of immunizations is allowed per the IACUC protocol.***
3. Use an emulsifying needle to aid in emulsification of the antigen and adjuvant. Make sure emulsifying needle is clean and sterilized. Make sure to add the **antigen** TO the **adjuvant** FIRST in the emulsifier.
4. The antigen and adjuvant are added at a ratio of 1:1. Titermax Gold is generally the adjuvant of choice, but ***Incomplete*** Freund’s adjuvant can be used (in IACUC protocol).
5. This final booster immunization is performed 8-16 days after the “Boost #2” or “Boost #3”, depending on how reactive the mice are after “test bleed #2”.
6. Qualify mouse serum via Western blotting **AND** indirect (colorimetric) ELISA for/after each bleed. Both immunoassays determine sufficient reactivity/immunization of mice.

References:

1. See... “Rsch-Dobos-Lab” → Steph’s (Propp) Folder → Antibodies Folder → Mpt51 Folder → **MAB Milestone 2_recMPT51 document** for an example of the immunization/bleed schedule
2. See... “Rsch-Dobos-TBContract” → Kala’s (Early) Folder → QC BEI Folder → Monoclonal Folder → GlnA REDO (peptide) Folder → Milestones Folder → **GlnApep_Milestone 2 document** for another example of the immunization/bleed schedule