

**APPENDIX 8****Immunofluorescent antibody test (IFAT)****Reagents***Phosphate buffered saline (PBS), pH 7.2*

NaCl	8 gm
KH <sub>2</sub> PO <sub>4</sub>	0.2 gm
Na <sub>2</sub> HPO <sub>4</sub> . 12H <sub>2</sub> O	2.88 gm
KCl	0.2 gm
Distilled water	up to 1 l

(Can be made up at a concentration of 10 x the above recipe for better long-term storage.)

*PBS/0.05 % Tween (PBS/T)*

PBS solution	99.95 ml
Tween 20	0.05 ml

*PBS/T/2 % milk powder (PBS/T/M)*

PBS/T solution	100 ml
Dried skimmed milk (low fat)	2.00 gm

*PBS/10 % glycerol (v/v)*

PBS solution	90.00 ml
Glycerol	10.00 ml

Cultured *L. donovani*, *L. infantum* or *L. chagasi* promastigotes are washed (x 3) with sterile phosphate buffered saline (PBS) by centrifugation and re-suspension, preferably at 4°C. The final pellet is re-suspended in PBS at  $5 \times 10^8$  promastigotes/ml and 5 µl volumes of cell suspension are immediately dispensed into each well of the IFAT microscope slides (e.g. from Henley, Essex, UK) and the slides are allowed to dry in air. The slides are wrapped in tissue paper, placed in plastic bags and stored at -20°C. Slides are retrieved from storage by allowing them to come to room temperature in a desiccator.

Serum samples are diluted in phosphate buffered saline/0.05 % tween/2 % milk (PBS/T/M). Serum sample dilutions, beginning with a dilution of 1:50 are added into each well and the slides are incubated for 30 min at room temperature in a humid chamber. After incubation the slides are washed (x 3) for 10 min with phosphate buffered saline/0.05 % tween (PBS/T). 5  $\mu$ l of a 1:50 dilution of anti-dog immunoglobulin fluorescein isothiocyanate conjugate (FITC) in PBST/M/1:10,000 Evans blue is added to each well and the slides are incubated for 30 min at 37°C in a humid chamber. After incubation, the slides are washed (x 5) in PBS and allowed to air-dry in the dark. Each slide is mounted in PBS/glycerol and observed at a x 1000 magnification under a fluorescent microscope in a dark room.