APPENDICES

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
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Appendix A
Histological preparation

Botanical microtechnique via paraffin embedding (Johansen, 1940; Sass, 1966)

Reagent preparation

1. Killing and fixation solution

FAA or Formalin-Acetic acid-Alcohol solution contains

95% ethyl alcohol	50	ml
glacial acetic acid	5	ml
formalin	10	ml
distilled water	35	ml

2. Dehydrating solution

Table A-1 Dehydrating solution

Solvent proportion	Concentration of alcohol				
	50%	**70%**	**85%**	**95%**	**100%**
95% ethyl alcohol	40	50	50	45	-
absolute alcohol	-	-	-	-	25
tertiary butyl alcohol	10	20	35	55	75
distilled water	50	30	15	-	-

3. Adhesive solution

stock solution : albumin 1 ml

distilled water 49 ml

when use, dilute 1 ml of the stock solution with distilled water to 50 ml

4. Stain

Table A-2 Compositions of Delafield's Hematoxylin

stain	chemicals		
Delafield's hematoxylin	aluminium sulfate ($Al_2(SO_4)_3.16H_2O$)	400	ml
	hematoxylin ($C_{16}H_{14}O_6$)	4	g
	95% ethyl alcohol	25	ml
	methyl alcohol	100	ml
	glycerol	100	ml

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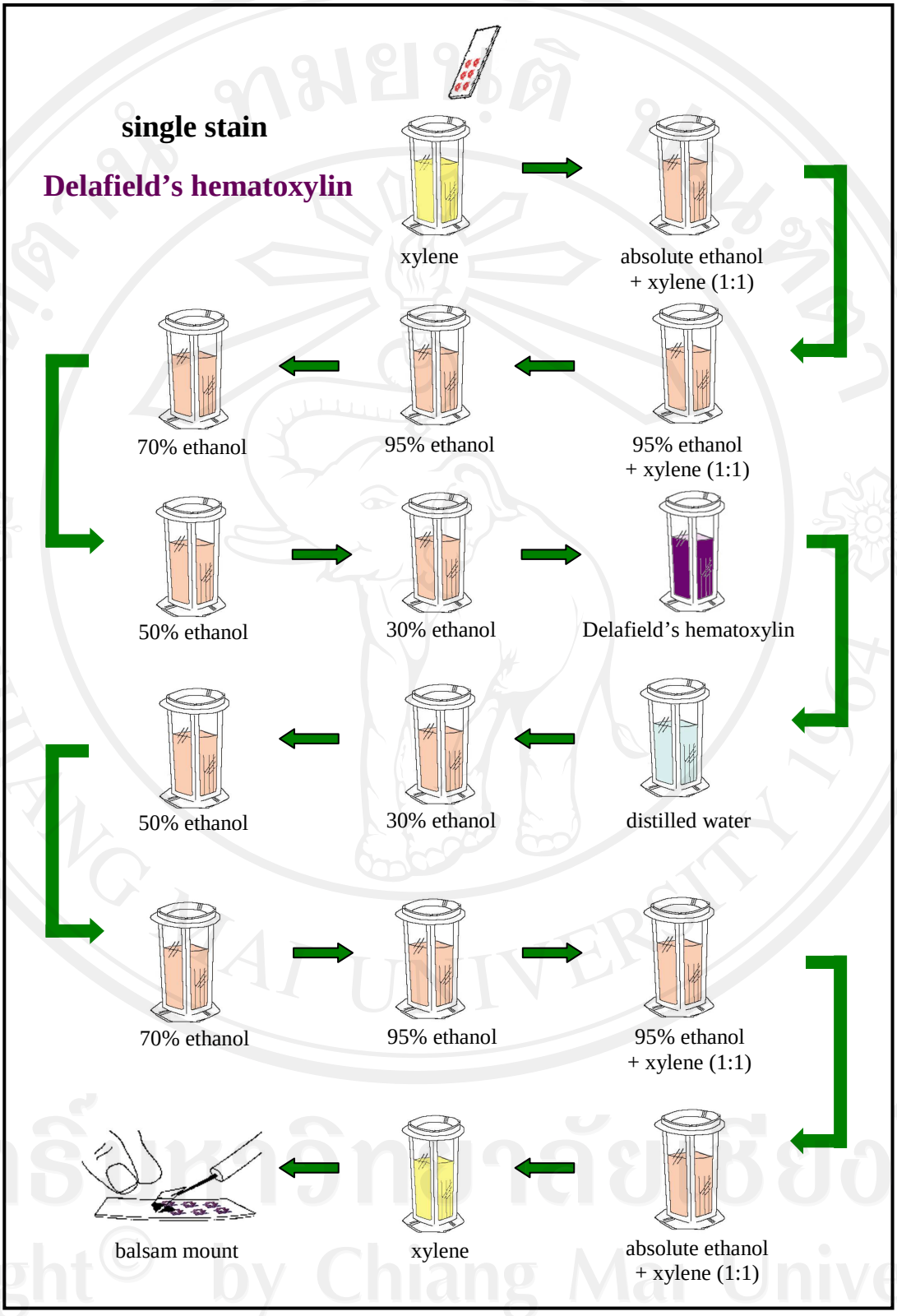

