APPENDIX B

Media and reagents for ELISPOT assay and indirect ELISA

1. Media and reagents for cell culture

1.1 Basic RPMI medium (serum free-RPMI)

The medium consisted of the following ingredients:

RPMI 1640 (Gibco Co., USA)	10.40 g
HEPES, MW 238.31	5.9525 g
(Sigma Chemical Co., USA)	
$NaHCO_3$	2.016 g
D-glucose	3.60 g
Sodium pyruvate	1.1005 g
(Sigma Chemical Co., USA)	
L-glutamine (Sigma Chemical Co., USA)	0.2923 g
UDW to make 1,000 ml	

The solutions of penicillin G, streptomycin and kanamycin were added to the final concentration of 20,000 units, 200 mg and 200 mg per liter, respectively. The medium was sterilized by filtering through a sterile Millipore membrane (pore size 0.22 μm). Aliquots of 100 ml were kept in steriled bottles at –20°C.

1.2 Complete RPMI medium

The medium consisted of the following ingredients:

Sterile, heat-inactivated bovine serum	5.0 ml
(Starrate Co., Australia)	
Basic RPMI medium	95.0 ml

1.3 Trypan blue solution (0.3%)

Sixty milligrams of trypan blue (Fluka, Switzerland) was dissolved in 20 ml of NSS.

2. Reagents for ELISPOT assay

2.1 Phosphate buffer (1 M PB, pH 7.4)

Solution A was prepared by dissolving 177.90 g of $Na_2HPO_4.2H_2O$ in 1,000 ml distilled water.

Solution B was prepared by dissolving 156.01 g of $NaH_2PO_4.2H_2O$ in 1,000 ml distilled water.

The solution B was slowly added to solution A for adjusting the pH to 7.4.

2.2 Phosphate buffered saline (0.01 M PBS, pH 7.4)

Ten milliliter of 1 M PB, pH 7.4 and 8.5 g NaCl were dissolved in 1,000 ml DW; the solution was sterilized by filtration through a sterile Millipore membrane (0.22 μm) and kept at 4 °C.

2.3 Washing solution (PBST)

The washing solution (PBST) was prepared by mixing Tween-20 in 0.01 M PBS, pH 7.4 to a 0.1% concentration.

2.4 Blocking solution (3% BSA)

Three grams BSA (Nacalai Tesdque, Japan) was dissolved in 100 ml 0.01 M PBS, pH 7.4.

2.5 Diluent (1% fetal bovine serum)

One milliliter fetal bovine serum was added to 100 ml 0.01 M PBS, pH 7.4.

3. Reagents for indirect ELISA

3.1 Coating buffer (carbonate-bicarbonate buffer, pH 9.6)

This buffer contained 2.93 g of $NaHCO_3$ and 0.53 g of Na_2CO_3 in 1 liter of water. The pH was adjusted to 9.6 with NaOH.

3.2 Washing solution (PBST)

The washing solution (PBST) was prepared by mixing Tween-20 in 0.01 M PBS, pH 7.4 to a 0.05% concentration.

3.3 Blocking solution (1% BSA)

BSA (Nacalai Tesdque, Japan) 1.0 g was dissolved in 100 ml 0.01 M PBS, pH 7.4.

3.4 Diluent (0.2% BSA, 0.2% gelatin)

The solution consisted of 0.2 g BSA (Nacalai Tesdque, Japan) and 0.2 g gelatin (Sigma Chemical Co, USA) in 100 ml of 0.01 M PBS, pH 7.4. The BSA was dissolved in the PBS and the gelatin was added. The solution was warmed up with stirring until the gelatin was completely dissolved. The gelatin was dissolved in 100 ml warm 0.01 M PBS, pH 7.4. Then the solution was cooled to room temperature before adding the BSA to dissolve.

3.5 Substrate buffer (0.1 M citrate buffer, pH 4.5)

Trisodium citrate ($Na_3C_6H_5O_7.H_2O$) (14.7 g) was dissolved in 500 ml DW. The pH of the solution was adjusted to 4.5 with 1 M HCl.

3.6 Substrate solution

The substrate solution consisted of 0.05% (0.05 g) of 1, 4- *p*- phenylenediamine-dihydrochloride (PPD) (Sigma Chemical Co, USA) in citrate buffer, pH 4.5 (10 ml), and 0.01% 30% H_2O_2 (10 µl). This solution was freshly prepared before use and always protected from light.

3.7 Stop solution (1 N NaOH)

NaOH (20 g) was dissolved in 500 ml distilled water.