Appendices

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Appendix A: Raw data

Table 9. Viral expansion, concentrations and number of aliquots (1st expansion)

Virus	Preparation	Day after Infection	Titer[a] (FFU/ml)	Volume (µl) 140	250	1,000
16681Nde(+)	C6-3(C6-2/3, D5, 22 Jan 04)	5	1.08×10^6	2	65	-
		7	6.27×10^7	2	44	-
JEVpr/16681	C6-3(C6-2, D5, 1 Jan 04)	5	1.38×10^6	-	16	12
		8	5.36×10^5	-	10	10
16681prE203A #1.1-10.1	C6-3/1(C6-2, D5, 26 May 05)	5	3.78×10^6	2	68	-
		7	3.42×10^7	2	46	-
16681pr(+4,-0)HS #5.4.11	C6-3(C6-2, D4, 20 Dec 03)	5	6.48×10^5	2	68	-
		7	1.65×10^7	2	41	-
16681pr(+7,-2) #4.4-2.1	C6-3(C6-2, D4, 20 Dec 03)	5	1.05×10^5	2	250	-
		7	1.32×10^7	2	48	-

Table 10. Viral expansion, concentrations and number of aliquots (2nd expansion)

Virus	Expansion code	Day after Infection	Titer[a] (FFU/ml)	Volume (µl) 100	500	1,000
16681pr(+6,-2)P8,10+	C6-2/1(C6-1, D7, Tx.)	5	4.45×10^3	-	5	12
		7	9.36×10^5	-	4	10
16681pr(+6,-2) P8,10+	C6-2/2(C6-1, D7, Tx.)	5	9.03×10^5	10	20	-
		6	1.06×10^6	10	20	-
16681pr(+6,-2)P8,P13+	C6-2(C6-1, D7, Tx.)	5	3.34×10^7	-	20	-
		7	1.71×10^6	-	20	-
16681pr(+6,-2)P10,13+	C6-2(C6-1, D7, Tx.)	4	2.30×10^5	10	20	-
		5	5.66×10^6	10	20	-

a is represented the average of titers from three separated experiments.

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Table 11. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Extracellular virus (FFU/ml) of 16681Nde(+)			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	1.35×10^3	1.30×10^2	81.82	$5.20 \times 10^2 \pm 4.14 \times 10^2$
4	90	1.54×10^2	1.00×10^2	$1.14 \times 10^2 \pm 19.88$
12	2.88×10^2	1.44×10^2	81.82	$1.71 \times 10^2 \pm 61.06$
14	5.82×10^3	6.32×10^2	5.13×10^2	$2.32 \times 10^3 \pm 1.75 \times 10^3$
16	1.22×10^4	1.00×10^4	2.59×10^3	$8.26 \times 10^3 \pm 2.91 \times 10^3$
20	3.91×10^5	1.60×10^5	9.00×10^4	$2.14 \times 10^5 \pm 9.09 \times 10^4$
24	1.21×10^6	5.64×10^5	1.03×10^6	$9.35 \times 10^5 \pm 1.92 \times 10^5$
36	7.00×10^6	4.00×10^6	7.66×10^6	$6.22 \times 10^6 \pm 1.13 \times 10^6$
48	2.00×10^7	1.52×10^7	1.14×10^7	$1.55 \times 10^7 \pm 1.49 \times 10^6$

Table 12. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Cell-associated virus (FFU/ml) of 16681Nde(+)			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	1.45×10^5	2.20×10^4	1.33×10^4	$6.01 \times 10^4 \pm 4.25 \times 10^4$
4	8.46×10^2	1.23×10^3	7.92×10^2	$9.56 \times 10^2 \pm 1.37 \times 10^2$
12	4.00×10^2	3.96×10^2	1.63×102	$3.19 \times 10^2 \pm 78.34$
14	7.00×10^3	4.68×10^3	1.58×10^3	$4.42 \times 10^3 \pm 1.57 \times 10^3$
16	6.60×10^4	5.73×10^4	1.60×10^4	$4.46 \times 10^4 \pm 1.54 \times 10^4$
20	6.64×10^5	9.01×10^5	6.45×10^5	$7.37 \times 10^5 \pm 8.23 \times 10^4$
24	1.24×10^6	2.18×10^6	4.05×10^5	$1.28 \times 10^6 \pm 5.13 \times 10^5$
36	7.63×10^6	9.73×10^6	4.00×10^6	$7.12 \times 10^6 \pm 1.67 \times 10^6$
48	5.27×10^6	1.93×10^7	8.91×10^7	$1.12 \times 10^7 \pm 3.00 \times 10^7$

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Table 13. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Extracellular virus (FFU/ml) of JEVpr/16681			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	10	2.00×10^2	36.36	82.12 ± 59.43
4	63.63	2.50×10^2	45.45	$2.42 \times 10^2 \pm 65.36$
12	20	90	10	34.24 ± 25.17
14	10	90.9	10	30 ± 26.97
16	10	5.54×10^2	10	$18.79 \pm 1.81 \times 10^2$
20	1.08×10^2	1.77×10^4	10	$62.67 \pm 5.88 \times 10^3$
24	1.65×10^3	1.00×10^5	50	$7.53 \times 10^2 \pm 3.31 \times 10^4$
36	1.14×10^5	1.89×10^6	2.09×10^3	$3.75 \times 10^5 \pm 6.12 \times 10^5$
48	4.50×10^5	5.31×10^6	7.09×10^4	$4.80 \times 10^5 \pm 1.69 \times 10^6$

Table 14. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Cell-associated virus (FFU/ml) of JEVpr/16681			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	3.00×10^3	2.78×10^4	1.62×10^4	$1.54 \times 10^4 \pm 7.16 \times 10^3$
4	2.61×10^2	2.32×10^3	4.59×10^2	$1.01 \times 10^3 \pm 6.55 \times 10^2$
12	50	63.63	10	41.21 ± 16.09
14	1.73×10^2	1.26×10^2	10	$1.03 \times 10^2 \pm 48.44$
16	8.09×10^2	1.14×10^4	6.48×10^2	$4.29 \times 10^3 \pm 3.56 \times 10^3$
20	2.85×10^4	1.85×10^5	1.75×10^4	$8.83 \times 10^4 \pm 7.80 \times 10^4$
24	1.33×10^5	1.51×10^6	5.00×10^5	$7.14 \times 10^5 \pm 4.12 \times 10^5$
36	1.39×10^6	1.66×10^7	2.18×10^5	$6.07 \times 10^6 \pm 5.28 \times 10^6$
48	5.55×10^6	1.43×10^7	1.01×10^7	$9.98 \times 10^6 \pm 2.53 \times 10^6$

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Table 15. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Extracellular virus (FFU/ml) of 16681pr(+4,-0)HS			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	10	2.36×10^2	63.64	82.12 ± 68.18
4	2.61×10^2	2.50×10^2	54.54	$2.42 \times 10^2 \pm 67.06$
12	50	90	30	34.24 ± 17.64
14	1.72×10^2	90.9	30	30 ± 41.13
16	8.09×10^2	5.54×10^2	54.54	$18.79 \pm 2.21 \times 10^2$
20	1.76×10^5	1.77×10^4	4.91×10^3	$62.67 \pm 5.50 \times 10^4$
24	1.16×10^6	1.00×10^5	6.13×10^4	$7.53 \times 10^2 \pm 3.60 \times 10^5$
36	1.05×10^7	1.89×10^6	7.27×10^5	$3.75 \times 10^5 \pm 3.08 \times 10^6$
48	6.09×10^6	5.31×10^6	2.21×10^6	$4.80 \times 10^5 \pm 1.18 \times 10^6$

Table 16. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Cell-associated virus (FFU/ml) of 16681pr(+4,-0)HS			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	2.43×10^4	2.48×10^4	2.08×10^2	$2.33 \times 10^4 \pm 8.12 \times 10^3$
4	1.43×10^3	1.73×10^2	1.68×10^3	$1.61 \times 10^3 \pm 4.66 \times 10^2$
12	1.54×10^2	1.00×10^2	80	$1.11 \times 10^2 \pm 22.10$
14	1.11×10^3	1.07×10^3	2.36×10^2	$8.05 \times 10^3 \pm 2.84 \times 10^2$
16	1.02×10^4	1.51×10^4	2.95×10^3	$9.42 \times 10^3 \pm 3.53 \times 10^3$
20	7.64×10^5	4.45×10^5	3.90×10^4	$4.16 \times 10^5 \pm 2.10 \times 10^5$
24	2.09×10^6	1.79×10^6	2.67×10^5	$1.38 \times 10^6 \pm 5.64 \times 10^5$
36	1.78×10^7	1.24×10^7	4.82×10^6	$1.17 \times 10^7 \pm 3.76 \times 10^6$
48	1.74×10^7	1.97×10^7	2.46×10^7	$2.05 \times 10^7 \pm 2.12 \times 10^6$

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Table 17. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Extracellular virus (FFU/ml) of 16681prE203A			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	30	80	10	40 ± 21.02
4	63.63	30	10	34.54 ± 15.65
12	20	30	10	20 ± 5.77
14	1.54×10^2	10	30	64.67 ± 45.04
16	8.06×10^2	1.20×10^2	2.27×10^2	$3.84 \times 10^2 \pm 2.13 \times 10^2$
20	8.82×10^4	1.37×10^4	1.18×10^4	$3.79 \times 10^4 \pm 2.52 \times 10^4$
24	4.46×10^5	1.44×10^5	2.21×10^5	$2.67 \times 10^5 \pm 9.06 \times 10^4$
36	4.73×10^7	2.79×10^6	6.67×10^6	$4.73 \times 10^6 \pm 2.00 \times 10^7$
48	1.88×10^7	1.61×10^7	1.55×10^7	$1.68 \times 10^7 \pm 1.01 \times 10^6$

Table 18. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Cell-associated virus (FFU/ml) of 16681prE203A			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	4.54×10^3	5.94×10^3	6.91×10^3	$5.80 \times 10^3 \pm 6.87 \times 10^2$
4	5.45×10^2	5.36×10^2	4.86×10^2	$5.22 \times 10^2 \pm 18.35$
12	60	50	90	66.67 ± 12.21
14	5.49×10^2	3.63×10^2	1.35×10^2	$3.49 \times 10^2 \pm 1.19 \times 10^2$
16	7.92×10^3	1.00×10^3	1.43×10^3	$3.45 \times 10^3 \pm 2.24 \times 10^4$
20	1.79×10^5	8.91×10^4	4.55×10^3	$9.09 \times 10^4 \pm 5.04 \times 10^4$
24	1.22×10^6	9.01×10^5	1.71×10^5	$7.64 \times 10^5 \pm 3.10 \times 10^5$
36	1.12×10^7	7.75×10^6	1.30×10^7	$1.07 \times 10^6 \pm 1.54 \times 10^6$
48	1.38×10^7	2.82×10^7	2.75×10^7	$2.32 \times 10^7 \pm 4.69 \times 10^6$

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Table 19. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Extracellular virus (FFU/ml) of 16681pr(+7,-2)			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	10	1.72×10^2	40	74 ± 49.76
4	60	3.72×10^2	1.45×10^2	$1.92 \times 10^2 \pm 93.12$
12	10	40	20	23.33 ± 8.82
14	90.9	72.73	50	71.21 ± 11.83
16	3.42×10^2	2.52×10^2	1.63×10^2	$2.53 \times 10^2 \pm 51.67$
20	1.30×10^4	1.00×10^4	4.64×10^3	$9.21 \times 10^3 \pm 2.45 \times 10^3$
24	6.13×10^4	8.11×10^5	3.41×10^4	$5.88 \times 10^4 \pm 2.55 \times 10^5$
36	2.20×10^5	1.64×10^6	3.64×10^5	$7.41 \times 10^5 \pm 4.51 \times 10^5$
48	1.17×10^6	1.68×10^6	1.34×10^6	$1.40 \times 10^6 \pm 1.50 \times 10^5$

Table.20. Virus titers, means and standard error of the means (SE) of three separated single-step kinetic experiments

Time (Hour)	Cell-associated virus (FFU/ml) of 16681pr(+7,-2)			
	1st Experiment	2ndExperiment	3rd Experiment	Mean ± SE
0	9.27×10^2	1.72×10^4	2.41×10^4	$1.41 \times 10^4 \pm 6.87 \times 10^3$
4	1.25×10^2	1.66×10^3	8.37×10^2	$1.25 \times 10^3 \pm 4.44 \times 10^2$
12	3.36×10^3	7.45×10^2	6.30×10^2	$1.58 \times 10^3 \pm 8.91 \times 10^2$
14	2.37×10^4	9.82×10^3	6.73×10^3	$1.14 \times 10^4 \pm 5.22 \times 10^3$
16	1.72×10^5	9.91×10^4	3.88×10^4	$1.03 \times 10^5 \pm 3.85 \times 10^4$
20	3.66×10^6	1.01×10^6	2.13×10^5	$1.63 \times 10^6 \pm 1.04 \times 10^6$
24	6.90×10^6	2.66×10^6	6.73×10^5	$3.41 \times 10^6 \pm 1.84 \times 10^6$
36	2.99×10^7	1.53×10^7	4.03×10^6	$1.64 \times 10^7 \pm 7.49 \times 10^6$
48	1.16×10^7	1.89×10^7	1.95×10^7	$1.32 \times 10^7 \pm 2.54 \times 10^6$

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Table 21. Virus titers from RNA transfection of a new set of double mutant at pr-M junction and 16681Nde(+) viruses

Day after transfection	Virus titers (FFU/ml)			
	16681pr(+6,-2)P8,10+	16681pr(+6,-2)P10,13+	16681pr(+6,-2)P8,13+	16681Nde(+)
0	0	0	0	0
4	1.10×10^2	1.30×10^2	2.45×10^2	6.63×10^2
7	1.14×10^5	6.75×10^5	2.12×10^5	7.57×10^5
11	1.89×10^6	2.58×10^6	1.25×10^6	8.09×10^6
14	7.00×10^6	7.47×10^6	7.32×10^6	2.28×10^6

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Table 22. Virus titers from the single-step kinetic experiment of 16681Nde(+), JEVpr/16681 and 16681pr(+7,-2) viruses.

