The Development and Application of Pyrosequencing Assays to Determine Antiviral Susceptibility of Influenza A to Neuraminidase Inhibitors.

MPhil in Medicine

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Summary:

Influenza A can result in complicated disease and death in high risk individuals, and can be treated with the neuraminidase inhibitors oseltamivir and zanamivir. During the 2007-2008 season a rise in oseltamivir resistance in H1N1 viruses was observed worldwide. This resistance was caused by a point mutation in the neuraminidase gene segment at position 274 causing an amino acid change from histidine to tyrosine. Other point mutations have also been observed conferring resistance to neuraminidase inhibitors in H1N1 and H3N2 viruses, such as D151, E119V, R292K and N294S. Assays for the detection of resistance to the neuraminidase inhibitors have been well documented, the most novel being pyrosequencing. The aim of this study was to optimise pyrosequencing assays for routine use in a diagnostic laboratory. Once optimisation and validation were achieved using reference strains of influenza A, clinical validation was performed on influenza A isolates collected during the 2008-2009 season. Optimisation and clinical validation of a SNP pyrosequencing assay for the detection of the H274Y mutation in H1N1pdm09 isolates was also performed on isolates collected during the 2009-2010 and 2010-2011 seasons. Routine diagnostic assays were optimised for the H274Y mutation in pre 2009 H1N1 viruses and H1N1pdm09 viruses, and for the E119V mutation in H3N2 viruses. Only SQA assays were able to be optimised for the D151, R292K and N294S mutations in H3N2 viruses. Clinical validation showed that all seasonal H1N1 viruses isolated before April 2009 possessed the H274Y mutation at nearly 100%. The E119V mutation was detected in nearly 50% of H3N2 samples tested at varying levels from 1% to 31%. The D151, R292K and N294S mutations were not detected in any of the samples and the majority of the H1N1pdm09 samples contained a low level (1-10%) of H274Y mutation in the viral quasi-species.

Declaration:

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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This thesis is being submitted in partial fulfillment of the requirements for the degree of MPhil.

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Contents:

1. Introduction:

Influenza A can result in complicated disease in high risk individuals and can lead to approximately 600 deaths in the UK each year (NHS, 2013). In the UK patients can be treated with the neuraminidase inhibitors, oseltamivir and zanamivir (Parry-Ford, 2013). Influenza viruses are highly prone to mutation due to their lack of a proof reading mechanism for RNA synthesis, during the replication process. When the neuraminidase inhibitors were first developed and introduced, single nucleotide point mutations were observed in challenge studies. The mutation of most interest was a change from CAT to TAT in codon 274, which resulted in an amino acid change form histidine (H) to tyrosine (Y), leading to a reduced susceptibility to oseltamivir in H1N1 viruses. An increasing number of clinical isolates containing this mutation was observed during the 2007-2008 season. Reference testing for the presence of this mutation in patient samples who do not improve when given neuraminidase inhibitor treatment can be performed, but can take at least 3 days for a result. It was agreed that it would be more cost effective and better for patient management if this testing could be performed on site at the Molecular Diagnostics Unit, part of Microbiology Cardiff at the University Hospital Wales. It was therefore decided to adapt and optimise pyrosequencing assays recently published by Deyde et al (2009b), and the assay in use by the Respiratory Virus Unit, at the Health Protection Agency, Colindale (now Public Health England). Performing this type of assay as part of the routine testing algorithm for influenza viruses would allow the administration of appropriate antiviral treatment, in a timely manner, leading to better care and outcomes for patient management.

1.1. Influenza Viruses:

Influenza viruses are members of the *Orthomyxoviridae* family. There are 3 genera of influenza, types A, B and C. The virions are pleomorphic and about 80-120 nm in diameter. They consist of a helical nucleocapsid encased in an envelope derived from the plasma membrane of its host-cell. The genome is segmented into 8 parts (7 for type C) of negative sense, single stranded RNA which code for the viral proteins (10 for type A, 11 for type B and 9 for type C).

Segment 1 codes for the PB2 protein which enables host-cell RNA cap binding. Segment 2 codes for the PB1 protein, which is involved in the initiation of transcription and catalyses nucleotide addition and segment 3 codes for the PA protein which enables the elongation of mRNA chains. These 3 proteins are all components of RNA transcriptase (Lamb & Choppin, 1983). Segment 4 codes for the surface protein Haemagglutinin (H). Segment 5 codes for the nucleocapsid protein (NP) which enables the RNA segments to form ribonucleoproteins (RNPs). Segment 6 codes for the other surface protein Neuraminidase (N). Segment 7 codes for the M1 proteins which line the viral membrane and the M2 ion channel proteins and segment 8 codes for the non-structural (NS) proteins, NS1 and NS2 (Figure 1) (Lamb & Choppin, 1983; White & Fenner, 1994; Wagner *et al*, 2008; Shors, 2013).

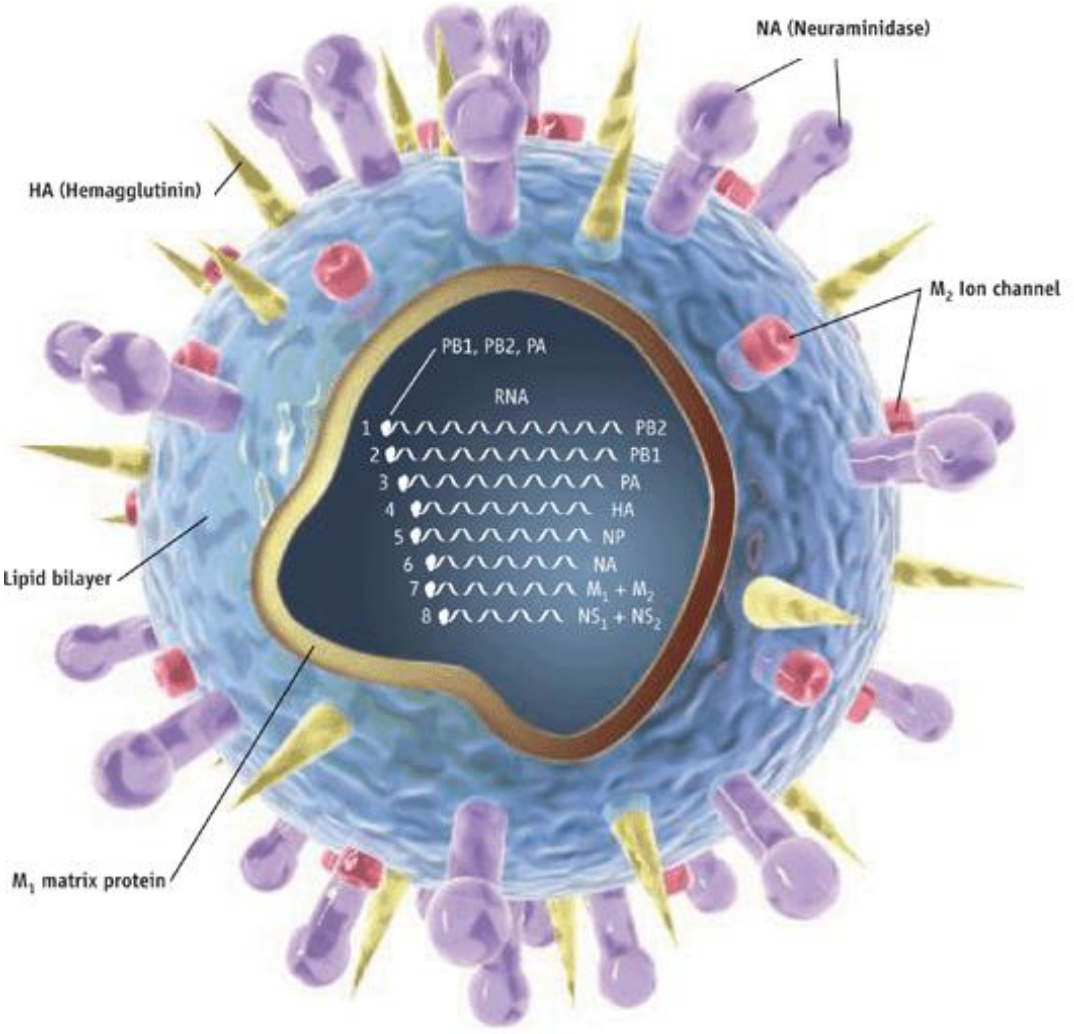

Figure 1 - Influenza virus (Kaiser, 2006). Illustration of generic influenza virus structure and genome segments.

Influenza A virus is further divided into subtypes. These are defined by the serotype of H and N proteins. There are 17 different H proteins and 10 N proteins (Shaw & Palese, 2013; Tong et al, 2013). The H protein is a 550 amino acid polypeptide which forms tetramers (spikes) on the cell surface (Figure 1) (Zambon, 1999). The N protein is a 200kDa tetrameric protein which forms mushroom shaped protrusions on the cell surface (Figure 1) (Colman, 1994). The amino acid sequence homology of the different subtypes can vary by more than 30-60% (Skehel, 2009).

The primary reservoir for influenza A viruses is waterfowl. Three of the H subtypes (H1, H2, H3) and only two of the N subtypes (N1 and N2) can naturally infect humans as they attach specifically to the α-2,6-galactose linkages on human epithelial cells rather than to the α-2,3-galactose linkages found in avian intestinal cells. Other H and N subtypes can also be found in pigs, horses, bats and seals (Shors, 2013; Tong et al, 2013).

Influenza A nomenclature is based on type, host, location, strain number and year of isolation for example for an avian strain of influenza isolated from a duck in 2009 in Cardiff would be given the name A/mallard/Cardiff/1/09 (H5N2). Human viruses are named in a similar way but without host type for example A/Cardiff/1/09 (H1N1).

Influenza B virus was isolated in 1940 (B/Lee/40) (Francis, 1940) and diverged into two lineages, Victoria and Yamagata in the 1980s (Rota et al, 1990) and is largely a human pathogen, although it has been reported to infect seals.

Influenza C virus was isolated in 1947 but is not considered to be a major human pathogen (Joosting et al, 1968), with 96% of individuals developing antibodies against influenza C by early adulthood (Wright et al, 2013).

1.2. Replication:

The H proteins on the surface of influenza A viruses bind to sialic acid molecules on the surface of ciliated columnar epithelial cells that line the sinuses and airway of humans with α-2,6 linkages or the intestines of fowl with α-2,3 linkages (Cross et al, 2001; Shaw & Palese 2013; Shors, 2013). Cell entry is predominantly achieved by clathrin-mediated endocytosis, but can also occur via a clathrin–

independent pathway (Lakadamyali et al, 2004; Shaw & Palese, 2013). The low pH of the endosome triggers cleavage of the H protein to expose the fusion peptide. The fusion peptide inserts itself into the membrane of the endosome fusing the virus and endosome membranes (Cross et al, 2001). H+ ions enter the virion, causing the release of the viral ribonucleoproteins (vRNPs), into the cytoplasm (Shaw & Palese, 2013; Shors, 2013).

The vRNPs are transported to the nucleus of the host cell, by nuclear localisation signals on the NS1 (Whittaker, 2001, Shaw & Palese, 2013)). The polymerase proteins PB1 and PA use nucleotides from the host cell as a primer to start viral RNA transcription, by first making a positive sense copy (complementary RNA (cRNA)), which is then used to make viral RNA (vRNA) (Shaw & Palese, 2013). The new viral segments are transported to the cytoplasm and translated into virus proteins. The new viral proteins are transferred to the cell membrane, where virion assembly takes place. One copy of each of the viral genome segments must be in each virion for it to be infectious. Release from the host cell is aided by the binding of H to the sialic acid-containing receptors on the host cell membrane. N binds to and cleaves the sialic acid from its receptor and releases the virus from the host cell (White & Fenner, 1994, Wagner *et al*, 2008, Shors, 2013).

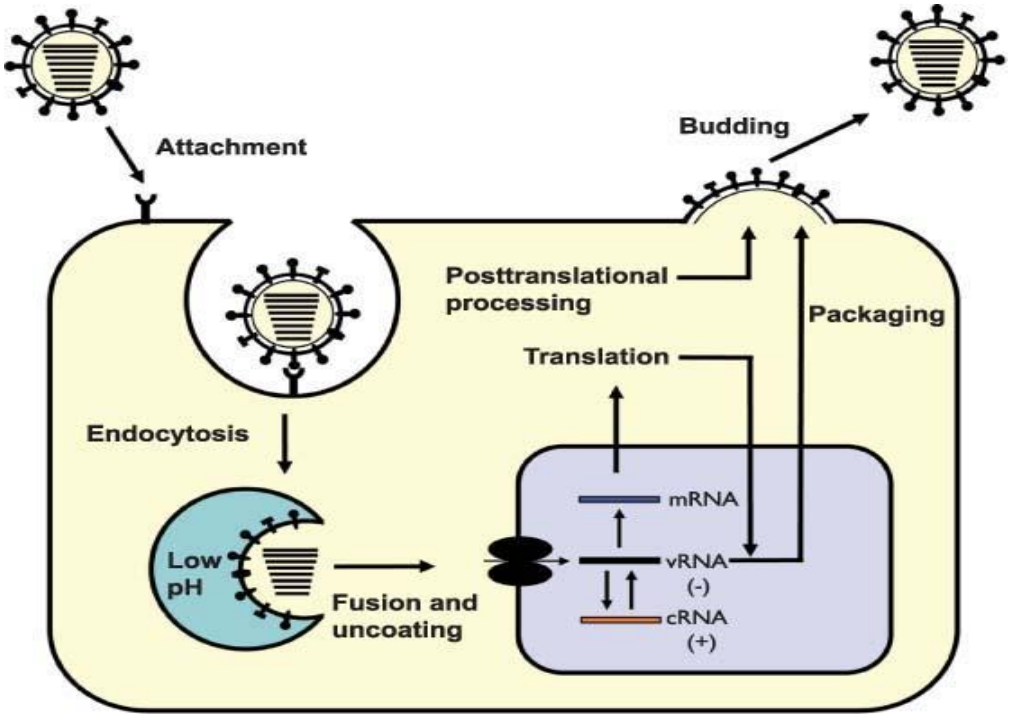

Figure 2 - Influenza Replication Cycle (Shaw & Palese, 2013). Diagramatic representation of the replication of influenza virus inside a host cell.

1.3. Disease:

Influenza A virus was first isolated from swine in 1930 (Shope, 1931) and from humans in 1933 (Smith et al, 1933). In 1947 the World Health Organization (WHO) set up an influenza surveillance programme to monitor global influenza activity (Zambon, 1999).

Classical influenza symptoms occur suddenly after a relatively short incubation period of 1-2 days and include; headache, high fever (pyrexia) (100-103^{o}F (37.8-39.4^{o}C) for 3 days), chills (rigors), weakness and lethargy (malaise), aching limbs and back (myalgia), a dry cough and sore throat (White & Fenner, 1994; Shors, 2013; Wright et al, 2013). Individuals may also suffer with blocked or runny nose with discharge (rhinorrhoea) and painful watery eyes (Carrat et al, 2008).

In uncomplicated influenza infections, symptoms usually resolve after 7 days but lethargy may last for a few weeks. The elderly can also present with lassitude, confusion and high fever without any respiratory symptoms (White & Fenner; 1994, Shors, 2013; Wright et al, 2013).

Secondary complications such as croup, ear infections, otitis media and secondary bacterial pneumonia, often caused by *Streptococcus pneumoniae* and *Haemophilus influenza* can be seen in high risk individuals such as children, the elderly, the immunocompromised and individuals with pre-existing medical conditions such as congestive heart failure and underlying conditions which affect the heart, lungs, liver, kidneys and the endocrine system (Shors, 2013; Wright et al, 2013).

Approximately 600 deaths each year in the UK are due to complications of influenza infection, and this can increase to around 13,000 during an epidemic (NHS, 2013).

1.4. Epidemiology:

The influenza season usually occurs in the winter months in temperate climates and during the rainy seasons in tropical climates. There are usually one or two strains that circulate in a particular region at any given time of the year (Shors, 2013). Localised epidemics occur due to the short incubation period of the

disease, and the high titre of viral shedding particularly in children (Carrat et al, 2008; Wright et al, 2013). Outbreaks are commonly associated with closed and semi-closed communities such as schools, hospital wards and nursing homes (Shors, 2013).

Due to the error prone nature of RNA polymerase activity the mutation rate of influenza is high at approximately 1.5×10^{-5} mutations per nucleotide per cycle. This is the equivalent of 2-3 amino acid changes in the H protein per year. This is known as antigenic drift and can occur in both influenza A and B viruses. When significant drift occurs in the extracellular H and N proteins this can give rise to a reduced immune response in the host leading to localised epidemics, even in populations with previous exposure to influenza A of similar sub-type or prior seasonal influenza vaccination (Hay *et al*, 2001; Shors, 2013; Zambon, 1999).

Antigenic shift is a dramatic change in the genome of influenza, caused by the reassortment of the genome segments. Only influenza A viruses can undergo antigenic shift due to their zoonotic nature. The most common mechanism for antigenic shift occurs when a human and non-human (usually avian) virus infect a pig simultaneously, as the sialic acid receptors for both human and avian influenza A viruses exist in the upper respiratory tract of swine. This can lead to the expression of new H and N proteins on the cell surface and cause pandemics if the general population have not been previously exposed to the new virus (Hay et al, 2001; Shors, 2013; Zambon, 1999). The last three pandemics have been due to re-assorted viruses (Wright et al, 2013).

1.5. Pandemics of the 20th and 21st Centuries:

There have been four major influenza A pandemics in the last 100 years, H1N1 in 1918 (“Spanish Flu”), H2N2 in 1957 (“Asian Flu”), H3N2 in 1968 (“Hong Kong Flu”) (Shors, 2013) and the recent H1N1pdm09 (“Swine Flu”) (Dawood et al, 2009). H1N1 re-emerged in 1977 and has since co-circulated with H3N2 as seasonal influenza (Hay et al, 2001) before being displaced by the emerging H1N1pdm09 strain in 2009.

1.5.1. "Spanish Flu" - 1918-19:

The origin of the H1N1 virus in 1918 is unknown but has been thought to be related to outbreaks of respiratory infections associated with high mortality and heliotrope cyanosis in Etaples, France, in 1916 and in Aldershot, UK in 1917 and an outbreak of influenza that led to pneumonia and death in young adults in Kansas, USA at the start of 1918 (Wright et al, 2013).

The first wave of the disease was mild, but highly contagious and spread via the railway and troopships across America and into Europe. The disease was named the "Spanish Flu" due to the unrestricted media coverage about the disease from Spain. A second wave started in late August 1918 with a high mortality rate, and a final wave began late 1918-early 1919 with morbidity rates similar to the second wave (Wright et al, 2013).

This pandemic was estimated to have killed 2.5% of infected individuals (the usual mortality rate for seasonal influenza is <0.1%), resulting in 20-50 million deaths worldwide (Shors, 2013, Wright et al, 2013). Influenza pandemics often cause a high mortality rate in children and young adults, and although the morbidity of the "Spanish Flu" was high in this age group the mortality rate was greatest in 15-35 year olds (Wright et al, 2013). This strain was closely related to the classic swine influenza virus and the sequences of the H and N proteins were related to an avian influenza virus that had not circulated in humans or pigs for many decades before the pandemic (Shors, 2013).

1.5.2. "Asian Flu"- 1957-58:

The "Asian Flu" first emerged in China in February 1957, and was isolated and identified as H2N2 in Japan, in May (Wright et al, 2013). It mainly infected 5-19 year olds and led to 1-4 million deaths worldwide. It was caused by a reassortment of avian H, PB1 and N segments with human influenza genes (Shors, 2013; Wright et al, 2013). This virus displaced the previously circulating influenza a H1N1 to become the seasonal influenza A type.

1.5.3. "Hong Kong Flu" - 1968:

During 1968 the H2N2 influenza A virus was displaced by an emerging H3N2 virus, which contained avian H and PB1 gene segments. The pathogenicity of this

virus was moderate compared to previous pandemics possibly as a consequence of residual antibodies to the N2 component in individuals exposed to the previously circulating H3N2 virus. As a result of this pandemic, an excess of 33000 deaths were recorded in the USA, and a high attack rate (40%) was observed in 10-14 year olds (Wright et al, 2013).

1.5.4. "Swine Flu" – 2009-10:

In April 2009, 2 unrelated cases of an influenza-like illness were reported in California, following reports of a major respiratory disease outbreak in Mexico (Dawood et al, 2009; WHO, 2009b). The virus causing these illnesses was discovered to be a new emerging triple-re-assorted strain of influenza A H1N1 (a mix of human (PB1 gene of H3N2), swine (NP and NS) and American (PB2 and PA) and Eurasian (N and M) avian influenza A gene segments (Wright et al, 2013)) which was genetically unrelated to the circulating human seasonal strain. The WHO announced a global pandemic on June 11, 2009. By the end of June 2009 this strain of virus had caused 70893 confirmed cases of influenza and 311 deaths worldwide (WHO 2009c).

1.5.5. Other Emerging Influenza Strains – 1997-2013:

In Hong Kong in the second half of 1997, 18 cases and 6 deaths of influenza caused by an avian strain of influenza A, H5N1 were reported. Until then there had never been a reported case of avian influenza that was fatal to humans. Unlike human adapted strains of influenza A direct human to human transmission was shown to be limited and as a consequence it was proven that for the first time an avian strain of influenza had jumped directly from bird to man without the intermediate swine host. This virus causes a severe influenza illness, and has been found in the stool and CSF of patients in addition to the respiratory organs demonstrating a more systemic infection. Due to the high mortality rate of 33% in 1997 (it is now 59%) concerns were raised that this virus could lead to a pandemic of great proportions. To date there have been 615 cases of avian flu in humans resulting in 364 deaths, throughout Asia, Southeast Europe and Africa (WHO, 2010; Wright et al, 2013).

A novel strain of "Avian Flu" has recently been identified as H7N9. The H7 subtype is often found in poultry worldwide but this is the first time it has been reported in humans. Since its isolation from 3 patients in March 2013, H7N9 has infected 132 individuals leading to 32 deaths. Human-human transmission has not yet been verified (Gao et al, 2013; WHO, 2013).

A triple-re-assorted H3N2 influenza A virus was identified in 2011, which included the M gene segment from the H1N1pdm09 strain and was antigenically distinct from seasonal influenza viruses (Lindstrom et al, 2012).

1.6. Laboratory Diagnosis and Detection of Influenza:

Clinical diagnosis of influenza can be difficult due to the similarity of symptoms to other respiratory infections and diseases. It is therefore preferable to have a laboratory diagnosis and confirmation of influenza or other respiratory infection so that appropriate action can be taken and where necessary effective treatment can be given to the patient.

Nasopharyngeal aspirates (NPA) and throat swabs are the preferred sample type for the growth of influenza in cell culture. Classical isolation techniques favoured cell lines such as primary monkey kidney cells and madin-derby canine kidney (MDCK) cells. Cytopathic effects or changes in the morphology of the infected cells are rarely seen in influenza so a marker of infection test was routinely performed called haemadsorption. When erythrocytes are exposed to viral haemagglutinin, which is expressed on the cell surface of the infected host cell or cell culture system, they become bound to the infected cell via the sialic acid receptor on the cell surface of the red cell. This phenomenon can then be visualised by standard microscopy (Figure 3) (Shelokov et al, 1958; White & Fenner, 1994).

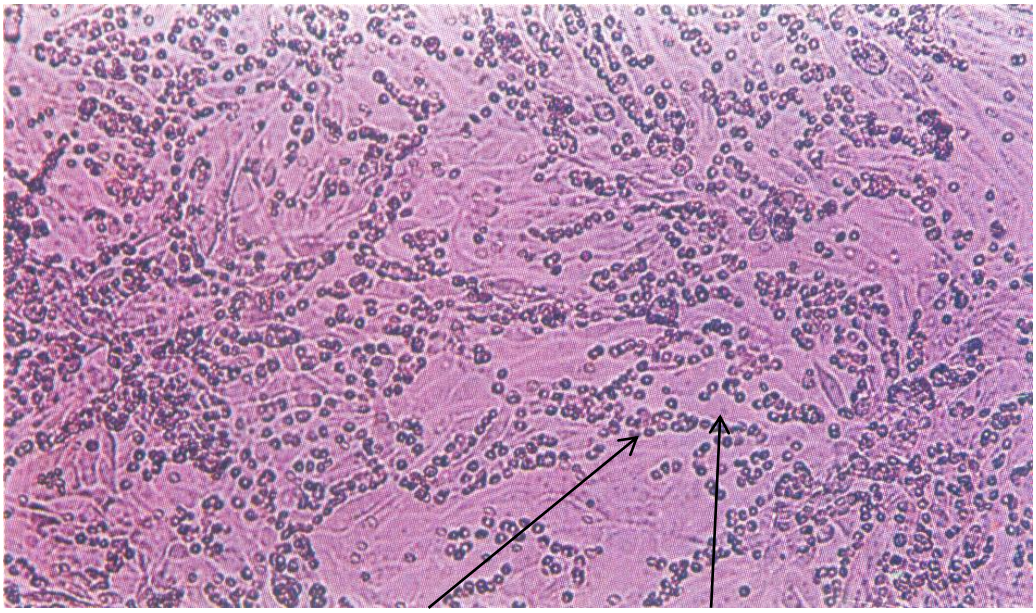

Figure 3 – Haemadsorption. A photograph of red blood cells adhering to cell culture infected with influenza. Magnification unknown. (http://dc231.4shared.com/doc/1OJVQm4w/preview.html).

A number of different viruses such as the parainfluenza viruses and mumps virus, also express haemagglutinin on the cell surface and so to confirm influenza infection a second confirmatory test was then performed, routinely using indirect or direct immunofluorescence, where a specific monoclonal antibody is tagged with a fluorophore and applied to the infected cell layer (Figure 4) (White & Fenner, 1994; Mims et al, 1999; PHW 2010; Shors, 2013).

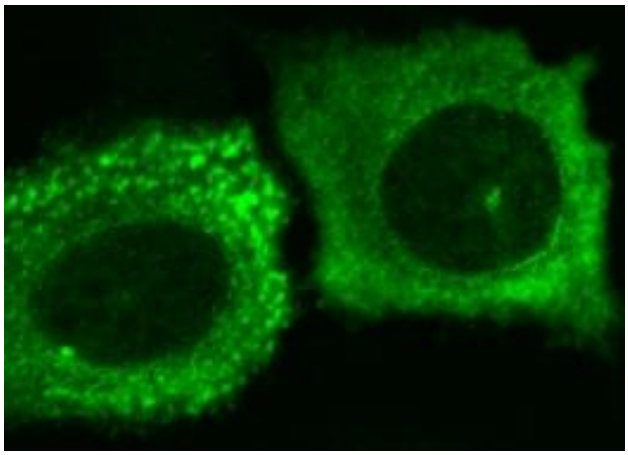

Figure 4 – Immunofluorescence. The staining of cells infected with influenza, using fluorescent labelled Anti-HA antibodies. Magnification unknown. (http://www.abcam.com/ha-tag-antibody-chip-grade-ab9110.html).

The whole process from inoculation of the cell line to confirmation by immunofluorescence could take from 5 -14 days, more recently rapid culture

techniques were developed to allow detection of respiratory viruses in 1-2 days. These are based on the principal of shell vial cell culture, the most commonly used commercial shell vial system is R-mix (Microgen Bioproducts, Camberley, UK), which are a mixture of human adenocarcinoma cells-A549 and mink lung cells. The cell monolayer is grown at the bottom of the vial and the sample is centrifuged onto it to enhance the infectivity. After incubation the samples are fixed and stained by immunofluorescence to target up to 8 different respiratory viruses (Landry, 2011).

More recently molecular based techniques have revolutionised the way influenza is diagnosed in the laboratory. The polymerase chain reaction (PCR) was first described by Kary Mullis in 1985 (Mullis & Faloona, 1987) and remains the basis for most routinely applied molecular assays. Although other molecular techniques have been described and applied for the detection of influenza viruses (Table 1).

Table 1 - Molecular methods suitable for the detection of influenza viruses (adapted from Ellis & zambon, 2002).

