Pre-pandemic Influenza A (H1N1) y (H3N2) virus genomic characterization.

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This protocol describes the PCR components and conditions used for amplifying the 8 genomic segments of Influenza virus type A subtypes (H1N1) y (H3N2). This method relies on a nested PCR approach in which the product of an initial PCR is used as a template for a second PCR using different (nested) oligonucleotides. This approach is more sensitive than single-pass PCRs and as such requires greater care and discipline to avoid contamination throughout setup. The nested strategy allows for better success at sequencing from samples having either low quality, concentration of template or viral load (in the case of RT-PCR).

Oligonucleotides

Nombre	Spec	PCR	Sequnce	bp	%GC	Tm	Position		Size	Ref
UniFlu-RT	All	1	5´-Agg-AAA-AgC-Agg-3´	12	50	38.1	-	-	-	1
PB2-F	PB2	1	5´-TAT-Tgg-TCT-CAg-ggA-gCg-AAAg-CAg-gTC-3´	28	53.6	63.3	10	24	2342	1
PB2-R	PB2		5´-ATA-Tgg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gTC-gTT-T-3´	34	35.3	58.7	2358	2376		1
SwPB2-F	PB2	2	5´-ATg-gAg-AgA-ATA-AAA-gAA-C-3´	19	31.6	44.1	38	56	833	3
PB2-R1	PB2		5´-gCT-AgT-ggA-TCT-gCY-g-3´	16	59.4	50.8	855	871		2
PB2-F2	PB2	2	5´-RAT-gTA-CAC-TCC-Agg-T-3´	16	46.9	46.7	764	779	942	2
PB2-R2	PB2		5´-RAT-TTC-TgA-TgA-TCC-A-3´	16	34.4	41.3	1692	1706		2
PB2-F3	PB2	2	5´-ggA-RgT-MAg-TgA-AAC-AC-3´	17	47.1	47.2	1585	1602	791	2
PB2-R	PB2		5´-ATA-Tgg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gTC-gTT-T-3´	34	35.3	58.7	2358	2376		2
PB1-F	PB1	1	5´-TAT-TCg-TCT-CAg-ggA-gCg-AAA-gCA-ggC-A-3´	28	53.6	64.7	8	21	2342	1
PB1-R	PB1		5´-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gCA-TTT-3´	33	33.3	57.6	2332	2350		1
SwPB1-F1	PB1	2	5´-ATg-gAT-gTC-AAT-CCg-ACT-C-3´	19	47.4	51.8	32	50	833	2
PB1-R1	PB1		5´-CAT-TAY-CYC-CAA-CYg-3´	15	50	44.2	847	865		2
PB1-F2	PB1	2	5´-CAC-RAT-gAC-CAA-AgA-Y-3´	16	43.8	45.1	706	723	849	2
PB1-R2	PB1		5´-CTC-CAT-gCT-RAA-ATT-Rg-3´	14	41.2	44.7	1540	1555		2
PB1-F3	PB1	2	5´-gAg-CAA-AAA-gAA-gTC-Y-3´	16	40.6	43.7	1463	1477	887	2
PB1-R	PB1		5´-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gCA-TTT-3´	33	33.3	57.6	2332	2350		2
PA-F	PA	1	5´-TAT-TCg-TCT-CAg-ggA-gCg-AAA-gCA-ggT-AC-3´	29	51.7	62.7	5	19	2233	1
PA-R	PA		5´-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gTA-CTT-3´	33	33.3	56.8	2222	2238		1
SwPA-F1	PA	2	5´-ggA-AgA-CTT-TgT-gCg-AC-3´	17	52.9	50.9	29	47	856	3
PA-R1	PA		5´-CCA-TCA-gSA-ggA-ATT-TKg-3´	18	47.2	49.5	868	885		2
PA-F2	PA	2	5´-gCT-RCA-TTg-Agg-gCA-Ag-3´	17	55.9	52.5	747	763	897	2
PA-R2	PA		5´-TCC-CAT-TTR-TgT-ggY-TC-3´	17	47.1	49.2	1626	1644		2
PA-F3	PA	2	5´-AAg-Agg-gAA-ggM-gAA-A-3´	16	46.9	48.1	1497	1514	741	2
PA-R	PA		5´-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gTA-CTT-3´	33	33.3	56.8	2222	2238		1

