

**APPENDIX 9****ELISA for anti-*Leishmania* antibodies****Reagents***Coating Buffer (CB)*

Na <sub>2</sub> CO <sub>3</sub>	1.59 gm
NaHCO <sub>3</sub>	2.93 gm
NaN <sub>3</sub>	0.2 gm
Distilled water	up to 1 l

(Can be made up at a concentration of 10 x for more convenient storage.)

*CB/2 % milk powder*

CB	100 ml
Dried skimmed milk (low fat)	2.00 gm

*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)*

PBST solution	100 ml
Dried skimmed milk (low fat)	2.00 gm

*Phosphate citrate buffer, pH 5.5*

## Solution A

Citric acid	2.1 gm
Distilled water	to 100 ml

## Solution B

Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O	3.5 gm
Distilled water	to 100 ml

Add 48.5 ml of solution A to 51.5 ml of solution B.

*Substrate solution (for peroxidase HRP conjugate)*

O-phenylenediamine hydrochloride (OPD)	0.040 gm
Phosphate citrate buffer, pH 5.5	100 ml
Hydrogen peroxide (3 %)	30 µl

*Preparation of ELISA antigen*

*L. donovani*, *L. infantum* or *L. chagasi* promastigotes are cultured in liquid medium and log-phase promastigotes harvested at a concentration of approximately  $1 \times 10^6$  cells/ml. The cell pellet is washed (x 3) preferably at 4°C, with sterile phosphate buffered saline (PBS) and frozen at -20°C. The frozen pellet is thawed to room temperature and re-suspended in sterile distilled water at 1:40 (v/v). The cells are disrupted by rapidly freezing in liquid nitrogen and thawing in water at 37°C (x 3) and if possible followed by ultrasonication for 15 seconds (x 5). The disrupted cells are centrifuged at high speed (e.g. 10,000 g) for 15 minutes at 4°C and the supernatant stored frozen at -20°C for use as the ELISA antigen.

### *Coating of ELISA wells*

Polystyrene microtitre plates or similar plates are used for ELISA but they must be plates that are specifically recommended for ELISA. The antigen dilution used for coating the wells is determined by a checker-board experiment using different antigen dilutions against standard positive and negative serum controls and several different test dilutions of the conjugate. Antigen dilutions are made in 0.05 M carbonate coating buffer, pH 9.6. To coat the wells 100  $\mu$ l of diluted antigen is pipetted into each well and left at 4°C overnight. The wells are then washed (x 3) with PBS and blocked with coating buffer/2 % milk powder for one hour at 37°C and washed again (x 3) with PBS.

### *Performing the ELISA*

Sera are diluted 1:200 in PBS/T/M. Further dilutions are made, e.g. doubling dilutions if serum titres are required. 100  $\mu$ l of serum dilutions are added to each antigen coated well of a 96 well ELISA plate. The plates are incubated for one hour at 37°C in a humid chamber and then washed (x 3) with PBS/T. Each well is immediately filled with 100  $\mu$ l of peroxidase conjugated, affinity purified, monoclonal anti-human immunoglobulin G (H + L) conjugate labelled with horseradish peroxidase (HRP) or alkaline phosphatase (ALP) diluted at approximately 1:1,000 or 1:2,000 in PBS/T/M and incubated for one hour at 37°C. The optimum conjugate dilution is determined by checker-board titration. For canine VL rabbit anti-dog immunoglobulin G (H + L) conjugate is used. After incubation plates are washed again (x 3) with PBS/T and 100  $\mu$ l of substrate solution added. The plates are left at room temperature for 15 minutes in the dark, after which the reaction is stopped by adding 50  $\mu$ l of 2.5 M H<sub>2</sub>SO<sub>4</sub>. The results are read on an ELISA plate reader using a 492 nm filter within half an hour of stopping the reaction. All plates must include negative and positive control sera. Tests are normally performed in duplicate. Day to day variations in the test conditions can be adjusted with a positive reference sample as follows:

(absorbance of the test sample/absorbance of the reference positive) x 1