Method	Advantages	Disadvantages
Hybridisation	High specificity Flexible formats Inexpensive	Sensitivity, Time-consuming
PCR	Sensitive and specific Allows further product analysis	Qualitative, not usually quantitative
Multiplex PCR	Sensitive and specific Tests >1 target per assay Allows further product analysis	Requires extensive optimisation to ensure no false negatives or primer competition May be limited by product
PCR EIA	Sensitive and specific High throughput Not limited by product size Can be multiplexed	Does not allow further product analysis Requires additional evaluation/validation before use
Real-Time PCR	Sensitive and specific Rapid Can be quantitative Can be multiplexed	Requires specialised equipment Product analysis not always feasible
NASBA	Sensitive and specific Can be quantitative	Three enzymes used Time-consuming RNA product analysis less feasible

The initial step for any molecular technique is to produce good quality nucleic acid from the starting sample. In 1990, Boom described a method where starting

clinical material was exposed to a chaotropic reagent (guanidinium thiocyanate) to simultaneously release nucleic acid from the cell and to inactivate nuclease enzymes thus preventing nucleic acid degradation. The nucleic acid was then bound to silica particles which could then be exposed to decreasing solutions of salt buffers to remove all contaminating material. The 'pure' nucleic acid could then be unbound from the silica by addition of a very low salt concentration of salt buffer or nuclease free water. This 'Boom principal' of nucleic acid extraction was later modified and adapted into various manual extraction methods and finally automated to allow rapid nucleic acid extraction with a higher throughput.

To apply PCR for the detection of influenza virus having an RNA genome, a preliminary reverse transcription step is required to form a complimentary strand of DNA (cDNA) from the target viral RNA. Previously this was performed using a separate step often using random primers to form cDNA strands of all RNA present in the extracted nucleic acid. Specialised reverse transcription enzymes were available including avian myoblastomavirus (AMV) reverse transciptase. The resulting cDNA could then be used as the starting template for the PCR reaction (Ellis & Zambon, 2002).

The PCR reaction itself is primer driven in that short complimentary DNA primers flanking the target region (often a highly conserved part of the genome) of interest are designed to initiate amplification. For influenza several targets are widely selected including the nucleoprotein and matrix genes. This allows for generic amplification of the genome of all known circulating types of influenza A. Sub-typing of influenza A requires the targeting of the external H gene and in some cases the N gene to differentiate the different types (Mahoney et al, 2011).

Simplification of the methods and development of 'all in one' mastermixes containing the RT and PCR enzymes, the deoxynucleotide triphosphates (dNTPs) and salts have enabled this two-step process to become a single-tube process where the reverse transcription step and then amplification are performed one after the other without any extra manipulation. By adding in target specific labelled probes which anneal to the target amplicon as it is produced in the PCR reaction, the amplification process can now be monitored in real-time. Where

development and or validation of the primer and probe sets are performed by the testing laboratory these are commonly called in-house or homebrew assays (Mahoney et al, 2011).

Commercially available molecular assays for influenza are now commonly available and are increasing in popularity. During 2008, 3 commercial real-time RT-PCR assays for the detection of influenza were authorised for use in the USA by the Food and Drug Administration (FDA) (Landry, 2011).

Even more rapid diagnostics tools have since been developed and are called Point Of Care Tests (POCT). These tests are based on immuno-fluorescent or enzyme-linked immuno-sorbent (ELISA) assays. They can give a result in 10-30 minutes and can distinguish between influenza A or B, but can have a false negativity rate of about 30% (Shors, 2013; Tayo et al, 2012). More POCT are being developed with the aim to increase their sensitivity and be able to distinguish between influenza subtypes. These include RT-PCR aspects and specialised equipment (likely immobile), which would increase the cost, and the subsequent turn-around time for a result would increase to 45-60 minutes (Tayo et al, 2012).

1.7. Prevention:

Vaccination remains the most effective method for prevention and control of the morbidity and mortality associated with influenza infection. In the UK, persons in high risk groups and healthcare workers are offered vaccination from September onwards in preparation for the forthcoming influenza season (Schuffham & West, 2002).

Inactivated influenza vaccines are routinely used in the UK during the seasonal influenza vaccine campaign. They can be derived from whole virus, split virus or purified surface antigen preparations (Pillay et al, 1998; Matthews, 2006). Seasonal influenza vaccines were first developed in the 1940s and were monovalent. Bivalent vaccines were introduced in the 1960s and trivalent vaccines were introduced in the 1970's, and remain the vaccine composition of choice. They are composed of a currently circulating H1N1 and H3N2 strain of influenza A virus and one lineage of influenza B virus (Matthews, 2006). Quadrivalent vaccines are now approved for use in the USA, and include both

influenza B lineages (Neuzil & Victor, 2014). Live, attenuated influenza vaccines administered intranasally have more recently been introduced as part of the influenza vaccine campaign in healthy children in the UK (NHS, 2014). The composition of viruses used in the seasonal influenza vaccine is based upon the global surveillance of circulating strains. The data used to help with this decision is taken from; antigenic analysis of recently isolated viruses, epidemiological data and post vaccine serological studies (Shors, 2013).

Vaccine efficacy varies from 70–80% in healthy young adults but is less effective in the elderly. Despite this, evidence shows that vaccines help to reduce the severity of the disease if they don't manage to prevent infection (Donaldson and Donaldson, 2006).

1.8. Treatment:

Healthy individuals presenting with influenza are advised to stay at home and take analgesics (not aspirin due to the risk of Reyes syndrome in the young). However for high risk individuals and in complicated cases of influenza, antiviral agents such as the adamantanes and neuraminidase inhibitors may help to reduce the length and severity of the infection.

1.8.1. Adamantanes:

The first antivirals developed for the treatment of influenza were the adamantanes, Amantadine and Rimantadine. These drugs inhibit the action of the M2 ion channel in the virus envelope, preventing the pH reduction inside the virus to allow release of the RNPs. In higher concentrations, the adamantanes can also affect the acidity of the endosome and Golgi apparatus of the host cell, preventing the action of the M2 ion channels and activating H proteins too early in the replication cycle thus producing non-infectious progeny (Wingfield et al, 1969; Hay et al, 1985; Whittaker, 2001). Adamantanes work most effectively when taken prophylactically and are effective against 50-90% of influenza A viruses (Douglas, 1990). However, the side effects associated with this class of antivirals are considerable and occur in around 30% of cases when given as prophylaxis, they include; insomnia, concentration difficulties, hallucinations,

seizures and other CNS related effects, renal dysfunction and gastrointestinal symptoms (Douglas, 1990; Shors, 2013).

1.8.2. Neuraminidase Inhibitors:

As mentioned previously N is important in the binding to and release from host cells. Due to this role it was targeted as a possible site for new antiviral drugs. This was first investigated by Edmond et al in 1966, but they were unable to identify any potent compounds. Newer techniques and greater awareness has allowed for rapid development in this field.

Neuraminidase (N) cleaves N-acetyl neuraminic (Sialic) acid from α2-3 or α2-6 linkages on the host cell, thus destroying the cell receptor and releasing the newly formed virus particle from the host cell (Colman, 2009). The catalytic site (Figure 5) of neuraminidase has four invaginations accommodating the substituents at C_2, C_4, C_5 and C_6 on the Sialic acid (Varghese et al, 1992). The C_2 carboxylase is bound in a twist boat formation to a cluster of 3 arginyl groups (R) at positions 118, 292 and 371 on the N sequence. The C_5 binds to a tryptophan residue (W), at position 178. A cluster of hydrogen bonds bind to the C_6 glycerol group and the hydroxyl group at C_4. The C_8 and C_9 hydroxyl groups are bound by hydrogen bonds to a Glutamic acid residue (E) at position 276. Another amino acid believed to be part of the catalytic reaction is a tyrosine (Y) residue at position 406 (Colman, 2009). The above mentioned amino acids are believed to be conserved due to their importance in the catalytic reaction to enable viral release (Colman et al, 1983) and are supported by the substrate and framework residues E119, R156, W178, S179, D/N198, I222, E227 N294 and E425 (Yen et al, 2006).

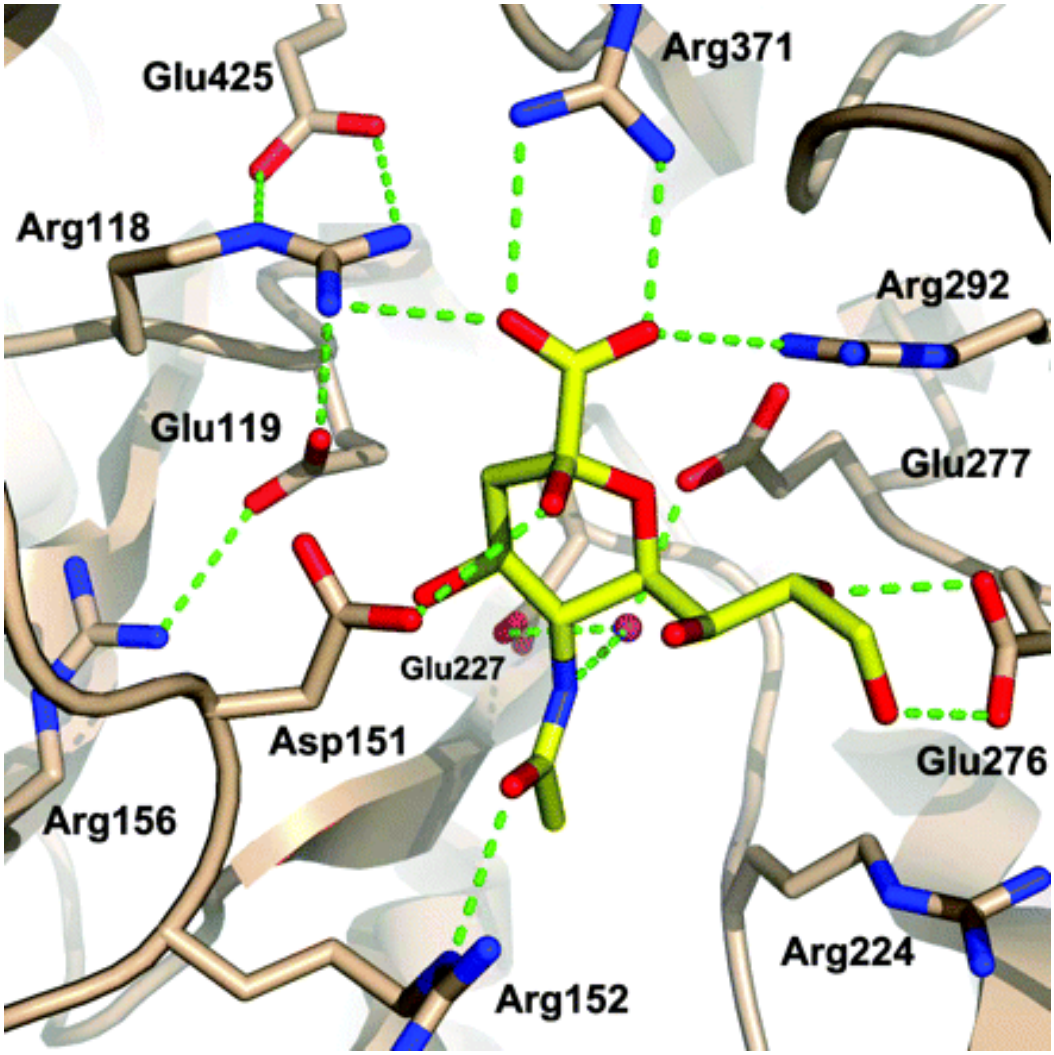

Figure 5- The active site of neuraminidase (Yen et al, 2006). A diagramatic representation of the active site of neuraminidase when it binds to sialic acid.

The product from the neuraminidase-sialic acid reaction produces α sialic acid or Neu5Ac (Figure 6). This product is the basis for the design of the modern neuraminidase inhibitors (NAIs), developed by rational, computer assisted drug design software such as GRID (von Itzstein et al, 1993). The action of the NAI's reduces the number of virus particles able to be released from the host cell to infect new cells (McKimm-Breschkin et al, 2003; Moscona, 2005).

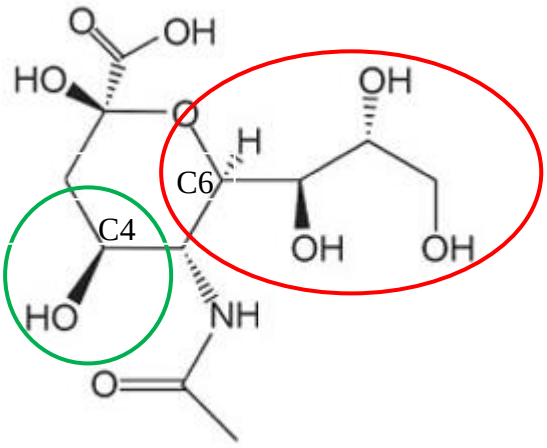

Figure 6 – Sialic acid (Das et al, 2010). The molecular structure of sialic acid.

1.8.2.1. Zanamivir:

Zanamivir (4-guanidino-Neu5Ac2en) (GG167) (Figure 7), commercially known as Relenza^{TM} (GlaxoSmithKline, London, UK), was the first neuraminidase inhibitor to be developed. It contains a guanidinyl group at C_4 (highlighted in Figure 7, the green circle) instead of the hydroxyl group of sialic acid (von Itzstein et al, 1993). This small structural change allows the inhibitor to bind in the active site and due to the lateral binding of the terminal nitrogen's of the guanidinyl group to E119 and E227 promotes better affinity (von Itzstein et al, 1993; Colman, 1994). Zanamivir is given by inhaler and has been shown to be approximately 1000 times more effective than amantadine and is effective against influenza A and B viruses (von Itzstein et al, 1993).

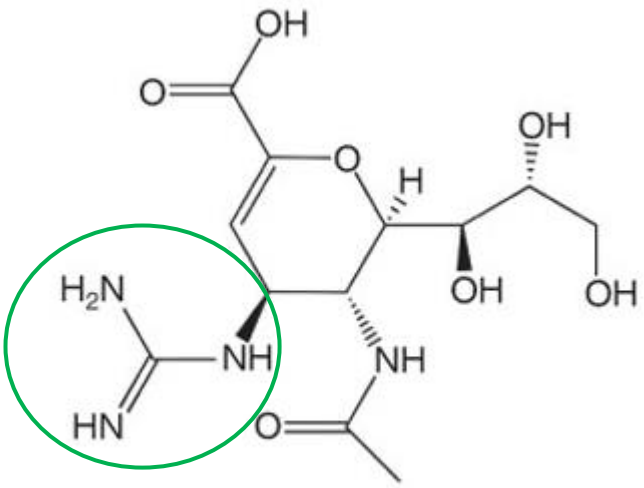

Figure 7 – Zanamivir (Das et al 2010). The molecular structure of zanamivir.

1.8.2.2. Oseltamivir:

Oseltamivir (GS 4104) (Figure 8), commercially known as Tamiflu™ (Roche, Basel, Switzerland), was developed in 1998 and was the first orally bioavailable NAI (Li et al, 1998). Oseltamivir has an amino group at C_4 (Figure 8 - green circle) and a 3-pentyloxy group instead of the glycerol group at C_6 (Figure 8 - red circle). Oseltamivir also promotes higher binding affinity than sialic acid. To allow oseltamivir to bind to the active site in the neuraminidase, the binding site must create a pocket for the large side chain of oseltamivir. This is achieved by the rotation of E276 which then binds to R224 (Moscona, 2005).

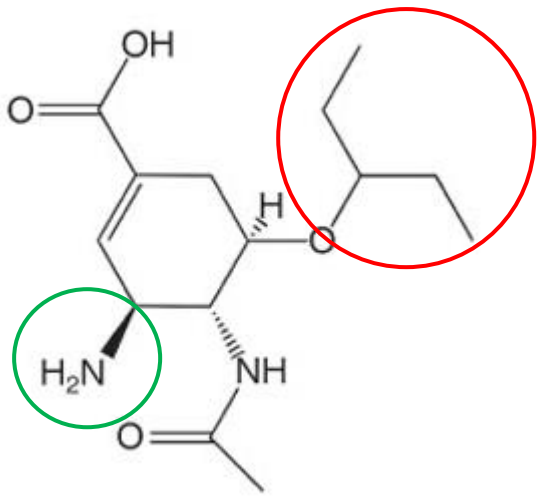

Figure 8 – Oseltamivir (Das et al 2010). Molecular structure of oseltamivir.

The similar modes of binding for oseltamivir (green) and zanamivir (yellow) to the sialic acid substrate (grey) can be seen in figure 9.

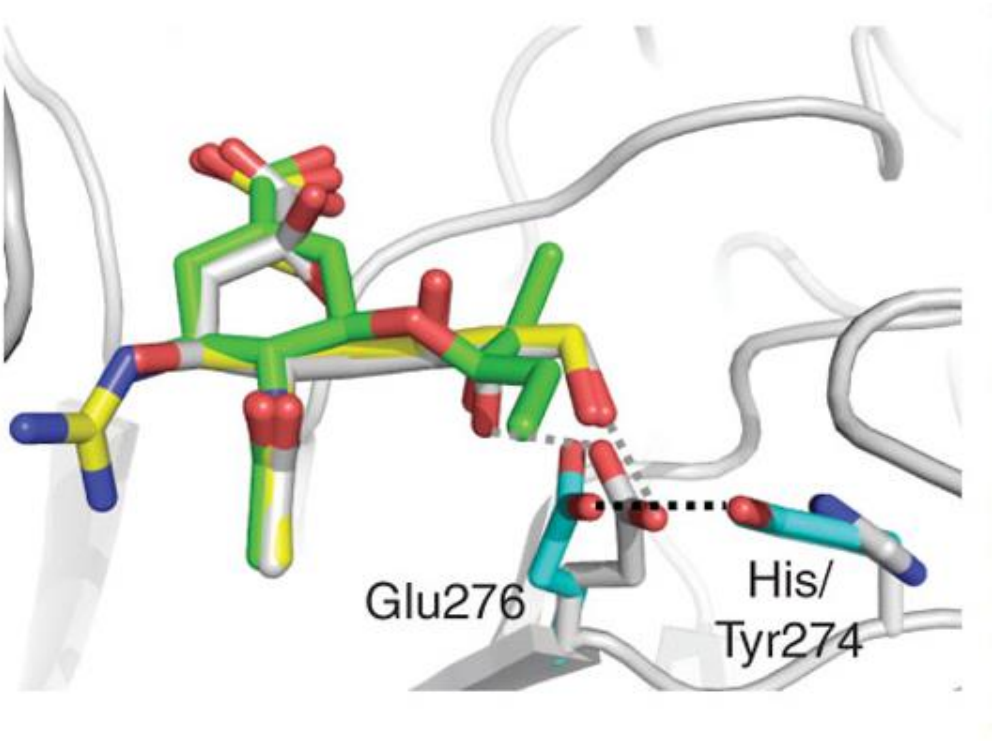

Figure 9 – Binding of neuraminidase inhibitors with neuraminidase (Das et al 2010). Diagramatic representation of the similar modes of binding for oseltamivir, zanamivir and sialic acid.

1.8.2.3. Use of NAIs:

NAI's were licensed and introduced between 1999 and 2002 for clinical use worldwide (McKimm-Breschkin et al, 2003).

For NAIs to be most affective they should be administered within 48 hours of symptom onset. As the drug itself is viral static preventing virion release, the drug needs to be given twice daily for a minimum of 5 days to allow for replication to be halted. The host immune response will then have time to develop antibodies to neutralise the virus and clear the infected cells.

In the UK, prescribing of NAIs is governed by the National Institute for Health and Care Excellence (NICE) who provide guidelines for the use of antivirals for

the treatment of influenza (TA168) (NICE, 2009). Prescribing is only recommended when influenza is circulating in the community and only given to patient groups who are at significant risk of severe infection, essentially all those groups who are entitled to receive annual seasonal influenza vaccination. Prophylaxis is only recommended in high-risk groups exposed to influenza and in outbreak management.

1.9. Resistance:

Resistance to the adamantanes was first described in H3N2 viruses during the 1970's and is associated with a point mutation at position 31 of the M2 gene which changes the amino acid residue from serine (S) to asparagine (N). This prevents binding of the drug to the M2 ion channel (Belshe et al, 1988). By the 2005-2006 influenza season in the US, 96.4% of H3N2 viruses tested were resistant to the adamantanes and the FDA no longer recommended the use of this class of antivirals for the treatment of H3N2 viruses. During this same season 15.5% of H1N1 viruses tested worldwide were also resistant to the adamantanes (Deyde et al, 2007).

The susceptibility of influenza to NAIs is usually measured as the drug concentration inhibiting neuraminidase activity by 50% (IC50). NAI resistance is defined as "a significantly raised IC50 value coupled with a characterised mutation in the neuraminidase gene" (Lackenby et al, 2008b).

Mutations that were noted to confer high level resistance or reduced NAI susceptibility from challenge studies and clinical isolates were H274Y (a point mutation at position 274 changing the amino acid code CAT for histidine (H) to TAT for tyrosine (Y)) in N1 viruses (Gubareva et al, 2001a), R292K (AGA for Arginine to AAA for Lysine) and to a lesser extent E119V (GAA for Glutamic acid to GTA for Valine) and N294S (AAC for Asparagine to AGC for Serine) in N2 viruses, (Gubareva et al, 1996; McKimm-Breschkin et al, 2003; Kiso et al, 2004). The most observed point mutation was at position D151 (Aspartic acid). This occurred in both N1 and N2 viruses and caused various amino acid changes (McKimm-Breschkin et al, 2003). At the time of these studies, it was hypothesised that these mutations would result in 'less-fit' viruses in that they would demonstrate poor growth and be poorly transmitted (Gubareva et al, 1997,

Tai et al, 1998, Covington et al, 2000, Wang et al, 2000, Gubareva et al, 2001a, Whitley et al, 2001, Carr et al, 2002, McKimm-Breschkin et al, 2003).

These preliminary studies demonstrated largely that all clinical isolates were susceptible to oseltamivir and zanamivir and there was no evidence for naturally occurring resistance. It was also shown that N1 viruses were more susceptible to zanamivir, and N2 viruses were more susceptible to oseltamivir (McKimm-Breschkin et al, 2003; Ferraris et al, 2005).

The Neuraminidase Inhibitor Susceptibility Network (NISN) was set up to monitor the development of resistance to NAIs after their introduction. Surveillance during the first 3 years of NAI use showed low levels of viruses with reduced susceptibility, 0.22% in the 1999-2000 season and 0.41% in the 2001-2002 season. The resistant viruses that were found showed a >10-fold decrease in their susceptibility to oseltamivir and in some cases to zanamivir (Monto et al, 2006). The amount of global resistance remained at a similar level over the next few years, 0% for the 2004-2005 season; 0.4% for the 2005-2006 season and 0.6% for the 2006-2007 season. A rise in resistance to oseltamivir was observed in H1N1 viruses from the start of the 2007-2008 season associated with the H274Y mutation (Sheu et al, 2008) with 14% of confirmed cases showing resistance in Europe (Lackenby et al, 2008a). Different geographical locations showed a range of 0-70% for H1N1 resistance to oseltamivir during the 2007-2008 season (Lackenby et al, 2008a; Deyde et al, 2009b) but by 2008-2009, 96% of all seasonal H1N1 viruses were demonstrated to have the H274Y mutation (NISN, 2010).

1.10. Detection of NAI Resistance:

Phenotypic drug susceptibility tests such as neuraminidase inhibition assays (NIA) and plaque reduction assays (PRA) are routinely used to monitor for the emergence of drug resistance. Tobita et al first described the growth of influenza in plaques in 1975. This method was modified by Hayden et al (1980) to develop drug susceptibility testing for Adamantanes. Influenza isolates are inoculated into and grown in cell culture. Once suspensions of 50-150 plague forming units (PFU) are present they are added to monolayers of MDCK cells grown in culture dishes and incubated for 60mins at 36^{o}C. An agarose overlay containing essential

medium and the appropriate dilution of the test drug is added and incubated for 36-48 hours at 36^{o}C. Plaques are then stained and compared to a control containing no drug (Hayden et al, 1980). Variations and modifications of these first assays have been performed to allow the phenotypic testing of neuraminidase inhibitors (Buxton et al, 2000; Gubareva et al, 2002; McSharry et al, 2004; Miller et al, 2006).

Genetic sequencing of the neuraminidase gene can be undertaken to determine the presence of mutations that commonly confer resistance to neuraminidase inhibitors predominantly using Sanger based sequencing methods (Sanger et al., 1977). Sanger sequencing is now largely performed on automated systems, following an initial PCR of the target region. These are divided into 2 types, gel based systems which separate fluorescently labelled DNA fragments by electrophoresis through a denaturing polyacrylamide gel and capillary based systems which electrophorese the samples through an array or single capillary which is filled with polyacrylamide or other specially developed polymers (Graham and Hill, 2001).

1.10.1. Pyrosequencing:

Pyrosequencing was developed by Ronaghi et al in 1996, this method was based on the enzymatic luminometric inorganic pyrophosphate (PPi) detection assay (ELIDA) developed by Nyren in 1987.

Before the pyrosequencing reactions can take place, the target sequence needs to be amplified using a primer set which has one primer biotinylated. The amplified product is then mixed with streptavidin beads. The biotin label will enable the binding of the double stranded target nucleic acid to the beads (figure 10a). The beads are captured onto filter probes using a vacuum (figure 10b). The double stranded nucleic acid is then washed and denatured to leave the biotin labelled nucleic acid strand attached to the beads (figure 10c). A sequencing primer is then annealed to the single stranded biotin labelled nucleic acid (figure 10d).

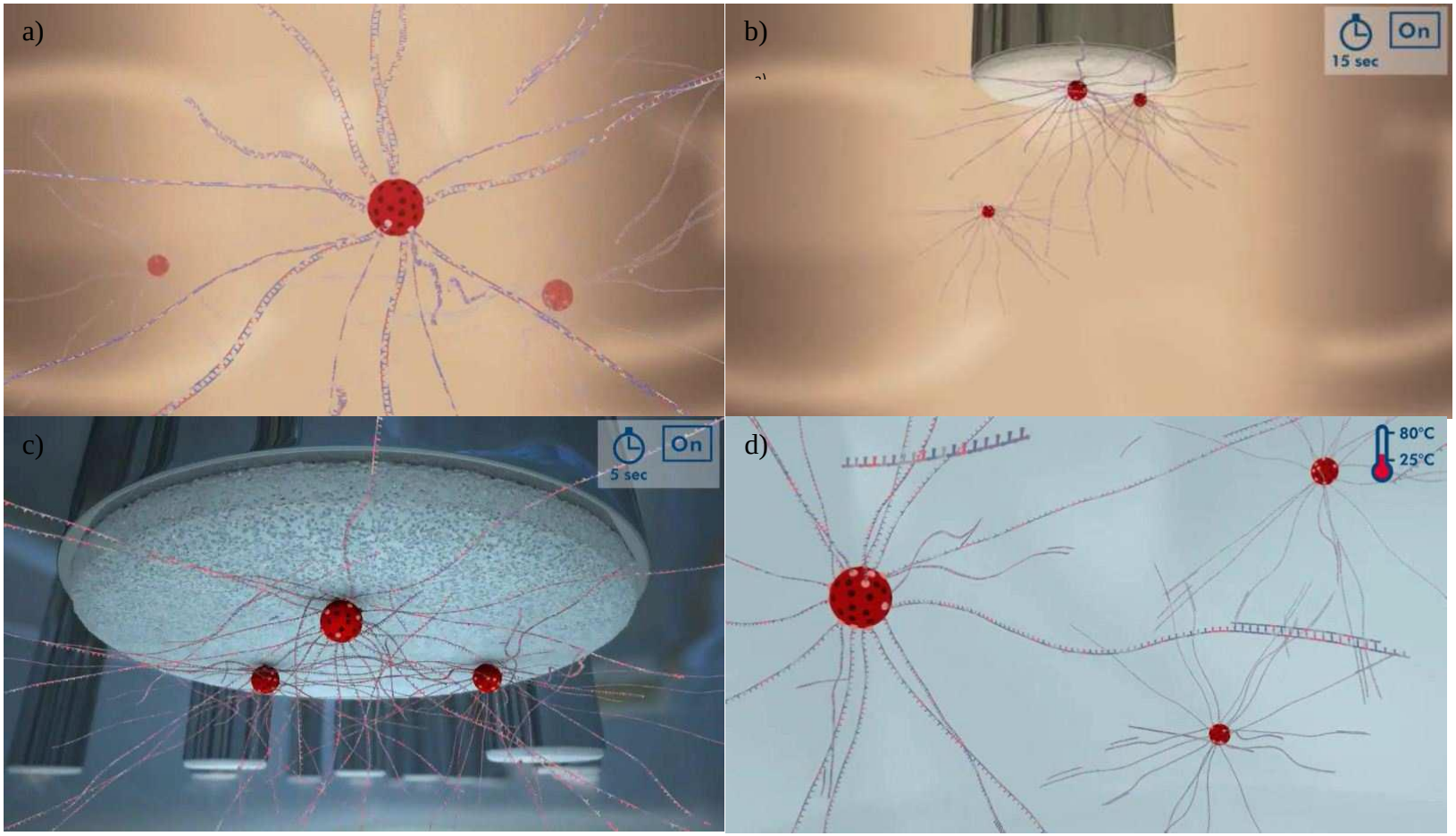

Figure 10 - Representation of the stages of DNA preparation before addition to the pyroseqencer (Qiagen, 2014).
a) Binding of biotinylated strands to streptavidin beads. b) Vacuum collection of streptavidin beads. c) Washing of beads to create single stranded DNA. d) Annealing of sequencing primer to single stranded DNA.

