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Set up and selection of qRT-PCR kits for the diagnosis of human respiratory syncytial virus and influenza viruses

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Influenssa- ja RS- virukset ovat merkittäviä ihmispatogeeneja, jotka aiheuttavat vuosittaisia epidemioita, ja influenssa A toisinaan pandemioita. Uusien molekyylibiologisten menetelmien kehitys on jatkuvassa nosteessa antiviraalisen hoidon kohdistamiseksi ja antibioottien väärinkäytön vähentämiseksi.

Tämän työn kokeellinen osuus suoritettiin Orion Diagnostica Oy:n tuotekehityslaboratoriossa ja työn tavoitteena oli pystyttää useita kaupallisia kvantitatiivisia käänteiskopiointi-PCR (qRT-PCR) kittejä respiratory syncytial- virukselle (RSV) ja influenssaviruksille. Valituista qRT-PCR kiteistä oli tavoitteena valita sopiva vertailumenetelmä Orion Diagnostica Oy:n isotermaaliselle geenimonistusmenetelmälle, SIBA®:lle (Strand Invasion Based Amplification).

Vertailua varten pystytettiin kuusi kvantitatiivista yksiputkimenetelmään perustunutta qRT-PCR kittiä. qRT-PCR kittien herkkyyttä mitattiin työtä varten eristetyillä virusnäytteillä, jotka kvantitoitiin ja joita käytettiin laimennossarjoina kokeissa. Myös kittien kantakattavuutta ja mahdollisia ristireaktioita testattiin muiden hengitystiepatogeenien kanssa NATtrol™ Flu Verification Panelista eritetyllä RNA:lla. Paneeli sisälsi yhteensä 16 erilaista virus- tai bakteeriperäistä mikrobikontrollia. Lopuksi, saatujen tuloksien perusteella qRT-PCR kittien suorituskykyä arvioitiin parhaimman kitin valitsemiseksi.

Työhön valituista qRT-PCR kiteistä, RealStar® S&T Influenza RT-PCR Kit 3.0 ja RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics) menestyivät parhaiten. Erot qRT-PCR kittien välillä olivat tilastollisesti merkitseviä, esimerkiksi parhaimpien ja huonoimpien Ct arvojen välinen ero oli jopa 67.6 %. Myös ristireaktioita esiintyi eri hengitystiepatogeenien välillä. Pitää kuitenkin huomioida, että työn aikana jokaista käytettyä qRT-PCR kittiä käytettiin vain kerran ja näin ollen luotettavampien tuloksien saamiseksi olisi hyvä jatkaa testausta etenkin RealStar® RT-PCR kittien kanssa. Jatkossa kokeita voitaisiin myös tehdä näytteillä, jotka on käsitelty käytettäväksi kylmäkuivatuissa SIBA® reaktioissa.

Avainsanat	qRT-PCR, influenza viruses, respiratory syncytial virus

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Respiratory syncytial virus (RSV) and influenza viruses are significant human pathogens causing annual epidemics and occasional pandemic outbreaks. Development of novel molecular diagnostics is of high importance in order to better and faster characterize the infection and use appropriate antiviral treatment and thus, prevent from misuse of antibiotics.

Experimental part of this study was executed in Research and Development laboratory of Orion Diagnostica Oy. The aim of this study was to set up several commercially available quantitative reverse transcription polymerase chain reaction (qRT-PCR) kits for diagnosis of RSV and influenza viruses. The aim was to select reference method for Orion Diagnostica's proprietary Strand Invasion Based Amplification (SIBA®) technology.

During this study, six commercially available one-step qRT-PCR kits were compared. In order to measure sensitivity of the assays, viral RNA samples were extracted and quantitated to be used as dilution series during the experiments. Inclusivity and possible cross-reactivity were elucidated by challenging the reactions with other respiratory pathogens from extracted commercial NATtrol™ FLU verification panel. Panel contained 16 different viral or bacterial particles. Lastly, the performance of the qRT-PCR kits was evaluated in order to select the best kits.

In conclusion, from the selected qRT-PCR kits, RealStar® S&T Influenza RT-PCR Kit 3.0 and RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics) were the most successful based on the results. Variability between the results was significant, up to 67.6 % difference in Ct values was detected between the best and the worst kit. Moreover, cross-reaction between respiratory pathogens occurred. Although it is important to keep in mind, that during this study, all of the kits were used only once. Therefore, to obtain more reliable results, testing with the RealStar® RT-PCR kits should continue. Furthermore, the follow-up step would be to test the kits with samples processed to be used in freeze-dried SIBA® reactions.

Keywords	qRT-PCR, influenza viruses, respiratory syncytial virus

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Abbreviations

ATCC®	American Type Culture Collection
cDNA	Complementary DNA
Ct	Cycle threshold
HA	Hemagglutinin
HEF	Hemagglutinin-esterase-fusion
RSV	Respritatory syncytial virus
IC	Internal Control
IVD	In Vitro Diagnostics
M1	Matrix protein
M2	Membrane protein
mRNA	Messenger RNA
NA	Neuraminidase
PA	Polymerase activity protein
PB1	Polymerase binding protein 1
PB 2	Polymerase binding protein 2
POCT	Point-of-care tests
RT	Reverse transcription
SIBA®	Strand invasion based amplification
qRT-PCR	quantitative reverse transcription polymerase chain reaction
vRNA	Viral RNA
WHO	World Health Organization

1 Introduction

Seasonal influenza, also known as the flu, is an acute viral infection caused by influenza viruses. This disease occurs globally and can affect all of the population making it a major health problem by causing annual epidemics and pandemics. Unlike common "cold", and many other respiratory infections, influenza viruses can cause life-threatening complications in high risk population. (WHO fact sheet N°211 2014.)

Whereas seasonal influenza may cause severe illness to population of all ages, human respiratory syncytial virus (RSV) is the main cause of severe lower respiratory tract infections in infants and young children. Additionally, RSV is a significant problem in the elderly people. As the clinical presentation usually does not differ from other respiratory tract infections such as influenza, it is difficult to distinguish RSV from other respiratory viruses without further analysis. (WHO 2015.) To guarantee the control of epidemics and to provide adequate treatment for the infected patients, it is essential to develop rapid and reliable ways of diagnosing infections caused by RSV and influenza viruses (Templeton – Scheltinga – Beersma – Kroes – Claas 2003). As the influenza viruses rapidly evolve, it is necessary to find sufficiently sensitive methods allowing accurate detection and differentiation of RSV and influenza virus subtypes from other respiratory tract infections.

Currently, patients with RSV and influenza infections can be treated with antiviral drugs. However, these treatments work optimally only when started within 30-36 hours after onset of the disease. (Stiver 2003; Mejias – Garcia-Maurino – Rodriguez-Fernandez – Peeples – Ramilo 2016.) Therefore it is important to find reliable and fast detection method for the diagnosis for both viruses. Point-of-care testing (POCT) methods are rapidly evolving in molecular diagnostics and we are in time where "heavy" instruments, such as PCR can be soon replaced with faster, easier and more accurate detection methods. Today, there are plenty of POC tests based on immunoassays, which contain monoclonal antibodies. Rapidity is the advantage when these tests can generate result in 15 minutes or less, although the drawback is their sensitivity is relatively poor compared to PCR method. (Kirkwood 2016; Hurt et al. 2009.)

The study was appointed by Orion Diagnostica Oy and the practical execution of this project was carried out during a ten-week practical training period, under the super-

vision of Laboratory Specialist Jenna Flinck. Practical training took place in Orion Diagnostica Oy Research and Development laboratory located in Espoo. During the practical training, the purpose was to set up several commercially available quantitative reverse transcription polymerase chain reaction (qRT-PCR) kits, which are able to detect RSV and influenza viruses. The request was to find qRT-PCR assay with desired level of performance and the assays were assessed for their sensitivity, inclusivity, and selectivity. Orion Diagnostica Oy has released the next analytes to their Orion GenRead Instrument, which are influenza A and B and RSV (SIBA® and Orion GenRead publications 2016). Tests are based on Strand Invasion Based Amplification (SIBA®) technology which is Orion Diagnostica's proprietary isothermal nucleic acid amplification technology. During development of new products it is required to have a reference method to compare to.

2 Influenza viruses

Influenza is a respiratory infection caused by influenza A, B and C viruses. Disease causes annual epidemics and high rate outbreak of infections, which can reach up to 5 million people. Moreover, influenza A is characterized by a high number of deaths per annum, approximately 250 000 to 500 000. Only influenza A virus causes significant human disease and potential pandemics like Spanish flu in 1918 and swine flu in 2009, which was caused by influenza A H1N1 strain. Influenza B and C viruses usually cause milder disease. (WHO factsheet; Tille 2004: 836-38.)

2.1 Structure of influenza viruses

Influenza viruses belong to the family of *Orthomyxoviridae* and are enveloped and have negative-sense, single stranded and segmented ribonucleic acid (RNA) genome. Influenza A and B viruses consist of eight unique genomic nucleocapsid segments, whereas influenza C has only seven. Segmentation increases potential variability of the virus when each negative-sense RNA is associated with the nucleoprotein (NP) and the polymerase components such as, PB1, PB2, and PA. Nucleocapsids are protected with the M1 matrix protein (M1) which is covered by lipid bilayer envelope. Membrane protein (M2) forms an ion channel through the envelope and is lined by M1 protein, transporting protons and promoting uncoating and viral release (Figure 1). (Murray – Rosen-

thal – Pfaller 2013: 524-25; Hedman et al. 2011: 470-72.) The M1, M2 and NP proteins are type-specific and for example influenza B viruses have their unique BM2 and NB proteins (Hatta – Goto – Kawaoka 2004).

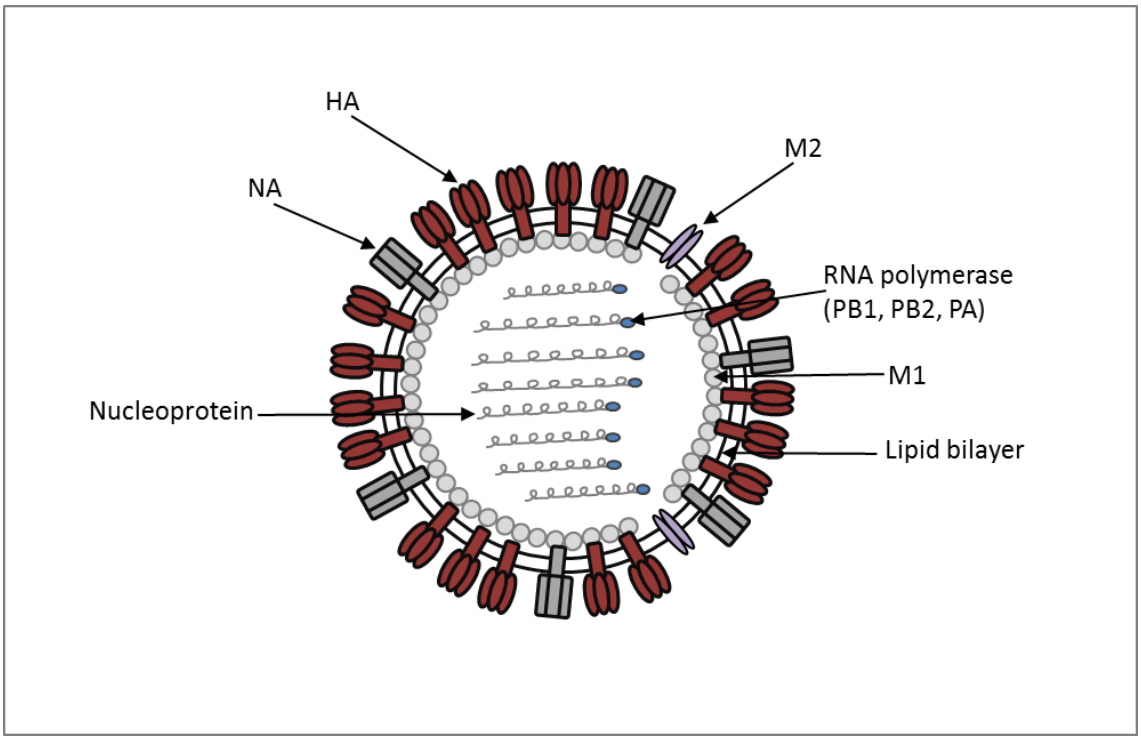

Figure 1. Model of influenza A virus. HA = hemagglutinin, NA = neuraminidase, M1 = matrix protein, M2 = membrane protein. (Modified from Hedman et al. 2013.)

Influenza A and B viruses have two glycoproteins in their envelope, trimer shaped hemagglutinin (HA) and tetramer shaped neuraminidase (NA). Influenza C virus has only one glycoprotein, hemagglutinin-esterase-fusion (HEF), which serves the functions of both the HA and the NA (Herrler – Dürkop – Becht – Klenk 1988). In case of the HA, two main functions can be distinguished, however the most important role is the facilitation of the virus binding to the host cell surface receptor. Subsequently, the HA binds to the sialic acid structures of the cell surface receptors. Different HA types bind to specific sialic acids. Another important function of the HA is to help virus to uncoat after delivering the nucleocapsid into the host cell. Moreover, enzymatic activity of the NA also plays an important role in this process. NA participates in removal of sialic acid from the glycoproteins, which prevents clumping and helps to release virus particles from the surface of the infected cell.

2.2 Influenza A life cycle

Viral RNA (vRNA) transcription and messenger RNA (mRNA) synthesis occur in a host cell. When HA molecule binds to the host cell glycoprotein receptor, virus gets an access into the cell with receptor mediated endocytosis. Low pH of the endosomal environment is crucial for occurrence of three dimensional changes in HA leading to fusion of viral and endosomal membranes. HA2 fusion peptide attaches specific structures of the virus surface to the endosome membrane causing holes in it. In low pH, M2 ion channels open and H^+ protons influx into the virus. This influx causes further decrease of the viral pH. That pH change initiate decomposition of the M1 protein and subsequent release of the ribonucleoprotein (RNP) complexes to the cytoplasm as well as their transport to the nucleus. (Brooks – Carroll – Butel – Morse 2013: 581-83.)

In the nucleus, the vRNA is synthetized to mRNA using transcriptase proteins (PA, PB1, and PB2). This is necessary to obtain methylated cap region of the RNA which is important sequence in order to bind to ribosomes. After transcription, the mRNAs are transported to the cytoplasm and translation occurs in ribosomes of endoplasmic reticulum. Surface proteins like HA, NA and M2 are processed in the Golgi apparatus and endoplasmic reticulum and then transported to the cell membrane. Virus exits from the cell by budding. The NA of newly formed viruses has important role facilitating the release of virus from a host cell. (Das – Aramini – Ma – Krug – Arnold 2010; Samji 2009.)

2.3 Influenza A subtypes and epidemiology

Glycoprotein HA can be classified into 16 subtypes (H1-H16) and NA to 9 subtypes (N1-N9). With different combinations of HA and NA, influenza A viruses can be divided in subtypes such as H1N1 or H5N1. With the HA and the NA, influenza A virus can undergo major (shift) or minor (drift) antigenic changes which helps virus to avoid immune system. Antibodies are specific for certain HA and NA types so rapid changing of antigens is necessary. This rapidity causes seasonal influenza and new vaccines have to be developed almost yearly. (Hedman et al. 2013: 473-74.)

Influenza viruses A, B and C are all from one original form which was divided thousands of years ago from each other. Influenza A occurs among humans, pigs, horses, sea mammals and birds of all kind. Based on laboratory tests, it has proven that only five influenza A virus subtypes has caused epidemics in human. These subtypes are

H8N8, H1N1, H2N2, H3N2 and H1N2. Influenza B on the other hand is almost only human virus with few exceptions. Classification of influenza A virus strains follows International standards. It contains type, place of original isolation, date of original isolation and antigen name. (Hedman et al. 2013: 473.)

3 Human respiratory syncytial virus

RSV is a significant lower respiratory pathogen dangerous especially in infants and young children. Moreover, RSV also causes infections in adults, even though clinical symptoms might be more variable and less distinctive. Most infants are infected before one year of age, and virtually everyone becomes infected by the age of two years. (Hall et al. 2009.) In a systematic review of Nair et al. (2010), RSV was estimated to be an important cause of the death in childhood from acute lower respiratory infection (ALRI) after pneumococcal pneumonia and *haemophilus influenzae* type b. RSV infections follow seasonal pattern causing epidemics during the winter season, although the virus appears throughout the year (Brooks et al. 2013: 600).

