

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

1. Harvest *in vitro Babesia* cultures from multiple flasks using a pipette and transfer the cell suspensions to 15 mL or 50 mL plastic centrifuge tubes. Cultures should be well established and growing vigorously with a parasitemia $\geq 4\%$.
2. Centrifuge at $1000 \times g$ for 5 minutes at room temperature.
3. Wash the pellet once with 10 or more volumes of incomplete RPMI 1640 medium. Centrifuge the cell suspension at $1000 \times g$ for 5 minutes. Remove the supernatant, leaving enough supernatant to resuspend the pellet. Estimate the volume of the remaining cell suspension.
4. To the volume of packed red blood cells, slowly add dropwise one volume of cold (4°C) Glycerolyte 57 solution (or equivalent). Allow to incubate for 5 minutes at room temperature.
5. Add dropwise an additional 4 volumes of cold Glycerolyte 57 solution to the pellet and mix well.
6. Dispense 0.5 mL aliquots into 1 mL to 2 mL sterile plastic screw-capped vials for cryopreservation.
7. Place the vials in a controlled rate freezing unit. From room temperature cool the vials at $-1^{\circ}\text{C}/\text{min}$ to -40°C . If the freezing unit can compensate for the heat of fusion, maintain rate at $-1^{\circ}\text{C}/\text{min}$ 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 to 2 hours and then plunge vials into liquid nitrogen.
8. 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.
9. Follow the protocol for *in vitro* propagation in the Growth Conditions section of the Product Information Sheet.