Real-time RT-PCR Allelic Discrimination Analysis for Detection of the Substitution at Amino Acid 275 in the Neuraminidase (NA) of A(H1N1)pdm09 Influenza Viruses

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The following protocol is for discriminating an oseltamivir-resistant A(H1N1)pdm09 virus possessing the H275Y substitution in the NA in clinical specimens or in clinical isolates. Two probes for detecting oseltamivir-susceptible H275 (VIC-labeled) and oseltamivir-resistant Y275 (FAM-labeled) were designed to discriminate C and T nucleotide of the A(H1N1)pdm09 virus NA gene (positioned at 823), respectively. The assay detects the H275Y substitution of the clinical isolates in cultured supernatants directly without RNA purification (0.5% TRBC HA titer >4). For preventing the effect of culture medium, ten times diluted cultured medium (containing virus) with distilled water is used for the reactions. RNA purification step is required for using the assay to detect the H275Y substitution in clinical specimens.

Specific considerations for real-time RT-PCR

• Ensuring appropriate equipment, software, and fluorescent based reagents are used and handled correctly.
• Ensuring appropriate training of personnel for interpretation of results.
• Validation in the laboratory and optimization of reactions are essential to making determinations.
• There is little likelihood of contamination when reactions are discarded after testing.

Equipment

Roche LightCycler® 480 II

Thermo Fisher Scientific ABI 7000, ABI 7300, ABI 7500, ABI 7500Fast, ABI 7900HT, ABI StepOne, ABI StepOnePlus, and ABI QuantStudio 12K Flex

** NIID also validated and optimized this method for Agilent MX3000P and Agilent MX3005P.* ***Please contact NIID, if you want to use these equipment****.*

Materials required

• QIAamp Viral RNA Mini Kit (QIAGEN Cat. No. 52904, 52906. Other extraction kits can be used after proper evaluation)
• QuantiTect Virus + ROX Vial Kit (QIAGEN Cat No. 211033, 211035) or QuantiTect Virus Kit (QIAGEN Cat No. 211011, 211013, 211015)
• RNase-free water

• Microcentrifuge (adjustable up to 13000 rpm)
• Adjustable pipettes (10, 20, 100, 200, and 1000 µl)
• Sterile, RNase-free pipette tips with aerosol barrier
• Vortex mixer
• Microcentrifuge tubes (0.2, 1.5 ml)
• Primers and probes (TaqMan® MGB Probe) sets
• Positive control (the two kinds of viral RNAs eluted from plaque-purified viruses, possessing H275 or Y275)

Primers and Probes

Names	Sequences (5'-3')	Position
H1N1NA-F690-719	ATGTGCATGTGTAAATGGTTCTTGCTTTAC	690-719
H1N1NA-R847-872	ACACATGTGATTTCACTAGAATCAGG	847-872
FAM-274Ya-swH1N1-F823-835 (for detecting resistant virus)	(FAM)**T**ACTATGAAGAAT(MGB)	823-835
VIC-H274a-swH1N1-F823-835 (for detecting susceptible virus)	(VIC)**C**ACTATGAAGAAT(MGB)	823-835

Because currently circulating A(H1N1)pdm09 viruses have a single nucleotide mutation at the underlined position, the probe sequence has been changed from G to A.

Procedure

1. Extract viral RNA from clinical specimen with QIAamp Viral RNA Mini Kit or equivalent extraction kit, according to manufacturer's instructions. (This step is required for clinical specimens, and not required for clinical virus isolates)

2. Perform real-time RT-PCR

- Take out the reagents from storage and thaw them. After they are thawed out, keep them on ice.
- Preparation of master mix. (Operate on ice)
- Add the following to microcentrifuge tubes and mix gently the master mix by turning up and down ten times. (To avoid localized differences in salt concentration, it is important to mix the solutions completely before use.)

i) For Roche LightCycler® 480 II

Use QuantiTect Virus + ROX Vial Kit

Reagent	**Volume (µl)**	**Final concentrations**
5×QuantiTect Virus NR Master Mix	4.0	1×
40×Primers&Probes Mix	0.5	0.6 µM each primers 0.1 µM each probes
QuantiTect Virus RT Mix	0.2	
RNase free Water	13.3	
Total	18	

ii) For ABI 7500, ABI 7500Fast, and ABI QuantStudio 12K Flex

Use QuantiTect Virus + ROX Vial Kit

Reagent	**Volume (µl)**	**Final concentrations**
5×QuantiTect Virus NR Master Mix	4.0	1×
40×Primers&Probes Mix	0.5	0.6 µM each primer 0.1 µM each probe
QuantiTect Virus RT Mix	0.2	
50×Rox Dye Solution	0.4	
RNase free Water	12.9	
Total	**18**	

iii) For ABI 7000, ABI 7300, ABI 7900HT, ABI StepOne, and ABI StepOnePlus

Use QuantiTect Virus Kit

Reagent	**Volume (µl)**	**Final concentrations**
5×QuantiTect Virus Master Mix	4.0	1×
40×Primers&Probes Mix	0.5	0.6 µM each primer 0.1 µM each probe
QuantiTect Virus RT Mix	0.2	
RNase free Water	13.3	
Total	**18**	

- Dispense 18 µl of the reaction mixture into each well of the reaction plate.
- Add 2 µl of the sample to the reaction mixture[1]. For control reactions, use 2 µl of distilled water for negative control and 2 µl of three different concentrations of viral RNAs for positive control, such as undiluted original RNA, and a tenfold dilution and 100-fold dilution of the same RNA.
- Program the thermal cycler according to the following thermal cycling conditions.