Time (Hour)	16681Nde(+) (FFU/ml)		JEVpr/16681 (FFU/ml)		16681pr(+7,-2) (FFU/ml)	
	Extracellular virus	Cell-associated virus	Extracellular Virus	Cell-associated virus	Extracellular virus	Cell-associated virus
0	10	0	1.60×10^2	60	0	0
4	20	0	1.80×10^2	1.00×10^2	20	0
12	10	0	1.00×10^2	70	0	0
14	1.00×10^2	0	20	60	10	0
16	36.36	0	10	1.00×10^2	0	10
20	2.88×10^2	4.95×10^2	10	3.36×10^2	20	30
24	8.01×10^2	9.64×10^3	5	3.45×10^3	1.09×10^2	9.90×10^3
36	2.73×10^5	2.36×10^5	1.80×10^2	2.41×10^5	8.46×10^2	2.49×10^5
48	9.72×10^5	6.49×10^5	6.76×10^3	3.74×10^5	3.85×10^4	8.18×10^5

Table 23. Virus titers from the single-step kinetic experiment of 16681pr(+6,-2)P8,10+, 16681pr(+6,-2)P8,13+ and 16681pr(+6,-2)P10,13+.

Time (Hour)	16681pr(+6,-2)P8,10+ (FFU/ml)		16681pr(+6,-2)P8,13+ (FFU/ml)		16681pr(+6,-2)P10,13+ (FFU/ml)	
	Extracellular virus	Cell-associated virus	Extracellular virus	Cell-associated virus	Extracellular virus	Cell-associated virus
0	10	10	5	0	0	10
4	3.77×10^2	20	90	0	40	0
12	1.68×10^2	0	0	10	25	10
14	2.91×10^2	0	10	0	27	0
16	33	0	40	10	98.64	10
20	3.76×10^2	63.63	1.77×10^2	6.94×10^3	1.87×10^2	3.73×10^3
24	9.59×10^2	3.62×10^4	1.77×10^4	6.49×10^4	8.05×10^2	1.91×10^4
36	2.97×10^3	3.82×10^5	5.36×10^5	9.73×10^5	6.53×10^4	6.58×10^5
48	1.06×10^5	3.84×10^5	1.04×10^6	1.46×10^6	2.13×10^5	2.55×10^6

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Table 24. Focus size of the parent, prM mutant and JEVpr/16681 viruses

Focus number	Focus size (cells/focus)				
	16681Nde(+)	JEVpr/16681	16681pr(+6,-2)P8,10+	16681pr(+6,-2)P8,13+	16681pr(+6,-2)P10,13+
1	251	10	32	361	299
2	232	11	53	131	244
3	225	10	66	345	200
4	261	18	60	91	239
5	281	25	60	158	366
6	259	11	50	28	590
7	269	19	93	244	273
8	146	11	35	206	280
9	251	16	63	203	257
10	223	38	16	242	115
11	81	21	39	211	252
12	207	30	54	118	396
13	216	20	32	129	332
14	286	35	63	246	437
15	335	17	64	87	482
16	235	41	15	127	130
17	305	18	36	149	375
18	117	18	91	155	579
19	136	21	16	213	340
20	456	20	84	128	355
21	224	40	23	68	611
22	359	36	76	240	279
23	125	18	60	333	116
24	154	29	66	114	280
25	203	20	68	75	236
26	169	35	84	317	265
27	247	38	62	234	253

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Table 24. Focus size of the parent, prM mutant and JEVpr/16681 viruses (continued)

Focus number	Focus size (cells/focus)				
	16681Nde(+)	JEVpr/16681	16681pr(+6,-2)P8,10+	16681pr(+6,-2)P8,13+	16681pr(+6,-2)P10,13+
28	324	51	45	79	110
29	206	22	73	292	99
30	395	18	39	178	111
31	273	21	24	115	184
32	259	18	40	260	385
33	159	47	54	207	197
34	294	26	26	508	251
35	296	17	74	271	258
36	290	35	90	254	226
37	221	18	23	112	294
38	203	28	34	268	320
39	196	24	58	87	157
40	245	10	52	223	170
Mean (cell/foci)	240.35	24.10	55.35	195.13	283.58
Standard deviation	75.43	10.53	24.99	98.29	128.41

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Appendix B: Chromatogram of nucleotide sequence

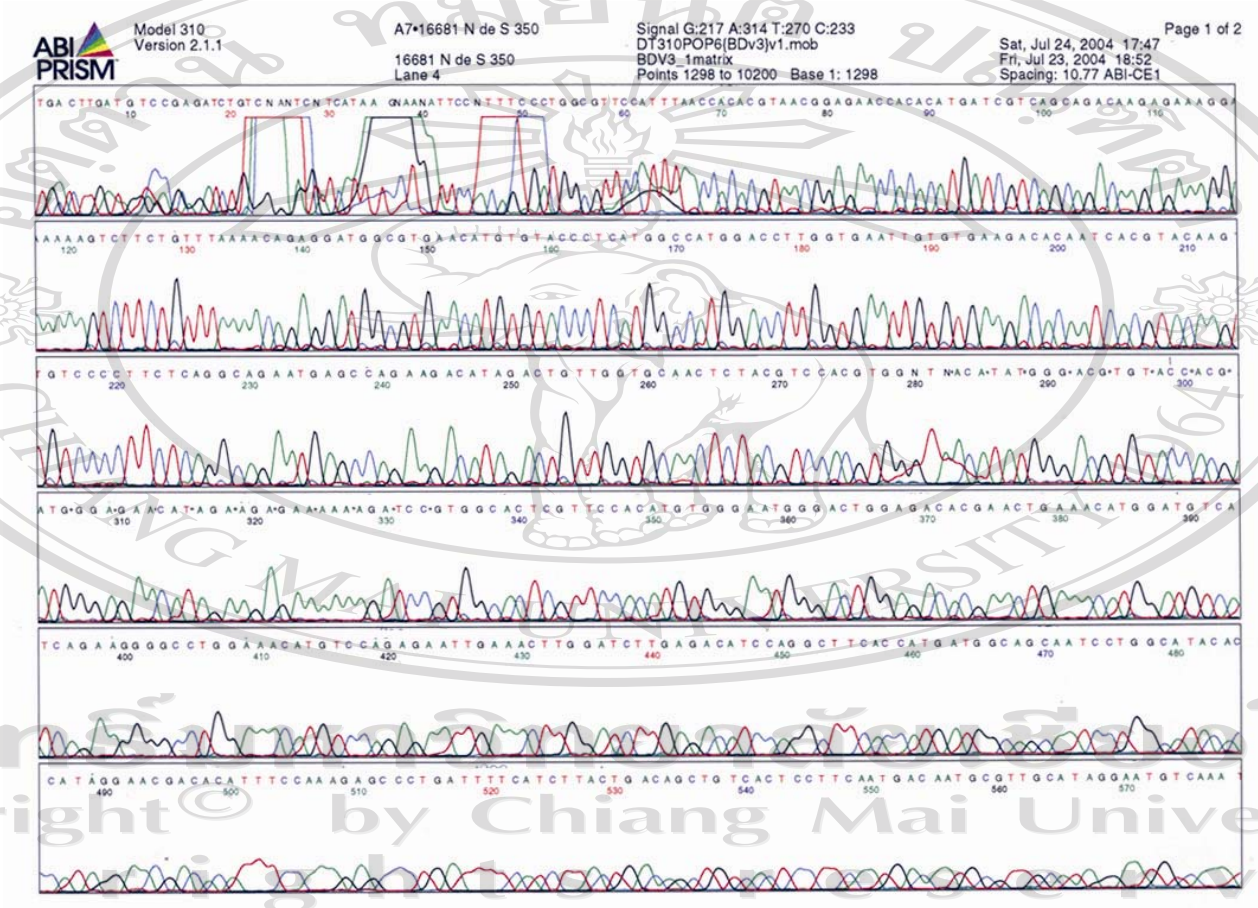

Figure 28. An entire sequence of the prM protein coding region of 16681Nde(+) virus

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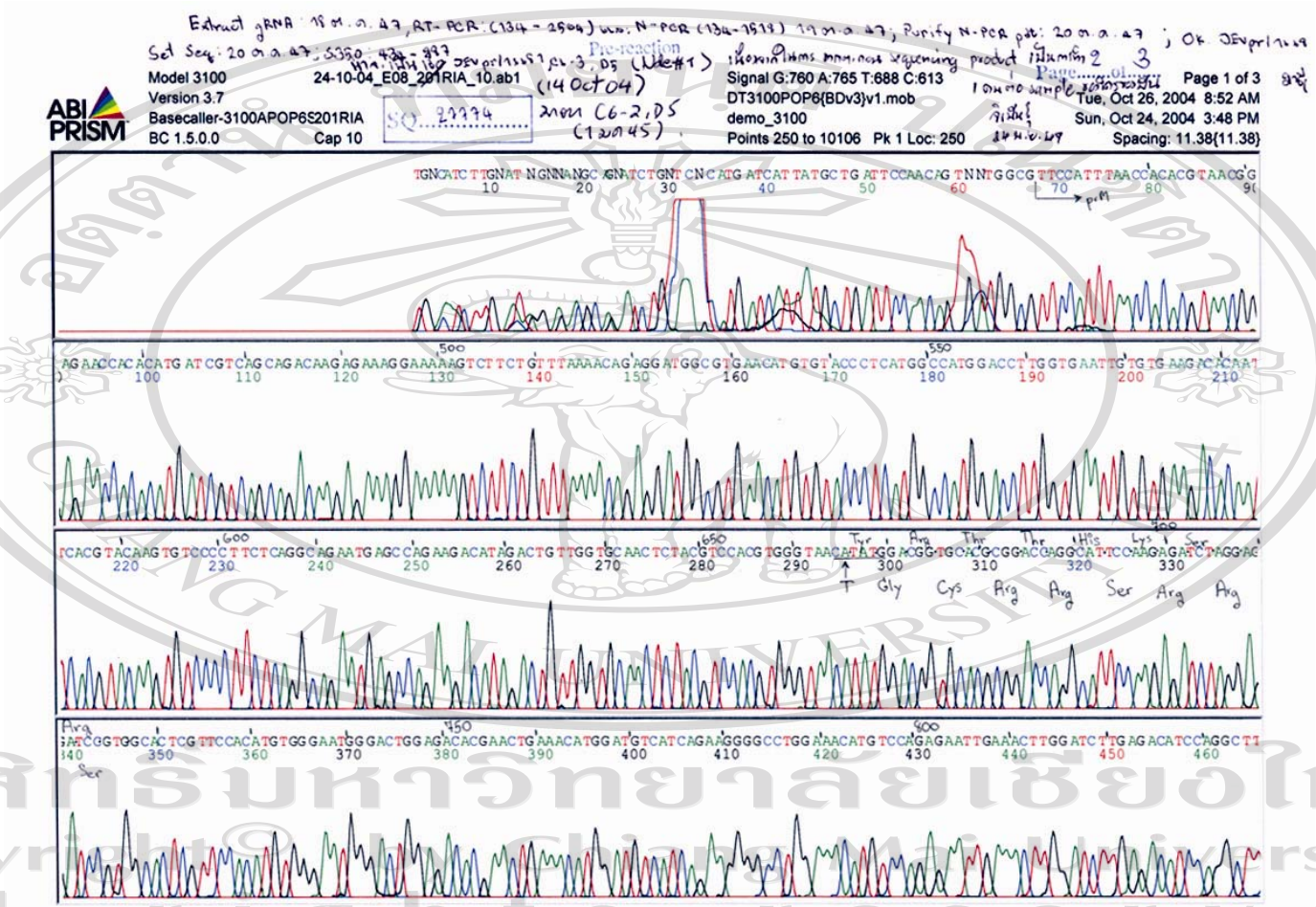

Figure 29. An entire sequence of the prM protein coding region of JEVpr/16681 virus