Pyrosequencing is a rapid, real time sequencing method involving a cascade of enzymatic reactions (Figure 11).

The sequencing primer and single stranded DNA target from the preparation stage is mixed with the substrates luciferin and adenosine 5' phosphosulfase (APS), and the enzymes apyrase, luciferase, adenosine triphosphate (ATP) sulfurylase and DNA polymerase. dNTP's are added to the reaction one at a time. If the dNTP is complementary to the base in the target it is incorporated into the DNA strand by the action of DNA polymerase. In response to each addition of dNTP to the DNA strand, pyrophosphate (PPi) is released. PPi is then converted by ATP sulfurylase into ATP in the presence of APS. ATP catalyses luciferase, leading to the conversion of luciferin to oxyluciferin, and the production of light. The amount of light produced is measured by a charge-coupled device (CCD) camera and is produced as a peak on a graph known as a pyrogram™ (Ronaghi et al, 1996; Ahmadin et al, 2000).

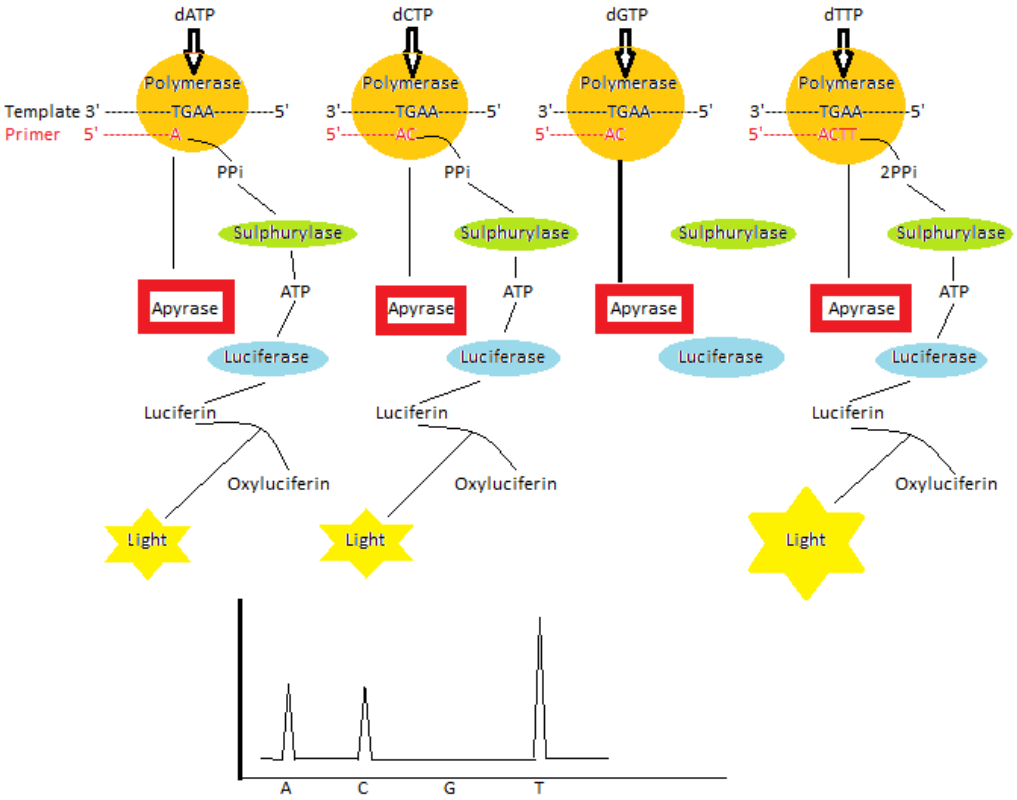

Figure 11- The pyrosequencing cascade (Adapted from Ahmadin et al, 2006). Schematic diagram of the enzyme cascade which occurs to produce a pyrogram.

A pyrosequencing assay was developed in 2005 for the detection of resistance to the adamantanes (Bright et al, 2005). This lead to the development of a similar assay for the detection of NAI resistance, once increased resistance to oseltamivir in H1N1 viruses was observed. The first pyrosequencing assays for NAI resistance have been concentrating on detecting only the H274Y mutation in H1N1 viruses (Duwe & Schweiger, 2008; Deyde et al, 2009a; Bolotin et al, 2009; NISN, 2010).

More recently Deyde et al (2009b) described pyrosequencing assays to detect all mutations known to confer reduced susceptibility to the NAIs in seasonal influenza A viruses (Deyde et al 2009b).

1.11. Aims and Objectives:

Due to the increased emergence of influenza viruses resistant to the neuraminidase inhibitors and the development of new rapid sequencing assays it was decided that these assays could be used as a routine diagnostic test.

The primary aims of this study were:

- to develop and optimise assays based on published methods for use as a series of rapid, routine diagnostic assays to determine the presence of mutations within the neuraminidase gene that confer resistance to oseltamivir and zanamivir in influenza A.
- to optimise the assays so that results could be achieved rapidly directly from clinical samples and not from cultured virus.

This would include assays for:

- The H274Y mutation in H1N1 viruses.
- The mutations at position D151 in H1N1 viruses.
- The E119V, D151 and R292/N294 mutations in H3N2 viruses.
- The H274Y mutation in H1N1pdm09 viruses.

Once assay optimisation was achieved, a clinical validation was performed on a series of clinical samples received during the 2008-2009, 2009-2010 and 2010-2011 seasons.

2. Materials and Methods:

2.1. Control Material:

Stock influenza A reference strains, A/Moscow/10/99 (H3N2) and A/New Caledonia/20/99 (H1N1) were inoculated into PLC cells (human liver hepatoma cells, Alexander cell line). Briefly; the reference strains were retrieved from long term storage in liquid nitrogen and thawed at 37^{o}C. 100µl of each reference strain was added to 2 vials containing PLC cells and growth medium. The inoculated cell vials were incubated in a sloped (approximately 80^{o}) stationary position to ensure inoculation of the cell line, at 37^{o}C for 6 days. The 2ml of media was changed every alternate day to maintain conditions for growth.

Haemadsorption was undertaken at 6 days to confirm the presence of influenza A virus (see Shelokov et al, 1958) using human O type red cells, supplied by the Blood Transfusion Services at University Hospital Wales (UHW), Cardiff. Briefly; approximately 15ml of human O type red blood cells were centrifuged at 1500rpm for 3 minutes. The supernatant was removed and the cells were re-suspended in sterile phosphate buffered saline (PBS). This stage was repeated 2 more times. After washing the supernatant was removed and discarded. 75µl of the packed red cells were added to 20ml of PLC maintenance media. The media from the influenza culture tubes was removed and saved. 0.5ml of the PLC media diluted O cells was added to the influenza culture tube. The tube was incubated at 4^{o}C in a sloped (approximately 80^{o}), stationary position for 20-30 minutes. The tube was then examined for the presence of haemadsorption using a light microscope at approximately 200x magnification. The infected cells were harvested to be used in the optimisation of the pyrosequencing assays. Briefly; the cell vials are scraped using a pastette to remove the cells from the vial. The cells are then suspended in maintenance media and frozen at -80^{o}C. One drop of the harvested cells was used to perform direct immunofluorescence to confirm the presence of influenza A. Briefly; a drop of suspended cells was placed into a well on a glass slide and allowed to dry on a heat plate. The slide was fixed in acetone for 2-5 minutes. Excess acetone was allowed to evaporate before the addition of 15µl of influenza monoclonal antibody. The slide was incubated in a moist box

for 15 minutes at 37^{o}C. The slide was rinsed in a steady stream of PBS and allowed to dry at 37^{o}C. Mounting fluid and a cover slip was applied before examination using an UV microscope.

The control material was also tested using the RT-PCR assays for influenza detection and the subtyping assay (see sections 2.3 and 2.4) to confirm influenza A and the subtype of each strain used and to record their crossing thresholds (which was taken as a semi quantitative measure of material concentration).

2.2. Clinical Isolates:

Clinical samples including upper and lower respiratory samples (nose and throat swabs and nasopharyngeal aspirates and bronchial alveolar lavages) received for routine diagnostic testing by molecular techniques in the PHW Microbiology Cardiff, Molecular Diagnostics Unit during the 2008-2009, 2009-2010 and 2010-2011 seasons which tested positive for influenza A virus were selected and anonymised for this study.

2.3. Nucleic Acid Extraction from Control Material and Clinical Samples:

Briefly, 200µl of control material, liquid sample or the dry respiratory swab was added to 0.9ml lysis buffer (bioMérieux, France), 200µl was then transferred to 2ml lysis buffer for total nucleic acid extraction using the NucliSENS® EasyMAG® (bioMérieux, France) automated extraction system, which is based on the Boom nucleic acid procedure as described previously. The purified nucleic acid was eluted into a volume of 60µl. The nucleic acid was stored at -80°C until processing.

2.4. Generic Influenza A Detection by RT-PCR:

During the course of this project a new in-house influenza A RT-PCR assay was introduced for the ABI Fast 7500 (Applied Biosystems®, Life Technologies™, California, USA), which was optimised from the protocol described by the Centers for Disease Control and Prevention (CDC) in 2009 utilising the TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems®, Life Technologies™, California, USA).

The assay was performed by adding 5µl of eluate to 20µl of RT-PCR mastermix containing; 62.5µl TaqMan® Fast Virus 1-Step master mix, primers at a final concentration of 0.8µM of each primer and a final concentration of 0.4µM of probe and 124µl RNAse free water (Ambion®, Life Technologies™, California, USA) (these values are for 10 reactions). Amplification was performed on the ABI Fast 7500 using the following amplification parameters 50°C for 5 minutes, 95°C for 20 minutes and 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

All samples and control material were tested by the new protocol to verify the previous results and to record the crossing threshold (CT) values of each sample. This assay is not quantitative, however the CT value can be used as an indicator as to the amount of virus present. A low CT value (e.g. 20) can mean the presence of a high number of virus in the sample, whereas a high value (e.g. 39) is suggestive of a low number of virus present or a poorly taken or stored sample.

Sub-typing of influenza A positive samples was achieved using an assay based on those described by the health protection agency (HPA, now Public Health England, PHE), specifically targeting the H3 and H1 genes and optimised to use the same reagents and cycling conditions as the generic assay above.

Table 2 – Primers and probes used for the identification and subtyping of influenza in clinical isolates using the ABI 7500 Fast.

Influenza Primers	Sequence name	Sequence
Generic influenza A	Forward Primer	5’ GACCRATCCTGTCACCTCTGAC
	Reverse Primer	5’ AGGGCATTYTGGACAAAKCGTCTA
	Probe	5’ 6-FAM-TCGCTCACTGGGCACG-MGB
H1 subtyping	Forward Primer	5’ GGAATAGCCCCCTACAATTG
	Reverse Primer	5’ AATTCGCATTCTGGGTTTCCTA
	Probe	5’ VIC-CGTTGCCGGATGGA-MGB
H3 subtyping	Forward Primer	5’ CCTTTTTGTTGAACGCAGCAA
	Reverse Primer	5’ CGGATGAGGCAACTAGTGACCTA
	Probe	5’ 6-FAM-CCTACAGCAACTGTTACC-MGB
H1 pdm09	Forward Primer	5’ TTACCAGATTTTGGCGATCTAYT
	Reverse Primer	5’ CCAGGGAGACTASCARTACCA
	Probe	5’ 6-FAM-ACWGTCGCCAGTTC-MGB

All assays used in clinical diagnostic laboratories are subjected to routine assay checks called proficiency panels. These panels check and ensure that all the laboratories across the country are detecting the correct results and that there are no faults or discrepancies in the assays. The PHW Microbiology Laboratories subscribe to 2 schemes, the National External Quality Assurance Scheme (NEQAS) and the Quality Control of Molecular Diagnostics Scheme (QCMD). The PHE also send out proficiency panels for the molecular detection of influenza during the respiratory season.

The QCMD and PHE proficiency panels for 2010 were tested by the above methods and also through the H1N1pdm09 pyrosequencing assay as a method of validation.

2.5. Amplification Primer Selection and Optimisation:

Primers were selected to allow the amplification of targeted regions of the neuraminidase gene segment which include the point mutations mentioned previously.

2.5.1. Amplification Primer Selection:

Amplification primers from several publications were selected for the study including H274Y and D151 point mutations in N1 viruses and E119V, D151 and R292/4 point mutations in N2 viruses described by Deyde et al (2009b). In addition, primers described by the HPA and those described by the WHO for the H274Y mutation in H1N1pdm09 viruses (WHO, 2009a). These primer sets allow the amplification of biotinylated DNA fragments of the neuraminidase gene segment. The annealing sites for the various primers were mapped to the neuraminidase gene sequences of the control influenza viruses and recently circulating clinical strains (see appendix I & II).

Table 3 – Amplification primers.

Influenza Mutation	Sequence name	Sequence
H1N1 H274Y	HuH1N1- 274-	5'-AGATCGAGAAGGGGAAGGTTACTA-3'

113bp	F770	
	HuH1N1- 274-R882-biot	5’-GTCYCTGCATACACACATCACT-3’
H1N1 D151 117bp	HuH1N1-151-F425	5’-ACAAACATTCAAATGGRACCG-3’
	HuH1N1-151-R521-biot	5’-CTGACCATGCAACTGATTCAA-3’
H3N2 E119V 584bp	HuH3N2-F333-biot	5’-TGGGGACATCTGGGTGACA-3’
	HuH3N2-R914	5’-ATATCTACTATGGGCCTATTGGA-3’
H3N2 D151 145bp	HuH3N2-F423	5’-CAACGTGCATTCAAATGACAC-3’
	HuH3N2-R567-biot	5’-CCAYGCTTTYCCATCRTG-3’
H3N2 R292/N294 584bp	HuH3N2-F333-biot	5’-TGGGGACATCTGGGTGACA-3’
	HuH3N2-R914	5’-ATATCTACTATGGGCCTATTGGA-3’
H1N1 H274Y (HPA) 301bp	H1N1 PCR3 Forward	5’-GGAGCCGTGGCTGTACTAAAATA-3’
	H1N1 PCR3 Reverse-biot	5’-CCACGTTTTGATTAAAAGACACC-3’
H1N1pdm09 H274Y 494bp	Uni-sw-N1-B-F780	5’-GGGGAAGATTGTYAAATCAGTYGA-3’
	Uni-sw-N1-B-R1273-biot	5’ CWACCCAGAARCAAGGYCTTATG-3’

2.5.2. Amplification Primer Optimisation:

All of the primers selected for the study were compared to consensus sequences of current circulating influenza A strains (see appendix). Briefly, N1 and N2 sequences were downloaded from Genbank and aligned using the online version of ClustalW http://www.ebi.ac.uk/Tools/msa/clustalw2/. The alignment of the

reverse E119V primer showed that some of the more recent circulating strains of H3N2 possessed a base change, C instead of A, in the middle of the binding site for the primer. Therefore a new primer was designed with the degeneracy K:

5'-ATATCTACKATGGGCCTATTGG-3'.

All primers used in the study were sourced from Eurogentec (Belgium).

2.5.3. Annealing Temperatures:

To further optimise the assay the annealing temperatures were calculated to ensure the correct PCR conditions were used for the primers that were utilised.

The Basic Melting Temperature (Tm) for sequences can be calculated approximately using the following calculation:

Tm= (wA+xT) * 2 + (yG+zC) * 4

where w,x,y,z represents the number of the bases A,T,G,C in the sequence, respectively.

Table 4 - Annealing temperatures for the primers used for amplification.

Influenza Mutation	Sequence name	Tm
H1N1 H274Y	HuH1N1-274-F770	5'-AGATCGAGAAGGGGAAGGTTACTA-3' =72
	HuH1N1-274-R882-biot	5'-GTCYCTGCATACACACATCACT-3' =64-66

H1N1 D151	HuH1N1-151-F425	5’-ACAAACATTCAAATGGRACCG-3’ =60-62
	HuH1N1-151-R521-biot	5’-CTGACCATGCAACTGATTCAA-3’ =60
H3N2 E119V	HuH3N2-F333-biot	5’-TGGGGACATCTGGGTGACA-3 =58’
	HuH3N2-R914	5’-ATATCTACTATGGGCCTATTGGA-3’ =64
H3N2 D151	HuH3N2-F423	5’-CAACGTGCATTCAAATGACAC-3 =60
	HuH3N2-R567-biot	5’-CCAYGCTTTYCCATCRTG-3’ =50-54
H3N2 R292/N294	HuH3N2-F333-biot	5’-TGGGGACATCTGGGTGACA-3 =58’
	HuH3N2-R914	5’-ATATCTACTATGGGCCTATTGGA-3’ =64

The annealing temperature stated in the amplification reagent kit protocols was 60^{o}C. This temperature is optimal for the majority of the primer sets but could possibly be too high for the N2 D151 primers. Therefore the annealing temperature for the amplification of the N2 D151 DNA fragment was altered to 55^{o}C to create better amplification conditions for this target.

2.5.4. Gel Electrophoresis:

Gel electrophoresis was performed on a small proportion of the N2 amplicons to check the size (584bp for the E119V and R292/N294 and 145bp for the D151) and quality of the amplified product.

A 2% agarose gel was made and loaded with 5µl of DNA ladder or product. The DNA was electrophoretically separated with a constant voltage of 90 Volts for 45-60 minutes.

2.6. Amplification Optimisation:

2.6.1. Superscript III RT/Platinum Amplification:

The Deyde study (2009b) recommended the use of Superscript III RT/Platinum reagent kit (Invitrogen™, Life Technologies™, California, USA).

Amplification was undertaken using the following reaction master mix: 1µl superscript enzyme, 25µl 2x reaction mix, 1µl of each 20µM primer (final concentration of 0.4µM), 17µl of RNAse free water and 5µl of template. Amplification was performed on a Veriti thermocycler (Applied Biosystems®, Life Technologies™, California, USA) using the cycling parameters: 50°C for 15 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds and then held at 4°C.

2.6.2. Superscript III HiFi Amplification:

To try to improve the quality of the amplicon used for sequencing, a high fidelity enzyme based kit was selected for evaluation, this was the Superscript III HiFi amplification reagent kit (Invitrogen™, Life Technologies™, California, USA).

Amplification was undertaken using the same reaction mastermix composition as for the Superscript III RT/platinum reagent kit. Amplification was performed on a Veriti thermocycler using the cycling parameters: 55°C for 30 minutes, 94°C for 2 minutes, 45 cycles of; 94°C for 15 seconds, 60°C for 30 seconds and 68°C for 30 seconds and held at 4°C. The D151 N2 target was amplified using slightly different amplification parameters: 55°C for 30 minutes, 94°C for 2 minutes, 45 cycles of; 94°C for 15 seconds, 55°C for 30 seconds and 68°C for 30 seconds and held at 4°C.

2.6.2.1. Further Optimisation:

Further optimisation of the N2 assays was undertaken by simply increasing the template DNA from 5µl to 10µl and finally to 15µl and decreasing the water within the mastermix.

2.6.3. Qiagen One-Step RT-PCR Amplification:

During the course of this study, Qiagen (Limburg, The Netherlands) developed amplification reagents specifically designed for the Pyrosequencer.

Amplification was performed using One-Step RT-PCR reagents (Qiagen, Limburg, The Netherlands).

Briefly amplification was undertaken as follows: 5µl of One step RT-PCR Buffer 5x, 1µl of each 20µM primer (0.8µM final concentration), 1µl of dNTP mix, 1µl of One step RT-PCR Enzyme mix, 11µl of RNAse free water, and 5µl of template. Amplification was performed on a Veriti thermocycler using the cycling parameters: 50^{o}C for 30 minutes, 95^{o}C for 15 minutes, 45 cycles of: 94^{o}C for 30 seconds, 60^{o}C for 30 seconds, 72^{o}C for 30 seconds; 72^{o}C for 10 minutes and held at 4^{o}C.

Amplification using the One-step RT-PCR reagents was further optimised by increasing the amount of eluate added to 10µl and reducing the water to 6µl.

This protocol was also used for the detection of the H274Y mutation in H1N1pdm09 viruses.

2.7. Pyrosequencer:

Pyrosequencing was performed on the PyroMark Q96 ID (Qiagen, Limburg, The Netherlands), using the PyroMark Q96 ID Gold Reagents (Qiagen, Limburg, The Netherlands), which included lyophilised Enzyme and Substrate, which were reconstituted with RNAse free molecular grade water (Sigma, Missouri, USA) on day of use and dNTPs. The denaturation solution (sodium hydroxide), wash buffer, annealing buffer and binding buffer were specific for use with the PyroMark Q96 ID and supplied by Qiagen (Limburg, The Netherlands). The streptavidin sepharose beads were supplied by GE Healthcare Life Sciences (UK). All RNAse free Molecular Grade Water was supplied by Sigma (Missouri, USA) and Stock solution of 70% Ethanol was made with Absolute Ethanol (Fisher Scientific, Massachusetts, USA) and RNAse free molecular grade water.

2.7.1. Setting up an Assay on the Pyromark ID Software:

The PyroMark ID software was used to set up a single nucleotide polymorphism (SNP) run or a general sequencing assay (SQA).

A run name (eg: H1N1 run 1 date) and the parameters stated on the reagent cartridge were selected in the General Tab. The reagent cartridge holds the Substrate, Enzymes and separate nucleotides for the assays (See Figure 12):

	G	
C		T
S	A	E

Figure 12 - Diagramatic representation of the pyrosequencing cartridge layout.

The reagent cartridge used for each run has a specific set of pressure, time and temperature parameters for the machine to adhere to.

The volumes required for the reagent cartridge are calculated based on the number of times each nucleotide appears in the dispensation order and the total number of sequencing reactions. A simplex single nucleotide polymorphism (SNP) entry was programmed on the PyroMark ID software for each point mutation. The entry includes the target sequence and the position where the point mutation occurs. From this the software calculates the best dispensation order for the nucleotides.

A general sequencing assay (SQA) was also performed for some of the mutations to allow for determination of other changes at the same position. This requires a user defined dispensation order and TCGACTGA was selected based on the most commonly used order for sequencing. Rather than identify the SNPs the sequencing assay required user interpretation.

The wells to be used for the assay were selected on the plate map and activated. The assay type (eg: H274Y) to be run and the sample numbers were selected and placed on the plate map. The reagent volumes were noted and the assay was then saved until required to be run.

2.7.2. Immobilisation of PCR Product by Streptavidin Sepharose HP Beads:

Pyrosequencing reagents were used at room temperature. The streptavidin sepharose HP beads were homogenised by shaking the solution. An immobilisation solution was made up as follows: 3µl of streptavidin sepharose HP beads, 40µl Binding buffer and 17µl of RNAse free molecular grade water.

60µl of immobilisation solution was added to the wells of a PCR plate corresponding to the plate map on the PyroMark ID software. 20µl of PCR product was added to the immobilisation solution in the same order as stated on the plate map on the PyroMark ID software. The plate was sealed with a plate sealer and placed on a shaker for 5-10 minutes at 1400rpm to allow the biotinylated DNA amplicon to bind to the streptavidin beads.

2.7.3. Sequencing Primers:

The sequencing primers matching the assays described previously for the H274Y and D151 point mutations in N1 viruses and E119V, D151 and R292/4 point mutations in N2 viruses by Deyde et al (2009b), the HPA primer for the H1N1 H274Y mutation and the WHO primer for the H274Y mutation in H1N1pdm09 viruses (WHO, 2009a) were used in the study. These primers bind to the biotinylated single stranded DNA immobilized by the sepharose beads and allow the binding of dNTPs to produce the DNA sequence.

Table 5 – Pyrosequencing Primers.

Influenza Mutation	Sequence name	Sequence
H1N1 H274Y	HuH1N1-274-F807-seq	5’-AAATGCACCCAAT-3’
H1N1 D151	HuH1N1-151-F425-seq	5’-ACAAACATTCAAATGGRACCG-3’

H1N1 H274Y HPA	HuH1N1-274-F807-seq	5’-AGTTGAATGCACCCAAT-3’
H3N2 E119V	HuH3N2-R377-seq	5’-GGATCGCATGACACATA-3’
H3N2 D151	HuH3N2-F423-seq	5’-CAACGTGCATTCAAATGACAC-3’
H3N2 R292/N294	HuH3N2-R905-seq	5’-ATGGGCCTATTGCAGCC-3’
H1N1pdm09 H274Y	Uni-sw-N1-B-F804-seq	5’GYTGAATGCMCCTAATT-3’

2.7.4. Strand Separation and Sequencing

To 438µl of annealing buffer, 2µl of 100µM sequencing primer was added to give a final concentration of 0.4µM. 40µl of annealing solution was added to wells of a pyrosequencing (PSQ) low plate which corresponded to the plate map in the PyroMark ID software.

The Vacuum Prep Workstation was prepared to the manufacturer’s protocol briefly; position 1 was filled with 110ml of 70% ethanol, position 2 with 90ml of denaturation solution, position 3 with 110ml wash buffer, and position 4 and the parking position were filled with RNAse free molecular grade water.

The filter probes were washed with RNAse free molecular grade water from the parking position, with the use of the vacuum.

The PCR plate and a PSQ low plate were placed on the vacuum prep workstation and the beads from the PCR plate were captured by vacuum onto the filter probes. The filter probes were then washed in ethanol at position 1 for 5 seconds, denaturation solution at position 2 for 5 seconds, and then in washing buffer at position 3 for 10 seconds. All the fluid from the filter probes was then allowed to flush away before the vacuum was turned off.

The filter probes were then placed into the PSQ low plate containing the annealing solution and sequencing primer and agitated to release the beads. The PSQ low plate was then transferred into the PSQ 96 sample prep thermoplate low

on a heating block and incubated at 80^{o}C for 2 minutes. The plate was then removed and allowed to cool.

The PSQ low plate and the reagent cartridge were placed into the pyrosequencer, and the cover closed. The assay was then carried out.

The stages of pyrosequencing were described previously (section 1.10.1).

2.7.6. Result Interpretation:

The PyroMark ID software was used to generate a pyrogram for each sample tested. The pyrogram is a reading of relative light intensity released as each nucleotide base is incorporated to the sequence.

The software uses a default set of criteria (including width of peaks, signal to noise ratio and possible dispensation errors) to read the sequence generated for each sample and the viability of each sequence produced. According to the match of the results to the criteria, the software assigns a colour to the results. Blue for passed, Amber for check results, ie a person is needed to verify the results and Red for failed.

The software is able to calculate allele quantification or the percentage of sequence type present. For example a clinical sample of influenza can contain viruses with H (CAT) at position 274 and also viruses with Y (TAT) at position 274. This dual or multi population is known as a quasi-species, meaning that you have a number of viruses with differing genetic make-up. Therefore for the H1N1 assays a reading of C 5% and T 95%, shows that 5% of the population possesses, H (CAT) at position 274 and the remaining 95% of the population possesses Y (TAT) at position 274.

3. Results:

3.1. Assay Optimisation:

In order to establish robust methods for determining the presence of neuraminidase mutations it was essential to optimise methods from cDNA generation to pyrosequencing, in relation to the scientific literature and the protocols established by the commercial companies.

H1N1 and H3N2 control material was used to optimise all the assays relevant to their subtype. Three aliquots of each subtype with varying CT values were put through each of the amplification assays stated below, and then through their subtype pyrosequencing assays. Any invalid results were repeated at least once to check results were due to amplification and not technical issues.

