

TABLE 3.1 Contents of the PCR reaction

Reagent	Volume
Takara 10× buffer	5 µl
dNTP Mixture (10 mM)	4 µl
Takara pro <i>Taq</i> (22 units/µl)	1 µl
Forward PCR primer (D26825F 50 µM)	1 µl
Reverse PCR primer (D27570R 50 µM)	1 µl
Den2 full-length cDNA	1 µl
Add ddH ₂ O to a final volume of 50 µl	

TABLE 3.2 Condition for the PCR reaction of NS4B

Segment	Cycles	Temperature	Time
1	1	94°C	3 minutes
2	30	94°C	30 seconds
		54.5°C	30 seconds
		72°C	1.5 minutes
3	1	72°C	15 minutes

TABLE 3.3 Comparison of the NS4B sequence variations between Taiwan local strain PL046 and New Guinea C strain (Accession No. M29095)

Nucleotide position	Nucleotide changes		Amino acid changes (NGC→PL046)	Identity of nucleotide sequences	Identity of amino acids
	NGC	PL046			
45	G	A	L 15 L	97.0 %	99.2 %
102	C	T	P 34 P		
126	T	C	A 42 A		
141	T	C	F 47 F		
162	C	T	H 54 H		
231	T	C	G 77 G		
250	T	C	L 84 L		
306	T	C	V 102 V		
318	T	A	T 106 T		
334	T	C	F 112 L		
340	C	T	L 114 L		
357	C	T	A 119 A		
390	C	T	T 130 T		
456	G	A	V 152 V		
531	C	T	C 177 C		
543	G	A	V 181 V		
593	C	T	T 198 I		
600	T	C	P 200 P		
603	C	T	I 201 I		
681	G	A	G 227 G		
693	C	T	A 231 A		
714	C	T	S 238 S		

TABLE 3.4 The number and size of plaques formed in the assay plates

Experiment Number	pcDNA3	NS2A	NS2B	NS4A	NS4B
1	133	85	116	117	—
2	134	89	97	112	—
3	129	148	74	114	—
4	110	85	104	73	93
5	108	105	105	103	92
6	96	80	80	94	90
7	100	104	104	110	104
Mean ± S.D.	115.7 ± 16	99.4 ± 23.5	97.1 ± 15	103.3 ± 15.4	94.8 ± 6.3
Percentage	100%	85.9%	84%	89.3%	81.9%
Plaque size* (mm)	0.52 ± 0.123 ^b	0.52 ± 0.099 ^b	0.35 ± 0.072 ^c	0.7 ± 0.14 ^a	0.51 ± 0.124 ^b

* Plaque size of cells transfected with each plasmid was compared with that of pcDNA3 using Duncan's test (n=65); letters marked next to mean plaque size indicate significantly different groups, such that groups which share a letter are not significantly different and groups which do not share a letter are significantly different ($P < 0.05$).

World Distribution of Dengue - 2005

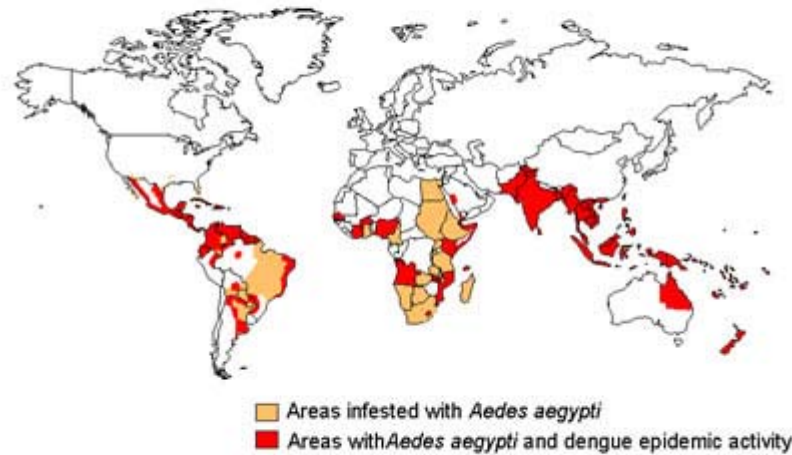


FIG. 1.1 World distribution of dengue viruses and their mosquito vector, *Aedes aegypti*, in 2005. (CDC, USA, 2005)

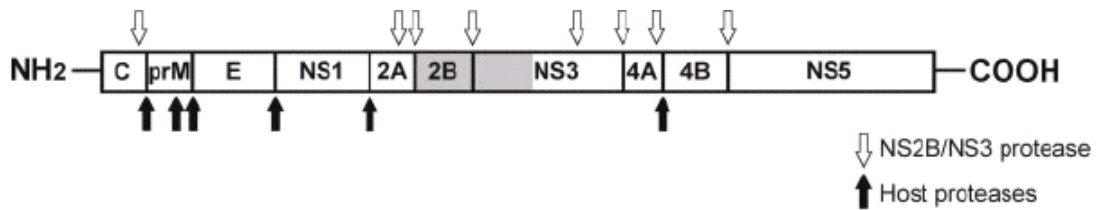


FIG. 1.2 Processing of Flavivirus polyprotein. (Leung, *et al.*, 2001)

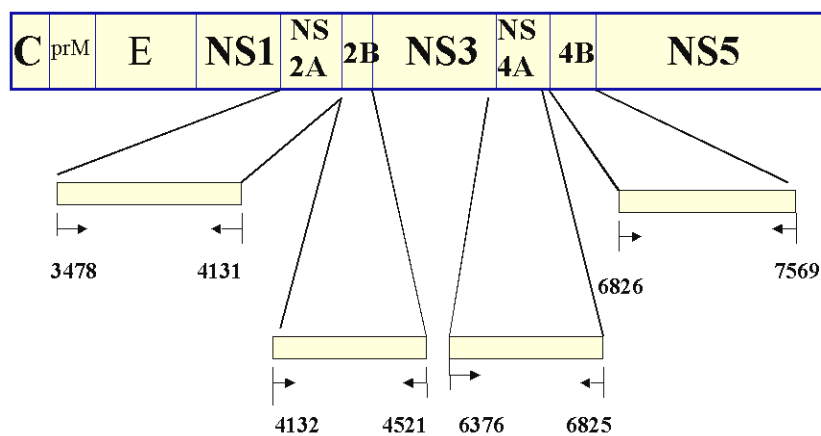


FIG. 1.3 Nucleotide mapping of NS2A, NS2B, NS4A and NS4B in dengue virus type 2 genome. Numbers indicate the nucleotide numbers starting from 5' UTR. (Gubler and Kuno, 1997)

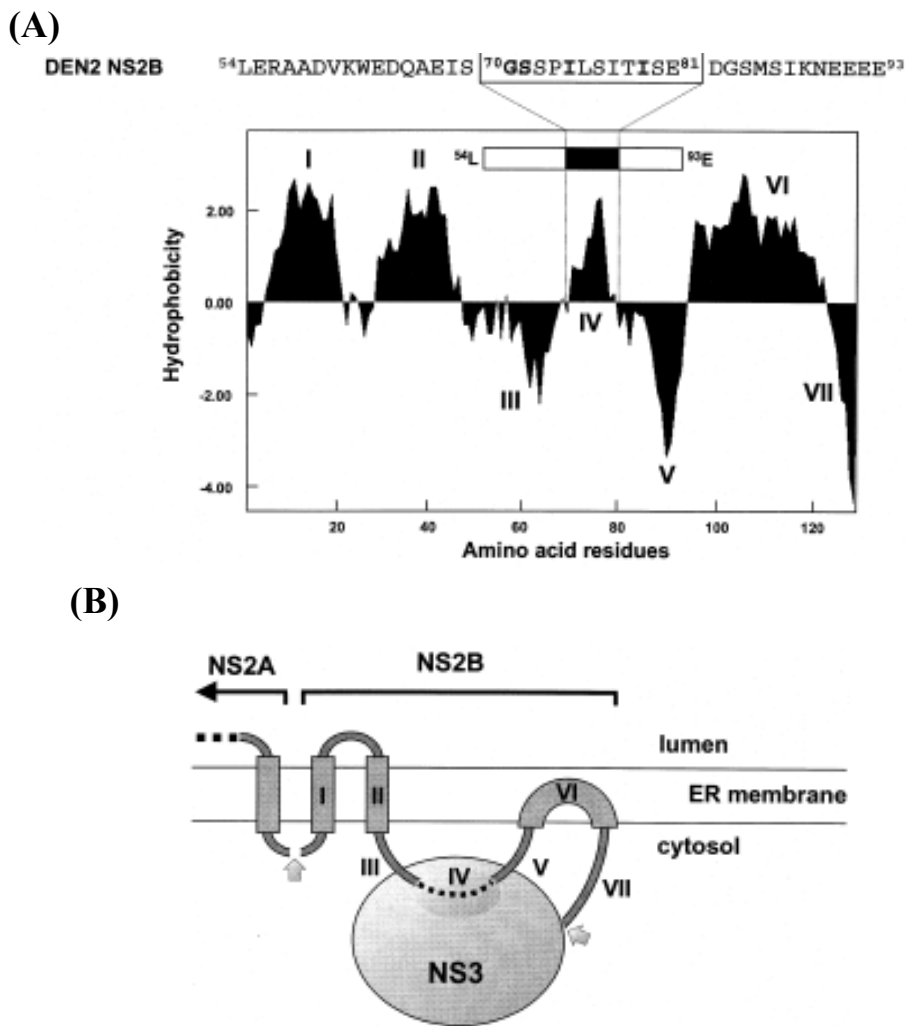


Fig. 1.4 NS2B hydrophobicity plots and a hypothetical model of NS2B-NS3pro association with membranes. (A) Hydrophobicity profiles [generated by the Kyte & Doolittle algorithm (Kyte & Doolittle, 1982) in the MacVector suite of programs] of the DEN2 cofactor NS2B. I–VII, regions of NS2B with either hydrophobic (I, II, IV and VI) or hydrophilic (III, V and VII) character. The 40 amino acid cofactor domain of NS2B (schematically represented as a boxed region) is as indicated, and the central hydrophobic 12 amino acid stretch of NS2B (70 GSSPILSITISE 81) modeled is also boxed. (B) Schematic representation of potential interactions between specific domains of the NS2B cofactor and either cellular membranes or NS3pro. Domains I–VII correspond to regions of hydrophobicity and hydrophilicity identified in (A). Shaded arrows identify sites of NS2B/NS3 cleavage.