3.1 Structure and life cycle of RSV

The RSV virus was recovered 1957 and systematically it was classified in *Pneumovirinae* genus of the *Paramyxoviridae* family. RSV has a negative sense, single-stranded and non-segmented RNA genome which is found in helical nucleocapsid. Nucleocapsid contains phosphoprotein (P) and large protein (L), which together are responsible for the polymerase activity of the virus. The virion is surrounded by the envelope containing two surface proteins, the binding glycoprotein (G) and fusion glycoprotein (F). The main function of the F protein is to promote fusion between the virion and the host plasma membrane. Glycoprotein G is an attachment protein, which replaces hemagglutinin-neuraminidase (HN) of other *Paramyxoviridae*. Matrix protein (M) is lining the inside of the virion envelope (Figure 2). (Versalovic et al. 2011: 1357.)

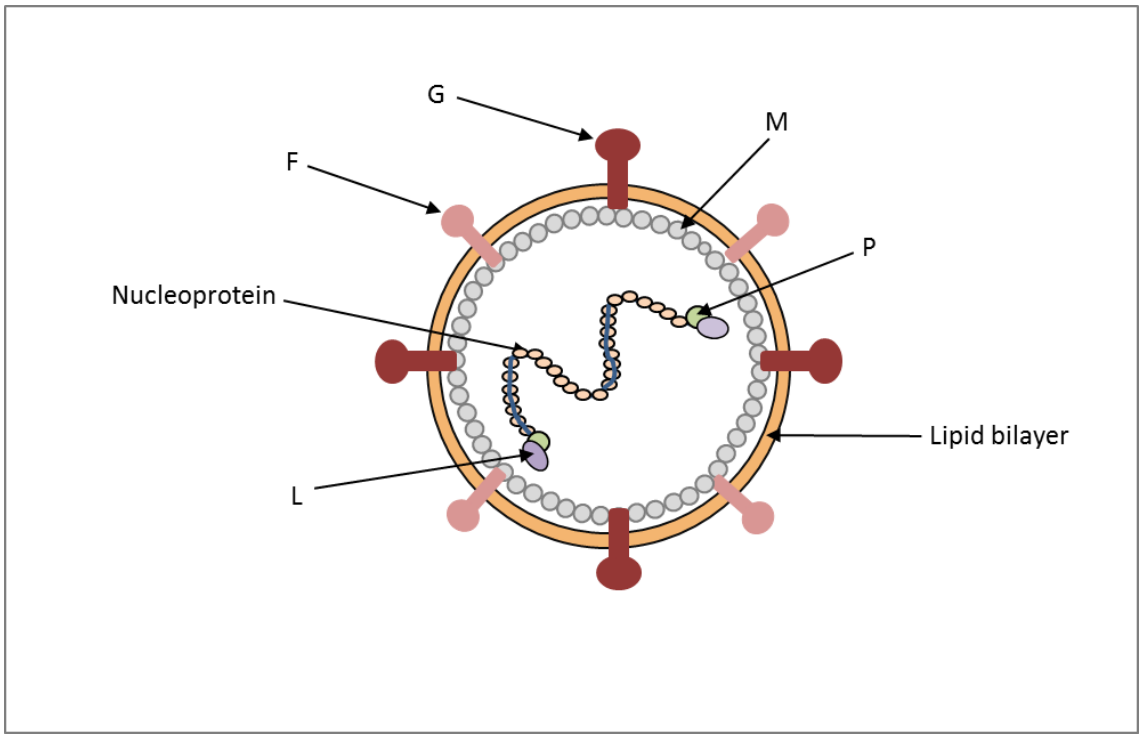

Figure 2. Model of respiratory syncytial virus. G = larger glycoprotein (attachment protein), F = smaller glycoprotein (fusion protein), M = matrix protein, P = phosphoprotein, L = RNA polymerase. (Modified from Murray et al. 2013)

Disease mechanism of RSV is similar to other members of the *Paramyxovirus* family; however there are so many differences in the structure that it is needed to classify RSV as a separate subgroup. RSV does not have hemagglutinin and neuraminidase like other paramyxoviruses, but there are many similar features in the structure. RSV enters the host cell by the fusion with plasma membrane after binding with G protein on the cell surface glycolipids and F protein promoting the fusion. Replication occurs in a similar manner to other negative-strand RNA viruses, in the cytoplasm of a host cell. Once the virus is in the cytoplasm of a host cell the genome is released and transcribed into mRNAs. The newly synthesized proteins are processed and mature virions leave the cell by budding without killing the cell. (Murray et al. 2013: 513-14.)

The pathological effect of RSV is its ability to infect respiratory epithelium, causing cell damage and host's immune response leading to the formation of lymphocytes, plasma cells and macrophages. Young children have already narrow airways and inflammation is decreasing ventilation causing wheezing and possibly asthma or allergic sensitization later in life. (Hedman et al. 2011: 497-98; Bacharier et al. 2012.)

3.2 RSV subgroups and epidemiology

Two antigenic subgroups of RSV can be distinguished, A and B. Subgroups can be identified on their variations in the structure of the glycoprotein G and other antigenic variability can occur with groups. During the annual epidemics, both groups can occur simultaneously, or other group dominates. Dominant subgroup usually alternates in cycles; therefore it varies yearly, which subgroup of RSV is more common. This yearly circulation, genetic and antigenic variation has been a challenge when attempting to develop functioning vaccine covering both subgroups. (Sullender 2000.)

4 Laboratory diagnosis of influenza and RSV

Diagnostic methods of RSV and influenza viruses have changed over time and new technologies are being developed (Figure 3). Typically, diagnosis of influenza is based on characteristic symptoms, season, and ongoing epidemic in the community. Distinguishing RSV and influenza viruses from other respiratory pathogens and identifying its type and strain give advantage to target correct treatment. Moreover, fast diagnostic methods are an essential contributor in infection control over pandemic strains. (Murray et al. 2013: 530-31.)

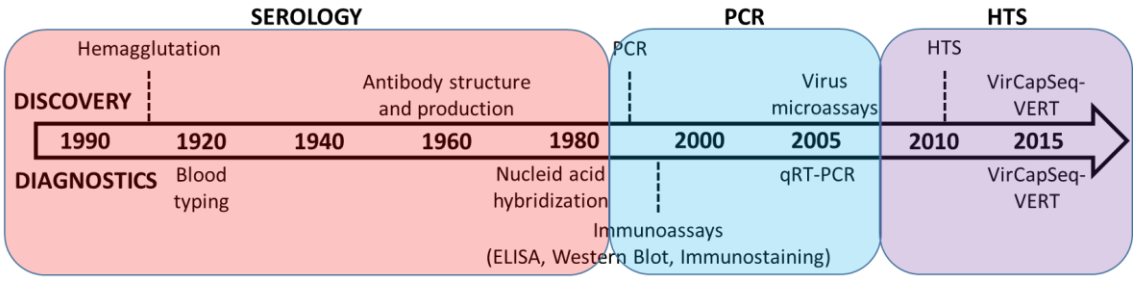

Figure 3. Evolution of virus diagnostics. Timeline is showing the development of different technologies. PCR = polymerase chain reaction, HTS = high-throughput sequencing (also known as next generation sequencing). (Modified from Rasmussen 2015.)

Laboratory diagnosis of influenza can be accomplished by the evaluation of the patients' immune response to the virus, detection of viral particles, isolation and growth of the virus, and establishing the viral components, such as antigens or genome (Murray et al. 2013). Influenza viruses are obtained from nasopharyngeal secretions as a nasopharyngeal aspirate or nasal/throat swab. Direct fluorescent antibody, polymerase

chain reaction, and viral culture are considered to be standard methods. However, viral culture is not rapid method and therefore, its clinical value is limited. Instead, qRT-PCR has been the benchmark for detection and quantification of respiratory RNA viruses as it offers more sensitive, specific, and rapid detection. (Templeton – Scheltinga – Beersma – Kroes – Claas 2003.) qRT-PCR can be used for diagnosis of a wider range pathogens and multiplex conventional qRT-PCR assays have been developed for simultaneous amplification of several viruses in a single reaction. Multiplex qRT-PCR assays are more cost-effective and faster when diagnosing different respiratory infections simultaneously. Moreover, the possibility to include multiple respiratory pathogens or for instance influenza A strains in one test is an advantage with multiplex qRT-PCR. (Ginocchio 2011.)

Alongside with qRT-PCR, POC tests have been used in order to make rapid diagnosis within 30 minutes. Commonly, POC tests are immunoassays based on antigen detection and they can detect and distinguish between influenza A and influenza B infections. Therefore, these tests are only qualitative. Epidemic and pandemic strains of influenza pose ongoing risk; therefore World Health Organization (WHO) has issued recommendation on the use of rapid testing for influenza diagnosis. Their sensitivity is suboptimal, usually < 80 % and, based on studies, detection of novel influenza subtypes is even lower. (Li-Kim-Moy et al. 2016; Petric – Comanor – Petti 2006.) Thus, in WHO recommendations, the both positive and negative results should be confirmed with immunofluorescent antibody test, culture or RT-PCR (WHO 2005).

Advanced diagnostic point-of-care tests started to flow on market 2015 when first molecular point-of-care test was publicized and it is expected, that respiratory infections are likely to be among leading applications by 2020. Now, when manufacturers have found a way of simplifying PCR and other nucleic acid amplification methods by creating easy-to-use portable form assays, the diagnosis process will improve significantly. Eventually, in the nearest future, there will be point-of-care tests, which are sensitive, rapid and do not require sophisticated instruments. (Drain – Garrett 2015; Brendish – Schiff – Clark 2015.)

5 Methods in molecular biology

5.1 Nucleic acid extraction

Nucleic acid extraction and purification from different sample material is the previous step to the qPCR itself. The variability in qPCR is often related to the nucleic acid purification and its quality is one parameter that can have significant impact to the results of qPCR. Therefore, carefully chosen extraction method is probably the most important step in qRT-PCR. Low-quality RNA can influence further parameters of the analysis including, the quality of detection and quantitation. It is very important to ensure the reproducibility of the extraction steps, when comparing biological samples. (Fleige – Pfaffl 2006.)

In this study, silica based nucleic acid purification method was used for nucleic acid extraction. Boom et al. introduced 1990 developed simple, rapid and cheap nucleic acid purification method which is based on vRNA binding to silica membrane in the presence of chaotropic agents and the lysates create optimal salt and pH conditions to ensure that protein or other contaminants are not retained on the silica membrane. Contaminants that might have remained on the sample are removed in two wash-steps. After wash-steps, pure vRNA is eluted in small volume of a low-salt buffer or RNAse-free water. (Boom et al. 2009.)

5.2 qRT-PCR

The quantitative real-time polymerase chain reaction (qPCR) is based on original PCR which relies on thermal cycling to amplify single copy of a piece of DNA to generate millions of copies of a specific DNA sequence (Madigan – Martinko – Bender – Buckley – Stahl 2015: 343). In original PCR, at the end of the amplification process, the product can be separated on a gel electrophoresis for detection of this specific product (Brown 2006: 57). In qPCR this step can be avoided since quantitation of the amount of the target leads to the immediate detection of the product in a single tube. Therefore, qPCR lowers the contamination risk caused by opening the tubes for post-PCR manipulation. Another advantage of the qPCR compared to gel based analysis is that it requires less time to be performed and it is able to generate quantitative results. qPCR

uses fluorescent probes to monitor the amplification process and the fluorescent signal reflects the amount of product formed. (Madigan et al. 2015: 345-46.)

Quantitative reverse-transcriptase polymerase chain reaction, qRT-PCR, is a quantitative real-time gene expression analysis method that allows amplification of a single-stranded RNA sequence. First RNA is converted to complementary DNA (cDNA) with reverse-transcriptase, which can use RNA as a template. qRT-PCR can be performed as a one-, or two-step method. The one-step qRT-PCR combines the reverse transcription (RT) to cDNA and PCR reaction in a simple, closed tube protocol. Therefore, the tube contains two enzymes. The first enzyme, reverse transcriptase, is an activation enzyme producing complementary cDNA strand. Temperature increase to 95°C inactivates the RT enzyme and simultaneously activates the DNA polymerase, which starts the amplification of the PCR products. (Nolte – Hill 2011: 1272-73.)

Using the one-step method is advantageous in various ways, for example it saves time when it is faster to set up in single tube and it reduces margin of error. Errors is reduced when there is less handling of the samples, less pipetting and less possibilities for contamination during the procedure. However, disadvantage for the one-step method is that it might affect to qRT-PCR efficiency when the cDNA might not be proportional or linear in each standard. (Wacker – Godard 2005.)

5.3 qRT-PCR amplification

Current detection methods are based on changes in fluorescence. In the qRT-PCR the product is fluorescently labelled and amount of fluorescence released during amplification is monitored during the whole PCR process. Moreover, there is a positive correlation between the initial number of RNA molecules in the sample and the increase of fluorescence during the PCR cycles. If a sample contains more targets, the fluorescent will be detected earlier. (Nolte – Hill 2011: 1274-76.)

There are several ways in which the amplified RNA is fluorescently labelled (probe- or none-probe chemistries) but in this study, all of the commercial qRT-PCR kits are based on TaqMan® Probe-based chemistry (Figure 4). In TaqMan® chemistry, after denaturation, an oligonucleotide probe is assembled. Fluorescent reporter dye is attached on the 5' end and a quencher dye on the 3' end of the probe. When the probe is intact, the reporter dye emission is quenched completely. If the target sequence is pre-

sent, the DNA polymerase cleaves the reporter dye from the probe with the activity of Taq DNA polymerase. When reporter dye is separated from the quencher dye, reporter dye signal starts to increase. Fluorescent intensity is directly proportional to the number of molecules released during that cycle. (Bustin – Mueller 2005; Kubista 2006: 98-103).

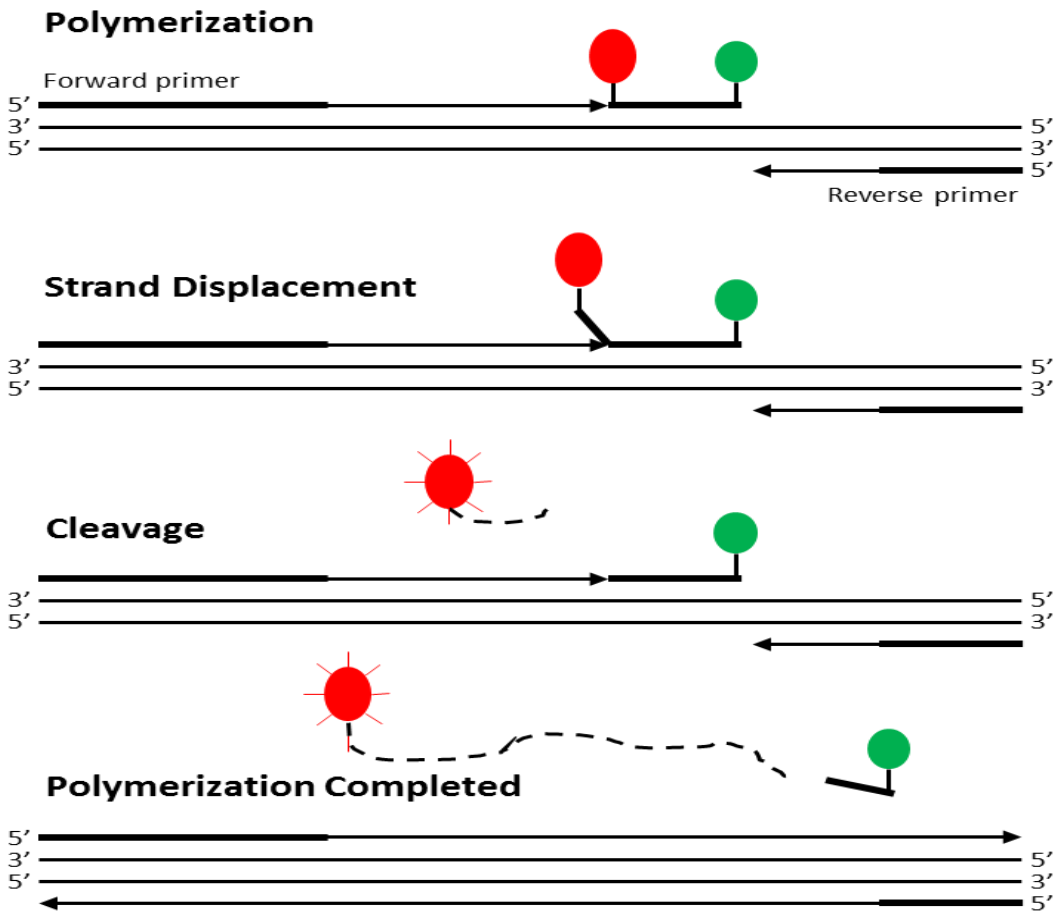

Figure 4. TaqMan principle. Reporter dye (fluorophore) = Red, Quencher dye = Green. (Modified from Wang et al. 2006.)