The H274Y was the first assay to be optimised due to this mutation being the most relevant.

The H274Y assay for H1N1pdm09 viruses was optimised using samples only due to the absence of stock control virus and the abundance of samples.

3.1.1. Superscript III RT/Platinum Amplification Reagents:

The study initially utilised the Superscript III RT/Platinum amplification reagents to generate cDNA, and the amplification of the target sequence down stream of pyrosequencing. This was found to produce unsatisfactory results. Samples treated in this way did not produce amplified products of good quality for either the N1 or N2 mutation targets. Figure 13 represents a pyrogram resulting from the use of amplified product produced using the Superscript III RT/Platinum amplification reagents. The results obtained indicate that the sample tested did not contain enough amplified product, and that an alternate method of amplification should be considered.

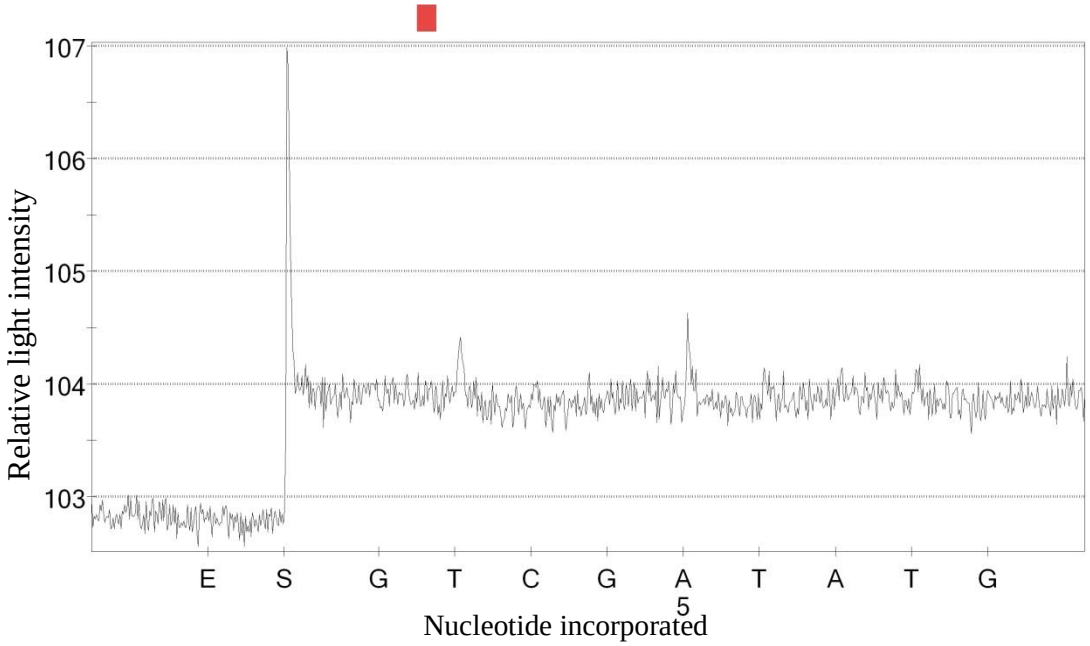

Figure 13 – Control sample 2 H1N1. A pyrogram representing the results obtained from using amplified product produced by the Superscript III RT/Platinum amplification reagents. This shows that a sequence has failed to be identified.

3.1.2. Superscript III HiFi Amplification Reagents:

In order to significantly improve the quality of the amplified product for downstream pyrosequencing, the use of Superscript III HiFi amplification reagents were assessed.

3.1.2.1. H274Y Assay for the Seasonal (pre 2009) N1 Viruses:

The H274Y assay for the seasonal (pre 2009) N1 viruses performed using the superscript III HiFi amplification reagents gave good quality reproducible results using the reference strains (Figure 14), indicating that this method was more effective in generating cDNA and high quality amplified products from clinical material than the previous method employed.

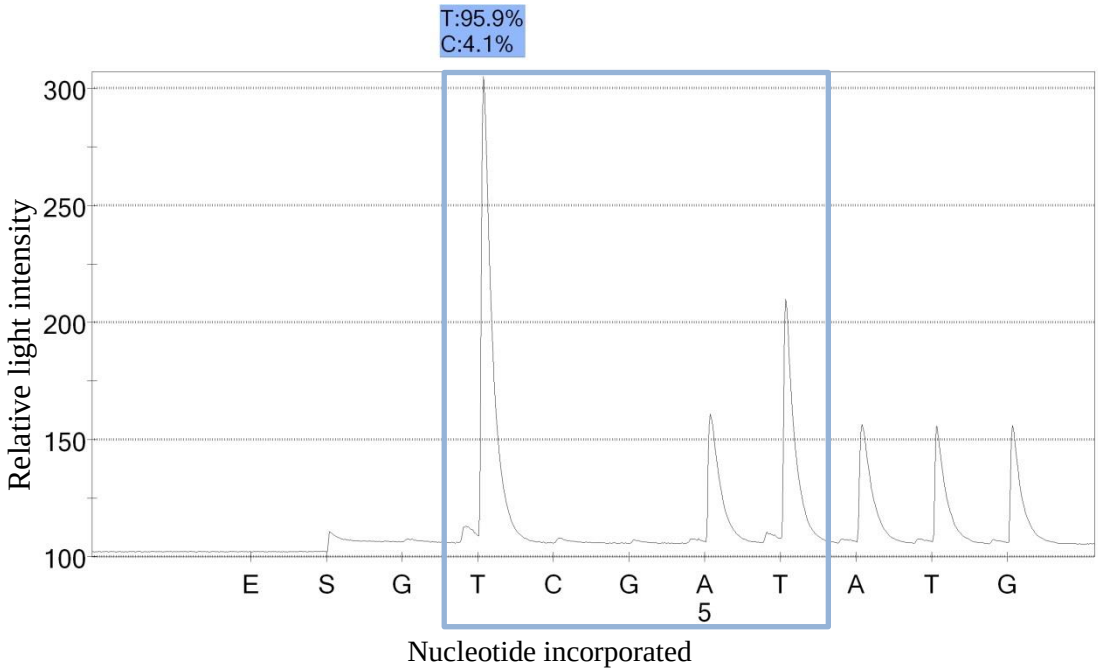

Figure 14 – Sample 25. A pyrogram showing successful amplification and pyrosequencing. Sequence readout: TTC/TATTATG, mutation present in 96% of the quasi-species.

3.1.2.2. D151 Assay for N1 Viruses:

The D151 protocol using the Superscript III HiFi amplification reagents could not be optimised for routine use. The SNP analysis produced erroneous pyrograms, with wide peaks (Figure 15). Further optimisation work could not be undertaken in the time-frame of the study.

This error usually results from the presence of too much nucleic acid for the used enzyme/substrate activity or a slow degradation of the pyrophosphate from the previous nucleotide incorporation, caused by decreased performance/activity of the enzyme (Qiagen 2013).

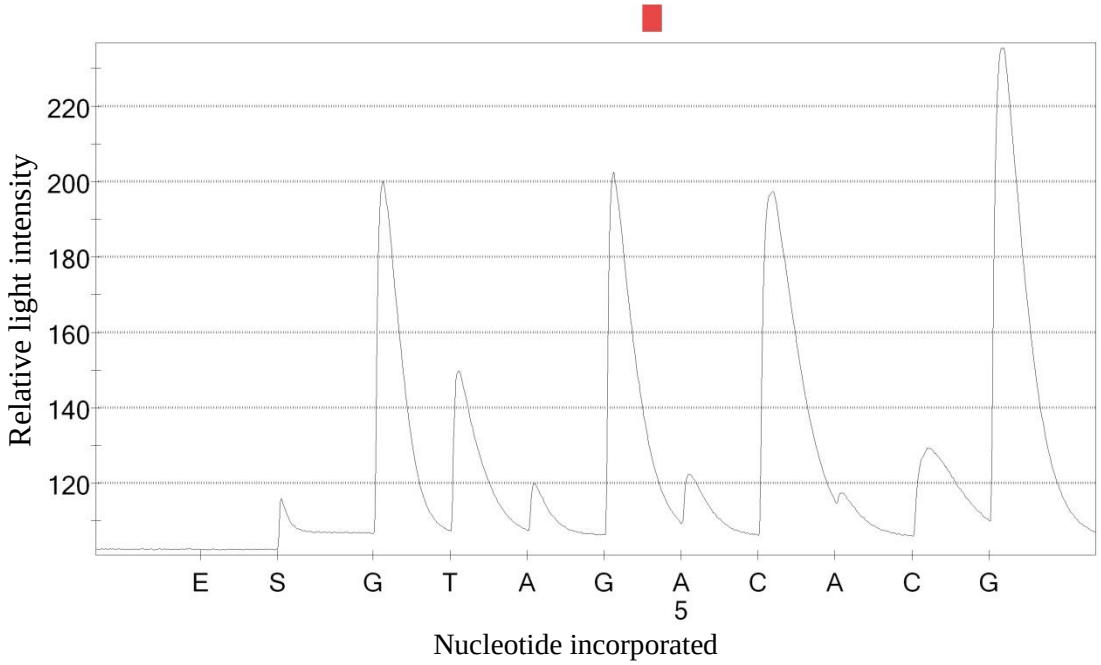

Figure 15 – Control sample 2 H1N1. Pyrogram results from the H1N1 D151 assay. This shows wide peaks which were unable to be interpreted by the pyrosequencing software.

3.1.2.3. Assays for the N2 Virus Mutations:

The use of the Superscript III HiFi amplification reagents for the H3N2 mutations was unsuccessful using the sample input volume of 5µl as the H1N1 viruses (see figure 16) .

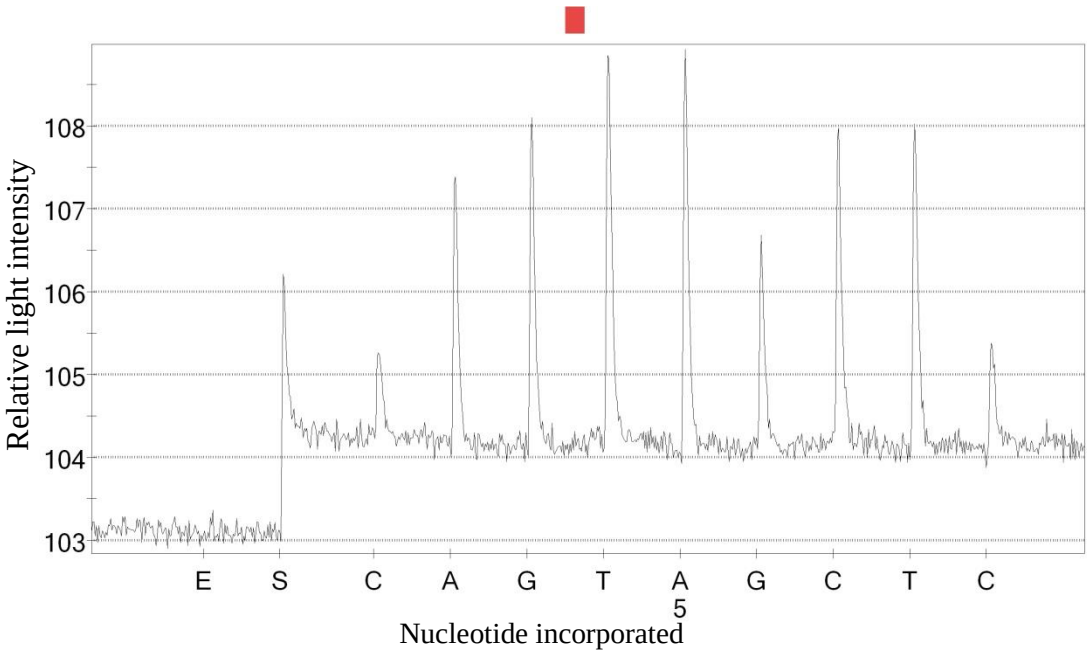

Figure 16 – Control sample 5 H3N2. Pyrogram results from the amplification of the E119V target using 5µl of eluate. This shows that not enough amplified product was present to produce interpretable results.

To optimise the assays for the H3N2 mutations using the Superscript III HiFi amplification reagents, the input volume of eluate for amplification was increased to 15µl (see figure 17).

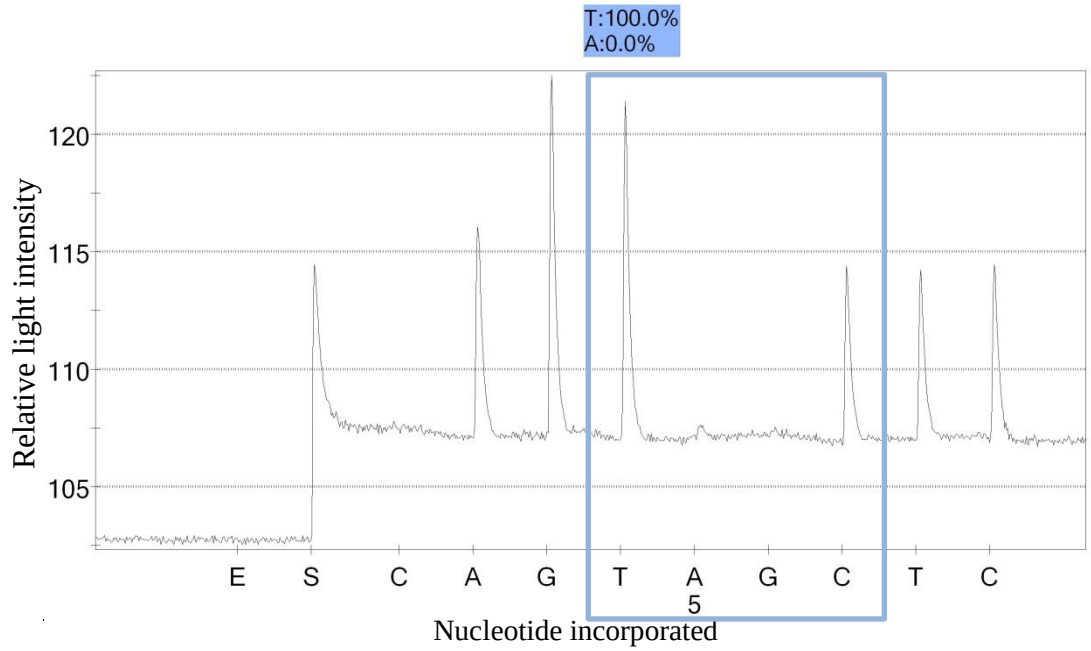

Figure 17 - Control sample 5 H3N2. Pyrogram results from the amplification of the E119V target using 15µl of eluate. Sequence readout: AGGTTCTC, no mutation present.

SNP analysis for the D151 and R292/4 point mutations for the N2 viruses could not be optimised and due to time constraints only SQA analysis was optimised for the clinical samples (Figure 18).

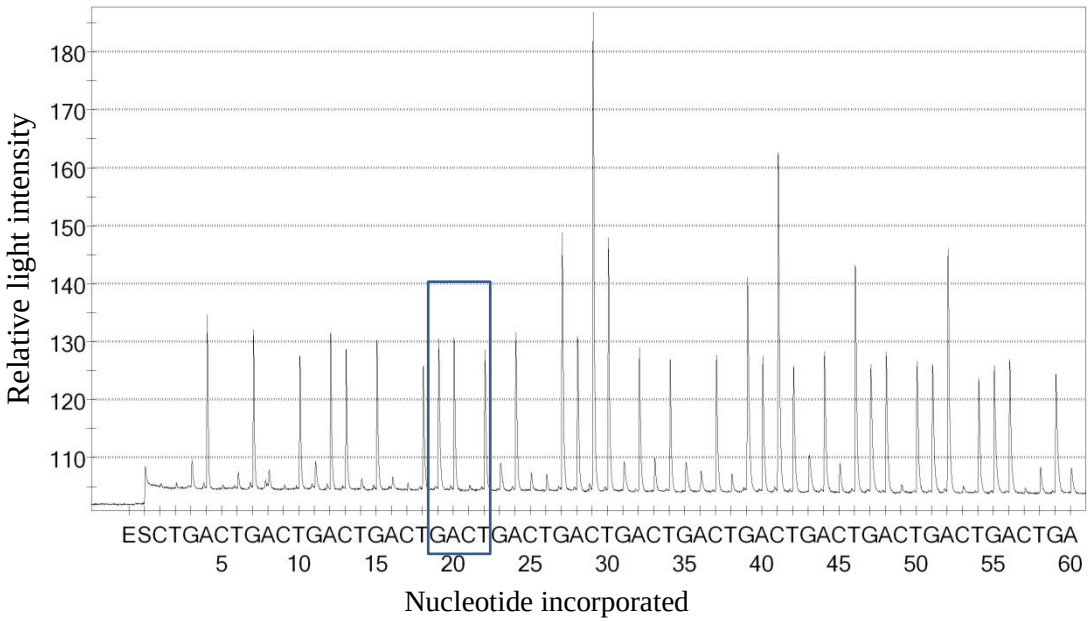

Figure 18 – Control sample 5 H3N2. An example of a SQA pyrogram from the H3N2 D151 assay. Sequence readout: AGTACGTGATAGGACCCCTTA TCGGACCCTATTGATGAATGAG. No mutation present.

3.1.3. Qiagen One-Step RT-PCR Amplification:

The Qiagen One-step RT-PCR amplification reagents were introduced during the course of this study to produce amplified product suitable for use with a pyrosequencer. These reagents were validated for use with the E119V assay developed by this study.

Eight samples were used to compare the HiFi reagents with the Qiagen One-step RT-PCR amplification reagents. The Qiagen One-step RT-PCR amplification reagents produced better results than the Superscript III HiFi amplification reagents (see figures 19-22). Three of the samples tested produced results from the product amplified using the One-step RT-PCR amplification reagents that did not produce results with the Superscript III HiFi products.

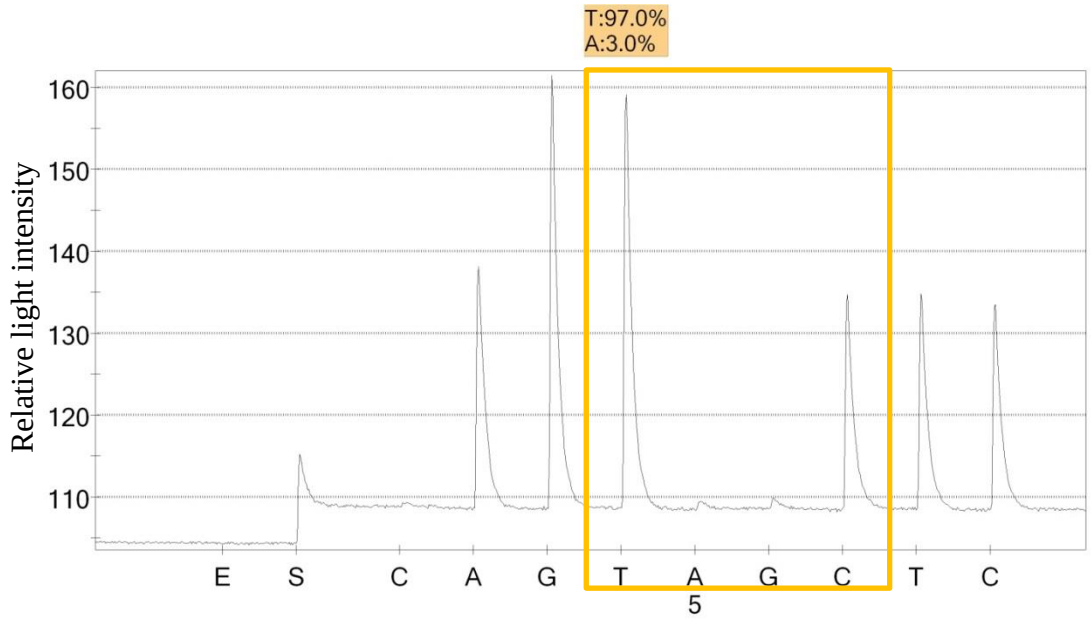

Figure 19 - Sample 28 (CT 29). pyrogram produced using Superscript III HiFi amplification reagents. The sequence was able to interpreted, but needed to be checked, AGGTT/ACTG.

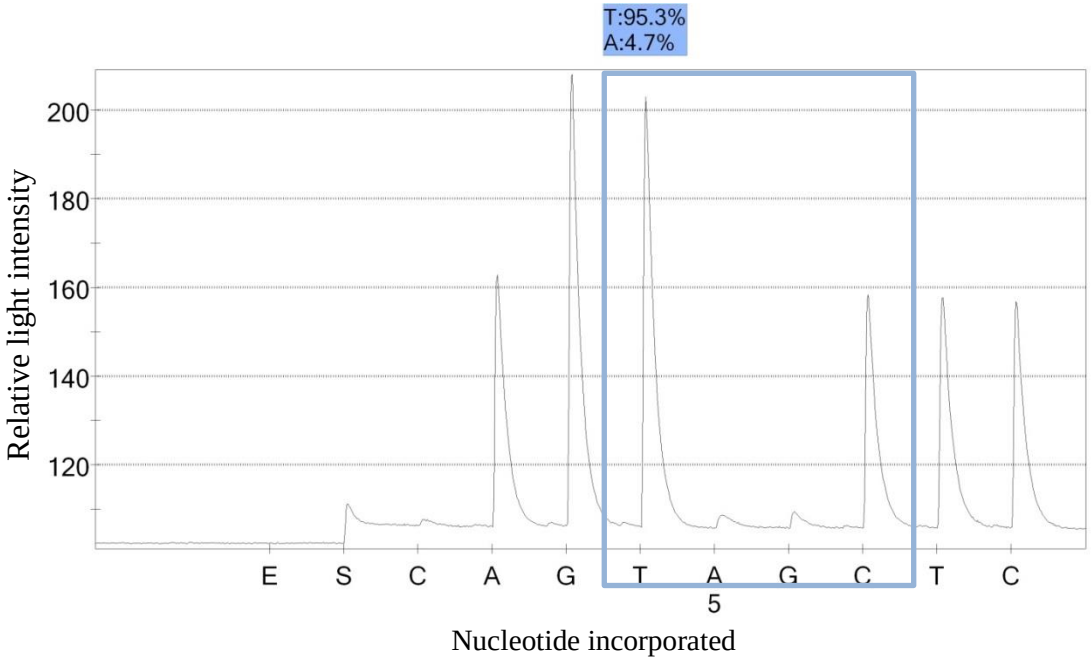

Figure 20 - Sample 28 (CT 29). Pyrogram produced using Qiagen One-stpe RT-PCR amplification reagents. Higher light intensity and better sequence viability, AGGTT/ACTC, 5% mutation present in quasi-species.

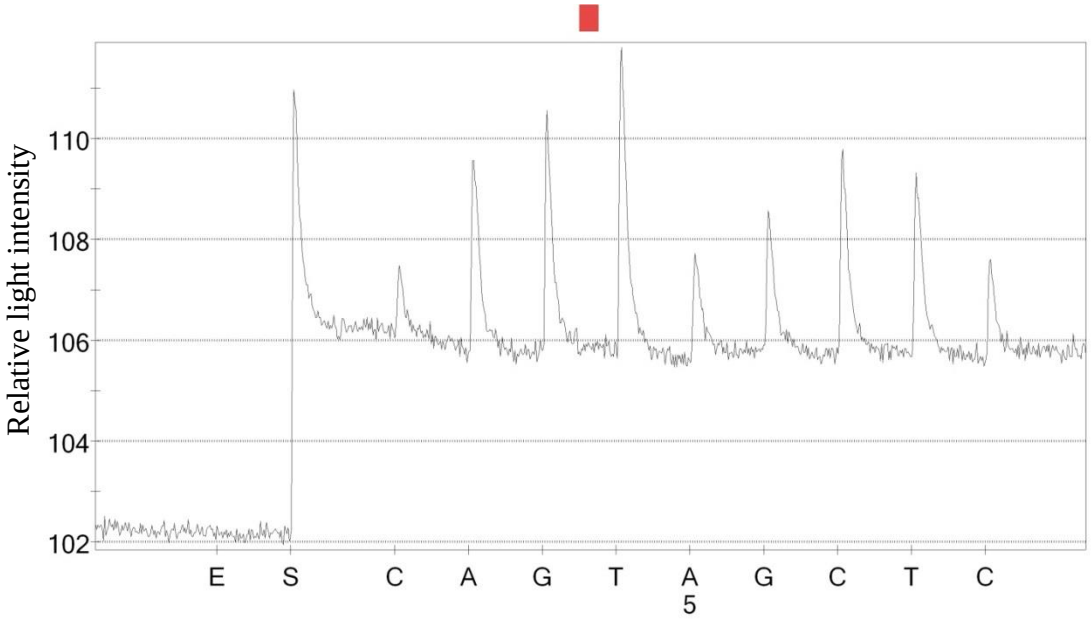

Figure 21 - Sample 7 (CT 40). Pyrogram results using Superscript III HiFi amplification reagents. Sequence unable to be interpreted.

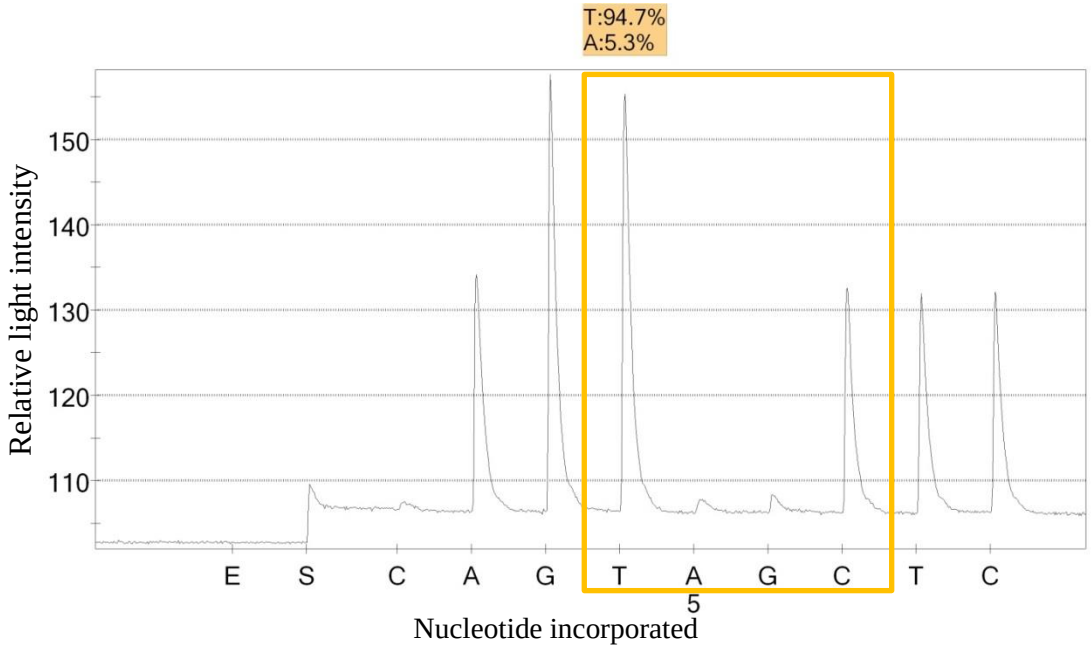

Figure 22 - Sample 7 (CT 40). Pyrogram results using Qiagen One-step RT-PCR amplification reagents. Sequence interpretable but needed to be checked, AGGTT/ACTC, 5% mutation present in quasi-species.

3.1.3.1. H274Y Mutation in H1N1pdm09 Viruses:

Similar good quality results for the H274Y mutation in the H1N1pdm09 virus were obtained using the Qiagen One-step RT-PCR amplification reagents with an increase in sensitivity. The only change to the manufacturer's protocols was the amount of template added (10µl instead of 5µl) to the amplification reagents.