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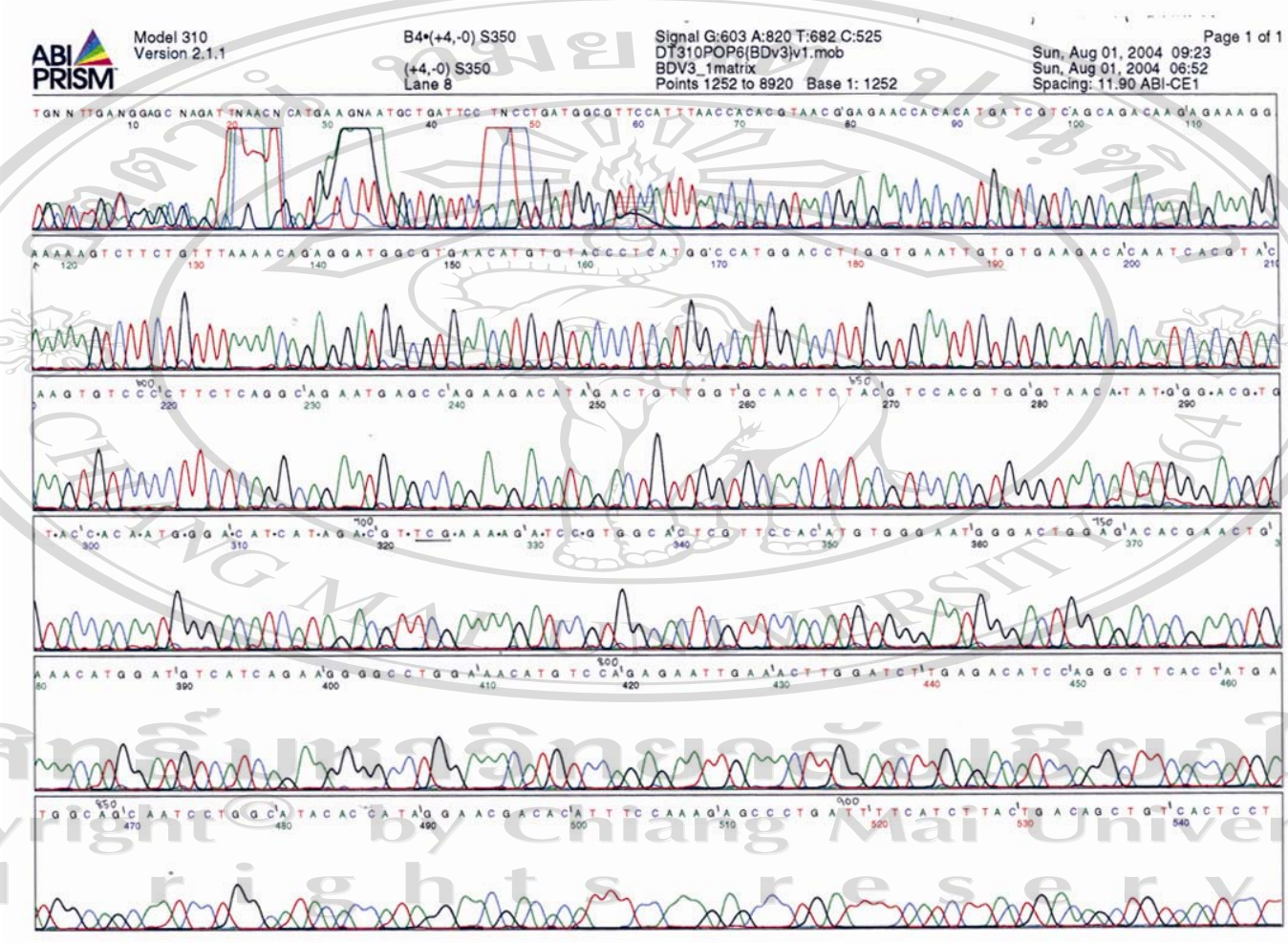

Figure 30. An entire sequence of the prM protein coding region of 16681pr(+4,-0)HS virus

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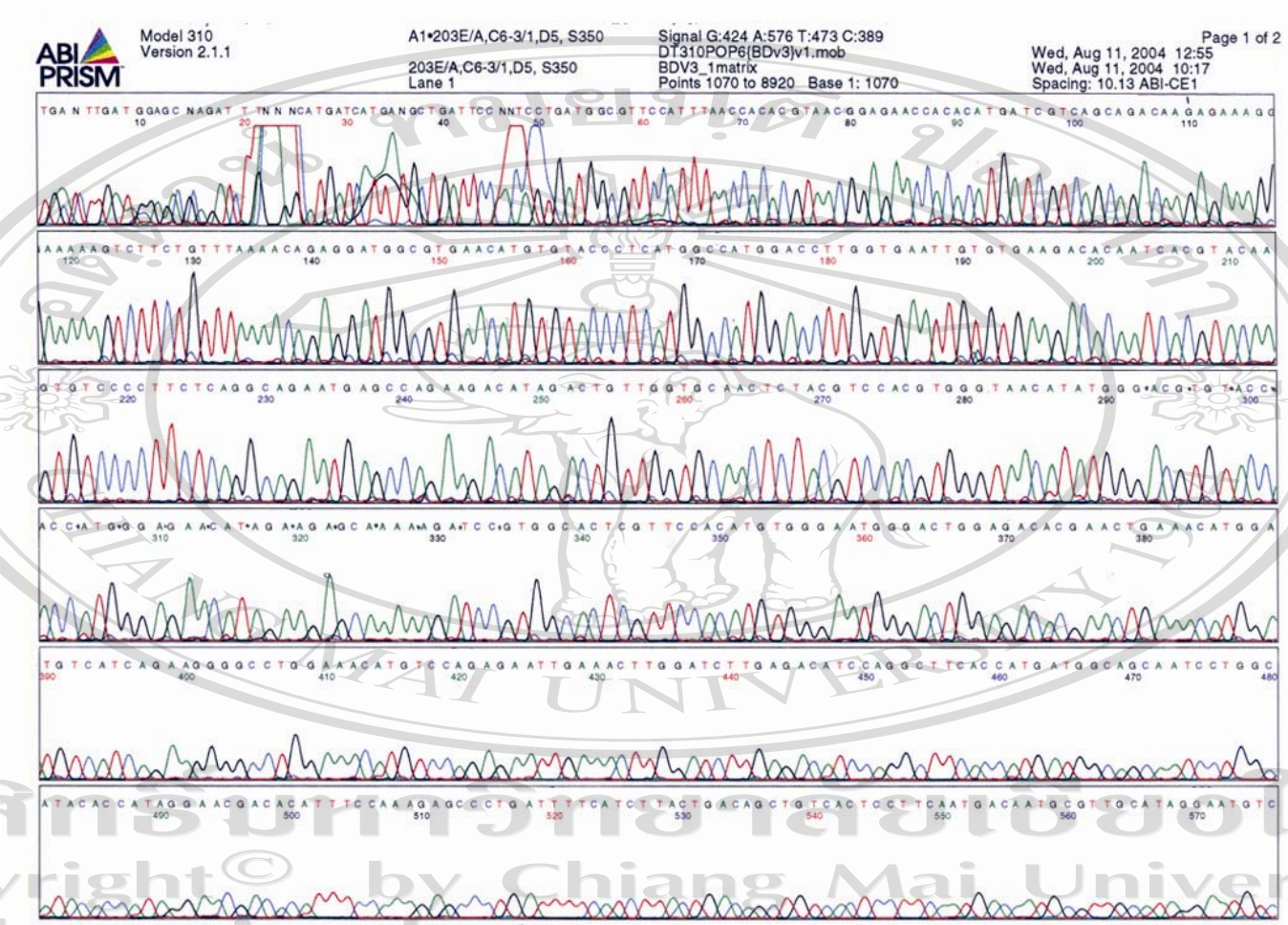

Figure 31. An entire sequence of the prM protein coding region of 16681prE203A

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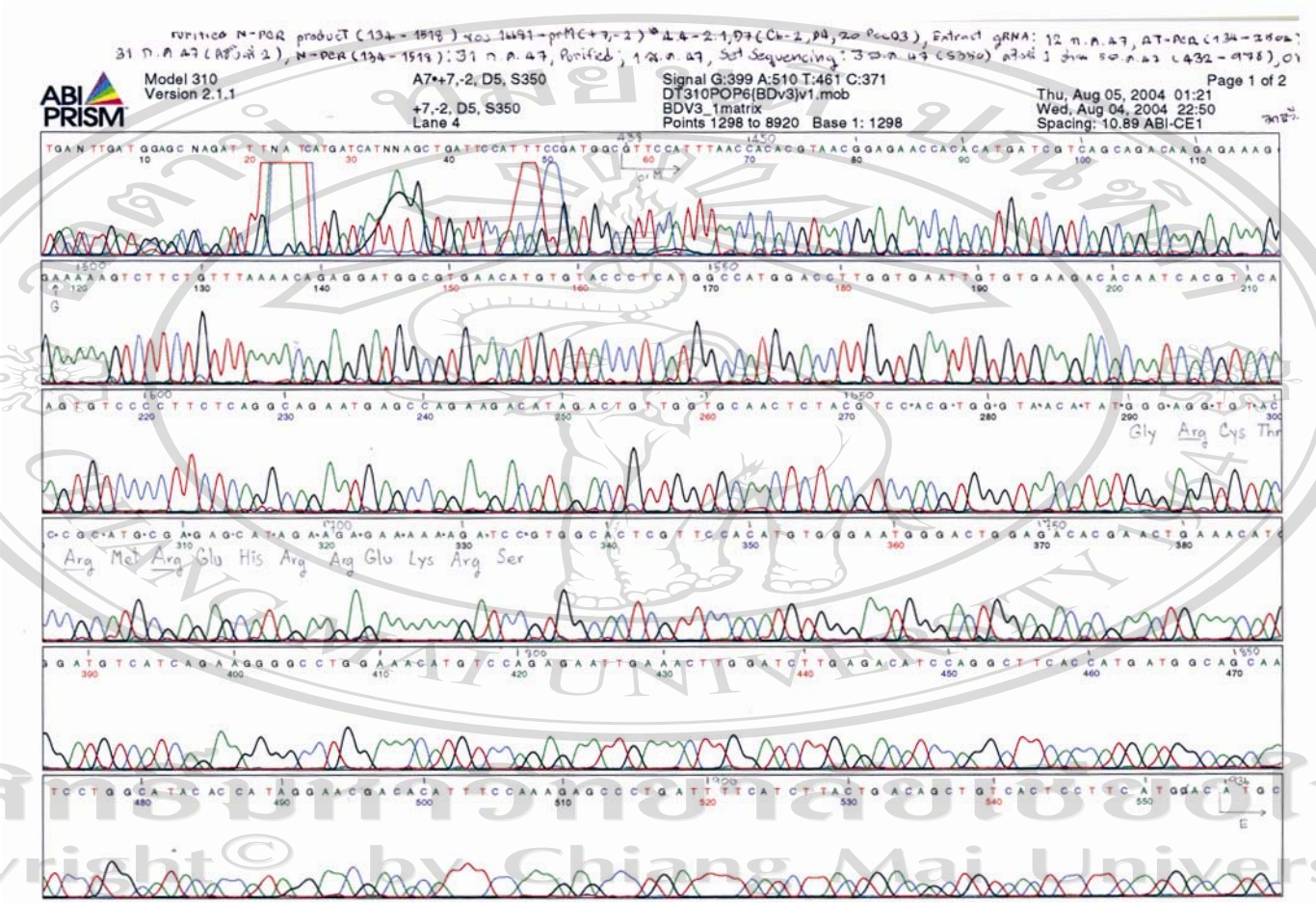

Figure 32. An entire sequence of the prM protein coding region of 16681pr(+7,-2) virus

110

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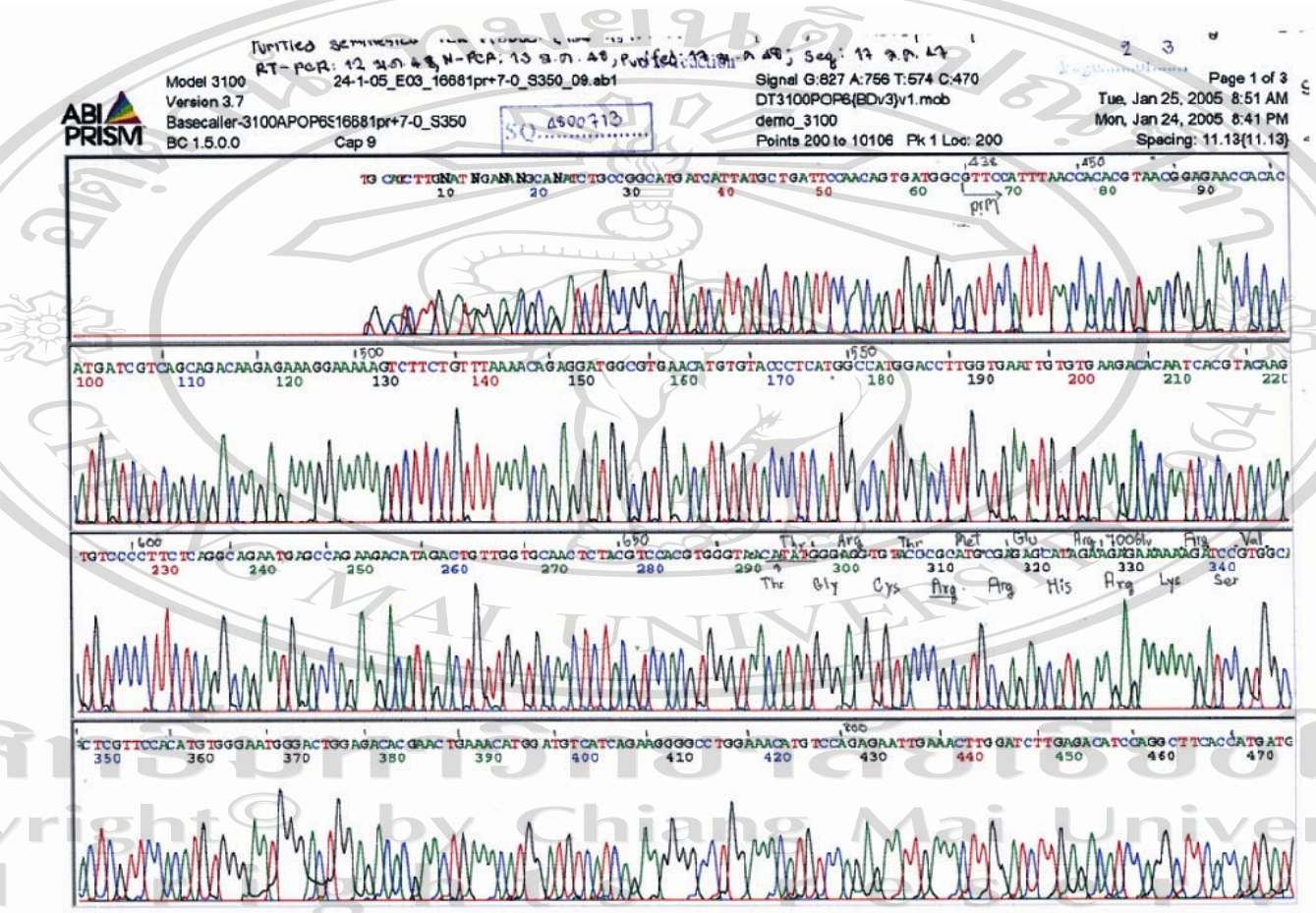