During the early cycles of qRT-PCR, there is very little of fluorescence and the signal is weak. Therefore, it cannot be distinguished from the background. Theoretically, after each cycle the amplification product doubles and for that reason, the fluorescence increases exponentially. After some time, exponential growth reaches plateau due to the lack of components required for the reaction to occur. (Kubista et al. 2006: 98) In order to be able to measure amount of amplified target, threshold line can be set to the qPCR programme manually or it can be calculated automatically. Threshold is set above the baseline, which defines the average background where no significant fluorescence is detected during the early cycles. When the fluorescent signal has increased significant-

ly and transcended the threshold, the point is defined as the Ct value (Figure 5). (Kubista et al. 2006: 98-101.)

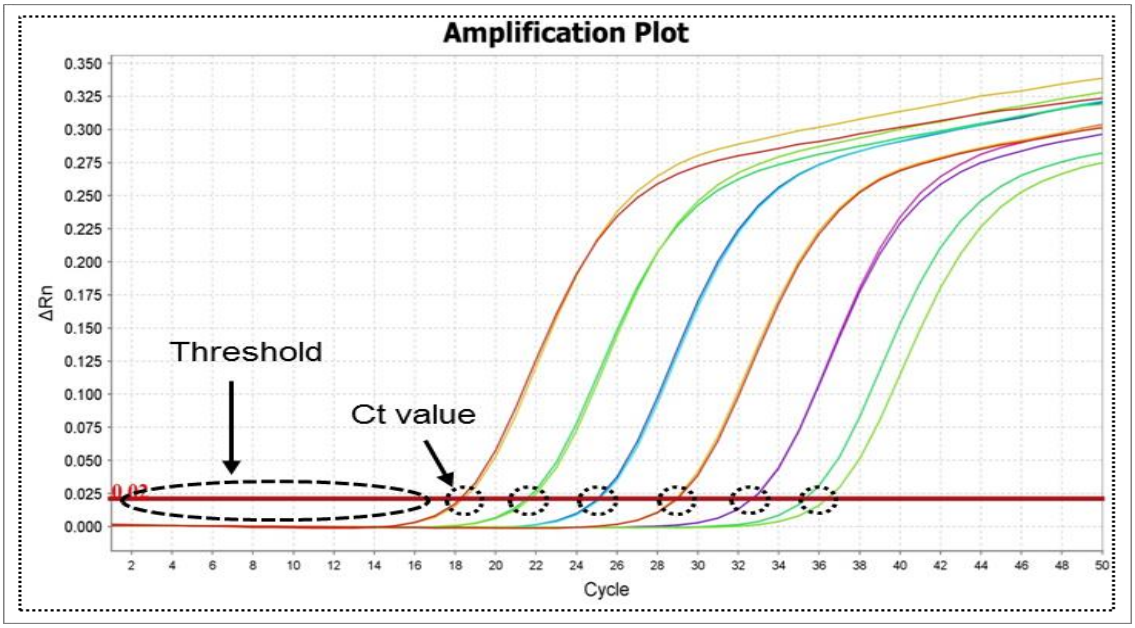

Figure 5. Example of threshold, which defines the Ct value of the target when increase of the reporter dye crosses the threshold. (Modified from qPCR guide.)

The determination of the amount of the viral RNA can be performed by absolute quantitation, where the exact number of copies of target is calculated. Absolute quantitation requires a standard sample of known concentration of RNA molecules and it can be constructed using serial dilution of known standard sample. After amplification, by the Ct values of the serial dilution, logarithmic standard curve can be generated (Figure 6). Quantitation of the target RNA is calculated by comparing the Ct values between the standard samples and the target amplification product. (Mackay et al. 2002; Bustin et al. 2005.)

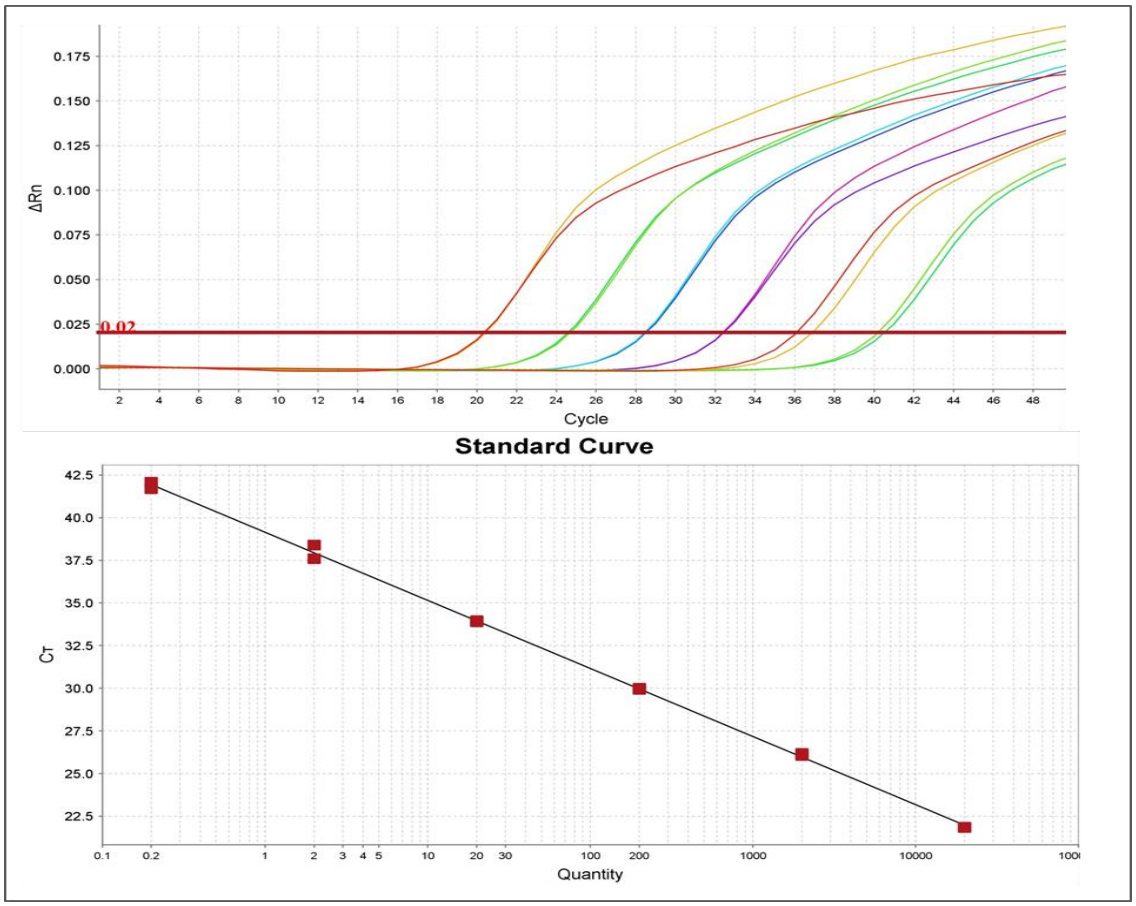

Figure 6. qRT-PCR standard curve. qRT-PCR response curves shown in logarithmic scale for six standard samples. (Modified from Tourinho et al. 2015.)

5.4 SIBA® technology

SIBA® (Strand Invasion Based Amplification) technology is an isothermal (the temperature never increases over 40 °C) nucleic acid amplification method developed by Orion Diagnostica Oy. The technology is based on the recombinase-dependent incorporation of a single-stranded invasion sequence into a complementary region of a DNA. SIBA® is in many aspect superior to qRT-PCR, which is used in similar applications. For example, unlike qRT-PCR, SIBA does not require thermal cycling and most importantly, it can be performed using standard laboratory equipment, therefore does not require expensive and sophisticated laboratory tools. It is well known, that analytical methods of this type, are prone to non-specific amplification, which reduces their sensitivity. Importantly, in case of SIBA, this issue is virtually non-existent, making it sensitive for a single target molecule. SIBA® is a real-time monitoring system, which utilizes fluorescent dyes and increases quality of the analysis. (Hoser – Mansukoski – Morrical – Eboigbodin 2014.)

For the detection of vRNA targets, reverse transcription SIBA (RT-SIBA) was developed to diagnose vRNA targets within point-of-care or central laboratory settings. RT-SIBA is one step method where a reverse transcriptase enzyme converts single-stranded RNA into cDNA, which can be amplified and detected by SIBA under isothermal reaction conditions. (Eboigbodin et al. 2016).

6 Aims of the study

Currently, Orion Diagnostica Oy is in the process of the publishing new analytes, influenza A and B and RSV to the Orion GenRead® platform (Orion Diagnostica Oy). The technology used in platform is based on the isothermal nucleic acid amplification technology, SIBA® (Strand Invasion Based Amplification). During the process of development and validation it is needed to have reference method. The aims of this study were to set up and test several commercial qRT-PCR kits and to select optimal assay to work as a reference method for SIBA®.

Main research questions of this study are:

- to find a reliable and sensitive reference qRT-PCR assay for tests based on SIBA® technology.
- to test whether selected assays also detect influenza virus subtypes.
- to investigate whether cross reactions between viruses occur.

All of these questions will be addressed and clarified during the progress of the studies.

The study was supervised by Laboratory Specialist Jenna Flinck from Orion Diagnostica Oy, as well as by Senior Lecturer Hannele Pihlaja from Metropolia University of Applied Sciences. Experimental part of this project was conducted at Orion Diagnostica Oy R&D laboratory in Espoo.

7 Materials and methods

For this study, viral RNA was extracted from selected sample population. Extracted viral RNA was then quantitated to be used with the qRT-PCR kits. After the set up of the qRT-PCR kits chosen for this study was accomplished, and results were obtained, the kits were evaluated based on their performance. These steps are shown schematically in Figure 7 and described in detail below.

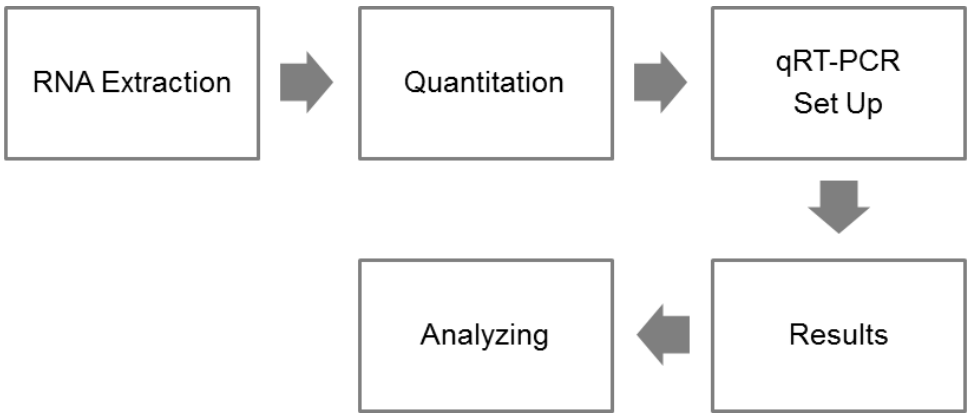

Figure 7. Workflow of the experimental part of the study.

7.1 Sample material

RSV and influenza virus strains (RSV subgroup A, RSV subgroup B, influenza A, influenza B) were obtained from the American Type Culture Collection (ATCC®). Table 1 shows strains that were used in this study.

Table 1. Respiratory pathogens from ATCC® used in this study.

	Strain	LOT
RSV A ATCC® VR-1540™	A2	60439286
RSV B ATCC® VR-1400™	B WV/14617/85	49609416
Influenza A ATCC® VR-1736™	A/Virginia/ATCC1/2009	59335953
Influenza B ATCC® VR-1813™	B/Massachusetts/2/2012	62482427

Later, extracted RNA from ATCC® influenza A VR-1540™ was replaced with Ampli-run® influenza A H1 RNA Control (Vircell). Basal concentration of the kit 14100 cp/µL was diluted to final concentration of 10000 cp/µL.

To be able to establish the inclusivity of the assays and possible cross-reactions between respiratory pathogens, they were performed using a set of purified and intact viral and bacterial targets of commercially available NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation), which mimics clinical specimen. Samples included to the panel should give positive results for different respiratory pathogens including, Rhinovirus, M. Pneumoniae, N. Meningitidis, RSV B, RSV A, Echovirus, Parainfluenza 1, Influenza A 2009 H1N1 strains New Cal and Brisbane, Influenza A H3N2 strains Wisconsin and Brisbane, Influenza A H1N1 09 strains Canada and NY, Influenza B strains Florida and Malaysia, Coxsackievirus. For details see Table 2.

Table 2. NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation) samples used in this study in order to determine inclusivity and cross-reactivity of the qRT-PCR kits.

Panel Member	Strain
Rhinovirus 1A	NA
M.Pneumoniae	M129
M. Meningitidis Serogroup A	NA
Respiratory Syncytial Virus B	CH93(18)-18
Respiratory Syncytial Virus A	NA
Echovirus Type 30	NA
Parainfluenza virus Type 1	NA
Influenza A H1N1	A/NewCaledonia/20/99
Influenza A H3N2	A/Wisconsin/67/05
Influenza A 2009 H1N1	A/Canada/6294/09
Influenza A H1N1	A/Brisbane/59/07
Influenza A H3N2	A/Brisbane/10/07
Influenza A 2009 H1N1	A/NY/02/09
Influenza B	B/Florida/02/06
Influenza B	B/Malaysia/2506/04
Coxsackievirus Type A9	NA

Later, actual clinical samples were included to the qRT-PCR assays (Table 3). Samples were either nasopharyngeal swabs or aspirates of influenza A and B from FIMLAB 2014 and RSV A and B (2007-2008) from Orion Diagnostica Oy own sample collection.

Table 3. Clinical samples used in this study.

Clinical sample	Pathogen
1	Influenza A
2	Influenza A H1N1
3	Influenza A
4	Influenza A
5	Influenza A H1N1 (weak positive)
6	Influenza B
7	RSV
8	RSV
9	RSV
10	RSV

7.2 RNA extraction

RNA was extracted with the commercially available QIAamp® Viral RNA Mini Kit (Qiagen). QIAamp® Viral RNA Mini Kit (Qiagen) uses Guanidinium isothiocyanate (GITC) - silica method. RNA extraction was carried out according to manufacturer's instruction with Spin Protocol, except for the step two where only 40 µL of sample (ATCC®, NATtrol® Flu Verification Panel) was used and rest of the volume (100 µL) was replaced with phosphate-buffered saline (PBS) to obtain total volume of 140 µL. 60 µL of the clinical samples was used and diluted into 80µL of PBS to obtain volume of 140 µL. This was done to save valuable sample material. Despite of the dilution, all of the samples contained large quantities of vRNA (cp/µL). After the two wash-steps, QIAamp Mini columns are instructed to perform one extra centrifugation at full speed for one minute. Full protocols are shown in the Appendix 1 and 2. After elution, purified viral RNA was collected to 1.5 ml microsentrifuge tubes and stored in - 70°C.