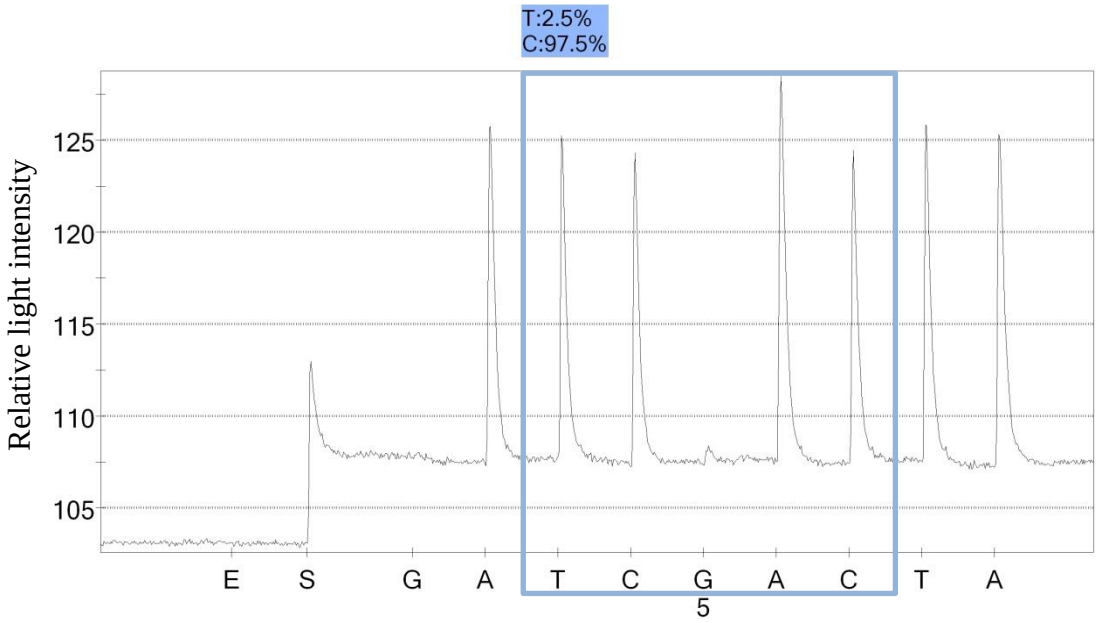

Figure 23 - Sample 103. Pyrogram result, sequence readout: ATC/TACTA, 2% mutation present in quasi-species.

3.2. Clinical Validation:

3.2.1 Antiviral Susceptibility Testing of Pre-Pandemic Seasonal Influenza A H1N1:

During the 2008-2009 season, 15 H1N1 samples were collected from the Welsh population and were tested for the H274Y mutation that confers high level resistance to oseltamivir.

3.2.1.1. H274Y Mutation:

Table 6 - Results of the clinical validation of the H274Y assay on H1N1 viruses pre-2009.

Sample ID	H274 C% (wildtype)	Y274 T% (mutation)
3	F	F
4	1	99
25	4	96
27	2	98
34	1	99
35	3	97
38	1	99
47	1	99
52	1	99
53	6	94
54	2	98
67	1	99
70	1	99
74	1	99
76	F	F

F= failed to produce interpretable pyrograms.

Following amplification and SNP analysis by pyrosequencing, 3 of the 15 samples produced invalid results. The remaining 12 samples were successfully amplified giving good results and possessed H274Y quasi-species of 94-99%. 8 of these samples were also tested using the HPA, Colindale primers. This assay showed all 8 samples to possess the H274Y mutation at a level of 100%.

Table 7 - Results of Colindale primers for the H274Y mutation in H1N1 pre-2009 viruses.

Sample ID	H274 C% (wildtype)	Y274 T% (Mutation)
47	0	100
52	0	100
53	0	100
54	0	100
67	0	100
70	0	100
74	0	100
76	0	100

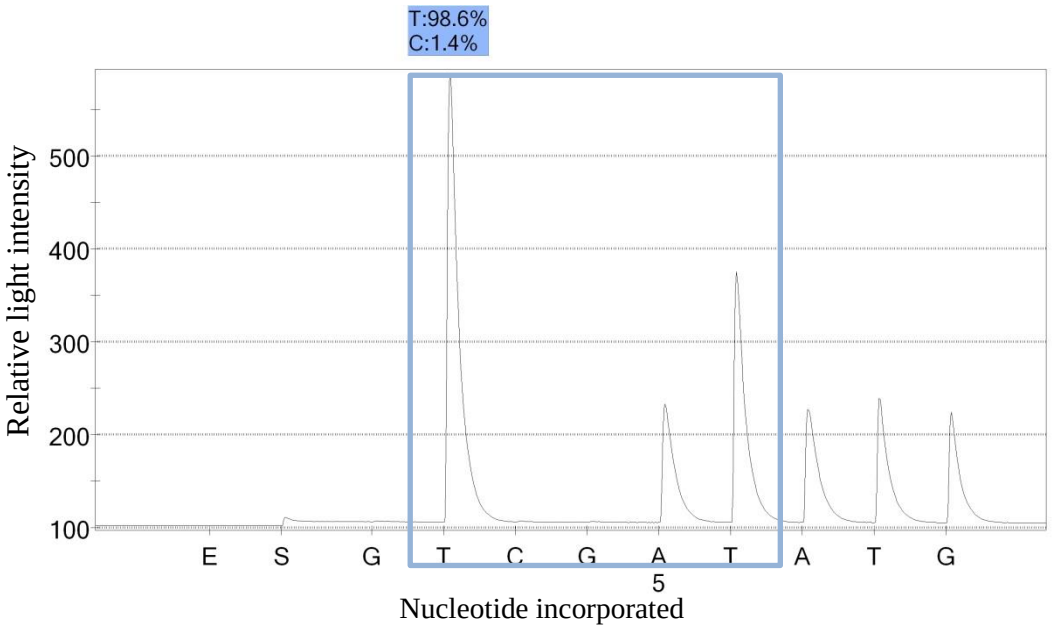

Figure 24 - Sample 4. Sequence readout: TTC/TATATG, 99% mutation present in the quasi-species.

3.2.2. Antiviral Susceptibility Testing of Influenza A H3N2 Viruses:

A total of 53 H3N2 positive samples were amplified and tested for the mutations associated with reduced sensitivity to the NAIs including E119V, and the mutations found at positions D151 and R292/N294.

3.2.2.1. E119V Mutation:

Table 8 - Results of the clinical validation of the E119V assay on H3N2 viruses.

Sample ID	CT value	E119 T% (wildtype)	V119 A% (mutation)
2	39	F	F
5		F	F
6	38	100	0
7	40	F	F
8	40	F	F
9	39	F	F
10	32	96	4
11	36	100	0
14	30	100	0
26		87.5	12.5
28	29	97	3
29	27	100	0
30	38	F	F
31	26	87	13
32		100	0
33	37	100	0
36	36	F	F
37	32	92	8
39	35	F	F
40	38	F	F
41	28	100	0
42	30	97	3
44	31	79	21
45	32	100	0
46	30	90	10
48	27	97	3
49	36	88	12
50	34	69	31
51	25	96	4
55	37	100	0
56	39	F	F
57	32	100	0
58	33	86	14
59	36	F	F
60	35	F	F
61	27	96	4
62	39	F	F
63	37	100	0
64	41	F	F

65	36	F	F
66	30	100	0
68	31	72	28
69	26	97	3
71	25	97	3
72	34	100	0
73	30	100	0
75	37	100	0
77	31	82	18
78	26	95	5
79		93	7

F = Failed to produce interpretable pyrograms, Sample = >10% mutation

Following amplification and SNP analysis for the E119V point mutation by pyrosequencing, 17 of the samples produced invalid results.

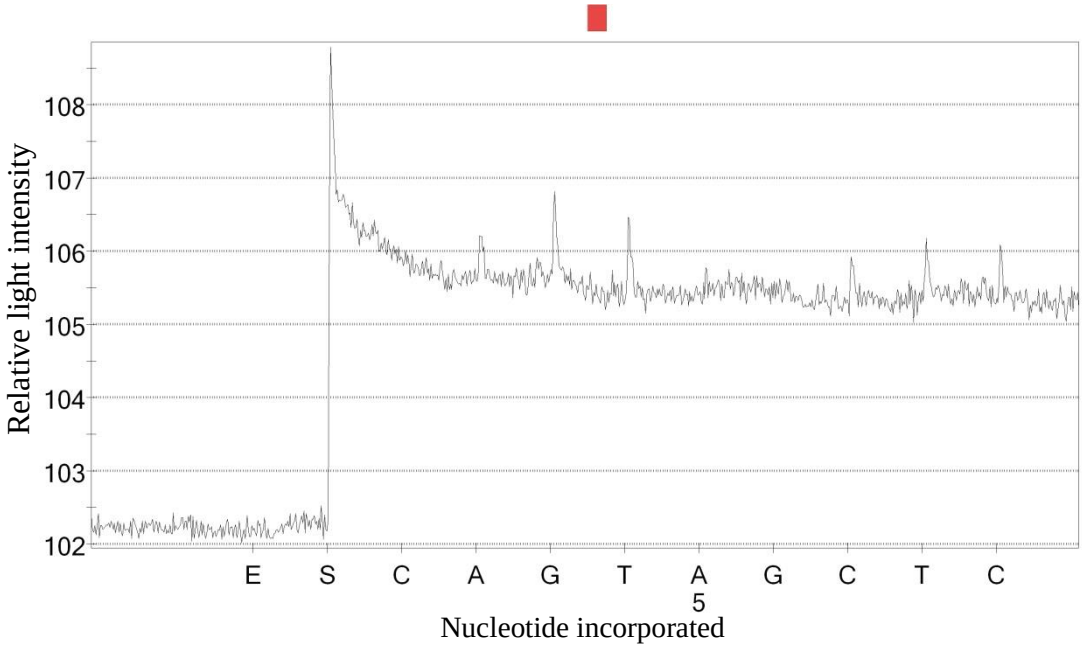

Figure 25 - Sample 56. Failed to produce interpretable results.

Of the remaining samples, 15 had no detectable E119V mutation present.

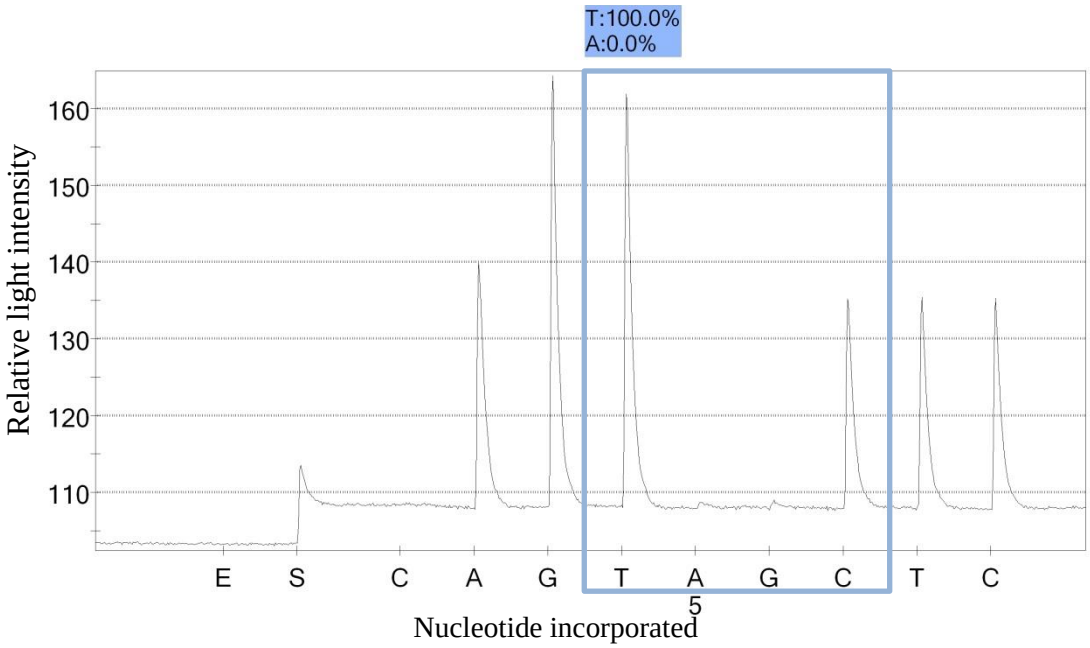

Figure 26 - Sample 41. Sequence readout: AGGTT/ACTC, no mutation present.

Using the allele quantification function on the pyrosequencer it was shown that 12 samples had a low level (1-7%) of detectable E119V mutation within the viral quasi-species.

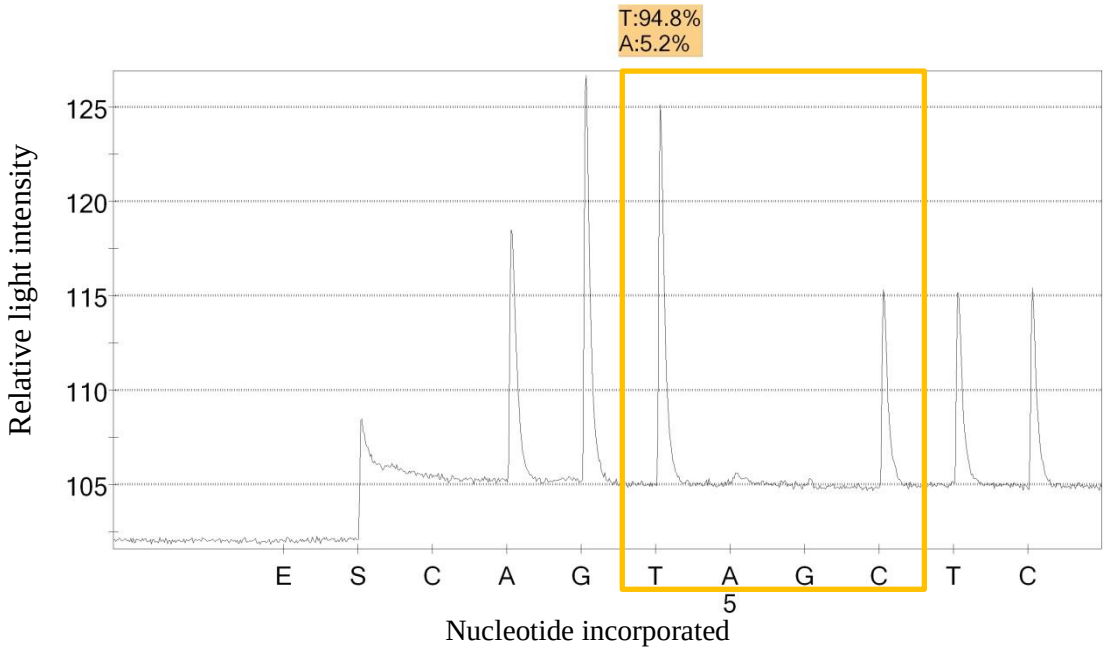

Figure 27 - Sample 78. Sequence readout: AGGTT/ACTC, 5% mutation present in the quasi-species.

Nine samples had an elevated population (8-31%) of E119V resistant virus in the quasi-species.

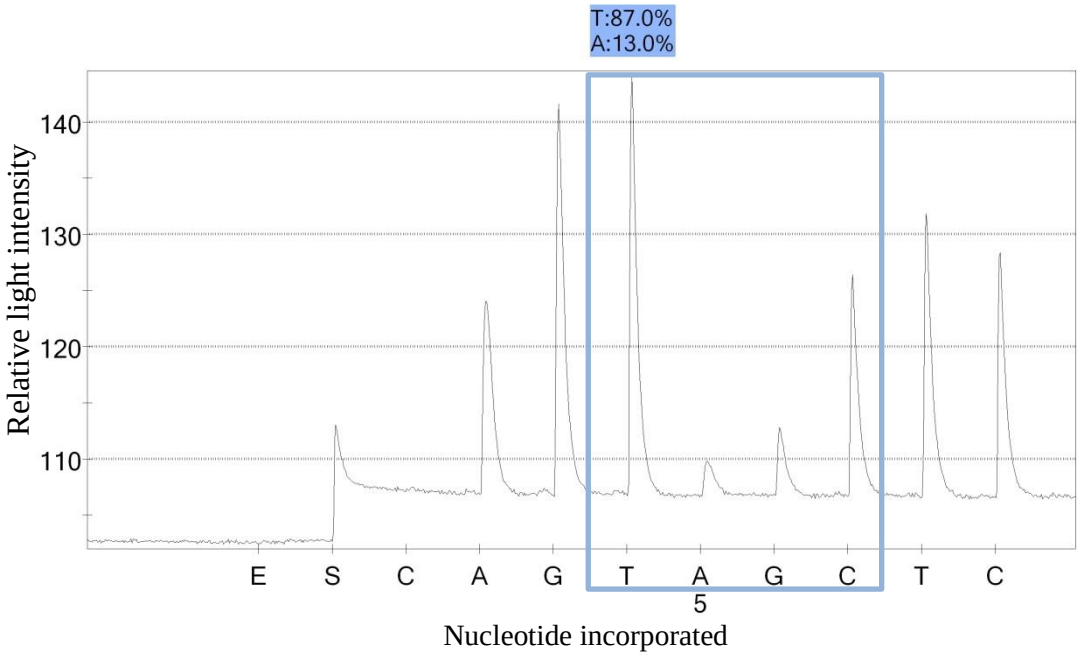

Figure 28 - Sample 31. Sequence readout: AGGTT/ACTC, 13% mutation present in the quasi-species.

3.2.2.2. D151 Mutation:

SQA analysis for the D151 mutations was performed on 26 of the 53 samples. Only 9 samples produced results and sequences that matched H3N2 N sequences. These 9 samples showed no mutations at position 151. The remaining samples produced invalid results.

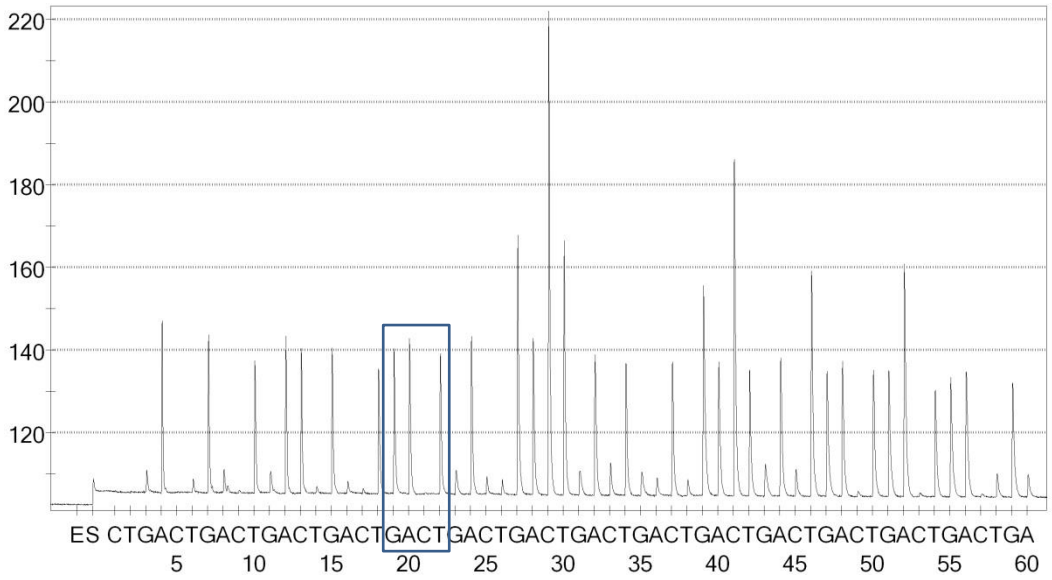

Figure 29 - Sample 51. Sequence readout: AGTACGTGATAGGACCCCTTATCG GACCCTATTGATGAATGAG, No mutation present.

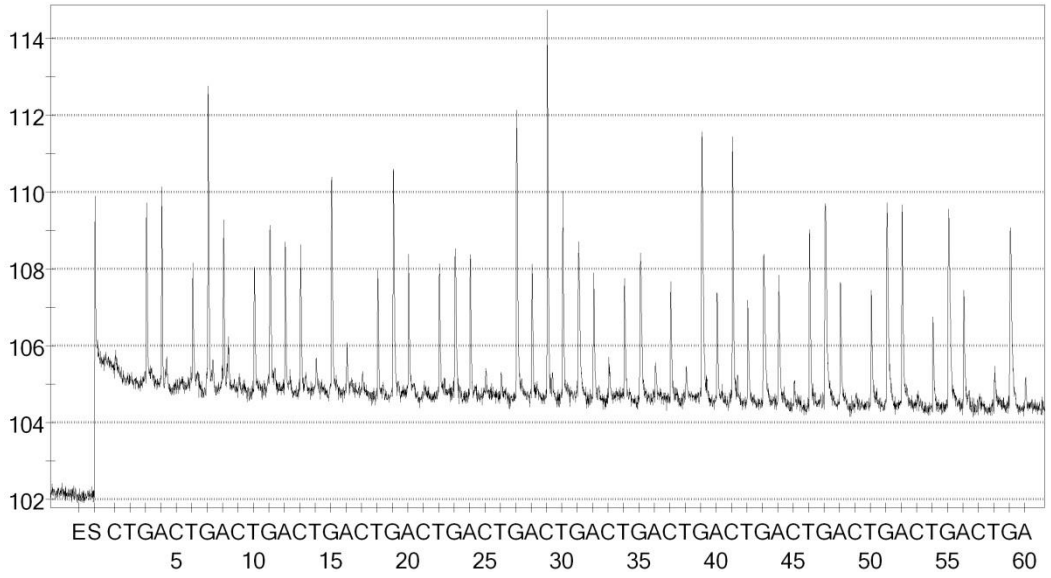

Figure 30 - Sample 30. Invalid SQA D151 assay. Sequence readout does not match H3N2 reference strains, GATGGATGACGGTGGATGAGGACCCTTGAT GCGGACCTGATGGATGGAATGGAGG

3.2.2.3. R292/N294 Mutation:

SQA analysis for the R292/N294 mutations was performed on 44 of the 53 samples. Only 16 samples produced results and sequences that matched H3N2 N sequences. These 16 samples showed no mutations at positions 292 or 294. The remaining samples gave invalid result.

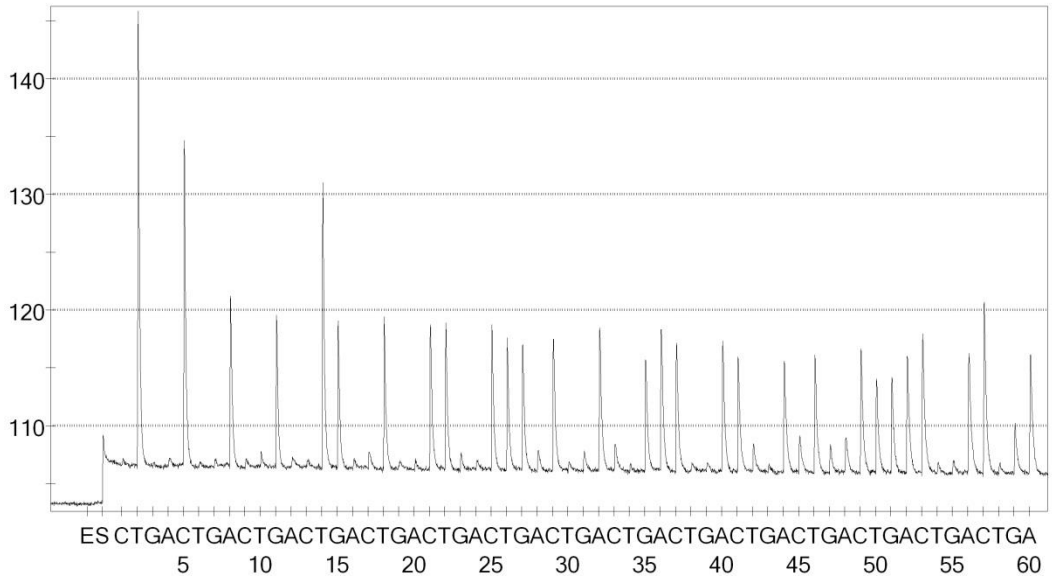

Figure 31 - Sample 14. Sequence readout: TTTCCAGTTGTCTCTGCAGACAC ATCTGACACCA, no mutations present.

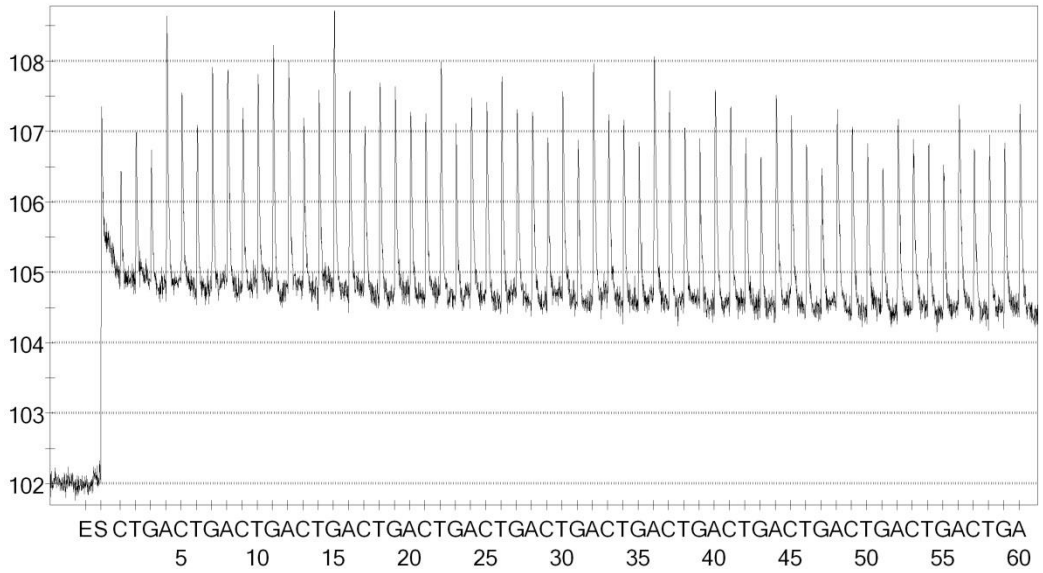

Figure 32 - Sample 30. Invalid SQA R292/N294 assay. Sequence readout does not match H3N2 reference strains: TGACTGACTGACTGGACTGACTGAC TGACTGACTGACTGACTGACTGACTGACTGA.

3.3. Clinical Validation of Influenza A H1N1pdm09 Viruses:

3.3.1. Antiviral Susceptibility Testing of H1N1pdm09 Viruses:

A cohort of 121 H1N1pdm09 samples were tested using the Qiagen One-Step RT-PCR (Limburg, The Netherlands) amplification reagents and SNP analysis on the pyrosequencer for the H274Y point mutation with primers described by the WHO (2009a).

Table 9 - Results of the clinical validation of the H274Y assay on H1N1pdm09 viruses.

Sample ID	CT	H274 C% (wildtype)	Y274 T% (mutation)
12		88	12
13		97	3
15		98	2
16		96	4
17		96	4
18		98	2
19		98	2
20		98	2
21		97	3
22		97	3
23		97	3
24		95	5
81		SQA	
82		SQA	
83		96	4
84		97	3
85		SQA	
86		SQA	
87		SQA	
88		SQA	
89		SQA	
90		SQA	
A	42	F	F
B		100	0
C	30	100	0
D		100	0
E	NEG	F	F
F		100	0
G		97	3
H		100	0
92		95	5
93		F	F
94		98	2
95		95	5
96		95	5
97		95	5

98		98	2
99		F	F
100		97	3
101		97	3
102		98	2
103		97	3
104		97	3
105		91	9
106		98	2
107		96	4
108		95	5
109		95	5
110		93	7
111		98	2
112		100	0
113		97	3
114		95	5
115		F	F
116		F	F
117		97	3
118		F	F
119		F	F
130		8	92
131		0	100
132		F	F
133		F	F
134		F	F
135		F	F
136		F	F
137		F	F
138		100	0
139		58	42
140		100	0
141		51	49
142		99	1
143		100	0
144		99	1
145	26	100	0
146	31	100	0
147	33	F	F
148	36	F	F
149	33	F	F
150	25	97	3
151	35	93	7
152	26	98	2
153	27	99	1
154	26	99	1
155	32	F	F
156	28	100	0
157	36	F	F
158	28	99	1
159	29	98	2

160	37	F	F
161	29	96	4
162	20	98	2
163	28	100	0
164	29	100	0
165	28	100	0
166	23	97	3
167	18	99	1
168	21	99	1
169	23	93	7
170	35	98	2
171	33	91	9
172	35	96	4
173		0	100
174		97	3
175		96	4
176		100	0
177		98	2
178		F	F
179		90	10
180		93	7
181		89	11
182		92	8
183		99	1
184		100	0
185		99	1
186		100	0
187		100	0
188		100	0
189		F	F
190		98	2

F = Failed to produce interpretable pyrograms,
Sample = polymorphism samples, Sample = >10% mutation
SQA results only.