(Brinkworth *et al.*, 1999)

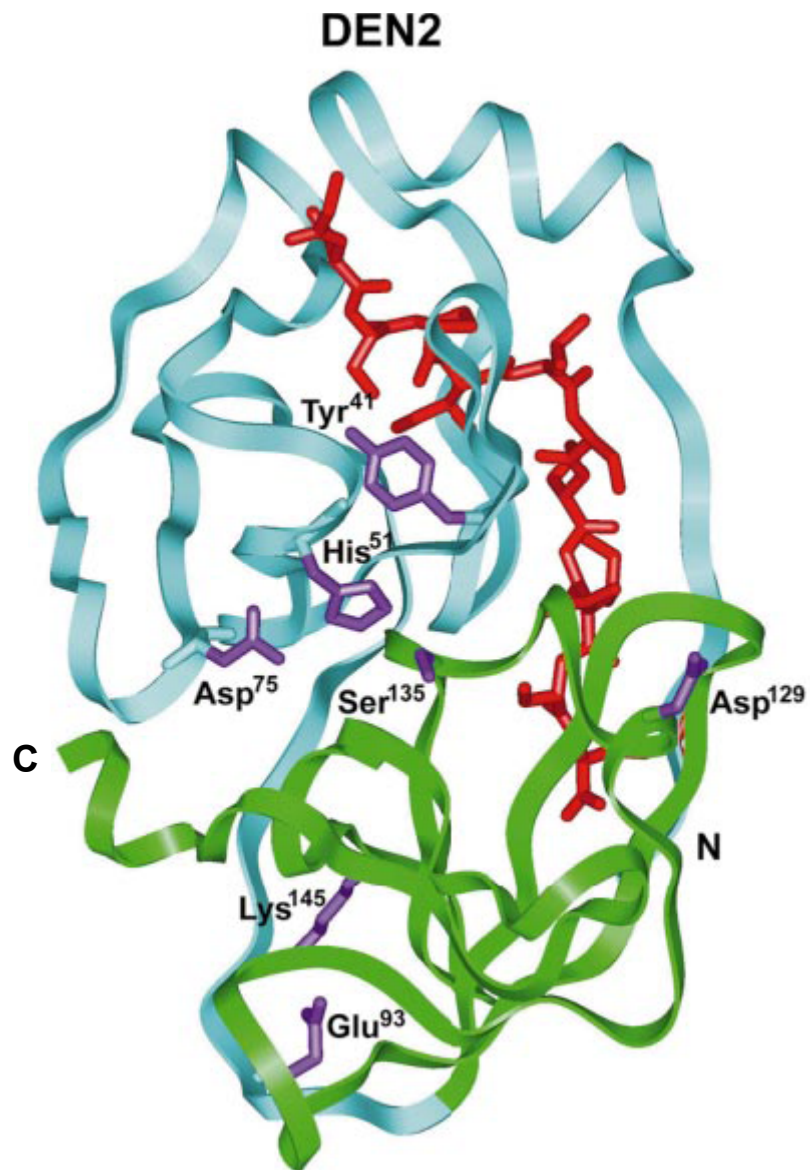
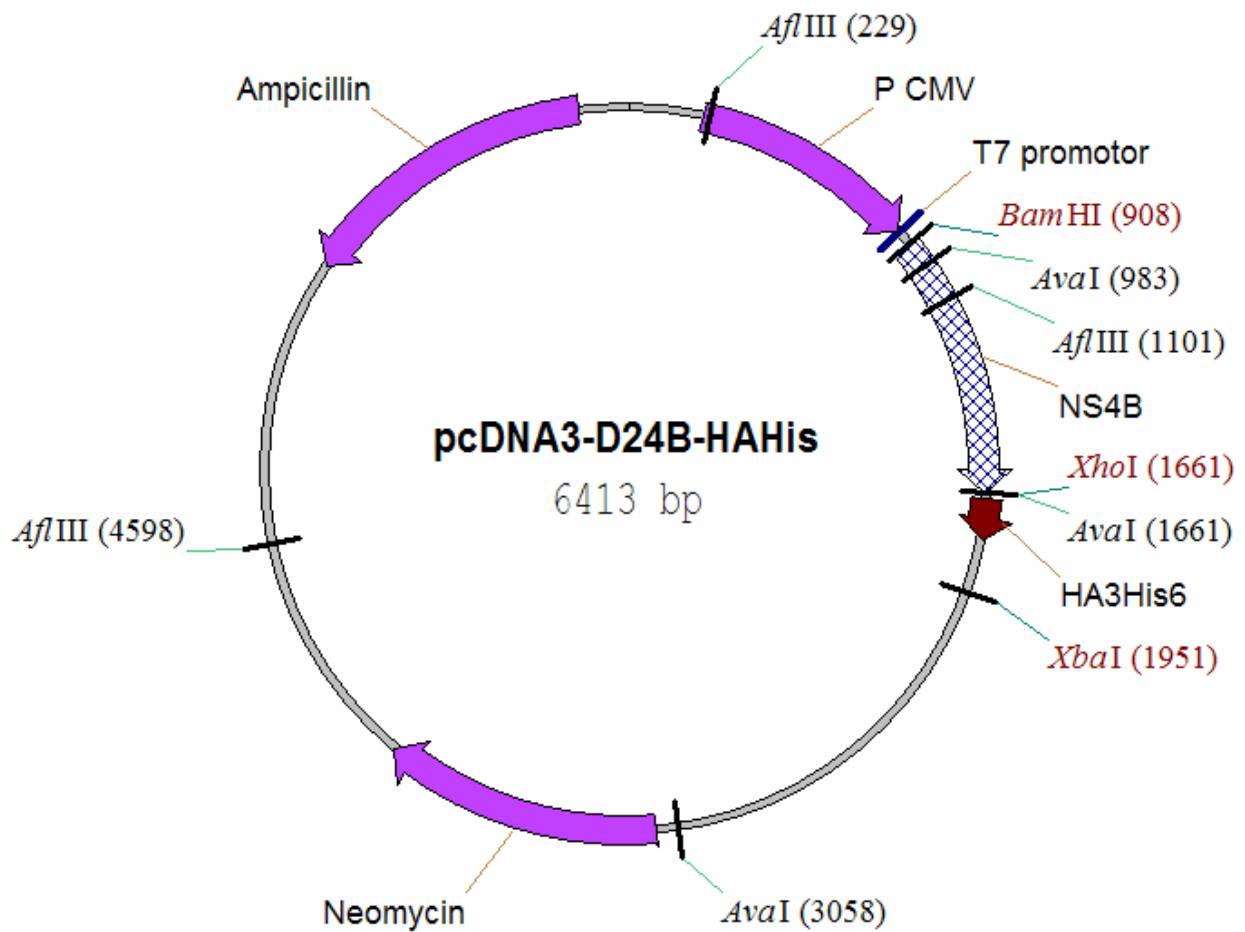


Fig. 1.5 The backbone structures of DEN2 NS3 protease with the cofactor, NS2B (the latter in red). The catalytic triad and the putative salt bridge in DEN2pro are shown in purple.

(Brinkworth *et al.*, 1999)

(A)



(B)

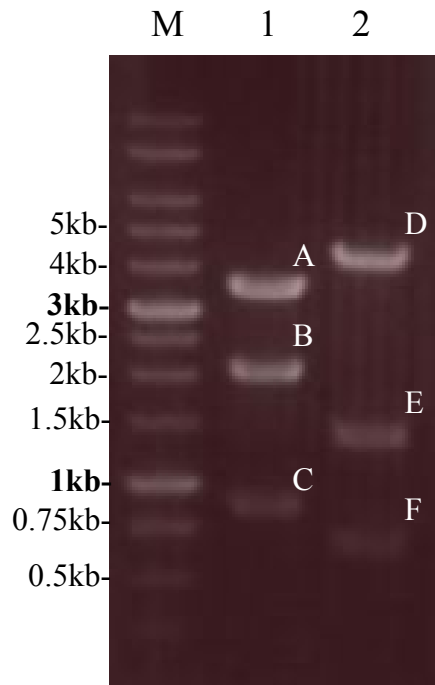


FIG. 3.1 Restriction enzyme digestion of pcDNA3-4B-HAHis. (A) Map of pcDNA3-4B-HAHis. (B) Lane 1, *Afl*III digested pcDNA3-4B-HAHis; lane 2, *Ava*I digested pcDNA3-4B-HAHis; M, marker. A, 3497bp; B, 2044bp; C, 872bp; D, 4338bp; E, 1397bp; F, 678bp.

