

APPENDIX: CRYOPRESERVATION OF *BABESIA* SPECIES *IN VIVO*

1. Prepare a 30% (v/v) sterile glycerol solution in Alsever's solution following the formulation below. Dissolve components in glass-distilled H₂O, adjust the pH to 6.1 with 10% (w/v) citric acid and filter sterilize. Alternatively, prepared Alsever's solution is available from commercial manufacturers.

Alsever's Solution

Sodium chloride	4.2 g
Trisodium citrate dihydrate (Na ₃ citrate • 2H ₂ O)	8 g
Glucose	20.5 g
Glass-distilled H ₂ O to	1 L

2. Dispense an appropriate amount of anticoagulant solution, based on the estimated amount of blood to be collected, into an appropriately sized conical tube. Add to the anticoagulant blood collected by a facility-approved method from infected animals that had reached the desired parasitemia. Invert the tube several times to mix the blood with the anticoagulant.
3. In a separate conical tube, add the anticoagulant blood dropwise to the 30% glycerol solution. Note that blood should be mixed with glycerol solution in a 2:1 ratio to obtain a final concentration of cryoprotectant of 10% (v/v). Mix slowly by inversion and place the tube on ice. The freezing process should start 15 to 30 minutes following the addition of the heparinized blood to the cryoprotectant solution.
4. Dispense 0.5 mL aliquots of blood suspension into 1 to 2 mL sterile plastic screw-capped cryovials for cryopreservation.
5. Place the vials in a controlled rate freezing unit. From room temperature, cool the vials at -1°C/minute to -40°C. If the freezing unit can compensate for the heat of fusion, maintain rate at -1°C/minute through this phase. At -40°C, plunge vials into liquid nitrogen. Alternatively, place the vials in a Nalgene 1°C freezing container. Place the container at -80°C for 1.5 to 2 hours and then plunge vials into liquid nitrogen.
6. To thaw a frozen vial, place in a 35°C to 37°C water bath, until thawed (2 to 3 min). Immerse the vial just sufficient to cover the frozen material. Do not agitate the vial.
7. Follow the protocol for *in vivo* propagation in the Growth Conditions section of the Product Information Sheet.