rhPCR Assays for Detection of the Substitution at Amino Acid 38 in the Polymerase Acidic Subunit (PA) of A(H1N1)pdm09, A(H3N2), and B Influenza Viruses

Research Center for Influenza and Respiratory Viruses
National Institute of Infectious Diseases
(NIID) Tokyo, Japan

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The following protocol is for discriminating a baloxavir marboxil-resistant seasonal A(H1N1)pdm09, A(H3N2) and B influenza viruses possessing the I38T substitution in the PA in clinical specimens or in clinical isolates. Three assays are developed based on RNase H2-dependent PCR (rhPCR). Each rhPCR assay consisted of one gene-specific primer and two allele-specific primers. One allele-specific primer was designed to detect a T at nucleotide 113 (encoding an Ile at amino acid residue 38: PA I38) and was detected with a Yakima Yellow-labeled universal probe, and the other was for detection of a C at nucleotide 113 (encoding a Thr at amino acid residue 38: PA T38) and was detected by a FAM-labeled universal probe.

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

- QIAcube (or other validated similar equipment)
- A broad class of Thermal Cycler
- Roche LightCycler® 480 II

or Thermo Fisher Scientific ABI 7500, ABI 7500Fast, and ABI QuantStudio 12K Flex

Materials required

- QIAamp Viral RNA Mini QIAcube Kit (QIAGEN Cat. No. 52926. Other extraction kits can be used after proper evaluation)
- PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara Cat. No. RR037A,

RR037B. Other RT reagent kits can be used after proper evaluation)

- rhAmp® Genotyping Master Mix (Integrated DNA Technologies Cat. No. 1076014, 1076015, 1076016, 1076017, 1076018)
- rhAmp® Reporter Mix (Integrated DNA Technologies Cat. No. 1076025, 1076026, 1076027, 1076028, 1076029) for Roche LightCycler 480 or rhAmp® Reporter Mix w/Reference (Integrated DNA Technologies Cat. No. 1076020, 1076021, 1076022, 1076023, 1076024) for Thermo Fisher Scientific ABI 7500, ABI 7500Fast, and ABI QuantStudio 12K Flex
- 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)
- PCR tubes, 8 well strips, or 96 well PCR plate

Primers for reverse transcription

Name	**Sequence (5′ -3′)**
Primer for A(H1N1)pdm09 and A(H3N2) viruses (Uni12 primer)	AGCAAAAGCAGG
Primer for B virus (Uni9 primer)	AGCAGAAGC

- rhAmp® SNP Assay* (Integrated DNA Technologies)

Name	**Sequence (5′ -3′)**	
Primers for A(H1N1)pdm09 virus (Assay ID: CD.GT.BSFN7032.1)		
A/H1pdm_PA rhAmp-FY	UFP1/AAACTTCCAAATGTGTGCAAArUTGCA	108-133
A/H1pdm_PA rhAmp-FF	UFP2/AAACTTCCAAATGTGTGCAAGrUTGCA	108-133
A/H1pdm_PA rhAmp-R	GCTGTGCGACAATGCTTCAATrCCAAT	12-35
Primers for A(H3N2) virus (Assay ID: CD.GT.BGPR9916.4)		
A/H3_PA rhAmp-FY	UFP1/CACCTCCAAGTGAGTGCATArUTGCT	108-132
A/H3_PA rhAmp-FF	UFP2/ACCTCCAAGTGAGTGCATGrUTGCT	108-131
A/H3_PA rhAmp-R	GCTGTGCGACAATGCTTCAACrCCGAT	12-35
Primers for B virus (Assay ID: CD.GT.BGDB2770.1)		
B_PA rhAmp-FY	UFP1/CCAGCAATGCTATTCAACATrCTGTG	94-118
B_PA rhAmp-FF	UFP2/CCAGCAATGCTATTCAACACrCTGTG	94-118
B_PA rhAmp-R	GCCTAATGCTGTATATGCTTTTCCTTrCTTCG	165-193

**an IDT account is required to order rhAmp® Genotyping Master Mix, rhAmp® Reporter Mix, and rhAmp® SNP Assay. You can order rhAmp® SNP Assay with Assay ID provided in the Table. "r" represented ribonucleotide.*

• Positive controls. Please order two synthetic oligonucleotide (single strand DNA)

containing target region of PA gene for each type/subtype viruses; the one possesses a T at nucleotide 113 and the other possesses a C at nucleotide 113 to an appropriate manufacturer. Sequence of positive controls used in Japan as follows:

