APPENDIX A

Reagent for dot-ELISA

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

Ten milliliters 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.

3. Washing solution (PBST)

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

4. Blocking solution (3% BSA)

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

5. 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.

6. Substrate buffer (0.15 M Tris-HCl, pH 9.6)

Trizma Base (Sigma Chemical Co, St. Louis, MO, USA, 18.16 g) was dissolved in DW. The pH was then adjusted to 9.6 with concentrated HCl. The final volume was brought up to 1,000 ml with DW.

7. Enzyme conjugate (goat anti-mouse immunoglobulins-alkaline phosphatase)

This was prepared by diluting goat anti-mouse alkaline phophatase (Dako Pattss, Denmark) with PBS, pH 7.4 to a final dilution of 1:4,000.

8. Substrate solution

The substrate of alkaline phosphatase enzyme was 5-bromo-4 chloro-3-indolryl phosphate (BCIP) and nitro-blue tetrazolime (NBT) which were obtained commercially in liquid; BCIP/NBT phosphatase substrate (Kirkegaard & Perry Laboratories, MD, USA). The substrate solution was prepared by adding three volumes of BCIP/NBT into five volumes of Tris buffer, pH 9.6. The substrate was freshly prepared before use, and kept under light protection during the reaction.