7.3 RNA quantitation

Genesig® qRT-PCR Pathogen kits (Primerdesign) contains a positive control template, which is quantitated. Therefore, it can be then used to generate standard curve. The determination of the amount of the pure RNA was performed by absolute quantitation, where the exact number of copies of interest was calculated. Absolute quantitation requires a standard curve of known number of copies and it can be constructed using several standards. The linear range of quantitation of the one-step qRT-PCR assay for

genomic RNA can be determined using 10-fold serial dilutions of the positive control template ranging from 20 to 2 × 10^5 copies/µL to determine the end-point limit of detection and the linearity of the assay (Table 4). Reason for choosing this quantitative method was that the qRT-PCR assay is more sensitive than fluorometric quantitation and less expensive than commercially available quantitated Vircell AMPLIRUN® RNA controls.

Table 4. Standard curve dilution series of Genesig® qRT-PCR pathogen kits positive control template in order to quantitate extracted vRNA from ATCC® samples.

Standard curve	Copy number per µL
Tube 1 Positive control (RED)	2 x 10^5
Tube 2	2 x 10^4
Tube 3	2 x 10^3
Tube 4	2 x 10^2
Tube 5	20

After quantitation, copy number of RNA samples extracted from ATCC® samples was known and samples were diluted to the desired concentration of 10 000 cp/µL and aliquoted into individual 10 - 30µl volumes before storage in - 70°C until used.

7.4 qRT-PCR assays used in this study

Six kits based on detection of RSV and influenza viruses and commercially available in Finnish market in 2016 were tested. In order to find an optimal reference method for SIBA® technology, several values were taken into consideration when selecting commercial qRT-PCR assays for this study. Ideal assay would be done only in one-step, which would save time on pipetting steps when RT and qPCR reactions are performed in same tube and the cross-contamination risk between RT and qPCR steps would be reduced to minimum. Also SIBA® technology has a one-step RT-SIBA method. It is desired that same assay would be able to detect multiple targets in single PCR experiment. In the current study, the targets were RSV and influenza viruses. Sensitivity and inclusivity should be high to meet the standards of Orion Diagnostica Oy requested for their purposes. Other parameter used to evaluate the assays in this study was to determine their usability with clinical diagnostics in EU (CE marked) and/or USA (FDA approval).

The qRT-PCR amplifications were carried out according to the manufacturer's instructions. For inclusivity testing, each sample was tested once with duplicate sample per used assay. All qRT-PCR kits, except Genesig® Pathogen kits (Primerdesign), included positive controls and required reagents to perform qRT-PCR. Negative controls (RNAse free water) were used in each reaction. Genesig® Pathogen kits (Primerdesign) required separate Oasig™ OneStep qRT-PCR Master Mix (Primerdesign).

Despite all used qRT-PCR kits included different reagents, different protocols were followed while preparing the Master Mix and during amplification process, the laboratory working routine was carried out under similar conditions. All of the used reagents and samples were handled on ice or cold blocks throughout the processing. Only necessary amount of samples or reagents were unfrozen to each qRT-PCR setup to avoid excessive freeze-thawing. Work was carried out using good laboratory practises and following unidirectional workflow in three different laboratory facilities (Figure 8).

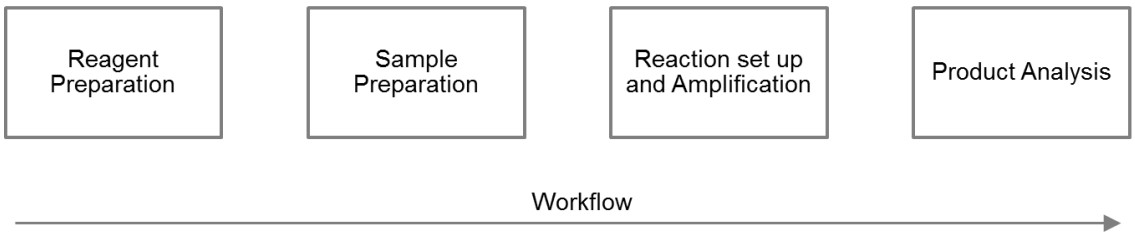

Figure 8. The workflow in PCR laboratory. Unidirectional workflow continues in one direction only from pre-PCR to post-PCR.

In the first laboratory the reagent preparation room, the PCR Reaction Mix was prepared and pipetted into 96-well PCR plate. Then, the plate was covered and moved to a template laboratory where samples and controls were added. Lastly, ready 96-well PCR plate was carried to the amplification room, where PCR instruments where housed. Before placing the 96-well PCR plate to qRT-PCR instrument, it was briefly centrifuged.

7.4.1 Genesig® Influenza B Virus Standard kit (Primerdesign)

This assay is designed for the detection of influenza B. The manufacturer assures more than 95% efficiency and detection of less than 100 copies of target template under optimal PCR conditions. A specific influenza B primer and probe mix, which is based on the TaqMan® principle, were provided with the kit. Fluorescent data can be detected through the FAM channel. Assay is not CE marked nor FDA approved, therefore, it can be used for research purposes only.

In this assay there is possibility to use either one-, or two-step qRT-PCR protocol, but the one-step method is recommended. Kit also provides positive control template, which can be used to generate standard curve of influenza B copy number /CT value. At least one positive control template should be also included in each qRT-PCR test to control the primers and probes for detecting the target influenza B. In this study, there were always two positive controls as well as negative control in each run.

The work was performed according to the manufacturer's instructions (Primerdesign™ 2016). Oasig™ OneStep qRT-PCR Master Mix (Primerdesign) was used. Amplification protocol can be seen in the Table 5 and full work protocol in the Appendix 5. Extracted active influenza B samples (ATCC) were diluted in a series from 1:10 up to 1:10000 dilutions. PCR instrument used in this experiment was Applied Biosystems™ ViiA™ 7 platform (Thermo Fisher Scientific Inc).

Table 5. Amplification protocol used with Genesig® Influenza B Virus Standard kit (Primerdesign).

Step	Time	Temp (°C)	Cycles
Reverse transcription	10 min	55	1
Enzyme activation	2 min	95	1
Denaturation	10 s	95	50
Data collection	60 s	60	

Genesig® human influenza B Virus Standard kit (Primerdesign) was only used for its feature to be able to create standard curve and use it to quantitate extracted vRNA (ATCC® samples) for this study.

7.4.2 Genesig® Respiratory Syncytial Virus (all species) Standard Kit (Primerdesign)

Assay is designed to detect both, A and B RSV subgroups and exclude closely related human Metapneumoviruses. Assay provides RSV specific primer and probe mix, which are based on the TaqMan® principle. Although, all of the RSV types can be detected, the method does not provide specification of the types. Fluorescent data can be detected through FAM channel. Under optimal PCR condition, the manufacturer assures more than 95% efficiency and detection of less than 100 copies of target template.

Genesig® Respiratory Syncytial Virus (all species) Standard Kit (Primerdesign) also offers possibility to carry out the experiment both with one-, or two-step protocol and for copy number determination assay contains a positive control template. The work was performed according to the manufacturer's instructions (Primerdesign™ 2016). Full work protocol can be seen in the Appendix 5 and amplification protocol is identical to Genesig® influenza B virus Standard kit (Primerdesign) (Table 5).

First phase was to quantitate obtained vRNA of the ATCC® RSV A and B samples. Quantitation was performed with standard curve dilution series (Table 4) and ATCC® samples were diluted from 1:10 up to 1:10000 dilutions. Following determination of the ATCC® samples, the assay was used to perform qRT-PCR with dilution series of RSV A and B (ATCC®), set of NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation) samples and the clinical samples 7-10 can be seen in the Appendix 5 and used samples in Table 13. In each amplification setup, two positive and negative controls were included into each run in order to verify, that the primers and probes are working correctly and to test, that the reagents have not become contaminated while setting up the protocol.

7.4.3 RealStar® S&T Influenza RT-PCR Kit 3.0 (altona Diagnostics)

The RealStar® Influenza S&T RT-PCR Kit 3.0 (altona Diagnostics) is able to detect and distinguish seasonal influenza A and B viruses as well as pandemic H1N1 strain. The assay contains Internal Control (IC) to identify possible qRT-PCR inhibition and/or work as a sample preparation procedure control. The probes were labelled with fluorescent reporter and quencher dyes specific for their respective target. Fluorophore Cy5 was specific for seasonal influenza A, FAM for pandemic influenza A H1N1, ROX

for influenza B and JOE for IC. The sequences of the primers and probes are listed below in the Table 6:

Table 6. Primers and probes used in RealStar® S&T Influenza RT-PCR Kit 3.0 (altona Diagnostics) for the specific amplification and detection of seasonal Influenza A and pandemic H1N1 strains.

Target	Sequence 5'-3'	Oligo
Matrix	AGAGACTTGAAGATGTATTTGCTGGGAAGAT	Probe 1
Matrix	TCCTGCAAAGACACTTTCCAGT	Probe 2
Matrix	CAGGCCCCCTCAAAGC	Primer 1
Matrix	CGTCAGGCCTCCTCAAAGC	Primer 2
Matrix	ATTCCATGAGAGCCTCAAGATC	Primer 3

In this study, IC was added to the master mix as a qRT-PCR inhibition control because viral RNA extraction was performed at once for all of the experiments. The work was performed according to the manufacturer's instructions (altona Diagnostics 2016). Amplification protocol can be seen in Table 7 and the full work protocol for the experiment in the Appendix 5. The whole set of different samples were added in one qRT-PCR experiment. The set up was with dilution series of influenza A and B (ATCC®), five different influenza strains from NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation) samples (Table 13) and clinical samples 1-6 containing different influenza strains.

Table 7. Amplification protocol used with RealStar® S&T Influenza RT-PCR Kit 3.0 (altona Diagnostics).

Step	Stage	Cycles	Temp (°C)	Time
Reverse transcription	Hold	1	55	10 min
Denaturation	Hold	1	95	2 min
			95	15 s
Amplification	Cycling	45	55	45 s
			72	15 s

7.4.4 RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics)

This assay is designed for the detection of respiratory syncytial virus specific RNA. Furthermore, the test allows the differentiation between RSV subgroup A and RSV subgroup B. Assay also includes IC to be used as a RT-PCR inhibition control and/or as a sample preparation procedure control. The probes were labelled with fluorescent re-

porter and quencher dyes specific for their respective target. Fluorophore Cy5 was specific for RSV A, FAM for RSV B and JOE for IC. Amplification protocol can be seen in Table 8.

Table 8. Amplification protocol used with RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics).

Step	Stage	Cycles	Temp (°C)	Time
Reverse transcription	Hold	1	55	10 min
Denaturation	Hold	1	95	10 min
			95	15 s
Amplification	Cycling	45	55	45 s
			72	15 s

In this study IC was added to the Master Mix and the experiment was performed according to the manufacturer's instructions (altona Diagnostics 2012). In the first phase of using RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics) the RT-PCR was run with dilution series of RSV A and B (ATCC) and set of other respiratory pathogens with NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation) samples (Table 13). In the second phase clinical samples 7-10 containing RSV was added. The full work protocol for the experiment can be seen in the Appendix 5.

7.4.5 RIDA®GENE Flu&RSV (R-Biopharm)

The RIDA®GENE Flu&RSV (R-Biopharm) is the only multiplex qRT-PCR for both, influenza and RSV used in this study and it is designed for detection and differentiation of influenza A and B, as well as RSV. The probes were labelled with fluorescent reporter specific for their respective target. Fluorophore Cy5 was specific for influenza A, ROX for influenza B and FAM for RSV. Assay provides IC, which can be used as an internal control for sample preparation procedure and for determination of possible RT-PCR inhibition control. Fluorophore specific for IC was VIC. Amplification protocol can be seen below in the Table 9:

Table 9. Amplification protocol used with RIDA®GENE Flu&RSV (R-Biopharm) kit.

Step	Time	Temp (°C)	Cycles
Reverse transcription	10 min	58	1
Initial denaturation	1 min	95	1
Denaturation	15 s	95	45
Annealing	30 s	55	

In this study, IC was added to the Master Mix and the experiment was performed according to the manufacturer's instruction (R-Biopharm 2014). Amplirun® influenza A H1 RNA Control was diluted as a dilution series with influenza B, RSV A and B (ATCC) and set of other pathogens with NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation) samples (Table 13) and real clinical samples 1-10 containing different influenza strains, as well as RSV. Full work protocol can be seen in the Appendix 5.

7.4.6 R-DiaFlu (Diagenode)

With the assay it is possible to detect and distinguish human influenza A and human influenza B. Target genes for influenza A are M1&2 and target gene for influenza B is NP. The probes were labelled with fluorescent reporter and quencher dyes specific for their respective target. Fluorophore FAM was specific for influenza A and fluorophore VIC for influenza B.

In case of this protocol, manufacturer's instruction were not completely followed, because it was the only qRT-PCR assay which is not designed to be used in neither Applied Biosystems® ViiA™ 7 Real-Time PCR System nor Applied Biosystems® 7500 Real-Time PCR Systems. Instead, the qPCR instrument listed in the manual was Applied Biosystems® 7500 Fast Dx Real-Time PCR System, which is very similar to Applied Biosystems® 7500 Real-Time PCR Systems. Diagenode kit has been used before in Orion Diagnostica Oy with these PCR instruments and has been proved working. Except used qPCR instrument, work protocol was following the manufacturer's instructions. Amplification protocol can be seen below in the Table 10.

Table 10. Amplification protocol used with R-DiaFlu (Diagenode) kit.

Step	Time	Temp (°C)	Cycles
Reverse transcription	30 min	50	1
Activation (Tag)	10 min	95	1
Denaturation	15 s	95	
Annealing	30 s	55	45
Elongation	30 s	68	

The RT-PCR set up was with dilution series of Amplirun® Influenza A H1 RNA Control and influenza B (ATCC), set of NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation) samples (Table 13) and clinical samples 1-6 containing different influenza strains. Full work protocol can be seen in the Appendix 5.

7.5 qPCR instruments

In this study, two of qPCR instruments were used including, Applied Biosystems® ViiA™ 7 Real-Time PCR System which uses ViiA™ 7 Software and Applied Biosystems® 7500 Real-Time PCR Systems which uses 7500 Software v2.0.6. Selected qPCR instruments utilize different software even though both of them are provided by Thermo Fisher Scientific.

qPCR instruments were chosen to this study based on manufacturer's instruction of qRT-PCR kits. Manufacturers generally validate their assays for few different qPCR instruments. Moreover, general usage in the laboratory of Research and Development at Orion Diagnostica Oy contributed to the options. Tight schedule of the study made it impossible to rely on only one qPCR instrument. Despite different software used, qPCR programme and data pre-processing pipeline in both instruments were similar.

8 Results

The results of this study are explained in work order and demonstrated with tables and pictures obtained from qPCR platforms. Overview of all the results can be seen in Table 12.

8.1 Quantitation

Based on created standard curve with Genesig® pathogen assays (Primerdesign), number of copies per each extracted vRNA sample was determined (Table 11). Results can be seen in Table 10. Due to the delay in order of correct influenza A kit, extracted ATCC influenza A stock was not used. Sample was replaced with Vircell AmpliRun® Influenza A H1 RNA control which was diluted from lyophilized form to meet quantity of 10 000 cp/µL.

Table 11. Results from quantitation of viral RNA with Genesig® qRT-PCR Pathogen kits (Primerdesign) presenting the number of copies per µL.

Pathogen	Quantity (cp/µL)
Influenza B ATCC® VR-1813™	1.81E+06
RSV A ATCC® VR-1540™	1.33E+05
RSV B ATCC® VR-1400™	8.46E+05

Standard curve included 6 points of 10-fold dilution, each of them was duplicated. Results obtained from the standard curve showed that the assays could be used for quantitation of the target RNA. Regarding the influenza B, the slope was - 3.657 and the correlation R^2 = 0.998. For RSV A the slope was − 4.009 and the correlation coefficient R^2 = 0.999 of the standard curve. For RSV B the slope was - 4.348 and the correlation coefficient R2 = 0.998. R^2 is a parameter, which indicates degree of overlap of data points with the standard curve. Values lower than 0.95 indicate no linear correlation between the Ct and the 10 log of the RNA concentration or that the reactions have not been pipetted accurately. (Eurogentec.) All the results were higher than 0.95 and therefore linear correlation between the Ct and the 10 log of the RNA occurred.