Figure 33. An entire sequence of the prM protein coding region of 16681pr(+7,-0) virus

111

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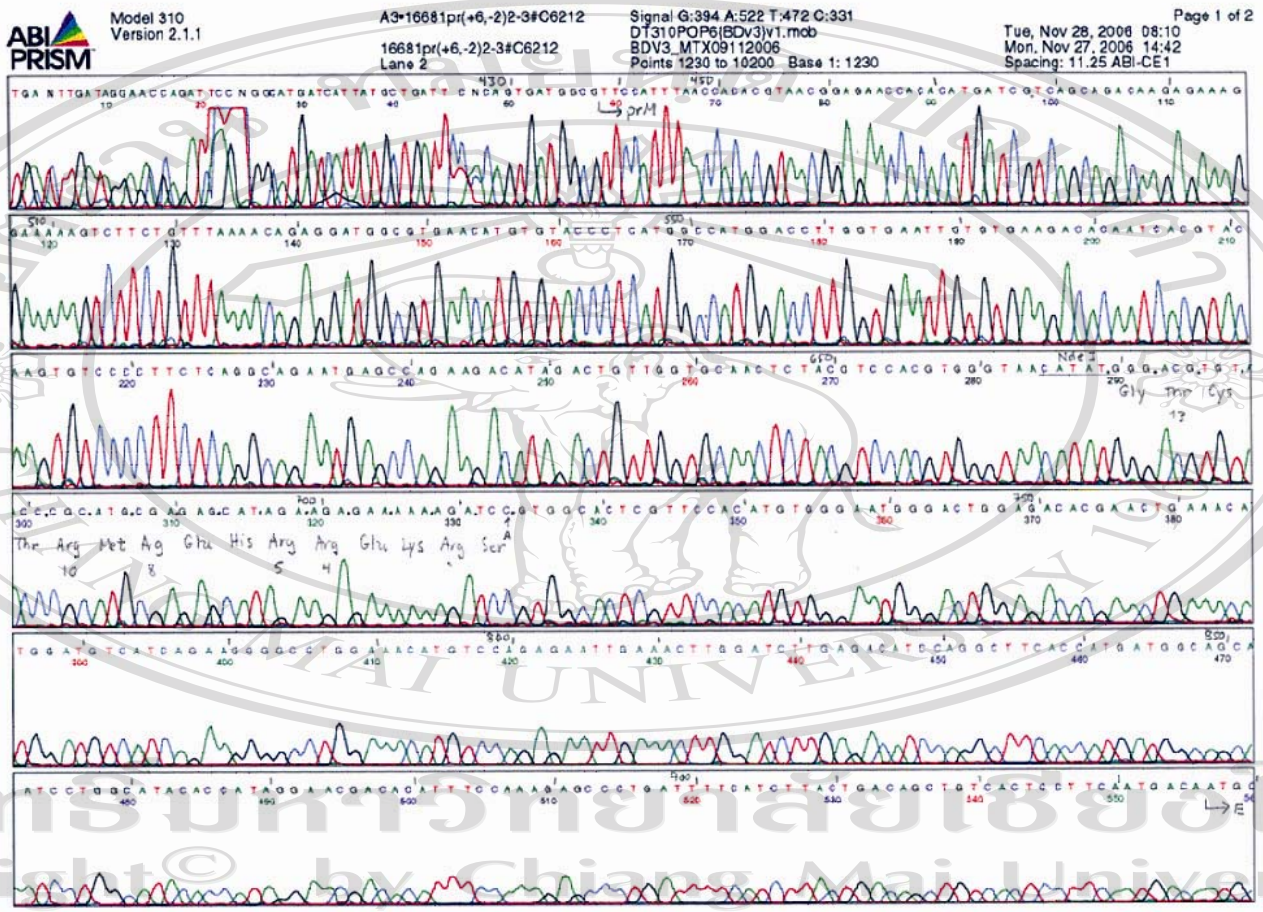

Figure 34. An entire sequence of the prM protein coding region of 16681pr(+6-2)P8,10+ virus

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112

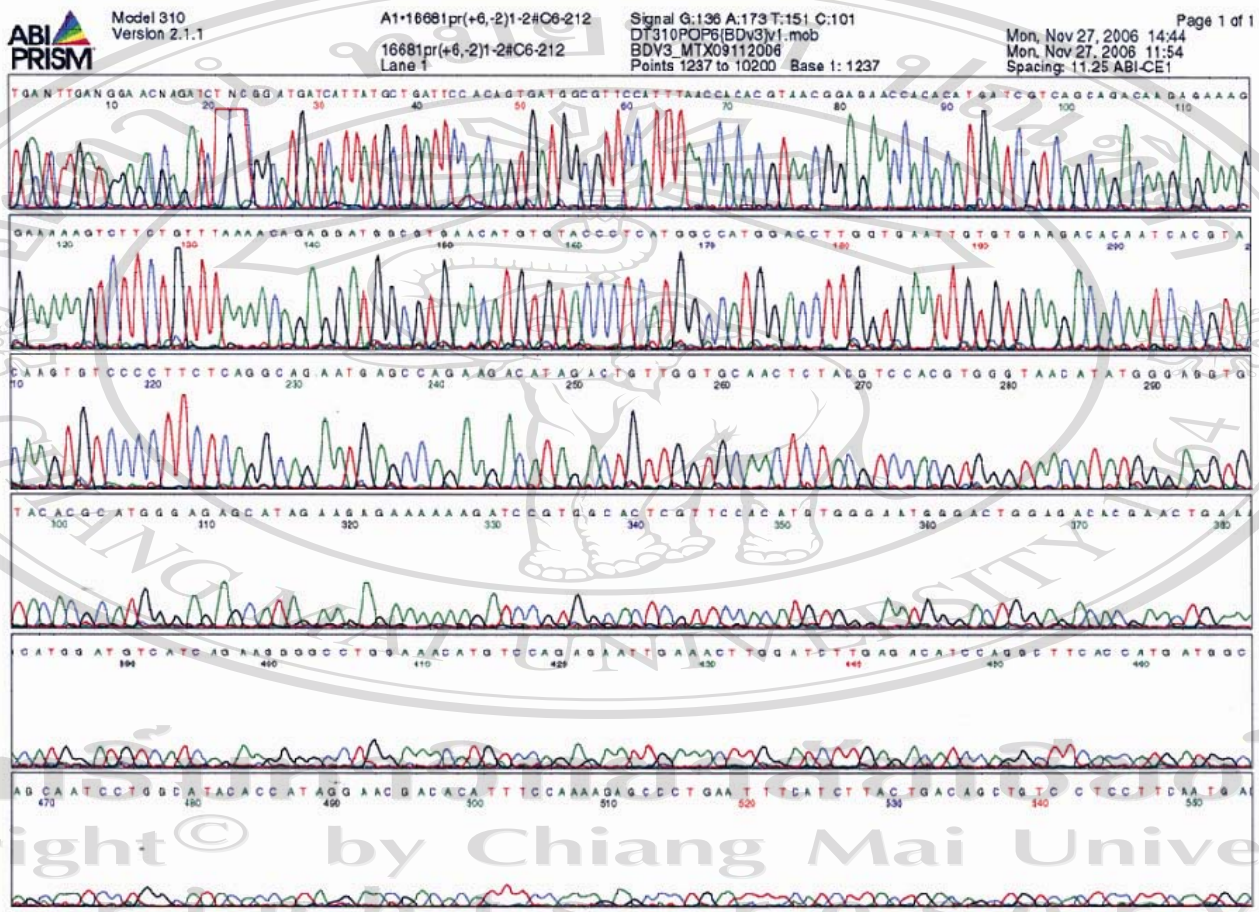

Figure 35. An entire sequence of the prM protein coding region of 16681pr(+6-2)P10,13+ virus

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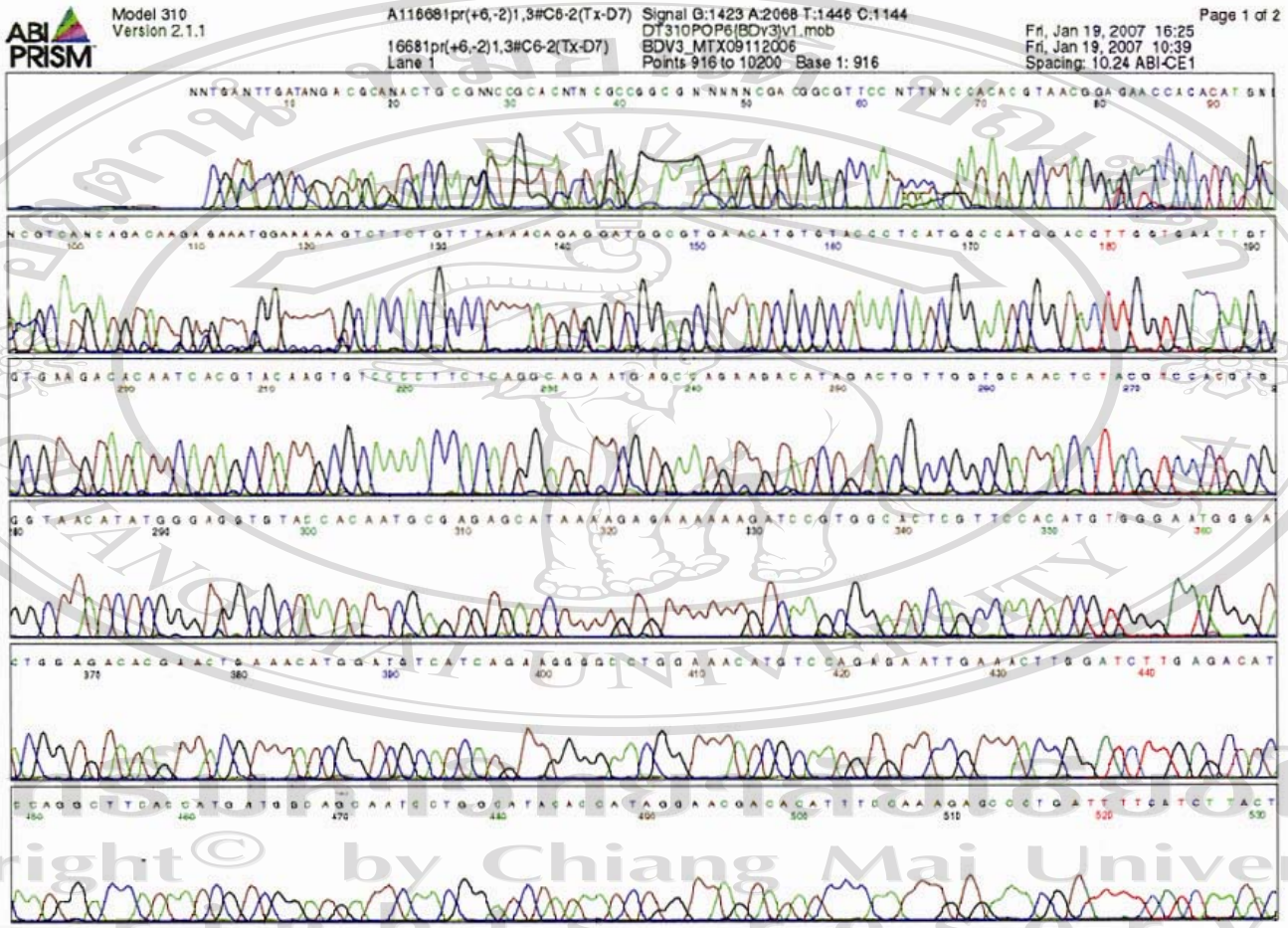

Figure 36. An entire sequence of the prM protein coding region of 16681pr(+6-2)P8,13+ virus

