APPENDIX A

Preparation of some reagents and buffer used in this study

Solution and Buffer for ELISA

1. Phosphate Buffer Saline (PBS)

NaCl	8.00	g
KCl	0.20	g
Na_2HPO_4	1.44	g
NaH_2PO4	0.24	g

All reagents were dissolved in distilled water and made up volume to 1,000 ml, adjusted the pH to 7.4 with HCl or NaOH and added distilled water to adjust volume to 1,000 ml and stored at room temperature. For PBS-Tween, Tween 20 was added 0.05 % v/v.

2. 1% Bovine Serum Albumin (blocking buffer)

BSA	0.2	g
PBS, pH 7.4	20	ml

3. PBS-Tween (0.05% Tween 20) (washing buffer)

PBS, pH 7.4	500	ml
Tween 20	250	µl

4. Carbonat-Bicarbonate Buffer pH9.6 (coating buffer and conjugation buffer)

Na_2CO_3	1.59	g
$NaHCO_3$	2.93	g
Distilled water	1,000	ml

5. 0.05 M Tris-HCl, pH 8.6 (0.05 % Tween 20) (FITC-HABP buffer)

Tris-HCl	1.97	g
Tween 20	125	µl
Distilled water	250	ml

6. Citrate phosphate buffer

Citric acid monohydrate	10.3	g
$Na_2HPO_4 \cdot 3H_2O$	18.16	g

All reagents were dissolved in 900 ml of distilled water, adjusted pH to 5.0 and made up volume to 1,000 ml and stored reagent at 4 °C.

7. Peroxidase substrate solution

O-phenylenediamine (OPD)	8	mg
Citrate phosphate buffer	12	ml
30 % H_2O_2	5	µl

Prepare reagent fresh for 1 plate; keep in dark before use.

SDS-PAGE analysis

1. Stock solution A: Separating gel buffer 1.5 mM Tris-HCl, pH 8.8

Tri-base	18.15	g
Deionize distilled water	80	ml

Adjusted pH to 8.8 then adjusted volume to 100 ml and filtrated any nonsoluble powder by filtration with membrane filter pore size 0.2 µm, collected in dark container.

2. Stock solution C: Stock acrylamide solution (30 % T, 2.67 %)

Acrylamide	29.2	g
N'N'-bis-methylene-acrylamide	0.8	g
Deionize distilled water	70	ml

Adjusted volume to 100 ml and filtrated any nonsoluble powder by filtration with membrane filter pore size 0.2 µm, collected in dark container.

3. Stock solution D: Stacking gel buffer 0.5 mM Tris-HCl, pH 6.8

Tris-base	6.05	g
Deionize distilled water	70	ml

Adjusted pH to 6.8 then adjusted volume to 100 ml and filtrated any nonsoluble powder by filtration with membrane filter pore size 0.2 μm, collected in dark container

4. Stock ammonium persulfate solution (10 % w/v APS in deionize water)

Ammonium persulfate	0.1	g
Deionize distilled water	1	ml

5. Electrode buffer

Tris-base	3.0	g
Glycine	14.4	g
SDS	1.0	g

Dissolved in deioniozed water 1,000 ml then filtrated by suction filter and stored at 4 °C

6. 5X nonreducing buffer

1.0 M Tris-HCl, pH 6.8	0.625	ml
Glycerol	1.0	ml
1 % Bromphenol blue	0.125	ml

Adjusted volume to 10 ml with distilled water.

7. 5X reducing buffer

5X nonreducing buffer	475	μl
2-mercaptoethanol	25	μl

8. Molecular weight marker

Marker	1	μl
5X reducing buffer	19	μl

9. Coomassie blue

Coomassie blue	0.25	g
Methanol	20	ml
Acetic acid	10	ml
Deionized water was top up to 100 ml		

10. Coomassie blue destaining solution

Methanol	100	ml
Acetic acid	50	ml
Deionized water was top up to 500 ml		

11. Stock 10 % SDS solution

SDS	0.2	ml
Deionize distilled water	1	ml

12. Separating gel 12 %

Deionize distilled water	1.7	ml
Tris-HCl, pH 8.8 (solution A)	1.25	ml
10 % SDS	50	µl
Acrylamide/Bis (solution C)	2	ml
10 % APS	50	µl
TEMED	10	µl