		Section 1					
	(1)	1	10	20	30	48	
PL046 NS4B	(1)	GCTTGGTNCGAGCTCGGATCCATGAACGAGATGGGTTTCCTGGAAAAA					
NGC NS4B	(1)	-----AACGAGATGGGTTTCCTGGAAAAA					
Consensus	(1)	AACGAGATGGGTTTCCTGGAAAAA					
		Section 2					
	(49)	49	60	70	80	96	
PL046 NS4B	(49)	ACGAAGAAAGATCTCGGATTAGGAAGCATTACAACCCAGCAACCCGAG					
NGC NS4B	(25)	ACGAAGAAAGATCTCGGATTGGGAAGCATTACAACCCAGCAACCCGAG					
Consensus	(49)	ACGAAGAAAGATCTCGGATTGGAAGCATTACAACCCAGCAACCCGAG					
		Section 3					
	(97)	97	110	120	130	144	
PL046 NS4B	(97)	AGCAACATCCTGGACATAGATCTACGTCCATGCATCAGCATGGACGCTG					
NGC NS4B	(73)	AGCAACATCCTGGACATAGATCTACGTCCATGCATCAGCATGGACGCTG					
Consensus	(97)	AGCAACATCCTGGACATAGATCTACGTCCGCATCAGCATGGACGCTG					
		Section 4					
	(145)	145	150	160	170	180	192
PL046 NS4B	(145)	TATGCTGTGGCCACAACATTCTGTCACACCAATGTTGAGACATAGCATT					
NGC NS4B	(121)	TATGCTGTGGCCACAACATTCTGTCACACCAATGTTGAGACATAGCATT					
Consensus	(145)	TATGCTGTGGCCACAACATTGTCACACCAATGTTGAGACATAGCATT					
		Section 5					
	(193)	193	200	210	220	230	240
PL046 NS4B	(193)	GAAAATTCCTCAGTGAACGTGTCCCTAACAGCTATTGCCAACCAAGCC					
NGC NS4B	(169)	GAAAATTCCTCAGTGAACGTGTCCCTAACAGCTATTGCCAACCAAGCC					
Consensus	(193)	GAAAATTCCTCAGTGAACGTGTCCCTAACAGCTATTGCCAACCAAGCC					
		Section 6					
	(241)	241	250	260	270	288	
PL046 NS4B	(241)	ACAGTGTTAATGGGCTTGGGAAAGGATGGCCAATGTCAAAGATGGAC					
NGC NS4B	(217)	ACAGTGTTAATGGGCTTGGGAAAGGATGGCCAATGTCAAAGATGGAC					
Consensus	(241)	ACAGTGTTAATGGGCTTGGGAAAGGATGGCCAATGTCAAAGATGGAC					
		Section 7					
	(289)	289	300	310	320	336	
PL046 NS4B	(289)	ATCGGAGTTCCCTTCTCGCCATTGGATGCTACTCACAAGTAAACCC					
NGC NS4B	(265)	ATCGGAGTTCCCTTCTCGCCATTGGATGCTACTCACAAGTAAACCC					
Consensus	(289)	ATCGGAGTTCCCTTCTCGCCATTGGATGCTACTCACAAGTAAACCC					
		Section 8					
	(337)	337	350	360	370	384	
PL046 NS4B	(337)	ATAACCTCACAGCAGCTCTTCTCTTATGGTAGCACATTATGCATC					
NGC NS4B	(313)	ATAACTCTCACAGCAGCTCTTCTCTTATGGTAGCACATTATGCATC					
Consensus	(337)	ATAACCTCACAGCAGCTCTTCTCTTATGGTAGCACATTATGCATC					
		Section 9					
	(385)	385	390	400	410	420	432
PL046 NS4B	(385)	ATAGGGCCAGGACTCCAAGCAAAAGCAACTAGAGAAGCTCAGAAAAGA					
NGC NS4B	(361)	ATAGGGCCAGGACTCCAAGCAAAAGCAACTAGAGAAGCTCAGAAAAGA					
Consensus	(385)	ATAGGGCCAGGACTCCAAGCAAAAGCAACTAGAGAAGCTCAGAAAAGA					
		Section 10					
	(433)	433	440	450	460	470	480
PL046 NS4B	(433)	GCAGCAGCGGGCATCATGAAAAACCCAACCTGTCGATGGAATAACAGTA					
NGC NS4B	(409)	GCAGCAGCGGGCATCATGAAAAACCCAACCTGTCGATGGAATAACAGTG					
Consensus	(433)	GCAGCAGCGGGCATCATGAAAAACCCAACCTGTCGATGGAATAACAGT					
		Section 11					
	(481)	481	490	500	510	528	
PL046 NS4B	(481)	ATTGACCTAGATCCAATACCCTATGATCCAAAGTTTGAAAAGCAGTTG					
NGC NS4B	(457)	ATTGACCTAGATCCAATACCCTATGATCCAAAGTTTGAAAAGCAGTTG					
Consensus	(481)	ATTGACCTAGATCCAATACCCTATGATCCAAAGTTTGAAAAGCAGTTG					
		Section 12					
	(529)	529	540	550	560	576	
PL046 NS4B	(529)	GGACAAGTAATGCTCCTAGTCCTCTGTGTGACTCAAGTATTGATGATG					
NGC NS4B	(505)	GGACAAGTAATGCTCCTAGTCCTCTGTGTGACTCAAGTATTGATGATG					
Consensus	(529)	GGACAAGTAATGCTCCTAGTCCTCTGTGTGACTCAAGTATTGATGATG					

									Section 13	
	(577)	577	590	600	610	624				
PL046 NS4B	(577)	AGGACTACATGGGCTCTGTGTGAGGCTTTAACCTTAGCGA					T	CGGGCC	C	
NGC NS4B	(553)	AGGACTACATGGGCTCTGTGTGAGGCTTTAACCTTAGCGA					C	CGGGCC	T	
Consensus	(577)	AGGACTACATGGGCTCTGTGTGAGGCTTTAACCTTAGCGA						CGGGCC		
									Section 14	
	(625)	625	630	640	650	660	672			
PL046 NS4B	(625)	AT	TCCACATTGTGGGAAGGAAATCCAGGGAGGTTTGGAACTACC							
NGC NS4B	(601)	AT	TCCACATTGTGGGAAGGAAATCCAGGGAGGTTTGGAACTACC							
Consensus	(625)	AT	TCCACATTGTGGGAAGGAAATCCAGGGAGGTTTGGAACTACC							
									Section 15	
	(673)	673	680	690	700	710	720			
PL046 NS4B	(673)	ATTGCAGTGTCAATGGCTAACATTTTATAGAGGA					AGTTACTTGGC	T	GGA	
NGC NS4B	(649)	ATTGCAGTGTCAATGGCTAACATTTTATAGAGGA					AGTTACTTGGC	C	GGA	
Consensus	(673)	ATTGCAGTGTCAATGGCTAACATTTTATAGAGG					AGTTACTTGGC		GGA	
									Section 16	
	(721)	721	730	740	750	768				
PL046 NS4B	(721)	GCTGGACTTCTCTTTTC					T	ATCATGAAGAACACAACCAACACGAGAAGG		
NGC NS4B	(697)	GCTGGACTTCTCTTTTC					C	ATCATGAAGAACACAACCAACACGAGAAGG		
Consensus	(721)	GCTGGACTTCTCTTTTC						ATCATGAAGAACACAACCAACACGAGAAGG		
									Section 17	
	(769)	769	780	790	800	816				
PL046 NS4B	(769)	CTCGAGCTCATCGAGGGCAGATACCCATACGATGTTTCCTGACTATGCG								
NGC NS4B	(745)	-----								
Consensus	(769)	<i>XhoI</i>							HA	
									Section 18	
	(817)	817	830	840	850	864				
PL046 NS4B	(817)	GGCTATCCCTATGACGTCCCGGACTATGCAGGCTATCCATATGACGTT								
NGC NS4B	(745)	-----								
Consensus	(817)								HA	
									Section 19	
	(865)	865	870	880	890	900	912			
PL046 NS4B	(865)	CCAGATTACGCAGGAGCTCACCATCACCATCACCATGGC					TAGTGAT	TGC		
NGC NS4B	(745)	-----								
Consensus	(865)							His6		

Fig. 3.2 Alignment of nucleotide sequences at NS4B of NGC and PL046 strain

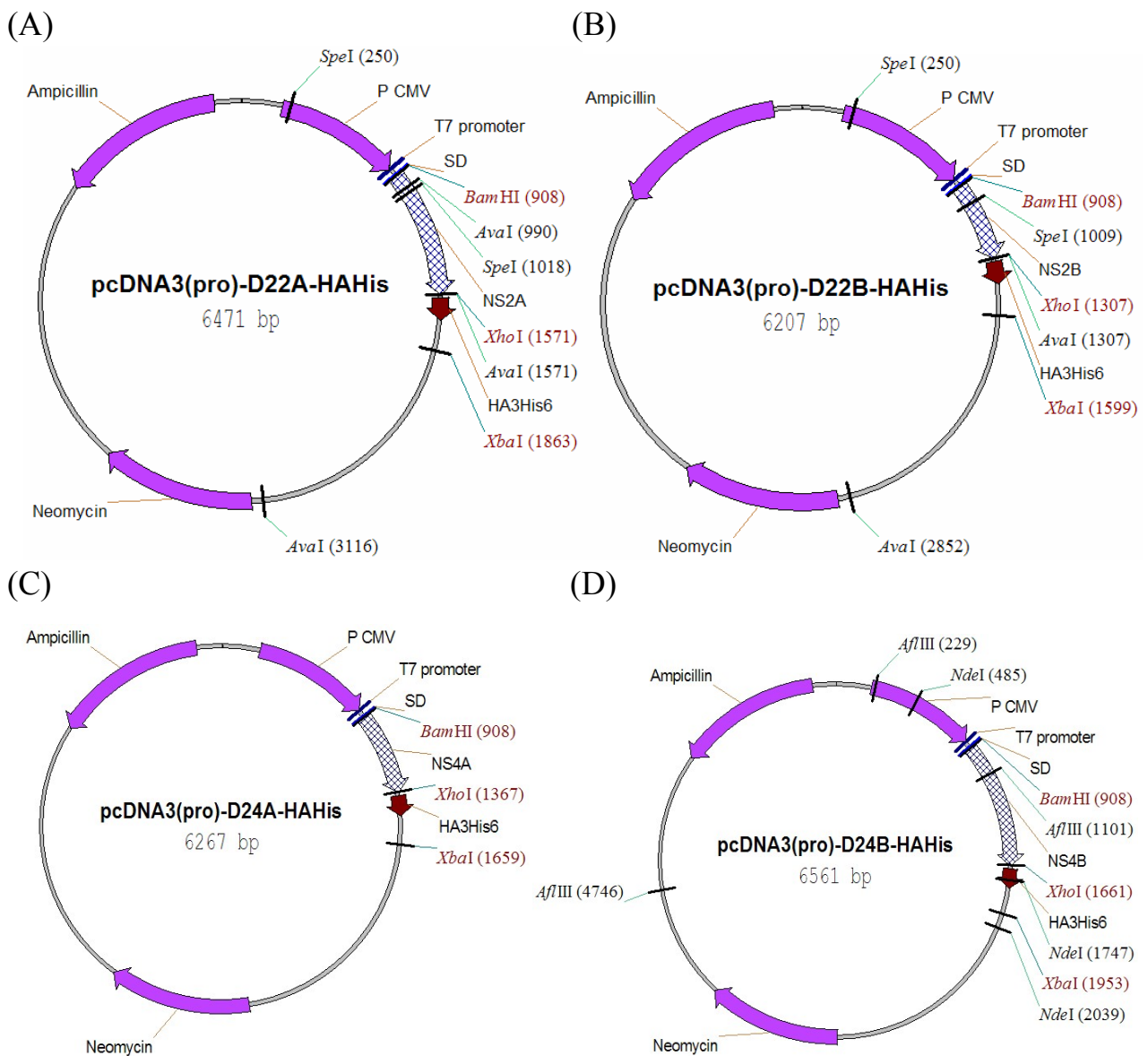


FIG. 3.3 Maps of **pcDNA3(pro)-D22A-HAHis**, **pcDNA3(pro)-D22B-HAHis**, **pcDNA3(pro)-D24A-HAHis**, and **pcDNA3(pro)-D24B-HAHis**. (A) Map of **pcDNA3(pro)-D22A-HAHis**. (B) Map of **pcDNA3(pro)-D22B-HAHis**. (C) Map of **pcDNA3(pro)-D24A-HAHis**. (D) Map of **pcDNA3(pro)-D24B-HAHis**.

