Appendix I, II, III for *Babesia* sp. *(in vitro)*

APPENDIX I: CRYOPRESERVATION

1. Harvest *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 ≥ 4%.
2. Centrifuge at 1000 × 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 × 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 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°C/min to -40°C. If the freezing unit can compensate for the heat of fusion, maintain rate at -1°C/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 days and then plunge vials into liquid nitrogen.
8. Store in either the vapor or liquid phase of a nitrogen refrigerator (-130°C or colder).

APPENDIX II: *BABESIA* GROWTH MEDIUM

1. Incomplete Medium: Used for many applications involving wash steps during preparation of parasites for culture or assay. The incomplete medium consists of RPMI 1640 medium supplemented with the following components[1]:

Incomplete Medium	
RPMI 1640 medium[2,3]	
Sodium bicarbonate ($NaHCO_3$)[4]	2.4 g/L
L-Glutamine	2 mM
HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]	25 mM
D-Glucose[5]	2 g/L
Hypoxanthine	27 µg/mL
Gentamicin (optional)	5 µg/mL

[1]Prepare sterile stock solutions at concentrations that are easily diluted into the liquid medium to obtain the appropriate user concentrations and add aseptically. Ready-made stock solutions for many of the components are available from numerous manufacturers.
[2]RPMI 1640 medium is available from numerous manufacturers as both a powder and a sterile, prepared liquid, with or without L-glutamine and HEPES. If using powdered RPMI 1640 medium, prepare the medium following manufacturer instructions, sterile filter using a 0.22 µm filter, then aseptically add the necessary components in the appropriate concentrations.
[3]If stock solutions were not sterile or aseptic techniques were not followed, sterile-filter the medium using a 0.22 µm filter after the addition of all components. Store at 4°C.
[4]Prepared, liquid medium typically contains sodium bicarbonate while powdered medium does not. A typical concentration of sodium bicarbonate in RPMI 1640 medium is 2 g/L, though some formulations contain different amounts.
[5]A typical concentration of D-glucose in RPMI 1640 medium is 2 g/L. The option to supplement with an additional 2 g/L yields a final concentration of 4 g/L D-glucose.

2. Complete Medium: consists of incomplete medium (above) supplemented with 10% heat-inactivated human serum. If necessary, filter the complete medium with a 0.22 µm filter. Since serum tends to clog sterilizing filters, a serum pre-filter may be used first, followed by a 0.22 µm sterilizing filter.

   Note: Human serum type A is used with washed type O blood. Serum substitutes may be used; however, they may not be acceptable for all parasite strains.

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APPENDIX III: PREPARATION OF HUMAN ERYTHROCYTES

1. Prepare the Puck's Saline Glucose (PSG) medium (see recipe below), mix well, adjust pH to 7.2, and adjust the volume to 1 L with distilled, deionized water. Filter sterilize using a 0.22 µm filter and store at 4°C.
2. Prepare the PSG+G solution (see recipe below), mix well, filter sterilize using a 0.22 µm filter and store at 4°C.

Puck's Saline Glucose Medium	
$CaCl_2 \cdot 7H_2O$	0.016 g
KCl	0.40 g
KH_2PO_4	0.15 g
$MgSO_4 \cdot 7H_2O$	0.15 g
NaCl	8.0 g
$Na_2HPO_4 \cdot 7H_2O$	0.29 g
D-glucose	1.10 g
Phenol red	0.005 g
Distilled, deionized water to	1 L

PSG+G Solution	
Puck's Saline Glucose Medium	500 mL
D-glucose	10 g
Antibiotic/Antimycotic Solution (ATCC® PCS-999-002™)	5 mL

3. Aseptically, wash donor blood three times by centrifugation at 600 to 800 × g for 15 minutes at 4°C in RPMI 1640 medium.
4. After each wash, aseptically remove the supernatant, consisting of the plasma and buffy (leukocyte) layers located on the top of the red blood cell (erythrocyte) pellet.
5. After the last wash, aseptically resuspend human erythrocytes in sterile PSG+G solution at a concentration of 50% erythrocytes. The human erythrocytes in PSG+G solution may be stored at 4°C until use, for a maximum of two weeks.

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