[1] For clinical isolates, ten times diluted cultured medium (containing virus) with distilled water is used for the reactions. For clinical specimens, purified RNA is used.

i) For Roche LightCycler® 480 II

	Analysis Mode	**Cycle**	**Temperature (°C)**	**Time**	**Ramp Rate (°C/sec)**	**Acquisition Mode**
RT	None	1	50	20 min	4.4	None
Denature	None	1	95	5 min	4.4	None
PCR	Quantification	45	95	15 sec	1.5	None
			56	45 sec	1	Single

ii) For all ABI equipment

	Temperature (°C)	**Time**	**Cycle**
Pre-read	60	1 min	1
Amplification	50	20 min	1
	95	5 min	1
	95	15 sec	40
	56	45 sec (Data collection)	
Post-read	60	1 min	1

Interpretation of results

Endpoint fluorescent signals were analyzed using the software with each equipment.

i) For Roche LightCycler® 480 II

Briefly, select positive controls and negative control and analyze the samples by "Endpoint Genotyping", according to manufacturer's instructions. The results will appear like **Figure 1** below. It is necessary to interpret the result by visual judgment.

ii) For all ABI equipment

Briefly, analyze the samples by "Allelic Discrimination" or "Genotyping" according to manufacturer's instructions and classify the samples into resistance, susceptible and mixed population not by automatic analysis but by visual judgment. Please make sure to read the manual about Allelic Discrimination or Genotyping Experiments supplied by ABI with the equipment. The results will appear like **Figure 1** below.

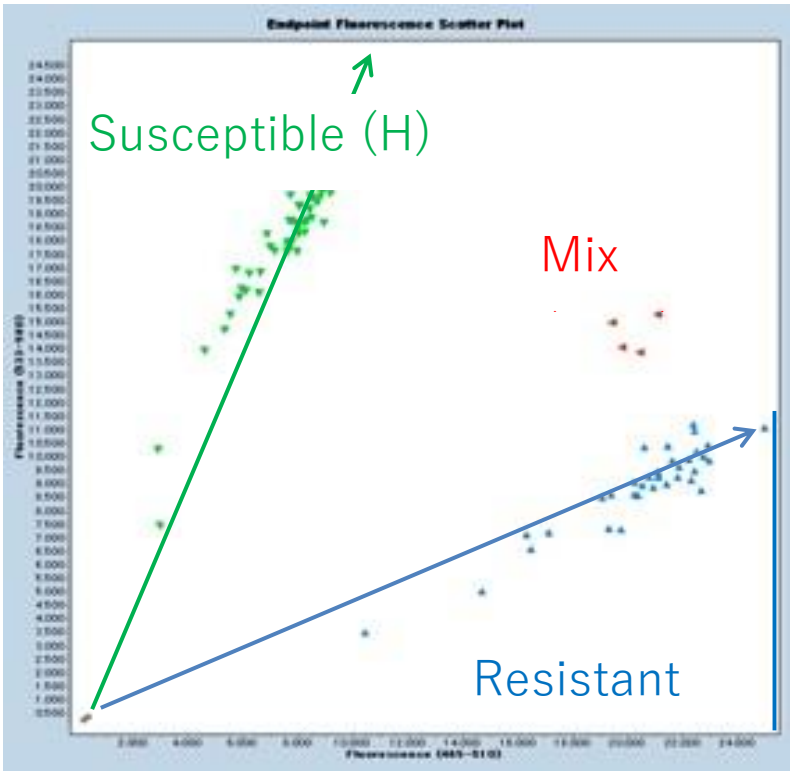

Figure 1.

Reference:

Rapid discrimination of oseltamivir-resistant 275Y and -susceptible 275H substitutions in the neuraminidase gene of pandemic influenza A/H1N1 2009 virus by duplex one-step RT-PCR assay. Nakauchi M, Ujike M, Obuchi M, Takashita E, Takayama I, Ejima M, Oba K, Konomi N, Odagiri T, Tashiro M, Kageyama T; influenza virus surveillance group of Japan. J Med Virol. 2011 Jul;83(7):1121-7. doi: 10.1002/jmv.22101.

Recommendations:

A Standard Operating Procedure (SOP) should be prepared when using own equipment by reference to this protocol.

Before using this assay for surveillance of H275Y substitution in A(H1N1)pdm09 neuraminidase, please contact NIID focal point below. NIID will help to build this assay and will support future updates. The person in charge of this assay; Dr Emi Takashita (emitaka@niid.go.jp).