8.2 Sensitivity and inclusivity

Assay sensitivity was defined with 10-fold dilution series from extracted ATCC® pathogen samples. Dilution series was performed with starting quantity of 10 000 cp/µL up to 1 cp/µL. All of the used kits, except RIDA®GENE Flu&RSV (R-Biopharm) met the highest possible sensitivity when there was detection in lowest dilution of 1 cp/µL. Regarding RIDA®GENE Flu&RSV (R-Biopharm), no amplification was detected in influenza A. All the results from ATCC® sample dilutions can be viewed in Table 12.

When comparing the Ct values of all used qRT-PCR kits, it is clear that both, RealStar® (altona Diagnostics) kits (Influenza S&T RT-PCR Kit 3.0 and RSV RT-PCR Kit 1.0) performed the best because the Ct values were the lowest. This means, that the PCR product starts to form earlier, and therefore assay is more sensitive. RIDA®GENE Flu&RSV (R-Biopharm) kit did not detect the lowest quantity of influenza A (1 cp/µL) and the Ct values were worst among all used pathogens for the lowest dilutions (1 cp/µL) from 31.8 – 37.6. Also, Genesig® Respiratory Syncytial Virus (all species) Standard Kit (Primerdesign) performed significantly worse particularly with detecting target RSV A with average 67.6 % difference in Ct values compared to RealStar® RSV RT-PCR Kit 1.0. Difference between RealStar® Influenza S&T RT-PCR Kit 3.0 and R-DiaFlu (Diagenode) was not significant, however, consistently in favor of the RealStar® assays.

Table 12. Comparison table of used qRT-PCR kits in order to evaluate sensitivity of the assays based on Ct values.

Pathogen	qRT-PCR Assay		
Influenza A	**RealStar Influenza 3.0 (Ct)**	**RidaGene Flu&RSV**	**R-DiaFlu**
10^4	23.4	25.9	24.4
10^3	26.2	29.4	27.6
10^2	28.8	32.8	30.8
10^1	31.7	37.7	35.3
10^0	35.2	Undetermined	37.4
Influenza A	**RealStar Influenza 3.0 (Ct)**	**RidaGene Flu&RSV (%)**	**R-DiaFlu (%)**
10^4	23.4	10.6	4.1
10^3	26.2	12.2	5.4
10^2	28.8	13.9	6.9
10^1	31.7	19.2	11.4
10^0	35.2	Undetermined	6.3
AVERAGE (%)		**14.0**	**7.3**
Influenza B	**RealStar Influenza 3.0**	**RidaGene Flu&RSV**	**R-DiaFlu**
10^4	20.6	25.4	20.8
10^3	24.3	28.4	23.8
10^2	27.2	31.2	27.3
10^1	30.1	34.8	29.8
10^0	32.3	37.4	33.7
Influenza B	**RealStar Influenza 3.0 (C_t)**	**RidaGene Flu&RSV (%)**	**R-DiaFlu (%)**
10^4	20.6	23.6	1.0
10^3	24.3	16.8	-2.1
10^2	27.2	14.7	0.1

10^1	30.1	15.8	-0.8
10^0	32.3	15.8	4.4
AVERAGE (%)		**17.3**	**0.5**
RSV A	**RealStar RSV 1.0 (C_t)**	**RidaGene Flu&RSV**	**Genesig RSV**
10^4	13.8	18.9	25.4
10^3	16.8	22.2	29.3
10^2	19.7	25.6	33.8
10^1	23.6	29.1	37.4
10^0	27.0	31.9	40.5
RSV A	**RealStar RSV 1.0 (C_t)**	**RidaGene Flu&RSV (%)**	**Genesig RSV (%)**
10^4	13.8	37.1	84.4
10^3	16.8	32.1	73.9
10^2	19.7	30.0	71.4
10^1	23.6	23.0	58.4
10^0	27.0	18.1	50.0
AVERAGE (%)		**28.1**	**67.6**
RSV B	**RealStar RSV 1.0 (C_t)**	**RidaGene Flu&RSV**	**Genesig RSV**
10^4	20.8	21.4	26.7
10^3	24.1	28.3	30.4
10^2	27.7	31.0	34.3
10^1	31.1	34.4	37.6
10^0	33.9	37.6	41.7
RSV B	**RealStar RSV 1.0 (C_t)**	**RidaGene Flu&RSV (%)**	**Genesig RSV (%)**
10^4	20.8	3.3	28.8
10^3	24.1	17.4	26.1
10^2	27.7	11.8	23.7
10^1	31.1	10.7	21.1
10^0	33.9	11.0	23.2
AVERAGE (%)		**10.8**	**24.6**

Although the RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics) is able to distinguish RSV subgroups A and B, it might be somewhat less sensitive when detecting target RSV B. When analyzing the amplification curve, there was minor “crawling” detected in probe labelled with fluorophore specific to RSV B (FAM) when using dilution series of ATCC® RSV A samples (Figure 9).

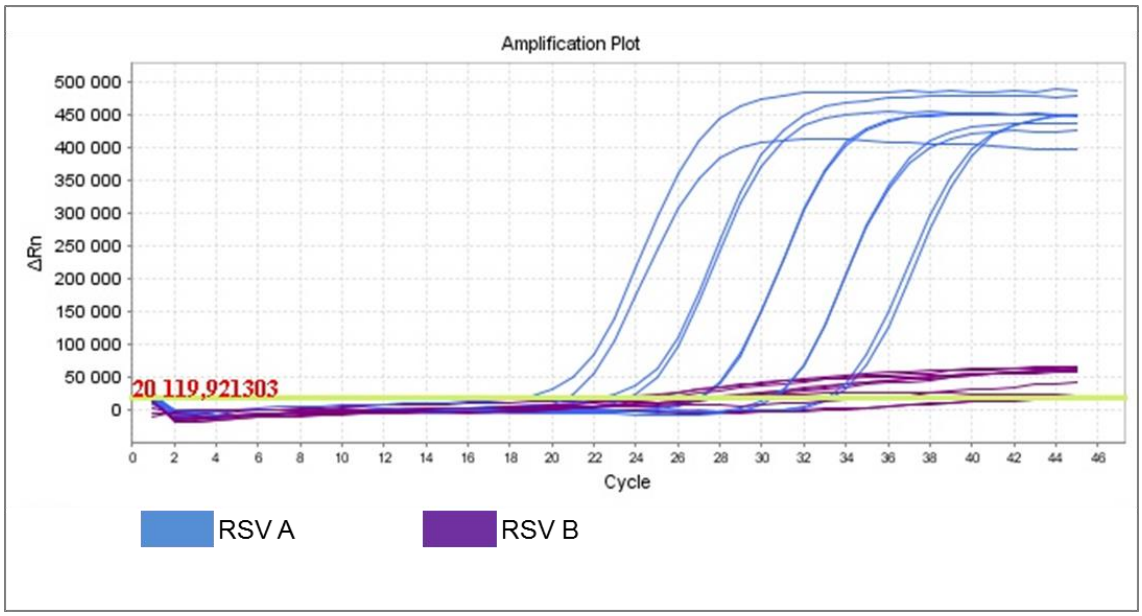

Figure 9. Amplification plot from RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics). ATCC® RSV A and B samples with 10-fold dilution series. Results of both RSV A (blue) and RSV B (purple) displayed in channel specific for RSV B (FAM).

The inclusivity of the qRT-PCR assays and possible cross-reaction between other respiratory pathogens were evaluated by their performance after adding nucleic acids from other common human respiratory pathogens using extracted NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation) samples (Table 13). The panel contained viral or bacterial particles and it contained six different influenza A virus strains, two influenza B strains, respiratory syncytial virus A, respiratory syncytial virus B, rhinovirus 1 A, parainfluenzavirus type 1, Echovirus Type 30, Coxsackievirus type A9, Mycoplasma pneumoniae strain M129, and Neisseria meningitidis Serogroup A.

Table 13. Inclusivity and possible cross-reactions of the used qRT-PCR kits were evaluated with NATtrol FLU verification panel containing other respiratory pathogens. IAV = influenza A, IBV = influenza B, RED = false positive, GREEN = false negative, GREY = was not tested.

NATtrol Flu	**RealStar**					**R-DiaFlu**		**RidaGene**			**Genesig**
Verification Panel	IAV	IAV H1N1	IBV	RSV A	RSV B	IAV	IBV	IAV	IBV	RSV	RSV
Rhinovirus Type 1A				-	-						-
M. Pneumoniae				-	-						-
N.Meningitidis				-	-						-
RSV A				-	+						+
RSV B				+	-						+
Echovirus Type 30				-	-						-
Parainfluenza 1				-	-						-
Influenza A H1N1 New Cal				-	-			+	-	-	-
Influenza A H3N2 Wisconsin	+	-	-			+	-	-	-	-	
Influenza A H1N1 09 Canada	+	+	-			+	-	+	-	-	
Influenza A H1N1Brisbane	+	-	-			+	-	+	-	-	
Influenza A H3N2 Brisbane	+	-	-			+	-	+	-	-	
Influenza A H1N1 09 Ny	-	+	+			+	+	+	-	(+)	
Influenza B Florida				-	-			+	+	+	-
Influenza B Malaysia	-	-	+			-	+	-	+	-	
Coxsackievirus Type A9				-	-						-

False positive results were obtained in three qRT-PCR assays, RIDA®GENE Flu&RSV (R-Biopharm), RealStar® Influenza S&T RT-PCR Kit 3.0 (altona Diagnostics) and R-DiaFlu (Diagenode). Regarding RealStar® assays (altona Diagnostics), all of the target RNA were detected correctly except influenza B virus, which incorrectly reported pres-

ence of influenza A H1N1 09 Ny virus. Also there was a slight ambiguity with influenza A H1N1 Brisbane, which was detected only in influenza A channel, but not in the H1N1 channel specifically designed for it. RSV A and RSV B were detected and divided into appropriate subgroups. Similarly, R-DiaFlu (Diagenode) reported detection of influenza A H1N1 09 on its influenza B specific channel. Otherwise, assigned target RNA was identified correctly. Genesig® Respiratory Syncytial Virus (all species) Standard Kit (Primerdesign) was able to detect the target RNA, even though it was not able to distinguish the subgroups of RSV and the results were positive for both, RSV A and RSV B sample.

False negative results were obtained in two qRT-PCR assays, RIDA®GENE Flu&RSV (R-Biopharm) and RealStar® Influenza S&T RT-PCR Kit 3.0 (altona Diagnostics). In RIDA®GENE Flu&RSV (R-Biopharm) kit there was no detection of influenza A H3N2 Wisconsin strain and with RealStar® Influenza S&T RT-PCR Kit 3.0 (altona Diagnostics) probe specific for influenza A H1N1 did not detect influenza A H1N1 Brisbane strain.

Regarding RIDA®GENE Flu&RSV (R-Biopharm), there was a lot more misdetection with target RNA, and cross-reactivity between respiratory pathogens occurred (Figure 10). Influenza A H3N2 Wisconsin was incorrectly identified as influenza B and influenza B Florida as influenza A. RSV channel gave strong positive readings in influenza B Florida and weak positive readings in influenza A H1N1 09 NY.

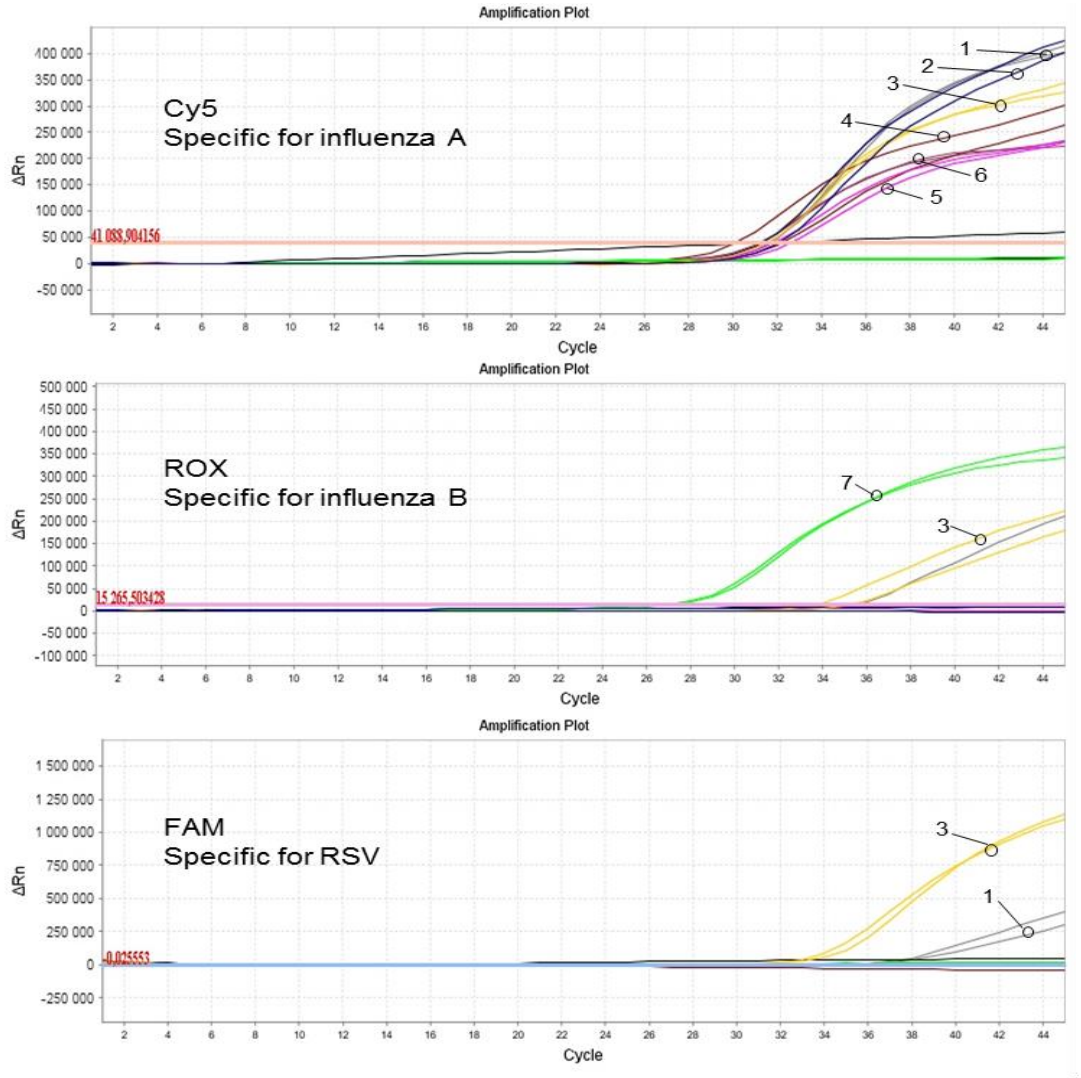

Figure 10. Amplification plots from RIDA®GENE Flu&RSV (R-Biopharm) qRT-PCR viewed from different channels specific for different target RNA. Samples extracted from NATtrol™ FLU Verification Panel (ZeptoMetrix Corporation). 1 = Influenza A H1N1 Ny, 2 = Influenza A H1N1 09 Canada, 3 = Influenza B Florida, 4 = Influenza A H1N1 New Cal, 5 = Influenza A H3N2 Brisbane, 6 = Influenza A H1N1 Brisbane, 7 = Influenza B Florida.

These findings suggest that the RealStar® assays (altona Diagnostics), R-DiaFlu (Diagenode) and Genesig® Respiratory Syncytial Virus (all species) Standard Kit (Primerdesign) are relatively specific for their respective target. On the contrary, RIDA®GENE Flu&RSV (R-Biopharm) cannot be described very specific and cross-reactions between respiratory pathogens occurred.

8.3 Clinical samples

Last step in the process of evaluating used qRT-PCR assays was to compare their performance with real clinical samples, which were extracted from nasopharyngeal swabs or aspirates. The results are listed in table 14. Usually, it is difficult to analyse real clinical samples because of the uncertainty about their content, however these samples have been identified in sending laboratories with validated methods, and therefore this issue was not of our concern.

Table 14. Results from qRT-PCR of clinical samples.