Of the 121 samples analysed, 23 samples produced invalid results. The majority of the remaining samples possessed a low detectable level (1-10%) of H274Y resistant virus in the quasi-species.

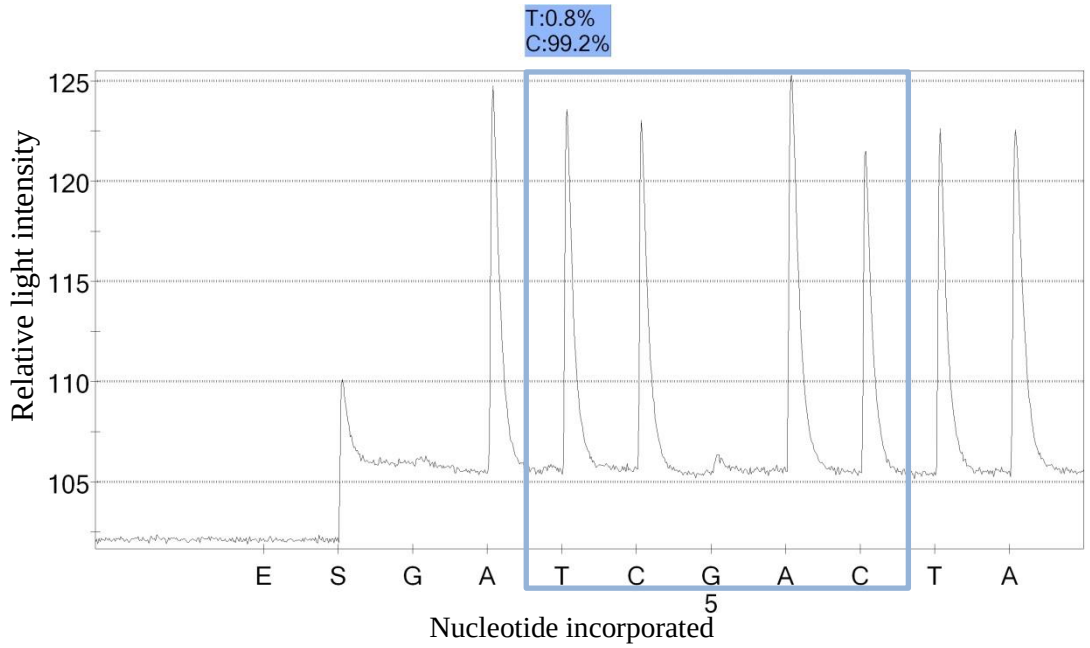

Figure 33 - Sample 144. Sequence readout: ATC/TACTA, showing 1% mutated virus in the quasi-species.

Only 18 samples produced results without any mutation present and one sample was shown to possess 100% resistant virus population.

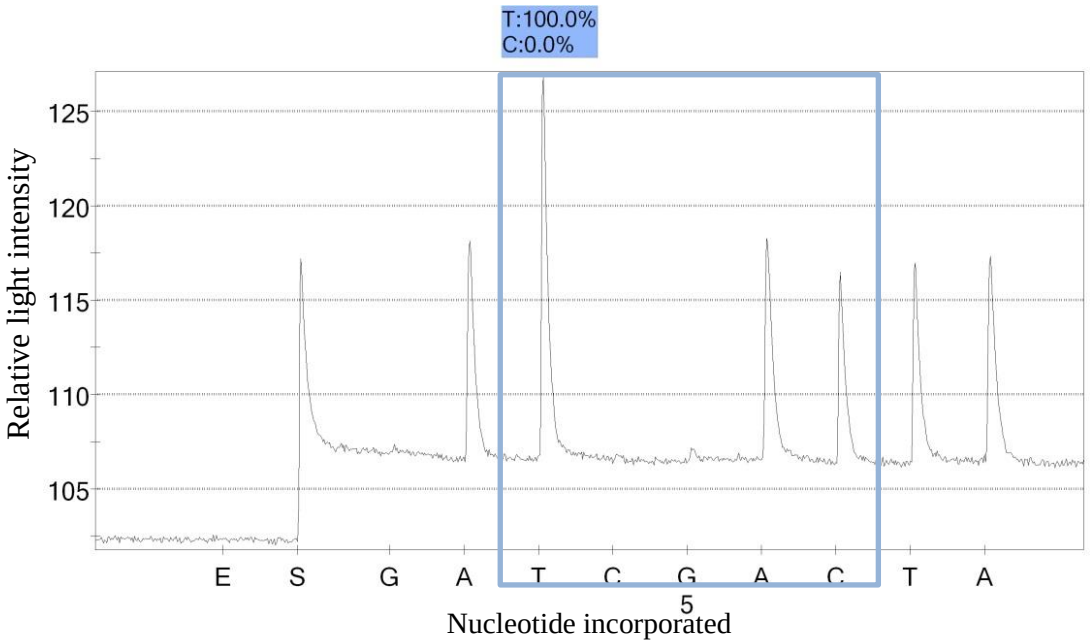

Figure 34 - Sample 173. Sequence readout: ATTACTA. 100% mutation present.

8 samples were also run through the SQA assay to check the assay quality. These samples showed no change in the neuraminidase sequence.

From the SNP analysis it was also observed that 3 samples (from the 2010-2011 season) had a sequence of TACCAC from position 273 instead of TATCAC, indicating a polymorphism in the H1N1pdm09 virus species.

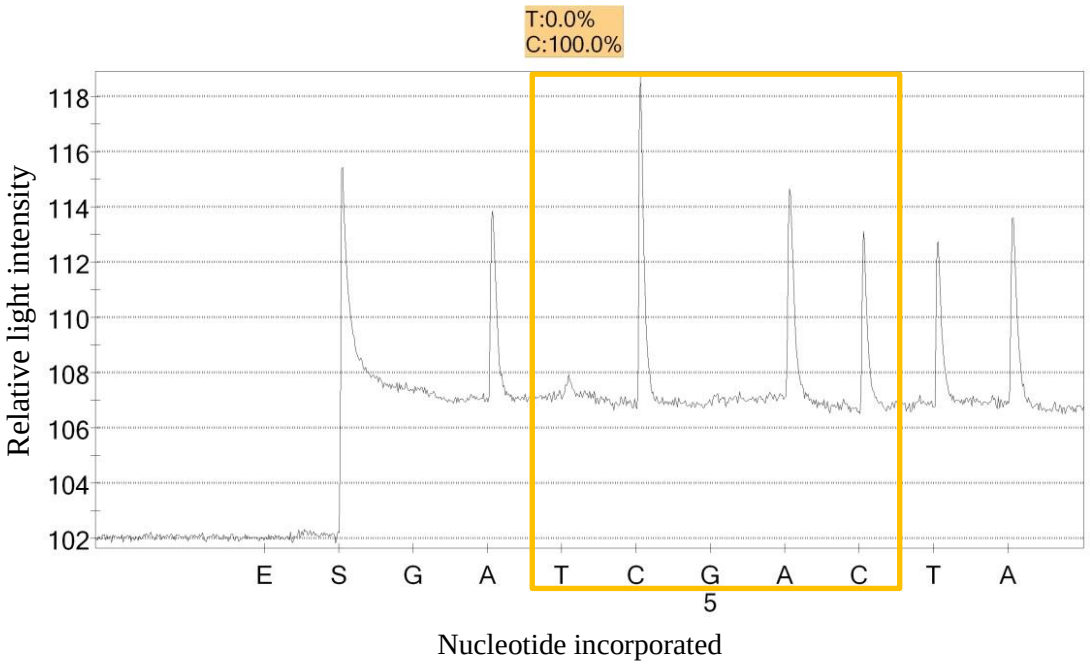

Figure 35 - Sample 176. Pyrogram showing the difference in sequence readout, ACCACTA instead of ATCACTA for the samples containing the polymorphism at position 273.

Consecutive samples were collected from 3 patients who were receiving oseltamivir therapy during the 2010-2011 season to look for the emergence of NAI resistance. The first samples from 2 of these patients showed the absence of resistant virus. In the consecutive samples from these 2 patients a detectable level of 42% and 49% resistant virus was observed. The samples from the 3rd patient showed an increase in resistant virus in the quasi-species from 1% in sample 1 (day 2 of illness), to 92% in sample 2 (day 10 of illness) to 100% in sample 3 (day 13 of illness) (See section 3.3.1.).

3.3.1. Case Study:

A 2 year old girl presented at A+E with status epilepticus. She had a 1 day history of feeling unwell and high temperature. She had had seizures during the day

which were treated with buccal midazolam but then developed an ongoing seizure which resulted in her removal to hospital. On admission to A+E she was treated for her seizures with 2 doses of rectal diazepam, 2 doses of intraosseal lorazepam; however she continued to fit so she was started on a phenytoin infusion. She developed bradycardia with a heart rate of 40/min requiring CPR for 1 minute. She was given a dose of adrenaline and an adrenaline infusion. She was also given100ml/kg IV fluid bolus and started on IV ceftriaxone.

She was intubated and transferred to PICU. The patient was sent for a chest x-ray which revealed bilateral airspace shadowing, which was more extensive on the right. This was indicative of oedema or infection. The patient was stared on bi-daily doses of 45mg oseltamivir.

An NPA was performed on day 1 and the sample sent for IF for respiratory viruses. This produced a positive result for Influenza A.

The patient was sedated with morphine, midazolam and chloral hydrate whilst she was ventilated.

A second chest x-ray was performed 3 days after admission after increased oxygen intake. It revealed progressive bilateral shadowing and a right-sided pleural effusion; therefore a chest drain was inserted.

The patient had a focal seizure on day 4 which was treated with IV lorazepam. On day 5 her chest drain was removed and she was extubated on day 6 and given a 60% oxygen facemask. Another chest x-ray was performed on day 7, which showed bilateral haziness.

Whilst on PICU the patient was given IV cefotaxine for her pyrexia and 15ml/kg packed red cells and 20ml/kg FFP. She was also given Vitamin K for prolonged PT.

After completing a 10 day course of oseltamivir and cefotaxine, and having reduced need for oxygen and an increase in feeding. The patient was transferred to a General Paediatric ward. Here she was given bi-daily dose of keppra 300mg, sodium valporate 360mg and lamotrigine 25mg. She was also kept on chloral hydrate 900mg every 4-6 hours. She was now on a self-ventilating 5L facial

oxygen mask and having chest physiotherapy. A nose swab was taken for respiratory virus testing and was positive for H1N1 pdm09 influenza virus.

The samples taken for respiratory testing were tested for influenza A, B and RSV by a real-time PCR assay performed on the Applied Biosystems Fast 9500. This found the samples to be positive for Influenza A. These samples were then typed by a similar assay performed on the Fast 9500, showing them to be positive for H1N1 pdm09.

After noting that the patient was still positive after 10 days of oseltamivir treatment the samples were tested for the point mutation H274Y which confers oseltamivir resistance by using the Qiagen Pyromark ID pyrosequencer.

The sample taken on day 1 contained 1% of virus species with the H274Y mutation (Figure 36). The next sample taken on day 9 contained 92% of virus species with the mutation (Figure 37). And a third sample taken on day 14 contained 100% virus species with the mutation (Figure 38).

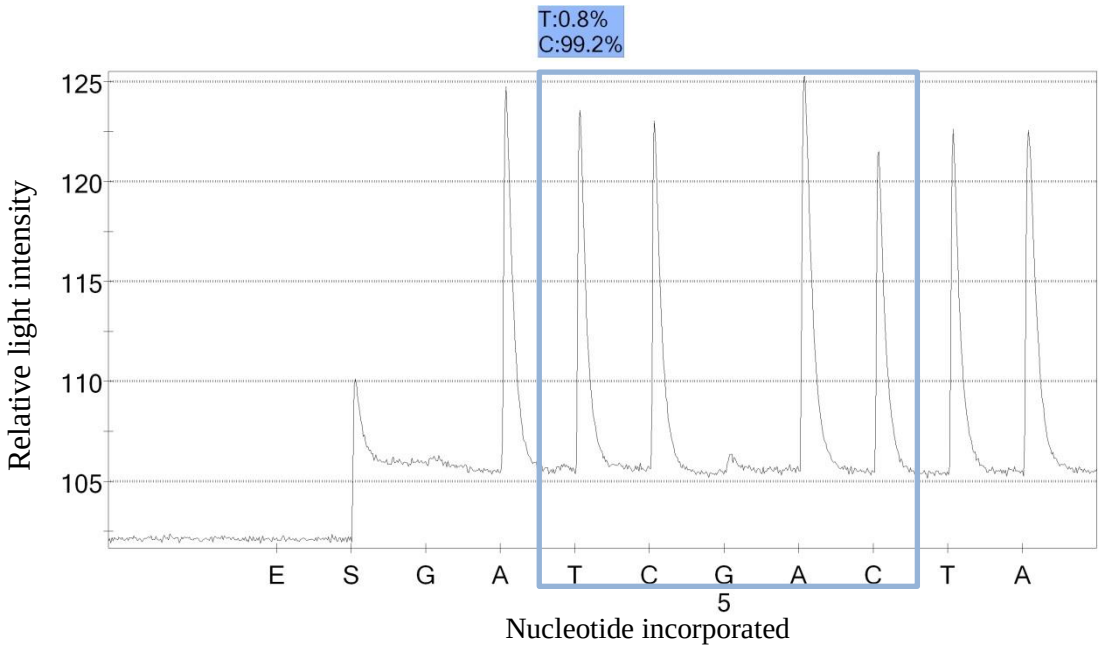

Figure 36 - Pyrogram from sample 1, taken on day 2 of illness, showing 1% mutated virus in the quasi-species.

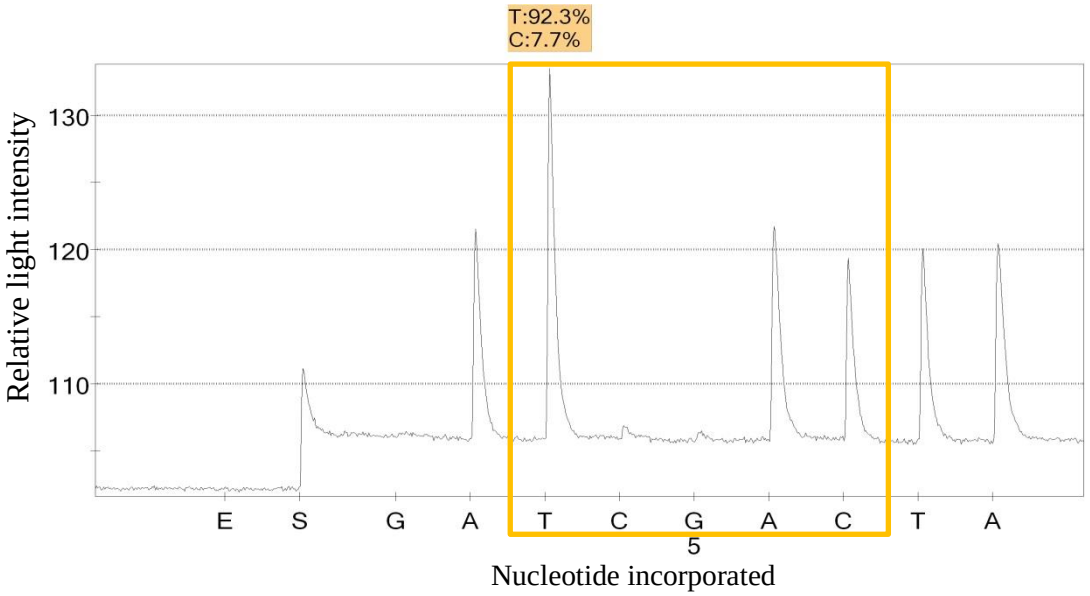

Figure 37 - Pyrogram of sample 2, taken on day 9 of illness, showing and increase to 92% mutated virus in the quasi-species.

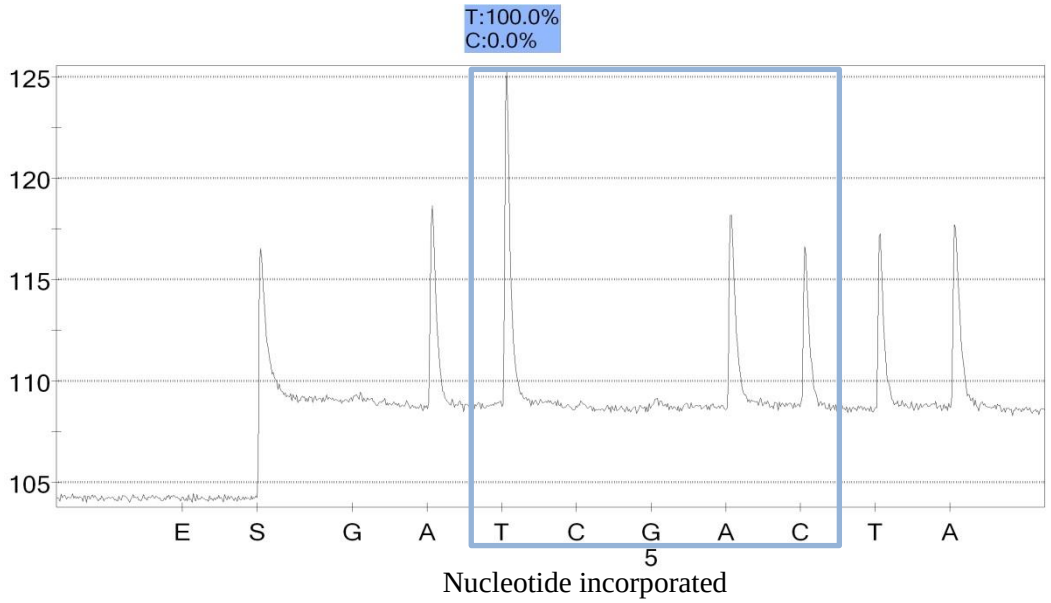

Figure 38 - Pyrogram of sample 3, taken on day 14 of illness, showing 100% mutated virus.

This patient had a previous history of epilepsy which had resulted in two admissions to hospital in the last year, and it was also noted that she was mildly delayed in her physical development. Apart from these factors there are no further medical history of importance and no history of reduced immunity.

Although the patient was extremely ill at the start of her illness, she had vastly improved when her second respiratory sample was taken (which showed 92%

resistance) and had been off oseltamivir for 3 days when her third sample was taken (100% resistant).

An underlying level of less than 10% mutated has been shown in the majority of H1N1 pdm09 samples tested in this study, but the relevance of this low level is at the moment uncertain. This case study has shown that there may be a need to monitor the patients who present with a low level of resistant virus at the start of their illness especially if they are given oseltamivir. The longevity of symptoms may have been reduced if her resistance level was regularly monitored from the onset of her illness and if she was able to be given zanamivir, as soon a rise in mutated virus was noted, if not at the very beginning of her illness.

4. Discussion:

4.1. Assay Optimisation:

4.1.1. H274Y Assay for Seasonal H1N1 Viruses (Pre 2009):

The H1N1 assay for the H274Y point mutation was optimised for routine diagnostic use, with one change to the protocol described by Deyde et al (2009b). The use of the Superscript III HiFi amplification reagent kit (Invitrogen™, Life Technologies™, California, USA), instead of the Superscript III RT/Platinum amplification reagents (Invitrogen™, Life Technologies™, California, USA). This reagent kit allowed the production of good quality amplified products which produced good results. This protocol was easy to perform and had a hands-on time, from amplification to results, of about 30 minutes and a total time of about 4 hours. This assay could therefore be performed on the same day as a positive influenza A H1N1 result was identified, especially as the same sample extract could be used.

The primers routinely used by HPA Colindale in a similar pyrosequencing assay, were validated in comparison with the Deyde et al (2009b) assay for 8 samples. The samples tested using the HPA Colindale primers gave results showing 100% levels of mutated virus. In comparison the same samples produced variable levels of 94-99% of mutated virus by the optimised Deyde et al (2009b) assay. This variation is minimal but could be due to differences in the target regions of the assays and how well each assay amplifies the entire quasi-species rather than simply the predominant species.

4.1.2. D151 Assay for Seasonal H1N1 Viruses (Pre 2009):

The D151 protocol described by Deyde et al (2009b) could not be optimised for routine use. The SNP analysis produced erroneous pyrograms, with wide peaks. This error usually results from the presence of too much nucleic acid for the used enzyme/substrate activity or a slow degradation of the pyrophosphate from the previous nucleotide incorporation, caused by decreased performance/activity of the enzyme (Qiagen, 2013). This error may be rectified by decreasing the amount of nucleic acid used as a template in the initial amplification step. Due to time

constraints this error was not able to be investigated further. From the point of view of the smooth running of laboratory assays, it is important to standardise sample preparation for downstream assays, but this is an area for potential future optimisation.

4.1.3. E119V Assay for H3N2 Viruses:

The E119V protocol described by Deyde et al (2009b) was easily optimised for routine use using the Superscript III HiFi amplification reagent kit (Invitrogen™, Life Technologies™, California, USA) and by adding a degeneracy to the reverse primer and the amount of target added for amplification. After optimisation this assay was easy to perform and had a hands-on time, from amplification to results, of about 30 minutes and a total time of about 4 hours. This assay could therefore be performed on the same day as a positive influenza A H3N2 result was identified, especially if the same sample extract could be used.

Consistent and reproducible results were also obtained using the Qiagen One-Step RT-PCR amplification kit (Limburg, The Netherlands). There was a notable increase in sensitivity as the assay was further optimised to double the amount of amplification primer and also the amount of starting template in the initial PCR. The significant reduction in the overall cost of the reagents when compared to the HiFi reagents means that the assay is more cost effective per sample sequenced.

4.1.4. R292K and N294S Assay for H3N2 Viruses:

The Deyde et al (2009b) assay for SNP analysis for the R292K and N294S mutations could not be optimised for routine use due to difficulties in SNP calling across the two positions. Therefore SQA analysis was performed for these mutations. This assay takes longer than the SNP analysis assays optimised for the H274Y and E119V mutations. However it does produce a gene sequence of up to 100bp. This analysis allowed known mutations to be identified and also has the potential to allow identification of new mutations in the target region.

4.1.5. D151 Assay for H3N2 Viruses:

SQA analysis was performed for the D151 mutations. This assay is most optimal for this mutation as it has been well documented that many different mutations

can occur at codon 151. However it has been documented that these mutations are usually cell culture derived and therefore unlikely to be seen when testing directly from the sample (Okomo-Adhiambo et al, 2010). The lack of the mutation in clinical samples was therefore not unexpected and in light of the requirement to culture the virus first, it is unlikely that this assay would be suitable for routine application.

4.1.6. Assessment of One-Step RT-PCR Amplification Kit:

The E119V assay was utilised to assess the Qiagen One-Step RT-PCR amplification kit (Limburg, The Netherlands). This amplification kit allowed the amplification of samples that were unable to be amplified using the Superscript III HiFi amplification reagent kit (Invitrogen™, Life Technologies™, California, USA). This is possibly due to the increased concentration (0.8µM compared to 0.4µM) of the amplification primers.

4.1.7. H274Y Assay for H1N1pdm09 Viruses:

The Qiagen One-Step RT-PCR amplification kit (Limburg, The Netherlands) was used for the amplification of the H1N1pdm09 isolates. This kit produced good quality amplified product in conjunction with the primers described by the WHO (2009a) and produced good results. This protocol was easy to perform and had a hands-on time, from amplification to results, of about 30 minutes and a total time of about 4 hours. This assay could therefore be performed on the same day as a positive influenza A H1N1pdm09 result was identified, especially if the same sample extract could be used.

This assay is now in routine use at PHW Microbiology Cardiff.

Assay optimisation for this study was achieved by making changes to the amplification kit reagents used and adjustment of the stated manufacturer's protocols and input volumes for amplification.

Further changes in optimisation could have been performed at many other steps in the process if time and money allowed. The main changes worth exploring would have been primer concentration. A more regimented approach of a standard

increase or decrease of 0.5µM intervals of concentration may have produced better comparisons for results.

Steps in the pyrosequencing protocol could also be regimented and small changes made to determine effect on the pyrosequencing results, such as length of incubation times and speed of mixing.

Invalid results were often observed due to technical issues with the vacuum station. A more advanced vacuum station with a reduction in pressure issues would be very valuable for this type of assay.

4.2. Clinical Validation:

All of the samples to be used for clinical validation were tested by the new In-house assay to determine the strength of the positive result of the samples. The In-house assay is not quantifiable but the CT value does give an indication of how much virus is present. It was found that the samples with a high CT value (greater than 39) were unable to produce enough amplified product to produce valid results, whereas samples with a CT value of 34 or lower were able to be amplified sufficiently. Samples with a CT value between 34 and 39 were sporadic in the production of sufficient amplified product to produce interpretable results. Therefore the majority of invalid results noted during clinical validation are due to the amount of virus present in the original clinical sample.

4.2.1. Antiviral Susceptibility in Seasonal H1N1 Viruses (pre 2009):

4.2.1.1. H274Y Mutation:

During the 2007-2008 respiratory season, an increase in the presence of oseltamivir resistant viruses of up to 70% was observed (Lackenby et al, 2008a). By the next respiratory season (2008-2009) the levels were nearly 96% in Britain (NISN, 2010). The results from this study reflect these findings and suggest that the levels of oseltamivir resistant H1N1 viruses in Wales (pre 2009), was close to 100%. Such information is important to clinicians in their assessment of patients and determination of appropriate treatments.

4.2.2. Antiviral Susceptibility of H3N2 Viruses:

4.2.2.1. E119V Mutation:

The E119V mutation was detected in 21 of the 53 samples tested. The amounts present in the virus quasi-species varied from 1-31%. These levels could suggest a possibility of resistance occurring in these individuals if they were treated with oseltamivir and/or were immunocompromised. The E119V mutation was identified early in the use of NAI's, and has only been documented outside the cell culture setting as the cause of oseltamivir resistance in several cases of treated individuals (Kiso et al, 2004; Sheu et al, 2008). Therefore it is possible that the samples with the presence of resistant virus could be from individuals already treated with oseltamivir. However this is unlikely, as most of the samples tested were initial diagnosis samples and therefore treatment would not have commenced at the time the sample was taken. Surveillance for this particular mutation is warranted as the results from this study suggest a possible increase in incidence.

4.2.2.2. R292K/N294S Mutations:

The mutation at R292K was one of the first mutations identified for H3N2 viruses in relation to the NAI's during challenge studies (Gubareva et al, 1996; Gubareva et al, 1997; McKimm-Breschkin et al, 1998). This mutation was associated with oseltamivir resistance in children infected with H3N2 viruses and treated with oseltamivir (Kiso et al, 2004).

The N294S mutation was also identified in children infected with H3N2 viruses and treated with oseltamivir (Kiso et al, 2004). However, this mutation has now been noted more frequently in H5N1 isolates (Le et al, 2005; Earhart et al, 2009).

Only 16 samples from this study produced valid sequences and none of these samples possessed R292K or N294S mutations at these positions. It is therefore suggested that these mutations only arise in H3N2 infected individuals when treated with NAIs.

These mutations have also been observed more recently, in oseltamivir treated immunocompromised patients after 8 (R292K in one patient) and 7 (N294S in a 2nd patient) days of treatment respectively (Piralla et al, 2013).

These cases are likely to have occurred due to the increased viral shedding observed in children and the immunocompromised.

4.2.2.3. D151 Mutation:

Only 9 samples produced results that matched the neuraminidase sequences of H3N2 viruses. The sequences produced from the SQA analysis for the D151 mutation showed no evidence of mutations at this position.

Again mutations at this position have mainly been observed in viruses grown in cell culture and have not been identified directly from clinical samples (Okomo-Adhiambo et al, 2010).

4.2.2.4. Invalid SQA results:

The SQA assays for the R292K/N294S and D151 assays for the H3N2 viruses were the last of the assays to be optimised. Use of sample in the E119V assays and the repeat assays performed due to the production of invalid results due to technical issues, caused a small amount of sample to be available for testing. Many of the SQA assays returned invalid results that could not be investigated further due to lack of sample, but due to the return of sequences that did not match the target sequence it is possible that the sample may have been degraded resulting in these unknown sequences.