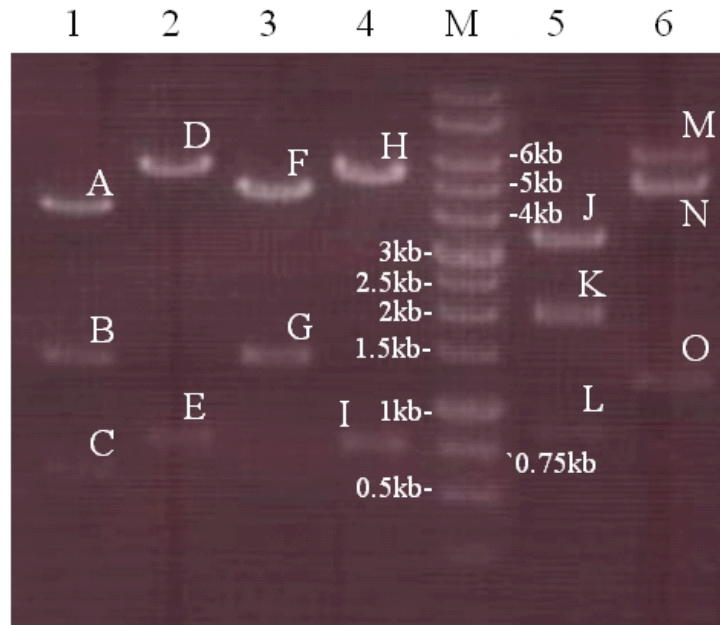


FIG. 3.4 Restriction enzyme digestion of pcDNA3(pro)-D22A-HAHis, pcDNA3(pro)-D22B-HAHis, and pcDNA3(pro)-D24B-HAHis. Lane 1, *Ava*I digested pcDNA3(pro)-D22A- HAHis; lane 2, *Spe*I digested pcDNA3(pro)-D22A- HAHis; lane 3, *Ava*I digested pcDNA3(pro)-D22B-HAHis; lane 4, *Spe*I digested pcDNA3(pro)-D22B-HAHis; lane 5, *Afl*III digested pcDNA3(pro)-D24B-HAHis; lane 6, *Nde*I digested pcDNA3(pro)-D24B-HAHis; M, marker. A, 4345bp; B, 1543bp; C, 581bp; D, 5701bp; E, 768bp; F, 4662bp; G, 1543bp; H, 5446bp; I, 759bp; J, 3645bp; K, 2044bp; L, 872bp; M, 6561bp; N, 5299bp; O, 1262bp.

		Section 1			
	(1)	1	10	20	39
pcDNA3(pro)-D24A-HAHis	(1)	GCTTGGTAAGGAGGTCGGATCCATGTC			
PL046 NS4A	(1)	-----ATGTCCTGACCCTGAA			
Consensus	(1)	SD BamHI ATGTCCTGACCCTGAA			
		Section 2			
	(40)	40	50	60	78
pcDNA3(pro)-D24A-HAHis	(40)	CCTAATCACAGAAATGGGAAGGCTTCCAAC			
PL046 NS4A	(18)	CCTAATCACAGAAATGGGAAGGCTTCCAAC			
Consensus	(40)	CCTAATCACAGAAATGGGAAGGCTTCCAAC			
		Section 3			
	(79)	79	90	100	117
pcDNA3(pro)-D24A-HAHis	(79)	TCAGAAGGCAAGAGACGCATTGGACAACCTGGCAGTGCT			
PL046 NS4A	(57)	TCAGAAGGCAAGAGACGCATTGGACAACCTGGCAGTGCT			
Consensus	(79)	TCAGAAGGCAAGAGACGCATTGGACAACCTGGCAGTGCT			
		Section 4			
	(118)	118	130	140	156
pcDNA3(pro)-D24A-HAHis	(118)	GCACACGGCTGAAGCAGGTGGAAGGGCGTACAATCATGC			
PL046 NS4A	(96)	GCACACGGCTGAAGCAGGTGGAAGGGCGTACAATCATGC			
Consensus	(118)	GCACACGGCTGAAGCAGGTGGAAGGGCGTACAATCATGC			
		Section 5			
	(157)	157	170	180	195
pcDNA3(pro)-D24A-HAHis	(157)	TCTCAGTGAAGTGGTGGAGACCCTGGAGACATTACTTTT			
PL046 NS4A	(135)	TCTCAGTGAAGTGGTGGAGACCCTGGAGACATTACTTTT			
Consensus	(157)	TCTCAGTGAAGTGGTGGAGACCCTGGAGACATTACTTTT			
		Section 6			
	(196)	196	210	220	234
pcDNA3(pro)-D24A-HAHis	(196)	ACTGACACTCCTGGCTACAGTCACGGGAGGAATCTTTTT			
PL046 NS4A	(174)	ACTGACACTCCTGGCTACAGTCACGGGAGGAATCTTTTT			
Consensus	(196)	ACTGACACTCCTGGCTACAGTCACGGGAGGAATCTTTTT			
		Section 7			
	(235)	235	240	250	273
pcDNA3(pro)-D24A-HAHis	(235)	GTTCTTGATGAGCGGAAGAGGTATAGGGAAGATGACCTT			
PL046 NS4A	(213)	GTTCTTGATGAGCGGAAGAGGTATAGGGAAGATGACCTT			
Consensus	(235)	GTTCTTGATGAGCGGAAGAGGTATAGGGAAGATGACCTT			
		Section 8			
	(274)	274	280	290	312
pcDNA3(pro)-D24A-HAHis	(274)	AGGAATGTGCTGCATAATCACGGCCAGTATTCTTCTATG			
PL046 NS4A	(252)	AGGAATGTGCTGCATAATCACGGCCAGTATTCTTCTATG			
Consensus	(274)	AGGAATGTGCTGCATAATCACGGCCAGTATTCTTCTATG			
		Section 9			
	(313)	313	320	330	351
pcDNA3(pro)-D24A-HAHis	(313)	GTACGCACAAATACAGCCACACTGGATAGCAGCTTCAAT			
PL046 NS4A	(291)	GTACGCACAAATACAGCCACACTGGATAGCAGCTTCAAT			
Consensus	(313)	GTACGCACAAATACAGCCACACTGGATAGCAGCTTCAAT			
		Section 10			
	(352)	352	360	370	390
pcDNA3(pro)-D24A-HAHis	(352)	AATATTGGAATTTTTTCTCATAGTTCTGCTTATTCCAGA			
PL046 NS4A	(330)	AATATTGGAATTTTTTCTCATAGTTCTGCTTATTCCAGA			
Consensus	(352)	AATATTGGAATTTTTTCTCATAGTTCTGCTTATTCCAGA			
		Section 11			
	(391)	391	400	410	429
pcDNA3(pro)-D24A-HAHis	(391)	ACCAGAAAAGCAGAGAACACCCCAAGATAACCAATTGAC			
PL046 NS4A	(369)	ACCAGAAAAGCAGAGAACACCCCAAGATAACCAATTGAC			
Consensus	(391)	ACCAGAAAAGCAGAGAACACCCCAAGATAACCAATTGAC			
		Section 12			
	(430)	430	440	450	468
pcDNA3(pro)-D24A-HAHis	(430)	CTACGTTGTCATAGCCATCCTCACAGTGGTGGCCGCAAC			
PL046 NS4A	(408)	CTACGTTGTCATAGCCATCCTCACAGTGGTGGCCGCAAC			
Consensus	(430)	CTACGTTGTCATAGCCATCCTCACAGTGGTGGCCGCAAC			

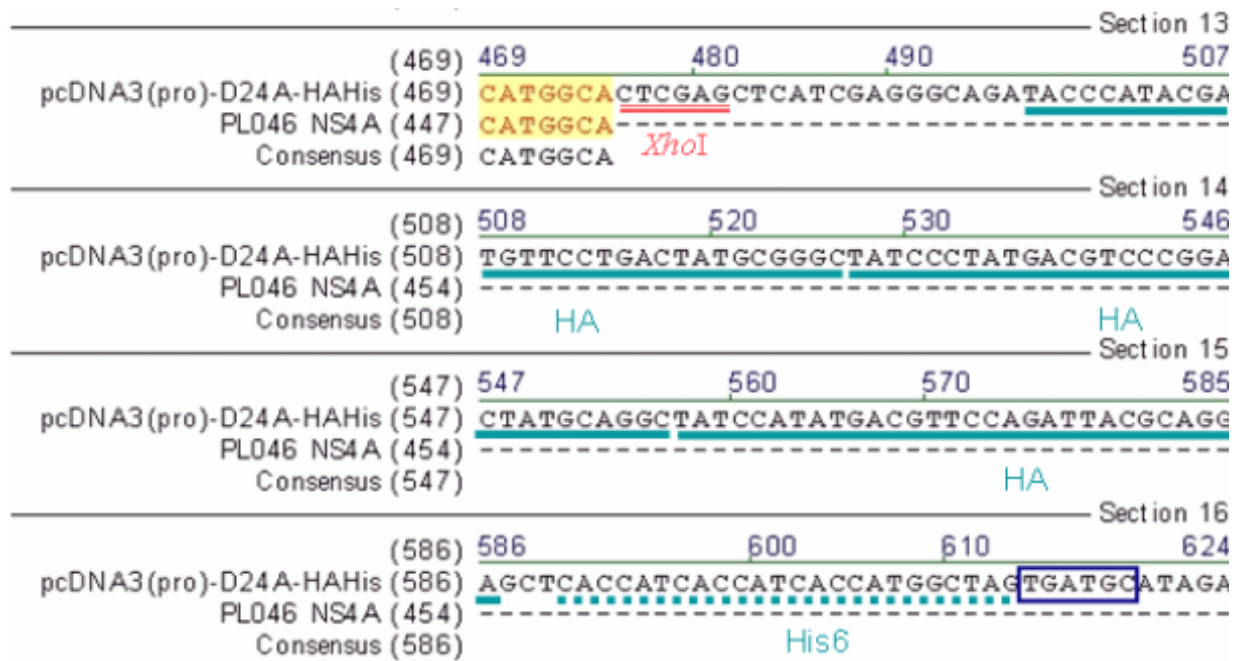


Fig. 3.5 Sequence analysis of pcDNA3(pro)-D24A-HAHis open reading frame.