H1pdm PA I38
ATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATCGTCGAGCTTGCGGAAAAGGCA ATGAAAGAATATGGGGAAGACCCCAAAATCGAAACTAATAAGTTTGCTGCAA**T**TTGCACA CATTTGGAAGTTTGTTTCATGTAT
H1pdm PA T38
ATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATCGTCGAGCTTGCGGAAAAGGCA ATGAAAGAATATGGGGAAGACCCCAAAATCGAAACTAATAAGTTTGCTGCAA**C**TTGCAC ACATTTGGAAGTTTGTTTCATGTAT
H3 PA I38
ATGGAAGATTTTGTGCGACAATGCTTCAACCCGATGATTGTCGAACTTGCAGAAAAAGCA ATGAAAGAGTATGGGGAGGATCTGAAAATTGAAACCAACAAATTTGCAGCAA**T**ATGCAC TCACTTGGAGGTGTGTTTCATGTAT
H3 PA T38
ATGGAAGATTTTGTGCGACAATGCTTCAACCCGATGATTGTCGAACTTGCAGAAAAAGCA ATGAAAGAGTATGGGGAGGATCTGAAAATTGAAACCAACAAATTTGCAGCAA**C**ATGCAC TCACTTGGAGGTGTGTTTCATGTAT
B PA I38
GTGAAGACCCTGAATTGCAACCAGCAATGCTATTCAACA**T**CTGTGTCCATCTAGAGGTTT GCTATGTAATAAGTGACATGAATTTTCTTGACGAAGAAGGAAAAGCATATACAGCATTAG AAGGACAAGGGAAAGAACAAAATT
B PA T38
GTGAAGACCCTGAATTGCAACCAGCAATGCTATTCAACAC**C**TGTGTCCATCTAGAGGTTT GCTATGTAATAAGTGACATGAATTTTCTTGACGAAGAAGGAAAAGCATATACAGCATTAG AAGGACAAGGGAAAGAACAAAATT

Procedure

1. Extract viral RNA from clinical specimen or clinical isolate with QIAamp Viral RNA Mini QIAcube Kit or equivalent extraction kit, according to manufacturer's instructions.

2. Perform reverse transcription
   - 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, then spin down. (To avoid localized differences in salt concentration, it is important to mix the solutions completely before use.)

Reagent	**Volume (μl)**	**Final concentrations**
5 × PrimeScript Buffer	2.0	1×
PrimeScript RT Enzyme Mix	0.5	
Uni12 or Uni9 Primer（2 μM）	0.5	0.1 μM
RNase free Water	2.0	
Total Volume	5.0	

- Dispense 5 μl of the reaction mixture into each well of the reaction tube/plate.
- Add 5 μl of purified RNA to the reaction mixture.
- Program the thermal cycler according to the following thermal cycling conditions.
  42℃ 15 min, 85℃ 5 sec, 4℃

3. Perform rhPCR
   - 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, then spin down. (To avoid localized differences in salt concentration, it is important to mix the solutions completely before use.)

i) For Roche LightCycler® 480 II

Reagent	**Volume (μl)**	**Final concentrations**
rhAmp® Genotyping Master Mix (2×)	5.0	1×
rhAmp® Reporter Mix w/Reference (40×)	0.25	1×
rhAmp® SNP Assay (20×)	0.5	1×
RNase free Water	2.25	
Total Volume	8	

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

Reagent	Volume (µl)	Final concentrations
rhAmp® Genotyping Master Mix (2×)	10.0	1×
rhAmp® Reporter Mix w/Reference (40×)	0.5	1×
rhAmp® SNP Assay (20×)	1.0	1×
RNase free Water	6.5	
Total Volume	18	

- Dispense 8 µl (for Roche LightCycler® 480 II) or 18 µl (for Thermo Fisher Scientific ABI 7500, ABI 7500Fast, and ABI QuantStudio 12K Flex) of the reaction mixture into each well of the reaction tube/plate.
- Add 2 µl of cDNA (from procedure 2) to the reaction mixture. For control reactions, use 2 µl of RNase free water for negative control and 2 µl of each positive control (1000 copies/reaction).

  *Be sure to prevent any contamination of cDNA when open cap/sheet of tube/plate and dispense of cDNA.
- Program the instrument according to the following thermal cycling conditions.

i) For Roche LightCycler® 480 II

	Analysis Mode	Cycle	Temperature (℃)	Time	Ramp Rate (℃/sec)	Acquisition Mode
Enzyme activation	None	1	95	10 min	4.4	None
PCR	Quantification	35	95	10 sec	4.4	None
			60	30 sec	2.2	None
			68	20 sec	4.4	Single