Figure A -1 Protocol for Delafield's hematoxylin staining
(Modified from Kermanee, 2008; Johansen, 1940; Sass, 1966)

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Appendix B
Preparation of carbol fucshin dye

Preparation of carbol fuchsin staining solution were conducted based on Chen's recipe (1992) as follows:

Fluid A: dissolve 3 g of basic fuchsin in 10 ml of 70% ethanol then add 90 ml of 5% phenol

Fluid B: mix 6 ml of glacial acetic acid and 6 ml of 37% formaldehyde into 55 ml of fluid A

The fluid B, 5 - 10 ml, was mixed with 95 ml of 45% acetic acid and 1.8 g sorbital then stirred with magnetic stirrer for several minutes.

Fresh carbol fuchsin stain fluid was ready after 30 - 45 days of incubation.

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Appendix C

Chemical reagent preparation for isozyme pattern investigation

1. extraction buffer (0.2M Tris buffer pH 8.4)

0.2M tris	75.000	ml
(tris 2.42 g + distilled water add to 100 ml)		
0.2M HCl	24.750	ml
(37% HCl 1.7 ml + distilled water add to 100 ml)		
distilled water add to	300.000	ml
pH adjust to 8.4 (with 0.2M HCl)		

2. electrode buffer

tris	3.000	g
glycine	14.400	g
distilled water add to	500.000	ml
pH adjust to 8.3		
(when use, electrode buffer : distilled water = 1 : 4)		

3. marker dye solution

bromophenol blue	0.050	g
glycerol	1.000	ml
tris-HCl buffer pH 6.7	10.000	ml
(1N HCl 48 ml + tris 5.98 g)		

marker dye solution : enzyme sample = 1 : 9

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4. stock solutions of acrylamide gel

(1) acrylamide/Bis

acrylamide	29.200	g
N,N'-methylene bisacrylamide	0.800	g
distilled water add to	100.000	ml

(2) 1M tris-HCl pH 8.8

1M tris	50.000	ml
(tris 12.11 g + distilled water add to 100 ml)		
1M HCl	8.000	ml
(37% HCl 8.35 ml + distilled water add to 100 ml)		
distilled water add to	100.000	ml
pH adjust to 8.8 (with 1M HCl)		

5. acrylamide gel 7.5%

acrylamide/Bis	10.000	ml
1M tris-HCl pH 8.8	10.000	ml
distilled water	19.400	ml
10% ammonium persulphate	400.000	µl
TEMED	20.000	µl

6. stock solutions of enzyme staining

(1) 0.1M tris-HCl (for GDH, GOT, SKD)

tris	12.110	g
37% HCl 8.35 ml + distilled water add to	100.000	ml
distilled water add to	1,000.000	ml
pH adjust to 7.5 for GDH, and 8.0 for SKD		