4.2.3. Antiviral Susceptibility of H1N1pdm09 Viruses:

4.2.3.1. H274Y Mutation:

In total, 98 samples produced valid results. Of these, 18 samples had no evidence of the H274Y mutation present in the virus, and 7 samples had evidence of resistant virus within the viral quasi-species of between 10% and 100%. Of these 7 samples, 4 were repeat specimens taken from patients who had had previous samples with no or low level resistant virus. In the UK, 72 cases of oseltamivir resistance in H1N1pdm09 viruses were recorded between May 2009 and February 2011. These cases were mainly in the immunocompromised or those who were exposed to oseltamivir (Lackenby et al, 2011).

One of these 7 samples showed 100% mutated virus. It is unknown whether this patient had contact with a H1N1pdm09 positive individual, who had had or was

taking oseltamivir, and whether they were known to be infected with resistant virus. During the 2010-2011 season, 3 cases of oseltamivir resistance were reported from community patients in the UK (Lackenby et al, 2011), and a case of H1N1pdm09 virus containing a mixed population of mutant (Y274-52.6%) and wild-type (H274-47.4%) virus from an immunocompetent, treatment naïve individual was reported in Hong Kong in 2009 (Chen et al, 2009).

The remaining 73 samples had a low level (1-10%) of resistant virus present. The significance of the presence of low levels of resistant virus is currently unclear. As seen in this study and worldwide, low level or even no resistant virus present at first sampling can increase up to 100% resistant virus in the presence of oseltamivir, which is most often observed in the immunocompromised (Inoue et al, 2010; Memoli et al, 2010; Nguyen et al, 2010a; Yi et al, 2010; Moore et al, 2011).

4.2.3.2. Polymorphism:

It was observed that 3 samples from the 2010-2011 season showed the presence of a base change in codon 273, TAT instead of TAC. This codon change is silent in that it does not produce a change in amino acid, and is unknown if it would have any positive or negative effect on the virus.

4.2.3.3. Patient Management:

This study did not have any impact on patient management during the period of testing, as the majority of testing was performed retrospectively, and the assays had not been validated to be used routinely.

The presence of low levels of resistant virus in patient samples has led to a discussion on whether or not it is suitable to keep these patients on oseltamivir. The agreed WHO strategy states that if the presence of resistant virus is >50% it is clinically and epidemiology relevant. It also suggests that quasi-species with <50% resistant virus present should be reported to the clinicians as resistant for patient management, but not for international records (Lackenby et al, 2011).

The PHE (formally HPA) in the UK released new guidance notes for healthcare practitioners on the use of antiviral agents for the treatment and prophylaxis of influenza from the start of 2014 (Figure 39) (Parry-Ford, 2013).

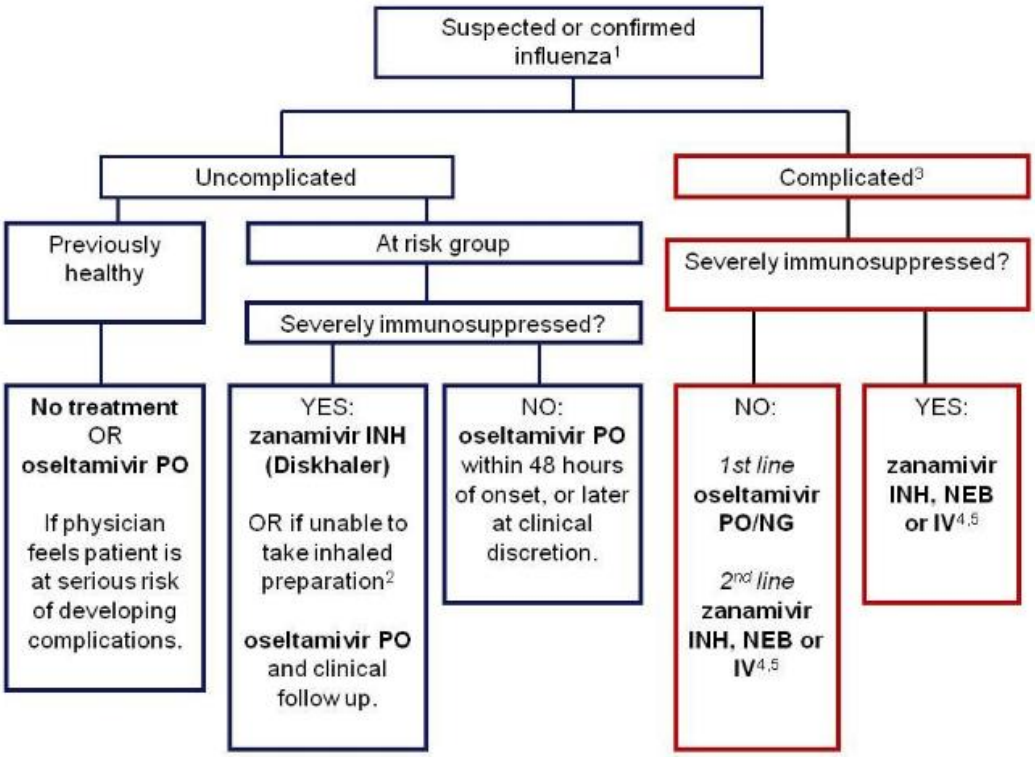

Figure 39 - Algorithm for the selection of antiviral therapy for the treatment of influenza (Parry-Ford, 2013).

NAI's are only suggested for use if the clinician feels the patient is at risk from complications.

Due to the observation of oseltamivir resistance developing in immunocompromised individuals these guidelines recommend the use of zanamivir instead of oseltamivir in this patient group. The emergence of oseltamivir resistant viruses should be tested for if the patient's condition has not improved after five days of treatment. These guidelines also warrant the use of zanamivir in its unlicensed forms via nebulisation and IV if the patient is unable to take or benefit from the disk-inhaler. It is also recommended that zanamivir be given for up to 10 days if resistance to oseltamivir is confirmed or suspected and also in patients who have been in contact with a confirmed or suspected case of oseltamivir resistant influenza (Parry-Ford, 2013).

4.2.4. Further Clinical Validation:

4.2.4.1. Untypeable Influenza A Samples:

The original subtyping assay performed by the Molecular Diagnostics unit during the 2008-2009 season was unable to type 3 of the influenza A isolates. These samples were put through all of the mutation assays to see if their subtype was identifiable. Only 1 sample was detected in any of the assays, the E119V SNP assay, which showed the sample to be 100% susceptible to NAI's and determined that this sample contained an N2 influenza virus.

4.3. Other Mutations:

Several new mutations have been noted on their own, or in conjunction with other already known mutations, to cause reduced susceptibility to NAI's. The most noted of these is I222 (223 in N1 numbering).

Isoleucine (I) 222 is a framework residue which helps to stabilize the formation of the binding site for sialic acid (Colman et al, 1983). Reverse genetics has shown that a mutation at this position can cause reduced susceptibility to NAIs. Depending on the amino acid the mutation results in, can cause varying amounts of reduction of susceptibility to NAI's (Richard et al, 2008). A change to leucine (L) can cause a reduction in sensitivity to zanamivir by up to 10 fold (compared to wild-type virus in inhibition assays) and to oseltamivir by 18 fold in N2 viruses (Richard et al, 2008). Baz et al (2006) noted a mutation of isoleucine to valine (V) at position 222 in conjunction with the E119V mutation. The virus was isolated from an immunocompromised child after 38 days of oseltamivir treatment and is believed to reduce the susceptibility of oseltamivir even further (IC_{50} value of 1011nmol/L) than if the virus possessed the E119V mutation alone (IC_{50} value of 452nmol/L) (Baz et al, 2006).

I222V has also been reported in H1N1pdm09 viruses on its own and in conjunction with the H274Y mutation (Garrison et al, 2009). A change to arginine (R) has also been reported in conjunction with the H274Y mutation in H1N1pdm09 viruses in immunocompromised and competent individuals, leading to multidrug resistance (Nguyen et al, 2010a; van der Vries et al, 2010; Eshaghi et al, 2011).

A point mutation at position 136, CAG to AAG, resulting in an amino acid change from glutamine (Q) to lysine (K) has been observed in H1N1 viruses, causing resistance to zanamivir (Hurt et al, 2009). However this was not observed directly from clinical samples. The Q136K mutation has also been discovered in H3N2 viruses causing resistance to zanamivir, from patients with no history of NAI usage (Dapat et al, 2010). A GenBank search performed by Dapat et al (2010) as part of their study, showed that the Q136K mutation could be a naturally occurring mutation as NA sequences with this mutation were isolated in 1995, before the worldwide use of NAI's, and in 2003, 2004 and 2007

A mutation at position 247 (N1 numbering) causing an amino acid change from serine (S) to asparagine (N) has been recorded in community patients in Australasia. This mutation causes a reduced susceptibility to oseltamivir and zanamivir, 6 and 3 fold difference versus wild-type virus, by NIA, respectively, and an increase in resistance to oseltamivir and peramivir when combined with the H274Y mutation (6000 fold difference versus wild-type virus compared to 600 fold difference for the H274Y mutation alone) (Hurt et al, 2011).

The amount of new and combination mutations being reported gives cause for concern especially as the only mutation tested for routinely is H274Y. It may be a better protocol to perform full influenza genome sequencing to highlight any mutations or deletions and also to perform inhibition assays to check the amount of susceptibility each mutation or combination of mutations results in.

4.4. New Techniques:

Since the start of this project in 2009 many genotypic protocols and techniques have been reported for the detection of the point mutations which can cause NAI resistance. These include real time RT-PCR assays (Bolotin et al, 2009; van der Vries et al, 2013), and other nucleotide based techniques (Steain et al, 2009; Liu et al, 2010; Duan et al, 2011; Miyoshi-Akiyama et al, 2012). However all these new genotypic techniques come to a similar conclusion in that they are limited by the knowledge of the mutations which cause resistance (Nguyen et al, 2012; Okomo-Adhiambo et al, 2012).

The WHO currently recommends; Sanger sequencing, Pyrosequencing or Real-time RT-PCR allelic discrimination for the detection of NAI resistance in H1N1pdm09 viruses (WHO, 2014). However quicker, high through-put and more cost-effective molecular sequencing methods are now available, known as next generation or deep sequencing. These techniques are now widely used in viral research especially for identification of new viruses or new strains of known viruses especially in outbreak situations and in drug resistance (Quiñones-Mateu et al, 2014). These new techniques may be useful in the future detection of new mutations in the neuraminidase gene, but the relevance of the mutations discovered could not be verified without still performing some form of phenotypic assay to confirm that the mutation causes reduced susceptibility to the NAI's.

4.5. New Treatments:

After the introduction worldwide of oseltamivir and zanamivir in 1999, further research has been conducted to develop other NAI's and to research other aspects for influenza antivirals to target.

4.5.1. Peramivir:

Peramivir is an NAI based on the structure of sialic acid. It was developed in 2000 as an alternative to oseltamivir and zanamivir (Babu et al, 2000).

Peramivir is currently undergoing phase 3 clinical trials in America and was approved for use in Japan and South Korea in 2011. It has been shown to reduce the longevity of symptoms, has demonstrated a higher efficacy than zanamivir for H1N1 and H3N2 viruses and an efficacy similar to oseltamivir and has been well tolerated (Bantia et al, 2001; Gubareva et al, 2001b; Hernandez et al, 2011). The FDA in the USA, awarded the intravenous use of peramivir a FDA Emergency Use Authorisation (EUA) for use during the 2009 influenza pandemic (FDA, 2009).

Neuraminidase inhibition assays were performed in 2001 to compare the effect of various point mutations on the efficacy of peramivir compared to oseltamivir and zanamivir. The mutation at position at R292 showed variable levels of resistance to each NAI, whereas the mutation at H274 showed resistance to Peramivir, but

susceptibility to zanamivir and various mutations at position E119 showed varying levels of resistance with a change to aspartic acid (D) causing the greatest resistance (Gubareva et al, 2001b). The H274Y mutation in H1N1pdm09 strains has shown resistance to peramivir especially when the I222R mutation is also present (Memoli et al, 2010; Nguyen et al, 2010a).

As peramivir is similar to the NAIs already licensed and has a varying effect on viruses containing the known mutations causing resistance to NAIs, it seems to only be useful if patients are not responding to other treatments and would benefit from intravenous administration. Zanamivir is now suggested to be used in an intravenous form (in the UK) if the patient is unable to tolerate inhalation (Parry-Ford, 2013) and it would seem a better idea to use this on patients who are unresponsive to oseltamivir treatment, especially if infected with the H1N1pdm09 strain, rather than peramivir.

The concentration of zanamivir in epithelial lining fluid after doses of either i.v zanamivir (600mg twice a day) or inhaled zanamivir (10mg twice a day) has been studied. The first aliquot of BAL samples performed 12 hours post dose, showed a higher zanamivir concentration in the subjects given inhaled zanamivir, but subsequent aliquots showed higher concentrations in the subjects given i.v zanamivir. This difference is believed to be due to the first wash collecting fluid from the areas closest to the broncoscope and also the areas that will be most affected by an inhaled drug, whereas the later aliquots would be from deeper in the lungs, areas which inhaled drugs would be less likely to reach. Therefore i.v zanamivir is likely to affect the whole of the lungs and target all infected cells, rather than just the upper areas where inhaled zanamivir is likely to reach (Shelton *et al*, 2011).

4.5.2. Laninamivir:

Laninamivir was developed in 2010 and has been commercially available as Inavir® in Japan since October 2010 (Yamishita et al, 2011). It is administered by inhalation like zanamivir but has shown greater efficacy with just one dose compared to the bi-daily dosing of oseltamivir and zanamivir. It has also been shown to be effective after one dose, up to 7 days pre-infection as prophylaxis in *in vivo* studies (Kubo et al, 2010). It has also shown great efficacy after single

doses of 40 and 20mg versus oseltamivir, against oseltamivir resistant H1N1 viruses in children. However no noted differences in efficacy compared to oseltamivir, were observed when used to treat H3N2 or B viruses, except of course the dosing regimen (Sugaya & Ohashi, 2010). In adults the efficacy of laninamivir was similar to oseltamivir (Watanabe et al, 2010).

4.5.3. Other Treatments:

Many different areas are now being researched to discover new antivirals for the treatment of influenza. A review by Hayden (2012) compiled a list of these agents (Table 10).

Some agents other than NAIs and their alternative routes of administration are in advanced clinical development. These include Favipiravir (T-705), DAS181 and Nitazoxanide. Favipiravir inhibits the RNA polymerase of influenza after it is metabolised by the infected cell (Furuta et al, 2009). It has shown equal efficacy to oseltamivir in phase 2 and 3 trials in Japan and other Asian countries, although an initial loading dose and dosage adjustments based on weight and maybe ethnicity maybe be required (Hayden, 2012). DAS181 is a combination of the catalytic site of the sialidase from *Actinomyces viscosus* and a cell surface anchoring domain of human amphiregulin (Malakhov et al, 2006). This sialidase removes the sialic acid receptors from both human and avian epithelial cells, thus preventing the binding of influenza. Nitazoxanide is a licensed anti-parasitic, which has shown inhibitory effects on the maturation of HA (Rossignol et al, 2009). In phase 2 trials it has shown a reduction in symptoms by up to 20 hours and even a reduction in viral load (Hayden, 2012). These agents clearly need further studies before they can be licensed for use for the treatment of influenza but they seem worth investigating and especially as none of them have shown any sign of resistance under pressure tests (Hayden, 2012).

Another treatment option is to combine treatments that are already available. A triple combination drug regimen (TCAD) of amantadine, ribavirin and oseltamivir has been shown to be highly synergistic *in vitro*, especially against amantadine and oseltamivir resistant isolates (Nguyen et al, 2010b). Some combinations have shown antagonistic effects, such as oseltamivir and zanamivir against H1N1pdm09 viruses *in vitro*, and in seasonal influenza in humans.

Table 10 - Investigational anti-influenza agents (adapted from Hayden, 2012)

Drug Class	**Agent-** IN-intranasal; IP-intraperitoneal; IV-intravenous; SC-subcutaneous
NA inhibitors (NAIs)	Peramivir (IV), zanamivir (IV), oseltamivir (IV)
	A-315675 (oral)(
Long-acting NAIs (LANIs)	Laninamivir (topical)
	ZNV dimers (topical)
Conjugated sialidase	DAS181 (topical)
Protease inhibitors	Aprotinin (topical, IV)
HA inhibitors and viral binding agents	Peptides- FluPep (topical), Entry Blocker (topical), HB80/36, Flufirvitide
	Arbidol (oral)
	Cyanovirin-N (topical)
	Iota-carrageenan (topical)
	Pentraxin PTX3 (IP)
	Polymer bound 6' sialyl-*N*-acetyllactosamine (topical)
	CYSTUS052 (topical)
	Recombinant human galectin-1 (topical)(
Polymerase inhibitors	Ribavirin (oral, IV, inhaled
	Favipiravir/T-705 (oral
	Viramidine (oral)
	Antisense oligonucleotides (IV, topical)
M gene	Antisense oligonucleotide (AVI-7100) (topical, IV)
NP inhibitors	Nucleozin (IP)
	Antisense oligonucleotides (IV)
Interferons	IFN inducers- poly-ICLC (topical), nitazoxanide (PO)
	RIG-I activator (5'PPP-RNA) (IV)
Antibodies to viral proteins	Convalescent plasma, hyperimmune globulin
	Anti-HA, M2e, NA, NP
Other topical agents	Cationic airway lining modulators (iCALM- topical)
	Surfactant nano-emulsions (topical)
	SOFA-HDV ribozymes targeting M, NS, NP
	Defective interfering particles (244 DI RNA in a cloned A/PR/8/34)

However this is likely to be due to the NAIs acting on the same target (Duval et al, 2010; Nguyen et al; 2010b).

Other treatment options include, antibody therapy, inhibition of RNA, adjunctive therapies to reduce the pro-inflammatory response, and host cell targets instead of influenza itself (Hayden, 2012).

Influenza treatment is still an ongoing discovery and maybe it will continue for another 50 years. Treatment needs to be found to help control disease progression and viral load especially in the immunocompromised, who are the worst effected, and most likely to develop drug resistance due to their long drug exposure. Treatment without the ability for resistance to occur would be the most beneficial. However maybe the fight for new treatment may come to an end with the development of a universal vaccine?

4.5.4. Universal Vaccine:

The H, M (M1 and M2), NP and N proteins (mainly in influenza A viruses) are being investigated for use as the target antigens for universal vaccines (Table 11) (Subbarao & Matsuoka, 2013).

Table 11 - Viral targets of universal vaccines (adapted from Subbarao & Matsuoka, 2013).

Protein	Target Site	Function of target	Proposed mechanism of protection
H	Stem	Fusion activity	Inhibition of fusion, maturation of H, viral egress, and ADCC*
M2	M2e	Ion channel	Alveolar macrophages and Fc, ADCC*, Ab dependent NK-cell activity, Complement-mediated lysis
NP	T-cell and Ab epitopes	T-cell stimulation and non-neutralising Ab	Cell lysis by $CD8^+$ cytotoxic T lymphocytes (CTL), $CD4^+$ T lymphocyte-mediated cytolysis and B cell stimulation
M1	T-cell epitopes	T-cell stimulation	Cell lysis by $CD8^+$ cytotoxic T lymphocytes (CTL), $CD4^+$ T lymphocyte-mediated cytolysis and B cell stimulation
N	Conserved sialidase active site	Sialidase	Inhibition of viral spread

*ADCC-Antibody-dependent cell cytotoxicity.

The sequence for the extracellular domain of the M2 protein, M2e is conserved among human influenza viruses and is not subject to drift or shift and has not significantly altered its genetic code since the 1918 pandemic (Fiers et al, 2004). The frequency and level of naturally occurring Abs to this region in infected individuals is very low, so studies into the use of this region for vaccine use have

concentrated on ways to overcome the poor immunogenicity (Subbarao & Matsuoka, 2013).

The NP and M1 proteins are conserved among influenza A viruses. These proteins are not extracellular and therefore only produce cellular immune responses (Subbarao & Matsuoka, 2013). Vector based and linear peptide-based antigen delivery, have shown to be promising with approximately 60% reduction in infection after a single intramuscular dose (Berthoud et al, 2011). Also a vaccine based on a recombinant protein containing multiple linear epitopes from H, NP and M1 proteins from both influenza A and B viruses has been well tolerated (Atsmon et al, 2012).

Virus-like particles (VLPs) containing N1 N protein have been shown to produce Abs in mice and protect them from H1N1 and H3N2 viruses, and have also shown a reduction in virus replication. However it is believed that vaccines that are based solely on the N protein would not prevent infection (Subbarao & Matsuoka, 2013).

5. Conclusions:

The aim of this study was to adapt and optimised a published protocol to detect the point mutations conferring NAI resistance in influenza A viruses, on a routine basis in a clinical setting. The assays for the detection of the H274Y mutation in H1N1 viruses and the detection of the E119V mutation in H3N2 viruses were able to be adapted for routine use. The other point mutations at positions D151 in H1N1 viruses and D151, R292 and N294 in H3N2 viruses were not able to be optimised to perform SNP analysis according to the protocol published by Deyde et al (2009b). SQA analysis was able to be performed for the H3N2 mutations. Clinical evaluation of these assays showed the presence of the H274Y mutation, 91-100%, in all the H1N1 viruses isolated from the welsh population during the 2008-2009 season. Nearly 50% of the H3N2 viruses detected during the 2008-2009 season had the E119V mutation, 3-31%, and none of the isolates had mutations at positions D151, R292 or N294. The assay for the detection of the H274Y mutation in H1N1pdm09 viruses, published by the WHO in 2009, was also adapted and optimised for routine use. A clinical evaluation of 121 samples collected between April 2009 and February 2011, was performed. The H274Y mutation was observed in the majority of samples but mainly at levels less than 10%.

Since the start of this assay the main circulating strain of influenza A has been H1N1pdm09. Therefore the detection of the H274Y mutation in this species can be performed routinely in a clinical setting based on this study. Being able to perform this assay, as part of a routine screen, will be beneficial for patient treatment, as a resistance result could be available with 24 hours from sample collection.

However, if a change in circulating strain occurs or a change in mutation conferring to NAI resistance then a new assay will need to be developed.

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Appendices:

Appendix I. Influenza Sequences:

Sequences with mutation and primers marked:

A/New Caledonia/20/99 (H1N1)

```
1    atgaatccaa atcaaaaaat aataaccatt ggatcaatca gtatagcaat cggaataatt
61   agtctaatgt tgcaaatagg aaatattatt tcaatatggg ctagtcactc aatccaaact
121  ggaagtcaaa accacactgg agtatgcaac caaagaatca tcacatatga aaacagcacc
181  tgggtgaatc acacatatgt taatattaac aacactaatg ttgttgctgg aaaggacaaa
241  acttcagtga cattggccgg caattcatct ctttgttcta tcagtggatg ggctatatac
301  acaaaagaca acagcataag aattggctcc aaaggagatg tttttgtcat aagagaacct
361  ttcatatcat gttctcactt ggaatgcaga acctttttc tgacccaagg tgctctatta
421  aatgacaaac attcaaatgg gaccgtaaag gacagaagtc cttatagggc cttaatgagc
481  tgtcctctag gtgaagctcc gtccccatac aattcaaagt ttgaatcagt tgcatggtca
541  gcaagcgcat gccatgatgg catgggctgg ttaacaatcg gaatttctgg tccagacaat
601  ggagctgtgg ctgtactaaa ataccaacggc ataataactg aaaccataaa aagttggaaa
661  aagcgaatat taagaacaca agagtctgaa tgtgtctgtg tgaacgggtc atgtttcacc
721  ataatgaccg atggcccgag taatggggcc gcctcgtaca aaatcttcaa gatcgaaaag
781  gggaaggtta ctaaatcaat agagttgaat gcacccaatt ttcattatga ggaatgttcc
841  tgttacccag acactggcac agtgatgtgt gtatgcaggg acaactggca tggttcaaat
901  cgaccttggg tgtcttttaa tcaaaacttg gattatcaaa taggatacat ctgcagtggg
961  gtgttcggtg acaatccgcg tcccaaagat ggagagggca gctgtaatcc agtgactgtt
1021 gatggagcag acggagtaaa ggggttttca tacaaatatg gtaatggtgt ttggatagga
1081 aggactaaaa gtaacagact tagaaagggg tttgagatga tttgggatcc taatggatgg
1141 acagataccg acagtgattt ctcagtgaaa caggatgttg tggcaataac tgattggtca
1201 gggtacagcg gaagtttcgt tcaacatcct gagttaacag gattggactg tataagacct
1261 tgcttctggg ttgagttagt cagaggactg cctagagaaa atacaacaat ctggactagt
1321 gggagcagca tttctttttg tggcgtaaat agtgatactg caaactggtc ttggccagac
1381 ggtgctgagt tgccgttcac cattgacaag tag
```

A/Hawaii/28/2007 (H1N1)

```
1    atgaacccaa atcaaaagat aataaccatt ggatcaatca gtatagcaat cggaataatt
61   agtctaatgt tgcaaatagg aaatattatt tcaatatggg ctagtcactc aatccaaact
121  ggaagtcaaa acaacactgg aatatgcaac caaagaatca tcacatatga aaacagcacc
181  tgggtgaatc acacatatgt taatattaac aacactaatg ttgttgctgg agaggacaaa
241  acttcagtga cattggctgg caattcatct ctttgttcta tcagtggatg ggctatatac
301  acaaaagaca acagcataag aattggctcc aaaggagatg tttttgtaat aagagaacct
361  ttcatatcat gttctcactt ggaatgcaga acctttttc tgacccaagg cgctctatta
421  aatgacaaac attcaaatgg gaccgtaaag gacagaagtc cttatagggc cttaatgagc
481  tgtcctctag gtgaagctcc gtccccatac aattcaaagt tcgaatcagt tgcatggtca
541  gcaagcgcat gccatgatgg catgggctgg ttaacaatcg gaatttctgg tccagacaat
601  ggagctgtgg ctgtactaaa ataccaacgga ataataactg gaaccataaa aagttggaaa
661  aagcaaatat taagaacaca agagtctgaa tgtgtctgta tgaacgggtc atgtttcacc
721  ataatgaccg atggcccgag taataaggcc gcctcgtaca aaattttcaa gatcgaaaag
781  gggaaggtta ctaaatcaat agagttgaat gcacccaatt tttattatga ggaatgttcc
841  tgttacccag acactggcat agtgatgtgt gtatgcaggg acaactggca tggttcaaat
901  cgaccttggg tgtcttttaa tcaaaacttg gattatcaaa taggatacat ctgcagtgga
961  gtgttcggtg acaatccgcg tcccgaagat ggagagggca gctgcaatcc agtgactgtt
1021 gatggagcaa acggagtaaa agggttttca tacaaatatg ataatggtgt ttggatagga
1081 aggaccaaaa gtaacagact tagaaagggg tttgagatga tttgggatcc taatggatgg
1141 acaaataccg acagtgattt ctcagtgaaa caggatgttg tagcaataac tgattggtcg
1201 gggtacagcg gaagtttcgt ccaacatcct gagttaacag gattggactg tataagacct
1261 tgcttctggg ttgagttagt cagagggctg cctagagaaa atacaacaat ctggactagt
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1321 gggagcagca tttctttttg tggcgttaat agtgatactg caaactggtc ttggccagac
1381 ggtgctgagt tgccgttcac cattgacaag tagttcgttg aaaaaaa
```

