

NITE/NIID Protocol for Sequencing Influenza A (H1N1) SWL Viral Genome Segments version 1.2 (30 May 2009)

National Institute of Technology and Evaluation (NITE) and National Institute of Infectious Diseases (NIID) of Japan provide the protocol for sequencing viral RNA segments of Influenza A (H1N1) of swine lineage. This protocol is optimized for sequencing human isolates, and may not be appropriate for sequencing swine isolates.

Protocol Outline

- RNA extracted from viral culture (either MDCK cells or egg).
- Reverse transcription performed for all 8 segments with 12-mer universal primer.
- Either full length DNA (for NA, MP and NS segments) or half-length DNAs (for HA, NP, PA, PB1 and PB2 segments) amplified with specific PCR primers.
- Product size verified on agarose gel (optional).
- Sequenced on ABI 3730XL sequencer with a set of 7-16 primers for each amplicon.

- * Details will be updated, if necessary, and made available on the following URL.
http://www.bio.nite.go.jp/ngac/flu_sequence_protocol.pdf
- * Send inquiries to Dr. Nobuyuki Fujita (fujita-nobuyuki@nite.go.jp)

1. Reverse transcription

Materials

SuperScript™ III Reverse Transcriptase (Invitrogen Corporation Cat. No. 18080-044)

RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen Corporation Cat. No. 10777-019)

dNTP mix (10mM) (Invitrogen Corporation Cat. No. 18427-013)

Procedure

1) RNA	10pg-5ug
RT primer (AGCAAAAGCAGG)	10 pmol
<u>dNTP(10mM)</u>	<u>1 ul</u>
Sterile distilled water	up to 13 ul

65°C for 5min.

on ice for at least 1min

2) 5×First-Strand Buffer	4 ul
0.1M DTT	1 ul
RNase OUT™	
Recombinant Ribonuclease Inhibitor (40U/ul)	1 ul
<u>Super Script™ III RT(200U/ul)</u>	<u>1 ul</u>
total	7 ul

mix 1) and 2)

50°C for 120min

70°C for 15min

2. PCR

Materials

KOD Dash DNA polymerase
(TOYOBO Code No. LDP-101)

Procedure

KOD Dash	0.2 uL
10xBuffer	1.0 uL
dNTPs (2mM each)	1.0 uL
Primer1	4.0 pmol
Primer2	4.0 pmol
RT product	1.0 uL
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Sterile distilled water	up to 10.0 uL

Step1	98°C for 2 min
Step2	98°C for 30 sec
Step3	45°C for 1 min
Step4	72°C for 2 min
Step5	Go to Step 2 29 X
Step6	72°C for 10 min
Step7	4°C hold

3. Sequencing

Materials

BigDye™ Terminator v3.1 Cycle Sequencing Kit
(Applied Biosystems Part Number 4337455)

BigDye™ Terminator v1.1/v3.1 Sequencing Buffer (5x)
(Applied Biosystems Part Number 4336697)

Procedure

purified PCR product	5-20 ng
BigDye™ Terminator v1.1/v3.1 Sequencing Buffer (5x)	1.88 ul
Primer	3.20 pmol
BigDye™ Terminator v3.1 Cycle Sequencing Kit	0.25 ul
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Sterile distilled water	up to 10.00 ul

Step1	96°C for 10 sec.
Step2	50°C for 5 sec
Step3	60°C for 2 min.
Step4	Go to Step 1 39 X
Step5	4°C hold