Clinical sample	**qRT-PCR assay**		
	RealStar Influenza S&T 3.0	**RidaGene Flu&RSV**	**R-DiaFlu**
1	27.8	32.8	28.7
2	27.4	28.9	27.8
3	20.4	23.3	20.1
4	26.5	32.5	26.4
5	30.0	32.1	32.1
6	32.2	38.2	34.9
	RealStar Influenza S&T 3.0 (C_t)	**RidaGene Flu&RSV (%)**	**R-DiaFlu (%)**
1	27.8	18.0	3.4
2	27.4	5.4	1.2
3	20.4	14.4	-1.6
4	26.5	22.5	-0.4
5	30.0	6.8	7.1
6	32.2	18.8	8.6
AVERAGE (%)		**14.3**	**3.1**
	RealStar RSV 1.0 (C_t)	**RidaGene Flu&RSV**	**Genesig RSV**
7	18.0	23.6	24.1
8	22.4	26.3	27.6
9	26.3	29.8	31.2
10	18.0	21.5	21.9
	RealStar RSV 1.0 (C_t)	**RidaGene Flu&RSV (%)**	**Genesig RSV (%)**
7	18.0	30.9	33.7
8	22.4	17.4	23.2
9	26.3	13.2	18.7
10	18.0	19.5	21.8
AVERAGE (%)		**20.2**	**24.3**

All the clinical samples were detected correctly, although samples 5 (average Ct 31.41) and 6 (Average Ct 35.11) were weak in all assays. However, from earlier experiments with these clinical samples sample 6 was registered as a weak influenza B virus. Both RealStar® (altona Diagnostics) kits (Influenza S&T RT-PCR Kit 3.0 and RSV RT-PCR Kit 1.0) performed the best because the Ct values were the lowest. In this assessment RIDA®GENE Flu&RSV (R-Biopharm) and Genesig® Respiratory Syncytial Virus (all species) Standard Kit (Primerdesign) performed significantly worse particularly with detecting RSV clinical samples with 20.2 % and 24.3 % difference in Ct values on average compared to RealStar® RSV RT-PCR Kit 1.0.

It is clear, that the RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics) performed significantly better than RIDA®GENE Flu&RSV (R-Biopharm) or Genesig® Respiratory Syncytial Virus (all species) Standard Kit (Primerdesign). With clinical samples registered as Influenza A and B virus, difference between RealStar® Influenza S&T Kit 3.0 (altona Diagnostics) and R-DiaFlu (Diagenode) was very small. R-DiaFlu (Diagenode) maybe transcends over the stronger samples, but in case of weaker samples, RealStar® Influenza S&T Kit 3.0 (altona Diagnostics) is superior.

8.4 Reliability of results

Reliability of the results is ensured by following good laboratory practises and research ethics. Professional ethics is intended to carry out the study professionally, in order to obtain honest, reliable, high quality results.

The experimental part of this thesis was carried out following the Orion Diagnostica Oy laboratory work instructions and aseptic approach was performed in order to avoid risk of contamination. During the work process no contamination was detected. All used laboratory equipment and facilities were cleaned as instructed before and after the work. Contamination of the reagents or samples was avoided by using appropriate laboratory clothing. Work with the samples and reagents was carried out in cold blocks or ice and long term storage of the used reagents was either in a freezer (- 20 °C) or in fridge temperature depending on manufacturers instruction. Used samples were always stored in - 70 °C before and after the use. Moreover, to avoid multiple unfreezing steps, after the extraction and quantitation samples were aliquoted to optimal amount. Therefore, for each experiment, samples were unfrozen only once. Traceability of the study was ensured by careful documentation of all of the experiments on the network

drives and in manual laboratory diaries. All the results were honestly obtained and reported in this thesis.

Reliability of the results from qRT-PCR assays was evaluated after each experiment. Two negative controls were included in each experiment and were confirmed not to be amplified after qRT-PCR assay. This was to detect possible contamination of the reagents while setting up the assay. Moreover, two positive controls were included in qRT-PCR run to ensure that primers and probes of the used assay were detecting their respective target. All the processes from the RNA extraction to the qRT-PCR were carried out according to the manufacturer's instructions as much as it was possible with given time and resources.

All the negative and positive controls worked correctly and based on these parameters the results can be considered reliable. However, repeatability and comparability have to be discussed when all of the qRT-PCR assays were tested only once. Therefore, statistics from the assays was relatively minor. Because of the limited data, the results are only suggestive. More data and testing are needed for reliable statistical analysis, for evaluating the assays and for reliable conclusions.

9 Discussion

The aim of the study was to set up several commercially available qRT-PCR kits for diagnosis of RSV and influenza viruses. Experiments were designed to evaluate the sensitivity and inclusivity of the qRT-PCR assays, as well as possible cross-reactions with other respiratory tract pathogens. Moreover, other features were taken into consideration during the process, such as the ease of use and the time of delivery. There were significant differences in the performance between used qRT-PCR assays.

All the used qRT-PCR kits performed with highest possible sensitivity except Rida®Gene Flu&RSV (R-Biopharm) where false negative result was obtained in lowest dilution from ATCC® sample dilution series. The best results on the other hand, were obtained when comparing the Ct values, Real®Star Influenza S&T RT-PCR Kit 3.0 and RealStar® RSV RT-PCR 1.0 (altona Diagnostics). The differences between Ct values deviated from 0.5 % up to 67.6 % on average. More significant results were obtained with tests containing samples from NATtrol™ FLU Verification Panel (ZeptoMetrix Cor-

poration). With pathogens from the panel there were abundant cross-reactions between influenza virus strains and even different respiratory pathogens. RIDA®GENE Flu&RSV (R-Biopharm) kit had probe specific for RSV, however it additionally gave weak positive result for influenza A H1N1 09 NY strain and strong positive result in Influenza B Florida strain. Also Real®Star Influenza S&T RT-PCR Kit 3.0 (altona Diagnostics) and R-DiaFlu (Diagenode) showed positive results for influenza A H1N1 09 NY strain in channel specific for influenza B. It was interesting to observe, that in all of the mentioned kits the influenza A H1N1 09 NY strain was detected incorrectly. Before accusing contamination during the extraction process, the results showed that the detection was not consistent. With Real®Star Influenza S&T RT-PCR Kit 3.0 (altona Diagnostics) and R-DiaFlu (Diagenode) the detection was done in influenza B specific channel, but with RIDA®GENE Flu&RSV (R-Biopharm) kit it was negative for influenza B and positive for RSV. Therefore, specificity of the assays can be questioned. qRT-PCR assays are limited by the quality of the chosen primers and probes. They have to be not only sensitive enough to match all target RNA, but also specific enough to exclude all others.

In previous study by Esposito et al. (2016), RIDA®GENE Flu & RSV (R-Biopharm) kit was shown to be highly sensitive and specific, unlike in my study. Assay was evaluated to be used in routine practise and samples used in that study were obtained from hospitalized patients with significant symptoms of either lower or upper respiratory infection. Also the sensitivity of the assay was calculated using synthetic RNA fragment and the results were ≥ 50 RNA copies per reaction or 10 copies/µL. Therefore, the results are not fully comparable with this study where lowest dilution to measure sensitivity was 1 copy/ µL. Also ReaStar® Influenza S&T RT-PCR Kit 3.0 (altona Diagnostics) was declared as an assay with high sensitivity in the previous study of Hatchette et al. (2013), although no specificity was tested. For that reason, it is difficult to evaluate if the cross-reaction is a problem with this study or the specificity of the assays is moderate in general. The problem with moderate specificity comes when qRT-PCR assay is used as a reference method for other gene amplification technology. At times clinical samples with no information of pathogens are screened and if qRT-PCR assay gives false negative or false positive results, it is impossible to know whether qRT-PCR or the new technology is responsible for the problem.

All of the selected qRT-PCR kits contained materials for Master Mix in different forms, positive control or positive controls together with negative control. Only Genesig® Res-

piratory Syncytial Virus (all species) Standard Kit (Primerdesign) was to be combined with separate Master Mix reagent (oasig™ Lyophilised OneStep or Precision™ OneStep 2x qRT-PCR MasterMix) and it did not contain internal control unlike other qRT-PCR assays. Preparation of the reaction mix was relatively simple for all used assays, although some of them required more steps and was not always straightforward. For instance, in Genesig® pathogen kits the Master Mix was in lyophilized form in a glass ampule. After re-suspending the Master Mix with the re-suspension buffer, appropriate amount of the Master Mix had to pipet from the glass ampule to the centrifugation tube with primer/probe mix and RNAse/DNAse free water. If the required volume was higher than 200 µL, the pipette tip was too big and the sample had to be pipetted twice with smaller volume pipette.

Based on the results, it can be concluded that the best qRT-PCR assays for diagnostics of influenza viruses and RSV were RealStar® Influenza S&T RT-PCR Kit 3.0 and RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics). The aforementioned assays met high sensitivity and RealStar® RSV RT-PCR Kit 1.0 (altona Diagnostics) also specificity criteria. Both RealStar® assays included IC and they were easy to use. RealStar® Influenza S&T RT-PCR Kit 3.0 can also distinguish pandemic influenza A H1N1 strain from seasonal influenza A, although there was some mismatch detected between pandemic influenza strain and influenza B. Moreover, these assays are CE-marked and can be used in *in vitro* diagnostics. Only clear disadvantage for this assay was that it requires 10 µL sample or control, whereas other assays require only 5 µL. This is especially important for research applications where clinical samples are not routinely available and reagents are usually harder to obtain and are relatively expensive. Regardless of these disadvantages, usability of these qRT-PCR assays is considerably better than with other assays.

In this study, the results were presented statistically in comparative table based on the Ct values obtained from the qRT-PCR. Ct values were also compared between qRT-PCR kits and presented as average percentage difference between Ct values. Generally, comparing the Ct values is not a standard method because amplification is logarithmic. However, in this study comparison was done this way to elucidate raw data.

The follow-up step would be to test RealStar® pathogen Kits with Orion Diagnostica Oy samples processed to be used in freeze-dried SIBA® reactions. Regrettably, as some of the extracted ATCC® RNA samples were weaker than first expected, we were not

able to determine the limit of detection in high concentrations. Also, the obtained data were relatively small because of the delay in order of correct Genesig® Influenza H1N1 Kit (Primerdesign). First ordered Kit was designed for influenza A H1 and it is targeted to M2 rather than M1 found in H1N1 specifically. Delay cost valuable time to test qRT-PCR Kits when extracted influenza A samples (ATCC®) could not be quantitated. As the subject could have expanded to almost unlimited, this effectively restricted the time and moreover, the used qRT-PCR Kits. I wish I had more time to carry out more tests with various qRT-PCR Kits and with different features. In the beginning I had more qRT-PCR kits to test and all together eight of them were set up. Two of eight did not work even with best effort to contact manufacturer and were excluded from the thesis. First kit was the Genesig® influenza A subtype H1 (Primerdesign) which turned out to be wrong type for tested strains and second was Lyra^{TM} Influenza A+B assay (Quidel®) with the FDA approval. It was unfortunate that the only qRT-PCR Kit with FDA approval did not work.

Even though the subject of the study was to test different qRT-PCR Kits, the motive was to find reference method for tests based on SIBA® technology. Faster and more sensitive methods for diagnosing of influenza viruses and RSV are necessary. Although, majority of respiratory tract infections are of viral etiology, they account for three-quarters of all antibiotic prescriptions. Antimicrobial drugs may have reduced the threat posed by infectious diseases; however, these advantages are now endangered by the emergence and spread of microbes that are resistant to effective first-choice drugs. (Low 2008.) Today, while fighting with increasing resistance to antibiotics, it is progressively important to develop sensitive and accurate infection diagnostic methods for infectious diseases. When source of the infection can be reliably diagnosed, correct treatment with antimicrobial drugs or antiviral drugs can be prescribed.

For influenza viruses, the most important benefits of rapid POC molecular diagnostics are better infection control over epidemic and pandemic influenza strains and lowering the hospitalization rate. RSV infections seem inevitable when virtually everyone becomes infected by the age of two therefore, it is reasonable to think benefits of early diagnostics of the infection. It has been shown in previous studies, that there is a positive correlation between severe RSV bronchiolitis and increase of early wheezing, asthma and even allergic sensitization later in life. Therefore, early diagnosis might reduce these risks when treatment with monoclonal antibodies can be started at earliest possible. (Blanken et al. 2013.)

Although, new period has begun in the ongoing development of POC molecular testing and diagnosis, surely the same challenges will have to be faced and similar problems overcome as in case of any other POC tests. While the molecular diagnostics show more sensitivity and specificity, there is higher chance of contamination and mistakes in pre-analytical phase. Careful introduction among users becomes more and more significant. Eboigbodin et al. (2016) are expecting the use molecular methods outside of centralized laboratories into smaller units, or even domestic households.

Along with this project, I created work instructions and comparison table for the used qRT-PCR assays to facilitate their use at Orion Diagnostica Oy. This reduces the time of assay set up when new user can follow the shorter instructions. This project at Orion Diagnostica Oy was enormously instructive and advantageous, because throughout the process I learned new techniques and methods. I am very satisfied with my new skills and I feel I received good skills for the future. In addition, the project helped me to develop practical skills important in laboratory environment work and gave me confidence to work more independently. At times, I had to tolerate uncertainty, find answers independently directly from the manufacturers and ask help, but as a reward I achieved valuable work experience in this field of research.

References

Altona Diagnostics 2016. Instruction for Use. RealStar® Influenza S&T RT-PCR Kit 3.0. Altona Diagnostics GmbH. Germany.

Altona Diagnostics 2012. Instruction for Use. RealStar® RSV RT-PCR Kit 3.0. Altona Diagnostics GmbH. Germany.

Bacharier, LB. – Cohen R. – Schweiger T. – Yin-Declue H. – Christie C. – Zheng J. – Schechtman KB. – Strunk RC. – Castro M. 2012. Determinants of asthma after severe respiratory syncytial virus bronchiolitis. J Allergy Clin Immunol, 130(1), 91-100.

Blanken, MO – Rovers, MM – Molenaar, JM – Winkler-Seinstra, PL – Meijer, A – Kimpen, JL – Bont, L 2013. Respiratory syncytial virus and recurrent wheeze in healthy preterm infants. N Engl J Med, 368(19), 1791-9.

Brendish, N – Schiff, H – Clark, T 2015. Point-of-care testing for respiratory viruses in adults: The current landscape and future potential. Journal of Infection, Volume 71, Issue 5, pages 501-510.

Boom – Sol – Salimans – Jansen – Wertheim-van Dillen – van der Noordaa 1990. Rapid and simple method for purification of nucleis acids. Journal of Clinical Microbiology, 28(3), 495-503.

Brooks, G – Carroll, K – Butel, J – Morse, S – Mietzner, T 2013. Jawetz, Melnick & Adelberg's Medical Microbiology. 26th Edition. The McGraw-Hill Companies. New York. United States.

Brown, T 2006. Genomes 3. Taylor & Francis Group. USA & UK.

Bustin, SA – Mueller, R 2005. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin Sci (Lond), 109(4), pp. 365-79.

Das, K – Aramini, JM – Ma, LC – Krug, RM – Arnold, E 2010. Structures of influenza A proteins and insights into antiviral drug targets. Nature Structural & Molecular Biology, 17(5), 530-538.

Drain, P – Garrett, N 2015. The arrival of a true point-of-care molecular assay—ready for global implementation? Lancet Global Health, Volume 3, Issue 11, e663-e664.

Eboigbodin, K – Filén, S – Ojalehto, S – Brummer, M – Elf, S – Pousi, K – Hoser, M 2016. Reverse transcription strand invasion based amplification (RT-SIBA): a method for rapid detection of influenza A and B. Appl Microbiol Biotechnol, 100(12), pp. 5559-67.

Esposito, S – Scala, A – Tagliabue, C – Zampiero, A – Bianchini, S – Principi, N 2016. Evaluation of a multiplex real-time polymerase chain reaction assay for the detection of influenza and respiratory syncytial viruses. Diagnostic Microbiology and Infectious Disease, 84(1), 40-2.

Eurogentec. qPCR guide. Online guide. Available <http://www.eurogentec.com/uploads/qPCR-guide.pdf>. Accessed 30.10.2016.

