

APPENDIX B

Reagents for indirect ELISA

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.

2. Phosphate buffer (1M PB, pH 7.4)

Solution A

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	177.90	g
DW to	1,000	ml

Solution B

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	156.01	g
DW to	1,000	ml

The solution B is added to solution A for adjusting the pH to 7.4

3. Phosphate buffered-saline (0.01 M PBS, pH 7.4)

Ten ml of the 1.0 M PB, pH 7.4 and 8.5 g of NaCl are dissolved in 900 ml of DW. After completely dissolving, DW is added to make 1,000 ml volume.

4. 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.

5. Blocking solution (1% BSA)

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

6. 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.

7. Substrate buffer (0.1 M citrate buffer, pH 4.5)

Trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$) (14.7 g) was dissolved in 500 ml DW. The pH of the solution was adjusted to 4.5 with 1 M HCl.

8. 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% 3 0% H_2O_2 (10 μl). This solution was freshly prepared before use and always protected from light.

9. Stop solution (1 N NaOH)

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