

VIRUS REFERENCE DIVISION

STANDARD OPERATING PROCEDURE

TITLE: Pyrosequencing of the Influenza A N1 (H1) gene for Neuraminidase Inhibitor Sensitivity

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SUMMARY

This SOP describes the method used to amplify the N1 gene from nucleotide 601 to 931. This region allows pyrosequencing of the mutation encoding the H274Y change reported to cause resistance to the neuraminidase inhibitor Oseltamivir.

These protocols should not be distributed and results obtained with these assays should not be published or presented in a public forum without the explicit consent of the Respiratory Virus Unit, Centre for Infections.

CROSS REFERENCE

- 1.1 SOP V5806 Viral RNA Extraction using the Qiagen QIAamp viral RNA mini kit
- 1.2 SOP V36 Operation of Roche MagNA Pure LC automated nucleic acid extraction robot
- 1.3 SOP **Preparation of DNA for Pyrosequencing: PyroMark Vacuum Prep Workstation**
- 1.4 Biotage manufacturers instructions for use of pyrosequencer

2 EQUIPMENT

- 2.1 Thermal Cycler
- 2.2 Microfuge
- 2.3 Picofuge
- 2.4 0.2ml and 1.5ml tubes
- 2.5 Single channel pipettes suitable for 1µl to 1000µl volumes and filtered tips
- 2.6 Pyrosequencer
- 2.7 Electrophoresis equipment and UV transilluminator (optional *See useful notes 5.1)

3 REAGENTS

- 3.1 Qiagen One-Step RT-PCR Kit (Cat no. 210210 or 210212)
- 3.2 Primers (MWG Biotech) 10µM working stock
H1N1 PCR3 Forward: GGAGCCGTGGCTGTACTAAAATA
H1N1 PCR3 Reverse: CCACGTTTTGATTAAGACACC (5'Biotinylated)
H1N1 274 Sequencing (forward direction): AGTTGAATGCACCCAAT

- 3.3 Pyrogold SNP kit and Reagent cartridge (Biotage)

The following reagents are optional * See useful notes 6.1

- 3.4 Multipurpose agarose (Roche)
- 3.5 10X Blue Juice Loading Buffer (Invitrogen)
- 3.6 DNA Molecular weight markers; 100bp ladder (New England Biolabs)
- 3.7 Running buffer (1x TBE) (Invitrogen)
- 3.8 Ethidium bromide solution (5mg/L) (Invitrogen)

4 PCR PROCEDURE

- 4.1 Extract 140µL of virus using the Qiagen Viral RNA mini Kit (SOP V5806) or MagNA Pure extraction robot (SOP V36). *See - Useful note 6.2).
- 4.2 Thaw primers, dNTP mix, 5x Qiagen One Step RT-PCR buffer and RNase-free water and place on ice.
- 4.3 Prepare an RT-PCR mastermix comprising the following per sample;
- 10µL 5x Qiagen One-Step RT-PCR Buffer
 - 2µL dNTP Mix
 - 3µL Forward primer (10µM)
 - 3µL Reverse primer (10µM)
 - 27.5µL RNase-free water
 - 2µL Qiagen One-Step RT-PCR Enzyme Mix
- 4.4 Add 2.5µL eluted RNA from step 4.1 to the RT-PCR mix prepared in step 4.3. Appropriate negative water controls should always be performed.
- 4.5 Place in thermal cycler and cycle using the following conditions;
- Incubate at: 50 °C for 30 minutes (Reverse transcription)
- 95 °C for 15 minutes (PCR activation)
- Then 40 cycles: 94 °C for 30 seconds
- 53 °C for 30 seconds
- 72 °C for 1 minute
- Final extension: 72 °C for 10 minutes and samples are cooled to 15 °C.
- 4.6 Samples should be prepared immediately for pyrosequencing according to SOP **Preparation of DNA for Pyrosequencing using the PyroMark Vacuum Prep Workstation** or can be stored at -20°C until required.