Fleige, Simone – Pfaffl, Michael 2006. RNA integrity and the effect on the real-time qRT-PCR performance. Molecular Aspects of Medicine, 27(2-3), 126-39.

Ginocchio, CC 2011. Strengths and weaknesses of FDA-approved/cleared diagnostic devices for the molecular detection of respiratory pathogens. Clinical Infectious Diseases, Suppl 4, S312-25.

Hall, CB – Weinberg, GA – Iwane, MK – Blumkin, AK – Edwards, KM – Staat, MA – Auinger, P – Griffin, MR – Poehling, KA – Erdman, D – Grijalva, CG – Zhu, Y – Szilagyi, P 2009. The burden of respiratory syncytial virus infection in young children. N Engl J Med, 360(6), 588-98.

Hatchette, TF – Drews, SJ - Bastien, N – Li,Y- German,G – Antonishyn, N – Charest,H - Mazzulli, T – Fonseca,K – Krajden,M – Petric, M – Dust, K – LeBlanca, JJ 2013. Detection of Influenza H7N9 Virus: All Molecular Tests Are Not Equal. Journal of Clinical Microbiology, 51(11), 3835-8.

Hatta, M – Goto, H – Kawaoka, Y 2004. Influenza B Virus Requires BM2 Protein for Replication. Journal of Virology, 78(11), 5576-83.

Hedman, Klaus – Heikkinen, Terho – Huovinen, Pentti – Järvinen, Asko – Meri, Seppo – Vaara, Martti 2011. Mikrobiologia 1. Mikrobiologia, immunologia ja infektiosairaudet. Duodecim. Bookwell Oy Porvoo.

Herrler, G – Dürkop, I – Becht, H – Klenk, HD 1988. The glycoprotein of influenza C virus is the haemagglutinin, esterase and fusion factor. J Gen Virol, 69 (Pt 4), 839-46.

Hoser, M – Mansukoski, H – Morrical, S – Eboigbodin, K 2014. Strand Invasion Based Amplification (SIBA®): A Novel Isothermal DNA Amplification Technology Demonstrating High Specificity and Sensitivity for a Single Molecule of Target Analyte. PLoS ONE, 9(11), e112656.

Hurt AC – Baas C – Deng YM – Roberts S – Kelso A – Barr IG 2009. Performance of influenza rapid point-of-care tests in the detection of swine lineage A(H1N1) influenza viruses. 3(4), 171-176.

Kirkwood, Julie 2016. The Next Step for Molecular Point-of-Care Testing. AACC. Clinical Laboratory News. Internet Article. <https://www.aacc.org/publications/cln/articles/2016/june/the-next-step-for-molecular-point-of-care-testing>. Accessed 3.11.2016.

Kubista, Mikael – Andrade, José Manuel – Bengtsson, Martin – Forootan, Amin - Jonák, Jiri – Lind, Kristina – Sindelka, Radek – Sjöback, Robert – Sjögreen, Björn – Strömbom, Linda – Ståhlberg, Anders - Zoric, Neven 2006. The real-time polymerase chain reaction. Molecular Aspects of Medicine, volume 27: 95–125.

Li-Kim-Moy, J – Dastouri, F – Rashid, H – Khandaker, G – Kesson, A – McCaskill, M – Wood, N – Jones, C – Zurynski, Y – Macartney, K – Elliott, EJ – Booy, R 2016. Utility of early influenza diagnosis through point-of-care testing in children presenting to an emergency department. Journal of Paediatrics and Child Health, 52(4), 422-9.

Low, D 2008. Reducing antibiotic use in influenza: challenges and rewards. Clin Microbiol Infect, 14(4), 298-306.

Madigan, M – Matinko, J – Blender, K – Buckley, D – Stahl, D 2015. Brock Biology of Microorganisms. Pearson Education, 14th Edition, USA, 343-346.

Mejias, A – Garcia-Maurino, C – Rodriguez-Fernandez, R – Peeples, ME – Ramilo, O 2016. Development and clinical applications of novel antibodies for prevention and treatment of respiratory syncytial virus infection. 410X(16), 30839-8.

Murray – Rosenthal – Pfaller 2013. Medical Microbiology, Seventh Edition, Elsevier Ltd.

Nair, H – Nokes, DJ – Gessner, BD – Dherani, M – Madhi, SA – Singleton, RJ – O'Brien, KL – Roca, A – Wright, PF – Bruce, N – Chandran, A – Theodoratou, E – Sutanto, A – Sedyaningsih, ER – Ngama, M – Munywoki, PK – Kartasasmita, C – Simões, EA – Rudan, I – Weber, MW – Campbell, H 2010. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. The Lancet, 375(9725), 1545-55.

Nolte, F – Hill, C 2011. Polymerase Chain Reaction and Other Nucleic Acid Amplification Technology. In publication of Henry's Clinical Diagnosis and Management by Laboratory Methods. 22nd Edition. Elsevier Saunders.

Petric M – Comanor L – Petti CA 2006. Role of the laboratory in diagnosis of influenza during seasonal epidemics and potential pandemics. The Journal of Infectious Diseases, 194, 98-110.

Primerdesign™ 2016. Genesig Standard kit handbook HB10.02.09. Human influenza B virus. Available online <http://www.genesig.com/assets/files/influenza_b_std.pdf>.

Primerdesign™ 2016. Genesig Standard kit handbook HB10.02.09. Respiratory syncytial virus. Available online <http://www.primerdesign.co.uk/assets/files/rsvspp_std.pdf>.

Rasmussen, Angela 2015. Probing the Viromic Frontiers. mBio, 6(6), e01767-15.

R-Biopharm 2014. Instructions. RIDA®GENE Flu&RSV. R-Biopharm AG. Germany. Available online <http://www.r-biopharm.com/wp-content/uploads/25649/PG0545RIDAGENE_Flu-RSV_ENG_2014_07_01.pdf>. Accessed 15.5.2016.

Samji, Tasleem 2009. Influenza A: Understanding the Viral Life Cycle. Yale Journal of Biology and Medicine, 82(4), 153–159.

SIBA® and Orion GenRead publications 13.4.2016. Orion Diagnostica Oy. <http://www.oriondiagnostica.com/Global/Documents%20and%20Materials/Orion%20GenRead/Orion%20GenRead%20and%20SIBA%20Publications.pdf> Accessed 13.11.2016.

Stiver, Grant 2003. The treatment of influenza with antiviral drugs. 168(1), 49–57.

Sullender, Wayne 2000. Respiratory Syncytial Virus Genetic and Antigenic Diversity. Clinical Microbiology Reviews, 13(1), 1-15.

Templeton, K. – Scheltinga, S. – Beersma, M. – Kroes, A. – Claas, E. 2004. Rapid and Sensitive Method Using Multiplex Real-Time PCR for Diagnosis of Infections by Influenza A and Influenza B Viruses, Respiratory Syncytial Virus, and Parainfluenza

Viruses 1, 2, 3, and 4. American Society for Microbiology. Journal of Clinical Microbiology, 42(4), 1564–1569.

Tille, P 2014. Bailey & Scott's Diagnostic Microbiology. 13th Edition. Mosby Inc an affiliate of Elsevier Inc.

Tourinho, R – Rodrigues de Almeida, C – Lemos, A – Gardinali, N – Vieira, Y – Schmidt-Chanasit, J – Salete de Paula, V 2015. Application of Synthetic Standard Curves for Absolute Quantification of Hepatitis A and E by Real-Time PCR. Journal of Genetics and Genome Research, 2:1.

Versalovic, J – Carroll, K – Funke, G – Jorgensen, J – Landry, M – Warnock, D 2011. Manual of Clinical Microbiology. 10th Edition. ASM Press. Washington, D.C.

Wacker, MJ – Godard, MP 2005. Analysis of One-Step and Two-Step Real-Time RT-PCR Using SuperScript III. Journal of Biomolecular Techniques, 16(3), 266–271.

Wang,Y – Barbacioru, C – Hyland, F – Xiao, W – Hunkapiller, KL – Blake, J – Chan, F – Gonzalez, C – Zhang, L – Samaha, RR 2006. Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays. Electronic supplementary material. MBC Genomics. Research Article.

World Health Organization 2014. Fact sheet N°211. Influenza (Seasonal). Available online. <http://www.who.int/mediacentre/factsheets/fs211/en/>. Accessed 16.3.2016.

World Health Organization 2005. WHO recommendations on the use of rapid testing for influenza diagnosis. Available online. <http://www.who.int/influenza/resources/documents/RapidTestInfluenza_WebVersion.pdf>. Accessed 6.11.2016.

Appendix 1. Work protocol for extraction of ATCC and NATtrol™ FLU Verification Panel with QIAamp® Viral RNA Mini Kit.

Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.

Add 40 µl sample and 100 µl of PBS to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing.

Incubate at room temperature (15–25°C) for 10 minutes.

Briefly centrifuge the tube to remove drops from the inside of the lid.

Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

Carefully open the QIAamp Mini column, and repeat step 6.

Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Minicolumn in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

To eliminate any chance of possible Buffer AW2 carryover place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min.

Store the obtained RNA in – 70 °C until use.

Appendix 2. Work protocol for RNA extraction from clinical samples with QIAamp® Viral RNA Mini Kit.

Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.
Add 60 µl sample and 80 µl of PBS to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing.
Incubate at room temperature (15–25°C) for 10 minutes.
Briefly centrifuge the tube to remove drops from the inside of the lid.
Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.
Carefully open the QIAamp Mini column, and repeat step 6.
Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Minicolumn in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
To eliminate any chance of possible Buffer AW2 carryover place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min.
Store the obtained RNA in – 70 °C until use.

Appendix 3. Content of the qRT-PCR kits: used reagents.

Genesig Respiratory Syncytial virus (all species) Standard Kit:

Reagent name	Batch
RSV_spp specific primer/probe mix	PD2492
RSV_spp positive control template	PC5053
RSV_spp RT primer mix	(in two-step protocols only)
RNAse/DNAse free water	H2O0162
Template preparation buffer	TPB063

Genesig Human Influenza B virus:

Reagent name	Batch
FluB spesific Primer/Probe mix	PD2638
FluB positive control template	PC5010
FluB RT primer mix	(in two-step protocols only)
RNAse/DNAse free water	H2O0157
Template preparation buffer	TPB061

Oasig lyophilized OneStep qRT-PCR Mastermix:

Reagent name	Batch
Lyophilized Mastermix	
Lyophilized ROX	OAS149
Re-suspension buffer	OAS166

RealStar Influenza S&T RT-PCR Kit 3.0:

Reagent name	LOT
Master A	MA1603
Master B	MB1603
Internal Control	IC022031504
Positive Control H1N1	
Positive Control Influenza A	LOTC1011601
Positive Control Influenza B	
PCR grade Water	W1514

RealStar RSV RT-PCR Kit 1.0:

Reagent name	LOT
Master A	MA1603
Master B	MB1603
Internal Control	IC022031504
Positive Control RSV A	C2011504
Positive Control RSV B	
PCR grade Water	W1514

RidaGene Flu&RSV:

Reagent name	LOT	REF
Reagent Mix	14485	PG0545
PP-Mix		
Enzyme Mix		
Internal Control RNA		
PCR Water		
Positive Control		

R-DiaFlu:

Reagent name	Batch
Optima AB Master Mix 4X RNA	2015D15/I
PPpathogen (Flu A/B Probes and primers)	15315J
H2O	
Flu A/B Positive Control	
Flu A/B Negative Control	

Appendix 4. Quantitation of influenza B and RSV A and B: results and work protocols.

Influenza B:

Genesig® Standard Kit: human influenza B virus (Primerdesign™)

Master Mix was not included to kit contents and it was reconstituted from oasig™ Lyophilized OneStep or Precision™ OneStep 2x qRT-PCR MasterMix.

Master Mix

1. For each glass ampule; Re-suspend lyophilized OneStep Mastermix in 525 µl of re-suspension buffer.
2. Add ROX if required following instructions below:

Real-time PCR Platform	ROX re-suspension volume	ROX addition per ampule
Applied biosystems 7700, 7000, and 7900, 7300, StepOne, StepOnePLUS and ViiA7 platforms, Roche capillary Lightcyclers.	100	20
All Stratagene platforms	200	15
Applied biosystems 7500 platform	700	10
All Other Machines	Not required	Not required

4. Re-suspend the lyophilized ROX (BROWN) in the correct volume of re-suspension buffer (table below)
5. Add re-suspended ROX to each ampule at the correct level

Reconstitution protocol for lyophilized primer and probe mix:

1 Pulse-spin each tube in a centrifuge before opening.
2. Reconstitute the positive control template in the template preparation buffer supplied, according to the table below:

Component - resuspend in water	Volume (µL)
fluB primer/probe mix	165
~~fluB RT primer mix~~	~~165~~

Reconstitution protocol for positive control template/standard curve:

1 Pulse-spin each tube in a centrifuge before opening.

2. Reconstitute the positive control template adding 500 µL of template preparation buffer supplied.

3. Vortex the tube thoroughly.

Standard curve dilution series:

1. Pipette 90 µl of template preparation buffer into 5 tubes and label 2-6.
2. Pipette 10 µl of Positive Control template (RED) into tube 2.
3. Vortex thoroughly.
4. Change pipette tip and pipette 10 µl from tube 2 into tube 3. Vortex thoroughly.
5. Repeat steps 4 and 5 to complete the dilution series.

Standard curve	Copy number per µL
Tube 1 Positive control (RED)	2 x 10^5
Tube 2	2 x 10^4
Tube 3	2 x 10^3
Tube 4	2 x 10^2
Tube 5	20

Sample dilution series:

1:10 dilution from 10^5 cp/µL

1. Pipette 45 µL RNAase free water
2. Pipette 5 µL sample
3. Change pipette tip and pipette 5 µL from10^4 tube to 10^3 tube
4. Continue to get dilutions of 10^1, 10, 1 (cp/µL)

Reaction mix:

Reagent	Volume/ 1 reaction (µL)	Volume/ 25 reactions (µL)
Oasig OneStep gRT-PCR Mastermix	10	20
fluB primer/probe mix	1	15
RNAse/DNAse free water	4	10
Total Volume	15	375

Reaction setup:

	µL
Master Mix	20
Sample or control	5
Total Volume	25

Amplification protocol:

Step	Time	Temp (°C)	Cycles
Reverse transcription	10 min	55	1
Enzyme activation	2 min	95	1
Denaturation	10 s	95	50
Data collection	60 s	60	

Results:

Estimated virus concentration based on qRT-PCR results was 1808820.313 copies/µL. 62 µL of sample was diluted in 1060 µL of RNAse-free water in order to get concentration of 100000 copies/µL. After this the sample was aliquoted into 1.5 ml microcentrifuge tubes and stored in - 70°C.

Raw data:

Sample Name	CT	Ct Mean	Ct SD	Quantity	Quantity Mean
INF B 1:10	19,193	19,130	0,089	180 882,031	188 356,797
STANDARD	18,844	19,117	0,386	200 000,000	
STANDARD	33,964	33,847	0,165	20,000	
INF B 1:10	19,066	19,130	0,089	195 831,563	188 356,797
STANDARD	19,390	19,117	0,386	200 000,000	
STANDARD	33,730	33,847	0,165	20,000	
INF B 1:100	22,672	22,647	0,036	20 347,354	20 678,205
STANDARD	22,793	22,808	0,020	20 000,000	
STANDARD	35,184	37,400	3,134	2,000	
INF B 1:100	22,621	22,647	0,036	21 009,057	20 678,205
STANDARD	22,822	22,808	0,020	20 000,000	
STANDARD	39,617	37,400	3,134	2,000	
INF B 1:1000	26,357	26,320	0,052	2 012,562	2 059,685
STANDARD	26,121	26,109	0,018	2 000,000	
NEGATIVE	Undetermined				
INF B 1:1000	26,284	26,320	0,052	2 106,807	2 059,685
STANDARD	26,096	26,109	0,018	2 000,000	
NEGATIVE	Undetermined				
INF B 1:10000	30,003	29,795	0,294	203,963	234,351
STANDARD	29,894	29,921	0,038	200,000	
INF B 1:10000	29,587	29,795	0,294	264,739	234,351
STANDARD	29,948	29,921	0,038	200,000	

RSV A and B:

Genesig® respiratory syncytial virus (all species) Standard Kit

Master Mix was not included to kit contents and it was reconstituted from oasig™ Lyophilized OneStep or Precision™ OneStep 2x qRT-PCR MasterMix.