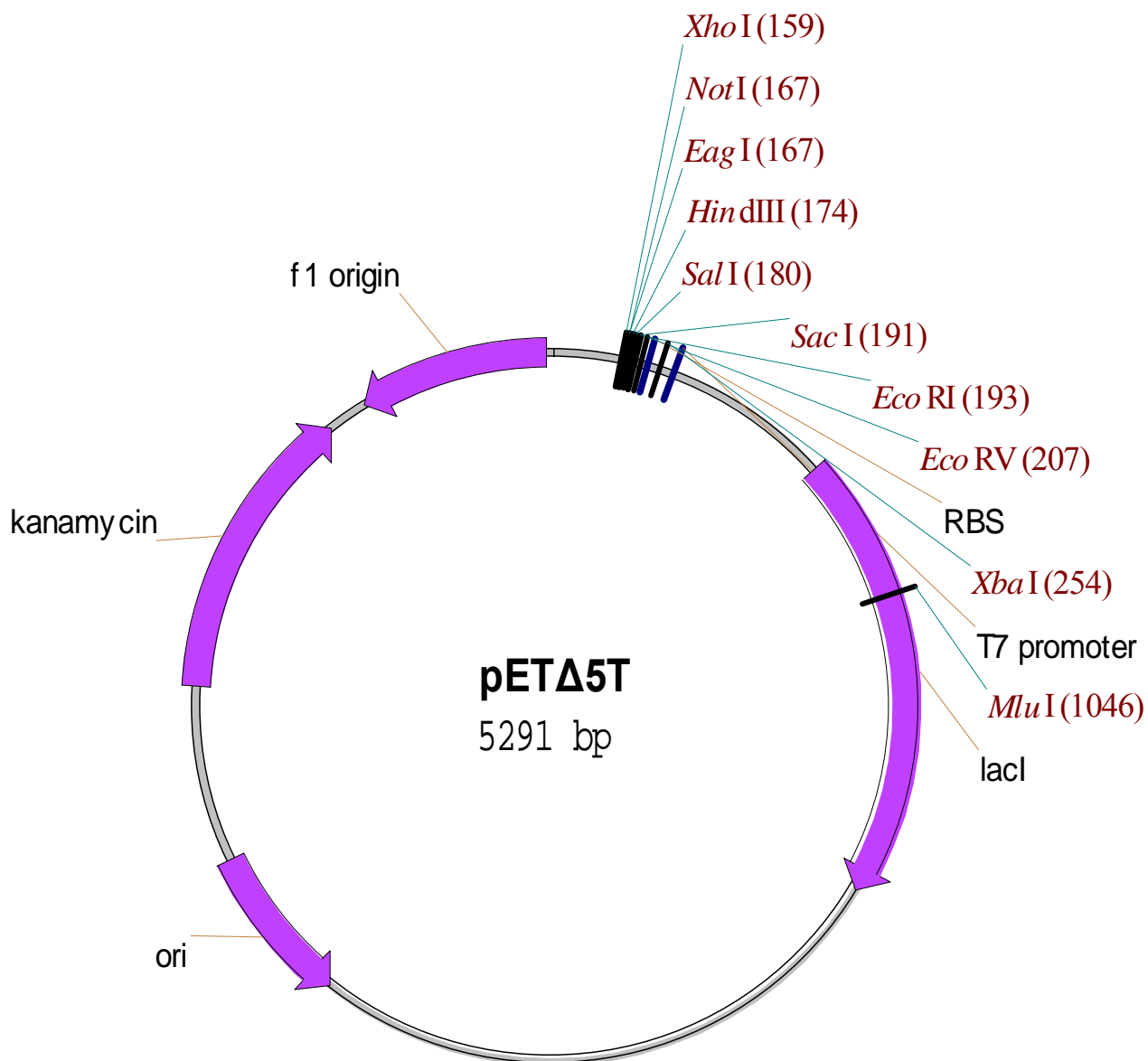


FIG. 3.6 Map of pETΔ5T. A pET-30a(+) plasmid was used as template for removal of 5' tag sequences in front of the multiple cloning site between *Nde*I and *Nco*I sites. The nucleotide numbers indicated are the numbers of new plasmid.

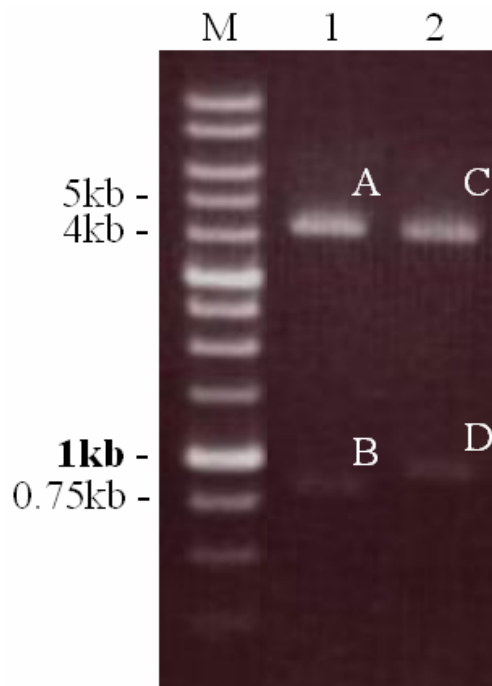


FIG. 3.7 Restriction enzyme digestion of pET Δ 5T. Lane 1, *Mlu*I and *Xho*I digested pET Δ 5T; lane 2, *Mlu*I and *Xho*I digested pET-30a(+); M, marker. A, 4403bp; B, 888bp; C, 4403bp; D, 1018bp.

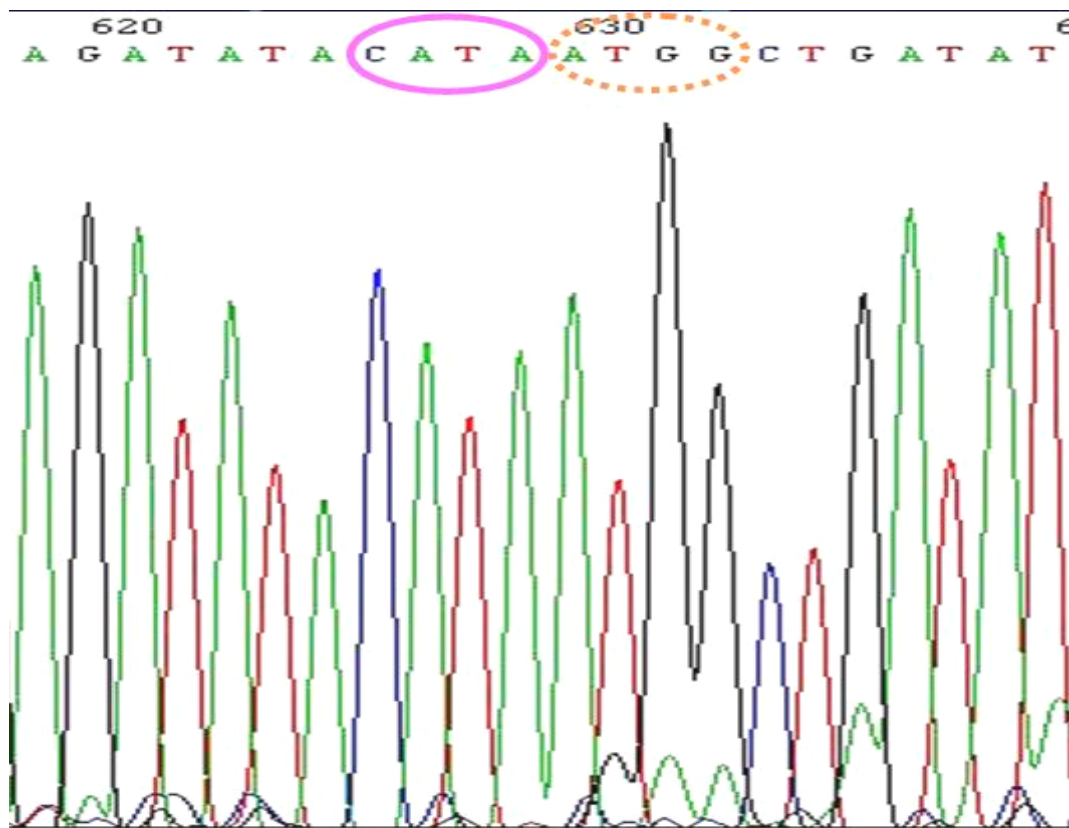
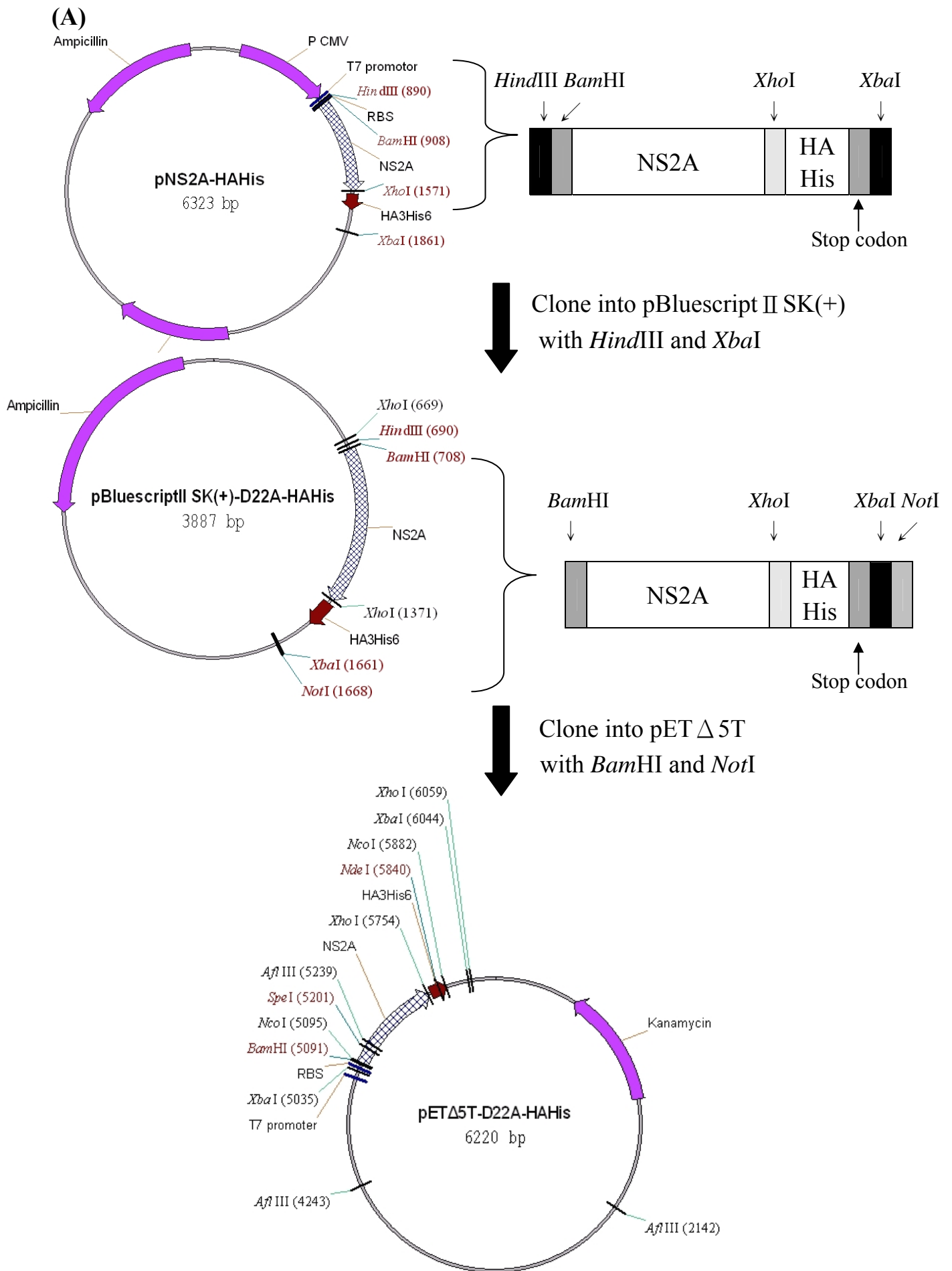
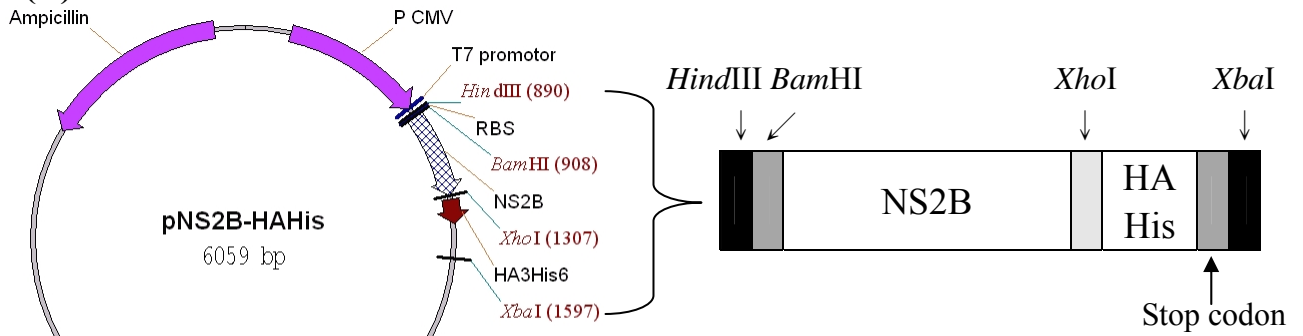