5 PYROSEQUENCING PROCEDURE

- 5.1 For both SQA and SNP pyrosequencing analysis 20 μ l of PCR product is sufficient
- 5.2 Prepare the PCR product for pyrosequencing as per the SOP: **Preparation of DNA for Pyrosequencing using the PyroMark Vacuum Prep Workstation.**
- 5.3 The H1N1 274 sequencing primer should be used at a final concentration of 0.44 μ mol/ μ l (i.e. 2 μ l of 10 μ M stock in 43 μ l pyrosequencing annealing buffer per sample).
- 5.4 Anneal the sequencing primer by heating the plate at 80°C for 2 minutes and then allow it to cool down to room temperature.
- 5.5 To perform quantitative analysis of the H274Y mutation, perform a SNP run using the N1 274 entry (see Appendix 1).
- 5.6 Prepare the pyrosequencer for the run by selecting the wells containing sample in the plate diagram, enter a run name, the reagent lot number, cartridge lot number under the 'general' tab. Under the 'set up' tab enter the entry to be carried out in each well (274 program) and input the sample names.
- 5.7 Click 'view run' to see the volumes of each base, enzyme and substrate to add to the cartridge.
- 5.8 Insert both the plate containing the DNA bound to beads in the sequencing primer solution and the reagent cartridge into the pyrosequencer and click run. Ensure a peak is seen when the substrate is added to confirm the enzyme and substrate are working correctly. The 274 program should take 10 minutes to run.
- 5.9 Expected results for a wild type and mutant virus are given in appendix 2. The expected sequences are:
Wild Type 274H: TTT**C**ATTATG
Mutant 274Y: TTT**T**ATTATG
- 5.10 Any virus which yields a percentage of >5% but less than 10% T (mutant) should be repeated or further more detailed analyses carried out.
- 5.11 A result of more than or equal to 10% is considered to be a true mixture of wild type and mutant virus.

6 USEFUL NOTES

- 6.1 Products can be visualised on an agarose gel to check size, quality and integrity. The PCR product should be 331 nucleotides in length. If samples are prepared for pyrosequencing according to SOP **Preparation of DNA for Pyrosequencing using the PyroMark Vacuum Prep Workstation** this step is not necessary.
- 6.2 Both the Magna pure and Qiagen methods of RNA extraction yield comparable results in this PCR reaction and in the subsequent pyrosequencing reaction. The method of RNA extraction is therefore at the user's discretion.

Appendix 1: H274Y SNP Entry

Define simplex SNP entry - N1_274 Current Isolates

PyroMark ID

SNP

Simplex Entries

Multiplex Entries

Sequences

SNP Runs

Result Browser

Entry ID: N1_274 Current Isolates

Sequence to analyze: Primers

Sequence name: TTTTC/TATTATGAGGAAT

Sequence: TTTTC/TATTATGAGGAAT

Primer: TTTTC/TATTATGAGGAAT

Sequence to analyze: TTTTC/TATTATGAGGAAT

Position names: 274H/Y

Dispensation order: GTCGATATG

Histograms

Warning (1): AQ may be uncertain (The polymorphism is part of a homopolymer >=3).

Error (0):

Reference p:

Notes:

Select histogram to view

No.	Polymorphisms
1	C/C
2	C/T
3	T/T

Export

Help Duplicate

SQA

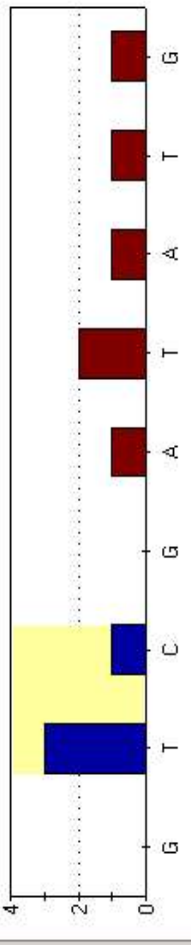
Samples

Instrument

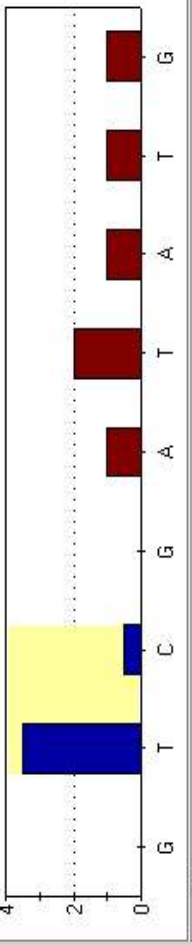
Tools

Default histograms

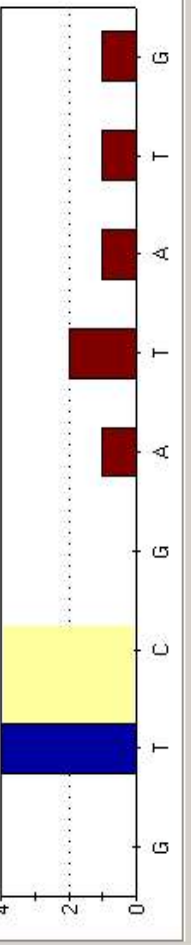
C/C



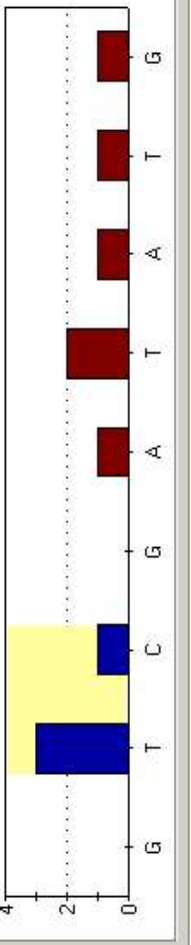
C/T



T/T



C/C



Save

Close

Appendix 2: Expected Results