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(2) 0.2M tris-HCl pH 8.0 (for MDH)

tris	2.420	g
distilled water add to	100.000	ml
pH adjust to 8.0 (with 1N HCl)		

(3) 0.2M tris-maleate pH 6.0 (for LAP)

tris	2.420	g
maleic acid	2.320	g
distilled water add to	100.000	ml
pH adjust to 6.0		

(4) 0.1M Tris buffer pH 4.0 (for POX)

tris	0.377	g
acetic acid	0.400	ml
distilled water add to	250.000	ml
pH adjust to 4.0		

(5) 0.2M acetate buffer pH 4.8 (for ACP)

0.2M acetic acid	40.000	ml
(acetic acid 1.15 ml + distilled water add to 100 ml)		
0.2M sodium acetate	60.000	ml
(sodium acetate 2.72 g + distilled water add to 100 ml)		
distilled water add to	200.000	ml
pH adjust to 4.8		

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(6) 0.2M phosphate buffer pH 6.0 (for EST)

0.2M monobasic potassium phosphate	87.800	ml
(monobasic potassium phosphate 2.72 g + distilled water add to 100 ml)		
0.2M dibasic potassium phosphate	12.300	ml
(dibasic potassium phosphate 1.74 g + distilled water add to 50 ml)		
distilled water add to	200.000	ml
pH adjust to 6.0		

(7) 1M L-malate (or 2M DL-malate; MDH substrate)

DL-malic acid	5.364	g
NaOH	3.000	g
distilled water add to	20.000	ml
pH adjust to 7.0		

(8) 2.5% leucine naphthylamide (LAP substrate)

L-leucine-2-naphthylamide · HCl	0.050	g
distilled water add to	2.000	ml

7. enzyme staining solutions

(1) acid phosphatase (ACP)

0.2M acetate buffer pH 4.8	200.000	ml
fast garnet GBC (sulfate salt)	0.200	g
α-naphthyl phosphate-sodium salt	0.100	g
filter in dark		

(2) esterase (EST)

A : 0.2M phosphate buffer pH 6.0	200.000	ml
B : fast blue B salt	0.300	g
C : α-naphthyl acetate (in absolute ethanol 6 ml)	0.006	g
A + B and then filter in dark, and add C		

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(3) glucose dehydrogenase (GDH)

0.1M tris-HCl pH 7.5	100.000	ml
D(+)-glucose monohydrate	16.000	g
10% NAD	400.000	µl
10% NBT in methyl alcohol	200.000	µl
10% PMS	40.000	µl

(4) glutamate oxaloacetate transminase (GOT)

0.1M tris-HCl	pH adjust to 7.4	100.000	ml
α-ketoglutaric acid		0.100	g
L-aspartic acid		0.200	g
10% pyridoxal-5'-phosphate		40.000	µl
fast blue BB		0.200	g

(5) leucine aminopeptidase (LAP)

0.2M tris-maleate pH 6.0	100.000	ml
LAP substrate	2.000	ml
1M $MgCl_2 \cdot 6H_2O$	2.000	ml
fast blue RR	0.100	g

(6) malate dehydrogenase (MDH)

0.2M tris-HCl pH 8.0	100.000	ml
1M L-malate (MDH substrate)	20.000	ml
1% NAD	4.000	ml
1% MTT	2.000	ml
1% PMS	0.400	ml

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(7) peroxidase (POX)

A : 0.1M tris buffer pH 4.0	160.000	ml
B : β-naphtol (in acetone 20 ml)	0.058	g
C : 3-amino-9-ethylcarbazole (in acetone 20 ml)	0.084	g
D : 3% H_2O_2	200.000	µl
A + B + C in dark, then add D		

(8) shikimate dehydrogenase (SKD)

0.1M tris-HCl pH 8.0	100.000	ml
shikimic acid	0.060	g
10% $NADP^+$	400.000	µl
10% NBT in methyl alcohol	200.000	µl
10% PMS	40.000	µl

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