**HEALTH PROTECTION AGENCY**

**CENTRE FOR INFECTIONS**

**VIRUS REFERENCE DEPARTMENT**

**STANDARD OPERATING PROCEDURE**

**TITLE: Pandemic 2009 H1N1 Influenza A H275Y PCR and Pyrosequencing**

**SOP NO.** **V-6777/01-10**

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**AUTHORISER:** **Joanna Ellis** **EFFECTIVE DATE:** **07.01.2010**

**ISSUED TO:** **REVIEW DATE:** **07.01.2013**


**SUMMARY**

This SOP describes the method for amplification and pyrosequencing of the 2009 pandemic influenza (H1) N1 gene to detect the H275Y mutation, which causes resistance to the neuraminidase inhibitor oseltamivir.


**SAFETY**

Good Laboratory Practice.

Biological COSHH assessment: VB-0022 Extraction of RNA for respiratory virus detection

Chemical COSHH assessment: VC-0896 RT-PCR

Chemical COSHH assessment: VC-0901 Pyrosequencing

## 1.0 CROSS REFERENCE

SOP V5806 Viral RNA Extraction using the Qiagen QIAamp viral RNA mini kit
SOP V36 Operation of Roche MagNA Pure LC automated nucleic acid extraction robot
SOP V5489 Biomérieux NucliSENS® easyMAG® system for automated isolation of total nucleic acid from biological specimens
SOP V-6778 PCR Product Preparation for Pyrosequencing
Qiagen manufacturers instructions for use of vacuprep and Pyrosequencer

## 2.0 PERSONNEL

All medical microbiologists, clinical scientists, biomedical scientists, and healthcare scientists with suitable training.

## 3.0 EQUIPMENT

3.1 Thermal Cycler
3.2 Microfuge
3.3 Picofuge
3.4 0.2ml and 1.5ml tubes
3.5 Single channel pipettes suitable for 1µl to 1000µl volumes and filtered tips
3.6 Pyrosequencer
3.7 8-well and/or12-well multi-channel pipettes suitable for 1µl to 50µl volumes
3.8 Electrophoresis equipment and UV transilluminator (optional *See - Useful notes 7.1)

## 4.0 REAGENTS

4.1 Qiagen One-Step RT-PCR Kit (Cat no. 210210 or 210212)
4.2 Primers 10µM working stock
PanSwH1N1 275Forward: GGGAAAGATAGTCAAATCAGTCGA (5'Biotinylated)
PanSwH1N1 275Reverse: TAGACGATACTGGACCACAACTG
PanSwH1N1 275 Sequencing (reverse direction): CAGGAGCATTCCTCA

The following reagents are optional * See useful notes 7.1

4.3 Multipurpose agarose (Roche)

4.4 10X Blue Juice Loading Buffer (Invitrogen)

4.5 DNA Molecular weight markers; 100bp ladder (New England Biolabs)

4.6 Running buffer (1x TBE) (Invitrogen)

4.7 Ethidium bromide solution (5mg/L) (Invitrogen)

## 5.0 PCR PROCEDURE

5.1 Extract RNA from 150µL of virus using an appropriate method (*See - Useful note 7.2).

5.2 Thaw primers, dNTP mix, 5x Qiagen One Step RT-PCR buffer and RNase-free water and place on ice.

5.3 Prepare an RT-PCR mastermix for the number of samples (+2) comprising the following per sample (*See- Useful note 7.3);

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

5.4 Aliquot 47.5µL of mastermix per tube into 0.2ml tubes or PCR plates.

5.5 Add 2.5µL eluted RNA from step 5.1 to the RT-PCR mix prepared in step 5.3. Appropriate negative water controls should always be performed.

5.6 Place in thermal cycler and cycle using the following conditions;

Incubate at: 50 $^{o}$C for 30 minutes (Reverse transcription)
95°C for 15 minutes (PCR activation)

Then 40 cycles: 94 $^{o}$C for 30 seconds
62 $^{o}$C for 30 seconds
72 $^{o}$C for 1 minute

Final extension: 72 $^{o}$C for 10 minutes and samples are cooled to 15 $^{o}$C.