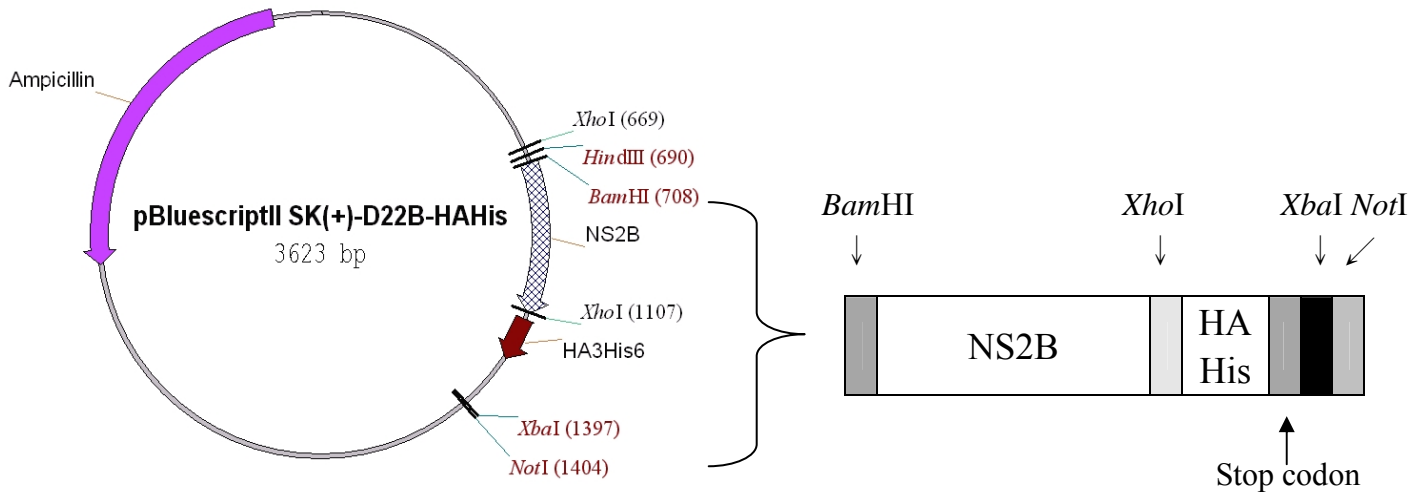
FIG. 3.8 Sequence of blunt-end ligated region on pET Δ 5T. CATA marked by solid circle is from *Nde*I digestion, and ATGG marked by dot-circle is from *Nco*I digestion.



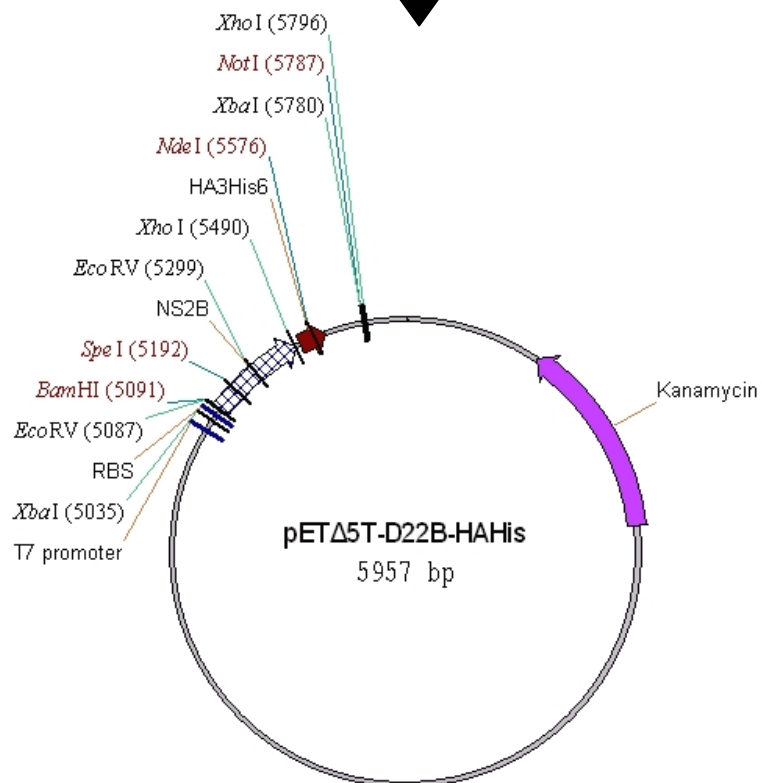
(B)



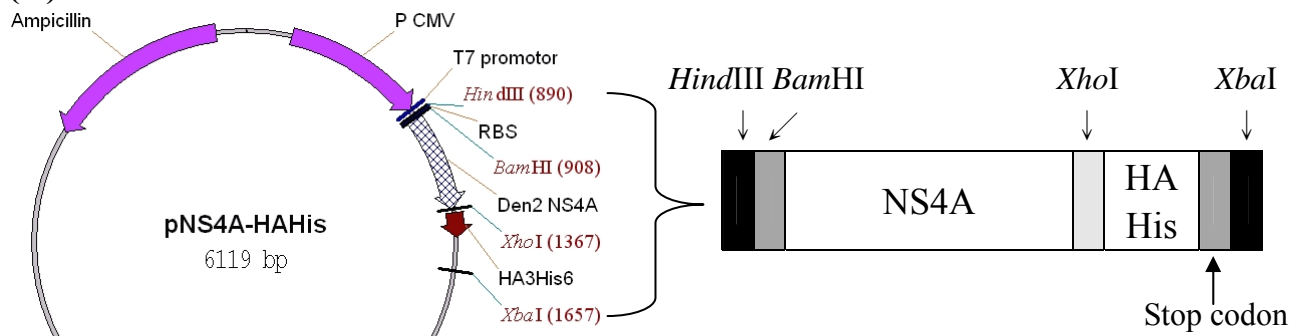
Clone into pBluescript II SK(+) with *Hind*III and *Xba*I



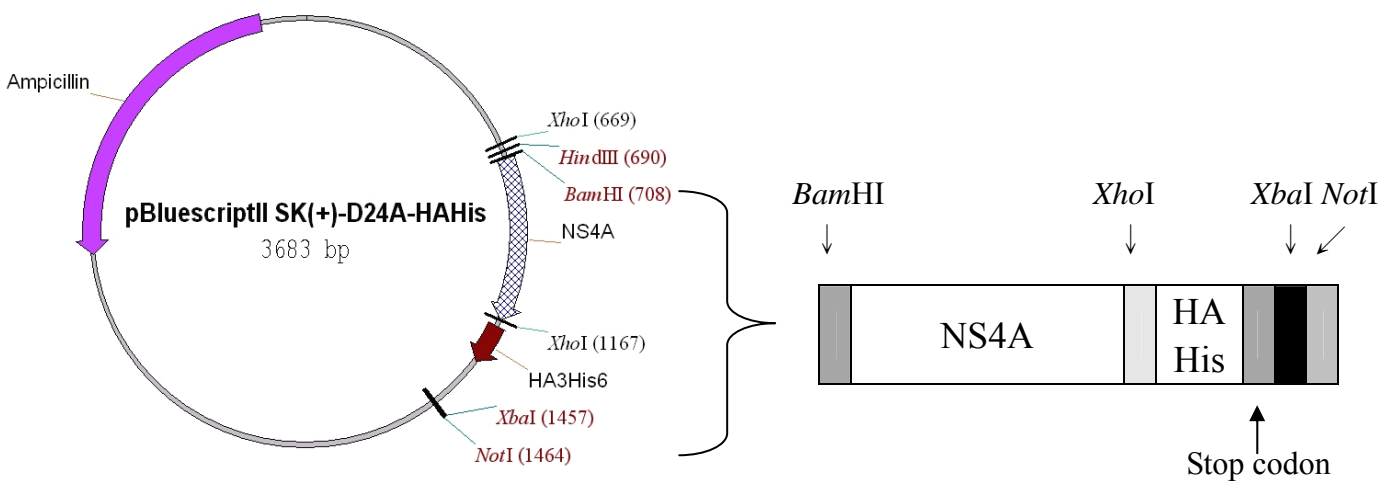
Clone into pET Δ 5T with *Bam*HI and *Not*I



