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APPENDICES

APPENDIX A

CHEMICAL AGENTS AND INSTRUMENTS

A. Laboratory supplies

Cylinders (Pyrex®, England)

Coplin staining jar

Dako pen (Dako, USA)

Disposable gloves (Latex, Thailand)

Eppendorf tube

Flask 1000 ml. (Pyrex®, England)

Glass pipettes (Witeg, Germany)

Glass slide (Soilbrand, China)

Humidified chamber

Microscopic glass cover slip (Chance, England)

Microtube

Reagent bottles: 250 ml, 500 ml, 1000 ml (Duran®, Germany)

Slide rack

Staining jars

Tip

B. Equipment

Autopipette (Gilson, France)

Balance (Precisa, Switzerland)

Freezer -20°C (SANYO, Japan)

Incubator (Heraeus)

Light microscope (Olympus, Japan)

Pressure cooker

Refrigerator 4°C (SANYO, Japan)

Rotary microtome

Timer

Vortex

C. General Reagents

Absolute Ethanol

Acetone (Merck, Germany)

Citric acid monohydrate

Clorox (Clorox, USA)

Conc. Hydrochloric

Diaminobenzidine tetrahydrochloride, anhydrous (DAB) (Sigma, USA)

EDTA

Eosin

Hematoxylin

Imidazole (Sigma, USA)

Lithium

Normal horse serum (NHS) (Gibco, USA)

Sodium Chloride

Sodium Hydrogen Phosphate

Sodium Hydroxide

Trisma base (Sigma, USA)

Triton X-100

Xylene (Sigma, USA)

3-aminopropyltriethoxysilane

30% Hydrogen Peroxide

D. Reagent kit

Apoptag® Peroxidase Kits (Intergen Company, USA)

Envision™ (Dako, USA)

E. Buffer and Reagent preparation

1. 30x Phosphate Buffered Saline (PBS), pH 7.4 1 liter

NaCl	263	g.
$NaH_2PO_4H_2O$	41.4	g.
5 NaOH	60	ml.

DW maker up to 1 liter.

2. 1X Phosphate Buffered Saline (PBS), pH 7.4 1 liter

30X PBS	70	ml.
DW	2030	ml.

3. Tris-HCl Buffer, pH 7.4

Stock A : 2.42 g. Trisma base in 100 ml DW

Stock B : 1.7 ml. conc. HCl in 100 ml DW

To make Tris-HCl buffer, pH 7.4

Stock A	2.5	ml.
Stock B	2.07	ml.

Make up to 10 ml. with DW.

4. 3,3' Diaminobenzidine tetrahydrochloride, anhydrous (DAB) 50 mg/ml

DAB	500	mg.
DW	10	ml.

Aliquot 100 ul. each microtubes (5mg.) and store below 0°C.

5. Working DAB Tris-HCl buffer, pH 7.4

DAB	1	microtube (5 mg.)

Tris-HCl buffer	10	ml.
30% H_2O_2	10	ul.
1M Imidazole	100	ul.

**Prepare freshly before use.

6. 3% NHS in PBS pH 7.4

1X PBS pH 7.4	100	ml.
NHS	3	ml.

7. 0.1% NHS + 0.01% Triton X-100 in PBS

1X PBS pH 7.4	1000	ml.
NHS	1	ml.
Triton X-100	100	ul.

8. 10 mM Citrate buffer pH 6.0

Citric acid monohydrate	2.1	g.
2M NaOH	13	ml.

Make up to 1000 ml. with DW.

9. 1M Imidazole

Imidazole	6.8	g.
DW	100	ml.

APPENDIX B

Coated Slides

Protocol

1. Rinse slides 2 changes in acetone .
2. Rinse slides in 2% 3-aminopropyltriethoxysilane 10 sec.
3. Quick rinse in acetone.
4. Rinse in deionized water.
5. Air-dried or in oven.

Immunohistochemistry by Envision™ system (Dako, USA)

Protocol

Slide preparation

1. Cut paraffin embedded tissue thick 3 μm by rotary microtome and put on coated slide.
2. Incubate tissue sections on coated slides 60°C 1 hr.

Deparaffinize tissue section (in a coplin jar)

3. Wash the specimen in 3 changes of xylene, dipping the slide 20 times each in first and second washes, followed by 10 min. in the third wash.
4. Wash the specimen in 3 changes of absolute ethanol, dipping the slide 10 times each in first and second washes, followed by 3 min. in the third wash.
5. Wash the specimen in 3 changes 95% ethanol, dipping the slide 10 times each in first and second washes, followed by 3 min. in the third wash.
6. Wash the specimen one change of distilled water for 1 min.