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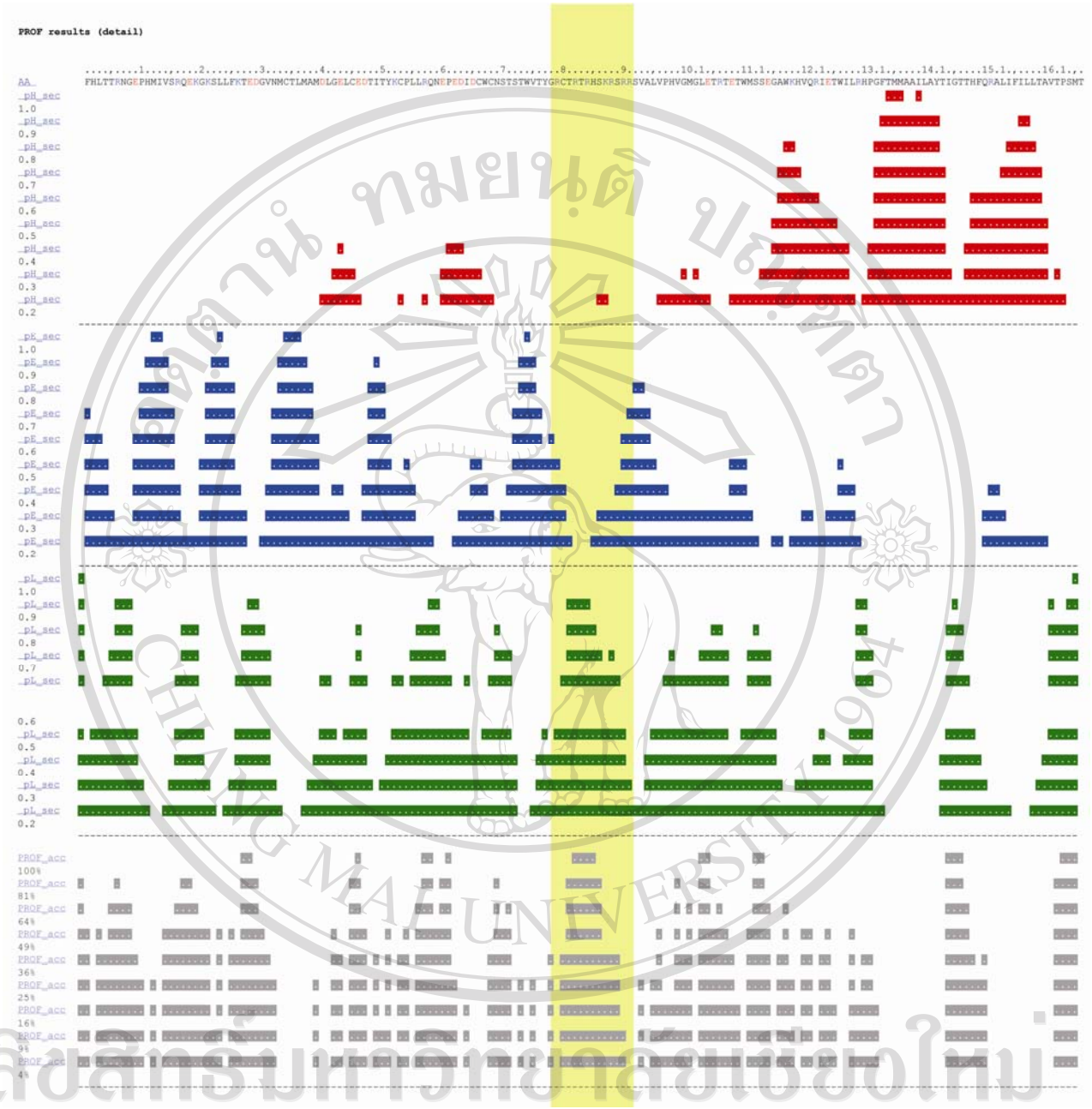

Figure 37. The secondary structure analysis of the amino acid sequences of the 16681Nde(+) prM protein by PHOFec (Rost et al., 2003)**.** An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org, and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 amino acid residues that changed at proximal to pr-M junction was marked with yellow.

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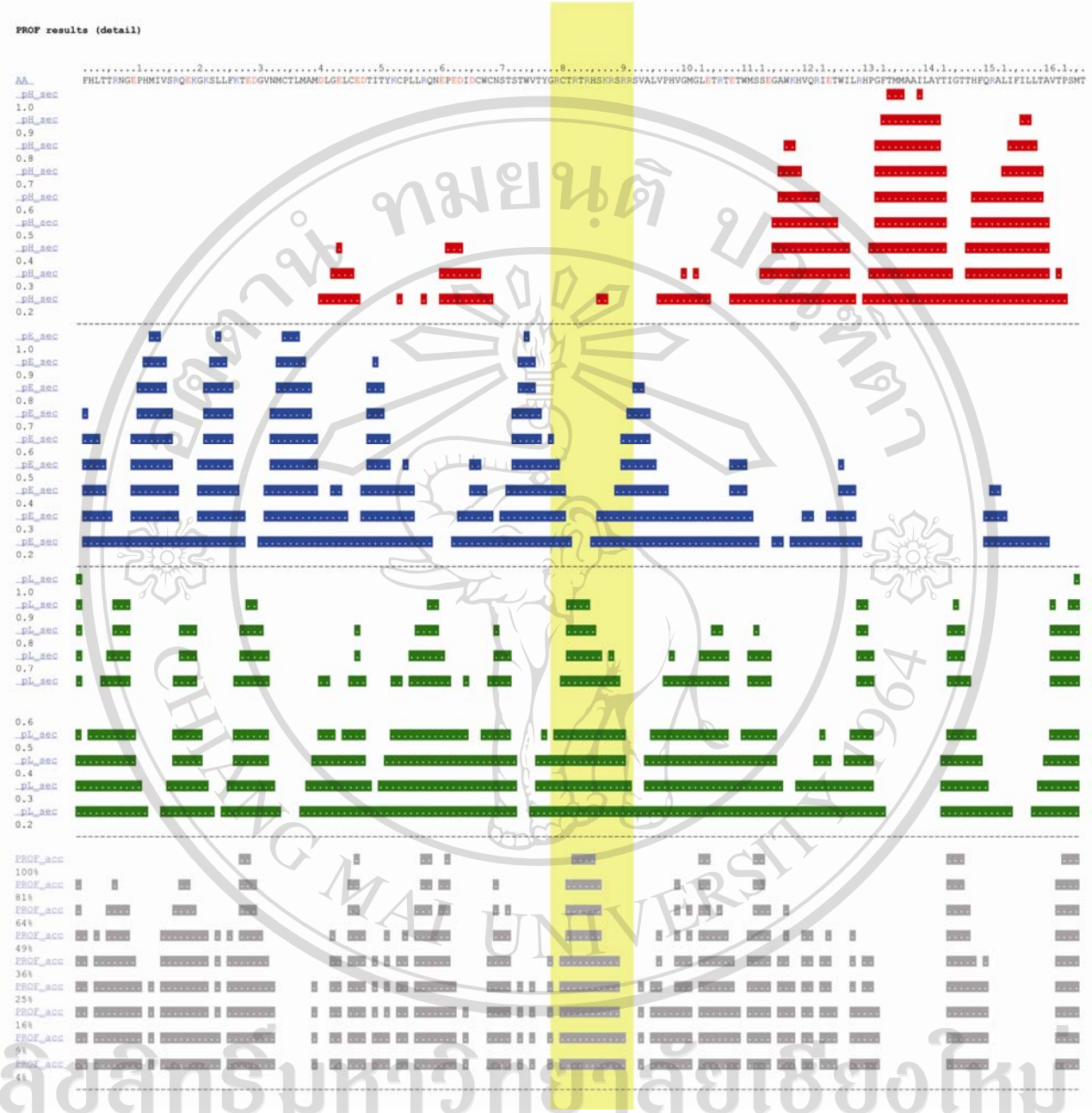

Figure 38. The secondary structure analysis of an amino acid sequences of the JEVpr/16681 prM protein by PHOFec (Rost et al., 2003)**.** An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org , and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 amino acid residues that changed at proximal to pr-M junction was marked with yellow.

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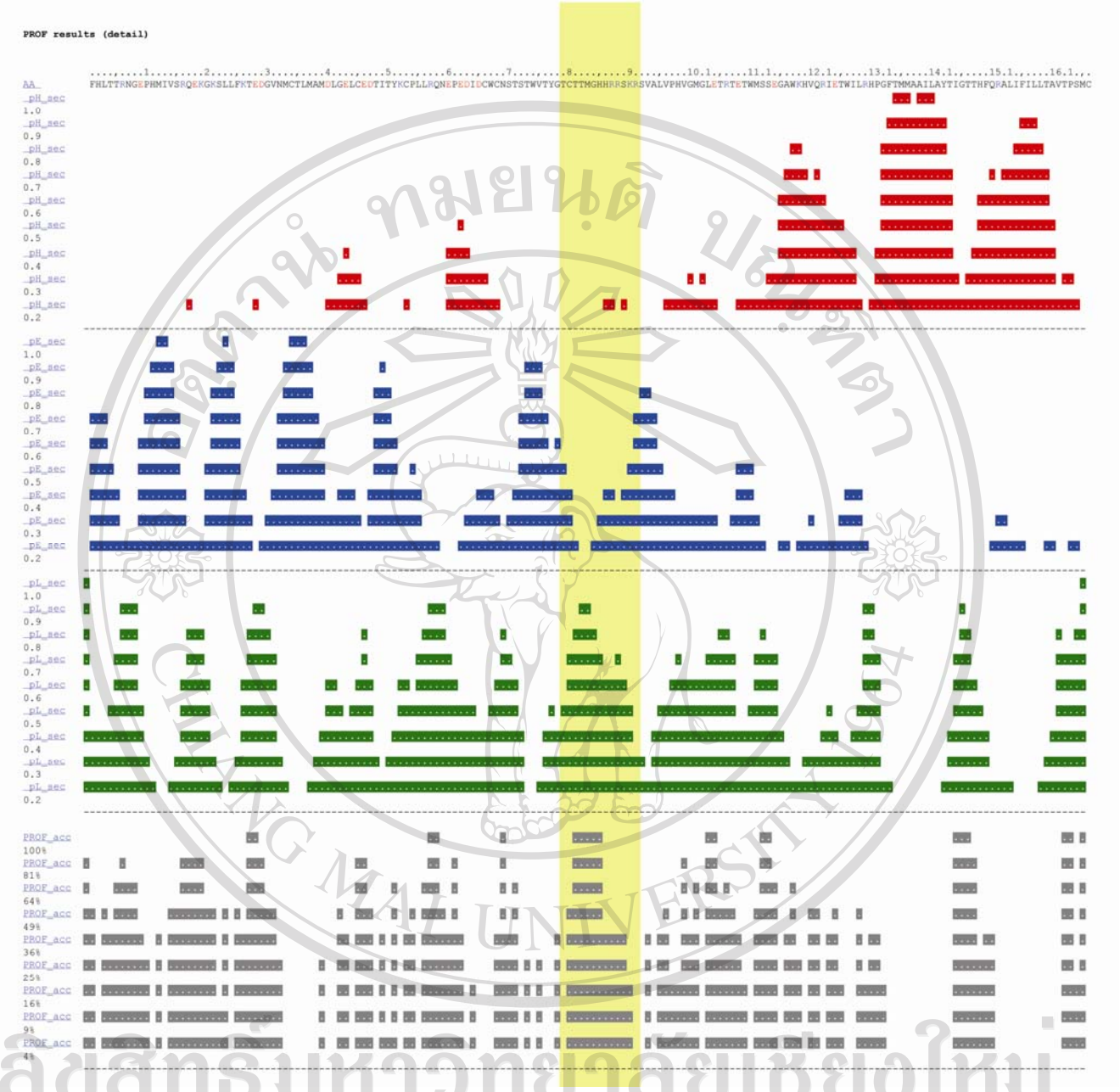

Figure 39. The secondary structure analysis of amino acid sequences of the 16681pr(+4,-0)HS prM protein by PHOFec (Rost et al., 2003)**.** An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org, and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 amino acid residues that changed at proximal to pr-M junction was marked with yellow.

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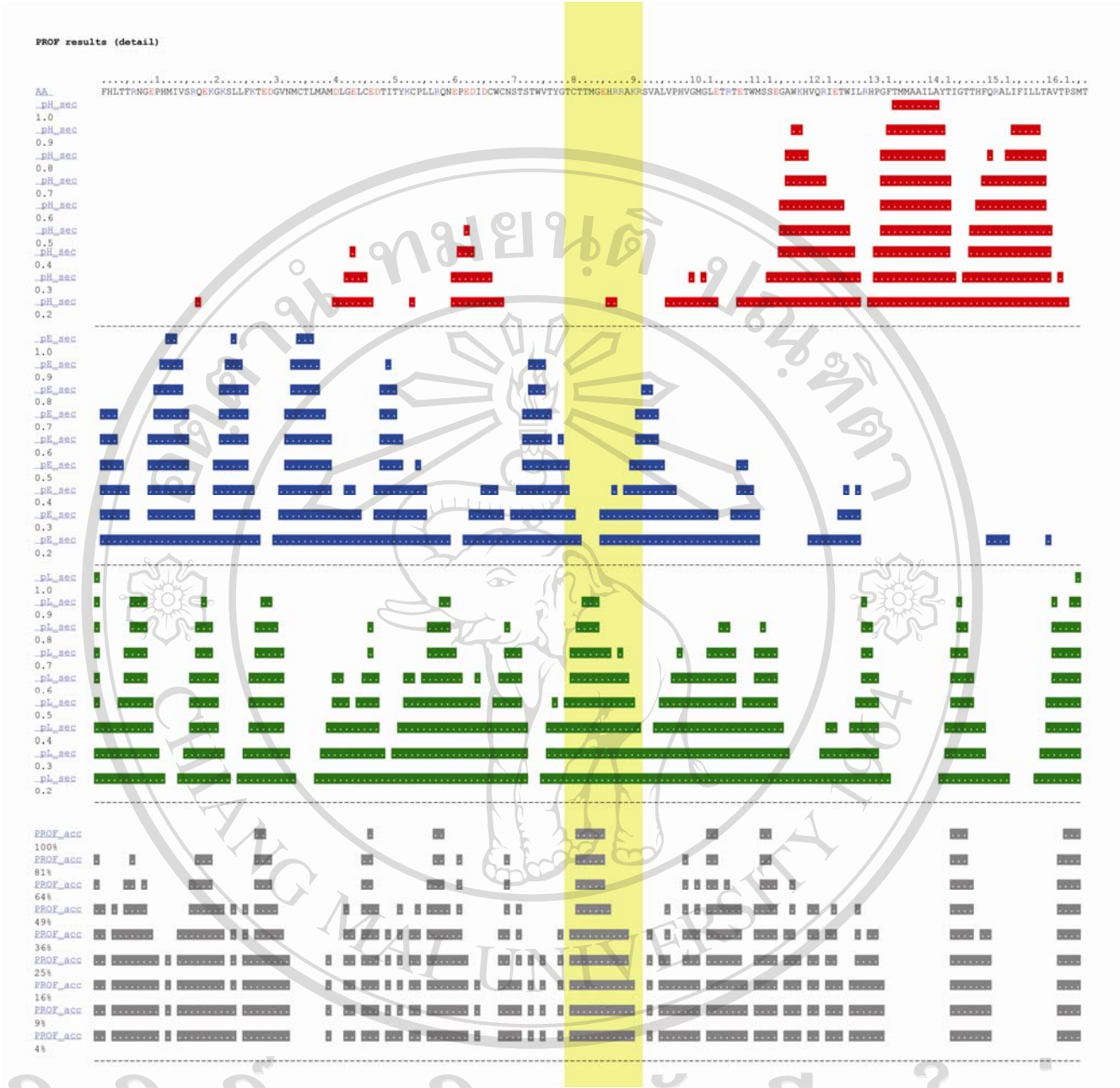

Figure 40. The secondary structure analysis of amino acid sequences of the 16681prE203A prM protein by PHOFec (Rost et al., 2003). An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org, and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 amino acid residues that changed at proximal to pr-M junction was marked with yellow.