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

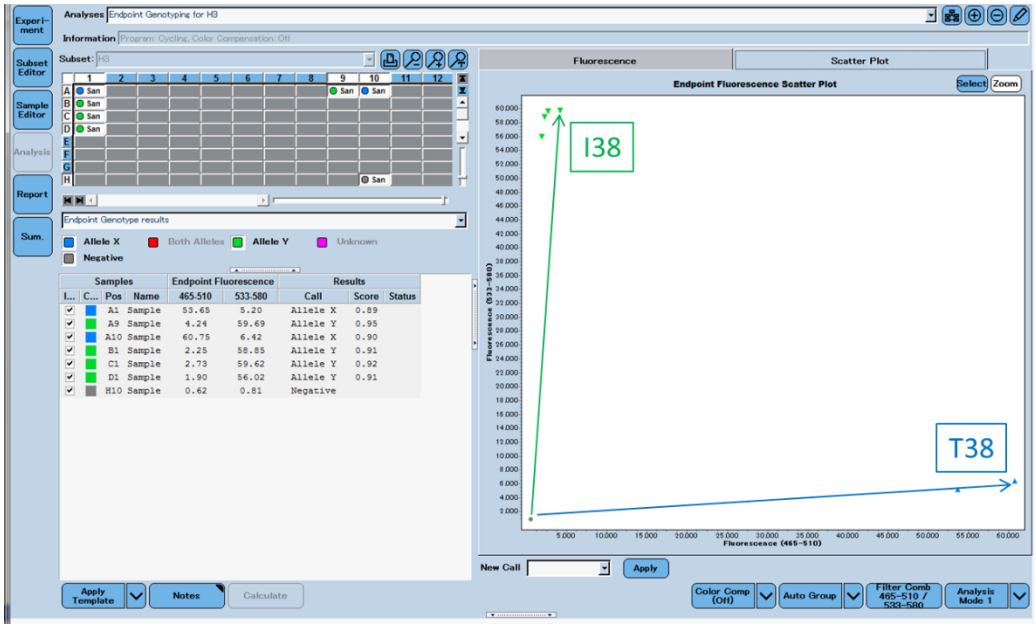
	Temperature (℃)	Time	Cycle
Pre-read	60	1 min	1
Amplification	95	10 min	1
	95	10 sec	35
	60	30 sec	
	68	30 sec (Data Collection)	
Post-read	60	1 min	

Interpretation of results

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

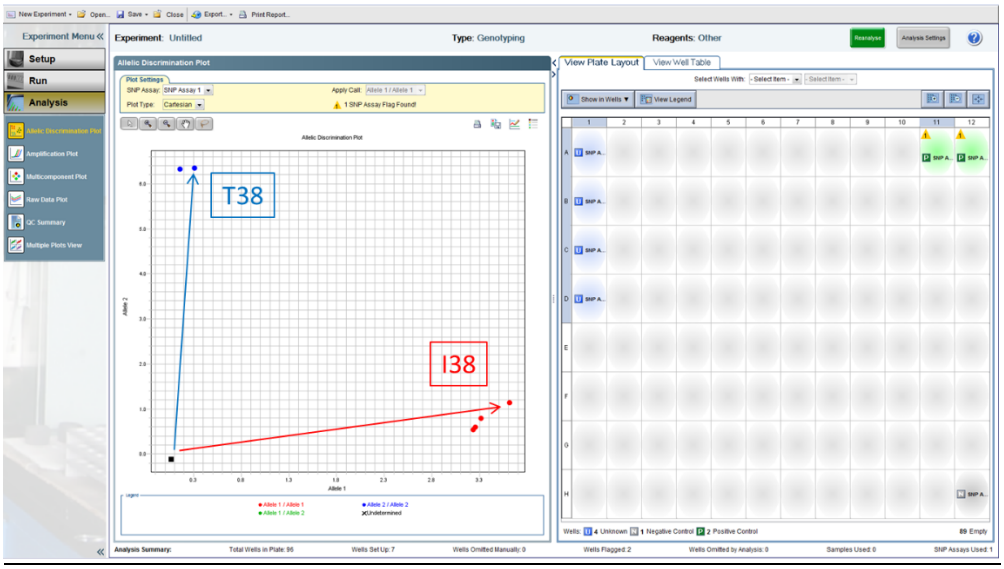
i) For Roche LightCycler® 480 II

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



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

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 equipment. The results will appear like the following figure.



Reference:

Rapid detection of an I38T amino acid substitution in influenza polymerase acidic subunit associated with reduced susceptibility to baloxavir marboxil. *Nakauchi M, Takashita E, Fujisaki S, Shirakura M, Ogawa R, Morita H, Miura H, Saito S, Watanabe S, Odagiri T, Kageyama T. Influenza Other Respir Viruses. 2020 Jul;14(4):436-443. doi: 10.1111/irv.12728.*

Recommendation:

A standard operation procedure should be prepared in each institute using own equipment by reference to this protocol. Please contact NIID[i], before using this assay for surveillance of I38T substitution in PA of seasonal influenza viruses. We will help to build this assay and support in future updates.

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[i] *WHO Collaborating Centre for Reference and Research on Influenza, National Institute of Infectious Diseases (NIID), Tokyo, Japan; Dr Emmi Takashita (emitaka@niid.go.jp)*