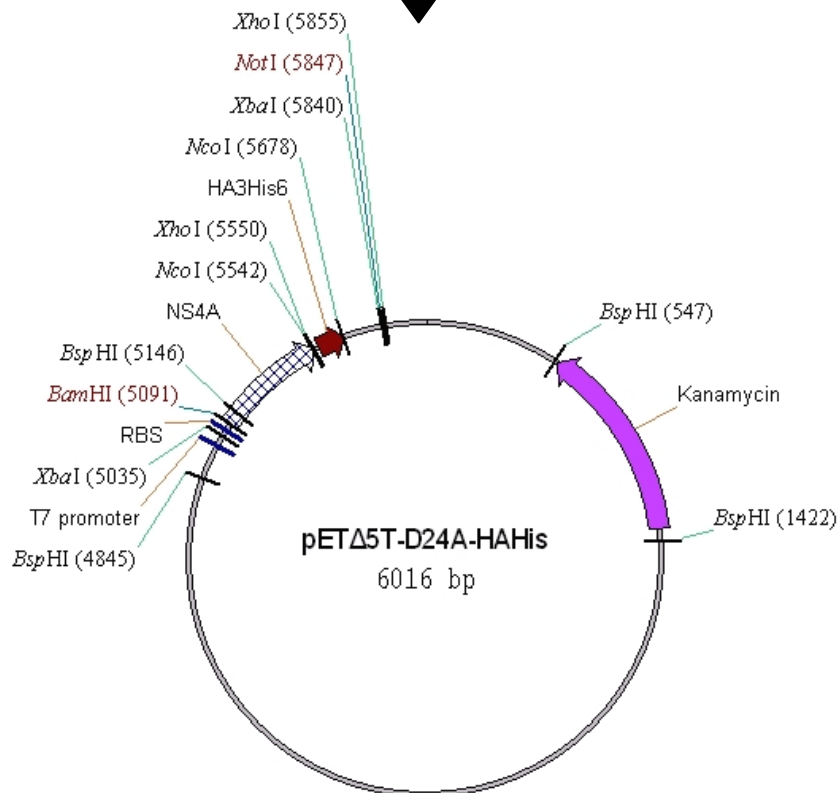
(C)

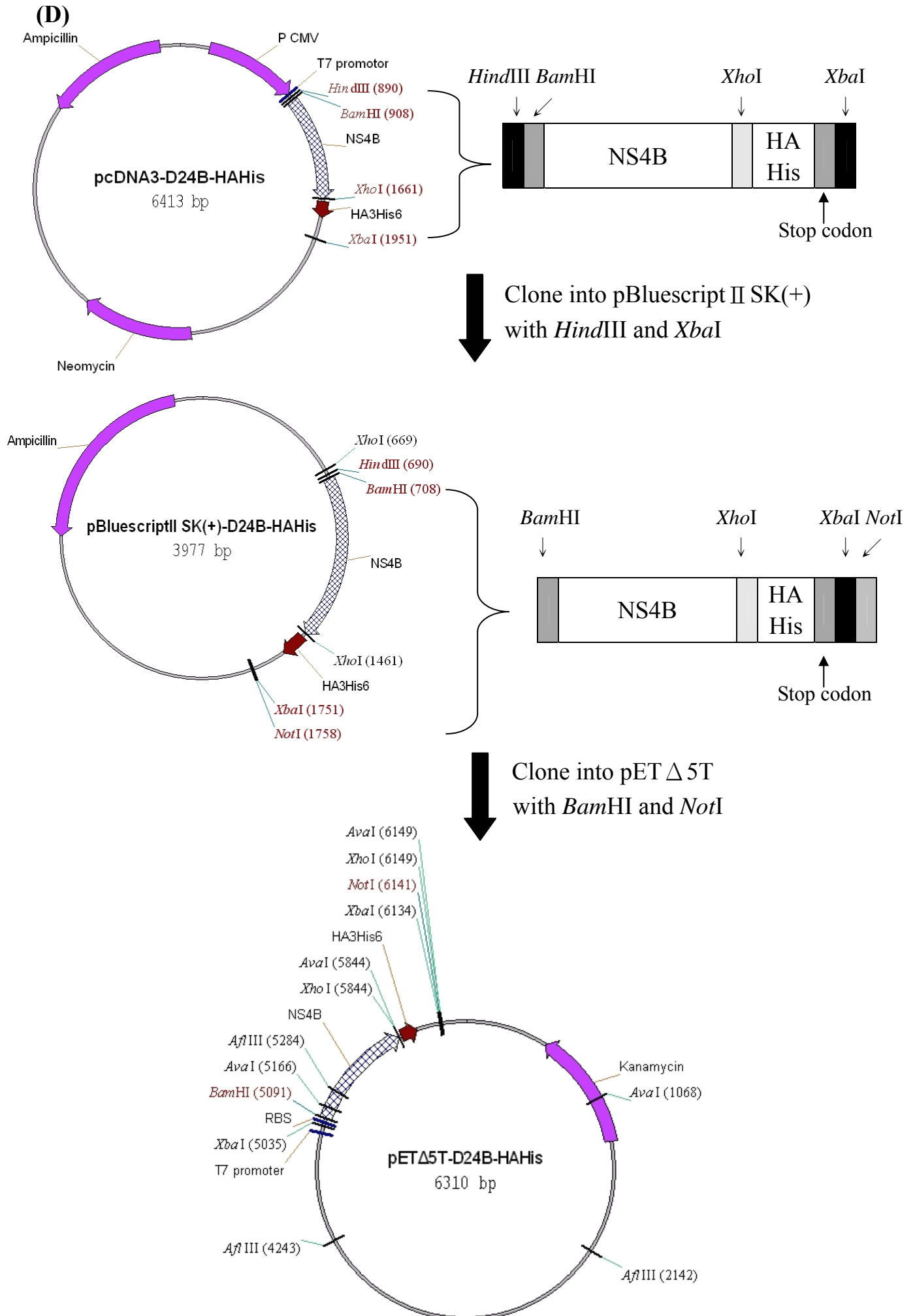


Clone into pBluescript II SK(+)
with *Hind*III and *Xba*I



Clone into pET Δ5T
with *Bam*HI and *Not*I





(E)

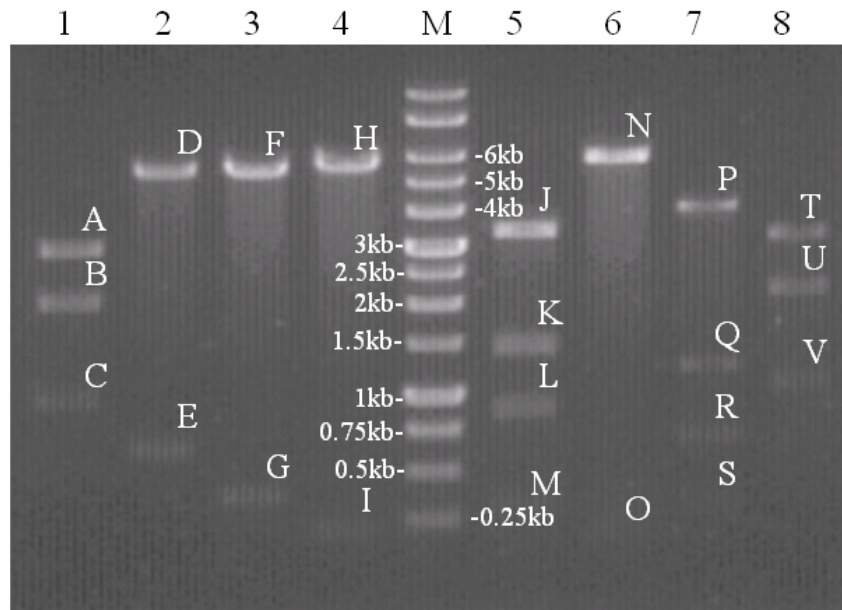


FIG. 3.9 Restriction enzyme digestion of pET Δ 5T-D22A-HAHis, pET Δ 5T-D22B-HAHis, pET Δ 5T-D24A-HAHis, and pET Δ 5T-D24B-HAHis. (A) Construction of pET Δ 5T-D22A-HAHis. (B) Construction of pET Δ 5T-D22B-HAHis. (C) Construction of pET Δ 5T-D24A-HAHis. (D) Construction of pET Δ 5T-D24B-HAHis. (E) Lane 1, *Afl*III digested pET Δ 5T-D22A-HAHis; lane 2, *Nde*I and *Spe*I digested pET Δ 5T-D22A-HAHis; lane 3, *Nde*I and *Spe*I digested pET Δ 5T-D22B-HAHis; lane 4, *Eco*RV digested pET Δ 5T-D22B-HAHis; lane 5, *Bsp*HI digested pET Δ 5T-D24A-HAHis; lane 6, *Nco*I digested pET Δ 5T-D24A-HAHis; lane 7, *Ava*I digested pET Δ 5T-D24B-HAHis; lane 8, *Afl*III digested pET Δ 5T-D24B-HAHis; M, marker. A, 3123bp; B, 2101bp; C, 996bp; D, 5581bp; E, 639bp; F, 5573bp; G, 384bp; H, 5745bp; I, 212bp; J, 3423bp; K, 1417bp; L, 875bp; M, 301bp; N, 5880bp; O, 136bp; P, 4098bp; Q, 1229bp; R, 678bp; S, 305bp; T, 3168bp; U, 2101bp; V, 1041bp.