PCR primers

PCR products	Primer 1(F)	sequence	Primer 2(R)	sequence
PB2-1	1F *	CTCGAGCAAAAGCAGGTCAA	3R	CACATTGTCGATGGATTCAATTC
PB2-2	2F	GATTGATCCAGTTGATAGTAAGC	1R *	TAGTAGAAACAAGGTCGTT
PB1-1	4F *	AGCAAAAGCAGGCAAACCAT	6R	TTGGTGCATTCACTATGAGAGC
PB1-2	5F	CGATGATTACATATATCACCAGA	4R *	AGTAGAAACAAGGCATTT
PA-1	7F *	AGCAAAAGCAGGTACTIONGAT	10R	GGACCCAGCTTGCTAGAGAT
PA-2	8F	GAGCCTAACATAGTCAAACCACA	8R	TTTGGACAGTATGGATAGCAAATAG
HA-1	9F *	ATACGACTAGCAAAAGCAGGGG	13R	TGCTCATTTTTGATGGTGATAACCG
HA-2	11F	ATCCGATCACAATTGGAAAATGTCC	11R *	GTGTCAGTAGAAACAAGGGTGTTT
NP-1	13F	AATGAGTGACATCGAAGCCA	15R	ATCCCGACCAGTGAGTACC
NP-2	14F	AATGACCGAAATTTCTGGAG	14R *	AGTAGAAACAAGGGTATTTTTTC
NA-1	16F *	AGCAAAAGCAGGAGT	16R *	AGTAGAAACAAGGAG
MP-1	18F *	AGCAAAAGCAGGTAG	17R *	AGTAGMAACAAGGTAGT
NS-1	20F *	AGCAAAAGCAGGGTGACAAAGACA	18R	GTAGAAACAAGGGTGTTTTTTATCA

* Based on those listed in the CDC sequencing protocol
 (http://www.who.int/csr/resources/publications/swineflu/sequencing_primers/en/index.html)

Sequencing primers

PCR products	primer	sequence
PB2-1	seq1	GGATGATGGCAATGAGAT
	seq2	GGTTGAAACATGGTACCT
	seq3	TGAAGTGTGGCACTTAACC
	seq4	AGACATATGCAAGGCAGC
	seq5	TCATACCCTTCATGTACTC
	seq6	CTCCTGGAGGTACATCT
	seq7	GTATCAACTCTCCTCCTT
	seq8	GAGGTGATACCATCACTC
	seq9	CTGAGAGATCTAATGTGCGAGTC
	seq10	TTGCCCTATTGACAAAGTTCAGA
PB2-2	seq11	GCATGATCAAGGCAGTTA
	seq12	AGATCAAAGAGGGAACG
	seq13	GACATTTGACACTGTCCA
	seq14	ACAGTTCTTGAAAAGGATG
	seq15	GCAAGATTGCTCAGTTCA
	seq16	ATCCTACTCTGTTCTGGTG
	seq17	CCACATCATTGATGACGA
	seq18	CATCACATTGTCGATGGA
	seq19	GATTGATCCAGTTGATAGTAAGC
	seq20	ATTGATGGCCATCCGAATCTTT
PB1-1	seq21	AGGATACCCATGGACAC
	seq22	ACATTAACAGAAATCAAC
	seq23	GATGCAGAGAGAGGCAAGT
	seq24	TGATTACATATATACCAG
	seq25	CCAAGATTCAGTATCGAG
	seq26	TGAGAACATTATGGGTGC
	seq27	GAAACCTCTAATCTGCATC
	seq28	ATTGATTCCATTACATCC
	seq29	TGTTGAACAACCTCCATT
	seq30	TGAATGGATGTCAATCCGACTC
PB1-2	seq31	GTCCAAGATTCAATATCGAGACT
	seq32	CGATACTGAATCTTGACA
	seq33	AGCTGACATGAGTATTGGA
	seq34	TCACATTCTGAAGTCTGC
	seq35	TCGTTCTATTCTCAACAC
	seq36	ACCATGCTAGAAATTTCCA
	seq37	TCATCATCCATTAGCTCC
	seq38	CCAATACTCATGTACGCT
	seq39	ATACCAGCAGAAATGCTAGCAAG
	seq40	GGTGAACAGATCTTCATGATCT
PA-1	seq41	ATGATCGTCGAGCTTG
	seq42	AGCCTTGAAAACCTTTAGA