13. Stacking gel 4 %

Deionize distilled water	3.05	ml
Tris-HCl, pH 6.8 (solution D)	1.25	ml
10 % SDS	50	µl
Acrylamide/Bis (solution C)	0.65	ml
10 % APS	50	µl
TEMED	10	µl

APPENDIX B

Conventional ELISA assay (Modified Yingsang, 1996)

HA content in individual sample was assayed in triplicate by ELISA assay. This involved competition for a fixed concentration of biotinylated HABP between HA bound to the wells of a microtiter plate, and HA in standard dilution ranging from 10-10,000 ng/ml (in serial five-fold dilutions) or in serum sample. The method was shown in Figure38 and 39. The inhibition immunoassay of HA had been employed in small polypropylene tubes. Samples containing unknown amounts of HA and standard (using known concentrations of highly purified HA (Healon) in PBS, pH 7.4, containing 6 % w/v BSA) were pipetted into small polypropylene tubes with biotinylated-HABP (equal volume of 180 ml/tube) in 0.05 M Tris-HCl, pH 8.6 and 0.05 % Tween. A vortex mixter was used prior to incubation at room temperature followed by 1 hour. Aliquots of 100 μl (triplicates of each samples) were then applied to HA coated and BSA blocked plates and incubated at room temperature for 60 minutes. The plate wells were washed three times with PBS-Tween 20 (0.05 % v/v), and 100 μl of peroxidase conjugated monoclonal anti-biotin (Zymed), was added to each well. Plates were incubated for 1 hour at room temperature and the wells were washed 3 times with PBS-Tween followed by drying. Peroxidase substrate was added to each well (100 μl) and incubation at room temperature. The reaction was stopped by the addition of 50 μl 4 M H_2SO_4. The chromogen was monitored by absorption at 492/690 nm in a plate reader (Titertek Multiskan M 340). A standard inhibition curve for HA was constructed, using log/linear coordinators, the HA levels in the test samples were determined by comparing their capacity to inhibit color development at OD 492/690 nm relative to standard curve. A standard curve for HA assay was constructed using a computer software (Genesis), the levels of HA in the samples were calculated automatically using 4-parameter curve fit.