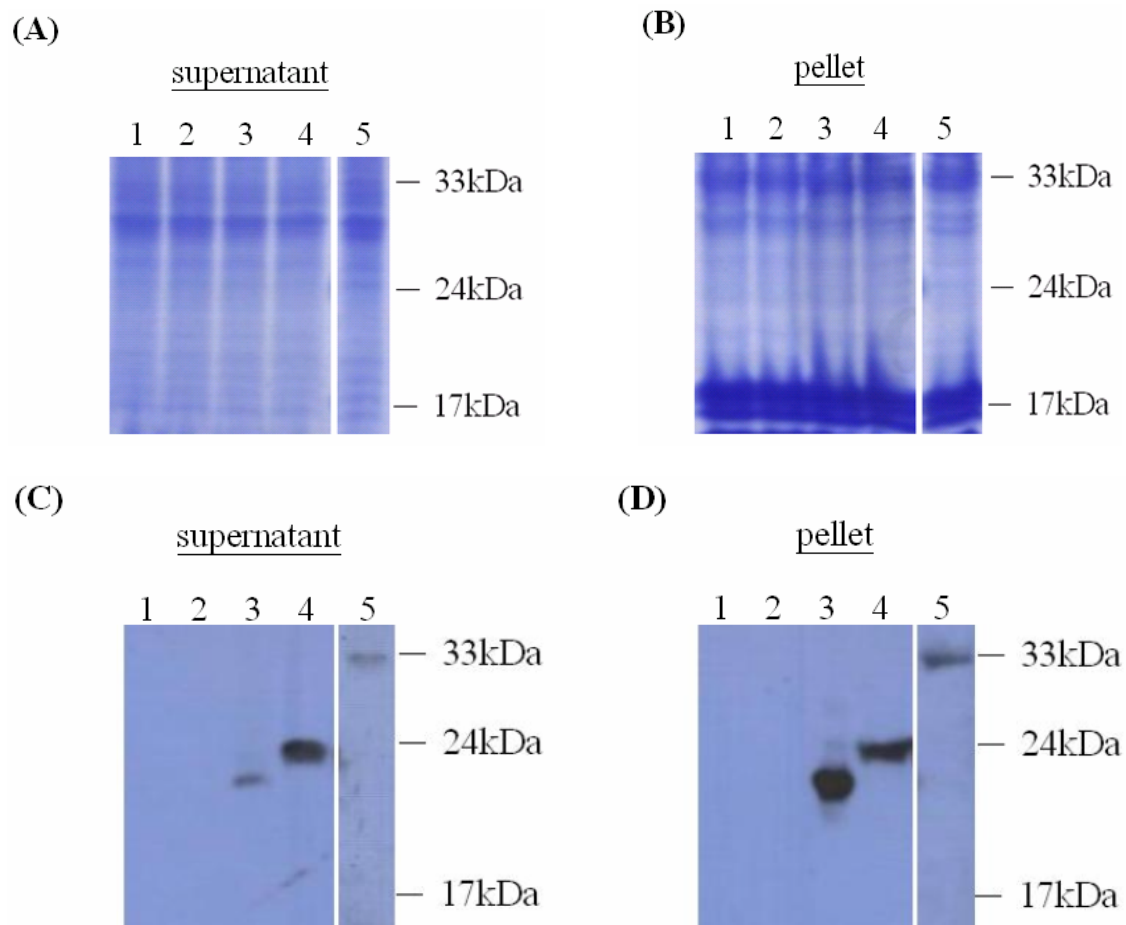


FIG. 3.10 Coomassie blue staining and Western blot of nonstructural proteins expressed in BHK-21. (A) Coomassie blue staining of the supernatant of BHK-21 transfected with different plasmid. (B) Coomassie blue staining of the pellet of BHK-21 transfected with different plasmid. (C) Western analysis with anti-HA antibody against supernatant of BHK-21 transfected with different plasmid. (D) Western analysis with anti-HA antibody against pellet of BHK-21 transfected with different plasmid. Lane 1, pcDNA3 (negative control); lane 2, pNS2A-HAHis; lane 3, pNS2B-HAHis; lane 4, pNS4A-HAHis; lane 5, pcDNA3-D24B-HAHis.

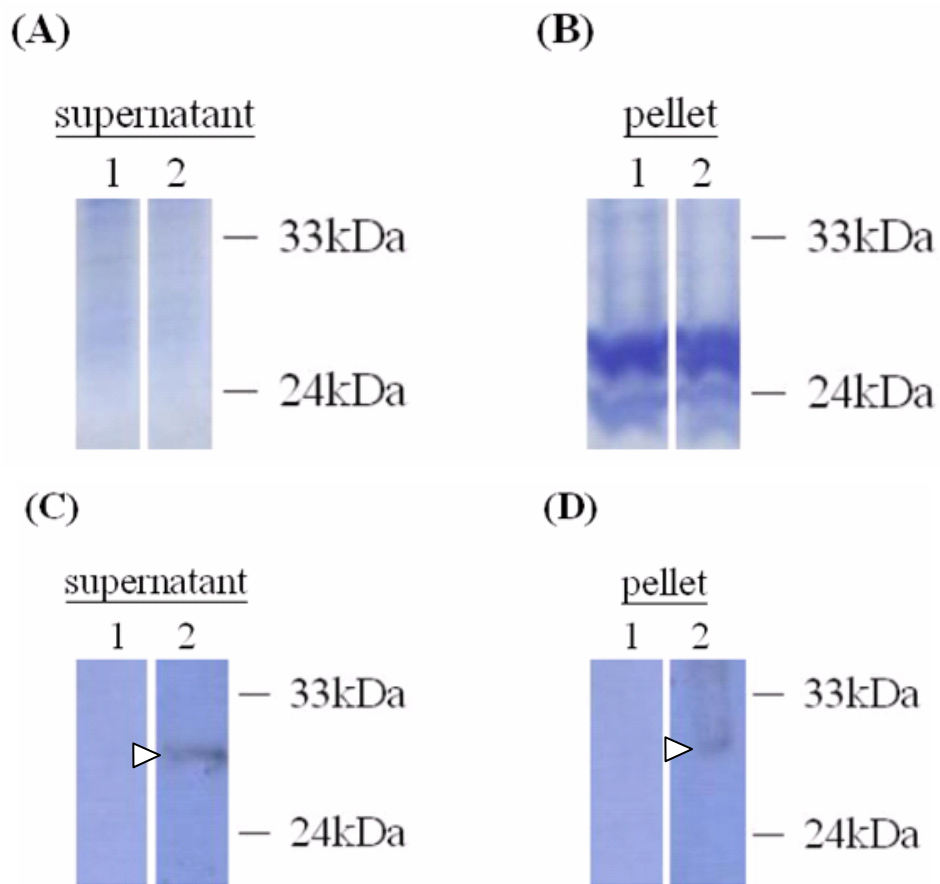


FIG. 3.11 Coomassie blue staining and Western blot of NS2A expressed in 293T. (A) Coomassie blue staining of the supernatant of BHK-21 transfected with different plasmid. (B) Coomassie blue staining of the pellet of BHK-21 transfected with different plasmid. (C) Western analysis with anti-HA antibody against supernatant of BHK-21 transfected with different plasmid. (D) Western analysis with anti-HA antibody against pellet of BHK-21 transfected with different plasmid. Lane 1, pcDNA3 (negative control); lane 2, pNS2A-HAHis.

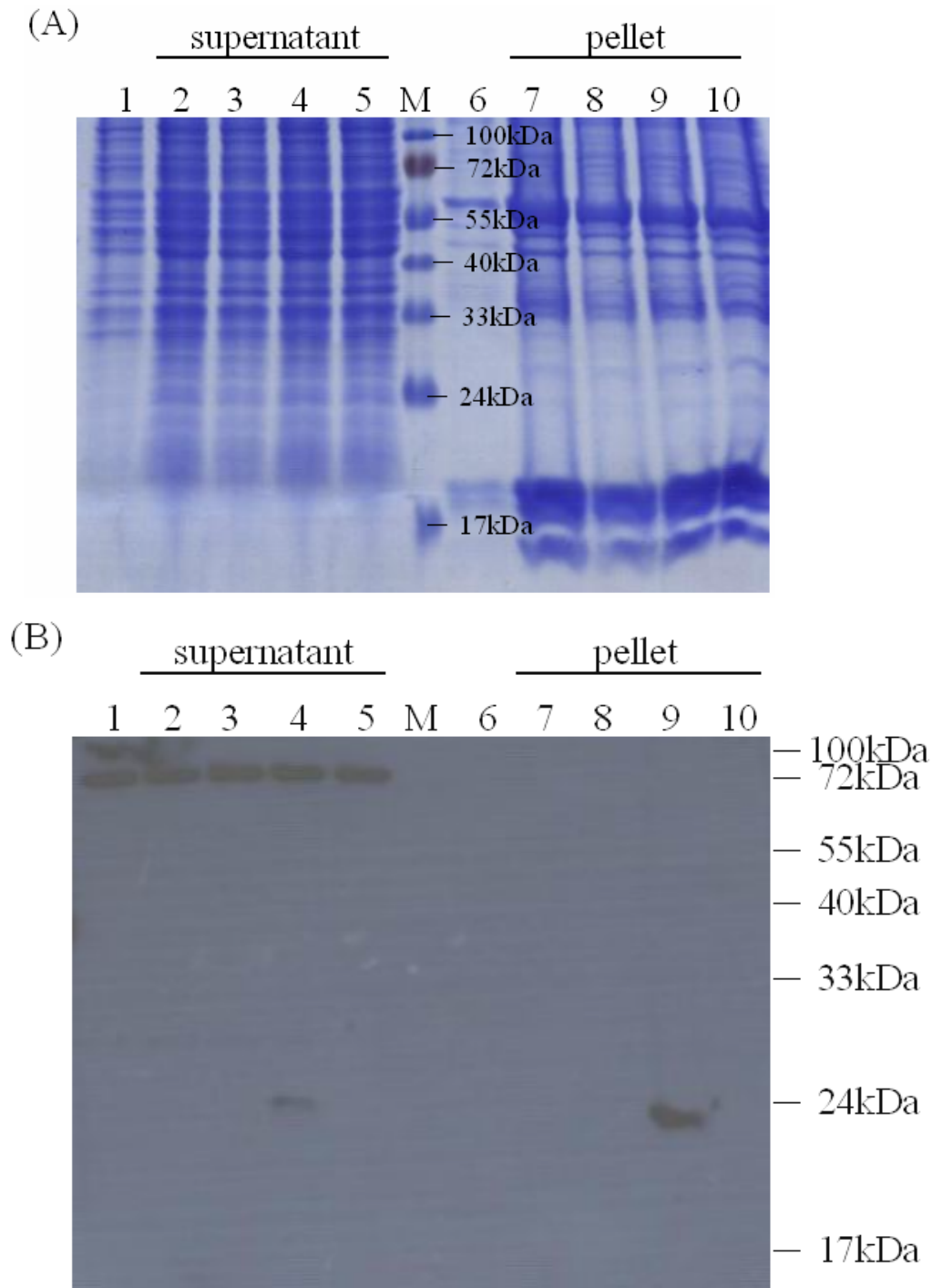


FIG. 3.12 BHK-21 expressed nonstructural proteins analyzed by coomassie blue staining and Western blot. (A) Coomassie blue staining of the supernatant and pellet of BHK-21 transfected with different plasmids. (B) Western analysis with anti-HA antibody against supernatant and pellet of BHK-21 transfected with different plasmids. Lane 1 and 6, pcDNA3 (negative control); lane 2 and 7, pcDNA3(pro)-2A-HAHis; lane 3 and 8, pcDNA3(pro)-2B-HAHis; lane 4 and 9, pcDNA3(pro)-4A-HAHis; lane 5 and 10, pcDNA3(pro)-4B-HAHis; M, marker.