5.7 Samples should be prepared immediately for pyrosequencing according to SOP V-6778 PCR Product Preparation for Pyrosequencing or can be stored at -20$^{o}$C until required.

## 6.0 PYROSEQUENCING PROCEDURE

6.1 For both SQA and SNP pyrosequencing analysis 20µl of PCR product is sufficient

6.2 The H1N1 275 sequencing primer should be used at a final concentration of 0.44pmol/µl (i.e. 2µl pf 10µM stock in 43µl pyrosequencing annealing buffer per sample).

6.3 Anneal the sequencing primer by heating the plate at 80$^{o}$C for 2 minutes and then allow it to cool down to room temperature.

6.4 Refer to Qiagen manufacturers instructions for use of vacuprep and pyrosequencer

6.5 To perform quantitative analysis of the H275Y mutation, perform a SNP run (see entry information in Appendix 1). Sequence changes other than the defined resistance SNPs occur relatively infrequently, but require modified dispensation to quantitate resistance in these cases.

6.6 Expected results for a wild type and mutant virus are given in appendix 2. The expected sequences are (*See – Useful note 6.4);

Wild Type 275H: TAGTGAT

Mutant 275Y: TAGTAAT

6.7 The limit of resistance mutation detection of the assay is 10%. Any virus which yields a percentage of less than 10% T (mutant) is considered to be wild type.

6.8 A result of more than 10% is considered to be a true mixture of wild type and mutant virus. All mixed samples should be repeated to confirm the proportion.

## 7.0 USEFUL NOTES

7.1 Products can be visualised on an agarose gel to check size, quality and integrity. The PCR product should be 242 nucleotides in length.