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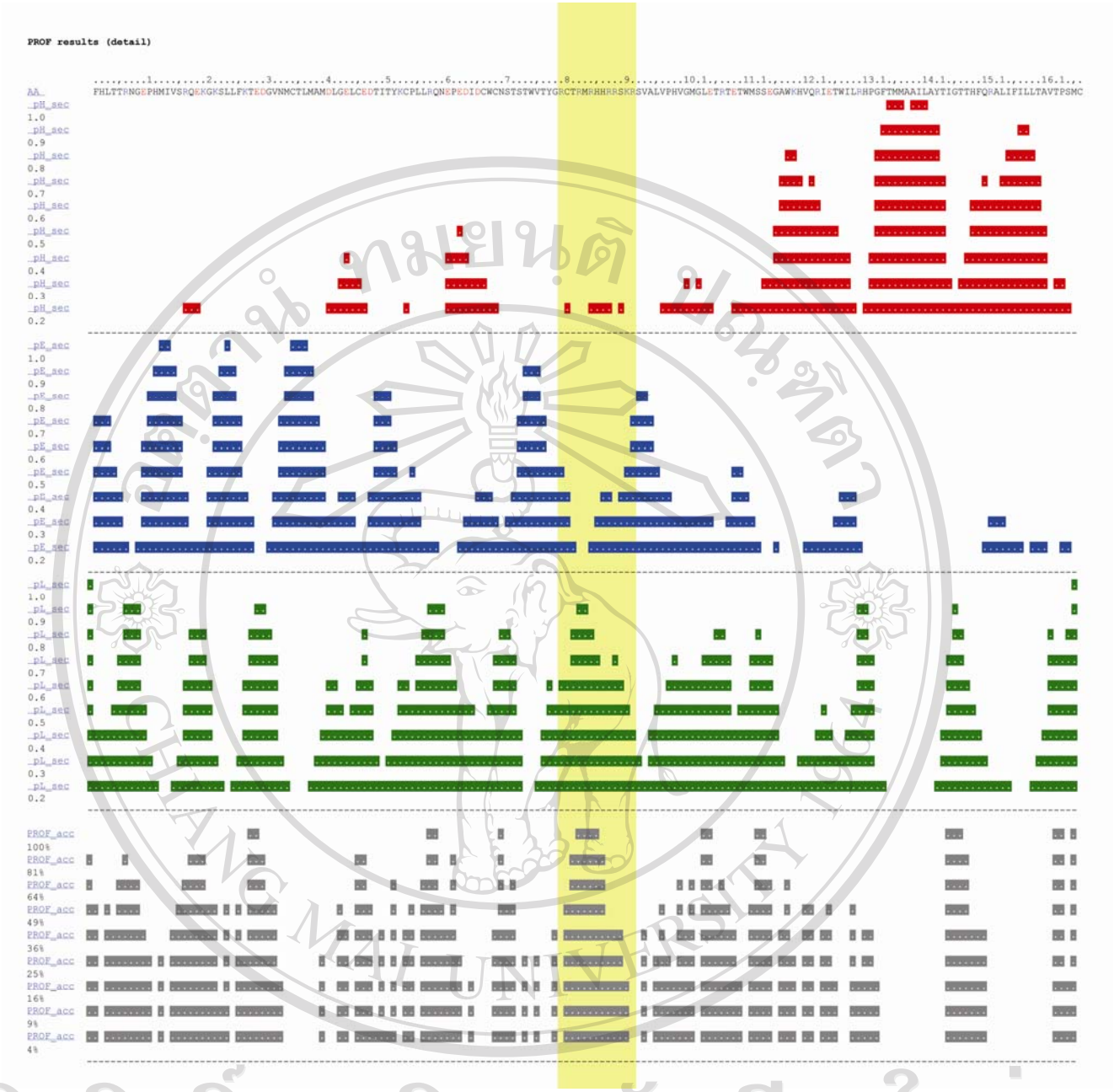

Figure 41. The secondary structure analysis of amino acid sequences of the 16681pr(+7,-0) prM protein by PHOFec (Rost et al., 2003)**.** An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org, and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 amino acid residues that changed at proximal to pr-M junction was marked with yellow.

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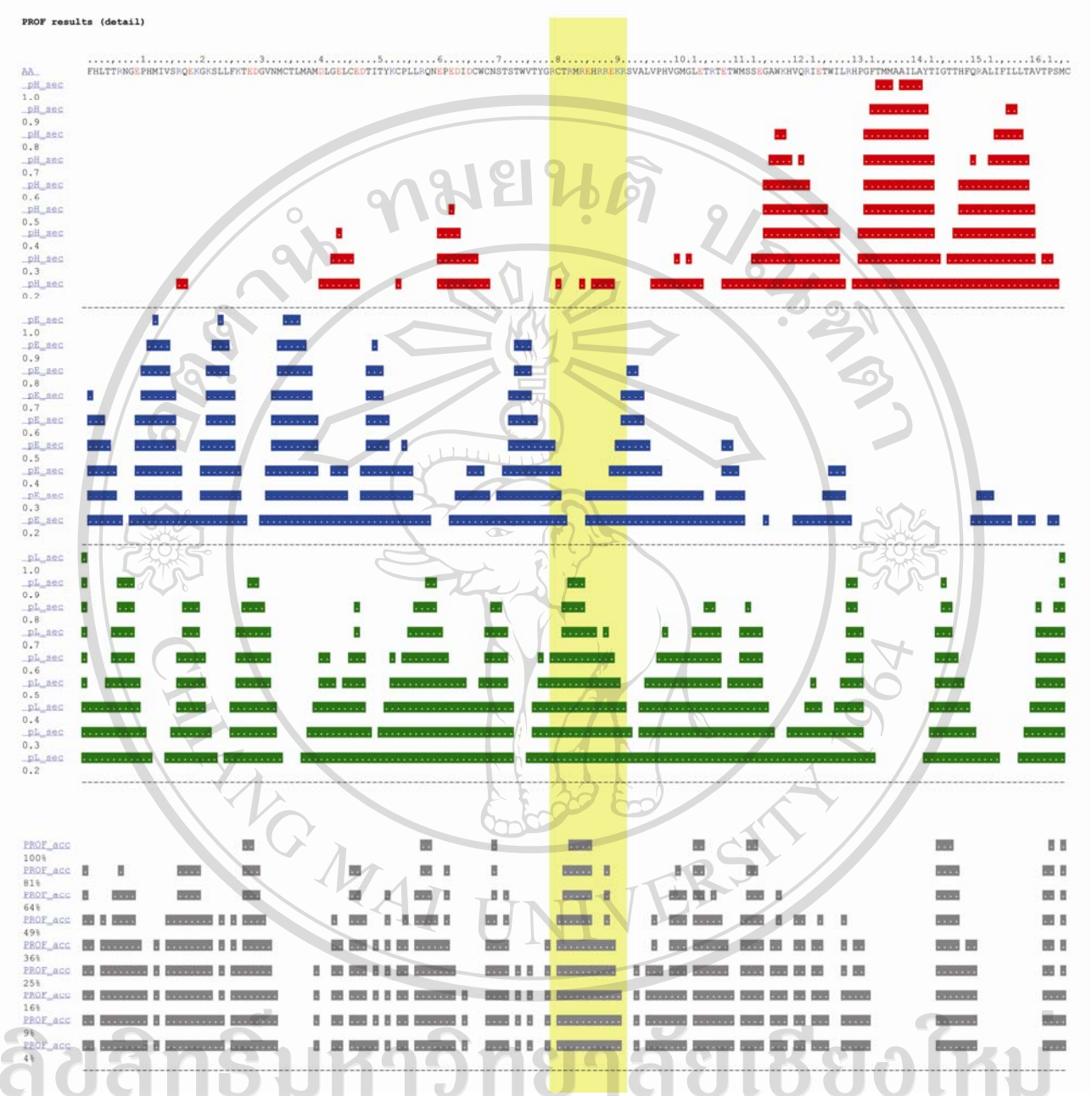

Figure 42. The secondary structure analysis of amino acid sequences of the 16681pr(+7,-2) prM protein by PHOFec (Rost et al., 2003)**.** An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org, and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 amino acid residues that changed at proximal to pr-M junction was marked with yellow.

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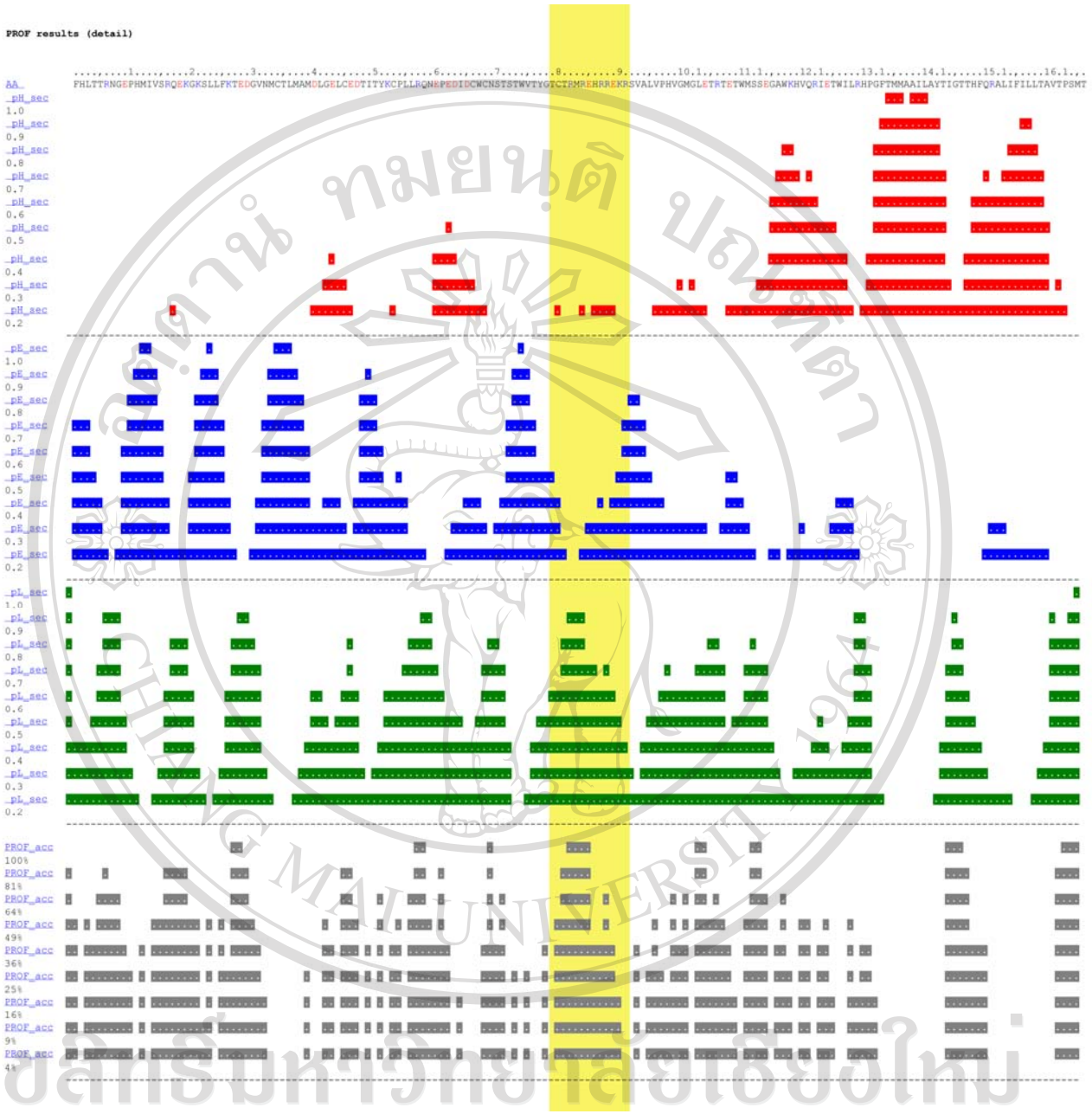

Figure 43. The secondary structure analysis of amino acid sequences of the 16681pr(+6,-2)P8,10+ prM protein by PHOFec (Rost et al., 2003)**.** An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org, and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 amino acid residues that changed at proximal to pr-M junction was marked with yellow.