Antigen retrieval

7. Antigen retrieved by pressure cooker with citrate buffer pH 6.0 1 min.
8. Put the specimen in coplin jar containing PBS for 5 min. at room temp.

Block endogenous peroxidase activity

9. Carefully blot around the section and circle with dako pen (Dako, USA).
10. Apply 3% H_2O_2 in distilled water to completely cover the specimen and incubate in a humidified chamber at room temperature for 10 min.
11. Gently tap excess liquid and wash the specimen with running tap water in a coplin jar for 5 min.
12. Put the specimen in a coplin jar containing PBS 3 min.

Block nonspecific background

13. Apply 3% normal horse serum (NHS) to completely cover the specimen and incubate in a humidified chamber at room temperature for 20 min.

Antibody application

14. Gently tap excess 3% NHS.
15. Apply room temperature primary antibody to the slide to completely cover the specimen and incubated 60 min. in a humidified chamber.
16. Gently tap excess liquid and wash the specimen in 2 changes of PBS in a coplin jar for 3 min. each wash.
17. Apply 1 drop or 200 µl. of visualization reagent (Envision™ system (Dako, USA)) and incubated for 30 min. in a humidified chamber at room temperature.
18. Gently tap off excess liquid and wash the specimen in 2 changes of PBS for 3 min. each wash.

Develop color in peroxidase substrate

19. Apply peroxidase substrate (freshly working DAB) to complete cover the specimen and stain for 10 min. at room temp.
20. Wash the specimen with running tap water in a coplin jar for 3 min.
21. Counterstain with hematoxylin.
22. Mount the specimen under a glass coverslip in a mounting medium (permount).

TUNEL assay by Apoptag® Peroxidase Kits (Intergen Company, USA)

Protocol

Slide preparation

1. Cut paraffin embedded tissue thick 3 μm by rotary microtome and put on coated slide.
2. Incubate tissue sections on coated slides 60°C 1 hr.

Deparaffinize tissue section (in a coplin jar)

3. Wash tissue section in 3 changes of xylene for 5 min. each wash.
4. Wash the specimen in 2 changes of absolute ethanol for 5 min. each wash.
5. Wash the specimen once in 95% ethanol and once in 70% ethanol for 3 min. each wash.
6. Wash one change of PBS pH 7.4 for 5 min.

Pretreat tissue

7. Carefully blot around the section and circle with dako pen (Dako, USA).
8. Apply freshly diluted proteinase K (20 μg/ml) to the specimen (12 μl/cm^2) for 10 min. at room temp. in moist chamber.
9. Wash the specimen in 2 changes of distilled water in coplin jar for 2 min. each wash.

Quench endogenous peroxidase

10. Drop 3% hydrogen peroxide in PBS pH 7.4 200 μl. for 5 min. at room temp.
11. Rinse the specimen twice with distilled water for 5 min. each time, in a coplin jar.

Apply equilibration buffer

12. Apply 20 μl. of equilibration buffer directly on the specimen and incubate for 10 sec. at room temp.

Apply working strength TdT enzyme

13. Gently tap off excess liquid.
14. Immediately pipette onto the section 11 $\mu l/cm^2$ of working strength TdT enzyme.
15. Incubate in moist chamber at 37°C for 1 hour.

Apply stop/wash buffer

16. Put the specimen in a coplin jar containing working strength stop/wash buffer, agitate for 15 sec., and incubate for 10 min. at room temp.

Apply anti-digoxigenin conjugate

17. Wash the specimen in 3 changes of PBS pH 7.4 for 1 min. each wash.
18. Apply room temp. anti-digoxigenin peroxidase conjugate to the slide 20 $\mu l/cm^2$ of specimen surface area.
19. Incubate in moist chamber for 30 min. at room temp.
20. Wash the specimen in 4 changes of PBS pH 7.4 in a coplin jar for 2 min. per wash at room temp.

Develop color in peroxidase substrate

21. Gently tap off excess liquid.
22. Apply peroxidase substrate (freshly working DAB) to complete cover the specimen and stain for 10 min. at room temp.
23. Wash the specimen with running tap water in a coplin jar for 5 min.
24. Counterstain with hematoxylin.
25. Mount the specimen under a glass coverslip in a mounting medium (permount).

BIOGRAPHY

Name	Miss Sasiwimon Chuntrakul	**Sex**	Female
Birth date	September 1, 1980	**Age**	24
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Home address	30, Moo 6, Bangsue, Bangkok, 10800 Thailand		
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