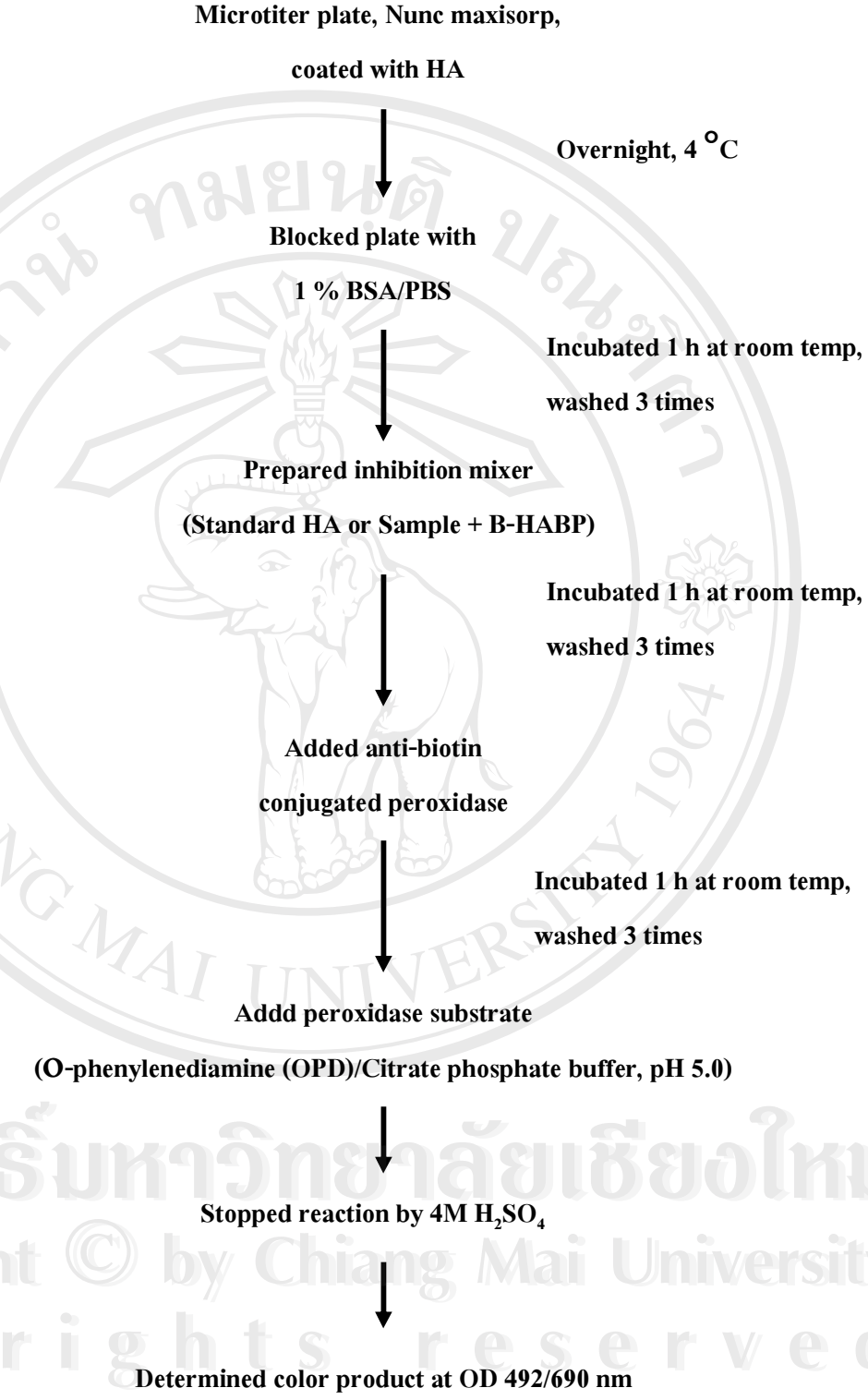

Figure 38. Diagram steps of the conventional ELISA method developed for hyaluronan determination.

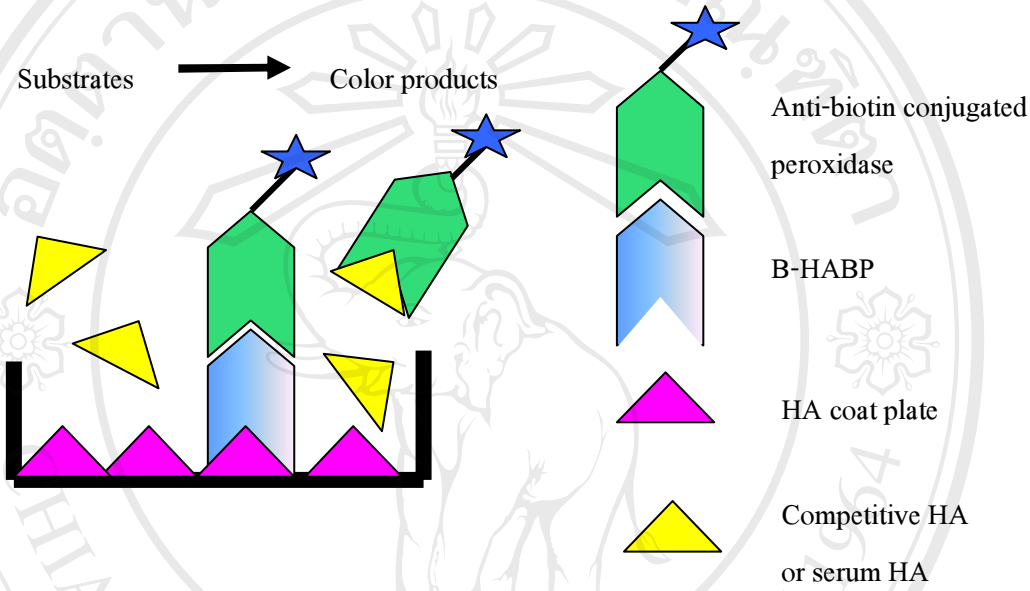

Figure 39. Principle of the conventional ELISA assay.

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