Master Mix

1. For each glass ampule; Re-suspend lyophilized OneStep Mastermix in 525 µl of re-suspension buffer.
2. Add ROX if required following instructions below:

Real-time PCR Platform	ROX re-suspension volume	ROX addition per ampule
Applied biosystems 7700, 7000, and 7900, 7300, StepOne, StepOnePLUS and ViiA7 platforms, Roche capillary Lightcyclers. **(RSV A)**	100	20
All Stratagene platforms	200	15
Applied biosystems 7500 platform **(RSV B)**	700	10
All Other Machines	Not required	Not required

4. Re-suspend the lyophilised ROX (BROWN) in the correct volume of re-suspension buffer (table below)
5. Add re-suspended ROX to each ampule at the correct level

Reconstitution protocol for lyophilized primer and probe mix:

1 Pulse-spin each tube in a centrifuge before opening.
2. Reconstitute the positive control template in the template preparation buffer supplied, according to the table below:

Component – resuspend in water	**Volume (µL)**
RSV_spp primer/probe mix	165
~~RSV_spp RT primer mix~~	~~165~~

Reconstitution protocol for positive control template/standard curve:

1 Pulse-spin each tube in a centrifuge before opening.
2. Reconstitute the positive control template adding 500 µL of template preparation buffer supplied.
3. Vortex the tube thoroughly.

Standard curve dilution series:

1. Pipette 90 µl of template preparation buffer into 5 tubes and label 2-6.
2. Pipette 10 µl of Positive Control template (RED) into tube 2.

3. Vortex thoroughly.
4. Change pipette tip and pipette 10 µl from tube 2 into tube 3. Vortex thoroughly.
5. Repeat steps 4 and 5 to complete the dilution series.

Standard curve	**Copy number per µL**
Tube 1 Positive control (RED)	2 x 10^5
Tube 2	2 x 10^4
Tube 3	2 x 10^3
Tube 4	2 x 10^2
Tube 5	20

Sample dilution series:

1:10 dilution from 10^5 cp/µL

1. Pipette 45 µL RNAse free water
2. Pipette 5 µL sample
3. Change pipette tip and pipette 5 µL from10^4 tube to 10^3 tube
4. Continue to get dilutions of 10^1, 10, 1 (cp/µL)

Reaction mix:

Reagent	Volume/ 1 reaction (µL)	Volume/ 25 reactions (µL)
Oasig OneStep gRT-PCR Mas-termix	10	20
RSV_spp primer/probe mix	1	15
RNAse/DNAse free water	4	10
Total Volume	15	375

Reaction setup:

	µL
Master Mix	20
Sample or control	5
Total Volume	25

Amplification protocol:

Step	Time	Temp (°C)	Cycles
Reverse transcription	10 min	55	1
Enzyme activation	2 min	95	1
Denaturation	10 s	95	50
Data collection	60 s	60	

Results:

Estimated virus concentration based on qRT-PCR results was for RSV A 132979.25 copies/µL. 68 µL of sample was diluted in 22 µL of RNAse-free water in order to get concentration of 100000 copies/µL.

As for the RSV B, the result was 845853.75 copies/µL. 71 µL of sample was diluted in 529 µL of RNAse-free water in order to get concentration of 100000 copies/µL.

After quantitation and dilution, the samples were aliquoted into 1.5 ml microcentrifuge tubes and stored in - 70°C.

Raw data (RSV A):

Sample Name	CT	Ct Mean	Ct SD	Quantity	Quantity Mean
RSV A 1:10	26,204	26,766	0,795	17 463,584	13 297,925
Standards	21,844	21,849	0,008	200 000,000	
Standards	37,592	37,991	0,565	20,000	
RSV A 1:10	27,328	26,766	0,795	9 132,266	13 297,925
Standards	21,855	21,849	0,008	200 000,000	
Standards	38,391	37,991	0,565	20,000	
RSV A 1:100	31,516	31,405	0,157	815,811	871,375
Standards	26,187	26,125	0,087	20 000,000	
Standards	41,697	41,885	0,267	2,000	
RSV A 1:100	31,295	31,405	0,157	926,938	871,375
Standards	26,064	26,125	0,087	20 000,000	
Standards	42,074	41,885	0,267	2,000	
RSV A 1:1000	35,951	35,589	0,512	63,209	79,588
Standards	29,927	29,964	0,052	2 000,000	
Negative	Undetermined				
RSV A 1:1000	35,227	35,589	0,512	95,966	79,588
Standards	30,000	29,964	0,052	2 000,000	
Negative	Undetermined				
RSV A	39,138	39,375	0,335	10,057	8,856

1:10000					
Standards	33,971	33,929	0,059	200,000	
RSV A					
1:10000	39,612	39,375	0,335	7,655	8,856
Standards	33,887	33,929	0,059	200,000	

Raw Data (RSV B):

Sample Name	Cт	Cт Mean	Cт SD	Quantity	Quantity Mean
RSV B 1:10	21,7222595	21,78255	0,08527	87297,60938	84585,375
Standard	20,0803051	20,0279	0,074106	200000	
Standard	37,9675484	37,594	0,528277	20	
RSV B 1:10	21,8428497	21,78255	0,08527	81873,14844	84585,375
Standard	19,9755039	20,0279	0,074106	200000	
Standard	37,2204514	37,594	0,528277	20	
RSV B 1:100	26,5688782	26,46874	0,141619	6625,997559	6998,472656
Standard	24,5385418	24,68242	0,203478	20000	
Standard	41,4561996	41,71004	0,358986	2	
RSV B 1:100	26,3685989	26,46874	0,141619	7370,947266	6998,472656
Standard	24,8263035	24,68242	0,203478	20000	
Standard	41,9638824	41,71004	0,358986	2	
RSV B 1:1000	30,9605942	30,64608	0,444785	640,6189575	767,9168701
Standard	28,7507896	28,77447	0,033492	2000	
RSVB1:1000	30,3315735	30,64608	0,444785	895,2147217	767,9168701
Standard	28,7981548	28,77447	0,033492	2000	
RSVB 1:10000	34,5868416	34,47871	0,152913	93,06798553	98,74144745
Standard	32,8781204	33,11982	0,341812	200	
NEG	Undetermined				
RSVB 1:10000	34,3705902	34,47871	0,152913	104,4149094	98,74144745
Standard	33,361515	33,11982	0,341812	200	
NEG	Undetermined				

Appendix 5. qRT-PCR protocols of used kits.

RealStar Influenza S&T RT-PCR 3.0 Kit

Primers and probes:

Target	Sequence 5'-3'	Oligo
Matrix	AGAGACTTGAAGATGTATTTGCTGGGAAGAT	Probe 1
Matrix	TCCTGCAAAGACACTTTCCAGT	Probe 2
Matrix	CAGGCCCCCTCAAAGC	Primer 1
Matrix	CGTCAGGCCTCCTCAAAGC	Primer 2
Matrix	ATTCCATGAGAGCCTCAAGATC	Primer 3

Master Mix setup:

reagent	Volume/1 reaction	Volume/49 reactions
Master A	5	245
Master B	10	490
Internal Control	1	49
Total Volume of the Master Mix	16	784

Sample dilution series:

1:10 dilution from 10^5 cp/µL

1. Pipette 45 µL RNAase free water
2. Pipette 5 µL sample
3. Change pipette tip and pipette 5 µL from10^4 tube to 10^3 tube
4. Continue to get dilutions of 10^1, 10, 1 (cp/µL)

Reaction setup/well:

Master Mix	15
Sample or control	10
Total Volume	25

qRT-PCR protocol:

Step	Stage	Cycles	Temp (°C)	Time
Reverse transcription	Hold	1	55	10 min
Denaturation	Hold	1	95	2 min
			95	15 s
Amplification	Cycling	45	55	45 s
			72	15 s

Fluorescent dyes	
INF A	Cy5
INF B	ROX
H1N1nv	FAM
IC	JOE
Quencher: NONE	

RealStar RSV RT-PCR Kit 1.0

Master Mix setup:

reagent	Volume (µL)/1 reaction	Volume/49 reactions
Master A	5	245
Master B	10	490
Internal Control	1	49
Total Volume of the Master Mix	16	784

Sample dilution series:

1:10 dilution from 10^5 cp/µL

1. Pipette 45 µL RNAase free water
2. Pipette 5 µL sample
3. Change pipette tip and pipette 5 µL from10^4 tube to 10^3 tube
4. Continue to get dilutions of 10^1, 10, 1 (cp/µL)

Reaction setup/well:

Master Mix	15
Sample or control	10
Total Volume	25

qRT-PCR protocol:

Step	Stage	Cycles	Temp (°C)	Time
Reverse transcription	Hold	1	55	10 min
Denaturation	Hold	1	95	10 min
			95	15 s
Amplification	Cycling	45	55	45 s
			72	15 s

Fluorescent dyes:	
INF A	Cy5
INF B	FAM
IC	JOE
Quencher: NONE	

RidaGene FLU&RSV

Master Mix setup:

reagent	Volume (µL)/1 reaction	Volume/85 reactions
Reaction Mix	12.5	1062.5
Pp Mix	6.9	586.5
Enzyme Mix	0.7	59.5
Internal Control	1	85
Total Volume of the Master Mix	21.1	1793.5

Sample dilution series:

1:10 dilution from 10^5 cp/µL

1. Pipette 45 µL RNAase free water
2. Pipette 5 µL sample
3. Change pipette tip and pipette 5 µL from10^4 tube to 10^3 tube
4. Continue to get dilutions of 10^1, 10, 1 (cp/µL)

Reaction setup:

	µL
Master Mix	20
Sample or control	5
Total Volume	25

qRT-PCR protocol:

Step	Time	Temp (°C)	Cycles
Reverse transcription	10 min	58	1
Initial denaturation	1 min	95	1
Denaturation	15 s	95	45
Annealing	30 s	55	

Fluorescent dyes:	
RSV	FAM
ICR	VIC
Flu B	ROX
Flu A	Cy5
Quencher: NONE	

R-DiaFlu

Master Mix setup:

reagent	Volume (µL)/1 reaction	Volume/52 reactions
Optima AB Master Mix 4X RNA	6.25	325
PPpathogen (Flu A/B Probes and primers)	2.5	130
H2O	11.25	585
Total Volume of the Master Mix	20	1040

Sample dilution series:

1:10 dilution from 10^5 cp/µL

1. Pipette 45 µL RNAase free water
2. Pipette 5 µL sample
3. Change pipette tip and pipette 5 µL from10^4 tube to 10^3 tube
4. Continue to get dilutions of 10^1, 10, 1 (cp/µL)

Reaction setup:

	µL
Master Mix	20
Sample or control	5
Total Volume	25

qRT-PCR protocol:

Step	Time	Temp (°C)	Cycles
Reverse transcription	30 min	50	1
Activation (Tag)	10 min	95	1
Denaturation	15 s	95	
Annealing	30 s	55	45
Elongation	30 s	68	

Appendix 6. qRT-PCR kit comparison table

Name	Manufacturer	PCR instruments	Extraction Systems	Pathogen
Genesig® Respiratory syncytial virus	PrimerDesign™	ALL	ALL	RSV A RSV B
Genesig® Influenza B Virus Standard Kit	PrimerDesign™	ALL	ALL	Influenza B
Genesig® H1N1 Influenza	PrimerDesign™	ALL	ALL	Influenza A subtype H1N1
RealStar® RSV RT-PCR Kit 1.0	Altona Diagnostics	m2000rt (Abbot Diagnostics) Mx 3005P QPCR System (Stratagene) Versant kPCR Molecular system AD ABI prism® 7500 SDS and 7500 Fast SDS (ABI) LightCycler® 480 Instrument II (Roche) Rotor-Gene 3000/6000 (Corbett Research) Rotor-Gene Q 5/6 plex Platform (QIAGEN)	KingFisher® Flex (Thermo Scientific) VERSANT Modular Sytem SP (Siemens) HighPure® Viral Nucleic Acid Kit (Roche) QIAamp® Viral RNA Mini Kit (QIAGEN)	RSV A RSV B
RealStar® Influenza RT-PCR Kit 1.0	Altona Diagnostics	Mx 3005P QPCR System (Stratagene) Versant kPCR Molecular system AD ABI prism® 7500 SDS and 7500 Fast SDS (ABI) LightCycler® 480 Instrument II (Roche) Rotor-Gene 3000/6000 (Corbett Research) Rotor-Gene Q 5/6 plex Platform (QIAGEN) CFX96/Dx Real-Time System (BIO-RAD)	KingFisher® Flex (Thermo Scientific) VERSANT Modular Sytem SP (Siemens) HighPure® Viral Nucleic Acid Kit (Roche) QIAamp® Viral RNA Mini Kit (QIAGEN)	Influenza A Influenza B

Name	Standard Curve	Internal control	CE/IVD/FDA	Reactions per Assay	Sample Volume
Genesig® Respiratory syncytial virus	YES	NO		150	5 µl
Genesig® Influenza B Virus Standard Kit	YES	NO		150	5 µl
Genesig® H1N1 Influenza	YES	NO		150	5 µl
RealStar® RSV RT-PCR Kit 1.0	NO	YES	CE	96	10 µl
RealStar® Influenza RT-PCR Kit 1.0	NO	YES	CE	96	10 µl

Name	Manufacturer	PCR instruments	Extraction Systems	Viruses
RealStar® Influenza S&T RT-PCR Kit 3.0	Altona Diagnostics	m2000rt (Abbot Diagnostics) Mx 3005P QPCR System (Stratagene) Versant kPCR Molecular system AD ABI prism® 7500 SDS and 7500 Fast SDS (ABI.) LightCycler® 480 Instrument II (Roche) Rotor-Gene 3000/6000 (Corbett Research) Rotor-Gene Q 5/6 plex Platform (QIAGEN) CFX96/Dx Real-Time System (BIO-RAD)	KingFisher®Flex (Thermo Scientific) VERSANT Modular Sytem SP (Siemens) HighPure® Viral Nucleic Acid Kit (Roche) QIAamp® Viral RNA Mini Kit (QIAGEN)	Influenza A Influenza A H1N1nv Influenza B
Lyra® RSV+hMPV Assay	Quidel®	Life Technologies Quantstudio™ Dx RT_PCR Applied Biosystems® 7500 Fast Dx Cepheid® Smartcycler II System	NucliSENS® easyMAG®	RSV hMPV
Lyra® Influenza A+B Assay	Quidel®	Life Technologies Quantstudio™ Dx RT_PCR Applied Biosystems® 7500 Fast Dx Cepheid® Smartcycler II System	NucliSENS® easyMAG®	Influenza A Influenza B
RIDA®GENE Flu & RSV	R-Biopharm	LightCycler® 480 Instrument II (Roche) Mx 3005P (Agilent Technologies) ABI 7500 (Applied Biosystems) m2000rt (Abbot Diagnostics) CFX96 (BIO-RAD)	RIDA®Xtract (R-Biopharm) Maxwell®16 (Promega) MagNA Pure 96 (Roche)	RSV Influenza A Influenza B
R-DIAFlu	Diagenode	ABI (7500 fast) Roche LightCycler® (480) Bio-Rad (CFX96) Qiagen Rotor-Gene (Q)	Nuclisens easyMAG® System MagNa pure LC 96 System (Roche)	Influenza A Influenza B

Name	Standard Curve	Internal control	CE/IVD/FDA	Reactions per Assay	Sample Volume	Target gene
RealStar® Influenza S&T RT-PCR Kit 3.0	NO	YES	CE	96	10 µl	
Lyra® RSV+hMPV Assay	NO	YES	FDA	96	5 µl	
Lyra® Influenza A+B Assay	NO	YES	FDA	96	5 µl	
RIDA®GENE Flu & RSV	NO	YES	CE	100	5 µl	M, NP1 F
R-DIAFlu	NO	YES	CE	100	5 µl	MP 1 &2 NP