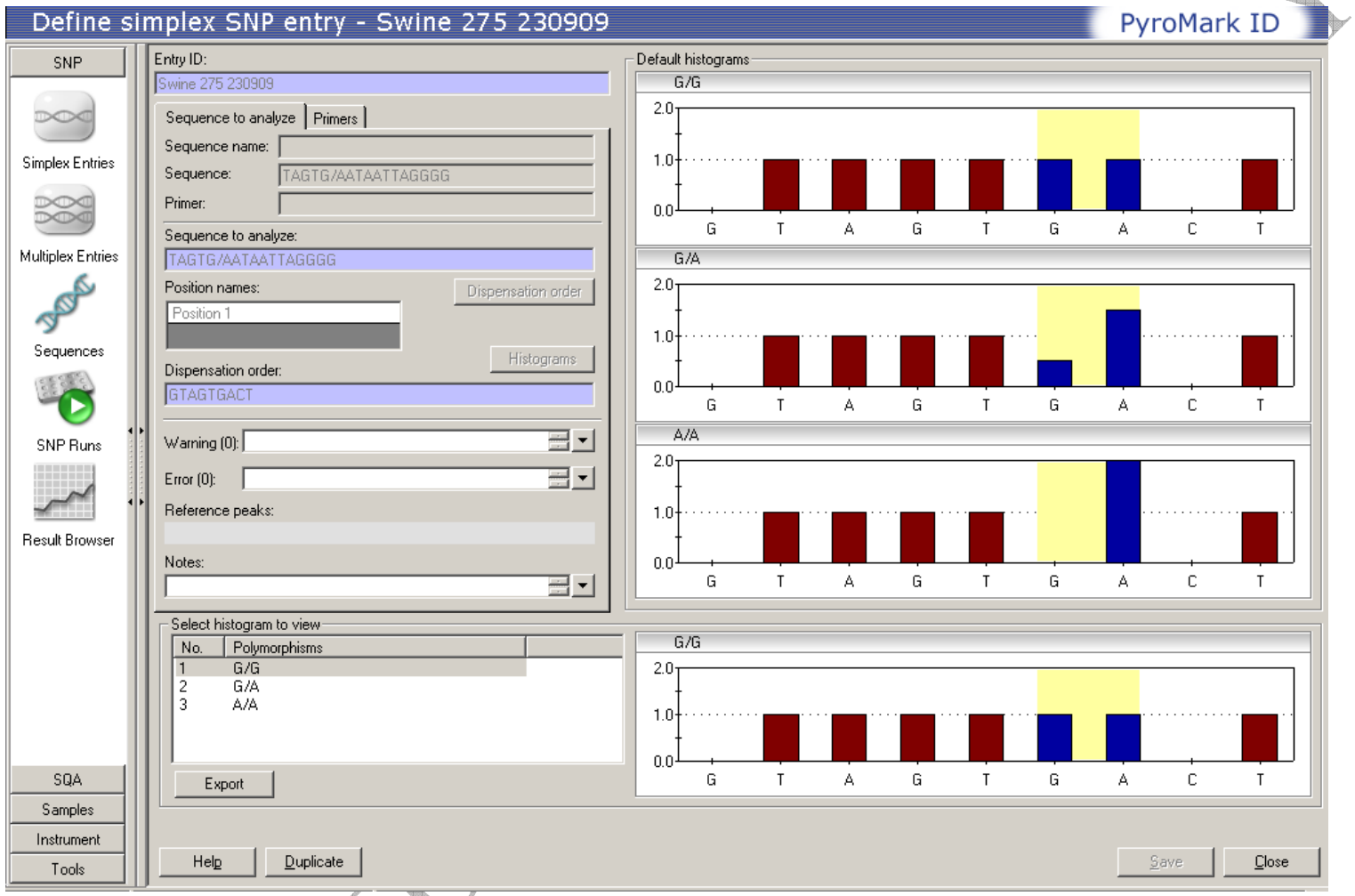
7.2 Magna pure, Biomérieux NucliSENS® easyMAG® and Qiagen methods of RNA extraction yield comparable results in this PCR reaction and in the subsequent pyrosequencing reaction. The method of RNA extraction is therefore at the user's discretion.

7.3 The total volume of the PCR reaction stated is 50µl. This method can be adapted to use a total PCR reaction volume of 25µl if required, by using half the volume of each reagent, including RNA, without loss of sensitivity.

## 8.0 SUMMARY OF REVISIONS

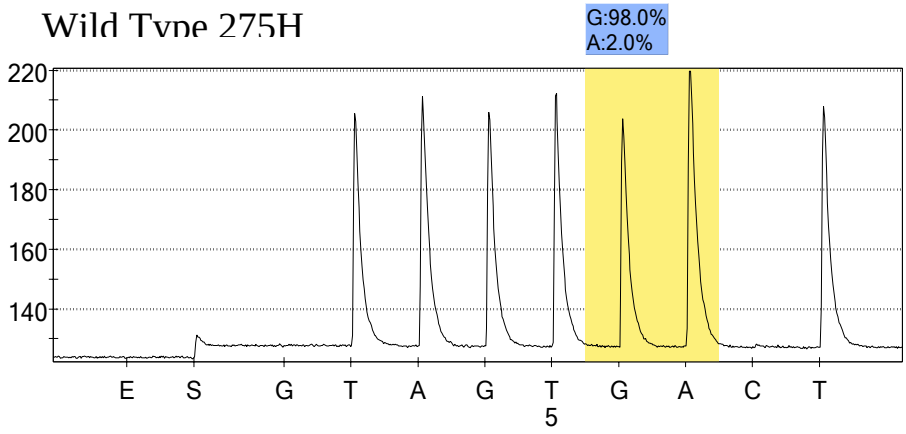
| Retraining Required | Yes ☐ | No ☐ |
|---|---|---|
| N/A | First Issue. | |

## Appendix 1: H275Y SNP Entry



## Appendix 2: Expected Results

Wild Type 275H




Mutant 275Y