PCR products	primer	sequence
PA-1	seq43	TACTGTTTAAGGTCTCCAA
	seq44	TAATTTTCCAGAGCATCCAT
	seq45	ACTGGCCATTTCTTGCT
	seq46	GGTCTCTTCTTCAATTAT
	seq47	GATCCAAAATGGAAGACTTTGTG
	seq48	TGGGCTCTGGCTCATCACT
	seq49	ATTACCTCATGGCTTGG
	seq50	ATTGAACATATCGCAAG
	seq51	ACTGACCCGAGACTGGA
	seq52	AGTGGAGGAAGGCTCTA
PA-2	seq53	AGCATTGGCCACAACACTACT
	seq54	CATGTTTCCGATTTGTT
	seq55	GACCTTCTTTTATAATGA
	seq56	ATGAGAAAAGGCATAAATCCCA
	seq57	TTTGACAGTATGGATAGCAAATAG
	seq58	ATGAAGGCAATACTAGTAGTT
	seq59	TGATAACCGTACCATCCATC
HA-1	seq60	TCCACAGCAAGCTCATGGTCC
	seq61	TACAGCAAGAAGTTCAAGCC
	seq62	ACCCAAAGCTCAGCAAATCC
	seq63	ATACACCAGTCCACGATTGC
	seq64	CAGCATTTCTTTCCATTGCG
	seq65	CTCTTAGCTCCTCATAATCG
	seq66	CAGCACTAGTAGATGGATGG
	seq67	GGTACCCTCTTAGTTGC
	seq68	TAAAAAGCACAAAATTGAGACTGGC
	seq69	TTAAATACATATTCTACACTGTAGAG
HA-2	seq70	CGAACTGTTGGTCTATTGG
	seq71	ATGAATACACAGTTCACAGC
	seq72	CTACCCAAAATACTCAGAGG
	seq73	TTCCATGCACGTGTTATCGC
	seq74	GTCCAAATGTCCAGGAAACC
	seq75	WCAGAAATCAGAGCATC
	seq76	AGCAAACAATGGCGGAA
NP-1	seq77	AGCCCATACACACAAG
	seq78	TGGGAAGTGTGAACTT
	seq79	ATTAGTCGTCCATCATAATC
	seq80	AATGAGTGACATCGAAGCCA
	seq81	GTCAATGCCACTTGTACT
NP-2	seq82	TTGCTTATGAAAAGATGT
	seq83	CACAAGAGTCAATTGGT
	seq84	GTTATGGCAGCATTACG

PCR products	primer	sequence
NP-2	seq85	CCCTTCATTACTCATGTCT
	seq86	CTTATGGCCCAGTATCT
	seq87	GGTGAAAATGGACGAAGGA
	seq88	GAAACAAGGGTATTTTTCTCAACT
	seq89	CAATTGGAATGGCTAAC
	seq90	TGGATGGCTATATACAG
	seq91	ATCGAACCCATATGAGC
	seq92	GAACACAAGAGTCTGAATG
NA-1	seq93	GATAACTGGCATGGCTC
	seq94	GTTTTGAGATGATTTGG
	seq95	TTCTGGTGTGAACATAATC
	seq96	CAAGACCAACCCACAG
	seq97	GCTTTATTGAGAAGTTATTG
	seq98	TGCATATGTATCCTATCTG
	seq99	GATCTTGTATGAGGCTCTG
	seq100	AATTGATGCCATCATGAC
	seq101	GAATGGTCCCTTATGAC
	seq102	AATCTGATTTTATTGTTCC
MP-1	seq103	ATGAATCCAAACCAAAGATAATAAC
	seq104	TTGTCAATGGTAAATGGCAAC
	seq105	CCCTCAAAGCCGAGAT
	seq106	CCTAAATGGGAATGGG
	seq107	GAACAGATTGCTGATTC
	seq108	GGGACTCATCCTAGCTC
	seq109	TTATCGTCGCTTAAATAC
	seq110	TCTGCTGTTCTCTGTTG
	seq111	CCATTGCTTCTGGTAG
	seq112	CAGCACCATTCTGTTTTT
NS-1	seq113	CGTTATTTCTCTTTTGAGC
	seq114	AGCCATTCATGAGAGC
	seq115	GAGTCTTCTAACCGAGGTCGAAAC
	seq116	CTCTAGCTCTATGTTGACAAAATGACC
	seq117	CACCATGTCAAGCTTTC
	seq118	GGTTCATGCTCATGC
	seq119	CGCTTGAGAAACTGT
	seq120	AGAAAGCTCTTATCTCTTG
	seq121	TAAGTATGCTGGAAGAG
	seq122	TCCATTCCACGATTTG
	seq123	ACATAATGGACTCCAACACC
	seq124	TCATTAATAAGCTGAAACGAGAA

Version history

version 1	27 May 2009	unpublished maintenance version
version 1.1	29 May 2009	first public version
version 1.2	30 May 2009	error correction in sequencing procedure