HA-F	HA	1	5′-TAT-TCg-TCT-CAg-ggA-gCA-AAA-gCA-ggg-g-3′	28	53.6	63.6	8	22	1782	1
NS-R	HA/NS		5′-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-ggT-gTT-TT-3′	35	34.3	58.9	1783	1790		1
HA-F	HA	2	5′-TAT-TCg-TCT-CAg-ggA-gCA-AAA-gCA-ggg-g-3′	28	53.6	63.6	19	32	862	1
HA1-R1	HA1		5′-AAg-CCT-CTA-CTC-ART-gCg-3′	18	52.8	53.1	864	881		2
HA1-F2	HA1	2	5′-CCR-ggg-ATA-CWA-TAA-TA-3′	17	38.2	42.2	804	820	986	2
NS-R	HA		5′-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-ggT-gTT-TT-3′	35	34.3	58.9	1783	1790		1
HA-F	HA	2	5′-TAT-TCg-TCT-CAg-ggA-gCA-AAA-gCA-ggg-g-3′	28	53.6	63.6	8	22	881	1
HA3-R1	HA3		5′-ATT-ATT-gAg-CTT-TTC-CC-3′	17	35.3	43.6	872	889		2
HA3-F2	HA3	2	5′-AAC-AgC-ACA-ggg-AAT-C-3′	16	50	48.9	817	832	952	2
NS-R	HA		5′-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-ggT-gTT-TT-3′	35	34.3	58.9	1750	1769		1
NP-F	NP	1	5′-TAT-TCg-TCT-CAg-ggA-gCA-AAA-gCA-ggg-TA-3′	29	48.3	62.5	9	23	1566	1
NP-R	NP		5′-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-ggT-ATT-TTT-3′	36	30.6	57.5	1555	1575		1
SwNP-F	NP	2	5′-ATg-gCg-TCT-CAA-gg-3′	14	57.1	47.7	54	67	816	3
NP-R1	NP		5′-TgA-gCA-ACT-gAT-CCT-CTC-3′	18	50	51.1	853	870		2
NP-F2	NP	2	5′-ggA-YCA-AgT-gAg-AgA-AAg -3′	18	47.2	48.3	705	722	870	2
NP-R	NP		5′-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-ggT-ATT-TTT-3′	36	30.6	57.5	1555	1575		1
NA-F	NA	1	5′-TAT-Tgg-TCC-Agg-gAg-CAA-AAg-CAg-gAg-T-3′	28	50	63.3	9	23	1486	1
NA-R	NA		5′-ATA-Tgg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gAg-TTT-TTT-3′	36	30.6	57.8	1475	1495		1
NA-F	NA	2	5′-TAT-Tgg-TCC-Agg-gAg-CAA-AAg-CAg-gAg-T-3′	28	50	63.3	9	23	821	1
NA-R1	NA		5′-AYY-TTY-CCC-TYY-TCR-AT-3′	17	41.2	47.3	812	828		2
NA-F2	NA	2	5′-ACM-CAR-gAg-TCW-gAA-T-3′	16	43.8	46.3	715	732	780	2
NA-R	NA		5′-ATA-Tgg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gAg-TTT-TTT-3′	36	30.6	57.8	1475	1495		1
M-F	M	1	5′-TATTCgTCTCAgggAgCAAAAgCAggTAg-3′	29	48.3	61.3	7	21	1126	1
M-R	M		5′-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-gTA-gTT-TTT-3′	36	30.6	57.4	1117	1133		1
NS-F	NS	1	5′-TAT-TCg-TCT-CAg-ggA-gCA-AAA-gCg-ggT-g-3′	28	53.6	63.7	4	19	965	1
NS-R	NS		5′-ATA-TCg-TCT-CgT-ATT-AgT-AgA-AAC-AAg-ggT-gTT-TT-3′	35	34.3	58.9	949	969		1

Note 1: Reverse oligonucleotide primer sequences given in this table are the reverse-complement of sequence present in alignments and as they should be ordered for synthesis; Hairpin (Hair), homodimer (HomD) and heterodimer (HetD) ΔQ's given in kcal/mol.

Components

First PCR		1 rx (µl)
dH_2O	C*f*	6.525
10x Buffer PCR	1X	1.25
$MgCl_2$ 50 mM	1.5 mM	0.375
4x dNTPs 10 mM	200 µM	0.25
Oligos Ext. 10 µM	1600 nM	2.0
Taq 5 UI/µL	0.04 UI	0.1
cDNA	-	2.0
	Vf	12.5

Nested PCR		1 rx (µl)
dH_2O	C*f*	8.525
10x Buffer	1X	1.25
$MgCl_2$ 50 mM	1.5 mM	0.375
4x dNTPs 10 mM	200 µM	0.25
Oligos Int 10 µM	800 nM	1.0
Taq 5 UI/µL	0.04 UI	0.1
Producto 1ra PCR 1:8	-	1.0
	Vf	12.5

Conditions (ETA 6.75 hrs).

Total time: 4:15 hrs		Denaturing		Hibridization	Extension		
HBV & HIV	Temperature	95°	95°	58°	72°	72°	4°
	Time	5 min	20 seg	30 seg	3 min	5 min	5 min
			x40 cycles				

Total time: 2:30 hrs		Denaturing		Hibridization	Extension		
HBV & HIV	Temperature	95°	95°	54°	72°	72°	4°
	Time	5 min	30 seg	30 seg	90 seg	5 min	5 min
			x40 cycles				

Notes

1. Clean workbench with 0.1% NaOCl 0.1% followed by 70% Ethanol before and after work.
2. Preparation of RT mastermix should only be performed in the RT-PCR room.
3. Preparation of PCR mastermix and addition of sample DNA should only be performed in the pre-PCR enclosure or area.
4. Addition of positive template DNA should be performed on instrument (post-PCR) area.
5. All mastermixes should be prepared on ice to prevent excess evaporation.
6. Vortex and spin all mastermixes before and after aliquoting to PCR tubes.

7. After first round PCR, dilute PCR product 1:8 by adding 100 µL of water to 12.5 µL of PCR product and then pipette mixing 10 times before adding 1 µL of this mix to the nested PCR.

References

1. Hoffmann, E., Stech, J., Guan, Y., Webster, R. G., and Perez, D. R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* **146**(12)**,** 2275-89.

2. Contreras-Trevino, H. I. (2010). *Laboratorio de Genómica Viral y Humana*.

3. García-Sepúlveda, C.A. (2009). *Laboratorio de Genómica Viral y Humana*.

Revision history

1.0 Original document.

2.0 Changes to document format only.

3.0 Translated to English.