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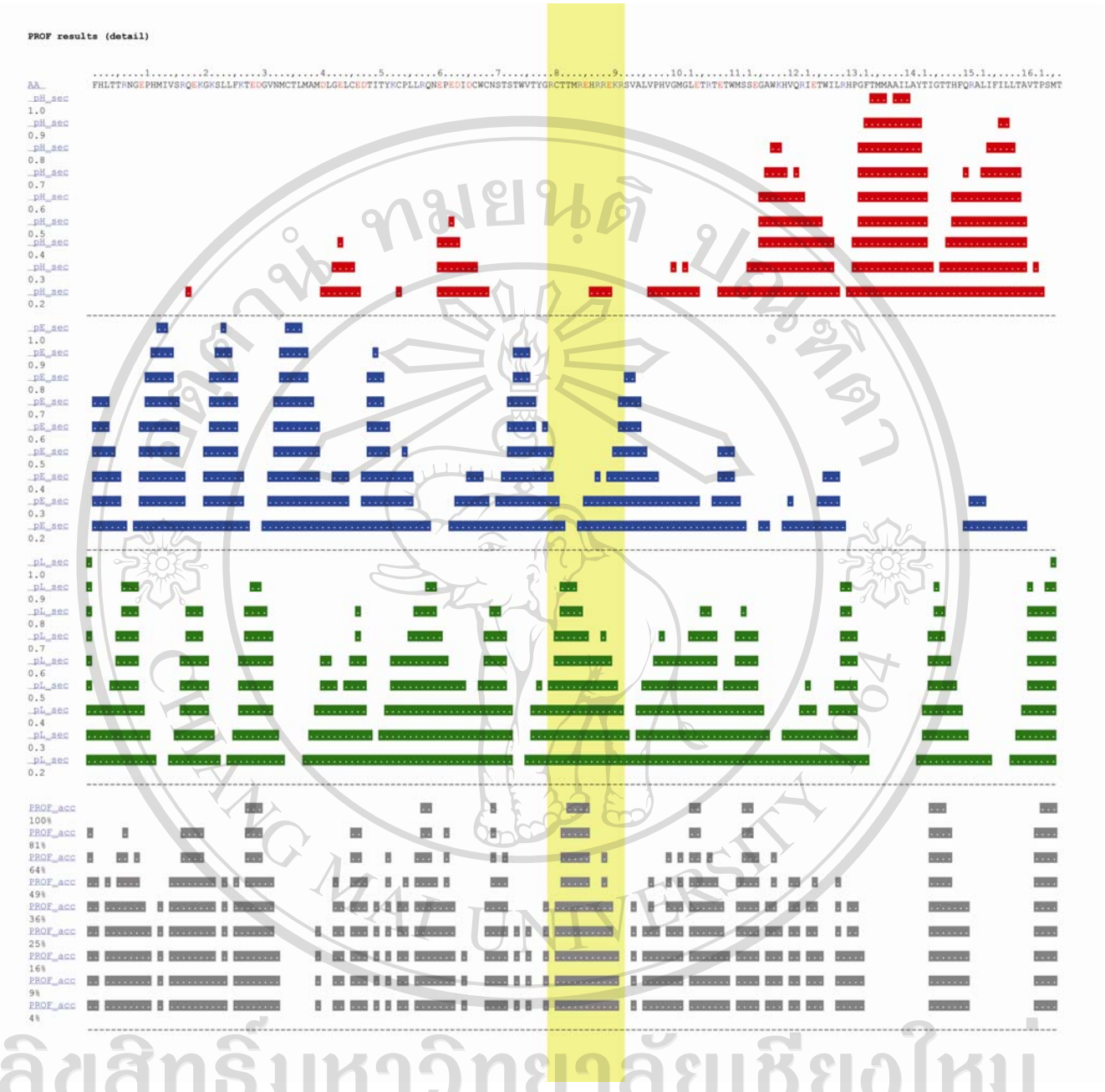

Figure 44. The secondary structure analysis of amino acid sequences of the 16681pr(+6,-2)P8,13+ prM protein by PHOFec (Rost et al., 2003)**.** An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org, and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 amino acid residues that changed at proximal to pr-M junction are marked with yellow.

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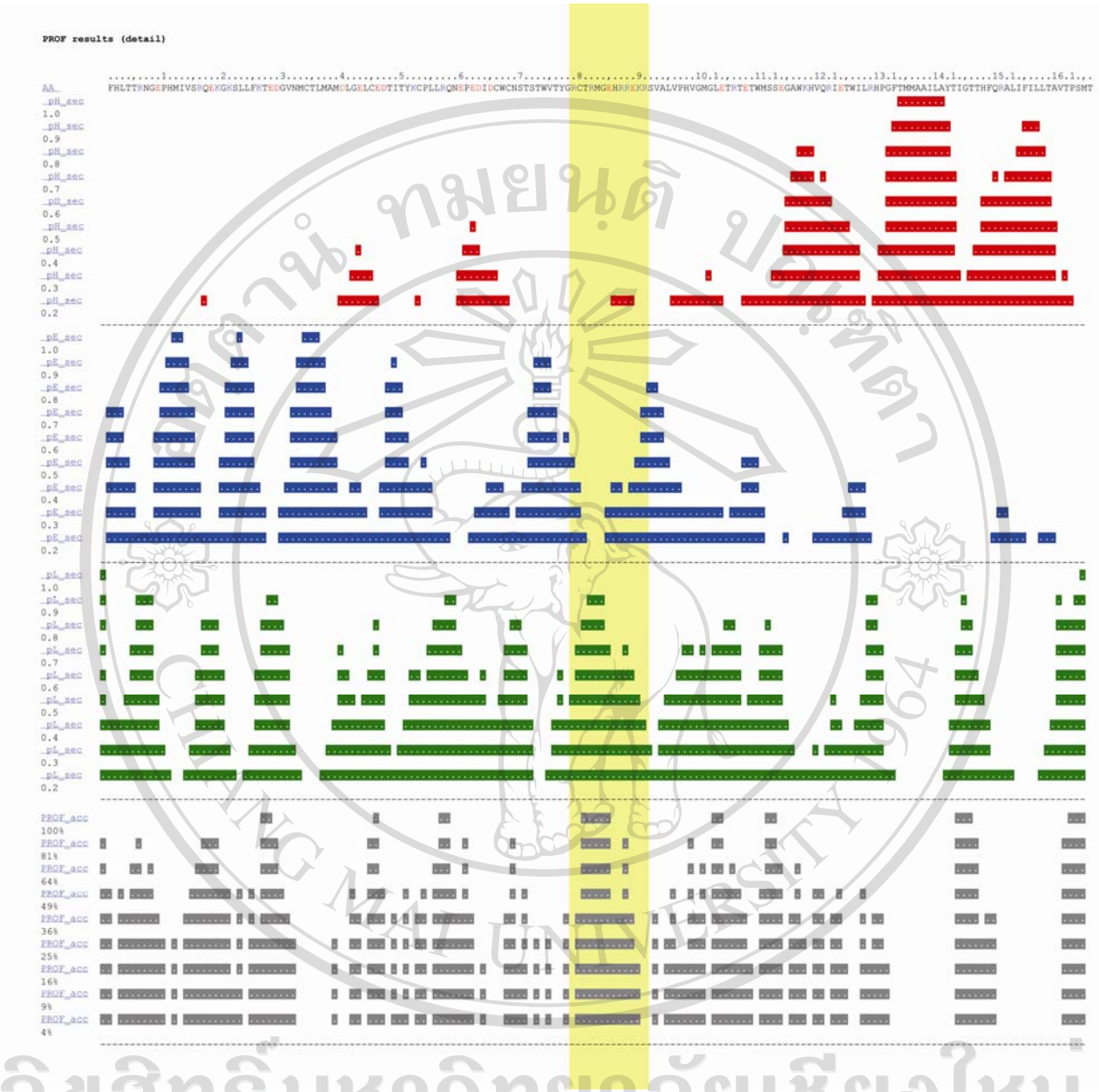

Figure 45. The secondary structure analysis of amino acid sequences of the 16681pr(+6,-2)P10,13+ prM protein by PHOFec (Rost et al., 2003)**.** An entire amino acid sequence of the prM protein encoded from the DNA sequence was submitted to www.propredict.org and the summary was shown: pH_sec, the score of helix (0.1-1.0); pE_sec, the score of the strand (0.1-1.0), pL_sec, the score of the loop (0.1-1.0) and PROF_acc, the solvent accessibility score (1-100%). The sequence of 13 acid amino residues that changed at proximal to pr-M junction was marked with yellow.

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Table 25. The summary of the secondary structure analyses of amino acid sequences of the mutant and wild type prM proteins by PROFec (Rost et al., 2003).

PrM mutant virus	Total helix in protein (%)	Total strand in protein (%)	Total loop in protein (%)	Exposition of amino acid residues (%)	All other residue (%)
16681Nde(+)	19.88	24.10	56.02	59.04	40.96
JEVpr/16681	19.88	27.11	53.01	59.64	40.36
16681prE203A	21.69	23.49	54.92	59.04	40.96
16681pr(+4,-0)HS	19.88	25.33	54.82	59.04	40.96
16681pr(+7,-0)	19.88	24.10	56.02	59.04	40.96
16681pr(+7,-2)	19.88	23.49	56.63	59.04	40.96
16681 pr(+6,-2)P8,10+	19.88	24.10	56.02	59.64	40.36
16681 pr(+6,-2)P10,13+	21.69	23.49	54.82	59.04	40.96
16681 pr(+6,-2)P8,13+	19.88	24.10	56.02	59.64	40.36

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Appendix C: Properties of amino acids

1. The codon dictionary.

First Position	Second Position: U	Second Position: C	Second Position: A	Second Position: G
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys
	UUA Lue	UCA Ser	UAA End	UGA End
	UUG Lue	UCG Ser	UAG End	UGG Trp
C	CUU Leu	CCU Pro	CAU His	CGU Arg
	CUC Leu	CCC Pro	CAC His	CGC Arg
	CUA Leu	CCA Pro	CAA Gln	CGA Arg
	CUG Lue	CCG Pro	CAG Gln	CGG Arg
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser
	AUC Ile	ACC Thr	AAC Asn	AGC Ser
	AUA Ile	ACA Thr	AAA Lys	AGA Arg
	AUG Met	ACG Thr	AAG Lys	AGG Arg
G	GUU Val	GCU Ala	GAU Asp	GGU Gly
	GUC Val	GCC Ala	GAC Asp	GGC Gly
	GUA Val	GCA Ala	GAA Glu	GGA Gly
	GUG Val	GCG Ala	GAG Glu	GGG Gly

The third nucleotide in red color of each codon is less specific than the first two, and codons are read in 5´ to 3´ direction.

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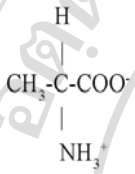
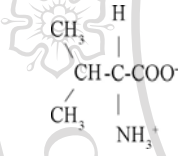
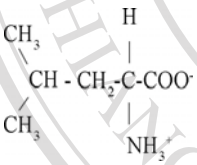
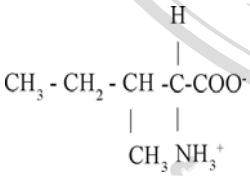
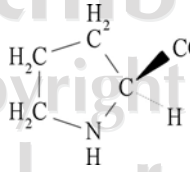
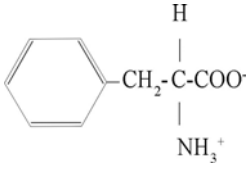
2. Abbreviation, pKa and molecular weight of amino acid residues

2.1 Amino acids with nonpolar R group. The amino acids are shown in ionizing forms at pH 6.0 to 7.0.

Chemical structure	Amino acids	Three-letter code	One-letter code	pKa of ionizing side chain	Residue mass (Daltons)
	Alanine	Ala	A	-	71.08
	Valine	Val	V	-	99.14
	Leucine	Leu	L	-	113.17
	Isoleucine	Ile	I	-	113.17
	Proline	Pro	P	-	97.12
	Phenylalanine	Phe	F	-	147.18

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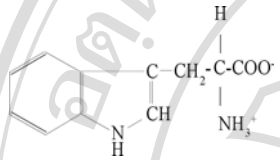
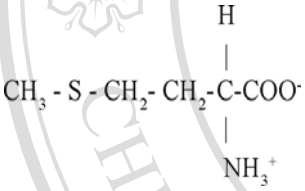
2.1 Amino acids with nonpolar R group. The amino acids are shown in ionizing forms at pH 6.0 to 7.0. (Continued)

Chemical structure	Amino acids	Three-letter code	One-letter code	pKa of ionizing side chain	Residue mass (Daltons)
	Tryptophan	Trp	W	-	186.21
	Methionine	Met	M	-	131.21

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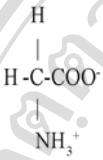
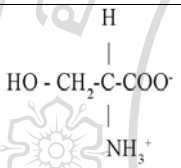
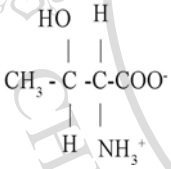
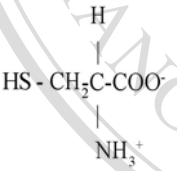
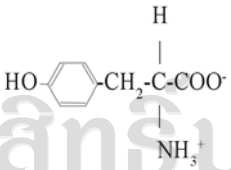
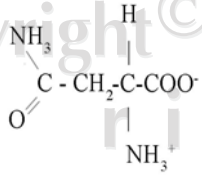
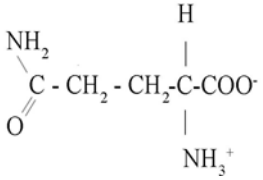
2.2 Amino acids with uncharged polar R group. The amino acids are shown in ionizing forms at pH 6.0 to 7.0.