A/Moscow/10/99 (H3N2)

```
1    atgaatccaa atcaaaagat aataacgatt ggctctgttt ctctcaccat tgccacaata
61   tgcttcctta tgcaaattgc catcctggta actactgtaa cattgcattt caagcaatat
121  gaatgcaact ccccccaaa caaccaagtg atgctgtgtg aaccaacaat aatagaaaga
181  aacataacag agatagtgta tctgaccaac accaccatag agaaggaaat atgccccaaa
241  ctagcagaat acagaaattg gtcaaagccg caatgtaaca ttacaggatt tgcacctttt
301  tctaaggaca attcgattcg gctttccgct ggtggggaca tctgggtgac aagagaacct
361  tatgtgtcat gcgatcctga caagtgttat caatttgccc ttggacaggg aacaacacta
421  aacaacgggc attcaaatga cacagtacat gataggaccc cttatcggac cctattgatg
481  aatgagttgg gtgttccatt tcatttggga accaagcaag tgtgcatagc atggtccagc
541  tcaagttgtc acgatggaaa agcatggctg catgtttgtg taacggggga tgatgaaaat
601  gcaactgcta gcttcattta caatgggagg cttgtagata gtattggttc atggtccaaa
661  aaaatcctca ggacccagga gtcggaatgc gtttgtatca atggaacttg tacagtagta
721  atgactgatg ggagtgcttc aggaaaagct gatactaaaa tactattcat tgaggagggg
781  aaaatcgttc atactagccc attgtcagga agtgctcagc atgtcgagga atgctcctgt
841  tatcctcgat atcctggtgt cagatgtgtc tgcagagaca actggaaagg ctccaatagg
901  cccatcgtag atataaacat aaaggattat agcattgttt ccagttatgt gtgctcagga
961  cttgttggag acacacccag aaaaaacgac agctccagca gtagccattg cttggatcct
1021 aacaatgagg aaggtggtca tggagtgaaa ggctgggcct ttgatgatgg aaatgacgtg
1081 tggatgggaa gaacgatcag cgagaagtta cgctcaggat atgaaacctt caaagtcatt
1141 gaaggctggt ccaaacccaa ctccaaattg cagataaata ggcaagtcat agttgacaga
1201 ggtaataggt ccggttattc tggtattttc tctgttgaag gcaaaagctg catcaatcgg
1261 tgcttttatg tggagttgat aaggggaagg aaacaggaaa ctgaagtctt gtggacctca
1321 aacagtattg ttgtgttttg tggcacctca ggtacatatg gaacaggctc atggcctgat
1381 ggggcggaca tcaatctcat gcctatataa
```

A/Illinois/14/2008 (H3N2)

```
1    atgaatccaa atcaaaagat aataacgatt ggctctgttt ctctcaccat ttccacaata
61   tgcttcttca tgcaaactgc catcttgata actactgtaa cattgcattt caagcaatat
121  gaattcaact ccccccaaa caaccaagtg atgctgtgtg aaccaacaat aatagaaaga
181  aacataacag agatagtgta tctgaccaac accaccatag agaaggaaat atgccccaaa
241  ctagcagaat acagaaattg gtcaaagccg caatgtgaca ttacaggatt tgcacctttt
301  tctaaggaca attcgattag gctttccgct ggtggggaca tctgggtgac aagagaacct
361  tatgtgtcat gcgatcctga caagtgttat caatttgccc tcggacaggg aacaacacta
421  aacaacgtgc attcaaatga cacagtacgt grtaggaccc cttatcggac cctattgatg
481  aatgagttag gtgttccttt tcatctgggg accaagcaag tgtgcatagc atggtccagc
541  tcaagttgtc acgatggaaa agcatggctg catgtttgta taacggggga tgataaaaat
601  gcaactgcta gcttcattta caatgggagg cttgtagata gtattgtttc atggtccaag
661  gaaattctca ggacccagga gtcagaatgc gtttgtatca atggaacttg tacagtagta
721  atgactgatg ggagtgcttc aggaaaagct gatactaaaa tactattcat tgaggagggg
781  aaaatcgttc atactagcac attgtcagga agtgctcagc atgtcgagga gtgctcctgc
841  tatcctcgat atcctggtgt cagatgtgtc tgcagagaca actggaaagg ctccaatagg
901  cccatcgtag atataaacat aaaggatcat agcattgttt ccagttatgt gtgttcagga
961  cttgttggag acacacccag aaaaaacgac agctccagca gtagccattg tttggatcct
1021 aacaatgaag aaggtggtca tggagtgaaa ggctgggcct ttgatgatgg aaatgacgtg
1081 tggatgggaa gaacgatcag cgagaagtca cgcttagggt atgaaacctt caaagtcatt
1141 gaaggctggt ccaaccctaa gtccaaattg cagataaata ggcaagtcat agttgacaga
1201 ggtgataggt ccggttattc tggtattttc tctgttgaag gcaaaagctg catcaatcgg
1261 tgcttttatg tggagttgat aaggggaaga aaagaggaaa ctgaagtctt gtggacctca
1321 aacagtattg ttgtgttttg tggcacctca ggtacatatg gaacaggctc atggcctgat
1381 ggggcggaca tcaatctcat gcctatataa
```

A/Oklahoma/1806/2008 (H3N2)

```
1     aataacgatt ggctctgttt ctctcaccat ttccacaata tgcttcttca tgcaaattgc
61    catcttgata actactgtaa cattgcactt caagcaatat gaattcaact ccccccaaa
121   caaccaagtg atgctgtgtg aaccaacaat aatagaaaga aacataacag agatagtgta
181   tctgaccaac accaccatag agaaggaaat atgccccaaa ctagcagaat acagaaattg
241   gtcaaagccg caatgtgaca ttacaggatt tgcacctttt tctaaggaca attcgattag
301   gctttctgct ggtggggaca tctgggtgac aagagaacct tatgtgtcat gcgatcctga
361   caagtgttat caatttgccc ttggacaggg aacaacacta aacaacgtgc attcaaatga
421   cacagtacgt ggtaggaccc cttatcggac cctattgatg aatgagttag gtgttccttt
481   tcatctgggg accaagcaag tgtgcatagc atggtccagc tcaagttgtc accatggaaa
541   agcatggctg catgtttgtc taacgggggа tgataaaaat gcaactgcta gcttcattta
601   caatgggagg cttgtagata gtattgtttc atggtccaaa gacatcctca ggacccagga
661   gtcagaatgc gtttgtatca atggaacttg tacagtagta atgactgatg ggagtgcttc
721   aggaaaagct gatactaaaa tactattcat tgaggagggg aaaatcgttc atactagcac
781   attgtcagga agtgctcagc atgtcgagga gtgctcctgc tatcctcgat atcctggtgt
841   cagatgtgtc tgcagagaca actggaaagg ctccaatagg cccatcgtag atataaacat
901   aaaggatcat agcattgttt ccagttatgt gtgttcagga cttgttggag acacacccag
961   aaaaaacgac agctccagca gtggccattg tttggatcct aacaatgaag aaggtggtca
1021  tggagtgaaa ggctgggcct ttgatgatgg aaatgacgng tggatgggaa gaacgatcag
1081  cgagaagtca cgcttagggt atgaaacctt taaagtcatt gaaggctggt ccaaccctaa
1141  gtccaaattg cagataaata ggcaagtcat agttgacaga ggtaataggt ccggttattc
1201  tggtattttc tctgttgaag gcaaaagctg catcaatcgg tgcttttatg tggagttgat
1261  aaggggaaga aaagaggaaa ctgaagtctt gtggacctca aacagtattg ttgtgttttg
1321  tggcacctca ggtacatatg gaacaggctc atggcctgat ggggcgga
```

Influenza A virus (A/California/07/2009(H1N1)) Pandemic:

```
1     atgaatccaa accaaaagat aataaccatt ggttcggtct gtatgacaat tggaatgg
61    aacttaatat tacaaattgg aaacataatc tcaatatgga ttagccactc aattcaactt
121   gggaatcaaa atcagattga aacatgcaat caaagcgtca ttacttatga aaacaacact
181   tgggtaaatc agacatatgt taacatcagc aacaccaact ttgctgctgg acagtcagtg
241   gtttccgtga aattagcggg caattcctct ctctgccctg ttagtggatg ggctatatac
301   agtaaagaca acagtgtaag aatcggttcc aaggggatg tgtttgtcat aagggaacca
361   ttcatatcat gctccccctt ggaatgcaga accttcttct tgactcaagg ggccttgcta
421   aatgacaaac attccaatgg aaccattaaa gacaggagcc catatcgaac cctaatgagc
481   tgtcctattg gtgaagttcc ctctccatac aactcaagat ttgagtcagt cgcttggtca
541   gcaagtgctt gtcatgatgg catcaattgg ctaacaattg gaatttctgg cccagacaat
601   ggggcagtgg ctgtgttaaa gtacaacggc ataataacag acactatcaa gagttggaga
661   aacaatatat tgagaacaca agagtctgaa tgtgcatgtg taaatggttc ttgctttact
721   gtaatgaccg atggaccaag taatggacag gcctcataca agatcttcag aatagaaaag
781   ggaaagatag tcaaatcagt cgaaatgaat gcccctaatt atcactatga ggaatgctcc
841   tgttatcctg attctagtga aatcacatgt gtgtgcaggg ataactggca tggctcgaat
901   cgaccgtggg tgtctttcaa ccagaatctg gaatatcaga taggatacat atgcagtggg
961   attttcggag acaatccacg ccctaatgat aagacaggca gttgtggtcc agtatcgtct
1021  aatggagcaa atggagtaaa agggttttca ttcaaatacg gcaatggtgt ttggataggg
1081  agaactaaaa gcattagttc aagaaacggt tttgagatga tttgggatcc gaacggatgg
1141  actgggacag acaataactt ctcaataaag caagatatcg taggaataaa tgagtggtca
1201  ggatatagcg ggagttttgt tcagcatcca gaactaacag ggctggattg tataagacct
1261  tgcttctggg ttgaactaat cagagggcga cccaaagaga acacaatctg gactagcggg
1321  agcagcatat ccttttgtgg tgtaaacagt gacactgtgg gttggtcttg gccagacggt
1381  gctgagttgc catttaccat tgacaagtaa
```

A/Aragon/RR3218/2008(H1N1): Proficiency panel Swine flu control.

```
1   aatggaacca ttaaggacag aagtccatat cgaaccttaa tgagctgtcc tattggtgaa
61  gtcccttctc catacaactc aaggtttgag tcagtcgctt ggtcagcaag tgcttgccat
121 gatggtacca gttggctaac aattggaatt tctggcccag ataatggggc agtggctgtg
181 ctgaaataca acggcataat aacagacact atcaagagtt ggagaaacaa catattaaga
241 acacaagagt ctgaatgtgc atgcgtgaat ggttcttgct ttactataat gaccgatgga
301 ccaagtaatg ggcaggcctc atacaagatc tttaagatag aaaggggggaa agtagtaaaa
361 tcagtcgagt tgaatgcccc taattaccac tatgaggaat gctcctgtta tcctgagtct
421 ggtgaaatta catgtgtgtg cagagataat tggcatggct caaatcggcc atgggtgtct
481 ttcaatcaga atctggaata taaaatagga tacatatgca gtgggatttt cggagacaat
541 ccgcgcccta atgatggaac aggcagttgt ggcccagtac tctctaatgg agcaaatggg
601 gtaaaaggat tttcatttaa atacggcaat ggtgtttgga tagggagaac taaaagcact
661 agctcaagga gcggttttga gatgatttgg gatccaaatg gatggaccag aacagacgat
721 aatttctcag taaagcaaga tatcatagga atgactcatt ggtcaggata tagcgggagt
781 tttgctc
```

Appendix II. Primers:

A(H1N1): 151 – GAC → GAA - AGGAC/AAG

HuH1N1-151-F425	5’-ACAAACATTCAAATGGRACCG-3’
HuH1N1-151-R521-biot	5’-CTGACCATGCAACTGATTCAA-3’
Strand binding point	3’- GACTGGTACGTTGACTAAGTT-5’
HuH1N1- 151-F425-seq	5’-ACAAACATTCAAATGGRACCG-3’

A(H1N1): 274 – CAT → TAT - TTTC/TATT

HuH1N1- 274-F770	5’-AGATCGAGAAGGGGAAGGTTACTA-3’
HuH1N1- 274-R882-biot	5’-GTCYCTGCATACACACATCACT-3’
Strand binding point	3’-CAGYGACGTATGTGTGTAGTGA-5’
HuH1N1-274-F807-seq	5’-AAATGCACCCAAT-3’

Colindale Primers

A(H1N1): 274 – CAT → TAT - TTTC/TATT

HuH1N1- 274-For	5’-GGAGCCGTGGCTGTACTAAAATA-3’
HuH1N1- 274-R882-biot	5’-CCACGTTTTGATTAAAAGACACC-3’
Strand binding point	3’-GGTGTCTTTTAATCAAAACGTGG-5’
HuH1N1-274-F807-seq	5’-AGTTGAATGCACCCAAT-3’

A(H3N2): 119 – TTC → TAC – AGGTT/ACTCT

HuH3N2-F333-biot	5’-TGCGGACATCTGGGTGACA-3’
HuH3N2-R914	5’-ATATCTACTATGGGCCTATTGGA-3’
Strand binding point	3’-TATAGATGATACCCGGATAACCT-5’

HuH3N2-R377-seq 5’-GGATCGCATGACACATA-3’

Strand binding point 3’-CCTAGCGTACTGTGTAT-5’

A(H3N2): 151 – V- GAT → GTT, A – GAT → GCT, N – GAT → AAT

V – TGA/TTAG, A – TGA/CTAG, N – TG/AATAG

HuH3N2-F423 5’-CAACGTGCATTCAAATGACAC-3’

HuH3N2-R567-biot 5’-CCAYGCTTTYCCATCRTG-3’

Strand binding point 3’- GGTYCGAAAYGGTAGRAC-5’

HuH3N2-F423-seq 5’-CAACGTGCATTCAAATGACAC-3’

A(H3N2): 292/294 – 292 – TCT → TTT – GTCTC/TTTC

HuH3N2-F333-biot 5’-TGCGGACATCTGGGTGACA-3’

HuH3N2-R914 5’-ATATCTACTATGGGCCTATTGGA-3’

Strand binding point 3’- TATAGATGATACCCGGATAACCT-5’

HuH3N2- R905-seq 5’-ATGGGCCTATTGCAGCC-3’

Strand binding point 3’-TACCCGGATAACGTCGG-5’

Pandemic Influenza A(H1N1) 2009: 274 – CAC → TAC - ATC/TACTATGAG

Uni-sw-N1-B-F780 5’-GGGGAAGATTGTYAAATCAGTYGA -3’

Uni-sw-N1-B-R1273-biot 5’-CWACCCAGAARCAAGGYCTTATG-3’

Strand binding point 3’- GWTGGGTCTTRGTTCCYGAATAC-5’

Uni-sw-N1-B-F804-seq 5’-GYTGAATGCMCCTAATT -3’

Appendix III. Worksheets:

Worksheet 1:

	Volume	n+ 1
2x Reaction mix	25µl	
Forward primer (20µM)	1µl	
Reverse primer (20µM)	1µl	
Superscript III RT/Platinum Taq Mix	2µl	
RNAse-free water	16µl	
Total	45 µl	

Add 5µl of eluate to 45µl of mastermix.

1.	9.	17.	25.	33.	41.
2.	10.	18.	26.	34.	42.
3.	11.	19.	27.	35.	43.
4.	12.	20.	28.	36.	44.
5.	13.	21.	29.	37.	45.
6.	14.	22.	30.	38.	46.
7.	15.	23.	31.	39.	47.
8.	16.	24.	32.	40.	48.

Sequencing worksheet:

Immobilisation mix:

Reagent	Volume	n+1
Streptavidin Beads	3ul	
Binding buffer	40ul	
Water	17ul	
Total	60ul	

Add 20ul of amplified product to 60ul immobilisation mix.

Reagent	Volume for 10 reactions	Reactions
Sequencing primer (100uM)	2ul	
Annealing buffer	438ul	
Total	440ul	

Cartridge Volumes:

	G	
C		**T**
S	**A**	**E**

1.	9.	17.	25.	33.	41.
2.	10.	18.	26.	34.	42.
3.	11.	19.	27.	35.	43.
4.	12.	20.	28.	36.	44.
5.	13.	21.	29.	37.	45.
6.	14.	22.	30.	38.	46.
7.	15.	23.	31.	39.	47.
8.	16.	24.	32.	40.	48.

New worksheet 18/04/10

	Volume	n+ 1
2x Reaction mix	25µl	
Forward primer (20µM)	1µl	
Reverse primer (20µM)	1µl	
Superscript III RT/Platinum Taq Mix	1µl	
RNAse-free water	12µl	
Total	40 µl	

Add 10µl of eluate to 40µl of mastermix.

1.	9.	17.	25.	33.	41.
2.	10.	18.	26.	34.	42.
3.	11.	19.	27.	35.	43.
4.	12.	20.	28.	36.	44.
5.	13.	21.	29.	37.	45.
6.	14.	22.	30.	38.	46.
7.	15.	23.	31.	39.	47.
8.	16.	24.	32.	40.	48.

New worksheet 12/05/10:

	Volume	n+ 1
2x Reaction mix	25µl	
Forward primer (20µM)	1µl	
Reverse primer (20µM)	1µl	
Superscript III RT/Platinum Taq Mix	1µl	
RNAsin	1µl	
RNAse-free water	6µl	
Total	35µl	

Add 15µl of eluate to 35µl of mastermix.

1.	9.	17.	25.	33.	41.
2.	10.	18.	26.	34.	42.
3.	11.	19.	27.	35.	43.
4.	12.	20.	28.	36.	44.
5.	13.	21.	29.	37.	45.
6.	14.	22.	30.	38.	46.
7.	15.	23.	31.	39.	47.
8.	16.	24.	32.	40.	48.

QIAGEN One step master mix worksheet:

	Volume	N+2
QIAGEN One step RT-PCR Buffer 5x	5µl	
Forward primer (20µM)	1µl	
Reverse primer (20µM)	1µl	
dNTP mix	1µl	
QIAGEN One-step RT-PCR Enzyme mix	1 µl	
RNAse-free water	6µl	
Total	15 µl	

Add 10µl of eluate to 15µl of mastermix.

1.	9.	17.	25.	33.	41.
2.	10.	18.	26.	34.	42.
3.	11.	19.	27.	35.	43.
4.	12.	20.	28.	36.	44.
5.	13.	21.	29.	37.	45.
6.	14.	22.	30.	38.	46.
7.	15.	23.	31.	39.	47.
8.	16.	24.	32.	40.	48.

Appendix IV. Raw data:

H1H1 pre 2009 samples:

	Typing	H274	Colindale
3	H1	F	
4	H1	R (T 99% / C 1%)	
25	H1	R (T 96% / C 4%)	
27	H1	R (T 98% / C 2%)	
34	H1	R (T 99% / C 1%)	
35	H1	R (T 97% / C 3%)	
38	H1	R (T 99% / C 1%)	
47	H1	R (T 99% / C 1%)	R (T 100%)
52	H1	R (T 99% / C 1%)	R (T 100%)
53	H1	R (T 94% / C 6%)	R (T 100%)
54	H1	R (T 98% / C 2%)	R (T 100%)
67	H1	R (T 99% / C 1%)	R (T 100%)
70	H1	R (T 99% / C 1%)	R (T 100%)
74	H1	R (T 99% / C 1%)	R (T 100%)
76	H1	F	R (T 100%)

H3N2 samples:

	Typing	CT	E119	R292	D151	E119 Qiagen
2	H3	39	F	F	F	S (T 95%/ A 5%)
5	H3		F		F	
6	H3	38	S (T 100%)	F		S (T 95%/ A 5%)
7	H3	40	F		F	S (T 95%/ A 5%)
8	H3	40	F	F	F	
9	H3	39	F	F	F	
10	H3	32	S (T 96%/A 4%)	F	F	
11	H3	36	S (T 100%)	F	F	
14	H3	30	S (T 100%)	S	S	
26	H3		S (T 87.5%/ A12.5%)	S	S	
28	H3	29	S (T 97% / A 3%)	S	S	S (T 95%/ A 5%)
29	H3	27	S (T 100%)		F	S (T 95%/ A 5 %)
30	H3	38	F	F	F	
31	H3	26	S (T 87% /A 13%)	F	F	
32	H3		S (T 100%)		S	
33	H3	37	S (T 100%)	S	F	
36	H3	36	F		F	
37	H3	32	S (T 92% / A 8%)	F	F	
39	H3	35	F	F	F	
40	H3	38	F		F	S (T 100%)
41	H3	28	S (T 100%)		S	S (T 95%/ A 5%)
42	H3	30	S (T 97% / A 3%)	S	S	S (T 95%/ A 5%)
44	H3	31	S? (T 79% / A 21%)	F	F	
45	H3	32	S (T 100%)			
46	H3	30	S (T 90% / A 10%)			
48	H3	27	S (T 97% / A 3%)	S		

49	H3	36	S (T 88% / A 12%)	F		
50	H3	34	S? (T 69% / A 31%)	F		
51	H3	25	S (T 96% / 4%)		S	
55	H3	37	S (T 100%)	F		
56	H3	39	F	F		
57	H3	32	S (T 100%)	S		
58	H3	33	S (T 86% / A 14%)	S		
59	H3	36	F	F		
60	H3	35	F	F	S	
61	H3	27	S (T 96% / A 4%)	S		
62	H3	39	F	F		
63	H3	37	S (T 100%)	F		
64	H3	41	F	F		
65	H3	36	F	F		
66	H3	30	S (T 100%)	S		
68	H3	31	S? (T 72% / A 28%)	F		
69	H3	26	S (T 97% / A 3%)	S		
71	H3	25	S (T 97% / A 3%)	S		
72	H3	34	S (T 100%)	S		
73	H3	30	S (T 100%)	F		
75	H3	37	S (T 100%)	S		
77	H3	31	S? (T 82% / A 18%)	F		
78	H3	26	S (T 95% / A 5%)	S		
79	H3		S (T 93% / A 7%)	F		

H1N1pdm09 samples:

		Typing	CT	Pandemic H274Y
12		H1 (Swine)		S (T12%/C 88%)
13		H1 (Swine)		S (T 3%/C 97%)
15		H1 (Swine)		S (T 2%/ C 98%)
16		H1 (Swine)		S (T 4%/ C 96%
17		H1 (Swine)		S (T 4%/ C 96%
18		H1 (Swine)		S (T 2%/ C 98%)
19		H1 (Swine)		S (T 2%/ C 98%)
20		H1 (Swine)		S (T 2%/ C 98%)
21		H1 (Swine)		S (T 3%/ C 97%)
22		H1 (Swine)		S (T 3%/ C 97%)
23		H1 (Swine)		S (T 3%/ C 97%)
24		H1 (Swine)		S (T 5%/ C 95%)
81		H1 (Swine)		SQA
82		H1 (Swine)		SQA
83		H1 (Swine)		S (T 4%/ C 96%)
84		H1 (Swine)		S (T 3%/ C97%)
85		H1 (Swine)		SQA
86		H1 (Swine)		SQA
87		H1 (Swine)		SQA
88		H1 (Swine)		SQA
89		H1 (Swine)		SQA
90		H1 (Swine)		SQA

A		H1 (Swine)	42	F
B		H1 (Swine)		S (C 100%)
C		H1 (Swine)	30	S (C 100%)
D		H1 (Swine)		S (C 100%)
E		H1 (Swine)	NEG	F
F		H1 (Swine)		S (C 100%)
G		H1 (Swine)		S (T 3%/ C 97%)
H		H1 (Swine)		S (C 100%)
92		H1 (Swine)		S (T 5%/ C 95%)
93		H1 (Swine)		F
94		H1 (Swine)		S (T 2%/ C 98%)
95		H1 (Swine)		S (T 5%/ C 95%)
96		H1 (Swine)		S (T 5%/ C 95%)
97		H1 (Swine)		S (T 5%/ C 95%)
98		H1 (Swine)		S (T 2%/ C 98%)
99		H1 (Swine)		F
100		H1 (Swine)		S (T 3%/ C 97%)
101		H1 (Swine)		S (T 3%/ C 97%)
102		H1 (Swine)		S (T 2%/ C 98%)
103		H1 (Swine)		S (T 3%/ C 97%)
104		H1 (Swine)		S (T 3%/ C 97%)
105		H1 (Swine)		S (T 9%/ C 91%)
106		H1 (Swine)		S (T 2%/ C 98%)
107		H1 (Swine)		S (T 4%/ C 96%)
108		H1 (Swine)		S (T 5%/ C 95%)
109		H1 (Swine)		S (T 5%/ C 95%)
110		H1 (Swine)		S (T 7%/ C 93%)
111		H1 (Swine)		S (T 2%/ C 98%)
112		H1 (Swine)		S (C 100%)
113		H1 (Swine)		S (T 3%/ C 97%)
114		H1 (Swine)		S (T 5%/ C 95%)
115		H1 (Swine)		F
116		H1 (Swine)		F
117		H1 (Swine)		S (T 3%/ C 97%)
118		H1 (Swine)		F
119		H1 (Swine)		F
130		H1 (Swine)		R (T 92%/ C 8%)
131		H1 (Swine)		R (T 100%)
132		H1 (Swine)		F
133		H1 (Swine)		F
134		H1 (Swine)		F
135		H1 (Swine)		F
136		H1 (Swine)		F
137		H1 (Swine)		F
138		H1 (Swine)		S (C 100%)
139		H1 (Swine)		R (T 42%/ C 58%)
140		H1 (Swine)		S (C 100%)
141		H1 (Swine)		R (T 49% / C 51%)
142		H1 (Swine)		S (T 1%/ C 99%)
143		H1 (Swine)		S (C 100%)
144		H1 (Swine)		S (T 1%/ C 99%)
145		H1 (Swine)	26	S (C 100%)

146		H1 (Swine)	31	S (C 100%)
147		H1 (Swine)	33	F
148		H1 (Swine)	36	F
149		H1 (Swine)	33	F
150		H1 (Swine)	25	S (T 3%/ C 97%)
151		H1 (Swine)	35	S (T 7%/ C 93%)
152		H1 (Swine)	26	S (T 2%/ C 98%)
153		H1 (Swine)	27	S (T 1%/ C 99%)
154		H1 (Swine)	26	S (T 1%/ C 99%)
155		H1 (Swine)	32	F
156		H1 (Swine)	28	S (C 100%)
157		H1 (Swine)	36	F
158		H1 (Swine)	28	S (T 1%/ C 99%)
159		H1 (Swine)	29	S (T 2%/ C 98%)
160		H1 (Swine)	37	F
161		H1 (Swine)	29	S (T 4%/ C 96%)
162		H1 (Swine)	20	S (T 2%/ C 98%)
163		H1 (Swine)	28	S (C 100%)
164		H1 (Swine)	29	S (C 100%)
165		H1 (Swine)	28	S (C 100%)
166		H1 (Swine)	23	S (T 3%/ C 97%)
167		H1 (Swine)	18	S (T 1%/ C 99%)
168		H1 (Swine)	21	S (T 1%/ C 99%)
169		H1 (Swine)	23	S (T 7%/ C 93%)
170		H1 (Swine)	35	S (T 2%/ C 98%)
171		H1 (Swine)	33	S (T 9%/ C 91%)
172		H1 (Swine)	35	S (T 4%/ C 96%)
173		H1 (Swine)		R (T 100%)
174		H1 (Swine)		S (T 3%/ C 97%)
175		H1 (Swine)		S (T 4%/ C 96%)
176		H1 (Swine)		S (C 100%)
177		H1 (Swine)		S (T 2%/ C 98%)
178		H1 (Swine)		F
179		H1 (Swine)		S (T 10%/ C 90%)
180		H1 (Swine)		S (T 7%/ C 93%)
181		H1 (Swine)		S (T 11%/ C 89%)
182		H1 (Swine)		S (T 8%/ C 92%)
183		H1 (Swine)		S (T 1%/ C 99%)
184		H1 (Swine)		S (C 100%)
185		H1 (Swine)		S (T 1%/ C 99%)
186		H1 (Swine)		S (C 100%)
187		H1 (Swine)		S (C 100%)
188		H1 (Swine)		S (C 100%)
189		H1 (Swine)		F
190		H1 (Swine)		S (T 2%/ C 98%)
	prof panel 2010 3	H1 (Swine)		S (T 4%/ C 96%)
	prof panel 2010 4	H1 (Swine)		F
	prof panel 2010 7	H1 (Swine)		R (T 100%)
	prof panel	H1 (Swine)		R (T 76%/ C 24%)

	2010 8			
120	QCMD INF03	H1		
121	QCMD INF09	H1		
122	QCMD INFHT06	H1		
123	QCMD INFHT09	H1		
124	QCMD INF02	H1 (Swine)		S (T 3.5%/ C 96.5%)
125	QCMD INF05	H1 (Swine)		S (T 1%/ C 99%)
126	QCMD INF08	H1 (Swine)		S (T 4%/ C 96%)
127	QCMD INF11	H1 (Swine)		S (T 4%/ C 96%)
128	QCMD INFHT03	H1 (Swine)		S (T 2%/ C 98%)
129	QCMD INFHT08	H1 (Swine)		S T 6%/ C 94%)