Chemical structure	Amino acids	Three-letter code	One-letter code	pKa of ionizing side chain	Residue mass (Daltons)
	Glycine	Gly	G	-	57.06
	Serine	Ser	A	-	87.08
	Threonine	Thr	T	-	113.17
	Cysteine	Cys	C	8.3	103.14
	Tyrosine	Tyr	Y	10.1	163.18
	Asparagine	Asn	N	-	114.11
	Glutamine	Gln	Q	-	128.14

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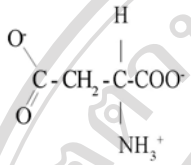
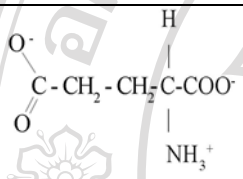
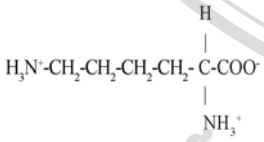
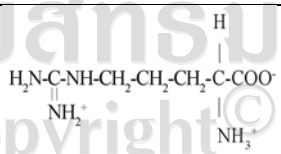
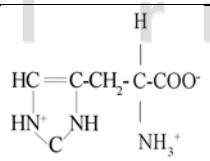
2.3 Acid amino acids (negatively charged at pH6.0)

Chemical structure	Amino acids	Three-letter code	One-letter code	pKa of ionizing side chain	Residue mass (Daltons)
	Aspatic acid	Asp	D	3.9	115.09
	Glutamic acid	Glu	E	4.2	129.12

2.4 Basic amino acids (Positively charged at pH 6.0)

Chemical structure	Amino acids	Three-letter code	One-letter code	pKa of ionizing side chain	Residue mass (Daltons)
	Lysine	Lys	K	10.0	128.18
	Arginine	Arg	R	12.5	156.20
	Histidine	His	H	6.0	137.15

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Appendix D: Reagents

1. Medium for bacterial growth

1.1 Luria-Bertani Broth (LB) Medium (per liter)

Tryptone	10.0 g
Yest Extract	5.0 g
NaCl	10.0 g

The powder of Luria-bertani medium is dissolved in 1,000 ml water, and pH is adjusted to pH 7.5 by 1 N NaOH. This medium was sterile by autoclave at 121^{O}C for 15 min.

1.2 Luria-Bertani Agar (per liter)

Tryptone	10.0 g
Yest Extract	5.0 g
NaCl	10.0 g
Agar	20.0 g

These compositions are dissolved with 1,000 ml of sterile water, and pH was adjusted to pH 7.5 by 1 N NaOH. This medium was sterile by autoclave. After sterilization, the medium was warmed to 55ºC, and they were poured into 100 mm Petri dishes.

1.3 LB-Ampicillin Agar plate (per liter)

250 µl of 100.0 mg/ml of ampicillin was added to 1,000 ml to Luria-Bertani agar. This medium was allowed to warm at 55ºC, and then the media were poured into 100 mm Petri dishes.

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1.4 SOB Medium (per liter)

Tryptone	20.0 g
Yeast extracts	5.0 g
NaCl	0.5 g

All the components were mixed in 1,000 ml of sterile distilled water to dissolve, and they were sterile by autoclave. Before use, 10 ml of 1.0 M $MgCl_2$ and 10 ml of 1 M $MgSO_4$ were added into the medium.

1.5 SOC Medium (100 ml)

Tryptone or peptone	2.0	g
Yeast extract	0.5	g
NaCl	0.005	g
KCl	0.0186	g
20% (w/v) glucose	2.0	ml

All components were dissolved in 100 ml distill water, and pH of the medium was adjusted to pH 7.0. This medium was sterile by autoclave. The working stock solution was prepared by adding of 1 ml of 1 M $MgCl_2$, 2 ml of 1 M glucose into this medium. The medium was stored at 4ºC until used.

2. Solution for plasmid DNA mini and midi-preparation.

2.1 Suspension Buffer (P1 Buffer)

Tris-base	6.06 g
EDTA.$2H_2O$	3.72 g

These two components were dissolved in 800 ml distill water, and then pH was adjusted to pH 8.0 with HCl. Total volume of solution was adjusted with distill water to make 1,000 ml. This medium was sterile by autoclave. Before use, RNase A was added to solution to be 100.0 µg/ml final concentrations.

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2.2 Lysis Buffer (P2 Buffer)

0.2 N NaOH

1% (W/V) SDS

NaOH pellets (8 g) and SDS were dissolved in 1,000 ml distilled water, and this solution was sterile by autoclave.

2.3 Neutralization Buffer (P3 Buffer)

The 294.5 g of potassium acetate were dissolved in 500 ml distill water. Glacial acetic acid (~110 ml) was added into solution to adjust pH to pH 5.5, and this solution was added by water to be 1,000 ml. The solution was sterile by autoclave.

2.4 Equilibration Buffer (QBT Buffer)

NaCl	43.83 g
Acid free MOPC	10.46 g
Isopropanol	150.00 ml
10% (V/V) Triton X-100	15.00 ml

All components were dissolved with 800 ml distill water, and pH was adjusted to pH 7.0. isopropanol and 10% (V/V) Triton X-100 were added into the solution. The volume was adjusted to 1,000 ml by distill water, and this solution was sterile by filtration through 0.2 µm pore size membrane.

2.5 Wash Buffer (QC Buffer)

NaCl	58.44 g
Acid free MOPC	10.46 g
Isopropanal	150.00 ml

Sodium chloride and MOPC were dissolved in 800 ml distill water, and pH was adjusted to pH 7.0. isopropanol was added into this solution. The final volume of solution was adjusted with distill water to be 1,000 ml, and this solution was sterile by filtration through the micropore (0.2 µm) membrane.

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2.6 Elution Buffer (QF Buffer)

NaCl	73.05 g
Tris-base	6.06 g
Isopropanol	150.00 ml

Sodium chloride and Tris-base were dissolved in 800 ml distill water, adjusted pH to 8.5. 150 ml of Isopropanol was added into solution. The volume was adjusted with sterile water to 1,000 ml and sterile though micropore filter.

3 Solution for DNA agarose gel electrophoresis

3.1 50X TAE Buffer (Stock solution)

Tris-HCl	242.0	g
Acetic acid	57.1	g
0.5 M EDTA	100.0	g

Tris-HCl was dissolved with 800 ml of distill water. After that, 0.5 M EDTA and acetic acid were added to the solution. The pH of solution was adjusted to pH 8.0 and final volume was made to 1,000 ml by adding of sterile water.

3.2 6X-loading buffer

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

45% (v/v) glycerol in sterile water

The 2.5 g of both bromophenol blue and xylene cyanol were dissolved in 350 µl of sterile water. Then 450 µl of 100% glycerol were added to the solution, and they were mixed by vertex. This solution was adjusted to one ml.

3.3 Ethidium bromide stock solution

Ethidium bromide	100.0 mg
Sterile distilled water	10.0 ml

Dissolved ethidium bromide into water, and this solution was mixed. The ethidium bromide solution was stored in dark bottle.

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4. Solution for *in vitro* transcription

4.1 RNase free water

Deithyl pyrocarbonate	0.2 ml
Deionized water	100.0 ml

Dethylpyrocarbonate was added to deionizing water, and this solution was mixed by stirred. The solution was incubated for overnight. Next day, the dethylpyrocarbonate remaining in this solution was destroyed by autoclave.

5. RNA agarose gel electrophoresis

5.1 RNA loading buffer

6.25%(v/v) Deionized formamide

1.14 M formaldehyde

200 µg/ml bromophenol blue

200 µl xylene cyanole

1.25X MOPE-EDTA-Sodium acetate buffer

Sample was added in to loading buffer, and they were heated at 65º C for 10 minutes.

5.2 10X MOPS-EDTA-sodium acetate buffer

MOPS 20.92 g

Sodium acetate 3.28 g

Sterile RNAse-free water 450 ml

All of the components were added in water, and 10 ml of 0.5 M EDTA (pH 8.0) were added to the solution. This solution was treated with 0.5 ml of DEPC, and they were incubated at room temperature for overnight. Next day, this solution was subjected to autoclave.

6. Reagents for focus immunological staining

6.1 10X phosphate buffer saline (PBS) for 1,000 ml (137.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 2.0 mM KH_2PO_4)

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	11.5 g
K_2HPO_4	2.0 g

All the components were dissolved in 800 ml of deionizing water. The pH of this solution was adjusted to pH 7.4 by 1 N HCl in 1,000 ml final volume. This solution was sterile by autoclave.

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6.2 2.0% (v/v) Triton-X 100

Triton-X 100	0.2 ml
1X sterile PBS	10.0 ml

0.2 ml of Triton-X 100 was added into ten ml 1X PBS, and they were gently mixed.

6.3 3.7% (v/v) Formaldehyde

37% Formaldehyde	2.0 ml
1X sterile PBS	18.0 ml

Two ml of 37% (v/v) of formaldehyde solution were added into 18.0 ml of 1X PBS, and they were mixed by inverting.

6.4 1X PBS-0.05% (v/v) Tween-20, 2.0% (v/v) Fetal bovine serum

1X sterile PBS	9.8 ml
Tween-20	2.5 µl
Fetal bovine serum	0.2 ml

All components were added to sterile PBS pH 7.4, and they were gently mixed by swirling.

6.5 Peroxidase substrate

6.0% (v/v) H_2O_2	200.0 ml
3,3 diaminobenzidine	500.0 mg
1X sterile PBS	9.0 ml

Those components are dissolved in 1X PBS to 10 ml final volume, mixed and kept away form light.

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6.6 Alkaline Phosphate buffer (AP buffer)

1 M Tris-HCl (pH 9.5)	.1.0 ml
1 M NaCl	1.0 ml
1 M $MgCl_2$	0.05 ml
Sterile water	7.95 ml

All the reagents were added in water, and they were mixed by inverting.

7. Reagents for cell culture

Leibovitz's L-15 Medium (1X)

The Leibovitz's L-15 Medium is contained of phosphates and free-base amino acids as the buffer. The compositions obtain: inorganic salts, i.e. 1.26 mM Calcium chloride ($CaCl_2$), 5.30 mM Potassium chloride (KCl), 0.441 mM Potassium phosphate monobasic (KH_2PO_4), 0.986 mM Magnesium chloride ($MgCl_2$), 0.814 mM Magnesium sulfate ($MgSO_4$), 138.00 mM Sodium chloride (NaCl), 1.34 mM Sodium phosphate (Na_2HPO_4).

The amino acids including in the medium were 2.252 mM L-Alanine, 2.87 mM Arginine, 1.89 mM L-Asparagine, 0.992 mM L-Cysteine, 2.055 mM L-Glutamine, 2.670 mM Glycine, 1.61 mM L-Histidine, 1.910 mM mM L-Isoleucine, 0.945 mM L-Leucine, 0.503 mM L-Lysine, 1.01 mM L- Methionine, 0.76 mM L-Phenylalanine, 1.90 mM L-Serine, 2.52 mM L-Threonine, 0.098 mM L_ Tryptophan, 1.66 mM L-Tyrosine, 0.85 mM L-Valine; vitamins, i.e. 0.002 mM L-pantothenate, 0.0071 mM Choline chloride, 0.0022 mM Folic acid, 0.011 mM i-Inositol, 0.0081 mM Niacinamide, 0.0048 mM Pyridoxine hydrochloride, 0.000209 mM Riboflavin 5´-phosphate, Na, 0.00226 mM Thiamine monophospate; Other components, i.e. 5.00 mM D Galactose, 5.00 mM Sodium pyruvate, 0.025 mM phenol red.

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Appendix E: Instruments

1. DNA thermal cycles, model 480 (Perkin Elmer, Foser City, California, USA)
2. Flip-Flop shaker (model FF 120 s, J.S.C. instrument)
3. Freezer, -20º C (Sanyo, Japan)
4. Freezer, -70º C (Forma Scientific Inc, USA)
5. Freezer, -70º C (Thermal Electron corporation, USA)
6. Gel documentary sytem, model gel doc 1000 (Bio-Rad Laboratories, Inc., RSA)
7. Genetic analyzer ABI PRISM 310 (Perkin Elmer, Foster City, California, USA)
8. High speed refrigerated microcentrifuge, model 4239R (ALC, Milano, Italy)
9. Incubator (Forma Scientific Inc, USA)
10. Incubator, model 1565 (Shellab, USA)
11. Laminar air flow cabinet, model NU-425-400 (NUAire, USA)
12. Microcentrifuge (ALC, Milano, Italy)
13. Microwave oven NN-6208 (Mutsushita Electric industrial Co., Ltd., Japan)
14. Orbital shaker bath Model 360 (Precision Scientific, USA)
15. Orbital shaker model Gyromax 737R (Amerex Instruments, Ins., USA)
16. pH meter model 661 (Orion Research Incorporated Laboratory product group, USA)
17. Superspeed refrigerated centrifuge, model Sorval RC-5 (E.I. Dupont Denemours & Co., USA)
18. Spectrophotometer (Spectronic Genesysz, UK)
19. Ultraviolet transilluminator (Vilber Lourmet, France)

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